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BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF NATURALLY OCCURRING RECOMBINANT AVIAN LEUKOSIS VIRUS ISOLATED FROM COMMERCIAL LAYERS AFFECTED WITH MYELOID LEUKOSIS

presented by

Ghida R. Banat

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Pathology

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Major Professor's Signature

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BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF NATURALLY OCCURRING RECOMBINANT AVIAN LEUKOSIS VIRUS ISOLATED FROM COMMERCIAL LAYERS AFFECTED WITH MYELOID LEUKOSIS

By

Ghida R. Banat

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Pathology

2009

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ABSTRACT

BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF NATURALLY OCCURRING RECOMBINANT AVIAN LEUKOSIS VIRUS ISOLATED FROM COMMERCIAL LAYERS AFFECTED WITH MYELOID LEUKOSIS

By

Ghida R. Banat

Avian leukosis virus (ALV) is an economically important poultry pathogen resulting in low productivity and tumor mortality in chickens. Limited information is currently available about natural recombination among different ALV subgroups and pathogenesis in chicken. Several biological and molecular studies were conducted to characterize seven isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) of a naturally occurring recombinant ALV from commercial layers affected with myeloid leukosis.

The first aim was to characterize seven isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) of a naturally occurring recombinant ALV by biological assays and polymerase chain reaction. Closely and distinctly related strains of naturally occurring recombinant ALVs were identified using various diagnostic techniques. The host range and antigenic relationships of the seven AF 115 isolates were comparable with those of the first characterized naturally occurring recombinant ALV-B/J (AF 115-4). However, the PCR amplification results, using different primer sets, indicated the presence of recombinant ALV-B/J sequences in proviral DNA of all AF 115 isolates. Moreover, three isolates (AF 115-5, AF 115-7 and AF 115-10) demonstrated different patterns of PCR amplification from the recombinant ALV-B/J (AF 115-4) and

the rest of the isolates, suggesting the presence of mutations and rearrangements in different genomic regions.

The second aim was to molecularly characterize these seven AF 115 isolates by DNA sequence analyses of various genomic regions. All AF 115 isolates were confirmed to be recombinant ALVs with different genomic regions representing different ALV subgroups. Five isolates (AF 115-1, AF 115-7, AF 115-13, AF 115-14 & AF 115-16) were closely related to the naturally occurring recombinant ALV-B/J, containing a subgroup B gp85, subgroup E gp37 and subgroup J 3' UTR (including DR1 and E element) and LTR. On the other hand, AF 115-5 had a subgroup A gp85 and is believed to be contain a mixture of two recombinant ALVs (ALV-A/J & ALV-B/J). The 3' UTR and LTR of AF 115-10 contained base deletions and insertions and were highly associated with a recombinant ALV isolate (ALV TymS_90). In addition, all AF 115 isolates contained important potential sites for protein structure, stability and function in the envelope glycoproteins as well as transcription regulatory elements in the 3' UTR and LTR. It is believed that recombination hotspots exist between SU and TM domains of the envelope, between the gp37 and the 3' UTR upstream of the DR1 region as well as 3' UTR and LTR.

Therefore, these research studies have added new information on ALV diversity (mutations, rearrangement and recombination) and should aid in improving the diagnosis and control of ALV infection in field flocks.

I would like to dedicate this work

to

my family especially my mother Mrs. Kamleh Ibrahim Al-Baitam
and my father Mr. Rakad Saleh Banat

ACKNOWLEDGMENTS

Thanks to God, The Greatest and The Merciful, who inspired and guided me through my entire life, especially through my Ph.D. journey.

I would like to express my gratitude and appreciation to my current academic advisor Dr. Scott Fitzgerald, my research advisor Dr. Aly Fadly and my previous academic advisor Dr. Willie Reed for providing me the opportunity to pursue my graduate Ph.D. studies in the collaborative program between USDA-ARS-ADOL and DCPAH. In addition, I would like to thank Dr. Robert Silva, Dr. John Gerlach and Dr. Steve Bolin for serving on my graduate guidance committee. I am grateful for all members of the graduate committee for their constant support, patience, encouragement, time, effort, shared knowledge and for reviewing the manuscript, making valuable suggestions and providing comments for improvement. I value the friendship, assistance, time and effort provided by all the scientists and staff at ADOL. In particular, I would like to thank Mr. Lonnie Milam, Dr. Jody Mays, Ms. Melanie Flesberg, Dr. Henry Hunt and Mrs. Pamela Campbell for their invaluable training, help, discussion and guidance in the laboratory. Thanks to all for this opportunity to grow professionally and personally.

I would also like to thank all my friends for all their constant help, support and encouragement. Profound appreciation and gratitude go to my dear mother for her unconditional love, patience, support and encouragement. I am forever grateful for my dear parents for providing me with the best education and opportunities. I am also appreciative to my late grandfather, sisters Dana, Doaa, and Areej, brothers Saleh and Ehab and their families for all their support and encouragement. I love you all so much.

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* Images in this dissertation are presented in color.

KEY TO SYMBOLS AND ABBREVIATIONS

%	Percentage
&	and
+ SS RNA	plus sense single-stranded RNA
®	registered trademark
° C	degree centigrade
µg	microgram
µl	microliter
966	Acute transforming avian leukosis virus subgroup J strain 966
A1/EBP	Leucine zipper A1/CCAAT enhancer binding protein
ABI	Applied Biosystems Inc.
AD1	Reverse PCR primer for ALV-A to ALV-E
ADOL	Avian Disease and Oncology Laboratory
AE/E	subgroup A gp85 and subgroup E gp37 and LTR
AE/J	subgroup A SU, subgroup E TM, subgroup J 3' UTR and LTR
AE/J,AEV,E	ALV-A SU, ALV-E TM, R & U5, ALV-J 3' UTR & AEV U3
AEV	avian erythroblastosis virus
AEV-ES4	Avian erythroblastosis virus strain ES4
AF 115	Aly Fadly 115 isolates of avian leukosis virus
AIDS	acquired immunodeficiency syndrome
ALSV	avian leukosis/sarcoma group of retroviruses
ALV	avian leukosis virus

alv6	Transgenic chicken line avian leukosis virus 6
ALV-A	avian leukosis virus subgroup A
ALV-A/J	avian leukosis virus with subgroup A env and subgroup J LTR
ALV-B	avian leukosis virus subgroup B
ALV-B/J	avian leukosis virus with subgroup B env and subgroup J LTR
ALV-BE/J	ALV with ALV-B SU, ALV-E TM & ALV-J 3' UTR & LTR
ALV-C	avian leukosis virus subgroup C
ALV-D	avian leukosis virus subgroup D
ALV-E	avian leukosis virus subgroup E
ALV-J	avian leukosis virus subgroup J
AMAV-2	Avian myeloblastosis-associated virus 2 subgroup B
AMC 29	avian myelocytomatosis virus strain 29
AMV	avian myeloblastosis virus
anti-Hc1	antibodies to Hc1 (American prototype of subgroup J ALV)
anti-RAV-2	Antibodies to RAV-2 (Rous-associated virus 2 or ALV-B)
ARK 4836	Arkansas 4836 forward PCR primer for all ALVs
ARS	Agricultura Research Service
ART-CH	avian retrotransposon from chicken genome
ASVs	avian sarcoma viruses
B LTR	subgroup B ALV long terminal repeat reverse primer
BAI-A AMV	Strain BAI-A of avian myeloblastosis virus
BBL	B-F/B-L region of chicken major histocompatibility complex
bet	cellular or viral oncogene bet

B-F	bursal-F chicken MHC class 1
BFV	bovine foamy virus
B-haplotype	bursal-haplotype or chicken major histocompatibility complex
BH-RSV	Bryan's high titer strain of Rous sarcoma virus
BIV	bovine immunodeficiency virus
B-L	bursal-L chicken MHC class II
BLAST	Basic Local Alignment Search Tool
BLV	bovine leukemia virus
Blym-1	A transforming gene or oncogene in Burkitt lymphoma
bp	base pair
bZip	Basic leucine zipper DNA binding protein or transcription factor
C/A	chickens or chicken cells resistant to ALV subgroup A infection
C/ABDE	chickens or chicken cells resistant to ALVs A, B, D & E
C/AE	chickens or chicken cells resistant to infection by ALV E & A
C/E	chickens or chicken cells susceptible to all exogenous ALVs
C/EBP	CCAAT/enhancer binding protein
C/EJ	chickens or chicken cells resistant to ALV subgroups E and J
C/O	chickens or chicken cells susceptible to infection by all ALVs
CA	capsid
CAEV	caprine arthritis encephalitis virus
CAR1	Chicken arginase 1 of TNFR family
c-bic	Cellular oncogene or proto-oncogene B-cell integration cluster
CEFs	chicken embryo fibroblasts

ChiRV1	Chicken retrovirus 1
CIAV	chicken infectious anemia virus
CMI	cell mediated immunity
CMII	avian carcinoma/myelocytoma virus strain CMII
CO_2	carbon dioxide
COFAL	complement-fixation test for avian leukosis virus
c-onc	cellular oncogenes
CR1	chicken repeat 1
CS	calf serum
CT10	avian sarcoma virus strain CT10
C-terminal	Carboxyl-terminal
CTL	cytotoxic T lymphocyte
D	aspartic acid
dATP	Deoxyadenosine triphosphate
DCPAH	Diagnostic Center for population and Animal Health
dCTP	Deoxycytidine triphosphate
DEAE	Diethylaminoethyl
DF-1	Douglas Foster-1, a spontaneously immortalized CEF cell line
DF-1/J	Genetically engineered DF-1 cell line resistant to ALV-J
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DR1	direct repeat 1

ds	double stranded
dTTP	Deoxythymidine triphosphate
E/B	subgroup E *env* (RAV-0) and subgroup B LTR (RSV-Pr-B)
E26	avian erythroblastosis virus strain E26
Ea-B	erythrocyte antigen B locus
EAV	endogenous avian virus
EAV-E51	endogenous avian virus element E51
EAV-HP	endogenous avian virus element HP
EB	erythroblastosis
EBP	enhancer binding protein
*Eco*R I	*E. coli* restriction enzyme I
EFI	enhancer factor I
EFIII	enhancer factor III
EFV	equine foamy virus
EGF	epidermal growth factor
EIAV	equine infectious anemia virus
ELISA	Enzyme-linked immunosorbent assay
env	envelope
erbA	erythroblastosis virus oncogene A
erbB	erythroblastosis virus oncogene B
ES4	avian erythroblastosis virus strain ES4
ev	endogenous viral loci
ev/J 4.1 Rb	endogenous avian virus element ev/J 4.1 Rb

F	forward primer
F1	fragment 1 (DR1)
F2	Fragment 2 (XSR or E element)
F5	Forward 5 PCR primer
FeLV	feline leukemia virus
FFV	feline foamy virus
FIV	feline immunodeficiency virus
FP	fusion peptide
fps	Fujinami avian sarcoma viral oncogene homolog
FuSV	Fujinami sarcoma virus
gag	group specific antigen
gp37	glycoprotein 37 or TM
gp85	glycoprotein 85 or SU
gPr92env	Glycoprotein 92 KDa of envelope
gsa	group specific antigen
H5	Hemagglutinin 5 forward PCR primer for all ALVs
H7	Hemagglutinin 7 reverse PCR primer for ALV-J
Hc1	USA prototype of ALV-J strain Hc1
HCl	Hydrochloric acid
HI	humoral immunity
HIV	Human immunodeficiency virus
HPRS-103	United Kingdom prototype of ALV-J strain HPRS-103
hr	hypervariable region

HTLV	Human T-lymphotropic virus
IA	intrabdominal
IAP	intracisternal A-type particle
IBD	infectious bursal disease
ICTV	International Committee on Taxonomy of Viruses
I^e	Independent locus for resistance to ALV-E or TVE
Ig	immunoglobulin
IN	integrase
IV	intravenous
JSRV	Jaagsiekte sheep retrovirus
kb	kilobase
KCl	Potassium chloride
L	Leader region
L	lysine
L/S	Leukosis/Sarcoma group
LDLR	low-density lipoprotein receptor
LL	lymphoid leukosis
LM	Leibowitz's L-15-McCoy's 5A tissue culture medium
LNGV	Langur virus
LR-9	Avian leukosis virus strain LR-9 or leukemia retrovirus 9
LTR	long terminal repeat
MA	matrix
MAV	myeloblastosis-associated virus

MAV-1	myeloblastosis-associated virus 1 subgroup A
MAV-2	myeloblastosis-associated virus 2 subgroup B
MAV-2 (O)	MAV-2 virus with osteopetrosis potential
MDV	Marek's disease virus
$MgCl_2$	Magnesium chloride
MH2	Avian carcinoma virus strain MH2
MH2E21	avian carcinoma virus strain MH2E21
MHC	major histocompatibility complex
ML	myeloid leukosis
mL	milliliter
ml	milliliter
MLV	murine leukemia virus
mm	millimeter
mM	millimolar
M-MLV	Moloney murine leukaemia virus
MMTV	mouse mammary tumour virus
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
myb	myeloblastosis oncogene
myc	myelocytomatosis oncogene
N	asparagine
Nab	neutralizing antibody
NC	nucleocapsid

NCBI	National Center for biotechnology Information
NCT	asparagine, cysteine and threonine amino acids
nef	negative factor gene of HIV
ng	nanogram
NJ	neighbor joining
NK	natural killer
N-linked	asparagine-linked
NP	non-producer
NS	non-synonymous nucleotide substitutions
NSS	asparagine and two serine amino acids
nt	nucleotide
N-terminal	Amine-terminal
NTRE-4	recombianat Rous sarcoma virus strain NTRE-4
NWS	asparagine, tryptophan and serine amino acids
NXS/T	aspargine, any amino acid, serine or threonine amino acids
ORF	open reading frame
p10	protein 10 KDa or additional gag protein
p12	protein 12 KDa or NC
p15	protein 15 KDa or PR
p19	protein 19 KDa or MA
p27	protein 27 KDa or CA
p32	protein 32 KDa or IN
p68	protein 68 KDa or RT

PAS	Polyadenylation signal
PBS	primer binding sequence
PCR	polymerase chain reaction
PDRC-3249	Poultry Diagnostic and Research Center 3249 isolate of ALV
PFU	archaeon *Pyrococcus furiosus* DNA polymerase
pH	Power of Hydrogen
PHV	Perch hyperplasia virus
PM	phenotypic mixing
pmoles	picomoles
pol	polymerase
PPT	polypurine tract
PR	protease enzyme
Pr180GAG-PR-POL	polyprotein 180 KDa gag-pr-pol
Pr76GAG-PR	Polyprotein 76 KDa gag-pr
Pr-B	Prague strain subgroup B Rous sarcoma virus
Pr-C	Prague strain subgroup C Rous sarcoma virus
PRE	pentanucleotide repeat element
pro	protease
Pr-RSV-C	Prague strain of Rous sarcoma virus subgroup C
PTC-100	Programmable Thermal Controler 100
PU	cis-acting element or gene in the E-element of some ALSV
QT6	Quail transformed Japanese fibrosarcoma cell line 6
R	avian erythroblastosis virus strain R

R allele	resistant allele
R primer	reverse primer
R region	repeat region
R5	Reverse 5 PCR primer for ALV-J
R8	Reverse 8 PCR primer for ALV-J
RAV	Rous-associated virus
RAV-0	Rous-associated virus 0 subgroup E
RAV-1	Rous-associated virus 1 subgroup A
RAV-2	Rous-associated virus 2 subgroup B
RAV-49	Rous-associated virus 49 subgroup C
RAV-50	Rous-associated virus 50 subgroup D
RAV-60	Rous-associated virus 60 subgroup E
RAV-7	Rous-associated virus 7 subgroup C
REV	Avian reticuloendotheliosis virus
rev	Regulator of virion gene of HIV
REVSP 6683	reverse PCR primer for all ALVs
RIF	resistance inducing factor
RNA	ribonucleic acid
RNase H	RNA endonuclease or ribonuclease H
ros	Oncogene encoding a receptor-type protein tyrosine kinase
RPL12	Regional Poultry Laboratory 12 strain of avian leukosis virus
R-RS-1	Regression of Rous sarcoma 1 gene in chickens
RS-6J	6J forward PCR primer for ALV-J

RS-7J	7J forward PCR primer for all ALVs
RSA	Rous sarcoma virus subgroup A
RS-A	Rous sarcoma virus subgroup A
RSV	Rous sarcoma virus
RSV-A	Rous sarcoma virus subgroup A
RSV-NTRE-7 td	Rous sarcoma virus strain NTRE-7 transformation defective
RSV-Pr-C	Rous sarcoma virus Prague strain subgroup C
RSV-SR-B	Rous sarcoma virus Schmidt-Ruppin subgroup B
RSV-SR-D	Rous sarcoma virus Schmidt-Ruppin subgroup D
RSV-td PR2257	RSV transformation defective Poultry Research 2257 strain
RT	reverse transcriptase enzyme
rTM	redundant transmembrane region insert
RT-PCR	reverse transcriptase-Polymerase Chain Reaction
S allele	susceptible allele Synonymous nucleotide substitutions
S	Synonymous nucleotide substitutions
S1	Smith 1 forward PCR primer for ALV-J
S2	Smith 2 reverse PCR primer for ALV-J
sag	superantigen
SB1	Marek's Disease virus serotype 2 strain SB1
SD0501	Chinese endogenous ALV strain SD0501
SDS	Sodium Dodecyl Sulfate
SFV	simian foamy virus
SIV	simian immunodeficiency virus

SMRV	Squirrel monkey retrovirus
SnRV	Snakehead retrovirus
SNV	spleen necrosis virus
SPF	specific-pathogen free
SRB-F	Forward PCR primer for ALV-B
SRB-R	Reverse PCR primer for ALV-B
src	sarcoma gene of Rous sarcoma virus encoding tyrosine kinase
ss	single stranded
STLV	Simian T-lymphotropic virus
SU	surface
T/BD	Turkey cells resistant to infection by ALV-B and ALV-D
TAE	Tris-Acetate-EDTA (ethylenediaminetetraacitic acid)
Taq	*Thermus aquaticus* thermostable DNA polymerase
tas/bel-1	Transcriptional transactivator gene of foamy viruses
tat	Trans-Activator of Transcription gene of HIV
TCID50	tissue culture infectious dose 50
TE	Tris-EDTA (ethylenediaminetetraacitic acid)
Th-1	Thymocyte 1, a T-lymphocyte antigen locus in chicken
TM	transmembrane
™	trademark
TMB	3, 3′, 5, 5′-tetramethyl benzidine
TNFR	tumor necrosis factor receptor
tRNA	transfer RNA

TVA	tumor virus A locus
TVB	tumor virus B locus
TVC	tumor virus C locus
TVE	tumor virus E locus
TymS_90	recombinant avian leukosis virus strain TymS_90
U3	unique 3' region
U5	unique 5' region
UD2	avian leukosis virus subgroup J strain UD2
UD-J1	avian leukosis virus subgroup J strain UD-J1
UR2	avian sarcoma virus strain UR2
US	United States
USDA	United States Department of agriculture
UTR	untranslated region
VBP	vitellogenin gene binding protein in chicken
vif	Viral infectivity factor gene of HIV
VN	virus neutralization
v-onc	viral oncogene
vpr	Viral protein R gene of HIV
vpu	Viral protein U gene of HIV
vr	variable region
WDSV	Walleye dermal sarcoma virus
WEHV	Walleye epidermal hyperplasia virus
XSR	Exogenous virus-specific region or E element

Y73	avian sarcoma virus strain Y73
YB-1	Y-box binding protein 1
yes	Yamaguchi sarcoma oncogene
Ψ	Psi element, an encapsidation signal

INTRODUCTION

Avian leukosis virus (ALV) is an important poultry pathogen resulting in economic losses due to low productivity and tumor mortality (Fadly and Payne, 2003). It causes a variety of transmissible benign and malignant neoplasms in mature and semimature chickens. According to the International Committee on Taxonomy of Viruses (ICTV), ALV is a member of the family *Retroviridae*, subfamily *Orthoretrovirinae* and genus *Alpharetrovirus* (Linial *et al.*, 2006). There are six subgroups of ALV in chickens, five exogenous (subgroups A, B, C, D and J) and one endogenous (subgroup E). Like other retroviruses, ALV is known for its high mutation and recombination rates constituting a constant threat to the poultry industry.

Genetic recombination has been reported between endogenous and exogenous ALVs leading to the generation of oncogenic mutants (Crittenden *et al.*, 1980; Tsichlis and Coffin, 1980; Weiss *et al.*, 1973). For example, ALV-J is a recombinant virus between endogenous (EAV-HP or ev/J) and undetermined exogenous ALV (Bai *et al.*, 1995b). This emerging recombinant ALV is being controlled by efficient diagnosis and eradication of infected broiler breeder flocks. In addition, recombinant ALV isolates were experimentally generated with subgroup A gp85, subgroup E gp37and subgroup J LTR through propagation of ALV-J in alv6 (C/AE) CEFs (Lupiani *et al.*, 2000).

Limited information is available about natural recombination among different subgroups of ALV and its impact on pathogenesis in chicken. Recombinant ALVs with genomic regions belonging to different subgroups may present new phenotypes and altered characteristics such as host range, antigenicity and pathogenicity. Genetic

diversity in ALV including mutations and recombination may confound its current field diagnosis by standard virological, serological and molecular methods. Such negative consequences may affect current efforts for prevention and control of this important virus infection.

The studies in this dissertation mainly focus on molecular and biological characterization of seven remaining isolates of a naturally occurring recombinant avian leukosis virus associated with myeloid leukosis in commercial white leghorn layers. The first chapter provides a review of the literature on family *Retroviridae*, diversity in retroviruses and avian leukosis-sarcoma viruses and diseases. The consequences of retroviral diversity including mutations and recombination are discussed. The second chapter describes the characterization of seven remaining isolates of a naturally occurring recombinant ALV by biological assays and polymerase chain reaction. Comparisons among the different isolates, a naturally occurring recombinant ALV and representative subgroups of ALV are demonstrated. Genetic diversity in different genomic regions of the various recombinant ALV isolates is evaluated using different PCR primer combinations. The third chapter illustrates the molecular characterization of these isolates by DNA sequencing. Sequence analyses of different genomic regions of these isolates were compared with the naturally occurring recombinant ALV-B/J, representative ALV subgroups and highly related ALV strains through NCBI blast search. In the fourth and final chapter, conclusions, significant findings, limitations, discrepancies, future studies and recommendations are discussed.

CHAPTER 1

LITERATURE REVIEW

I. RETROVIRIDAE

A. Introduction

Members of the family Retroviridae are animal enveloped viruses known as retroviruses, which contain two identical single stranded ribonucleic acid (RNA) molecules. In general, retroviruses share characteristics with other deoxyribonucleic acid (DNA) and RNA viruses as well as with cellular movable genetic elements including retrotransposons and retroposons (Temin, 1992). Some of these characteristics are similar genomic organization and fundamental replication strategy such as reverse transcription and transcription. In fact, retroviruses replicate efficiently in living cells through a DNA intermediate and are considered successful obligate parasites.

Retroviruses can be divided into simple or complex viruses with common basic genomic features. Both simple and complex retroviruses contain 5' repeat (R) region, unique 5' (U5) region, group specific antigen (*gag*), protease (*pro*), polymerase (*pol*), and envelope (*env*) genes and unique 3' (U3) region and 3' repeat (R) region. Complex retroviruses have larger genomes since they have additional regulatory genes; sometimes between *pol* and *env*, or *env* and 3' long terminal repeat (LTR) or both (Temin, 1992).

Retroviruses have significant agricultural impact on production of farm animals and devastating medical and economic consequences in humans. In fact, retroviruses have been linked to various disease conditions such as malignancies, neurological disorders and immunodeficiencies in many species (Coffin, 1992). On the other hand,

they have been used in biotechnology and molecular biology to study other viruses and the roles of various genes. In addition, retroviruses are being studied for their role and use in somatic gene and cancer therapies.

B. Morphology

The morphology of retroviruses was studied in transmission electron micrographs and has been useful in their previous classification. Every retrovirus virion consists of an envelope, a nucleocapsid (or core) and a nucleoid. There are small densely dispersed surface projections (8 nm long) or glycoprotein spikes, which cover the surface evenly. A schematic illustration of the known structure of the retrovirus virion, specifically avian leukosis virus (ALV) is shown in Figure 1.1 (Fadly and Payne, 2003). In fact, retrovirus particles range in size from 80 nm to 130 nm. Four distinct types of retrovirus particles exist and they are based on mode of assembly, location and shape of the nucleocapsid in mature virions and the appearance of the surface glycoproteins (Coffin, 1992).

Type A particles are strictly intracellular, either intracytoplasmic or intracisternal. They consist of ring-shaped nucleoid surrounded by a membrane. On the other hand, B-type virions assemble through budding of A partciles. Mature virions have eccentrically located doughnut shaped cores and prominent surface projections. C particles have no known cytoplasmic intermediates and form directly on the cell surface. They mature into centrally located condensed spherical cores with visible, but less prominent surface projections. The morphology of type D virions is intermediate between B and C virions with elongated or cylindrical dense cores. Virions of *Lentivirus* and *Spumavirus* have unique morphologies and are surrounded by envelopes. While spumaviruses contains

prominent spikes on the surface, lentiviruses are slightly pleomorphic and spherical with small or barely visible surface projections and evenly dispersed spikes (Gifford and Tristem, 2003). Nucleocapsids of lentiviruses are isometric and nucleoids are concentric and shaped like a rod, truncated cone or ice cream cone. On the other hand, spumaviruses have central but uncondensed cores (Goff, 2007).

C. *Components of Retroviruses*

1. Retroviral Virion

Although retroviruses exhibit differences in morphology and biology, they share similar set of virion components. Typically, a retrovirus virion is composed of 2% nucleic acids (RNA), 60% proteins, 35% lipids, and 3% carbohydrates (Donovan and Fuller, 2004). All retroviruses are surrounded by a lipid bilayer envelope consisting mainly of phospholipids. This envelope is formed through budding from the target cell plasma membrane. Moreover, retroviral genome is made up of two identical plus sense single-stranded RNA molecules (+ SS RNA) non-covalently joined near their 5' ends. Each RNA monomer is 7-10 kb in length and has a poly (A) tract at the 3' terminus and a methylated nucleotide cap at the 5' terminus. The retroviral particle contains nucleic acids (RNA and DNA) from the host cell such as transfer RNA (tRNA). This host tRNA is associated with the 5' end of viral RNA and serves as a primer for the synthesis of provrial DNA by the viral reverse transcriptase enzyme (RT).

2. Retroviral Genome and Major Viral Genes

The genome of all retroviruses generally consists of similarly arranged terminal noncoding regions and diverse internal coding regions. The internal coding regions encode structural and non-structural virion proteins as well as regulatory proteins in complex retroviruses (Coffin, 1992). The retroviral genome usually contains three open reading frames (ORF) encoding structural and nonstructural proteins, which are essential for the virus life cycle. The first ORF is in the group specific antigen (*gag*) gene, which codes for retrovirus structural proteins. The polymerase (*pol*) gene is in the second ORF and codes for reverse transcriptase and integrase enzymes. A third ORF is that of the envelope (*env*) gene, which codes for retroviral coat proteins, surface and transmembrane glycoproteins. Nevertheless, the protease (*pro*) gene coding for protease enzyme is found in a separate ORF or in the ORF of *gag* or *pol*.

In each retroviral RNA strand, the order of the structural genes is *gag-pol-env* from the 5' end to the 3' end. Key features of retroviral genome are illustrated in Figure 1.2, showing genomic organization of RNA and DNA forms of ALV (Fadly and Payne, 2003). In addition to the previously described genomic features, highly oncogenic retroviruses have oncogenes either in the *pol* or *env*. For example, Rous sarcoma virus (RSV) has a *v-src* gene at the end of ORF of *env*. Moreover, complex retroviruses usually have additional genes, which regulate retroviral genome expression and other accessory functions. Human immunodeficiency virus (HIV) genome contains *tat* gene between *pol* and *env* encoding transactivator protein.

On both sides of the retroviral genome, important cis-acting sequences play essential roles in the process of reverse transcription (Coffin, 1992). The primer binding sequence (PBS) is found downstream of U5 region in the 5' end of retroviral genome and

is complementary to the 3' end of the tRNA primer. An untranslated leader (L) region is downstream of PB and upstream of *gag* at the 5' end of the genomic RNA. It usually includes a specific packaging signal and the splice donor site for generation of subgenomic mRNA (messenger RNA), responsible for *env* expression. Polypurine tract (PPT) is an AG-rich sequence and is the primer for plus-strand DNA synthesis during the process of reverse transcription.

3. Proviral DNA and Long Terminal Repeats

The retroviral RT enzyme reverse transcribe genomic RNA into proviral DNA, which is then covalently integrated into the infected host cell DNA. The proviral DNA is usually longer than the genomic RNA due to the duplication in the repeated (R) and unique (U5 and U3) sequences during the process of reverse transcription. These duplicated terminal sequences are called long terminal repeats (LTRs), which are noncoding regions essential for viral replication in *cis*. They are usually composed of three regions in the following order U3-R-U5 (Coffin, 1992). U3 region contains enhancer, promoter and regulatory sequences for transcription initiation. On the other hand, R region contains signals for cleavage and poly (A) addition required for transcripts processing. In addition, R region determine the 5' and 3' end of retroviral genomes and all mRNA and ensure correct end-to-end transfer of the growing chain during reverse transcription. Figure 1.2 shows the differences in the terminal sequences in genomic RNA and proviral DNA (Fadly and Payne, 2003).

4. Major Viral Encoded Proteins

GAG and ENV are retroviral structural proteins encoded by *gag* and *env* genes respectively. On the other hand, POL is non-structural protein encoded by *pol* gene. The virion core of all retroviruses is generally formed of three common GAG proteins; matrix, capsid and nucleocapsid (Coffin, 1992). The matrix (MA) protein joins the retroviral core with the envelope and is involved in the virus assembly and budding. The capsid (CA) protein constitutes the major structural element of the core shell or capsid. In many retroviruses, CA antigen is mostly used in retroviral immunoassays for detection of the virus. The nucleocapsid (NC) is a basic protein made of cysteine and histidine aminoacids, resembling "zinc finger" and closely associated with genomic RNA. It is often needed for correct packaging and it promotes RNA-RNA duplex formation for genome dimerization and genome-primer association. In some retroviruses, additional small core proteins may be present between MA and CA, CA and NC, and downstream of NC (Donovan and Fuller, 2004). In most cases, these are encoded by *gag* and their function is poorly understood.

Two ENV proteins are noncovalently linked glycoproteins and needed for specific host cell receptor recognition and entry. Surface (SU) protein is a knob-like glycoprotein, which is exposed on the surface to the external environment. It directly binds cellular receptors during infection and is recognized by neutralizing antibodies. The host ranges are encoded within SU and are useful in the classification of closely associated retrovirus species into subgroups (Fadly and Payne, 2003). The other glycoprotein, transmembrane (TM), is a spike-like structure anchoring SU to retroviral envelope. It also has an amino-terminal hydrophobic region, which mediates fusion of retroviral envelope with the host cell membrane (Coffin, 1992).

Retroviral enzymes such as protease, integrase and reverse transcriptase are essential for replication, but are less abundant in the virion than structural proteins. The protease enzyme (PR) is encoded by *pro* while the reverse transcriptase (RT) and the integrase (IN) are encoded by *pol*. PR is usually inactive until activated through dimerization after virion budding. It is responsible for all the proteolytic cleavages and generation of mature proteins during virion maturation (Coffin, 1992). Highly conserved among retroviruses, RT uses genomic single stranded RNA as a template to synthesize the retroviral double stranded DNA. It has active sites, which can function as RNA- and DNA-directed DNA polymerases, as well as RNA endonuclease or ribonuclease H (RNase H). The RNase H function allows the degradation of retroviral RNA template and primers during proviral DNA synthesis. Similarly, IN has multiple functions, cleavage and ligation of newly synthesized linear proviral DNA. It recognizes the ends of the two linear proviral DNA, removes two nucleotides from the 3′ end of each strand, and cleaves the DNA target. Afterwards, it joins the trimmed proviral DNA end to target host cell DNA at random sites.

D. *Classification of Retroviruses*

Similar to all viruses, taxonomical classification of retroviruses is developed and reviewed by a subcommittee of the International Committee on Taxonomy of Viruses (ICTV). It was reported that retroviral classification is largely based on genome structure, nucleic acid sequence and sequence similarity within the *pol* gene (Coffin *et al.*, 1997). Since retroviruses comprise a diverse group of viruses, they can be described by several criteria other than their evolutionary relationships. These include the type of diseases they

induce, tropism for certain host species or target cell types, virion morphology, simple versus complex lifestyles, mechanism of transmission, serology and biochemical features (Donovan and Fuller, 2004).

Previously, retroviruses were classified under the family *Retroviridae*, which was subdivided into three subfamilies; *Oncovirinae*, *Lentivirinae* and *Spumavirinae*. However, this taxonomical classification of retroviruses is no longer appropriate. Following recent ICTV reclassification, *Retroviridae* is currently subdivided into two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae* in addition to unclassified retroviridae (Linial *et al.*, 2006). Each subfamily is further subdivided into different genera and species, which contain mostly exogenous retroviruses. Within *Orthoretrovirinae*, there are six genera namely *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus* and *Lentivirus*. On the other hand, there is only one genus, *Spumavirus*, in the subfamily *Spumaretrovirinae* (Fauquet *et al.*, 2005). The unclassified retroviridae contains endogenous and other unclassified exogenous retroviruses from different species including humans, primates, amphibians, fish, avian and others. Moreover, alpharetroviruses, betaretroviruses, gammaretroviruses and epsilonretroviruses have simple lifestyles while lentiviruses, deltaretroviruses and spumaviruses have complex lifestyles (Weiss, 2006).

Retroviruses can be exogenous or endogenous and they can infect somatic and germline cells. When retroviruses infect germline cells, they are transmitted to the next generation and are termed endogenous retroviruses. Endogenous retroviruses can persist in the host genome for a long time. However, they are only infectious for a short time after integration since they acquire knockout mutations during host DNA replication.

Moreover, endogenous retroviruses can be partially excised from the genome by a process known as recombinational deletion. Gifford and Tristem (2003) reported that endogenous retroviruses are involved in viral ecology, evolution and host disease.

Classification of endogenous retroviruses has been developed separately from that of exogenous retroviruses. In general, endogenous forms of exogenous retroviruses are grouped within the seven recognized genera. Based on their relatedness to exogenous genera, endogenous retroviruses are classified into three classes (Jern *et al.*, 2005). Classes I, II and III contain endogenous retroviruses most similar to *Gammaretrovirus*, *Betaretrovirus* and *Spumavirus* respectively (Figure 1.3).

E. Seven Genera of Retroviruses

1. Alpharetrovirus

Exogenous and endogenous retroviruses of *Alpharetrovirus* have only been identified in wild and domestic birds. Members of this genus have C-type retroviral morphology and simple genome organization. Within *Alpharetrovirus*, viruses are classified into species according to differences in the genome, gene product sequences, natural host range and nature of incorporated oncogenes. Examples of species belonging to this genus are avian leukosis virus (ALV), avian myeloblastosis virus (AMV), avian myelocytomatosis virus (AMC 29) and Rous sarcoma virus (RSV). Members of the avian leukosis/sarcoma group of retroviruses (ALSV) are alpharetroviruses. ALV is a simple slow transforming retrovirus and is considered the type species of this genus.

2. Betaretrovirus

Viruses in *Betaretrovirus* have only been found in mammals and they mostly have simple genomic structures. These retroviruses have type-B or type-C morphology and do not possess viral oncogenes. Two former retroviral genera form this genus and these include the mammalian B-type murine exogenous and endogenous retroviruses and D-type retroviruses from primates and sheep (Gifford and Tristem, 2003). The mouse mammary tumour virus (MMTV), Jaagsiekte sheep retrovirus (JSRV), Langur virus (LNGV), Mason-Pfizer monkey virus (MPMV), and Squirrel monkey retrovirus (SMRV) are species in this genus. The prototype of betaretroviruses is MMTV representing endogenous and exogenous retroviruses of mice. These murine retroviruses have an accessory gene encoding a superantigen (sag) and widely divergent and distinct *env* genes. Moreover, they have the ability to induce mammary carcinomas by activating cellular oncogenes (Linial *et al.*, 2006). Murine endogenous retroviruses called intracisternal A-type particle (IAP) are deleted or truncated in the mouse genomes and sometimes lack *env* genes (Goff, 2007).

3. Gammaretrovirus

Gammaretrovirus is considered a unique genus because it includes the highest number of known members. Initially, gammaretroviruses have been associated with sarcomas and leukemias in mice. However, retroviruses within this genus belong to many vertebrate classes including mammals, avian, reptiles and amphabians. Similar to alpharetroviruses, gammaretroviruses are simple endogenous and exogenous retroviruses, replication competent or defective viruses with C-type morphology (Goff, 2007). In

mammals, replication competent gammaretroviruses lack oncogenes and replication defective viruses have acquired a variety of cell-derived oncogenes from their hosts.

Gammaretroviruses are subdivided into mammalian, reptilian and avian virus groups (Linial *et al.*, 2006). Differentiation into species is based on genome sequence, presence of oncogene, antigenic properties, natural host range and pathogenicity. Examples include murine leukemia virus (MLV), avian reticuloendotheliosis virus (REV) and feline leukemia virus (FeLV). MLV is the type species of *Gammaretrovirus* and is used in gene therapy and cancer mutagenesis studies. Endogenous forms of these viruses are widespread in the genomes of mammals, birds, reptiles and amphabians (Martin *et al.*, 1999). Yet, ICTV recognizes only a small number of endogenous retroviruses closely related to their exogenous counterparts (Fauquet *et al.*, 2005).

4. Deltaretrovirus

Members of *Deltaretrovirus* are oncogenes-free exogenous retroviruses with complex genomic structure and C-type morphology (Goff, 2007). No endogenous counterparts of Deltaretroviruses exist, but exogenous retroviruses are horizontally transmitted and rarely found in few mammals such as primates and cattle. Deltaretroviruses are classified into species based on genome sequence, absence of viral oncogene, antigenic properties, natural host range and pathogenicity (Linial *et al.*, 2006). Bovine leukemia virus (BLV) is the prototype of *Deltaretrovirus* and other species include Human T-lymphotropic virus (HTLV) and Simian T-lymphotropic virus (STLV). In the host, these retroviruses are indefinitely persistent often causing slow or chronic infections, characterized by long incubation period.

5. Epsilonretrovirus

Epsilonretrovirus contains complex exogenous viruses characterized by C-type morphology in fish and reptiles. Duplicated viral homologues of the host cyclin D gene, which may regulate the cell cycle, are the only known accessory gene products found in these retroviruses (Fauquet *et al.*, 2005). Classification into species is based on genome and gene product sequences, viral oncogene and natural host range (Linial *et al.*, 2006). Walleye dermal sarcoma virus (WDSV) is a fish retrovirus and the prototype of the genus. Other retroviruses in this genus include Walleye epidermal hyperplasia viruses (WEHV) type 1and 2, Snakehead retrovirus (SnRV) and Perch hyperplasia virus (PHV).

6. Lentivirus

Lentivirus mainly includes exogenous complex mammalian viruses characterized by unique virion morphology. Endogenous retroviruses show low level of homology to their exogenous counterparts. Lentiviruses are often associated with anemia, cancer, immune dysfunction and central nervous system disorders (Temin, 1992). Infection with these retroviruses cause slow or chronic pattern of disease and long incubation period and involve diverse organ systems (Gifford and Tristem, 2003). On the other hand, lentiviruses can be useful in gene therapy research experiments through introducing a gene product into in vitro systems or animal models.

Lentivirus is subdivided into primate, ovine/caprine, equine, feline and bovine lentivirus groups reflecting the vertebrate hosts they infect. Species of lentiviruses are classified according to genome and gene product sequences, antigenic properties, natural

host range and pathogenicity (Linial *et al.*, 2006). Examples of lentivirus species are bovine immunodeficiency virus (BIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), caprine arthritis encephalitis virus (CAEV) and simian immunodeficiency virus (SIV).

The type species of the genus is human immunodeficiency virus 1 (HIV-1), a complex retrovirus containing additional regulatory genes (*vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*). These accessory genes control transcription, RNA processing, virion assembly, gene expression and other replication functions (Goff, 2007). Within the primate lentivirus group, HIV-1 shows sequence divergence of more than 50% when compared to HIV-2, which contain a *vpx* gene (Linial *et al.*, 2006). However, these viruses result in progressive decrease in CD4 T cell count, increase viral load and various life threatening opportunistic infections, characteristics of acquired immunodeficiency syndrome (AIDS).

7. Spumavirus

The genus *Spumavirus* contains complex exogenous retroviruses with unique virion morpholog. These retroviruses are widespread in mammals, but they are not associated with any disease condition. While spumaviruses are non-oncogenic and non-pathogenic, they establish persistent infections in many animal species. In addition, they contain at least two accessory genes known as *tas/bel-1*, which encodes a transcriptional transactivator and *bet* downstream of *env*. Although there are no endogenous equivalents, vertebrate genomes commonly contain distinctly related viruses.

Species are differentiated according to their genomes, gene product sequences and natural host ranges (Linial *et al.*, 2006). Examples within *Spumavirus* include simian

foamy virus (SFV), bovine foamy virus (BFV), equine foamy virus (EFV), and feline foamy virus (FFV). The prototype example of spumaviruses is SFV, which is closely related to HIV. SFV was named after the foamy bubbles appearing under the microscope when infected cells fuse and form syncythia or giant cells.

II. DIVERSITY IN RETROVIRUSES

A. Introduction

Various species of viruses including RNA viruses and retroviruses mutate at a very high rate (Svarovskaia *et al.*, 2003). These mutations are manifested as variations in nucleotide sequences, which enable viral populations to evade host immune responses and develop resistance to antiretroviral drugs. Different steps of replication cycle contribute to genetic variability in retroviruses such as reverse transcription, DNA integration, RNA transcription and virion assembly. However, the major factor in retroviral variation is the low proofreading activity of reverse transcriptase (RT) enzyme resulting in polymerization errors during the process of reverse transcription.

In retroviruses, genetic diversity is usually manifest by acquisition of cellular genes and alteration of host range and considerable variations in incidence, severity and symptoms of diseases. Therefore, studying retroviral genetic diversity is crucial to understand the association between retrovirus variation and disease progression. Mutations usually occur during the early and late stages of replication and result in subtle genetic variability. On the other hand, recombination causes major genetic changes and usually takes place during the early stage of replication (Katz and Skalka, 1990). Moreover, the relatively high rates of mutation and recombination of retroviruses

influence virus diversity, evolution, virulence, pathogenesis and the development of effective antiviral drugs and vaccines (Mansky, 1998).

Retroviral diversity may result in the emergence of new strains (mutants or recombinants) and subgroups of viruses. Genetic diversity arises when retroviruses are passaged in tissue culture or animals (Katz and Skalka 1990). For example, Rous sarcoma virus (RSV) was the first retrovirus to exhibit genetic variations and result in different varieties of tumors in chickens such as rapidly growing and late-developing tumors (Duran-Reynals, 1942; Rous and Murphy, 1913). On the other hand, it was reported that naturally occurring strains of viruses are more likely to cause severe illness than others (Roizman, 1996).

Evolution of retroviruses is influenced by selection of particular variants with specific types of mutations in the surface and transmembrane envelope proteins (Fleischmann Jr., 1996). Selection for these mutations is based on the interaction between virus load, host immune responses and disease progression (Mansky, 1998). Thus, to understand the effect of retroviral diversity on immune escape, drug resistance and disease progression, we must investigate the genetic nature of variants produced by different selective forces.

B. General Principles

Retroviruses are known to exhibit high rates of mutation resulting in their rapid evolution (Coffin *et al.*, 1980; Gojobori and Yokoyama, 1985, 1987; Hahn *et al.*, 1986; Monk *et al*, 1992; O'Rear and Temin, 1982). These genetic variations lead to changes in the function of virion envelope proteins and the production of new retroviral serotypes

with altered virulence (Fleischmann Jr., 1996). In addition, mutations can lead to epitope deletion, failure of antigen processing, loss of major histocompatibility complex (MHC) class I binding, impaired recognition by the T cell receptor resulting in viral persistence and avoidance of the immune system.

In retroviruses, replication generates large numbers of genetically diverse populations known as swarms or quasispecies (Payne, 2001). Thus, an individual virus is made up of heterogeneous population of similar genomic variants, where the wild-type sequence can only be defined by consensus (Katz and Skalka, 1990). Selective forces generate mutations, but the genome of mutant population can not be defined since retroviral population exists in a dynamic equilibrium. A mutation frequency of 10^{-3} to 10^{-4} misincorporations per genome per replication cycle results in a significant number of mutant genomes even in biologically cloned retroviral stocks (Katz and Skalka, 1990).

During the evolution of retroviruses, cellular movable genetic elements (transposons) evolved into endogenous retroviruses permenantly present in the host genome. On the other hand, various mutations over time have created degenerate proviral forms of exogenous retroviruses, which are unable to produce infectious virions (Best *et al.*, 1997; Lower, 1999). In Eukaryotes, endogenous retroelements are present in multiple copies and constitute more than 10% of the host genome. In addition, several classes of endogenous retrovirus-related elements exist in the genome of chickens (Fadly and Payne, 2003). It is important to note that endogenous retroviruses can cause mutational changes in exogenous retroviruses through recombination.

Different species of retroviruses are compared through analyses of their genetic structure and nucleotide sequences. Evolutionary trees are constructed based on

nucleotide or inferred amino acid sequences of different retroviral genes (Page and Holmes, 1998). Sequence data are analyzed and trees are developed by computer programs using various mathematical models of sequence variations. Evolutionary trees include cladogram, additive trees and ultrametric or dendrugram (Payne, 2001). Cladograms are the most basic trees depicting relative recency of common ancestry. Additive trees describe amount of evolutionary change represented by length of the branches. Ultrametric trees (or dendrograms) are special kinds of additive trees where the tips of the branches are at an equal distance from the root. They express evolutionary time indirectly by the degree of sequence divergence. Phylogenic trees show evolutionary and epidemiological relationships among retroviruses and may reflect mutations and recombination in retroviral genes over time.

C. Mutations

Mutations in retroviruses are very common and result in permenant changes in the genetic material. In RSV, the frequency of mutation in viral genomes is estimated to be 10^{-4} - 10^{-5} mutation per incorporating nucleotide per viral replication cycle (Preston and Dougherty, 1996). Most mutations are deleterious and are removed; others are mainly advantageous and are selected and some of the neutral ones become fixed by genetic drift (Page and Holmes, 1998). Mutations that do not interfere with normal functions are inherted and persist in a virus population. Viral mutations have been used by virologists in developing attenuated live viral vaccines despite the possibility of back-mutations or re-versions (Fleischmann Jr., 1996). On the other hand, conserved sequences or motifs can be useful for developing new vaccines or antiretroviral drugs (Jacobs *et al.*, 2008).

Nucleotide substitutions and rearrangement such as simple deletions, deletions with insertions and duplications of nucleotides in retroviral genomes represent point mutations (Payne, 2001). The main source of point mutations is the error-prone replication due to the lack of proofreading activity (activity of 3'-5' exonuclease) of RT. Nucleotide substitutions (A to G) are the most common point mutations, 80% of which are transitions whereas the remaining 20% are transversions (Figlerowicz *et al.*, 2003). Transition is the exchange of pyrimidine into pyrimidine or purine into purine. On the other hand, transversion is the exchange of the pyrimidine into purine and vice versa.

Nucleoide subsitutions are more common during transcription of an infectious avian retrovirus than replication of defective endogenous retrovirus (Payne, 2001). BLV and HTLV replicate in the proviral forms through clonal expansion of infected cells and have fewer mutations than otherretroviruses, which replicate by reverse transcription such as HIV (Wain-Hobson, 1996). Moreover, substitution mutations occur in the variable regions of gp85 SU sequence of the *env* gene of exogenous ALVs (Bai *et al.*, 1995a; Bova *et al.*, 1986; Venugopal *et al.*, 1998). These mutations may influence antigenic determinants, pathogenicity, host range or target cell specificity. The *env* gene of ALVs shows the highest diversity compared to *gag* and *pol* genes; while LTR sequences are relatively variable (Payne, 2001).

Various molecular approaches have been used for investigating retrovirus mutations and variations (Mansky, 1998). Molecular cloning and analysis of restriction enzyme digestion patterns of proviruses of spleen necrosis virus (SNV) allowed the study of their biological and molecular properties (O'Rear *et al.*, 1980). The polymerase chain reaction (PCR) was used to isolate HIV and SIV variants and analyse variations in the SU

region of their *env* and resulting amino acid changes by DNA sequencing (Burns and Desrosiers, 1991; Johnson *et al.*, 1991; Overbaugh *et al.*, 1991). In addition, DNA sequence analysis was used to study the association among different Moloney murine leukaemia virus (M-MLV) strains (Weiss *et al.*, 1985).

D. Recombination

Many investigators reported that the high level of retroviral recombination is attributed to the diploid nature of retroviruses (Coffin *et al.*, 1997; Hu and Temin, 1990a and b; Nichol, 1996; Zhang and Ma, 2003). Recombination usually takes place when two related parental RNA genomes exchange genetic materials during reverse transcription. On the other hand, it can result from physical interaction between retroviral and host genomes. If the host genes code for growth factors and growth factor receptors, the recombinant retroviruses may be oncogenic, carrying cellular oncogenes.

Recombination contributes to retroviral diversity and its frequency exceeded 10% of the progeny (Alevy and Vogt, 1978; Blair, 1977; Kawai and Hanafusa, 1972; Linial and Brown, 1979; Vogt, 1971; Wyke and Beamand, 1979). Recombination is mainly responsible for emergence of new viral strains and species with new viral phenotypes and altered host range and virulence. On the other hand, it allows the introduction of foreign genes coding for a specific immunogen, normal human or animal genes into retroviral genomes. These recombinant viruses can be used experimentally as vaccines or can be useful for gene therapy of a wide range of diseases (Fleischmann Jr., 1996).

Recombination was first observed after cell co-infection with two genetically distinct avian tumor viruses (Vogt, 1971). It was observed that murine endogenous

retroviruses recombine and form pathogenic strains (Stoye *et al.*, 1991). Recombination between exogenous and endogenous avian retroviruses generates viruses with new host ranges, encoded in the *env* SU (Rein, 1982; Weiss *et al.*, 1973) and altered cell-specific transcription in the LTR (Boral *et al*, 1989; Celander and Haseltine, 1984; Golemis *et al.*, 1990). This results in a more efficient replication of these pathogenic viruses and activation of cellular oncogenes causing host cell transformation and tumor formation.

The novel ALV subgroup J may have resulted from either a single or multiple recombination events between the endogenous and exogenous ALVs (Venugopal, 1999). Three retroviruses ADOL 5701A, ADOL 5701AΔ, and ADOL 6803A are recombinants between exogenous subgroup J ALV and a defective endogenous ALV expressing subgroup A envelope (Lupiani *et al.*, 2000). In addition, a naturally occurring ALV isolate was molecularly characterized as a recombinant with a subgroup B gp85 region and a subgroup E gp37 region and subgroup J LTR (Lupiani *et al.*, 2006).

Genetic recombination in retroviruses can be either homologous or non-homologous. Homologous recombination involves two similar RNA molecules possessing long region of homology (Payne, 2001; Figlerowicz *et al.*, 2003). It can occur by intramolecular (more efficient) or intermolecular template switching (Hu *et al.*, 1997). Forced-copy choice and strand displacement-assimilation are two models propoed to define homologous recombination in retroviruses (Coffin, 1979; Hunter, 1978; Katz and Skalka, 1990; Skalka *et al.*, 1982).

The forced copy-choice model (Figure 1.4) proposes that recombination occurs when RT jumps and switchs retroviral RNA templates during minus strand DNA synthesis (Coffin, 1979). On the other hand, the strand displacement-assimilation (minus-

strand exchange) model proposes that recombination occurs when plus strand DNA synthesis takes place on two templates (Junghans *et al.*, 1982). Each model predicts the nature of the recombinant product, a homoduplex for the forced copy-choice and a heteroduplex for the strand-assimilation model.

In 1979, Wyke and Beamand reported that homologous recombination frequently occur between exogenous ALSVs under experimental conditions. In the field, homologous recombination between exogenous and endogenous avian retroviruses is possible. For example, ALV-J is shown to have *gag* and *pol* genes closely related to those of exogenous ALSV and an *env* gene nearly identical to that of EAV-HP, a member of the EAV family of endogenous retroviruses (Bai *et al.*, 1995b; Smith et *al.*, 1999). Moreover, recombination between two types of endogenous retroviruses is also possible as suggested by the high identity of the R and U5 regions of the LTRs of EAV-HP and ART-CH (Sacco *et al.*, 2000).

Non-homologous recombination takes place between unrelated sequences in the same or distinct retroviral genomes (Cheslock, 2000; Figlerowicz *et al.*, 2003; Zhang and Temin, 1994). In addition, non-homologous recombination occurs between retroviral sequences and non-homologous or unrelated host sequences. This results in transduction of cellular genes and the formation of highly oncogenic retroviruses. For example, acute avian leukemia and sarcoma retroviruses such as RSV, FuSV, UR2, Y73, AEV-ES4, AMV and MC29 have acquired cellular oncogenes (*src*, *fps*, *ros*, *yes*, *erbA* and *erbB*, *myb* and *myc*) and enhanced oncogenicity (Payne, 2001). The mechanisms of non-homologous and homologous recombinations are similar (Mansky, 1998). However, non-homologous

recombination occurs at about 0.1-1% the rate of homologous recombination (Zhang and Temin, 1993).

E. Rearrangements

Deletions and duplications are two types of rearragement in retroviral genomes. In retroviruses, small deletions in the order of several nucleotides occur due to template slippage or mispriming at direct repeats. Large deletions up to thousands of nucleotides result from homologous recombination between direct repeats (Bizub *et al.*, 1984). In fact, homologous recombination is the most frequent source of genetic rearrangements in retroviruses (Negroni and Buc, 2001). Yet, non-homologous recombination in the same retroviral genome allows more extensive genomic rearrangements such as deletions, duplications and inversions (Negorni and Buc, 2001; Pathak and Temin, 1990a and b).

Large deletions affecting structural and enzyme genes generate replication-defective mutants (Katz and Skalka, 1990). Moreover, Voynow and Coffin (1985) observed deletions in RSV by a pathway independent of homologous recombination. Deletions are also possible due to the spurious endonucleolytic action of ASLV IN near the internal border (U5) of the upstream LTR (Olsen and Swanstrom, 1985). Duplications of retroviral sequences usually result from polymerization errors during reverse transcription and from non-homologous recombination (Coffin, 1986).

F. Role of Selective Forces

Various models of molecular evolution have been proposed to explain the behavior of mutating retroviral populations. In the founder effect model, few (founder)

individuals that carry some of the genetic variations of the parent population are responsible for the establishment of a new population (Payne, 2001). Muller's ratchet model states that accummalating deleterious mutations lead to the loss of fitness and survival of retroviral population (Maynard Smith, 1998). Red Queen hypothesis proposes that as species evolve in a population, change will always occur even in a constant physical environment (Maynard Smith, 1998). In the competitive exclusion model, one competing species will always overcome and eliminate other species in a population (Payne, 2001).

Selection of viral variations often takes place in four stages generating infectious, replicating, non-neutralizable and drug-resistant viral variants (Figlerowicz *et al.*, 2003). During the first stage, viral particles must be infectious to enter a cell. These viruses replicate and generate a population of diversified viral particles during the second stage of selection. The third stage of selection is that of the host immune responses, where immune escape mutants are generated. In the fourth stage of selection, the selective forces of antiviral therapy generate replicating and spreadable drug-resistant mutants.

Replication is a potential selective force generating retroviral diversity. Selection for more efficient replication increases retroviral pathogenicity through the action of viral gene products or activation of cellular oncogenes (Stoye and Coffin, 1985; Teich *et al.*, 1982 and 1985). Tissue-specific replication in retroviruses has been linked to LTR, 5' leader, *gag* and *env* and possibly other genomic regions (Robinson *et al.*, 1985). In murine pathogenic viruses, sequence changes in the LTR increase the activity of the transcriptional promoter and enhancer in the U3 region (Boral *et al.*, 1989; Celander and

Haseltine, 1984; Golemis *et al.*, 1990). Replication in new hosts influence evolutionary and recombination rates as well as selection profiles (Poss *et al.*, 2008).

Selective forces target retroviral envelopes and generate genotypic and phenotypic changes. For example, selection for tissue-specific replication results in mutations in the receptor binding region (SU protein of *env*), expanded host range and increased pathogenicity (Dorner and Coffin, 1986; Dorner *et al.*, 1985). Furthermore, the ability of retroviruses to replicate in different host species is a selective pressure to cause changes in the *env* sequences and the recognition of new receptors (Rein, 1982; Stoye and Coffin, 1985). Selective proliferation of transformed host cells causes oncogenes mutations and promotes the capture and activation of cellular oncogenes.

It is well documented that retroviral *env* gene products are targets for selective forces of neutralizing or humoral immune response (Ho *et al.*, 1988; Katz and Skalka, 1990; Skinner *et al.*, 1988; Weiss *et al.*, 1986). Moreover, retroviral diversity reflects selection of *env* variants that can evade the neutralizing immune system. On the other hand, the neutralizing immune response is not a selective force in congenitally ASLV-infected chicken due to immunological tolerance (Teich *et al.*, 1985). RT-inhibitor drugs are potential selective forces resulting in variations in *pol* gene and eventually generating drug- resistant retroviruses (Larder and Kemp, 1989).

Natural selection of variant viruses can be detected by comparing the numbers of non-synonymous (NS) and synonymous (S) nucleotide substitutions within a gene (Payne, 2001). NS substitutions alter the antigenicity or other properties of the viral proteins since they involve changes in the first or second bases in a codon, which will modify the amino acid product. On the other hand, S substitutions do not change the

amino acid produced since they entail a change to the third base of the codon. Positive natural selection is said to be operating on viral variants when the number of NS codons produced as a result of mutation exceeds the number of S codons (or the NS/S ratio is >1). Analysis of *env* sequences has allowed the detection of the survival and selection of ALV and HIV immune escape mutants (Mansky, 1998; Venugopal *et al.*, 1998).

G. Evolution and Retroviruses

For decades, scientists have investigated the origin of retroviruses and the relationship between cellular moveable genetic elements, endogenous and exogenous retroviruses. Retroviruses are believed to have evolved from ancient transposable genetic elements based on RT (*pol*) sequences from various retroviruses and retrovirus-like elements (retrotransposons) of different species (Doolittle *et al.*, 1989 and 1990; Temin, 1985). These transposable elements insert DNA sequences into non-homologous genomic DNA with the help of the enzyme reverse transcriptase in the process of DNA transposition. Furthermore, they are important mutagens and source of genetic variation.

Retroviruses have evolved over time as either exogenous infectious agents or endogenous proviruses (Katz and SKalka, 1990). The retroviral genome is influenced by opposing selective forces for passive replication as endogenous proviruses versus efficient replication as exogenous retroviruses. These selective forces generate variations in clustered areas of functional or non-essential regions of retroviral genomes. Germline infection provides a selective advantage over the course of evolution since the host survival ensures the survival of the endogenous provirus. On the other hand, selection for high proliferative rate of exogenous retroviruses is not advantageous if it kills one host

before the virus is transmitted to another. Segments of these endogenous entities are perpetuated through recombination with exogenous retroviruses.

Homologous recombination is a highly frequent event shaping retroviral evolution (Casci, 2000; Schierup and Hein, 2000a and b). Around 10% of infectious strains of human immunodeficiency virus (HIV) are the result of recombination among different viral subtypes (McCutchan, 2000; Peeters and Sharp, 2000). Moreover, pathogenic retroviral strains in mice are generated from recombination among endogenous retroviruses (Stoye *et al.*, 1991). On the other hand, non-homologous recombination is effective at several turning points of retroviral evolution such as the capture of oncogenes by transduction of cellular sequences and the generation of gene duplications (Goldfarb and Weinberg, 1981; Swain and Coffin, 1992; Swanstrom *et al.*, 1983).

Mutations in retroviruses contribute to the emergence of new virus strains and new disease expressions. Retroviral replication generates new variants through point mutations, rearrangements and recombination. Studies on mutations of many different retroviruses have contributed to understanding how avian retroviruses mutate, how mutants may be selected to become dominant forms, and the disease consequences in the host (Payne, 2001).

III. AVIAN LEUKOSIS-SARCOMA VIRUSES AND DISEASES

A. Introduction

The viruses in the avian leukosis-sarcoma (L/S) group are alpharetroviruses associated with various neoplastic diseases and production problems in birds mainly chickens. Avian leukosis virus (ALV) is a member of this group and most commonly

causes lymphoid leukosis (LL) and myeloid leukosis (ML) in field flocks. Infection and diseases induced by ALV result in millions of U.S. dollars of economical losses each year due to tumor mortality and reduced productivity (Fadly and Payne, 2003).

B. Etiology

1. Classification

According to the new classification of the International Committee on Taxonomy of Viruses (ICTV), the avian leukosis-sarcoma (L/S) group of viruses (ALSV) belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae* and genus *Alpharetroviruses* (Fauquet *et al.*, 2005). Members of this group share common physical, chemical and molecular characteristics and group-specific antigen (Fadly and Payne, 2003). Under the new taxonomy, the type species of the genus is avian leukosis virus (ALV). Other species include Rous sarcoma virus (RSV) and other replication defective viruses such as avian erythroblastosis virus (AEV) and avian myeloblastosis virus (AMV), carrying oncogenes.

ALSVs are classified into several categories depending on their virological, immunological and pathological characteristics including their modes of transmission. Within a species like ALV, viruses are grouped based on envelope glycoprotein into subgroups, which include viral types depending on antigenic variations and strains depending on oncogenic spectrum in various host tissues. Most viral strains are subgroup-pathotype combinations containing mixtures of viruses (Payne, 1987) and inducing a variety of tumors, which often overlap with other strains.

Based on the rate of tumor induction in susceptible chickens, strains of ALSV can also be placed into 2 major classes, acute and slow transforming viruses (Fadly and

Payne, 2003). Acute transforming viruses carry viral oncogenes and cause acute leukemia, leukosis and solid tumors usually sarcomas within few days or weeks (Enrietto and Wyke, 1983; Graf and Beug, 1978; Moscovici and Gazzolo, 1987). The type of neoplasm produced by a certain virus strain depends on the target cell transformed by the viral oncogene (*v-onc*). These viruses are genetically or/and replication defective, requiring a helper leukosis virus for replication. Examples include Rous sarcoma virus (RSV), MC29, 966 and MH2 (Fadly and Payne, 2003).

Conversely, slow transforming viruses lack oncogenes and induce transformation by promoter insertion or another mechanism near a host proto-oncogene (Weiss *et al.*, 1985). They are replication competent and usually induce various leukoses, leukemias and sarcomas weeks or months after infection (Coffin *et al.*, 1997; Cooper, 1982). Examples include Rous-associated virus 1 (RAV-1, subgroup A ALV), RAV-2 (subgroup B ALV), RAV-49 (subgroup C ALV), RAV-50 (subgroup D ALV), and ADOL-Hc1 (subgroup J ALV).

The mode of transmission and the nature of replication of ALSVs are used in their classification into exogenous and endogenous viruses. Exogenous ALVs constitute free infectious viral particles, which spread through vertical and horizontal routes (Payne, 1987). On the other hand, endogenous ALVs comprise incomplete to complete viral particles making up part of their host genomes or free infectious entities respectively (Rovigatti and Astrin, 1983). Therefore, they can be transmitted genetically, horizontally and vertically (Robinson and Eisenman, 1984; Rubin *et al.*, 1961; Smith *et al.*, 1986).

2. Subgroups

There are six ASLV envelope subgroups (A, B, C, D, E and J) in chickens based on the diversity in the viral envelope glycoproteins. Subgroups A, B, C, D and J are all exogenous, while subgroup E is endogenous. Moreover, Subgroups F, G, H and I are endogenous ALVs present in the genomes of pheasants, partridge and quail (Payne, 1992). The viral subgroup is determined by assays of antigenicity, viral interference patterns and host range (Weiss *et al.*, 1985).

Antigenicity is associated with viral envelope antigens neutralized by known subgroup-specific antibodies detected by viral and serum neutralization tests respectively. In general, viruses belonging to the same subgroup cross-neutralize each other to varying degrees, but do not cross-neutralize viruses from other subgroups. Moreover, viruses tend to neutralize homologous viruses more strongly than heterologous viruses within the same subgroup (Chubb and Biggs, 1968). Subgroups B and J viruses appear to be more heterogeneous than other ALV subgroups. On the other hand, some subgroup J viruses do not always cross-neutralize or cross-neutralize in one direction (Fadly and Smith, 1999; Fadly *et al.*, 2000; Venugopal *et al.*, 1998). Moreover, there is a partial cross-neutralization between subgroups B and D viruses (Payne, 1992).

Viral interference pattern is established in susceptible avian embryo fibroblast cultures by infection with unknown virus and challenge with different RSV subgroups. Comparison of RSV foci reduction between infected and uninfected controls indicates the presence of viral interference.Host range is defined by viral SU protein interaction with a specific host cell receptor. Host range is determined by virus growth in embryo fibroblasts from various avian species or transformed cell lines (Payne *et al.*, 1992b). The host range of ALV is investigated in chicken embryo fibroblasts (CEFs) belonging to

distinct genetic lines and different phenotypes. Viral interference pattern and host range are more reliable for subgroup classification than antigenicity assays, which are used for strain classification (Fadly and Payne, 2003).

Furthermore, several strains of ALSVs are genetically defective, lacking an envelope gene and thereby have no subgroup designation. Nevertheless, they require the complement function of the helper leukosis virus present in the viral mixture for replication. Thus, they take on the designation of the helper leukosis virus envelope subgroup such as BH-RSV (RAV-1), a Bryan's high titer strain of RSV grown with Rous-associated virus 1 (subgroup A).

3. Endogenous Leukosis Viruses

The normal chicken genome contains various classes of endogenous retroviruses inherited in a Mendelian fashion (Crittenden, 1991; Fadly and Payne, 2003). These families include the endogenous viral (*ev*) loci, the moderately repetitive elements EAV (endogenous avian virus) and ART-CH (avian retrotransposon from chicken genome) and the highly repetitive elements CR1 (chicken repeat 1). The prototype virus of subgroup E, Rous-associated virus (RAV-0), was spontaneously produced in line 7 chicken embryo cells (Vogt and Friis, 1971). Endogenous ALSVs rarely cause tumors apparently due to the weak promoter activity of their LTRs (Crittenden *et al.* 1979; Fadly and Payne, 2003; Motta *et al.*, 1975). They can be beneficial and stimulate immunity or detrimental and induce tolerance to tumor virus antigens (Fadly and Payne, 2003).

The *ev* loci family consists of complete or defective proviral DNA sequences of subgroup E ALV integrated in the chicken genome (Sacco *et al.*, 2000). They are found

on certain chromosomes (Tereba *et al.*, 1981) of somatic and germ line cells and are transmitted genetically by both sexes (Astrin *et al.*, 1979; Crittenden *et al.*, 1977; Payne and Chubb, 1968). More than 30 *ev* loci have been documented in different chicken strains (Sabour *et al.*, 1992). On average, there are about 5 *ev* loci in each bird with variable phenotypic expression (Rovigatti and Astrin, 1983). The *ev* loci have low copy numbers segregated in the population and restrictly distributed in various species of *Gallus* (Sacco *et al.*, 2000).

Some *ev* loci produce defective non-infectious viral particles (*ev*3, *ev*6 and *ev*9) phenotypically expressed. Others generate complete infectious virions (*ev*2, *ev*7, *ev*10, *ev*11, *ev*12, *ev*14, *ev*18 and *ev*21) spontaneously produced as subgroup E ALVs. For example, *ev*21 encodes a complete virus known as EV21 and induces immunological tolerance and increases the susceptibility of chicks to exogenous ALV (Bacon *et al.*, 1988; Harris *et al.*, 1984; Smith and Fadly, 1988; Smith *et al.*, 1991). Moreover, it renders White Leghorn chickens more susceptible to ALV infections resulting in reduced egg production, increased incidence of viremia and subsequently higher tumor mortality (Bacon *et al.*, 1988; Harris *et al.*, 1984; Smith and Fadly, 1988). Nevertheless, certain *ev* loci (*ev*1, *ev*4, *ev*5, *ev*8, and *ev*17) do not produce viral products since they are transcriptionally silent (Coffin, 1982).

The protein expression of some *ev* loci will give false positive reactions and can interfere with the interpretation of diagnostic tests such as enzyme-linked immunosorbent assay (ELISA) and complement-fixation test for ALV (COFAL). Line 0 chickens are devoid of any *ev* loci (Astrin *et al.*, 1979; Crittenden and Fadly, 1985) and provide useful research tools for chicken experiments and biological assays (line 0 CEFs) since they are

resistant to infection with subgroup E ALV (C/E). Many *ev* loci are associated with decreased productivity, reduced immunity and disease resistance to varying degrees (Crittenden, 1991). For example, chicken cells expressing envelope proteins of some *ev* genes are dominantly resistant to infection by subgroup E ALV by blocking virus receptors on cells (Payne *et al.*, 1971; Robinson *et al.*, 1981). Moreover, the presence of ev2 or ev3 protects chickens from a unique non-neoplastic syndrome caused by infection with ALV subgroup A (Crittenden *et al.*, 1982 and 1984).

The EAV class is a heterogeneous group of highly diverged retroviral elements found in chickens and other species of *Gallus* (Sacco *et al.*, 2000). EAV-0 elements were first discovered in line 0 chickens and have typical proviral structure with deletions in the *env* region (Boyce-Jacino *et al.*, 1989). There are about 50 copies of EAV in a normal chicken genome. Howeve, EAVs do not produce infectious virions, but they express RT activity especially in live virus vaccines and cell cultures (Hussain *et al.*, 2001; Robertson *et al.*, 1997; Tsang *et al.*, 1999; Weissmahr *et al.*, 1997). EAV-HP is believed to be the origin of the *env* gene of subgroup J ALV (Bai *et al.*, 1995a and b; Benson *et al.*, 1998a and b; Ruis *et al.*, 1999; Smith *et al.*, 1999). In addition, ALV J and EAV-0 element ev/J 4-1 Rb might share the same receptor (Denesvre *et al.*, 2003)

The ART-CH family belongs to the defective retrotransposon class, requiring the use of a helper virus to complement its replication. There are approximately 50 copies per haploid genome, each copy is composed of functional LTRs and short regions of homology to the ALV-related gene sequences (Nikiforov and Gudkov, 1994). The 5' end sequences of ART-CH highly resemble those of EAV-HP (Sacco *et al.*, 2000).

The CR1 class consists of short interspersed repetitive DNA sequences, which belong to the non-LTR class of retrotransposons possessing RT sequences (Stumph *et al.*, 1984). CR1 sequences are highly numerous, about 7000 to 20,000 repeats per haploid genome (Crittenden, 1991). They have conserved 3' ends and variable 5' truncations and are not functionally expressed. CR1 sequences are identified in several avian and reptilian species representing ancient and primitive sequences preceding the evolution of birds and reptiles (Crittenden, 1991; Vandergon and Reitman, 1994).

Recently, an endogenous retrovirus ChiRV1 was identified in the chicken genome (Borysenko *et al.*, 2008). It clusters with murine leukemia virus (MLV)-related viruses, but is not related to exogenous MLV-related retroviruses infecting chicken. It is 9133 bp long and contains 90% identical LTRs, *gag*, *pol* and *env* genes. It is tanscriptionally active but genetically defective; thus, it is unlikely to produce viral particles.

4. Virus Structure and Composition

ALSVs have an outer membrane, an intermediate membrane and an inner centrally located electron-dense core with a diameter of 35-45 nm (Coffin, 1996). ALVs are approximately 80-120 nm in diameter and have a type C morphology (Fadly and Payne, 2003). On the outer membrane, each transmembrane glycoprotein (projection or spike) is 7 nm long and 8 nm diameter with a 6 nm knob like surface glycoprotein at the tip (Beard, 1963). ALVs have a buoyant density of 1.15-1.17 g/cm^3 in sucrose, a characteristic of type C viruses (Bauer, 1974) and a sedimentation rate of about 60S-70S representing the diploid viral genome. The virions are susceptible to heat, detergent and formaldehyde (Fadly and Payne, 2003).

Each ALSV particle is composed of 30-35% lipid, 60-65% protein (of which 5-7% is glycoprotein), 2.2% RNA and small amounts of DNA of host cell origin (Bauer, 1974; Beard, 1963). Since the virion envelope is derived from host outer cell membrane, it is a bilayer made mainly of phospholipids (Bauer, 1974; Bolognesi, 1974). The viral proteins are encoded by *gag/pro*, *pol* and *env* genes present in the retroviral genome (Weiss *et al.*, 1985; Fadly and Payne, 2003). Four non-glycosylated proteins are encoded by *gag* namely capsid (CA or p27), matrix (MA or p19), nucleocapsid (NC or p12) proteins and an additional gag protein (p10). CA is the major group specific antigen (gsa); MA lines the inner surface of the virion envelope; NC is a basic protein associated with genomic RNA; and p10 is located between MA and CA. The protease (PR or p15) is an enzyme encoded by *pro* gene, which lies between the *gag* and *pol* genes. The *pol* gene encodes the enzymes, reverse transcriptase (RT or p68) and integrase (IN or p32). The *env* gene encodes 2 glycoproteins (Coffin, 1992), the surface envelope protein (SU or gp85) and the transmembrane protein (TM or gp37).

The ALV genome is a dimer of linear single stranded plus-sense RNA, 7.8 kb in length (Coffin, 1992). The retroviral RNA consists of a 5' cap and 3' poly (A) tail generated by the host transcriptional machinery. The viral genome is surrounded by 2 repeate regions (R) made of 20-nucleotides (nt), one immediately after the 5' cap and one immediately before the 3' poly (A) tail (Coffin, 1992). Furthermore, an 80-nt unique 5' sequence (U5) lies downstream of 5' R and contains one of the attachment (*att)* sites required for proviral integration. The U5 region precedes a primer binding site (PBS), an 18-nt sequence, which hybridizes to the host tRNA and initiates minus-strand DNA synthesis during reverse transcription (Coffin, 1992). Downstream of the PBS is the Psi

element (Ψ), a major encapsidation signal for the viral RNA. The genes coding for structural proteins i.e. *gag/pro-pol-env* follow the Ψ element. Downstream of the structural genes is a short polypurine tract (PPT), a run of at least nine A and G residues and the initiation site for the plus-strand DNA synthesis (Coffin, 1992). Subsequent to the PPT lies a 200-nt unique 3' sequence (U3), containing a number of *cis*-acting elements for viral gene expression and one of the *att* sites required for proviral DNA integration.

Exogenous ALV subgroups (A, B, C and D) share 96-97% sequence homology with subgroup J ALV in the *gag/pro* and *pol*, but not in the *env*. On the other hand, the transmembrane envelope glycoproteins of ALV A to E are homologous and highly related while that of ALV J is very different (Melder *et al.*, 2003). Moreover, ALV-J share 97%, 95% and 75% sequence homology in the *env* gene with EAV-HP, ev/J and EAV-E51 respectively (Benson *et al.*, 1998a; Smith *et al.*, 1999). Downstream to the gp37 of the *env*, the 3' noncoding region or untranslated (3' UTR) of ALV-J consists of a redundant transmembrane region insert (rTM), a direct repeat (DR1) element and an E element (XSR). rTM of ALV-J is more homologous (97%) to other exogenous ALVs than to other ALV-J viruses. In addition, ALV J contains a single copy of the DR1 element found 3' to the rTM, but avian sarcoma viruses contain two copies flanking the *src* gene. Downstream of DR1, a copy of the E element is found in replication-competent RSVs, but not in replication competent ALVs (Bai *et al.*, 1995a).

5. Antigenic Variation

Antigenic variation has been documented in retroviruses from avian, animal and human sources. In ALV, antigenic variation can be divided into 2 categories namely

subgroup-specific and type-specific based on the use of host cell receptors. ALVs use different host cell receptors when subgroup-specific antigenic variations occur while they use the same receptor when type-specific antigenic variations arise. In ALV infections, subgroup-specific and type-specific antigenic variations have been reported (Fadly and Smith, 1999; Venugopal *et al.*, 1998). In general, subgroup-specific variation results in the development of a novel ALV subgroup (Fadly and Payne, 2003). Moreover, type-specific antigenic variation has been observed to be much higher in infections with subgroup J than with other ALV subgroups (Silva *et al.*, 2000; Venugopal *et al.*, 1998).

Sequence variations in gp85 (SU) and gp37 (TM) of the *env* influence both virus-host cell receptor interactions and neutralizing antibody (Nab) responses. The SU protein interacts with the host receptor while the TM protein anchors the SU protein to the viral envelope membrane and is responsible for the fusion of viral and host membranes (Coffin, 1992). The gp85 contains 2 hypervariable and 3 less variable regions organized as *vr1-vr2-hr1-hr2*-vr3. The gp85 protein is more variable than the gp37 protein, which is composed of fusion peptide and membrane anchor domains. Clusters *hr1* and *hr2* determine the specificity of receptor binding and host range in all ALV subgroups (Dorner *et al.*, 1985; Dorner and Coffin, 1986). In addition, *hr2* and *vr3* regions of ALV J *env* gene may be targets for immune selection since they exhibited high sequence variations along with very high NS/S ratios (Venugopal *et al.*, 1998). The *env* of ALV-J differs more extensively from that of other subgroups since it contains regions of higher sequence variability designated as hypervariable regions 1-4 (Silva *et al.*, 2000). The *vr3* domain is responsible for receptor recognition specificity but not receptor binding

affinity. On the other hand, *vr1* and *vr2* do not seem to be crucial for receptor specificity or binding affinity (Dorner *et al.*, 1985).

Commercial ELISA kits have been widely used to identify and eradicate ALV in chickens (Venugopal *et al.*, 1997). They can also be used to monitor new emerging viruses including recombinants. However, they have limited value due to the high level of antigenic variation in the *env* (Silva *et al.*, 2000; Venugopal, 1999; Venugopal *et al.*, 1998). Moreover, polymerase chain reaction (PCR) based tests have been used to monitor flocks for known and new ALV subgroups and strains using different primers specific for the variable regions in the *env* and LTR (Fadly and Payne, 2003; Silva *et al.*, 2000; Smith *et al.*, 1998a and b). Nevertheless, they are not entirely reliable since the primers can not be specific and generic at the same time. This may result in false negatives if mutations arise in proviral DNA sites, where primers are designed to anneal. Therefore, constant monitoring of the specificity and sensitivity of all diagnostic methods is indispensable for the successful prevention, control and eradication of ALV.

6. Virus Replication

ALSVs including ALVs adsorb to the host cell membrane in a nonspecific manner even in cells resistant to infection (Piraino, 1967). However, they enter the host cells based on the specific interaction of their envelope glycoproteins (SU and TM) with the host cell membrane receptors. In fact, different ALV subgroups have different SU glycoproteins and enter the infected cells through exploiting receptors of other molecules. For example, the receptor for ALV subgroup A is genetically related to the human low-density lipoprotein receptor, LDLR (Bates *et al.*, 1993; Young *et al.*, 1993). Moreover,

Subgroups B and D ALV share the same receptor CAR1, a member of the tumor necrosis factor receptor (TNFR) family (Brojatch *et al.*, 1996). On the other hand, the receptor for ALV-E is almost identical to CAR1, only differing in amino acids at residue 62 (Adkins *et al.*, 2000). The receptor for subgroup C ALSV is related to mammalian butyrophilins, members of the immunoglobulin superfamily (Elleder *et al.*, 2005). In addition, Chai and Bates (2006) identified the receptor for ALV J to be a Na+/H+ exchanger type 1 in chickens similar to its mammalian version, which is a housekeeping protein, regulating intracellular pH and cell volume.

After SU glycoprotein binds the appropriate receptor, a conformational change in the virion envelope glycoprtoteins occur leading to viral envelope fusion with host cell membrane (Gilbert *et al.*, 1995). Viral entry into the host cell is facilitated via endosomes where the virion core is released into the cytoplasm. The early phase of the retroviral life cycle continues with the process of reverse transcription. RNA: DNA hybrid is formed when the plus-strand viral RNA is reverse transcribed into a minus-strand viral DNA by RT. While the RNase H activity of RT facilitates the cleavage of RNA from the hybrid, the RT DNA polymerase activity generates plus-strand DNA complimentary to the minus-strand DNA. The end products are linear DNA duplexes flanked by LTRs on both ends. The process of reverse transcription of ALV by RT is depicted in Figure 1.5. The linear double-stranded DNA migrates into the nucleus and is randomly integrated into the host cell DNA at multiple sites by the viral enzyme integrase (Fadly and Payne, 2003).

The host cell RNA polymerase II carries out the transcription of proviral DNA template into viral RNA, which is exported back to the cytoplasm. The resulting viral RNA is packaged as genomic RNA in newly formed virions. On the other hand, the viral

RNA forms messenger RNA (mRNA), which is translated by polyribosomes into several polyprotein precursors namely Pr180GAG-PR-POL, Pr76GAG-PR and gPr92env (Fadly and Payne, 2003). Viral and cellular proteases cleave these precursors to yield the viral proteins and glycoproteins (Luciw and Leung, 1992). The viral proteins localize directly at the host cell plasma membrane and crescent-shaped structures develop into immature virions, which bud off from the cell. The resultant immature virions have large, open spherical cores and undergo maturation to become infectious (Vogt, 1996). The viral protease (PR) cleaves GAG polyprotein into three major proteins, CA, MA, NC and the mature particles are characterized by centrally located condensed cores with small surface projections (Coffin, 1992). The retroviral life cycle is depicted in Figure 1.6 (Coffin *et al.*, 1997).

C. *Epizootiology*

1. Virus Infection Incidence

Infection of chickens with ALSVs is associated with delayed sexual maturity, reduced egg production and quality, reduced body weight and increase in mortality (Spencer *et al.*, 1984). It is an economic problem to layer and broiler poultry breeders in most areas of the world. In spite of the eradication efforts implemented by many primary breeding companies, ALV is still widespread in commercial poultry flocks. Egg-type and meat-type chickens, pheasants, partridges and quail are naturally susceptible to ALV infections; however, other avian and mammalian species can be experimentally exposed (Geryk *et al.*, 1996; Kumanishi *et al.*, 1973; Nehyba *et al.*, 1990; Salter *et al.*, 1999; Trejbalova *et al.*, 1999; Vogt, 1965). In fact, all chicken flocks are infected with ALV by

sexual maturity except those genetically resistant or virus-free through successful eradication programs (Fadly and Payne, 2003). ALV infections were reported to be more prevalent in egg-type chickens compared to meat-type chickens.

ALV-A is the most frequent encountered ALV subgroup in field outbreaks in many countries, followed by subgroup B ALV (Calnek, 1968). Subgroups C and D are extremely rare in the USA but have been isolated from commercial flocks in Finland (Sandelin and Estola, 1974). Most chicken lines and some species of galliform birds contain endogenous retroviral genes (Crittenden, 1981 and 1991; Dimcheff *et al.*, 2000; Robinson, 1978; Smith, 1987a). The susceptibility to infection with exogenous ALVs increases upon congenital infection with subgroup E ALVs such as EV21 and RAV-0 (Crittenden *et al.*, 1987; Smith and Fadly, 1988). Fadly and Smith (1999) estimated the incidence of ALV-J infections in meat-type breeders to be 87%. ALV subgroups F and G have been isolated from pheasants, while H and I from partridges and quails respectively. Furthermore, the incidence rates of infection with recombinant ALVs belonging to different ALV subgroups have been reported to be high in natural and experimental settings (Gingerich *et al.*, 2002; Lupiani *et al.*, 2003 and 2006).

2. Disease Incidence

ALV subgroups A, B, C and D usually induce a variety of neoplasms; predominantly lymphoid leukosis; less commonly erythroblastosis, myeloblastosis and myelocytomatosis and occasionally hemangiomas, nephroblastomas, osteopetrosis and histiocytic sarcomas (Fadly and Payne, 2003; Perek, 1960; Purchase, 1987). On the other hand, endogenous ALVs (subgroups E) such as RAV-0 cause little or no oncogenicity in

chickens (Crittenden *et al.*, 1979; Fadly and Payne, 2003; Motta *et al.*, 1975). In the late 1980s, subgroup J ALV was first isolated and associated with myelocytomatosis in meat-type chickens (Payne *et al.*, 1991). It resulted in significant economic losses for broiler breeders and poultry industry in the US and abroad (Fadly, 1998; Fadly and Smith, 1997 and 1999; Payne *et al.*, 1992a). In addition, high incidence rates of tumors mostly LL have been observed in chickens naturally or experimentally infected with recombinant ALVs (Gingerich *et al.*, 2002; Lupiani *et al.*, 2003 and 2006).

Although lymphoid leukosis (LL) is the most common neoplasm encountered in infected flocks, its incidence varies from to 1 or 2% up to 20%. Extensive losses are rare, but can occasionally be up to 30%, which have been reported in commercial layers and breeder flocks (Fadly and Payne, 2003; Jordan, 1990; Payne and Venugopal, 2000; Purchase *et al.*, 1972). In fact, the incidence of LL is influenced by chicken genetics, infectious bursal disease (IBD), and vaccinations with serotype 2 MDV (SB1). Following ALV exposure at hatch, the incidence of LL is inversely correlated with the widespread incidence of IBD (Purchase and Cheville, 1975) and positively correlated with serotype 2 MDV (SB-1) vaccination (Bacon *et al.*, 1989; Fadly and Witter, 1993).

Before ALV-J isolation, the incidence of myelocytomatosis was infrequent to rare in commercial flocks. Nevertheless, the incidence of myelocytomatosis induced by ALV-J strain HPRS-103 was estimated to be around 27% in broilers (Payne *et al.*, 1992a). Fadly and Smith (1999) found losses due to tumor mortality in commercial meat-type breeders to be 1.5% higher per week. In chickens infected with ALV-J, the incidence of erythroblastosis, hemangiomas, nephroblastomas and connective tissue tumors varies and can be seen either alone or along with other neoplastic conditions. Complete eradication

of subgroup J ALV is a very difficult due to its persistence, sporadic isolation from ALV-free broiler-breeder flocks and mixed infections with chicken anemia virus, MDV and other poultry pathogens (Cui *et al.*, 2003b).

Many solid tumors belonging to avian leukosis complex occur naturally in field or experimental infections. These include connective tissue tumors, nephromas, nephroblastoma, other epithelial, endothelial and neural tumors (Fadly and Payne, 2003). For example, hemangiomas (25%) and renal tumors (19%) are frequently found in broilers infected with ALV (Campbell and Appleby, 1966). Moreover, sporadic cases of osteopetrosis occur more frequently in males than females and some have been associated with LL (Fadly and Payne, 2003).

3. Transmission

ALV is naturally transmitted either horizontally (contact), vertically (congenital) or genetically. Exogenous and complete infectious endogenous ALVs are transmitted by contact among birds through feces, saliva, skin and fomites (Burmester and Gentry, 1954; Crittenden *et al.*, 1987; Spencer *et al.*, 1977). For example, chickens infected *in ovo* or during the first 2 weeks shed ALV in saliva and meconium and spread it by contact to hatch-mates and pen-mates (Burmester, 1956; Burmester *et al.*, 1960). In fact, horizontal transmission maintains high levels of infection within the flock, thereby increasing the possibility of vertical transmission. ALV-J is known to be more efficient in horizontal transmission than other ALV subgroups while endogenous ALVs are less efficient (Payne, 1987; Robinson and Eisenman, 1984).

The rate of vertical transmission is variable, but it sustains the infection between generations. Congenitally infected chicks contribute to the early ALV horizontal transmission and increased disease incidence in the flock (Fadly and Payne, 2003; Weyl and Dougherty, 1977). In fact, the efficiency of congenital transmission of ALV is mainly influenced by the frequency and amount of virus shed in the albumen, the presence of NAbs in the yolk and the thermo sensitivity of the virus (Fadly and Payne, 2003). In addition, the infection status and age of the dam are the two main factors responsible for efficient congenital transmission of ALV. Viremic dams transmit ALV to progeny at high levels, while infected non-viremic dams spread it at intermittent and lower levels. The shedding of ALV into eggs is less consistant and at lower proportions in older (2-3 years) than younger (<18 months) dams (Burmester and Waters, 1956).

Conversely, the sire does not influence ALV vertical transmission since the male germinal cells can not be infected (Distefano and Dougherty, 1966 and 1968). For example, spermatozoa were not infected and genetic transmission through semen was not observed when testes were directly inoculated with RAV-1 (Afanassieff *et al.*, 1996). Moreover, Smith and Fadly (1994) suggested that the sire venereally transmits the virus to the dam and does not directly influence the rate of congenital transmission. In some ALV-shedding males, semen production was not reduced but abnormal spermatozoa and reduced fertility were found (Segura *et al.*, 1988). However, the quality and quantity of semen production were not affected by infection with ALV-J (Hudson *et al.*, 2002).

D. *Pathobiology*

1. Introduction

The natural host for ALSVs is the domestic chicken, but there is a wide host range under experimental conditions. Members of L/S group are commonly associated with more than one type of neoplasm, a predominant type characteristic of a certain viral strain in addition to other tumors. Most chicken neoplastic diseases usually have nonspecific clinical signs such as inappetance, weakness, diarrhea, dehydration and emaciation (Fadly and Payne, 2003). Birds with LL sometimes have abdominal enlargement, pale shriveled and cyanotic combs (Kahn, 2005). In myelocytomatosis, some chickens have protuberances on the head, thorax and shanks, hemorrhage in the orbits of the eyes and blindness. In addition, hemorrhage from the feather follicles may occasionally occur in erythroblastoisis and myeloblastosis while hemangiomas are characterized by blood blisters, which may burst and cause hemorrhage in the skin. Renal tumors sporadically cause paralysis while osteopetrosis result in stunted pale limping birds.

The incubation period for diseases induced by ALSVs depends on virus strain and dose, route of and age at exposure and host genetic makeup (Fadly and Payne, 2003). Acute transforming viruses such as RSV induce tumors (4-7 weeks) more rapidly than slow transforming viruses (14-20 weeks) such as ALV (Beard, 1963; Fadly and Smith, 1999; Kung and Maihle, 1987; Payne *et al.*, 1993). Moreover, certain laboratory recombinant viruses cause LL within 5-7 weeks (Kanter *et al.*, 1988), while naturally occurring recombinant ALV induced LL within 30 weeks postinoculation (Lupiani *et al.*, 2006; Mays *et al.*, 2006). The incubation period of ALSVs is inversely proportional to the virus dose and host age at exposure (Beard, 1963; Burmester *et al.*, 1959b and 1960; Eckert *et al.*, 1954; Franklin and Martin, 1980). Intravenous (IV) inoculation of birds

induces earlier tumors and mortality than intrabdominal (IA) inoculation (Burmester *et al.*, 1959a and 1960; Fadly and Payne, 2003; Mladenov *et al.*, 1967).

2. Pathogenicity

There are many factors influencing ALSV pathogenicity in chickens such as virus subgroup, strain, dose, and route of exposure, host sex, age at exposure and genotype, environmental factors and stress. ALV subgroup affects tumor incidence and type, which mainly depend on LTR (Payne, 1987) and envelope respectively (Brown and Robinson, 1988; Chesters *et al.*, 2002; Jaffredo *et al.*, 1993). Commonly, all ALV subgroups predominantly cause tumors of lymphoid lineage except subgroup J, which mainly results in tumors of myeloid lineage. Conventionally, most viral strains were named after the predominant tumor they induce at high doses such as RSV (sarcomas), AMV (myeloblastosis) and AEV (erythroblastosis). However, viral strains within a certain ALV subgroup have varying capacities to induce LL, myeloid leukosis (ML) or other tumors.

The inoculated viral dose greatly influences the type of tumors produced. For example, high doses of certain ALV-A strains predominantly induce erythroblastosis (Burmester *et al.*, 1959a) whereas low doses predominantly induce LL (Purchase, 1987). In general, the route of exposure to ALV determines the effective viral dose and the type of target cell exposed to the virus. For instance, subcutaneous or intramuscular inoculations with ALV primarily induce sarcomas and LL while intravenous inoculation mostly induces erythroblastosis and myeloblastosis (Fredrickson *et al.*, 1965). Furthermore, resistance to tumor development is acquired more quickly when exposure to

the virus is through the natural fecal-oral routes than parenteral routes due to the low effective viral dose (Burmester *et al.*, 1959a).

The gender of the host has an important effect on the incidence of certain tumors. Male chickens are less susceptible to LL than female and castrated male chickens probably due to the protective nature of testosterone, which results in the regression of target cells (Burmester and Nelson, 1945; Cooper *et al.*, 1968). On the other hand, males appear to be more susceptible to osteopetrosis than females (Smith, 1987a). ALV-J induces myelocytomatosis, several types of leukemias and sarcomas, which are independent of the sex of the host.

The host age at exposure influences both the course of infection and disease incidence (Piraino *et al.*, 1963; Rubin and Vogt, 1962; Rubin *et al.*, 1962). Younger hosts (up to 2 weeks) tend to be more susceptible to viral persistence and tumor mortality than older hosts. This is certainly true in infections with subgroup J ALV and certain ALV strains such as RPL12 (Burmester *et al.*, 1960; Witter *et al.*, 2000).

The genotype of the host has a strong influence on resistance to cell infection and transformation. Genetic resistance to viral infection is dictated by the absence of specific viral receptors on surface of host cells. Commercial chicken lines are characterized by a variable frequency of alleles encoding cellular resistance to infection (Crittenden and Motta, 1969; Motta *et al.*, 1973). On the other hand, resistance to cell transformation depends on many alleles and varies among chicken lines (Purchase, 1987). For example, line 6 chickens are resistance to tumor formation while line 7 is susceptible.

Several environmental factors have an effect on the incidence of certain tumor types. For example, factors that deplete bursa of Fabricius may reduce LL incidence but

may increase the incidence of osteopetrosis. Certain management practices such as manual vent sexing and lack of biosecurity increase the rate of virus exposure and tumor incidence. Environmental factors inducing stress increase tumor incidence by negatively influencing the immune system of the host (Fadly *et al.*, 1989).

3. Viral and Cellular Oncogenes

Tumors in chickens can result from the direct transcription of viral oncogenes in acute transforming ALSV viruses. On the other hand, they can be induced by activation and overexpression of cellular oncogenes through insertional mutagenesis of slow transforming viruses. Viral oncogenes (*v-onc*) are usually expressed at high levels while cellular oncogenes (*c-onc*) are expressed at low levels. Viral oncogenes can encode growth factors, growth factor receptors, transcription factors or various proteins of signal transduction cascades. These proteins normally function in normal cell growth, proliferation and differentiation (Gordon, 1985; Lewin, 1994). Viral oncogenes are associated with cellular oncogenes through transduction subsequent to proviral DNA integration into the host DNA. This integration sometimes leads to rearrangement of viral geneome and the consequent incorporation of a cellular oncogene.

Some examples of *v-oncs* in avian retroviruses include *src*, *myc*, *myb* and *erb*. Rous sarcoma virus and its associated *v-src* were the first to be identified and characterized (Bishop and Varmus, 1982). In fact, c-SRC protein is membrane associated non-receptor or intracellular tyrosine kinase (Lewin, 1994). It is mainly involved in the regulation of cell proliferation, migration, differentiation and survival (Brown and

Cooper, 1996). On the other hand, v-SRC protein causes unrestricted cell proliferation and leads to tumor formation.

The *v-myc* was originally identified in the avian myelocytomatosis virus MC29 associated with myelocytomatosis in chickens. MC29 is a transforming defective retrovirus lacking part of *gag*, all of *pol* and most of *env* (Enrietto and Hayman, 1987; Reddy *et al.*, 1983; Sheiness *et al.*, 1978). The porotoncogene *c-myc* is a highly regulated gene, which belongs to the *myc* family of nuclear DNA-binding/transcription factor genes (Sheiness *et al.*, 1980). The c-MYC protein functions as a transcription factor, interacts with other proteins and targets genes that regulate and control gene expression and cell growth (Dang, 1999). Altered expression of disrupted *c-myc* has been associated with LL upon infection with ALV-A or B mainly in layers and ML in broilers infected with ALV-J. In addition, overexpression of v-MYC causes uncontrolled cell proliferation and blocks terminal differentiation of hematopoietic cells (Lee and Reddy, 1999).

Avian myeloblastosis virus (AMV) and avian E26 leukemia virus contain the oncogene *v-myb* responsible for transformation of myeloid cells (Duesberg *et al.*, 1980; Introna *et al.*, 1990). The protooncogene *c-myb* encodes a transcription factor, which is involved in cell cycle regulation via control of intracellular calcium concentration (Introna *et al.*, 1994; Oh and Reddy, 1999). Moreover, *c-myb* is highly expressed on all immature cells of hematopoietic and lymphoid systems and its expression is down regulated during differentiation (Duprey and Boettiger, 1985; Golay *et al.*, 1997). Truncation of N-terminal of *c-myb* is highly transforming while C-terminal truncation is weakly transforming (Lipsick and Wang, 1999). Overactivation of *myb* causes LL upon

infection with ALV subgroups A or B mainly in egg type chickens (Kanter *et al.*, 1988; Pizer and Humphries, 1989).

Avian erythroblastosis virus (AEV) contains *v-erbA* and *v-erbB*, which are responsible for full transformation of erythroblasts (Lewin, 1991). The *v-erbA* encodes a nuclear protein belonging to the superfamily of nuclear hormone receptors. The *v-erbB* encodes a truncated form of the epidermal growth factor (EGF) receptor with a constitutively activate tyrosine kinase function (Kris *et al.*, 1985). The cellular homologs of *v-erb* and *v-erbB* encode the nuclear receptor for thyroid hormone (Sap *et al.*, 1986; Weinberger *et al.*, 1986) and the epidermal growth factor receptor respectively (Sawyers *et al.*, 1991). Slow transforming ALV strains induce the activation of the cellular oncogenes *erbA* and *erbB* by LTR insertion (Fadly and Payne, 2003). This causes maturation arrest, proliferation and transformation of erythroblasts and results in erythroblastosis (erythroid leukosis) in chickens.

4. Nonneoplastic Conditions

Infection of chickens with ALSV results in several nonneoplastic conditions and many neoplastic diseases. Some of the nonneoplastic conditions are caused by certain strains of ALSV whereas others are more generic. At an early age, subclinical infection of chickens with high doses of ALV is characterized by absence of clinical signs, low viral loads at fewer sites in the body and detectable antibody response (Arshad *et al.*, 1997). In addition, infection with subgroup J ALV sometimes causes depressed body weight, reduced growth rate, stunting, non-uniform flock size in broiler breeders and

meat-type chickens probably due to low feed consumption, conversion and efficiency (Crittenden *et al.*, 1983; Gavora, 1987; Gavora *et al.*, 1982; Stedman and Brown, 1999).

In ALV infections, the productivity of egg-laying chickens is negatively affected and observed as reduced rate, size, quality (softer shells), number, fertility and hatchability of eggs, delayed sexual maturity and age at first egg in addition to nonneoplastic mortality (Fadly and Payne, 2003; Gavora *et al.*, 1980). In roosters shedding ALV, the semen quality and fertility are reduced but the semen quantity is not affected (Segura *et al.*, 1988). On the other hand, ALV-J infection does not influence the quality or quantity of semen production (Hudson *et al.*, 2002). Congenital infection with subgroup J ALV sometimes resulted in depressed body weight in broilers due to decreased serum thyroxine T4 levels (Brown *et al.*, 2000).

Other nonneoplastic conditions in experimental ALV infection include immune, circulatory and nervous system disorders. Early exposure to RAV-1 (Rous-associated virus subgroup A), myeloblastosis-associated virus or avian osteopetrosis virus MAV-2 (O), RAV-60 (subgroup E) and ALV subgroups B and D results in anemia, hepatitis, immunodepression, ascites and wasting in chickens (Crittenden *et al.*, 1982; Cummins and Smith, 1988, Smith and Van Eldik, 1978). Immunodepression is exhibited as atrophy of lymphoid organs and tissues, lower antibody production and reduced mitogen-induced lymphoblastic response (Smith, 1987b).

Infection with RAV-1 (subgroup A) sometimes causes myocarditis and chronic circulatory syndrome in adult chickens (Gilka and Spencer, 1990). Moreover, chickens inoculated with RAV-7 (subgroup C) can show neurological signs of infection such as ataxia, lethargy and imbalance. Moreover, RAV-7 results in stunting, atrophy of the

lymphoid organs, obesity (high cholesterol and triglycerides), hypothyroidism (lower thyroid hormones) and increased insulin levels (Carter and Smith, 1983; Carter *et al.*, 1983; Fadly and Payne, 2003). Congenital infection with subgroup J ALV is sometimes associated with abnormal feather development in broilers (Landman *et al.*, 2001).

5. Neoplastic Diseases

The various neoplastic diseases induced by members of the L/S group depend on many factors. These factors are the site of integration of slow transforming ALVs in the host's genome, the type of oncogene carried by acute transforming ALVs and ALV envelope glycoproteins. Generally, ALV infection can cause different kinds of benign or malignant tumors affecting lymphoid, myeloid, epithelial (carcinoma) or endothelial (sarcomas) cells and tissues.

L/S associated neoplastic diseases include lymphoid leukosis (LL), myelcytomatosis (myeloid leukosis, ML), myeloblastosis, erythroblastosis, connective tissue tumors such as fibroma and fibrosarcoma, osteoma and osteosarcoma, myxoma and myxosarcoma, chondroma and chondrosarcoma, renal tumors including nephroma and nephroblastoms, adenoma and carcinoma, hemangioma, hemangiosarcoma, osteopetrosis and other rare neoplasms (Fadly and Payne, 2003). The most commonly occurring neoplasms associated with ALV-related viruses in chickens are LL, ML, myeloblastosis and erythroblastosis.

Lymphoid leukosis is usually observed in chickens older than 14 weeks of age, peaking at sexual maturity (Fadly and Payne, 2003). Thus, it is a significant problem for broiler breeders and layers, but not commercial broilers. LL is usually caused by slow

transforming ALV strains such as subgroups A and B. Although subgroup J ALVs are seldom associated with LL (Payne, 2000), bursal transformation was detected in infected white leghorns (Williams *et al.*, 2004).

Clinical signs of LL are non-specific such as emaciation, pale or cyanotic comb inappetance, enlarged abdomen and organ dysfunction leading to death. LL is often associated with gross or/and microscopic lesions in the bursa of Fabricius and other visceral organs. The principle bursal cells for ALV infection are the medullary macrophages, which transmit infection to lymphoid cells (Gilka and Spencer, 1987). The target cells for transformation are bursal lymphocytes or stem cells expressing surface IgM (Purchase and Gilmour, 1975).

ALV provirus is often inserted next to a cellular oncogene in infected B lymphocytes commonly *c-myc* and rarely *c-myb* (Kanter *et al.*, 1988; Pizer and Humphries, 1989). This insertion allows the enhancer elements in the U3 region to drive the transcription, activation and overexpression of cellular oncogenes. The altered expression of the oncogene products causes proliferation of B cells and maturation arrest of transformed B cells blocking intraclonal Ig class switch from IgM to IgG (Crittenden and Kung, 1984). Progression of the transformed follicle is mediated by additional genetic changes and other cellular oncogenes such as *Blym-1* (Goubin *et al.*, 1983) and *c-bic* (Clurman and Hayward, 1989).

In chickens infected *in ovo* or at hatch, hyperplasia or neoplastic transformation of individual bursal follicles can be observed as early as 4 weeks in experimental studies (Nieman *et al.*, 1980). By 7 weeks, these chickens have one or more abnormal, altered or transformed bursal follicles (Cooper *et al.*, 1968). However, most of the hyperplastic and

neoplastic follicles regress due to anti-tumor immune response (Purchase, 1987). After 12 weeks, transformed B cells in the clonal bursal tumors metastasize to different viscera such as the liver (mostly), spleen, kidney, lung, gonad, heart, bone marrow, digestive tract and mesentery leading to terminal disease (Fadly and Payne, 2003).

Myelocytomatosis occur naturally in immature susceptible chickens upon infection by acute or slow transforming strains of ALV (Purchase, 1987). The incubation period is usually variable depending on ALV strain and is often shorter than LL, but longer than erythroblastosis and myeloblastosis. Acute transforming ALV strains cause myelocytomas within 4-6 weeks (Chester *et al.*, 2001), while slow transforming ALV strains such as HPRS-103 have a latent period of about 9-20 weeks in meat-type chickens (Payne, 1998). Acute transforming strains of ALV such as MC29, CMII and 966 carry *v-myc* and induce neoplastic transformation of the target myelocyte (Enrietto and Hayman, 1987; Moscovici and Gazzolo, 1987). On the other hand, slow transforming strains of ALV such as HPRS-103 and ADOL-Hc1 induce transformation of the target cell by insertional activation of *c-myc* (Chester *et al.*, 2001; Fadly and Payne, 2003).

Clinical signs include bony protuberances on the skull, ribs and other periosteal surfaces. Gross lesions are characterized by moderate to massive enlargement of the liver and myelocytomas of the bones in the skull, sternum and pelvis (Mladenov *et al.*, 1967). Gross and microscopic lesions are usually observed in the bones and some internal organs. The target cell for infection and transformation is the myeloid stem cell in the bone marrow. The neoplastic myelocytes arise from the bone marrow stem cell, proliferate and infiltrate the bone marrow sinusoids. Tumors are generally found in the bones, liver, spleen, ovary, thymus, oral cavity, trachea and eye (Fadly and Payne, 2003).

Natural field cases of myeloblastosis are rare and are generally seen in adult chickens prior to 24 weeks of age (Purchase, 1987). Nevertheless, acute transforming strains of ALV such as E26 and BAI-A AMV can cause the disease within 10 days (Fadly and Payne, 2003). Moreover, ALV subgroups A, B, and J are usually associated with myeloblastosis. The target cell is the myeloblast in the bone marrow and the v-*myb* oncogene of AMV or insertional activation of *c-myb* is responsible for its transformation (Enrietto and Hayman, 1987).

Differentiation of the transformed cell is arrested and proliferation occurs in the bone marrow. This is followed rapidly by leukemia and infiltration of different parts of the body such as the liver, spleen, kidney and other organs (Purchase, 1987). Clinical signs are similar to LL in addition to severe leukemia, thrombocytopenia and anemia (Payne, 1992). Gross lesions are characterized by massive enlargement of the liver, mottling of the visceral organs and presence of solid, yellowish-gray tumor nodules in the bone marrow (Purchase, 1987).

Erythroblastosis usually occurs between 12-24 weeks of age in susceptible chickens. It is not restricted to a certain subgroup, but its incidence in ALV-J infections is variable. However, erythroblastosis incidence is high in chickens experimentally infected with acute transforming stains of ALV-J such as strain 966 (Venugopal *et al.*, 2000). In slow transforming ALV infections, the incidence of erythroblastosis depends on the proviral LTR insertional activation of a cellular oncogene c-*erb*B (Fung *et al.*, 1983; Kurth and Bauer, 1972). On the other hand, some acute transforming viruses possess the viral oncogene *v-erbA* or *v-erbB* such as avian erythroblastosis viruses (AEV) isolates

ES4 and R. These ALV strains induce erythroblastosis and mortality after a short incubation period of 7-14 days (Graf and Beug, 1978).

The target cells for transformation are the erythroblasts in the bone marrow sinusoids (Purchase, 1987). The altered expressions of the cellular and viral oncogenes lead to proliferation, maturation arrest and transformation of the target cells. The transformed erythroblasts enter the circulating blood causing erythroblastic leukemia and metastasize to visceral organs forming solid tumors in the liver and spleen. Moreover, erythroblasts continue to accumulate in different organs until death of the host (Ponten and Thorell, 1957).

Clinical signs of erythroblastosis are similar to LL in addition to anemia. The most characteristic gross lesions are diffuse enlargement of the liver, spleen and sometimes the kidneys. These organs are usually soft and cherry red to mahogany in color due to infiltration of neoplastic erythroblasts (Fadly and Payne, 2003). The affected birds are anemic and have petechial hemorrhages in various visceral organs (Jordan, 1990). The bone marrow is replaced by proliferating erythroblasts and is hyperplastic, semi-solid and red in color.

E. Immunology

1. Humoral Immunity

Humoral immune response plays vital role in protection against ALSV infection through reducing virus replication and neoplasia. The source of passive immunity in chicks is maternal antibodies (IgG), which are passed from the hen through the egg yolk. It lasts 3-4 weeks after hatch and delays the onset of ALV infection, diminishes the levels

of viremia and shedding, and eventually reduces the incidence of tumors (Burmester, 1955; Fadly, 1988; Witter *et al.*, 1966).

Most chicks develop transient viremia and neutralizing antibodies (Nabs), which persist for life after infection with ALV (Rubin *et al.*, 1962; Solomon *et al.*, 1966). The age of the bird at exposure to ALV is inversely proportional to the duration of viremia and the delay in development of Nab. When birds are infected before or 2 weeks post hatch, they develop permenant viremia and no Nabs and are referred to as tolerant viremics. In fact, seroconverted and tolerant viremic chickens are both susceptible to tumor mortality. However, the incidence of tumors in tolerant viremic birds is likely to be higher than in immune birds.

After chickens are infected with ALV, antibodies are raised against different viral proteins and have different roles in protection against the virus. For example, Nabs are directed against virus envelope glycoproteins namely SU (gp85) and TM (gp37). They are highly specific for each subgroup as well as strains within each subgroup (Meyers, 1976; Qualtiere and Meyers, 1983; Vogt and Ishizaki, 1965). They restrict the amount of ALV circulating and shedding in birds and thus limit tumor formation. Antibodies produced against gsa (p27) are not likely to participate in virus neutralization and have no effect on tumor growth (Roth *et al.*, 1971; Sigel *et al.*, 1971). Moreover, antibodies against RT have been detected in ALV infected and uninfected chickens (Graevskaya *et al.*, 1982). Chickens with LL usually have high titers of IgM, which do not protect against ALV (Cooper *et al.*, 1974; Smith *et al.*, 1980).

2. Cell-Mediated Immunity

In ALSV infections, cell-mediated immunity (CMI) is thought to be directed against virus antigens and tumors. Cytotoxic T lymphocytes (CTL) against viral envelope antigens were observed in some ALV infections (Bauer *et al.*, 1976; Kurth and Bauer, 1972; Schat, 1987). Moreover, studies on regressing avian sarcomas demonstrated that T cells are important in CMI against ASV-induced tumors (Perry *et al.*, 1978; Wainberg *et al.*, 1979). Chicken MHC class I amino acid residues were shown to be essential in antigen presentation to ALV-induced CTL responses (Fulton *et al.*, 1995; Thacker *et al.*, 1995). In addition to T cells, natural killer (NK) cells and macrophages were implicated in anti-tumor immunity (Qureshi and Taylor, 1993; Wainberg *et al.*, 1983 and 1987).

F. Genetic Resistance

1. Cellular Resistance to Infection

Cellular resistance to virus infection is one of two levels of genetic resistance to ALSV-induced tumors. In fact, cellular responses to infection by ALSV subgroups are controlled by independent autosomal loci, which are inherited in a simple Mendelian fashion (Crittenden, 1975). In 1994, the Poultry Committee of the United States Department of Agriculture (USDA) National Animal Genome Research Program has adopted a new nomenclature system for genes controlling cellular susceptibility to ALSVs (Fadly and Payne, 2003). The new gene designations are tumor virus followed by the subgroup such as TVA, TVB, TVC and controversial TVE (Adkins *et al.*, 2000; Bates *et al.*, 1998; Crittenden, 1991; Crittenden *et al.*, 1973; Pani, 1974 and 1976).

In chickens, TVA and TVC loci are genetically linked and TVB locus controls responses to subgroups B and D ALVs (Crittenden and Motta, 1975; Pani, 1975; Payne

and Pani, 1971). Suceptibility to ALV subgroup E depends on the interaction between genes at two loci, TVE and I^e. Moreover, I^e is a dominant resistance gene, which acts epistatically and blocks susceptibility by TVE locus (Payne and Pani, 1971). However, some studies suggested that I^e is an *ev* locus encoding ENV glycoproteins, which block ALV subgroup E receptor (Robinson *et al.*, 1981). Genetic resistance to cellular infection by subgroup J ALV has not yet been documented in chickens. Nevertheless, several avian species are known to be resistant to ALV J infection such as pheasants, Japanese quail, guinea fowl, ducks and geese (Payne *et al.*, 1991 and 1992b).

Alleles for susceptibility encode subgroup-specific virus receptors on host cell surface. In susceptible cells, the subgroup-specific virus receptors interact with viral envelope glycoproteins and allow viral penetration and cell infection. Alternatively, no subgroup-specific virus receptors are expressed by resistance alleles. At each locus, susceptibilty alleles (S) are always dominant over resistance (R) alleles (Crittenden, 1968). Host cells or birds can be resistant to infection by one or several members of ALV subgroups. The phenotype is designated according to the cell or bird species (C for chicken) and the subgroup to which they are resistant (/).

The phenotype C/A denotes a cell or chicken resistant to ALV subgroup A infection and susceptible for the other subgroups. On the other hand, the C/O phenotype denotes cells or chickens susceptible to all ALV subgroups. Currently, there are no chickens or chicken cells naturally resistant to subgroup J ALV infection. However, a new genetically engineered cell line, DF-1/J (C/EJ) was developed to be resistant to subgroup J and E ALV infection (Hunt *et al.*, 1999). These cells and chickens can be

considered useful diagnostic tools for screening, isolation and identification of field and laboratory isolates of ALSV.

2. Resistance to Tumor Formation

The second level of genetic resistance to ALSV-induced tumors is that to tumor induction and formation. Genetically resistant birds can not be infected or develop tumors when exposed to a certain ALSV subgroup (Crittenden, 1975; Crittenden and Okazaki, 1966). Regression of Rous sarcoma is controlled by *R-RS-1*, a dominant gene located in the BBL region within the chicken MHC complex or B-haplotype (Collins *et al.*, 1977; Gebriel *et al.*, 1979; Schierman *et al.*, 1977). MHC and non-MHC genes in the B-L/B-F regions or closely linked regions may be responsible for Rous sarcoma regression (Auclair *et al.*, 1995; Taylor Jr., 2004). It is believed that these genes induce tumor regression through immune recognition of a tumor-specific antigen or by influencing viral replication. Genetic resistance to LL tumor development has been associated with MHC locus of B erythrocyte antigen (*Ea-B*), non-MHC lymphocyte locus (*Th-1*) and immunoglobulin genes (Bacon *et al.*, 1981, 1985 and 1986). Nevertheless, the basis for resistance to LL tumor formation seems to be the inherent inability of the bursal target cells to be infected or transformed (Purchase *et al.*, 1977).

G. Diagnosis

1. Virus Isolation and Identification

ALV infections are detected by assays for virus isolation in infected commercial, pathogen-free and breeder flocks. ALV detection tests can also identify and classify new

isolates and test vaccine safety. There are various sample materials suitable for ALV detection. The most common ones are blood, plasma, serum, meconium, cloacal and vaginal swabs, oral washings, egg albumin, embryos and tumors (Crittenden and Smith, 1984; Fadly, 2000; Fadly and Witter, 1998; Fadly *et al.*, 1981a; Spencer *et al.*, 1977). ALV can also be isolated from saliva, feces (Burmester, 1956), feather pulp (Spencer *et al.*, 1983) and semen (Segura *et al.*, 1988).

Most ALV diagnostic tests depend on the detection of specific gene products or proviral DNA or viral RNA sequences. Currently, virus isolation is carried out in cell culture, which is fast, economical and suitable for large-scale use. Since most ALV strains do not produce cytopathic changes in cell cultures, indirect virus detection methods are essential. Different biological assays can be used to detect ALV p27, the most common of which is ELISA (Crittenden *et al.*, 1987; Fadly and Witter, 1998; Smith *et al.*, 1979; Tsukammoto *et al.*, 1991). Commercial ELISA kits for p27 detection are available, but in-house preparation is possible using commercially available anti-p27 antibodies and conjugates.

In all biological assays, using the correct chicken embryo fibroblasts (CEFs) with specific host range is necessary. To detect any ALV, CEFs that are susceptible to infection with all ALV subgroups (C/O) are employed. On the other hand, CEFs of genotype C/E, which are resistant to infection with endogenous ALV are necessary to isolate and detect exogenous ALVs. Moreover, different CEFs and cell lines that are resistant to different ALV subgroups can be utilized to confirm the subgroup of the isolated ALV such as alv6 (C/AE), 7_2 (C/ABDE) and DF-1/J (C/EJ) cell line (Crittenden and Salter, 1990; Fadly and Payne, 2003; Hunt *et al.*, 1999; Salter and Crittenden, 1991).

They have also been used to isolate and characterize recombinant ALVs from field and laboratory isolates (Gingerich *et al.*, 2002; Lupiani *et al.*, 2003 and 2006).

Several tests have been utilized for the detection of ALV nucleic acids such as the widely used polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR). PCR-based methods have been reported for detection of different subgroups of ALV isolated from contaminated vaccines, infected cell cultures, plasma, tumors, feather follicles and eggs (Gingerich *et al.*, 2002; Hauptli *et al.*, 1997; Pham *et al.*, 1999; Silva *et al.*, 2000 and 2007; Smith *et al.*, 1998a and b; Van Woensel *et al.*, 1992; Zavala *et al.*, 2002). Furthermore, different primers specific for different ALV subgroups were used to identify the nature of recombinant ALVs (Lupiani *et al.*, 2000). Most ALV sequences used for designing PCR primers are located in the *env* and LTR regions (Fadly and Payne, 2003). If mutations occur where the developed primers anneal, the desired sequences will not be amplified, not all variant viruses will be detected and false negatives will be reported.

2. Serology Tests

For the detection of antibodies to ALV, samples of plasma, serum or egg yolk can be utilized (Fadly and Payne, 2003). Many cell culture assays based on the reaction between the antibodies and ALV or RSV have been described such as ELISA (Mizuno and Itohara, 1986; Smith *et al.*, 1986; Tsukamoto *et al.*, 1985 and 1991) and flow cytometry (Hunt *et al.*, 1999). In fact, several ELISA kits for detecting antibodies to subgroups A, B and J are commercially available (Fadly and Payne, 2003; Lee *et al.*, 1986; Venugopal *et al.*, 1997). The most commonly used virus neutralization (VN) test

assays for residual virus and indirectly detect antibodies to ALV (Fadly and Witter, 1998). This microneutralization test has been used in many studies to detect and quantitate antibodies to different ALV subgroups including recombinant ALVs (Fadly and Smith, 1999; Lupiani *et al.*, 2003, Mays *et al*, 2005 and 2006; Venugopal *et al.*, 2000; Witter *et al.*, 2000)

3. Differential Diagnosis

Differential diagnosis of lymphomas in chickens is essential for two important reasons. First, different avian oncogenic viruses may cause similar symptoms, tumors and lesions. For example, lymphoid tumors may be observed in Marek's disease (MD), LL and Reticulendotheliosis virus (REV) infections (Fadly, 1987 and 1997; Purchase and Sharma, 1973; Wakenell, 1997). In addition, avian oncogenic viruses are widespread in poultry even in the absence of tumors. This renders standard virological, serological and molecular assays of little value in diagnosis of virus-induced lymphoma. However, molecular assays such as Southern blots and PCR tests for detection of proviral DNA in tumors can be helpful (Fadly, 1997). In most cases, differential diagnosis is based on flock history, age, clinical signs, gross and microscopic lesions and cytological examination of tumors including target cell characteristic and cell surface markers (Fadly and Payne, 2003).

H. Intervention Strategies

1 . Immunization and Treatment

Vaccination is often used to elicite an efficient immune response and to offer protection to chickens against ALV infections and induced lymphoma. On the other hand, congenitally infected chicks are immunologically tolerant and thus are not good candidates for immunization. Moreover, they are viremic and they shed and spread ALV to other chicks. In fact, congenital transmission of ALV is best prevented by maternal antibodies passed from the hen to the progeny. Several attempts have been made to elicit similar immune response, but none has been promising so far. The use of replicating retrovirus as a vaccine is not reasonable because of its integration into the host genome and the increased chance of congenital transmission. On the other hand, Burmester (1952) showed that viruses in inactivated ALV vaccines lost their antigenicity and their ability to elicit an immune response.

Induction of immunity in chickens through the use of CEFs or quail QT6 cells expressing ALV envelope glycoproteins was not successful (Chebloune *et al.*, 1991; Hunt *et al.*, 1999). The efficacy of some subunit vaccines containing envelope glycoprotein gp85 in different expression systems was not satisfactory (Lee *et al.*, 1998; Nazerian and Yanagida, 1995; Noteborn *et al.*, 1990). Nevertheless, Bennett and Wright (1987) reported some protection of subunit vaccines containing viral envelope glycoproteins against RSV-induced sarcomas.

Recombinant live ALV vaccines expressing exogenous ALV *env* gene and LTRs showed some protection (Robinson *et al.*, 1985). However, recombinant viruses in these vaccines may persist in chickens or mutate into more pathogenic or virulent forms. Moreover, recombinant subunit vaccines made of immunogenic endogenous envelope gp85 proteins can induce some degree of protection. On the other hand, they may result

in decreased Nabs production or immunological tolerance to exogenous ALV (Crittenden *et al.*, 1984). Therefore, recombinant ALVs expressing exogenous gp85 proteins might be successful in controlling ALV in commercial poultry operations (Fadly and Payne, 2003). Thus far, there are no treatments of virus-induced neoplasms of chickens.

2. Prevention and Control by Eradication

To date, the most effective means for controlling ALV infection in chickens is eradication. Previously, ALV eradication was only applied to experimental and specific-pathogen free (SPF) flocks used for vaccine production. Development of more feasible and practical testing methods made eradication possible in broader range of flocks including commercial flocks (Chase, 1991; Okazaki *et al.*, 1975; Payne and Howes, 1991; Rispens *et al.*, 1970; Spencer, 1997; Spencer and Gilka, 1982). So far, several breeding companies have significantly reduced or eradicated ALV subgroups A, B and J from their lines (Fadly and Payne, 2003).

ALV eradication programs maily depend on the elimination of vertical and horizontal transmission of ALV in all flocks. Therefore, eradication protocols involved accurate ALV diagnosis in hens, hatching eggs, embryos and chicks (Fadly and Payne, 2003). Several test materials are used for ALV detection, but vaginal swabs, egg albumin and plasma are most commonly used. Detection of ALV-infected chicks is facilitated by testing with more than one technique such as biological assays and PCR. ALV detection is followed by culling of ALV-positive dams, hatching and rearing ALV-free chickens in total isolation in small groups (Spencer *et al.*, 1977).

ALV-positive hens are culled by frequent testing of adequate number of samples for viremia, antibody and shedding in eggs (albumin gsa) and embryos (Fadly and Payne, 2003). Negative dams are selected for producing ALV-free fertile hatching eggs by vaginal swabs and albumin tests and include immune non-shedders and non-immune non-shedders and non-immune or immune non-viremic (Fadly *et al.*, 1981a; Hughes *et al.*, 1963; Zander *et al.*, 1975).

To avoid contact transmission, ALV-free chicks are hatched and reared in small groups in isolation following biosecurity procedures (Fadly *et al.*, 1981b; Witter and Fadly, 2001). Groups of ALV-free chicks are selected for producing future generations through discarding the entire lot containing ALV-positive chicks (Okazaki *et al.*, 1979). In general, chicks are more susceptible to infection immediately after hatching from congenitally infected hatchmates and later from penmates, fomites and the environment.

Good management practices should be implemented to reduce or eliminate ALV transmission from various sources such as other chicks and the environment (Fadly and Payne, 2003). General management procedures to be implemented are many and include using eggs and chicks from one source, using disposable chick boxes, rearing one chicken age group, mechanical vent sexing, MDV vaccination with separate needles and rearing on wire-floored cages. Furthermore, all farm equipment including the ones used for incubation, hatching, and brooding of fertilized eggs and chicks should be thoroughly fumigated, cleaned and disinfected with detergent after each use.

3. Selection for Genetic Resistance

Artifical selection for the two levels of genetic resistance is difficult in chickens for several reasons. Cellular resistance to virus infection is mainly controlled by single dominant susceptibility alleles to certain ALV subgroup. Alternatively, cellular resistance alleles are recessive and more prevalent in meat-type chickens (Crittenden and Motta, 1969). Moreover, commercial chicken lines differ in the frequency of resistance alleles and breeding for resistance mostly depends on crosses between several lines (Crittenden, 1983). On the other hand, genetic resistance to tumor development is controlled by multiple alleles not related to ALV subgroups (Crittenden, 1975). Selection for resistance to ALV infection may be overcome by mutations while resistance to development of neoplasms is based on selection for host viability (Fadly and Payne, 2003).

Artifical poultry breeding is aimed toward selection for genetic resistance to the predominant subgroup A and sometimes subgroup B ALV. For example, one primary broiler breeder company has successfully selected ALV subgroup A-resistant chickens (McKay and Rosales, 2000). Moreover, the search for chicken lines genetically resistant to ALV-J infection has failed thus far. During ALV-J eradication, culling tolerant viremic birds from the already limited broiler breeder stocks may result in the loss of genes linked to some production traits (McKay, 1998). Crittenden suggested that the control of ALV infections will be possible by the development of ALV resistant breeder stocks by transgenesis (1991).

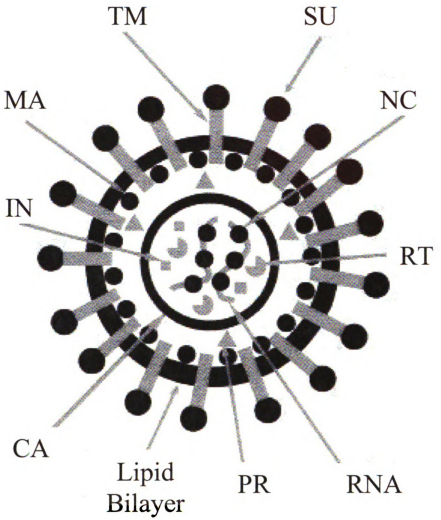


Figure 1.1. Schematic diagram of a retrovirus virion (Avian leukosis virus particle). The viral envelope is a lipid bilayer expressing the gp37 transmembrane (TM) and the gp85 surface (SU) glycoproteins, encoded by the *env* gene. Internal components encoded by the *gag/pro* gene are p19 matrix (MA) protein, p27 capsid (CA) protein, p12 nucleocapsid (NC) protein, and p15 protease (PR). The *pol* gene encodes the reverse transcriptase (RT) and p32 integrase (IN). The core of the particle contains 2 viral RNA strands. Adapted from Diseases of Poultry, 11th edition (Fadly and Payne, 2003).

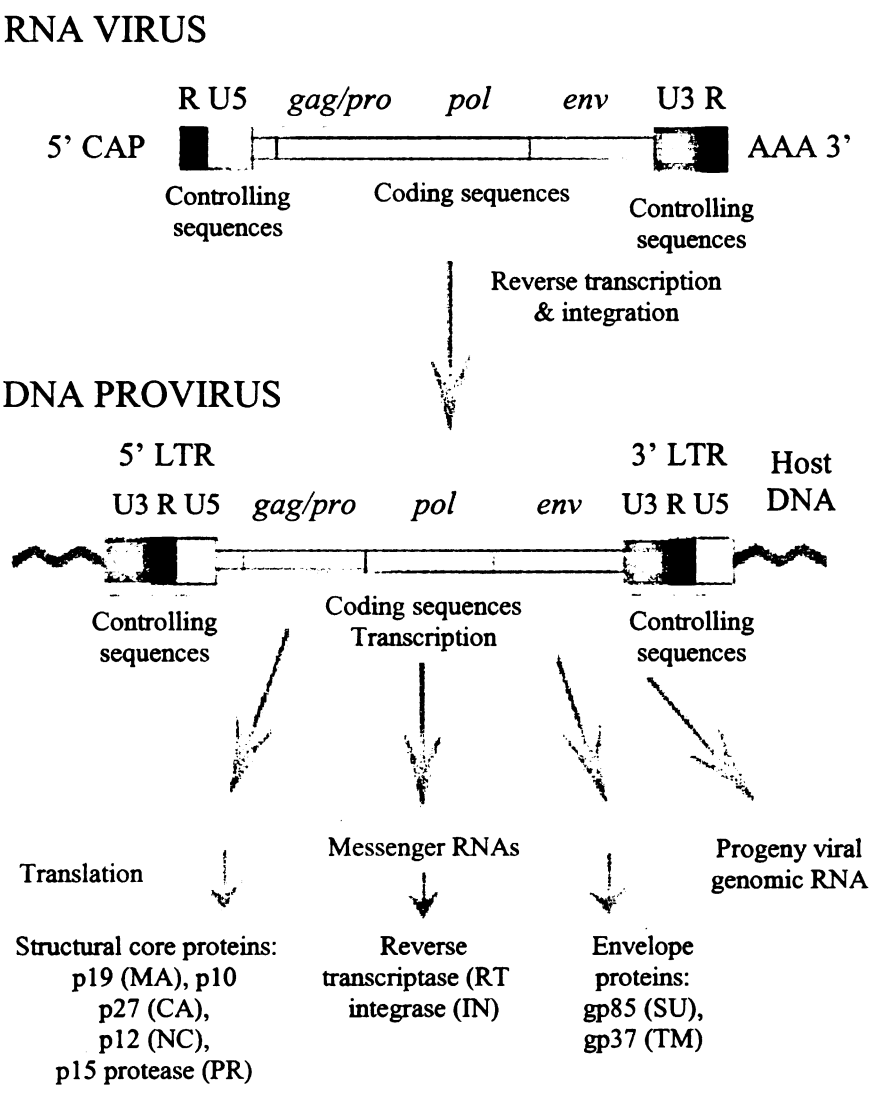


Figure 1.2. Key features of viral genomic RNA and proviral DNA of ALV genome. The RNA genome of ALV has a methylated nucleotide CAP at the 5' end and a poly (A) tract (AAA) or polyadenylation at the 3' terminus. It is surrounded by repeat (R) sequence on both sides. It also contains unique 5' end sequence (U5) and unique 3' end sequence (U3). These three regions form the long terminal repeat (LTR) flanking the DNA provirus. ALV also contains three genes (*gag/pro*, *pol* and *env*) encoding various structural core proteins, viral enzymes and envelope glycoproteins respectively. For other abbreviations, see Figure 1.1 and the text. Adapted from Diseases of Poultry 11th edition (Fadly and Payne, 2003).

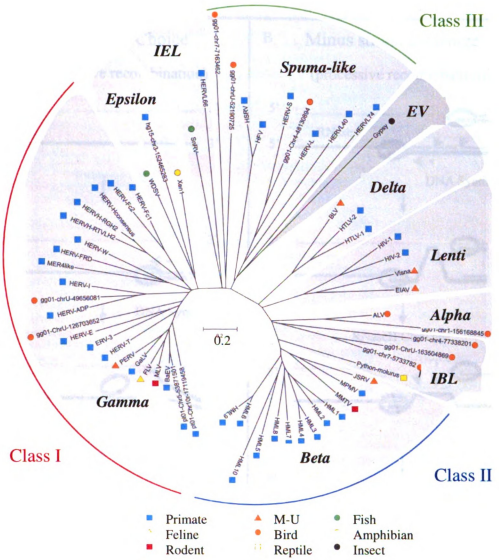


Figure 1.3. Representative unrooted pol neighbour joining (NJ) dendrogram (500 bootstraps consensus) of the seven retroviral genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Lentivirus* and *Spumavirus*. The endogenous retroviral classes (I, II and III) are indicated in the periphery and most similar to *Gammaretrovirus*, *Betaretrovirus* and *Spumavirus* respectively. Some retrovirses belong to *Intermediate Beta-like* (*IBL*), *Intermediate Epsilon-like* (*IEL*) and *Errantiviruses* (*EV*). Adapted from *Retrovirology* (Jern *et al.*, 2005).

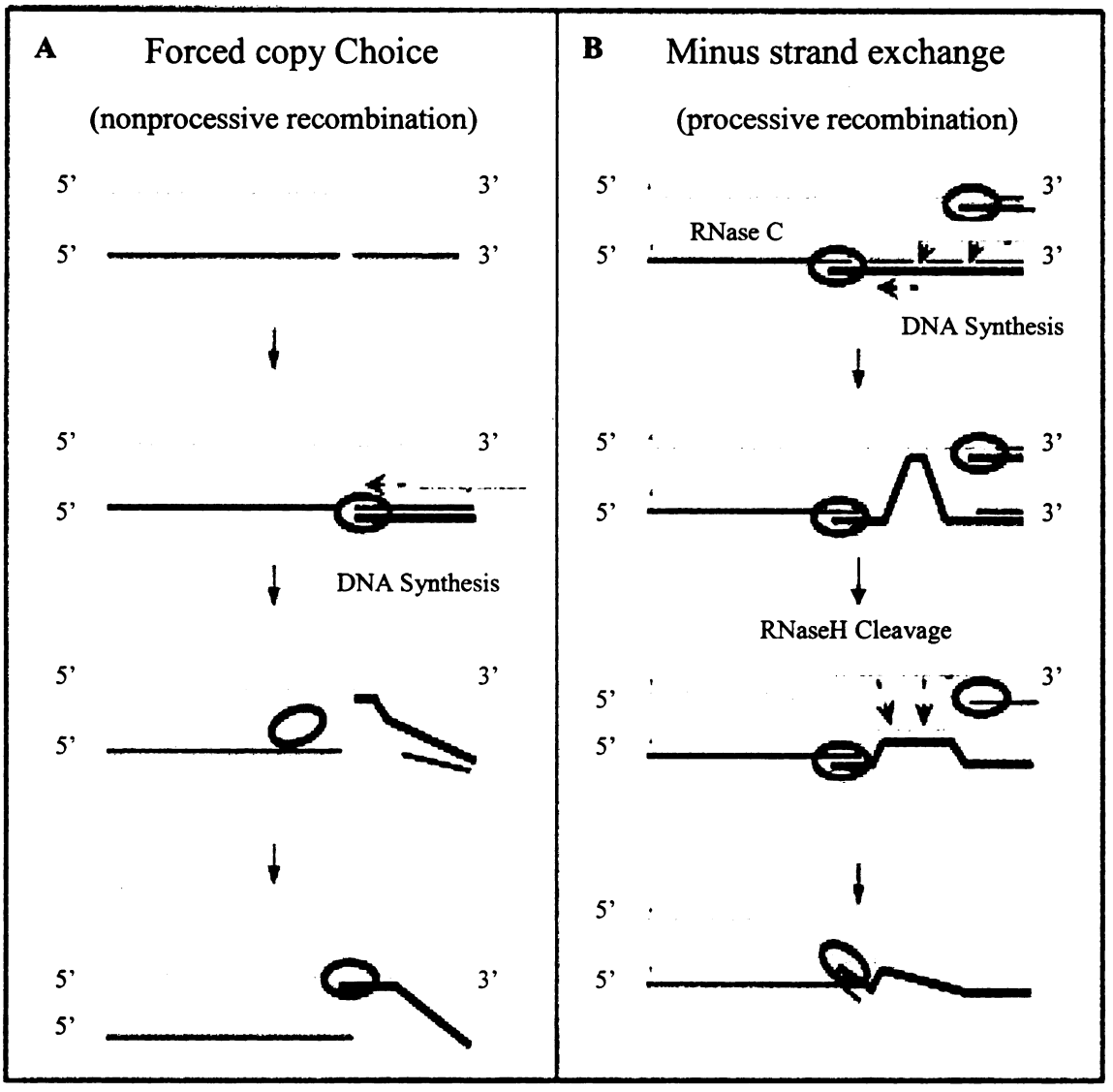


Figure 1.4. Two models of homologous genetic recombination in retroviruses during minus-strand synthesis. (A) Forced copy choice model or nonprocessive recombination is believed to occur when a break in one RNA template (donor template, thin black line) forces reverse transcriptase (RT) to continue DNA polymerization using the homologous region of the copackaged RNA (acceptor template, thin grey line) as a template. (B) During processive recombination or minus strand exchange, DNA synthesis (thick black line) proceeds farther along on one of the RNA molecules (thin black line) than the other (thin grey line). RNase H cleavage (C) of the associated donor RNA template (thin black line) produces internal regions of single-stranded DNA which can then base pair with the other copackaged RNA molecule (acceptor template; thin grey line). Recombination results from a repositioning of the nascent DNA (primer strand) terminus onto the acceptor template strand. Adapted from *Journal of Virology* (Brincat *et al.*, 2002).

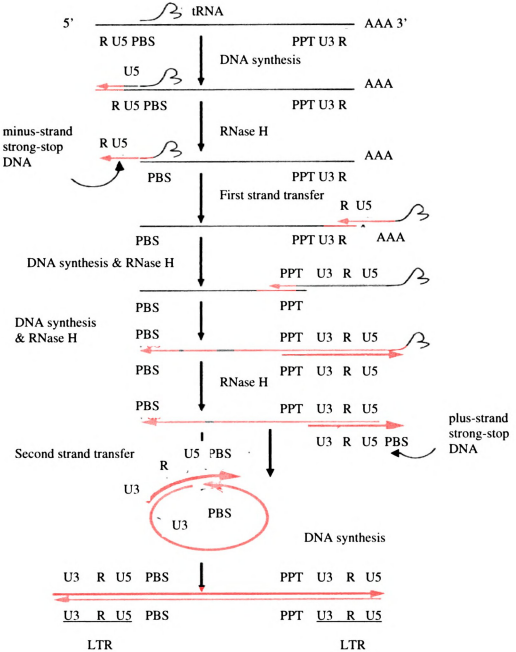


Figure 1.5. Replication of ALV genome through the process of reverse transcription. Black line represents RNA while the light and dark colored lines correspond to minus-strand and plus-strand DNAs respectively. PBS stands for primer binding sequence and PPT corresponds to polypurine tract. For other abbreviations, see Figure 1.2 and the text for a description of this process. Adapted from Retroviruses (Coffin *et al.*, 1997).

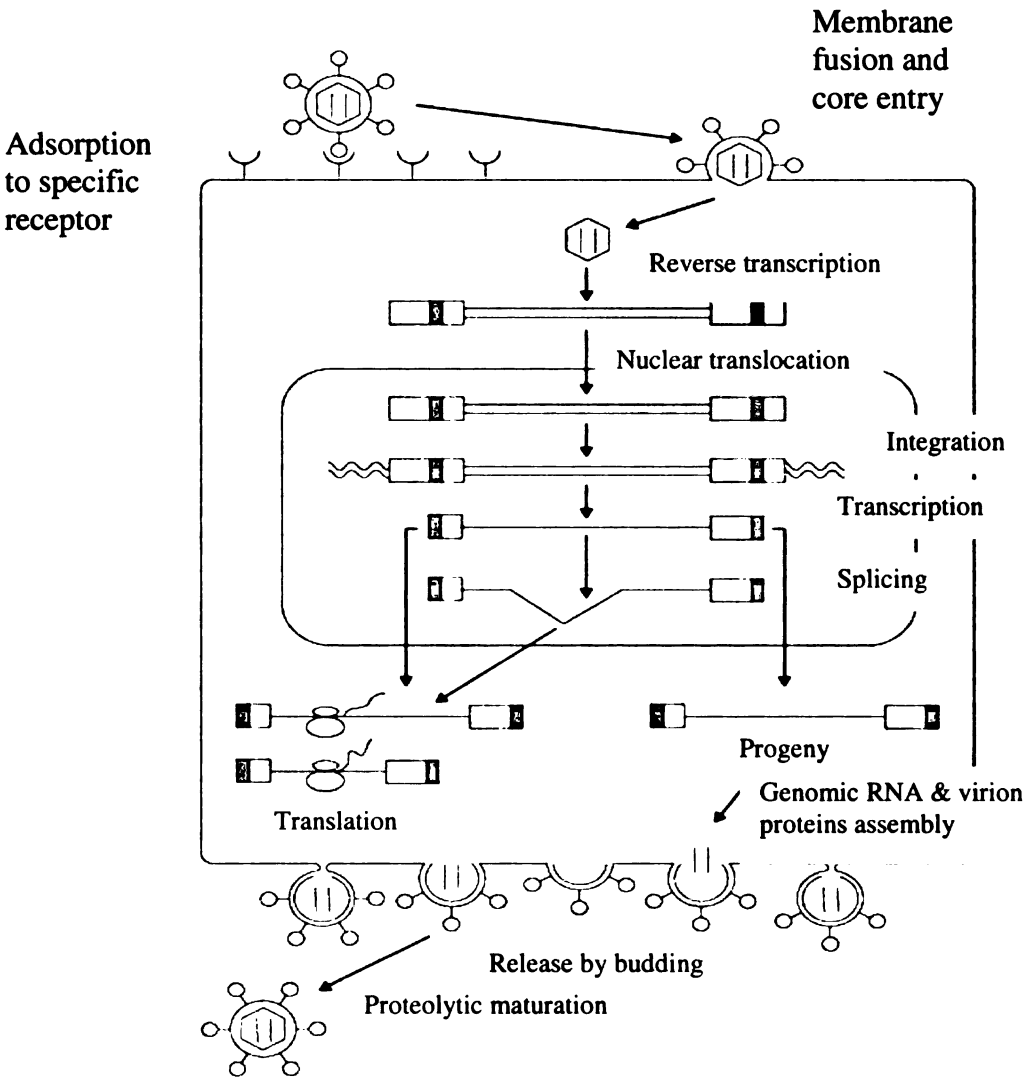


Figure 1.6. The life cycle of retroviruses including ALSVs. The retrovirus particle interacts with specific host cell membrane receptors, fuses directly with the plasma membrane and the viral core gains entry into the cyoplasm. Then, the ss RNA genome is reverse transcribed by retroviral enzymes into ds DNA, which is transported into the nucleus and integrated into the host DNA. The integrated viral DNA or provirus is transcribed by the host RNA polymerase, generating mRNAs and genomic RNA progeny. The host cell machinery translates the spliced mRNAs into glycoproteins and nucleocapsid proteins. The latter assemble with genomic RNA to form progeny nucleocapsids, which interact with the membrane-bound viral glycoproteins. Eventually the progeny virions are released by budding from the host cell membrane followed by proteolytic maturationof virion proteins. Adapted from Retroviruses (Coffin *et al.*, 1997).

CHAPTER 2

Characterization of various isolates of a naturally occurring recombinant avian leukosis virus using biological assays and polymerase chain reaction

ABSTRACT

This study was conducted to evaluate the effect of natural recombination between different ALV subgroups on the relatedness of the resulting strains or isolates. Moreover, in an attempt to develop specific diagnostics, biological assays and polymerase chain reaction were used to characterize seven isolates of a naturally occurring recombinant avian leukosis virus from commercial layer flocks affected with myeloid leukosis (ML). Biological assays of AF 115 isolates were performed to exclude the presence of a mixture of ALV subgroups. Host range utilizing two chicken embryo fibroblasts (line 0 and 7_2 CEFs) and two cell lines (DF-1 and DF-1/J) was examined to propagate and identify ALV subgroups in the seven AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16). Moreover, antigenic relationships of all seven AF 115 isolates with known ALV subgroups were studied by virus neutralization assays. In order to identify the nature of the ALV sequences in the AF 115 isolates, a series of PCR reactions was performed on proviral DNA isolated from line 0 and 7_2 CEFs and DF-1/J cell line infected with the isolates using different combinations of primers specific for different ALV subgroups.

All the AF 115 isolates tested positive for ALV by p27 ELISA on line 0 (C/E) CEFs, DF-1 (C/E) and DF-1/J (C/EJ) cell lines; but tested negative on 7_2 (C/ABDE)

CEFs. Thus, the host range indicated the presence of exogenous ALV subgroups other than subgroup J. Data from virus neutralization assays revealed antigenic relationships of all AF 115 isolates primarily with ALV-B, suggesting that ALVs in AF 115 isolates were of subgroup B envelope. The PCR amplification results, using different primer sets, indicated the presence of ALV-J like and ALV-B like sequences as well as recombinant ALV-B/J sequences in all the AF 115 isolates grown in line 0 CEFs and DF-1/J cell line. Four of the seven AF 115 isolates (AF 115 -1, AF 115-13, AF 115-14 and AF 115- 16) propagated in line 0 CEFs showed identical amplification patterns to the naturally occurring recombinant ALV-B/J (AF 115-4) in PCR tests using different primer sets. This implied that these four isolates may be closely related to the naturally occurring recombinant ALV-B/J, which will be later confirmed by DNA sequencing. The remaining three isolates (AF 115-5, AF 115-7 and AF 115-10) demonstrated different patterns of PCR amplification and may thus be distant from the naturally occurring recombinant ALV-B/J (AF 115-4) and the rest of the AF 115 isolates.

INTRODUCTION

Avian leukosis virus (ALV) is the one of the most common naturally occurring avian retroviruses associated with neoplastic diseases in chickens (Fadly and Payne, 2003). Infection with ALV continues to be an economical threat to the poultry industry due to reduced productivity, high tumor mortality and emergence of new mutant and possibly recombinant viral strains. In chickens, ALVs are classified into six subgroups (A, B, C, D, E and J) depending on viral envelope glycoproteins, which determine host range, antigenicity and viral interference (Coffin, 1992). Based on their mode of

transmission, exogenous ALVs (subgroups A, B, C, D and J) are transmitted vertically and horizontally; while endogenous ALV (subgroup E) is transmitted mainly genetically and sometimes like exogenous viruses (Payne, 1998). In addition, exogenous viruses are usually highly oncogenic while endogenous viruses are non-pathogenic.

In egg-type chickens, subgroups A and B ALVs are most commonly associated with lymphoid leukosis (LL). On the other hand, ALV subgroup J is mainly implicated in causing myeloid leukosis (ML) in meat-type chickens. Currently, ALV infection is largely controlled by identification and eradication of breeder dams and progeny chicks positive for the virus (Spencer, 1984). Yet, high level of horizontal and vertical transmission as well as significant genetic and antigenic variations in different strains and field isolates of ALV have made control and prevention of ALV infection more difficult (Fadly and Smith, 1999; Hunt *et al.*, 1999; Silva *et al.*, 2000; Venugopal *et al.*, 1998). Moreover, the emergence of new mutants and recombinants may confound current field diagnosis of ALV infection by standard biological and molecular methods. Such limitations may have a negative impact on prevention and control of this economically important virus infection in chickens.

Eradication programs have been established and proven effective for controlling exogenous ALV subgroups (Payne and Howes, 1991; Spencer *et al.*, 1977; Venugopal, 1999; Witter and Fadly, 2001). In general, multiple diagnostic tests are used and repeated to identify shedder hens and ALV-positive progeny chicks reared in isolated small groups (Witter *et al.*, 2000). These tests may include isolation of the virus in CEFs of different phenotypes, detection of ALV by ELISA and PCR (Venugopal *et al.*, 1997). Direct ELISA measures p27 group specific antigen (gsa) levels in vaginal and cloacal swabs,

plasma, egg albumin and other clinical specimens (Smith *et al.*, 1979), while indirect ELISA measures antibodies to the gp85 envelope glycoprotein (Hunt *et al.*, 2000). The polymerase chain reaction (PCR) usually utilizes primers specific for different regions of ALV genome mainly *env* and LTR (Silva *et al.*, 2000; Smith *et al.*, 1998a and b).

Chickens and CEFs with different phenotypes constitute essential diagnostic tools in detection of known and new emerging isolates of ALSV. p27 ELISA tests have been used in eradication programs to recognize and remove the chickens exposed to ALV and to screen for new emerging viruses (Venugopal *et al.*, 1997). However, the results from these tests may be confounded by the high antigenic and genetic diversity in the *env* gene of ALV (Silva *et al.*, 2000; Venugopal, 1999; Venugopal *et al.*, 1998). Moreover, the application of polymerase chain reaction (PCR) procedures using primers specific for different regions of the retroviral genomes have contributed to the effectiveness of ALV eradication programs (Fadly and Payne, 2003; Silva *et al.*, 2000; Smith *et al.*, 1998a and b). Nevertheless, these tests may result in false negatives when mutations arise in regions, where primers anneal. Therefore, continuous testing and evaluation of diagnostics especially on new ALV isolates, mutants and recombinants will aid in the present efforts of ALV eradication.

Genetic diversity in retroviruses is mainly attributed to low fidelity of the reverse transcriptase enzyme and the diploid nature of retroviral genome. In fact, retroviruses are known for their high mutation and recombination rates (Elena *et al.*, 2000; Hu and Temin, 1990a and b; Katz and Skalka, 1990; Mansky, 1998; Nichol, 1996). Exogenous retroviruses have been reported to recombine with other exogenous and endogenous retroviruses or nonhomologous cellular genes (Alevy and Vogt, 1978; Blair, 1977; Kawai

and Hanafusa, 1972; Linial and Brown, 1979; Rein, 1982; Svoboda *et al.*, 1986; Weiss *et al.*, 1973; Wyke and Beamand, 1979). For example, subgroup J ALV has *gag* and *pol* genes closely related to those of exogenous ALSV and an *env* gene nearly identical to that in EAV-HI, a member of the EAV family of endogenous retroviruses (Bai *et al.*, 1995b; Benson *et al.*, 1998a; Silva *et al.*, 2000; Smith *et al.*, 1999).

Three ALV isolates were characterized as recombinants with subgroup A gp85, subgroup E gp37 and subgroup J LTR (Lupiani *et al.*, 2000). These viruses were experimentally induced by propagating ALV-J field samples in *alv6* CEFs and they mainly caused LL in layers (Lupiani *et al.*, 2003). Information is currently limited on natural recombination among different ALV subgroups and its impact on virus isolation, diagnosis and pathogenesis in chickens. In 1997, three commercial white leghorn layer flocks used for human vaccine production were hatched in a hatchery used for hatching broiler breeder eggs. They were diagnosed with ML at 33 weeks of age based on gross and microscopic lesions. This was the first reported field case of ML in commercial egg-type chickens (Gingerich *et al.*, 2002). In 2004, Xu and coworkers reported field cases of ML caused by ALV-J in commercial layers from 12 farms in China.

Eight of the seventeen plasma samples (AF 115-1, AF 115-4, AF 115-5, AF115-5, AF115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) tested positive for ALV using p27 ELISA on CEFs of both line 0 (C/E) and *alv6* (C/AE), indicating the presence of an exogenous ALV probably other than subgroup A (Gingerich *et al.*, 2002). Initial characterization of these isolates by PCR analysis of the long terminal repeat (LTR) suggested that they were similar to subgroup J (Gingerich *et al.*, 2002). However, further sequence analysis of one isolate AF 115-4 revealed that it is a recombinant virus

expressing a subgroup B gp85, a subgroup E gp37 and a subgroup J LTR (Lupiani *et al.*, 2006). Inoculation of AF 115-4 into susceptible experimental egg-type chickens and different parental lines of commercial white leghorn layers induced predominately LL (Lupiani *et al.*, 2006; Mays *et al.*, 2006).

The aim of this study was to characterize the seven remaining AF 115 isolates of a naturally occurring recombinant ALV by biological assays and polymerase chain reaction. Two hypotheses were tested in this research work. 1) Closely and distinctly related strains of recombinant ALV can arise from natural recombination between different ALV subgroups in the same outbreak. 2) The annealing location and sequence of PCR primers will reveal variations in different genomic regions of recombinant ALVs. The objectives were to: 1) Compare the host range and antigenic relationship of the seven AF 115 isolates with those of the naturally occurring recombinant ALV-B/J. 2) Show that PCR analysis of DNA of different CEFs and cells infected with AF 115 isolates can reveal the nature or subgroup of ALV sequences present in AF 115 isolates when different primer combinations are used. 3) Demonstrate that PCR amplification patterns of AF 115 isolates with different primers can indicate mutations in different genomic regions and may reveal different viral strains.

MATERIALS AND METHODS

A. Origin of Viruses

Prototypes from various ALV subgroups maintained at Avian Disease and Oncology Laboratory (ADOL) were used as controls in virus propagation and identification using cell cultures, virus neutralization, p27 ELISA and PCR. These

included RAV-1 (subgroup A), RAV-2 (subgroup B), ADOL Hc1 (subgroup J, Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b). In fact, ADOL-Hc1 is the earliest ALV J viral strain isolated in the United States and is considered as the American prototype ALV J (Fadly and Smith, 1999). AF 115-4 is a recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-B/J, Lupiani *et al.*, 2006). The seven remaining AF 115 isolates are AF 115-1, AF 115-5, AF115-5, AF115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16 (Gingerich *et al.*, 2002). Viral stocks of all AF 115 isolates were obtained from line 0 CEFs inoculated with the original AF 115 samples and stored at -80° C. The virus titers of all AF 115 isolates were determined by limiting dilution after a couple of passages in tissue culture using ADOL line 0 secondary chicken embryo fibroblasts (CEFs) that support all ALVs except for the endogenous viruses (Subgroup E ALVs). The titers of AF 115 isolates varied from $10^{5.0}$ to $10^{7.5}$ infectious units per milliliter ($TCID^{50}$/ml) using p27 ELISA performed on supernatants of infected cells in 12 well plates.

B. Biological Assays

Biological assays using CEFs and cell lines with different phenotypes (Fadly and Smith, 1999; Fadly and Witter, 1998) were used to determine ALV subgroup and host range of the different AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16). The cells used for virus propagation were line 0 (C/E) CEFs (Crittenden *et al.*, 1987), 7_2 (C/ABDE) CEFs, DF-1 (C/E) and DF-1/J (C/EJ) cell lines. DF-1 (C/E) is a fibroblastoid cell line developed spontaneously from high density seeding of fibroblasts derived from line 0 chicken embryos (Himly *et al.*, 1998;

Maas *et al.*, 2006). On the other hand, DF-1/J is a genetically engineered cell line expressing envelope protein from ADOL-Hc1 strain of ALV-J (Hunt *et al.*, 1999).

All seven AF 115 samples, recombinant ALV-B/J (AF 115-4), negative (uninfected media) and positive (appropriate prototypes of ALV subgroups) reference virus controls were assayed for virus replication in different cells in duplicates using virologic assays according to the procedures described earlier (Fadly and Witter, 1998). Briefly, around 100 µl of each undiluted viral stock or control (kept at - 80° C) was added to 0.5 x 10^6 secondary CEFs suspended in 4% calf serum (CS, Sigma Chemical Co, St. Louis, MO) in 1:1 Leibowitz's L-15-McCoy's 5A tissue culture medium (LM, Sigma Chemical Co, St. Louis, MO) containing penicillin/streptomycin (Sigma Chemical Co, St. Louis, MO), 2 µg/ml amphotericin B and 2 µg/ml DEAE-Dextran in 35-mm tissue culture plates. Twenty four hours later, the 4% CS LM media was replaced with 1% CS LM media and the plates were incubated in 4% CO_2 at 37° C for 7-9 days.

C. p27 ELISA Assay

All the infected and uninfected cells (CEFs and cell lines) described earlier were completely lysed with 50 µl of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and two alternate cycles of freezing at - 80° C and thawing at 37° C. About 100 µl of the cell lysate was used to test for p27 group-specific antigen (gsa) by enzyme-linked immunosorbent assay (ELISA) (Smith *et al.*, 1979). The p27 gsa ELISA was carried out using rabbit anti-p27 polyclonal antibody (IgG) coated immunolon® plates (Dynatech, Chantilly, VA), rabbit anti-p27 IgG antibody conjugated to horse-radish peroxidase (SPAFAS, Storrs, CT) and TMB substrate (3, 3′, 5, 5′-tetramethyl benzidine, BD

Biosciences Pharmingen, San Diego, CA). The plate was read at an absorbance of 630 nm within one hour of stopping the reaction with stop buffer (Sigma Chemical Co, St. Louis, MO) using a MRX microplate reader (Dynex, Chantilly, VA).

D. Virus Neutralization

Virus neutalization tests were performed for typing and determining the antigenic relationship of all seven AF 115 isolates as well as recombinant ALV-B/J (AF 115-4) to ALV subgroups B and J as described earlier (Fadly and Witter, 1998). Subgroup B ALV (RAV-2) and ALV-J (Hc1) were included to confirm neutralization by the respective subgroup-specific antibody controls. As a negative control, negative sera and media were used to assess for cross contamination of samples. Specific antibodies to gp85 of ALV subgroups B (anti-RAV-2) and J (anti-Hc1) or negative sera were diluted 1:5 in serum-free LM media and incubated at 56° C for 30 minutes to denature the complement factors. About 500 - 1000 ALV viral particles from each AF 115 sample, ALV-B (RAV-2) and ALV-J (Hc1) in 50 µl LM media were incubated with 50 µl of heat-denatured 1:5 diluted antibodies or negative controls in duplicates in 96-well flat bottomed tissue culture plates for 45 minutes at 37° C and 4% CO_2.

After the incubation, about $5x10^4$ cells of line 0 CEFs in 150 µl of 4% CS LM media were added into each of the 96 wells and incubated in 4% CO_2 at 37° C for a day and later replaced by 1% CS LM media and incubated in 4% CO_2 at 37° C for 7-9 days. At the end of incubation, the cell cultures were completely lysed with 20 µl of 0.5% tween 80 (Sigma Chemical Co, St. Louis, MO) and were subjected to two alternate cycles of freezing at -70° C and thawing at 37° C. The cell lysates were tested for p27 gsa

ELISA as described earlier (Smith *et al.*, 1979). Samples with a chromogenic reading of 1 or negative on the p27 gsa ELISA read-out was considered to be positive for neutralization by the reference antibodies and samples with a chromogenic reading of ≥ 2 on the p27 gsa ELISA read-out was considered to be negative.

E. DNA Isolation

After 7-9 days of virus replication in 35-mm plates of tissue culture, total DNA was extracted from uninfected and infected cells (line 0 and 7_2 CEFs and DF-1/J cells) using standard proteinase K, phenol-chloroform extraction procedures (Maniatis *et al.*, 1982). Briefly, the medium was removed and the cells were scraped off the plate and pelleted by centrifugation. The pellet was treated with lysis buffer (10 mM TRIS, 1 mM EDTA, 100 mM NaCl and 0.5% SDS) and proteinase K (100 µg/ml) overnight at 56°C. DNA was extracted twice with phenol-chloroform, treated with RNAse (2 µl/ml) at 37°C for an hour, precipitated with ice-cold absolute ethanol, dried at room temperature, dissolved in TE buffer and stored at 4°C.

F. Oligonucleotide Primers

Most of the primer sets specific for one or different ALV subgroups were adapted from the literature (Silva *et al.*, 2000 and 2007; Smith *et al.*, 1998a and b) and used for PCR detection and identification of ALV subgroups in the AF 115 isolates. To generate PCR primers for subgroup B LTR, assembly and alignment of available subgroup B viral strains and isolates contained in GenBank were performed. Based upon the alignment and

the common sequences, PCR primers (Table 2.1) were designed based on U3 and U5 common sequences of LTR of subgroup B ALV isolates using Oligo 4.0-s software (National Biosciences, Plymouth, MN). The forward and reverse PCR primers adapted from other studies, their nucleotide sequences, their annealing locations in the ALV genome and their ALV subgroup specificities are listed in Tables 2.2 and 2.3 respectively. The PCR primer pairs and PCR cycle used, the estimated amplified PCR product size, the ALV subgroup specificity are listed in Table 2.4.

G. Polymerase Chain Reaction (PCR)

In each PCR assay, positive and negative controls were included with the AF 115 samples. Negative controls consisted of molecular grade water and uninfected cells in order to test for cross contamination. At least duplicate PCR reactions were carried out for each primer set used to amplify ALV sequences in the AF 115 isolates. Each 50-ml PCR reaction contained 1x reaction buffer (A, B or C, Table 2.5), 25 mM of each dNTP, 25 pmoles of each primer, 2.5 units Taq polymerase, and around 100 ng of template DNA or water. PCR reaction buffer systems A and D were used with most of the primer sets for screening under six different PCR cycles or programs. On the other hand, PCR buffer reaction systems B and C were employed to resolve the problem with extra bands for two primer pairs, RS-7J and R8 and SRB-F and R8 (Table 2.5). Moreover, buffer system D was mainly used for the designed subgroup B ALV-specific PCR primer pairs and it contained reaction buffer, dNTPs, Taq and tracking dyes.

The following PCR conditions (cycle A, Table 2.6) were used with most of the primer pairs; following an initial template melting or preheating step at 95°C for 3 min,

the DNA was amplified during 30 cycles with denaturation at 95°C for 1 min, primer annealing at 57°C for 1 min, and amplification or extension at 72°C for 2 min, followed by a final elongation step at 72°C for 10 min and cold storage at 4°C in PCR thermocycler, PTC-100 Programmable Thermal Controler, Peltier-effect Cycling (MJ Research, Inc., Waltham, MA). Five other PCR cycles performed for some PCR primer sets were similar to the previously mentioned PCR conditions except for differences in the temperatures and times of primer annealing and extension (Table 2.6). Amplified products were resolved by electrophoresis through 1% agarose gel in 1X TAE buffer containing 200 ng/ml ethidium bromide and examined under ultraviolet transillumination.

RESULTS

A. Host range of AF 115 isolates

Host range of each of the seven AF 115 isolate was determined in different CEFs and cell lines. Line 0 (C/E) is obtained from chicken lines known to be sensitive to all ALV subgroups except E. On the other hand, 7_2 (C/ABDE) is resistant to subgroup A, B, D and E. Cell lines DF-1 (C/E) is sensitive to all ALV subgroups except E, while DF-1/J (C/EJ) is resistant to subgroups J and E ALVs. As seen in Table 2.7, no ALV was detected in 7_2 CEFs inoculated with any of the seven AF 115 isolates or the recombinant ALV-B/J (AF 115-4), while ALV was detected by p27 ELISA in line 0 CEFs, DF-1 and DF-1/J infected cells.

Results obtained from cell culture analysis indicated the presence of an exogenous ALV subgroup (Subgroup A, B or D) and the absence of ALV-J in the AF 115 isolates. In addition to the seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4),

negative (uninfected media) and positive (appropriate prototypes of ALV subgroups) virus controls were used to test the functionality of the assay and assess cross contamination. Infectivity of different cells with the negative and positive controls were as expected and verified the functionality of the procedures and confirmed the absence of any cross contamination.

B. Antigenicity of AF 115 isolates

Virus neutalization tests were performed for typing the seven remaining AF 115 isolates (subgroup classification) and determining the antigenic relationship of AF 115 isolates to ALV subgroups B and J. As noticed in Table 2.8, all AF 115 isolates as well as recombinant ALV-B/J (AF 115-4) were neutralized by ALV subgroup B gp85-specific antibodies, but not by ALV-J gp85-specific antibodies. In other words, no ALV was detected by p27 ELISA in line 0 CEFs inoculated with AF 115 isolates or recombinant ALV-B/J (AF 115-4) and ALV subgroup B gp85-specific antibodies (anti-RAV-2).

On the other hand, ALV was detected in line 0 CEFs inoculated with AF 115 isolates and recombinant ALV-B/J (AF 115-4) incubated with ALV subgroup J gp85-specific antibodies (anti-Hc1). The functionality of the test was verified through neutralization of subgroup B ALV (RAV-2) and ALV-J (Hc1) by the respective subgroup gp85-specific antibodies. The absence of neutralization of all viruses used when incubated with the negative control (negative sera and media) confirmed that no cross contamination among samples took place.

C. PCR amplification of DNA from AF 115-infected line 0 CEFs

1. Detection of ALV sequences

In order to identify and confirm ALV subgroups present in the seven AF 115 isolates, a series of PCR reactions was performed using different sets of primers specific for all ALV subgroups or certain ALV subgroups such as ALV-B, ALV-J and ALV-B/J. In fact, it is critical to establish background or endogenous levels of PCR product, which may be spuriously or non-specifically amplified from cells proposed for use in ALV isolation and propagation. In each PCR run, water and uninfected line 0 CEFs were used to assess cross contamination, background levels and proper procedure or technical errors. As expected, there were no detectable PCR products in any of the negative controls in any of the PCR runs performed (Figures 2.1 - 2.10).

To confirm the presence of ALVs in these isolates, DNA prepared from line 0 CEFs infected with AF 115 isolates were amplified using two all-ALV subgroup primer pairs (ARK 4836 & REVSP 6683 and RS-7J & REVSP 6683) and primers specific for ALV A-E (H5 & AD1). Table 2.9 demonstrated that the three different primer sets amplified different PCR products or DNA fragments from line 0 CEFs inoculated with all seven AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16). It was not expected to observe amplification in DNA of Hc1-infected line 0 CEFs using primer pair H5 & AD1. In addition, DNA from all ALV positive controls including naturally occurring recombinant ALV-B/J (AF 115-4), RAV-2 (subgroup B ALV) and Hc1 (subgroup J ALV) tested positive for the respective ALV PCR products. Figure 2.1 represented a successful amplification of ALV sequences in all ALV infected samples by all-ALV subgroup primer pair (RS-7J & REVSP 6683).

2. Detection of recombinant ALV sequences

To find out whether recombinant ALVs were present in the seven AF 115 isolates, DNA extracted from these isolates were PCR amplified using two primer combinations (SRB-F & S2 and SRB-F & R8), which detect sequences from recombinant ALV-B/J (AF 115-4). SRB-F was chosen since it annealed to gp 85 of *env* of subgroup B ALV and S2 and R8 were selected because they annealed to LTR of subgroup J ALV. PCR analysis of DNA from all seven AF 115 isolates-infected line 0 CEFs (SRB-F & R8) showed a recombinant-specific fragment or an amplified product of the same size as that from the naturally occurring recombinant ALV-B/J (Table 2.10 and Figure 2.2).

As shown in Table 2.10, DNA from line 0 CEFs inoculated with four AF 115 isolates (AF 115-1, AF 115-13, AF 115-14 and AF 115-16) showed the presence of the expected PCR products using SRB-F & S2 primer pair. On the other hand, no fragments were obtained from proviral DNA of AF 115-5 and AF 115-10. A very faint band of PCR product was observed in AF 115-7-infected line 0 CEFs using SRB-F & S2 primer pair (Figure 2.3). As predicted, no amplification products of expected sizes with any of the two PCR primer sets were detected in ALV belonging to subgroups B (RAV-2) and J (Hc1). However, faint non-specific band of a smaller size (0.6 Kb) was detected in DNA of RAV-2-infected line 0 CEFs when SRB-F & R8 primers were used. Moreover, using different buffer systems and PCR cycles did not remove or resolve the non-specific binding in RAV-2, the naturally occurring recombinant ALV-B/J and AF 115 isolates.

3. Detection of ALV-B sequences

To test the presence of subgroup B ALV-like sequences in the seven AF 115 isolates, DNA prepared from these isolates were PCR amplified using different primer combinations specific for subgroup B ALV sequences, which were already published or newly designed. Amplification of RAV-2 (subgroup B ALV)-infected line 0 CEFs DNA using all ALV-B-specific primer sets resulted as predicted in all cases in significant yield of the expected size fragments. None of the primer sets produced amplification products in Hc1-infected and uninfected line 0 CEFs (Table 2.11).

The first two primer pairs (ARK 4836 & SRB-R and SRB-F & REVSP 6683) amplified PCR products when all seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4) were propagated in line 0 CEFs (Table 2.11). Figure 2.4 showed a successful PCR reaction of subgroup B (RAV-2), naturally occurring recombinant ALV-B/J and all seven AF 115 samples with a representative primers ARK4836 & SRB-R.

Similar PCR analysis results were observed with primer SRB-F in combination with one of two of the newly designed primers B LTR 3.3 and B LTR 6.3. Amplified products yielding the expected sizes were successfully obtained from proviral DNA of all seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4) grown in line 0 CEFs (Table 2.11). On the other hand, three primer sets (SRB-F & B LTR 2.3, SRB-F & B LTR 4.3 and SRB-F & B LTR 8.3) detected RAV-2 (subgroup B ALV), but none of the seven AF 115 isolates or the naturally occurring recombinant ALV-B/J. Figure 2.5 denoted a representative of a successful amplification of RAV-2 using subgroup B ALV-specific primers (SRB-F & B LTR 8.3).

Using SRB-F and B LTR 1.3 primers, amplification of DNA of AF 115-1, AF 115-5, AF 115-7, AF 115-10 and the naturally occurring recombinant ALV-B/J (AF 115-4) propagated in line 0 CEFs produced a faint band of expected size. However, AF 115-13, AF 115-14 and AF 115-16 were not amplified successfully when the same primers were utilized (Table 2.11). PCR with SRB-F & B LTR 5.3 primers resulted in amplification of fragments from the naturally occurring recombinant ALV-B/J (AF 115-4) and all AF 115 isolates except AF 115-5 and AF 115-10. Moreover, when SRB-F & B LTR 7.3 primer combination were used, faint PCR products were detected from DNA of the naturally occurring recombinant ALV-B/J (AF 115-4) and all AF 115 isolates except AF 115-5, AF 115-10 and AF 115-14, which were not amplified (Figure 2.6).

4. Detection of ALV-J sequences

PCR analysis was performed to examine the presence of subgroup J ALV-like sequences in the seven AF 115 isolates using different already published primer sets specific for subgroup J ALV sequences. As expected, PCR using the different primer combinations amplified fragments with the expected sizes from line 0 CEFs inoculated with Hc1 (subgroup J ALV). None of the primers produced PCR products in RAV-2-infected and uninfected line 0 CEFs (Table 2.12).

Five primer pairs (H5 & H7, RS-6J & REVSP 6683, RS-6J & S2, RS-6J & R8 and RS-6J & R5) produced specific fragments of the expected sizes only from DNA of Hc1-infected line 0 CEFs (Table 2.12). However, PCR failed to amplify products from DNA of the AF 115 isolates or the naturally occurring recombinant ALV-B/J (AF 115-4). Figure 2.7 designated positive amplification results of subgroup J ALV (Hc1) using

subgroup J ALV-specific primers (RS-6J & S2). Conversely, PCR products were amplified from all seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4) when three primer sets (ARK 4836 & R8, F5 & R8 and RS-7J & R8) were employed. Figure 2.8 referred to a successful PCR amplification of Hc1, the naturally occurring recombinant ALV-B/J and all seven AF 115 samples using primer pair RS-7J & R8. A faint non-specific band of smaller size (1.2 Kb) was observed in RAV-2-infected line 0 CEFs in addition to extra faint non-specific bands in AF 115 isolates. In addition, the non-specific binding in RAV-2, the naturally occurring recombinant ALV-B/J and AF 115 isolates did not disappear when different buffer systems and PCR cycles were utilized.

PCR tests using primer combinations (RS-7J & S2 and S1 & S2) revealed that AF 115-1, AF 115-13, AF 115-14 and AF 115-16 in addition to the naturally occurring recombinant ALV-B/J (AF 115-4) were positive. On the contrary, AF 115-5, AF 115-7 and AF 115-10 gave a negative PCR tests (Table 2.12). However, we were not able to replicate the amplification results of these AF 115 isolates with S1 & S2 primers. Figure 2.9 illustrated PCR assay results of AF 115 isolates and other ALV subgroups with primers RS-7J & S2. Furthermore, PCR amplification of all AF 115 isolates except AF 115-10 revealed fragments similar in sizes to those of the naturally occurring recombinant ALV-B/J (AF 115-4) when primer sets, RS-7J & R5 and F5 & R5, were used. Figure 2.10 displayed PCR analysis results of all seven AF 115 isolates and representatives of ALV subgroups employing primer pair RS-7J & R5.

D. PCR reactivity of AF 115 isolates in 7_2 infected CEFs

PCR analysis was conducted on DNA extracted from 7_2 CEFs inoculated with each of the seven AF 115 isolates, the naturally occurring recombinant ALV-B/J (AF 115-4), positive (Hc1) and negative (RAV-2) control ALVs. The primer combinations utilized were either all-ALV subgroup primer pairs (ARK 4836 & REVSP 6683) or subgroup J ALV-specific primer sets (S1 & S2, RS-6J & S2, H5 & H7 and F5 & R8) since it was expected that only subgroup J ALV will be able to infected these CEFs. Since 7_2 CEFs are resistant to subgroup B ALV, primer specific for ALV-B were not utilized in the PCR assays. These PCR tests were performed in order to investigate whether the AF 115 isolates could infect 7_2 CEFs and if they contain mixture of ALV subgroups as well as to confirm the biological assays results.

The results shown in Table 2.13 indicate that no PCR products or fragments were detected in the DNA prepared from 7_2 CEFs infected with any of the seven AF 115 isolates, the naturally occurring recombinant ALV-B/J (AF 115-4) or RAV-2 (subgroup B ALV) or uninfected 7_2 CEFs controls with any of the primer sets used. As predicted, successful amplification of DNA of Hc1-infected 7_2 CEFs was observed with all the primers employed. Figure 2.11 revealed PCR amplification results of proviral DNA of all seven AF 115 isolates employing primer pair ARK 4836 & REVSP 6683.

E. PCR reactivity of AF 115 isolates in DF-1/J infected cells

1. Detection of ALV sequences

PCR analysis results of DNA prepared from AF 115-infected DF-1/J cells using different primer combinations were similar to those of line 0 infected CEFs (Table 2.14). Similarly, there were no observable PCR fragments in any of the negative controls or

uninfected samples including water and uninfected DF-1/J cells in any of the PCR runs performed (Figures 2.12 - 2.16). However, ALV was not detected from DNA extracted from Hc1-infected DF-1/J cells in any of the PCR reactions utilizing different primers (Table 2.14).

In order to confirm the infection of DF-1/J cells with ALVs in the AF 115 isolates, DNA of DF-1/J cells inoculated with all seven AF 115 isolates were amplified using an all-ALV subgroup primer pair (ARK 4836 & REVSP 6683). Figure 2.12 verified that this primer set amplified DNA fragments from DF-1/J infected with all seven AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16). Amplifiable DNA was also observed from all ALV positive controls including naturally occurring recombinant ALV-B/J (AF 115-4) and RAV-2 (ALV-B).

2. Detection of ALV-B sequences

To verify the existence of subgroup B ALV-like sequences in DNA prepared from DF-1/J infected with the seven AF 115 isolates, the naturally occurring recombinant ALV-B/J (AF 115-4) and subgroup B ALV (RAV-2), PCR assay was carried out employing subgroup B ALV-specific primers (ARK 4836 & SRB-R). Products of identical sizes were obtained from all seven AF 115 isolates, ALV-B/J and RAV-2 (Table 2.14 and Figure 2.13).

3. Detection of ALV-J sequences

PCR analysis was performed to study subgroup J ALV-like sequences in AF 115 isolates-infected DF-1/J using different primer pairs (S1 & S2, H5 & H7 and F5 & R8).

Since DF-1/J is resistant to infection by subgroup J ALV, DNA from Hc1-infected line 0 CEFs were used in PCR as a positive control. PCR products were only observed in DNA prepared from Hc1-infected line 0 CEFs when primer pairs (S1 & S2, and H5 & H7) were used. Figure 2.14 revealed PCR amplification results of all seven AF 115 isolates and representatives of ALV subgroups employing primer pair H5 & H7. On the other hand, primers F5 & R8 amplified fragments from all seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (Figure 2.15). In all cases, PCR failed to amplify DNA extracted from RAV-2-infected DF-1/J cells.

4. Detection of recombinant ALV sequences

To determine if recombinant ALV-B/J propagated in DF-1/J cells, DNA of DF-1/J infected cells with the seven AF 115 isolates were PCR amplified using primers SRB-F & R8. Positive PCR signals of the expected size were observed in all seven AF 115 isolates as well as the positive control, the naturally occurring recombinant ALV-B/J (Table 2.14 and Figure 2.16). As predicted, no amplification products were detected in the negative controls, ALV belonging to subgroups B (RAV-2) and J (Hc1).

DISCUSSION

ALV infections can be mainly controlled through the early detection and removal of virus-shedding birds to reduce the congenital and contact infection to other birds (Smith *et al.*, 1998b; Witter *et al.*, 2000). Diagnosis of ALV infections is based on assays for virus isolation, which identify and classify new isolates. Currently, virus isolation and propagation are carried out in cell culture, followed by indirect biological assay such as

p27 ELISA for ALV (Crittenden *et al.*, 1987; Fadly and Witter, 1998). In all biological assays, using chicken embryo fibroblasts (CEFs) with different phenotpyes helps in characterizing the new isolates. It was shown by many investigators that CEFs resistant to different ALV subgroups can be utilized to confirm the subgroup of the isolated ALV (Crittenden and Salter, 1990; Fadly and Payne, 2003; Hunt *et al.*, 1999; Salter and Crittenden, 1991).

Various approaches were used to characterize and identify ALV subgroups present in the seven AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16). In fact, host range pattern constitutes the most reliable method for subgroup classification (Fadly and Payne, 2003). Propagation of these isolates was investigated in line 0 CEFs (C/E), 7_2 CEFs (C/ABDE), DF-1 (C/E) cells and DF-1/J (C/EJ) cells to exclude the presence of a mixture of ALV subgroups. The host range or cell culture analysis of the seven AF 115 isolates suggested that ALVs from all isolates belonged either to ALV subgroup A, B or D, but not to J (Table 2.7).

Previous observations showed that all AF 115 isolates tested positive for ALV on CEFs of line 0 (C/E), 15 B1 (C/O), 7_1 (C/A) and cell lines of DF-1 (C/E) and DF-1/J (C/EJ); but tested negative on 7_2 (C/ABDE) CEFs (Lupiani and Fadly, unpublished data). Analysis of all data obtained from current and previous virus isolation and characterization assays indicated that all AF 115 isolates may have subgroup B or D ALV and no subgroup J ALV. The fact that subgroup D ALV is rarely found in the field suggested that subgroup B ALV may be the ALV subgroup present in these isolates. The subgroup of ALV present in these isolates will be later confirmed by sequence analysis.

Antigenicity is usually determined by virus neutralization with known subgroup-specific antibodies (Fadly and Payne, 2003). It can also be used for strain classification, but is less reliable than host range assay. The finding that all AF 115 isolates were neutralized by ALV subgroup B gp85-specific antibodies, but not by ALV-J gp85-specific antibodies suggested antigenic relationships of all AF 115 isolates primarily with ALV-B (Table 2.8). Moreover, earlier flow cytometry results indicated that DF-1 cells infected with the different AF 115 isolates reacted with chicken polyclonal serum specific for ALV subgroup B but not subgroups A and J specific anti-sera (Lupiani and Fadly, unpublished data).

Analysis of the virus neutralization results in addition to previous flow cytometry data confirmed the host range data, indicating the presence of a subgroup B exogenous ALV and the absence of subgroup J ALV. Even though there is a partial cross-neutralization between ALV subgroups B and D, presence of subgroup D ALV in AF 115 isolates may be excluded since it is rarely found in the field and this will later be confirmed by DNA sequence analysis.

Molecular techniques such as PCR have been proven to be rapid, specific, sensitive and effective in ALV detection (Garcia *et al.*, 2003; McKay, 1998; McKay and Rosales, 2000; Payne, 2000; Smith *et al.*, 1998a and b). Currently, PCR technology could be used to characterize and establish relationships among emerging ALV field isolates including mutants and recombinants. Available sequence data about ALV genome has been used to design primers for detection of ALV by PCR (Bai, 1995a and b; Coffin, 1992; Silva *et al.*, 2000 and 2007; Smith *et al.*, 1998a and b; Van Woensel *et al.*, 1992). In addition, most newly designed primers anneal at *env* and LTR regions.

The continuous variations exhibited by ALV resulted in the development of new specific reagents for the amplification and characterization of new emerging isolates (Garcia *et al.*, 1998; Lupiani *et al.*, 2000 and 2006; Silva *et al.*, 2000; Venugopal, 1999; Venugopal *et al.*, 1998). For example, specific primer pairs were developed for the detection of recombinant subgroup J ALVs expressing subgroup A envelope (Lupiani *et al.*, 2000). However, if mutations occur in ALV genome where primers anneal, the desired sequences will not be amplified, not all variant viruses will be detected and false negatives will result.

The data obtained from PCR analysis of genomic DNA extracted from line 0 CEFs and DF-1/J cells inoculated with the seven AF 115 isolates using all-ALV primers (ARK 4836 & REVSP 6683 and RS-7J & REVSP 6683) indicated the presence of ALV sequences in these isolates. The finding that ALV sequences were detected in DNA prepared from line 0 CEFs (Table 2.9; Figure 2.1) and DF-1/J cells (Table 2.14 and Figure 2.12), but not 7_2 CEFs (Table 2.13; Figure 2.11) confirmed the host range and antigenicity data, indicating the presence of subgroup B ALV in AF 115 isolates.

Collectively, analysis of all PCR assays conducted here revealed that there no cross contamination among samples, background or endogenous levels of ALV in uninfected line 0 or 7_2 CEFs or DF-1/J cells. This conclusion is supported by the absence of any observable background or any detectable PCR products in any of the negative controls used including uninfected line 0 CEFs (Table 2.9 and Figures 2.1 - 2.10), 7_2 CEFs (Table 2.13; Figure 2.11) and DF-1/J cells (Table 2.14; Figures 2.12-2.16).

Given that an earlier study indicated that H5 and AD1 primers were designed to detect ALV subgroup A-E, but not ALV-J (Smith *et al.*, 1998b). In our study, we were

not expecting to detect PCR products from DNA isolated from Hc1-infected line 0 CEFs using these primers (Table 2.9). The reasons for this discrepant result were not clear; but they might be due to differences in the ALV-J isolate used in the earlier study (HPRS-103). Different strains of ALV-J may have variable nucleotide sequences in the *env* gene (gp 85 or SU), where AD1 primer can anneal to one, but not the other.

Analysis of amplification results attained from line 0 CEFs and DF-1/J cells infected with all seven AF 115 isolates using primer set SRB-F & R8 showed a recombinant-specific fragment of the same size as that from the naturally occurring recombinant ALV-B/J (Tables 2.10 and 2.14; Figures 2.2 and 2.16). The results presented here demonstrated that primers SRB-F & R8 are specific for recombinant ALV-B/J found in all seven AF 115 isolates propagated in line 0 CEFs and DF-1/J cells. In addition, no spurious or non-specific amplification were observed in uninfected line 0 CEF and DF-1/J cells respectively indicating the absence of endogenous sequences that may cross-hybridize with these PCR primers. Therefore, these primers constitute the principle tools for confirming the isolation and detection of recombinant ALV-B/J from field isolates as well as experimental settings.

Although the recombinant ALV-B/J-specific primer set SRB-F & S2 detected most of the AF 115 isolates, certain isolates such as AF 115-5, AF 115-7 and AF 115-10 were not detected, confirming molecular variation among AF 115 isolates (Table 2.10; Figure 2.3). We hypothesize that sequence analysis of these later isolates may show nucleotide sequence changes in their LTRs where S2 primer anneals (U5 region). Thus, the annealing location and sequence of the primers can provide indications for subgroup

designation of field isolates. They may also provide information about mutations in ALV genomes and variations among various ALV strains.

All primer combinations used to amplify subgroup B ALV sequences were specific since they amplified PCR fragments from RAV-2-infected, but not Hc1-infected or uninfected line 0 CEFs and DF-1/J cells (Tables 2.11 and 2.14). However, some of the primer pairs were also able to detect the naturally occurring recombinant ALV-B/J (AF 115-4) and all seven AF 115 isolates since the forward and reverse primers were able to anneal to complementary sequences in different locations of the ALV genomes of these isolates (Tables 2.11 and 2.14). These primer sets included ARK 4836 & SRB-R, SRB-F and REVSP 6683, SRB-F & B LTR 3.3 and SRB-F & B LTR 6.3 (Figures 2.4 and 2.13). Other primers such as SRB-F & B LTR 1.3, SRB-F & B LTR 5.3 and SRB-F & B LTR 7.3 amplified PCR products from some of the AF 115 isolates to varying degrees since the reverse primers were not able to anneal to LTR sequences of all AF 115 isolates (Figure 2.6). This may be due to the presence of sequence variations in the LTR sequences of AF 115 isolates where these primers are designed to anneal. Data from PCR assays presented here clearly document that all AF 115 isolates contain subgroup B ALV-like sequences, thus confirming host range and antigenicity results. However, BLAST search of GenBank indicated that some of the primers might anneal to sequences from ALV subgroups A, C, D and J to varying degrees. On the other hand, three primers (SRB-F & B LTR 2.3, SRB-F & B LTR 4.3 and SRB-F & B LTR 8.3) were very specific since they only detected DNA of RAV-2-infected line 0 CEFs (Figure 2.5). The failure of detection of ALV sequences in AF 115 isolates by these primers may be due to the differences in the LTR sequences of RAV-2 (ALV-B) and AF 115 isolates (ALV-B/J).

Analysis of PCR assays with five primer sets specific for ALV-J (H5 & H7, RS-6J & REVSP 6683, RS-6J & S2, RS-6J & R8 and RS-6J & R5) revealed positive reaction with DNA obtained from line 0 and 7_2 CEFs, but not DF-1/J cells inoculated with Hc1 (Tables 2.12-2.14; Figures 2.7 and 2.14). Since DF-1/J is resistant to infection by subgroup J ALV, ALV was not detected from DNA extracted from Hc1-infected DF-1/J cells in any of the PCR reactions utilizing different primers (Table 2.14). These primers seem to be very specific to ALV-J since the forward and reverse primers anneal only to the envelope and LTR regions of ALV-J. In addition, there were no amplifications from DNA extracted RAV-2-infected or uninfected line 0, 7_2 CEFs and DF-1/J cells using all the primer combinations for detection of ALV-J-like sequences (Tables 2.14-2.15; Figures 2.7-2.10 and 2.14). PCR products of expected sizes were found in AF 115 isolates-infected in addition to the naturally occurring recombinant ALV-B/J (AF 115-4)-infected line 0 CEFs and DF-1/J when primer pairs ARK 4836 & R8, F5 & R8 and RS-7J & R8 were utilized (Tables 2.12 and 2.14; Figures 2.8 and 2.15). These amplifications were expected since two of the forward primers used (ARK4836 & RS-7J) were generic and able to anneal to all ALV subgroups. On the other hand, F5 is a forward primer, which usually anneals to ALV-J envelope, but it annealed to ALV-B/J and other AF 115 isolates since the 10 nucleotide at the 3' end of the primer has 100% homology with ALV-B envelope. Moreover, the reverse primer (R8) is specific to LTR of ALV-J and hence the amplification of ALV-J, ALV-B/J and all isolates. These results significantly establish that all seven AF 115 isolates contain subgroup J ALV-like sequences.

Four primer combinations (RS-7J & S2, S1 & S2, RS-7J & R5 and F5 & R5) resulted in variable patterns of amplifications of AF 115 isolates. This may be due to the

difference in the nucleotide sequences in different regions of ALV sequences in our isolates affecting the annealing of the different primers. Moreover, four of the seven AF 115 isolates, namely AF 115 -1, AF 115-13, AF 115-14 and AF 115- 16 showed identical amplification patterns in PCR tests using these primer combinations to that of the naturally occurring recombinant ALV-B/J (Table 2.12; Figures 2.9-2.10) in infected line 0 CEFs. This revealed that these four isolates may be identical or closely related to the naturally occurring recombinant ALV-B/J (AF 115-4) which will be later confirmed by DNA sequencing. The remaining three isolates, AF 115-5, AF 115-7 and AF 115-10 demonstrated different pattern of PCR amplification and may thus be different or distantly related to the naturally occurring recombinant ALV-B/J (AF 115-4) and the rest of the AF 115 isolates. While a more thorough study of the molecular makeup of these isolates through sequence analysis is necessary, the results observed here along with our earlier findings suggest that these isolates may display genomic diversity or nucleotide-level differences including mutations, deletions and insertions.

There was discrepant results regarding the detection of ALV-J sequences in DNA prepared from AF 115 isolates-infected line 0 CEFs, but not in that of DF-1/J cells using primers S1 & S2 for unknown reasons. However, we noticed that S1 & S2 primers were able to amplify DNA from four of the AF 115 isolates-infected line 0 CEFs in the first trial, but not in later trials. All the data from PCR analysis with subgroup J ALV sequence-specific primers (F5 & R5, RS-6J & S2, RS-7J & S2 and RS-7J & R5) were consistent with earlier studies on AF 115 isolates inoculated into line 0 CEFs (Lupiani and Fadly, unpublished data). The only exception was amplification of all AF 115 isolates with S1 & S2 primers (Gingerich *et al.*, 2002; Lupiani and Fadly, unpublished

data). It is worth mentioning that Lupiani and Fadly were not able to amplify all the AF 115 isolates from the first PCR trial. The length or sequences of the primers (S1 & S2), the buffer system and amplification conditions used may have been different than the ones utilized in our study.

PCR analysis in conjunction with sequence analysis of these AF 115 isolates can yield important information about identity and genomic-level natural variations within these recombinants. Design of future primers would be improved as more information is accumulating about ALV diversity. These new primers may be able to detect specifically a wider range of new and emerging mutant and recombinant ALVs in field flocks and experimental settings.

Table 2.1. *Reverse PCR primers designed to detect U3 or U5 common sequences of LTR of subgroup B ALV*

Primer[1]	Nucleotide Sequence[2]	Annealing Location	Product Size (~kb)[3]
B LTR 1.3	AGCCTTCTGCTTCATGC	LTR (U5)	2.2
B LTR 2.3	GTAGTCGTCAGGGAATCA	LTR (U5)	2.1
B LTR 3.3	AACCCAGGTGCACACCAA	LTR (U5)	2.1
B LTR 4.3	TGAATGGTCAAATGGCGT	LTR (U3)	2.1
B LTR 5.3	TTCGTCCAATCCGTGTTAG	LTR (U3)	2.0
B LTR 6.3	ACCACGATCATACCACCTT	LTR (U3)	1.9
B LTR 7.3	ACGGTGCTTTTTCTCTCCT	LTR (U3)	1.9
B LTR 8.3	ACAAGAGTATTGCATAAGA	LTR (U3)	1.8

[1] Each of the PCR reverse primers was designed using Oligo 4.0-s software (National Biosciences, Plymouth, MN).
[2] Nucleotide sequence of each primer was based on the common sequences of available subgroup B viral strains from GenBank,
[3] Each one of these reverse primers was coupled with forward primer SRB-F to amplify ALV subgroup B and the product size is estimated. PCR was run using buffer reaction system D at G cycle, which was chosen based on the Tm of the primers.

Table 2.2. *Forward PCR primers for different ALV subgroups*

Primer	Nucleotide Sequence	Annealing Location	ALV Subgroup Specificity
5258.5 F11 (H5)[1]	GGATGAGGTGACTAAGAAAG	*pol* (RT)	All subgroups
RS-7J[2]	CCCAAAAGGATGAGGTGACTAAGA	*pol* (RT)	All subgroups
ARK 4836[3,4]	CGAGAGTGGCTCGCGAGATGG	*pol* (RT)	All subgroups
SRB-F[5]	TTACCCCATACGATAGTCCGGCT	*env* (SU, gp85)	Subgroups B and D
848.5 F5[2]	GGTATTTTCTTGATTTGTGGGG	*env* (SU, gp85)	Subgroup J
RS-6J[4,6]	CTTGCTGCCATCGAGAGGTTACT	*env* (SU, gp85)	Subgroup J
S1[7]	AATTCTGCTTGAAATATG	UTR (E)	Subgroup J

[1] Smith *et al.*, 1998b
[2] Lupiani *et al.*, 2000
[3] Fadly *et al.*, 2006
[4] Silva *et al.*, 2007
[5] Silva, unpublished data
[6] Hunt *et al.*, 1999 and Silva *et al.*, 2000
[7] Smith *et al.*, 1998a

Table 2.3. *Reverse PCR primers for different ALV subgroups*

Primer	Nucleotide Sequence	Annealing Location	ALV Subgroup Specificity
REVSP 6683[1,2]	ACACTACATTTCCCCCTCCCTAT	*env* (TM, gp37)	All subgroups
5345.3 AD1[3]	GGGAGGTGGCTGACTGTGT	*env* (SU, gp85)	Subgroups A-E
SRB-R[2]	AGCCGGACTATCGTATGGGGTAA	*env* (SU, gp85)	Subgroups B and D
H7[3]	CGAACCAAAGGTAACACACG	*env* (SU, gp85)	Subgroup J
1868.3 R5[4]	GATTCGCAAGTTTCATACCCCT	UTR (DR1)	Subgroup J
2351.3 R8[5]	CAATGTGGTGGGAGGTAAAATGG	LTR (U5)	Subgroup J
S2[6]	AGTTGTCAGGGAATCGAC	LTR (U5)	Subgroup J

[1] Fadly *et al.*, 2006
[2] Silva *et al.*, 2007
[3] Smith *et al.*, 1998b
[4] Lupiani *et al.*, 2000
[5] Silva, unpublished data
[6] Smith *et al.*, 1998a

Table 2.4. Forward and reverse primer combinations and their characteristics

Primer Pair[1]	ALV Subgroup[2]	Product Size[3] (~kb)	PCR Cycle[4]
ARK 4836 & REVSP 6683	All	2.4	A and E
RS-7J & REVSP 6683	All	2.4	A and C
H5 & ADI	A-E	0.3	B and H
ARK 4836 & SRB-R	B	1.0	A and F
SRB-F & REVSP 6683	B	1.8	A
H5 & H7	J	0.5	B and H
S1 & S2	J	0.4	B and H
ARK 4836 & R8	J	2.9	A
F5 & R5	J	1.0	A
F5 & R8	J	1.5	A
RS-6J & REVSP 6683	J	2.1	A
RS-6J & R5	J	1.9	A
RS-6J & R8	J	2.3	A
RS-6J & S2	J	2.3	A
RS-7J & S2	J	2.5	A
RS-7J & R5	J	2.0	A
RS-7J & R8	J	2.5	A, C, D and E
SRB-F & S2	B/J	2.1	A
SRB-F & R8	B/J	2.1	A, D and E

[1] The PCR forward and reverse primers were chosen either from already published sequences or primers available at ADOL.

[2] ALV subgroup specificities of the PCR primer sets were determined based on the annealing location of each primer in the ALV genome and from already published use of these primer combinations for certain ALV subgroups.

[3] The PCR amplification product size was estimated according to the annealing locations of primer pairs in the respective ALV subgroups.

[4] The PCR cycle of each primer pair was chosen based on the Tm of the primers and published procedures. Some primer sets were run in more than one cycle to resolve the problems of non-specific binding and extra bands or for better resolution and amplification.

Table 2.5. PCR reaction buffer systems

Buffer System	Contents
A[1]	100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin
B[2]	10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton® X-100
C[3]	100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin
D[4]	2X Green GoTaq® Reaction Buffer (pH 8.5), yellow and blue dyes, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$

[1] Buffer system A is a 10x buffer for Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO)
[2] Buffer system B is a 10x buffer for Taq DNA polymerase in storage buffer B (Promega, Madison, WI)
[3] Buffer system C is a 10x buffer for Jumpstart™ Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO)
[4] Buffer system D is a GoTaq® Green Master Mix contains Taq and Taq buffer, dNTPs and tracking dyes (Promega, Madison, WI)

Table 2.6. PCR cycles used with different primer sets

PCR Cycle[1]	Conditions[2]
A	Primer annealing at 57°C for 1 min, and amplification or extension at 72°C for 2 min
B	Primer annealing at 49°C for 15 sec, and amplification or extension at 72°C for 15 sec
C	Primer annealing at 60°C for 1 min, and amplification or extension at 72°C for 2.5 min
D	Primer annealing at 60°C for 1 min, and amplification or extension at 72°C for 2 min
E	Primer annealing at 63°C for 30 sec, and amplification or extension at 72°C for 2 min
F	Primer annealing at 60°C for 30 sec, and amplification or extension at 72°C for 2 min
G	Primer annealing at 53°C for 1 min, and amplification or extension at 72°C for 2 min
H	Primer annealing at 53°C for 15 sec, and amplification or extension at 72°C for 15 sec

[1] The following PCR conditions were used in each cycle; following an initial template melting or preheating step at 95°C for 3 min, 30 cycles with denaturation at 95°C for 1 min, variable primer annealing and amplification or extension temperature and time according to the specific cycle, followed by a final elongation step at 72°C for 10 min and cold storage at 4°C.

[2] The PCR conditions in each cycle were programmed into PTC-100 Programmable Thermal Controller, Peltier-effect Cycling (MJ Research, Inc., Waltham, MA).

Table 2.7. *Infectivity or host range of AF 115 isolates in cells of different phenotypes using virologic p27 ELISA*

Viral Isolate[1]	CEFs or Cell lines (Phenotypes of Cells)[2]			
	line 0 (C/E)[3]	7_2 (C/ABDE)[4]	DF-1 (C/E)[5]	DF-1/J (C/EJ)[6]
AF 115-1	+	-	+	+
AF 115-5	+	-	+	+
AF 115-7	+	-	+	+
AF 115-10	+	-	+	+
AF 115-13	+	-	+	+
AF 115-14	+	-	+	+
AF 115-16	+	-	+	+
AF 115-4 (ALV BE/J)	+	-	+	+
RAV-2 (ALV-B)	+	-	+	+
RAV-1 (ALV-A)	+	-	+	+
Hc1 (ALV-J)	+	+	+	-
Media (Negative Control)	-	-	-	-

[1] Viral stocks were obtained from stored vials of supernatants of viral isolates inoculated in line 0 CEFs at -80° C.
[2] Cells obtained from chicken lines with different subtype susceptibility were used to determine host range. The cell phenotype designation denoted chickens (C) cells resistant to (/) the specified subgroup (A, B, D, E and J).
[3] line 0 (C/E) CEFs are known to be sensitive to all ALV subgroups except subgroup E ALV (Crittenden *et al.*, 1987).
[4] 7_2 (C/ABDE) CEFs are resistant to ALV subgroups A, B, D and E.
[5] DF-1 (C/E) is a fibroblastoid cell line derived from line 0 chicken embryos (Himly *et al.*, 1998; Maas *et al.*, 2006) and thus is susceptible to all ALV subgroups except subgroup E ALV.
[6] DF-1/J (C/EJ) is a genetically engineered cell line expressing the envelope protein isolated from ADOL-Hc1 strain of subgroup J ALV (Hunt *et al.*, 1999) and thus is resistant to subgroups J and E ALVs.
+ & – respectively indicated ALV presence & absence in the corresponding cells (run in duplicates) using virologic assays (Fadly and Witter, 1998) and p27 ELISA (Smith *et al.*, 1979) or cells are susceptible or resistant to infection by the corresponding viral isolate.

Table 2.8. *Antigenic relationships of AF 115 isolates to ALV subgroups B and J using virus neutralization test*

Viral Isolate[1]	Subgroup-Specific Antibodies or antisera[2]			
	Anti-RAV-2[3]	Anti-Hc1[4]	Negative sera[5]	Media (Negative Control)[6]
AF 115-1	+	-	-	-
AF 115-5	+	-	-	-
AF 115-7	+	-	-	-
AF 115-10	+	-	-	-
AF 115-13	+	-	-	-
AF 115-14	+	-	-	-
AF 115-16	+	-	-	-
AF 115-4 (ALV BE/J)	+	-	-	-
RAV-2 (ALV-B)	+	-	-	-
Hc1 (ALV-J)	-	+	-	-
Media (Negative Control)	-	-	-	-

[1] Subgroup B ALV (RAV-2) and ALV-J (Hc1) were included to confirm neutralization by the respective subgroup-specific antibody controls. Media was used as a negative control to assess for cross contamination of samples.
[2] Virus neutralization of each viral isolate was determined using known subgroup gp85-specific antibodies (Fadly and Witter, 1998).
[3] Anti-RAV-2 is a subgroup B ALV gp85-specific antibody or antisera.
[4] Anti-Hc1 is a subgroup J ALV gp85-specific antibody or antisera.
[5] Negative sera are ALV-free sera obtained from chickens known to be free from ALV.
[6] Media was used as a negative control to assess for cross contamination of samples.
+ indicated virus neutralization by the corresponding antibodies or absence of ALV detection by p27 ELISA (run in duplicates).
- indicated lack of virus neutralization by the corresponding antibodies or ALV detection by p27 ELISA (run in duplicates).

Table 2.9. PCR detection of ALV sequences in DNA of AF 115-infected line 0 CEFs using all-subgroup primer pairs

Primer Set[1]	Genomic DNA from line 0 CEFs infected with									
	Hc1[2]	**RAV-2[3]**	**ALV-BE/J (AF 115-4)[4]**	**AF 115-1**	**AF 115-5**	**AF 115-7**	**AF 115-10**	**AF 115-13**	**AF 115-14**	**AF 115-16**
ARK 4836 & REVSP 6683	+	+	+	+	+	+	+	+	+	+
RS-7J & REVSP 6683	+	+	+	+	+	+	+	+	+	+
H5 & ADI	+	+	+	+	+	+	+	+	+	+

[1] Each primer set is a combination of forward and reverse primers, which amplify all ALV subgroups except H5 & AD1, which amplifies ALV A-E.
[2] Hc1 is a positive control and is a prototype of ALV subgroup J (Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b).
[3] RAV-2 is Rous-associated virus 2, a positive control and a prototype of subgroup B ALV.
[4] AF 115-4 isolate is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-BE/J, Lupiani *et al.*, 2006).
The negative controls are the water and uninfected line 0 CEFs DNA (not shown).
+ stands for amplification and – stands for no amplification.

Table 2.10. PCR detection of ALV sequences from recombinant ALV-B/J in DNA of AF 115-infected line 0 CEFs

Primer Set[1]	Genomic DNA from line 0 CEFs infected with									
	Hc1[2]	RAV-2[3]	ALV-BE/J (AF 115-4)[4]	AF 115-1	AF 115-5	AF 115-7	AF 115-10	AF 115-13	AF 115-14	AF 115-16
SRB-F & S2	-	-	+	+	-	+f	-	+	+	+
SRB-F & R8	-	-	+	+	+	+	+	+	+	+

[1] Each primer set is a combination of forward and reverse primers, which are believed to amplify recombinant ALV with subgroup B envelope and subgroup J LTR.
[2] Hc1 is the prototype of ALV subgroup J (Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b). Hc1 is a negative control since it will not be detected or amplified by either primer pairs. The reverse primers (S2 & R8) can anneal to Hc1 since they are specific for ALV-J LTR. However, the forward primer (SRB-F) only anneals and detects subgroup B ALV envelope.
[3] RAV-2, Rous-associated virus 2, is a prototype of subgroup B ALV. It is also considered to be a negative control since it will not be detected or amplified by either primer pairs. The forward primer (SRB-F) will detect and anneal to subgroup B ALV envelope such as RAV-2. However, the reverse primers (S2 & R8) will not anneal to RAV-2 since they are specific for ALV-J LTR.
[4] AF 115-4 is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-B/J, Lupiani *et al.*, 2006) and a positive control for the amplification by these two primer pairs.
Water and uninfected line 0 CEFs were used for assessment of contamination and proper procedure (not shown).
+ stands for amplification, – stands for no amplification and +f stands for a very faint band.

Table 2.11. PCR detection of subgroup B ALV-like sequences in genomic DNA of AF 115-infected line 0 CEFs

Primer Set[1]	Genomic DNA from line 0 CEFs infected with									
	Hc1[2]	**RAV-2[3]**	**ALV-BE/J (AF 115-4)[4]**	**AF 115-1**	**AF 115-5**	**AF 115-7**	**AF 115-10**	**AF 115-13**	**AF 115-14**	**AF 115-16**
ARK 4836 & SRB-R	-	+	+	+	+	+	+	+	+	+
SRB-F & REVSP 6683	-	+	+	+	+	+	+	+	+	+
SRB-F & B LTR 3.3	-	+	+	+	+	+	+	+	+	+
SRB-F & B LTR 6.3	-	+	+	+	+	+	+F	+	+	+
SRB-F & B LTR 2.3	-	+	-	-	-	-	-	-	-	-
SRB-F & B LTR 4.3	-	+	-	-	-	-	-	-	-	-
SRB-F & B LTR 8.3	-	+	-	-	-	-	-	-	-	-
SRB-F & B LTR 1.3	-	+	+F	+F	+F	+F	+F	-	-	-
SRB-F & B LTR 5.3	-	+	+	+	-	+	-	+	+	+
SRB-F & B LTR 7.3	-	+	+F	+F	-	+F	-	+F	-	+F

[1] Each primer set amplifies subgroup B ALV-like sequences. Water and uninfected line 0 CEFs were used to assess contamination and proper procedure (not shown).
+ stands for amplification, +F stands for a faint band and – stands for no amplification.
[2] Hc1 is a negative control and a prototype of ALV subgroup J (Fadly and Smith, 1997 & 1999; Smith *et al.*, 1998b).
[3] RAV-2 is Rous-associated virus 2, a positive control and a prototype of subgroup B ALV.
[4] AF 115-4 is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-BE/J, Lupiani *et al.*, 2006).

Table 2.12. PCR detection of subgroup J ALV-like sequences in genomic DNA of AF 115-infected line 0 CEFs

Primer Set[1]	Genomic DNA from line 0 CEFs infected with									
	Hc1[2]	RAV-2[3]	ALV-BE/J (AF 115-4)[4]	AF 115-1	AF 115-5	AF 115-7	AF 115-10	AF 115-13	AF 115-14	AF 115-16
H5 & H7	+	-	-	-	-	-	-	-	-	-
RS-6J & REVSP 6683	+	-	-	-	-	-	-	-	-	-
RS-6J & S2	+	-	-	-	-	-	-	-	-	-
RS-6J & R8	+	-	-	-	-	-	-	-	-	-
RS-6J & R5	+	-	-	-	-	-	-	-	-	-
ARK 4836 & R8	+	-	+	+	+	+	+F	+	+	+
F5 & R8	+	-	+	+	+	+	+	+	+	+
RS-7J & R8	+	-	+	+	+	+	+	+	+	+
RS-7J & S2	+	-	+	+	-	-	-	+	+	+
S1 & S2	+	-	+	+	-	-	-	+	+	+
RS-7J & R5	+	-	+	+	+	+	-	+	+	+
F5 & R5	+	-	+	+	+	+	-	+	+	+

[1] Each primer set amplifies subgroup J ALV-like sequences.
[2] Hc1 is a positive control and a prototype of ALV subgroup J (Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b).
[3] RAV-2 is Rous-associated virus 2, a negative control and a prototype of subgroup B ALV.
[4] AF 115-4 is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-BE/J, Lupiani *et al.*, 2006).
Water and uninfected line 0 CEFs were used to assess contamination and proper procedure (not shown).
+ stands for amplification, +F stands for a faint band and – stands for no amplification.

Table 2.13. PCR detection of ALV sequences in genomic DNA of AF 115-infected 7_2 (C/ABDE) CEFs

Primer Set[1]	Genomic DNA from 7_2 CEFs[2] infected with									
	Hc1[3]	RAV-2[4]	ALV-BE/J (AF 115-4)[5]	AF 115-1	AF 115-5	AF 115-7	AF 115-10	AF 115-13	AF 115-14	AF 115-16
ARK 4836 & REVSP 6683	+	-	-	-	-	-	-	-	-	-
S1 & S2	+	-	-	-	-	-	-	-	-	-
RS-6J & S2	+	-	-	-	-	-	-	-	-	-
H5 & H7	+	-	-	-	-	-	-	-	-	-
F5 & R8	+	-	-	-	-	-	-	-	-	-

[1] Each primer set amplifies subgroup J ALV-like sequences except the first (ARK 4836 & REVSP 6683), which amplifies all ALVs.
[2] 7_2 CEFs have the phenotype C/ABDE which stands for chicken cells resistant to ALV subgroups A, B, D and E.
[3] Hc1 is a positive control, which infects 7_2 CEFs and is a prototype of ALV subgroup J (Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b).
[4] RAV-2 is Rous-associated virus 2, which does not infect 7_2 CEFs and thus is a negative control and a prototype of subgroup B ALV.
[5] AF 115-4 is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-BE/J, Lupiani *et al.*, 2006), which does not infect 7_2 CEFs since it has a subgroup B ALV.
Water and uninfected 7_2 CEFs were used to assess contamination and proper procedure (not shown).
+ stands for amplification and – stands for no amplification.

Table 2.14. PCR detection of ALV sequences in genomic DNA of AF 115-infected DF-1/J (C/EJ) cells

Primer Set[1]	**Genomic DNA from DF-1/J cells**[2] **infected with**									
	Hc1[3]	**RAV-2**[4]	**ALV-BE/J (AF 115-4)**[5]	**AF 115-1**	**AF 115-5**	**AF 115-7**	**AF 115-10**	**AF 115-13**	**AF 115-14**	**AF 115-16**
ARK 4836 & REVSP 6683[6]	-	+	+	+	+	+	+	+	+	+
ARK 4836 & SRB-R[7]	-	+	+	+	+	+	+	+	+	+
S1 & S2[8]	-	-	-	-	-	-	-	-	-	-
H5 & H7[8]	-	-	-	-	-	-	-	-	-	-
F5 & R8[8]	-	-	+	+	+	+	+	+	+	+
SRB-F & R8[9]	-	-	+	+	+	+	+	+	+	+

[1] Primer sets used to assess the presence of a mixture of ALV subgroups.
[2] DF-1/J (C/EJ) is a genetically engineered cell line expressing the envelope protein isolated from ADOL-Hc1 strain of subgroup J ALV (Hunt *et al.*, 1999) and thus is resistant to subgroups J and E ALVs.
[3] Hc1 does not infect DF-1/J cells and is a negative control for replication in these cells and is a prototype of ALV subgroup J (Fadly and Smith, 1997 and 1999; Smith *et al.*, 1998b).
[4] RAV-2 infects DF-1/J cells and thus is a positive control for some of the first two primer sets (ARK 4836 & REVSP 6683 and ARK 4836 & SRB-R). It is called Rous-associated virus 2 and is a prototype of subgroup B ALV.
[5] AF 115-4 is a naturally occurring recombinant ALV with subgroup B gp 85, subgroup E gp 37 and subgroup J LTR (ALV-BE/J, Lupiani *et al.*, 2006), which infects DF-1/J cells since it has a subgroup B ALV and is a positive control for SRB-F & R8.
[6] This primer set were used to amplify all ALVs.
[7] This primer set were used to amplify subgroup B ALV-like sequences.
[8] These primer sets were used to amplify subgroup J ALV-like sequences. DNA from Hc1-infected line 0 CEFs were used in PCR as a positive control (not shown). Water and uninfected DF-1/J cells were used to assess contamination and proper procedure (not shown).
[9] This primer set were used to amplify recombinant ALV-B/J-like sequences.
+ stands for amplification and – stands for no amplification.

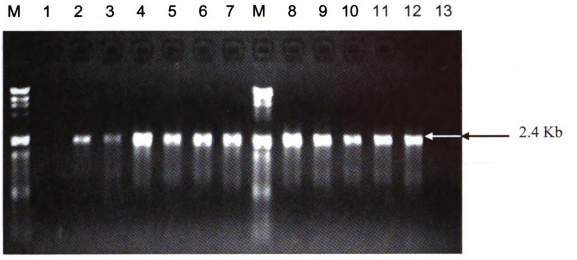


Figure 2.1. Detection of ALV in proviral DNA prepared from line 0 CEFs infected with AF 115 isolates by PCR using all-ALV primers RS-7J & REVSP 6683. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, positive control); 3 = RAV-2 (ALV-B, positive control); 4 = RAV-2 (ALV-B, positive control) DNA; 5 = Naturally occurring recombinant ALV-B/J (AF 115-4, positive control); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16; 13 = water (no DNA). The approximate 2.4 Kb hybridizing fragments of DNA correspond to all-ALV amplified fragments and are characteristics of ALV DNA.

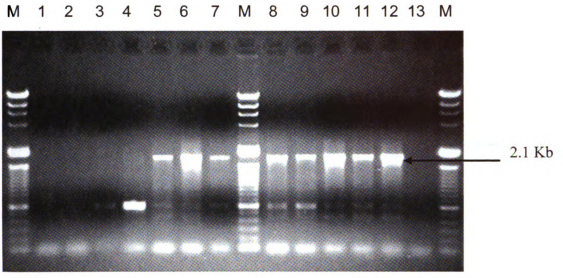


Figure 2.2. Analysis of PCR amplification products from line 0 CEFs inoculated with AF 115 isolates utilizing recombinant ALV-B/J-specific primer pair SRB-F & R8. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, negative control); 3 = RAV-2 (ALV-B, negative control); 4 = RAV-2 (ALV-B, negative control) DNA; 5 = Naturally occurring recombinant ALV-B/J (AF 115-4, positive control); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16; 13 = water (no DNA). Amplification products of around 2.1 Kb were obtained for DNA templates of recombinant ALV-B/J.

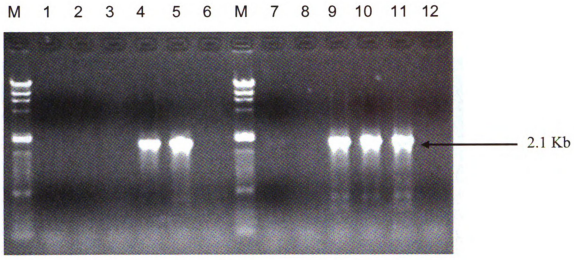


Figure 2.3. Agarose gel showing the PCR products obtained by employing SRB-F & S2 primer set on DNA samples extracted from AF 115 isolates-infected line 0 CEFs. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, negative control); 3 = RAV-2 (ALV-B, negative control); 4 = Naturally occurring recombinant ALV-B/J (AF 115-4, positive control); 5 = AF 115-1; 6 = AF 115-5; 7 = AF 115-7; 8 = AF 115-10; 9 = AF 115-13; 10 = AF 115-14; 11 = AF 115-16; 12 = water (no DNA). Successful amplification of DNA templates in various samples produced recombinant ALV-B/J-specific fragments of approximately 2.1 Kb.

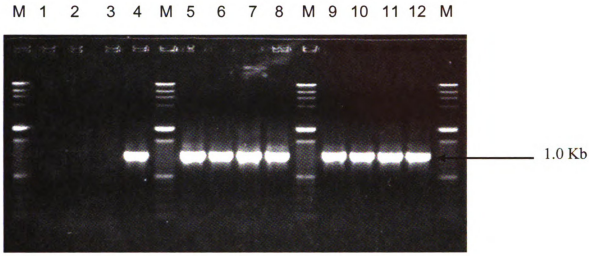


Figure 2.4. PCR amplification of DNA isolated from AF 115 isolates-infected line 0 CEFs utilizing primer combination specific for subgroup B ALV sequences (ARK 4836 & SRB-R). Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected line 0 CEFs (negative control); 3 = Hc1 (ALV-J, negative control); 4 = RAV-2 (ALV-B, positive control); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. The estimated 1.0 Kb PCR products correspond to ALV-B-specific amplified fragments.

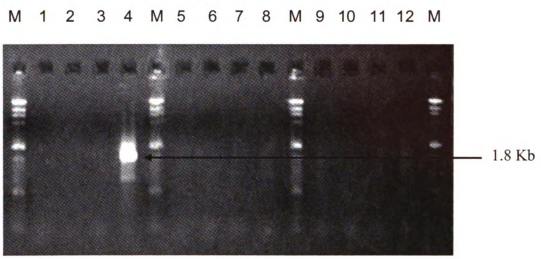


Figure 2.5. PCR for specific detection of subgroup B ALV with line 0 CEFs inoculated with AF 115 isolates using primer pair specific for subgroup B ALV (SRB-F & B LTR 8.3). Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected line 0 CEFs (negative control); 3 = Hc1 (ALV-J, negative control); 4 = RAV-2 (ALV-B, positive control); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. Successful amplification of DNA templates in RAV-2-infected line 0 CEFs formed subgroup B ALV-specific fragment of approximate size of 1.8 Kb.

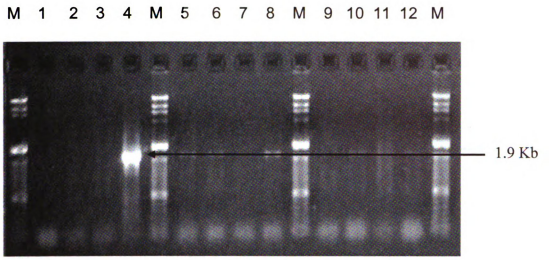


Figure 2.6. PCR reaction of genomic DNA from line 0 CEFs inoculated with AF 115 isolates and amplified with primers specific for subgroup B ALV (SRB-F & B LTR 7.3). Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected line 0 CEFs (negative control); 3 = Hc1 (ALV-J, negative control); 4 = RAV-2 (ALV-B, positive control); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. The near 1.9 Kb DNA fragments correspond to ALV-B like sequence-specific amplified fragments.

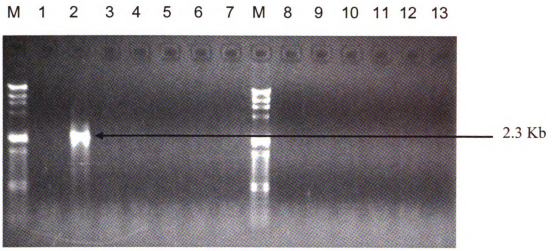


Figure 2.7. Specific PCR detection of subgroup J ALV in DNA extracted from line 0 CEFs infected with AF 115 isolates utilizing ALV-J-specific primer set RS-6J & S2. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, positive control); 3 = RAV-2 (ALV-B, negative control); 4 = RAV-2 (ALV-B, negative control) DNA; 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16; 13 = water (no DNA). Successful amplification of DNA templates in Hc1-infected line 0 CEFs created subgroup J ALV-specific fragment of rough size of 2.3 Kb.

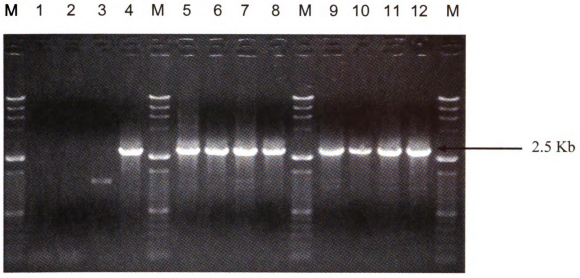


Figure 2.8. Analysis of PCR carried out on proviral DNA extracted from line 0 CEFs inoculated with AF 115 isolates and amplified using primer pair specific for ALV-J sequences (RS-7J & R8). Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected line 0 CEFs (negative control); 3 = RAV-2 (ALV-B, negative control); 4 = Hc1 (ALV-J, positive control); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. PCR products of estimated sizes of 2.5 Kb correspond to ALV-J like-specific amplified fragment.

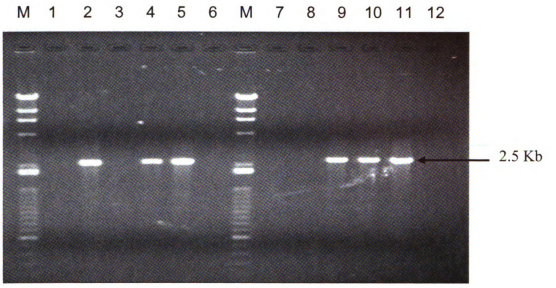


Figure 2.9. PCR analysis of DNA isolated from AF 115 isolates propagated in line 0 CEFs and amplified by ALV-J sequence-specific primer combination RS-7J & S2. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, positive control); 3 = RAV-2 (ALV-B, negative control); 4 = Naturally occurring recombinant ALV-B/J (AF 115-4); 5 = AF 115-1; 6 = AF 115-5; 7 = AF 115-7; 8 = AF 115-10; 9 = AF 115-13; 10 = AF 115-14; 11 = AF 115-16; 12 = water (no DNA). The nearly 2.5 Kb bands correspond to ALV-J like sequence-specific amplified fragments.

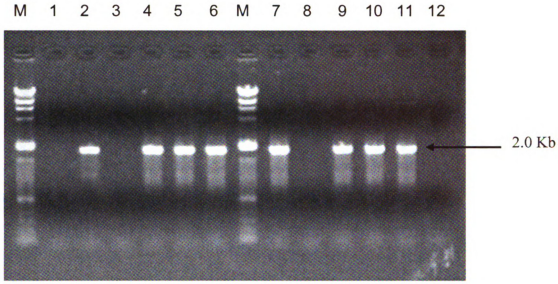


Figure 2.10. Ethidium bromide –stained agarose gel with PCR products of DNA prepared from AF 115-infected line 0 CEFs obtained by amplification with ALV-J sequence-specific primers RS-7J & R5. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected line 0 CEFs (negative control); 2 = Hc1 (ALV-J, positive control); 3 = RAV-2 (ALV-B, negative control); 4 = Naturally occurring recombinant ALV-B/J (AF 115-4); 5 = AF 115-1; 6 = AF 115-5; 7 = AF 115-7; 8 = AF 115-10; 9 = AF 115-13; 10 = AF 115-14; 11 = AF 115-16; 12 = water (no DNA). The roughly 2.0 Kb PCR products correspond to ALV-J like sequence-specific amplified fragments.

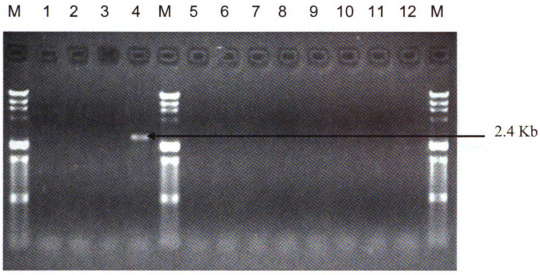


Figure 2.11. PCR amplification from isolated proviral DNA samples of 7_2 CEFs inoculated with AF 115 isolates employing primer pair ARK 4836 & REVSP 6683, which can detect all ALV subgroups. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected 7_2 CEFs (negative control); 3 = RAV-2 (ALV-B, negative control); 4 = Hc1 (ALV-J, positive control); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. Amplified product of about 2.4 Kb represents all-ALV fragment.

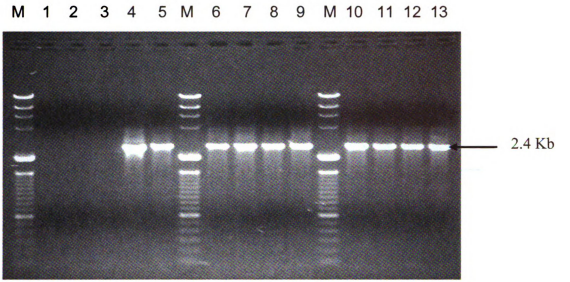


Figure 2.12. Genomic DNA from AF 115 isolates-infected DF-1/J cells using all-ALV primer set ARK 4836 & REVSP 6683. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected DF-1/J cells (negative control); 3 = Hc1 (ALV-J); 4 = Hc1 (ALV-J propagated in line 0 CEFs); 5 = RAV-2 (ALV-B); 6 = Naturally occurring recombinant ALV-B/J (AF 115-4); 7 = AF 115-1; 8 = AF 115-5; 9 = AF 115-7; 10 = AF 115-10; 11 = AF 115-13; 12 = AF 115-14; 13 = AF 115-16. Amplified products of around 2.4 Kb characterize all-ALV fragment.

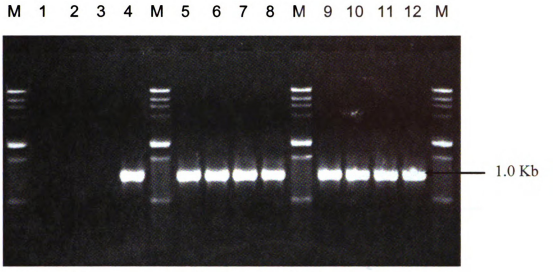


Figure 2.13. PCR analysis of DNA extracted from DF-1/J cells inoculated with AF 115 isolates utilizing ALV-B specific primers ARK 4836 & SRB-R. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected DF-1/J cells (negative control); 3 = Hc1 (ALV-J); 4 = RAV-2 (ALV-B); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. The estimated 1.0 Kb PCR fragments signify ALV-B-specific amplified fragments.

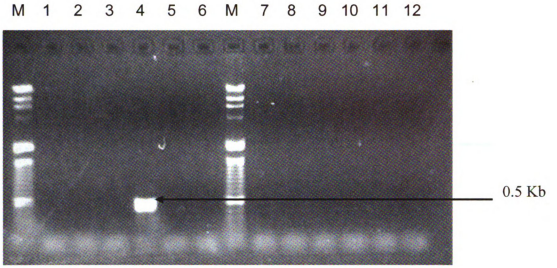


Figure 2.14. Specific PCR amplification of subgroup J ALV in DNA isolated from DF-1/ J cells infected with AF 115 isolates with ALV-J-specific primer set H5 & H7. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected DF-1/J (negative control); 2 = RAV-2 (ALV-B, negative control); 3 = Hc1 (ALV-J); 4 = Hc1 (ALV-J propagated in line 0 CEFs, positive control) DNA; 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. Successful amplification of DNA templates in Hc1-infected line 0 CEFs produced subgroup J ALV-specific fragment of approximately 0.5 Kb.

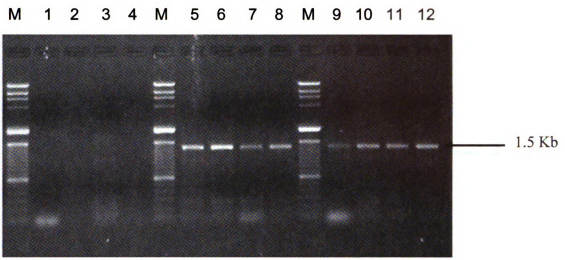


Figure 2.15. Detection of ALV-J sequences in proviral DNA isolated from AF 115 isolates-infected DF-1/J cells through PCR analysis using primer combination F5 & R8. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = water (no DNA); 2 = uninfected DF-1/J cells (negative control); 3 = Hc1 (ALV-J); 4 = RAV-2 (ALV-B); 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16. The estimated 1.5 Kb PCR products correspond to ALV-J sequences-specific amplified fragments.

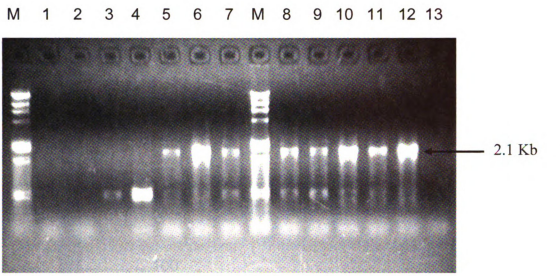


Figure 2.16. PCR analysis done to detect recombinant ALV-B/J sequences in genomic DNA prepared from DF-1/J cells incubated with AF 115 isolates employing primers SRB-F & R8. Lanes and respective samples: M = DNA molecular weight marker (Figure 2.17); 1 = uninfected DF-1/J cells (negative control); 2 = Hc1 (ALV-J); 3 = RAV-2 (ALV-B); 4 = RAV-2 (ALV-B propagated in line 0 CEFs) 5 = Naturally occurring recombinant ALV-B/J (AF 115-4); 6 = AF 115-1; 7 = AF 115-5; 8 = AF 115-7; 9 = AF 115-10; 10 = AF 115-13; 11 = AF 115-14; 12 = AF 115-16; 13 + water (no DNA). Successful amplification of DNA templates in AF 115 isolates-infected DF-1/J cells formed recombinant ALV-B/J-specific fragments of around 2.1 Kb.

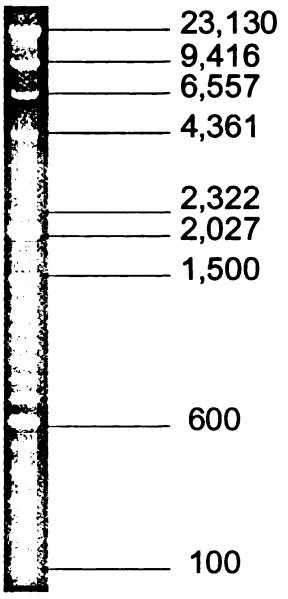


Figure 2.17. DNA molecular weight marker as visualized by ethidium bromide staining. It consists of 1:1 of lambda DNA-Hind III-digested and 100 bp DNA marker or ladder. Values are in base pairs (bp).

CHAPTER 3

Comparison of proviral DNA sequences of seven isolates of naturally occurring recombinant avian leukosis virus and representative ALVs

ABSTRACT

This study was performed in order to molecularly characterize seven AF 115 isolates of a naturally occurring recombinant avian leukosis virus associated with myeloid leukosis in commercial layers. Sequence analyses of different genomic regions of these AF 115 isolates were carried out to confirm the identity of the subgroup of these recombinant viruses. The deduced amino acid sequences of gp85 and gp37 and the DNA sequences of 3' UTR and LTR of the seven isolates were compared with the naturally occurring recombinant ALV-B/J. These sequences were also evaluated for variations and relationship with prototypes of various ALV subgroups and related ALV strains through NCBI blast search. Sequence alignment, % identity, divergence and phylogenetic trees were studied to draw associations and identify important potential sites for protein structure and retrovirus transcription. Moreover, potential conserved sequences in different regions of these isolates were assessed for predicted stability and functions.

Six AF 115 isolates (AF 115-1, AF 115-7, AF 115-10, AF 115-13, AF 115-14 & AF 115-16) showed high sequence identity to the naturally occurring recombinant ALV-B/J, subgroup B ALV and RAV-2-like viruses in the SU domain of the envelope. On the other hand, the gp85 of AF 115-5 shower lower sequence identity to that of recombinant ALV-B/J and was more similar to that of ALVs subgroup A such as those of MAV-1-like viruses and recombinant ALVs with MAV-1-like gp85. Moreover, the TM domains of all

AF 115 isolates showed similar sequences to that of ALV-B/J, subgroup E ALVs and different ALV-E isolates and recombinant ALVs with subgroup E gp37. The 3' UTR of all AF 115 isolates showed high sequence similarity to those of recombinant ALV-B/J, different ALV-J isolates and recombinant ALVs with subgroup J 3' UTR containing a DR1 region and E element. Moreover, all AF 115 isolates except AF 115-10 had a 3'LTRs, which were similar to those of ALV-B/J, ALV-J viruses, recombinant ALV-A/J. On the other hand, AF 115-10 contained a 3' LTR with similar sequences to that of recombinant ALV among endogenous and different exogenous viruses. Some sequence rearrangements including base insertions and deletions as well as mutations were observed in the 3' UTR and LTR of AF 115-10 exhibiting the addition and loss of potential enhancer elements.

All AF 115 isolates contained conserved cysteine residues and potential N-linked glycosylation sites in the SU and TM envelope glycoproteins needed for their proper structure and function. The fusion peptides of all AF 115 isolates were similar to that of ALV-B/J as well as other endogenous and exogenous viruses reflecting its importance in retroviral life cycle. The putative transcription regulatory elements in the 3' UTR and LTR of all AF 115 isolates were mostly conserved. These included CCAAT motif, PU1 and PU2 core regions in the 3' UTR and TATA box, PAS signal, CCAAT motif and CCAAT-related LTR enhancer motif and two boxes of Y, CArG and PRE in the 3' U3 region. These elements in all AF 115 isolates were similar to those of exogenous ALVs in number and sequence. When compared with ALV-J, these isolates showed deletion in one of two adjacent repeated motifs in the U3 region of the 3' LTR. AF 115-10 contained

extra enhancer elements in the rearranged sequences (base insertions) of U3 region of the 3' LTR and had more mutations in the enhancer elements than the rest of the isolates.

INTRODUCTION

Avian leukosis viruses (ALVs) belong to the avian leukosis-sarcoma group of viruses (ALSVs). They are classified into exogenous subgroups A, B, C, D, J and endogenous subgroup E in chickens (Fadly and Payne, 2003). Subgroup A is frequently detected in commercial layers while ALV-B is occasionally encountered and both mainly cause lymphoid leukosis (LL). Subgroups C and D are rare in field flocks and the endogenous subgroup E is found in most commercial chickens. Since its discovery, subgroup J has caused huge economical losses to the poultry industry and is usually associated with myeloid leukosis (ML) in broilers (Payne, 1998).

Viral strains in ALSV are divided into acute or slow transforming viruses (Fadly and Payne, 2003). Acute transforming viruses such as Rous sarcoma virus (RSV) and avian myeloblastosis virus (AMV) are defective and require helper viruses for replication (Dougherty, 1987). On the other hand, Rous associated viruses 1 (RAV-1) and 2 (RAV-2) are slow transforming viruses and prototypes of subgroup A and B helper viruses for RSV respectively (Hanafusa, 1965). Myeloblastosis associated virus-1 (MAV-1) is a subgroup A helper virus for AMV (Baluda *et al.*, 1983; Moscovici and Vogt, 1968).

Like other retroviruses, ALV is known for its genetic diversity including mutations and recombinations (Coffin, 1992; Katz and Skalka, 1990; Mansky, 1998). This diversity is exhibited by emergence of new mutant and recombinant strains with altered characteristics. For example, variations in the *env* gene and long terminal repeat

(LTR) may affect virus-host receptor interaction and virus replication respectively (Boral *et al*, 1989; Celander and Haseltine, 1984; Golemis *et al.*, 1990). In addition, molecular variations in different regions of ALV genome may lead to difficulties in diagnosis, prevention and control of ALV infections (Fadly and Smith, 1999; Silva *et al.*, 2000; Venugopal *et al.*, 1998).

Subgroup J ALV is a recombinant ALV with *gag* and *pol* genes similar to exogenous ALSV and an *env* gene nearly identical to an endogenous retrovirus EAV-HI (Bai *et al.*, 1995b; Benson *et al.*, 1998a; Silva *et al.*, 2000; Smith *et al.*, 1999; Venugopal, 1999). Three experimental recombinant ALVs with recombinant envelopes of subgroups A and E and subgroup J LTRs were previously described (Lupiani *et al.*, 2000). A naturally occurring recombinant ALV (AF 115-4) containing the a subgroup B gp85 region and a subgroup E gp37 region and a subgroup J LTR (ALV-B/J) was isolated from commercial layer flocks affected with ML (Gingerich *et al.*, 2002; Lupiani *et al.*, 2006).

We previously showed that seven other isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) from the same field case were similar to the naturally occurring recombinant ALV-B/J by biological assays and PCR. Biological assays suggested the presence of an exogenous ALV other than subgroup J probably having a subgroup B envelope. AF 115 isolates were PCR amplified with different primer sets specific for ALV-J, ALV-B and ALV-B/J sequences. Moreover, PCR data suggested that four isolates (AF 115-1, AF 115-13, AF 115-14 and AF 115-16) were similar to ALV-B/J while AF 115-5, AF 115-7 and AF 115-10 were more diverse.

Various molecular approaches have been used for studying retroviral diversity and characterizing new and emerging viral isolates (Mansky, 1998). Polymerase chain

reaction (PCR) can be used to specifically amplify different regions of ALV genome, which can then be cloned and sequenced. DNA sequence analysis has been used to identify, characterize and establish relationships among ALV variants (Bai *et al.*, 1995b; Reddy *et al.*, 1983; Venugopal *et al.*, 1998). Moreover, it can facilitate the study of the genetic variations within different regions of ALV genome (Bai *et al.*, 1998a and b; Bova *et al.*, 1986 and 1988; Chesters *et al.*, 2002 and 2006; Hue *et al.*, 2006; Zachow and Conklin, 1992).

The main aim of this study is to molecularly characterize the seven AF 115 isolates of a naturally occurring recombinant ALV by DNA sequence analysis. Sequences of various genomic regions of seven AF 115 isolates including *env* (gp85 and gp37), 3'UTR and LTR will be compared with prototypes of ALV subgroups. These molecular analyses may reveal how and to what extent different ALV subgroups have contributed to the origin and unique features of these viruses. It is also necessary to compare both envelope SU and TM amino acid sequences and 3' UTR and LTR DNA sequences with related sequences through NCBI blast search. This will be carried out to understand the molecular basis and significance of the emergence of these recombinants. Moreover, it will more accurately establish the evolutionary relationships of AF 115 isolates with other related viruses as well as the classical ALV subgroups.

Three general hypotheses were tested in this research work. 1) The subgroup designation of different recombinants will be identified and confirmed by sequencing different genomic regions of these viruses. 2) Rearrangements (deletions and insertions) and mutations in different genomic regions of recombinant ALV can predict associations with different ALV strains. 3) Sequences in different genomic regions of recombinant

ALVs can be used to speculate their stability, presence of potential regulatory elements and recombination hotspots or boundaries among different ALV subgroups. The objectives were to: 1) Compare the sequences of various genomic regions of seven AF 115 isolates including *env* (gp85 and gp37), 3'UTR and LTR with prototypes of ALV subgroups to identify and confirm the subgroups of these recombinant viruses. 2) Compare SU and TM amino acid sequences and 3' UTR and LTR DNA sequences of these isolates with similar blasted ALV strains to predict relevant associations and relationships. 3) Compare cysteine residues and predicted N-linked glycosylation sites in the SU and TM glycoprtoeins as well as transcription regulatory elements in the 3' UTR and LTR of these isolates with those of other ALVs to draw relationships and predict stability and recombination hotspots.

MATERIALS AND METHODS

A. Viruses and Cells

All seven AF 115 isolates (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) were propagated in line 0 CEFs as described previously (Fadly and Witter, 1998). Briefly, around 100 µl of each undiluted viral stock (kept at -80° C) was added to 0.5 x 10^6 secondary CEFs suspended in 4% calf serum (CS, Sigma Chemical Co, St. Louis, MO) in 1:1 Leibowitz's L-15-McCoy's 5A tissue culture medium (LM, Sigma Chemical Co, St. Louis, MO) containing penicillin/streptomycin (Sigma Chemical Co, St. Louis, MO), 2 µg/ml amphotericin B and 2 µg/ml DEAE-Dextran in 35-mm tissue culture plates. Twenty four hours later, the 4% CS LM media

was replaced with 1% CS LM media and the plates were incubated in 4% CO_2 at 37° C for 7-9 days.

B. DNA Extraction

Total genomic DNA was extracted from infected cells using standard proteinase K, phenol-chloroform extraction procedures (Maniatis *et al.*, 1982). Briefly, the medium was removed and the cells were scraped off the plate and pelleted by centrifugation. The pellet was treated with lysis buffer (10 mM TRIS, 1 mM EDTA, 100 mM NaCl and 0.5% SDS) and proteinase K (100 µg/ml) overnight at 56°C. DNA was extracted twice with phenol-chloroform, treated with RNAse (2 µl/ml) at 37°C for an hour, precipitated with ice-cold absolute ethanol, dried at room temperature, dissolved in TE buffer and stored at 4°C. The extracted DNA from the seven different samples was used as templates for polymerase chain reaction (PCR).

C. Amplification of Proviral DNA

To broadly and more extensively investigate the relationship between the seven AF 115 isolates, the naturally occurring recombinant ALV-B/J and known prototypes of various ALV subgroups, PCR was performed on extracted DNA using primer set (RS-7J & R8). DNA from AF 115-1 and AF 115-10 isolates were additionally amplified using two other sets of primers (ARK 4823 & SRB-R, SRB-F & R8). This was done to ensure that similar sequences were obtained. The sequence and annealing locations of these primers are shown in Table 3.1. The primer sets in combination (ARK 4823 & SRB-R, SRB-F & R8) or alone (RS-7J & R8) were expected to yield PCR amplified fragments

extending roughly from near the 3' end of the polymerase gene to the 5' end of U5 of LTR. These will amplify sequences spanning all of the variable and hypervariable regions of gp85, the relatively conserved gp37 region and 3' UTR and LTR regions.

Three independent PCR reactions are carried out on each of the DNA samples of line 0 CEFs inoculated with AF 115 isolates. Each 50-ml PCR reaction contained 1x reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin), 25 mM of each dNTP, 25 pmoles of each primer, 2.5 units Taq polymerase, and around 100 ng of template DNA. The same PCR conditions were used with all the primer pairs. Following an initial template melting or preheating step at 95°C for 3 min, the DNA was amplified during 30 cycles with denaturation at 95°C for 1 min, primer annealing at 57°C for 1 min, and amplification or extension at 72°C for 2 min, followed by a final elongation step at 72°C for 10 min and cold storage at 4°C. This was carried out in PCR thermocycler, PTC-100 Programmable Thermal Controler, Peltier-effect Cycling (MJ Research, Inc., Waltham, MA).

D. Subcloning of PCR Products

Amplified PCR products were resolved by electrophoresis through 1% agarose gel in 1X TAE buffer containing 200 ng/ml ethidium bromide and examined under ultraviolet transillumination. PCR products were purified by agarose gel separation and extraction from gel slices using the GeneClean®111 kit (MP Biomedicals, Solon, OH). Then, the purified PCR fragments were blunt-ended using PFU polymerase and subcloned into pCR®-Blunt vector (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The ligated PCR insert into the vector was transformed using One Shot ®

TOP10 competent cells (*E. coli*) following manufacturer's instructions. Several antibiotic-resistant colonies or transformants were selected and plasmid DNA isolated. Restriction analysis using *Eco*R I was used to confirm the presence of the desired insert. Three individual positive clones or transformants from each of three independent PCR products of AF 115 isolates were selected for sequence analysis. Plasmid DNA was isolated and purified using PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA).

E. Sequence Determination

The DNA sequences of each clone were determined using several forward and reverse primers designed with Oligo 4.0-s software (National Biosciences, Plymouth, MN). DNA sequencing was carried out using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The sequence contigs were constructed using Sequencher™ version 4.5 (Gene Codes Corp., Ann Arbor, MI).

F. Analysis of Sequence Data

The proviral DNA sequences of 3' UTR and LTR and deduced amino acid sequences of SU (gp85) and TM (gp37) of the envelope of all viruses were compared using ClustalW multiple sequence alignment algorithm of MegAlign program (DNASTAR Inc., Madison, WI). The ClustalW slow accurate method was used for sequence analysis since it is the most accurate and effective for typical multiple alignment when the similarities among sequences extend over a majority of their length.

It also leads to refinement of sequence alignments for phylogenetic analysis. The DNA sequences and deduced amino acid sequences of all AF 115 isolates were compared among each others, with the naturally occurring recombinant ALV-B/J (AF 115-4), with prototypes of various ALV subgroups and with related published sequences of viral strains from GenBank using the BLAST program from NCBI.

Phylogenetic relationships of the isolates were generated using the neighbor-joining (NJ) distance-based method utilized in MegAlign. The sequence identity and divergence in sequence distances between viruses were used for the construction of a cladogram. These phylogenetic trees (cladograms) are rooted trees, where branch distances correspond to sequence divergence. The length of each pair of branches represents the distance between sequence pairs, while units at the bottom of the tree indicate the number of substitution events. The relevant accession numbers for sequences of viruses used in the analyses are found in Table 3.2.

RESULTS

A. Analysis of sequence data

PCR products of DNA of line 0 CEF infected with the AF115 isolates using all primer sets were of expected sizes. To characterize the genomic structure of seven AF 115 isolates, proviral DNA sequencing of PCR products spanning the *env* gene (gp85 and gp37) and 3' UTR and LTR was performed and analyzed. The DNA sequences of AF 115-1 and AF 115-10 using the primer sets (ARK 4823 & SRB-R, SRB-F & R8 in combination or RS-7J & R8 alone) were identical.

B. Sequence comparisons among AF 115 isolates

1. Comparison of the envelope SU glycoprotein (gp85)

Compared to ALV-B/J, the amino acid sequences of gp85 region of all AF 115 isolates except AF 115-5 were found to be conserved but scattered amino acid substitutions were observed (Figure 3.1). The N- and C-terminal SU amino acid sequences of all AF 115 isolates were conserved and identical to ALV-B/J (AF 115-4). Conversely, the middle sequences of the isolates showed varying degree of amino acid substitutions in conserved and variable (vr1, vr2 and vr3) and hypervariable regions (hr1 and hr2). For example, AF 115-16 showed sequence diversity only in vr1. On the other hand, AF 115-5 showed the most sequence variations in all variable and hypervariable regions when compared with the rest of the isolates and ALV-B/J.

There were 14 conserved cysteine residues in the gp85 of all seven AF 115 isolates as well as ALV-B/J (Figure 3.1). One of the conserved cysteine residues was in hr1 and two were in hr2 in all AF 115 isolates. Moreover, the SU envelope glycoproteins of three AF 115 isolates (AF 115-7, AF 115-13 and AF 115-16) contained 14 potential N-linked glycosylation sites (NXS/T). The remaining four AF 115 isolates (AF 115-1, AF 115-5, AF 115-10 and AF 115-14) in addition to ALV-B/J (AF 115-4) contained 15 possible N-linked glycosylation sites (NXS/T) in the SU envelope glycoproteins (Figure 3.1). Twelve of these likely glycosylation sites were conserved in all AF 115 isolates and ALV-B/J, one of which was in hr1 (NSS).

Two of the conserved probable glycosylation sites were consecutive just upstream of hr1 of all isolates and ALV-B/J. Upstream of vr1, a possible glycosylation site (NCT) was found in all isolates and ALV-B/J except AF 115-7, where asparagine (N) was

replaced by lysine (L). One potential site (NWS) was in hr2 of all isolates and ALV-B/J except AF 115-5. Another likely glycosylation site was observed downstream of hr2 in five AF 115 isolates and ALV-B/J. However, AF 115-13 and AF 115-16 had an amino acid substitution of aspartic acid (D) instead of asparagine (N). Downstream of vr3 of AF 115-5, a probable glycosylation site not present in the other isolates of ALV-B/J was observed where N substituted for serine (S).

Simple comparison of sequence distances displaying the divergence and percent identity values among gp85 amino acid sequences of seven AF 115 isolates and ALV-B/J are shown in Table 3.3. The SU-encoding sequences of six AF 115 isolates showed 95.7%-99.7% identity to each other and 96.3%-99.1% to naturally occurring recombinant ALV-B/J (AF 115-4). However, AF 115-5 was a unique isolate, having very low gp85 sequence identity with the other six AF 115 isolates (79.4%–81.8%) and ALV-B/J (79.1%). The degree of similarity ranged from 79.1% (AF 115-5 and ALV-B/J) up to the highest similarity between isolates AF 115-10 and AF 115-14 at 99.7%. Conversely, the highest divergence was observed when AF 115-5 was compared with the rest of the AF 115 isolates and ALV-B/J (20.9%-24.6%).

Figure 3.2 demonstrate the relationships of gp85 amino acid sequences of seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4). All AF 115 isolates and ALV-B/J (AF 115-4) formed a separate group with AF 115-16 clustering the furthest and AF 115-5 being in a distinct group.

2. Comparison of the envelope TM glycoprotein gp37

The alignment of TM or gp37 amino acid sequences of the seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4) are shown in Figure 3.3. Compared to ALV-B/J, all amino acid sequences of gp37 region of all AF 115 isolates were conserved and identical to ALV-B/J. Compared to ALV-B/J. Single amino acid substitution was observed in gp37 of four of the isolates (AF 115-7, AF 115-10, AF 115-14 and AF 115-16). There were 9 conserved cysteine residues in the gp37 of all seven AF 115 isolates as well as ALV-B/J (Figure 3.3). Moreover, all TM envelope glycoproteins studied contained only 3 potential conserved N-linked glycosylation sites (NXS/T).

By analogy with the conserved sequence of fusion peptide (FP) in ALSV subgroup A envelope, a conserved sequence was observed in the TM domain of all AF 115 isolates and ALV-B/J (Figure 3.3). Similar to ALSV-A, two conserved cysteines flanking the internal fusion peptide were observed in all AF 115 isolates and ALV-B/J. In addition, a conserved proline residue was observed in the middle of the predicted fusion peptides of all AF 115 isolates and ALV-B/J.

The percentage identity and divergence among gp37 amino acid sequences of all AF 115 isolates and ALV-B/J are shown in Table 3.4. The TM-encoding sequences of all seven AF 115 isolates showed 99%-100% identity to each other and 99.5%-100% to naturally occurring recombinant ALV-B/J (AF 115-4). Figure 3.4 demonstrate the relationships of TM glycoprotein sequences of seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4). All AF 115 isolates clustered very close to ALV-B/J (AF 115-4).

3. Comparison of the 3' UTR and LTR

Compared with the naturally occurring recombinant ALV-B/J, all AF 115 isolates had 3' UTR containing a single DR1 region and an E element (Figure 3.5). The DNA sequences of 3' UTR of all AF 115 isolates were very similar to those of ALV-B/J. However, scattered transition and few transversion nucleotide substitutions were observed in most isolates particularly AF 115-10. Interestingly, AF 115-10 had a 5-nucleotide deletion and a 6-base insertion in the E element. A putative CCAAT enhancer (ACAAT) was observed in all AF 115 isolates and ALV-B/J at the junction of DR1 and the E element.

Three potential C/EBP binding sites with one mismatch (in bold letter) compared to the consensus sequence were identified in all AF 115 isolates and ALV-B/J (Figure 3.5). The two 5' potential sites (core region of PU1) were overlapping with conserved sequences (ATGGCCAAAT and ATAGAGCAAG) in six AF 115 isolates and ALV-B/J. On the other hand, AF 115-10 had the following respective ATGGCTAAAT and ATAGGGTAAG sequences for these sites. The 3' possible C/EBP binding site (core region of PU2) had the following conserved sequence GCTGCGAAAT in all AF 115 isolates and ALV-B/J except AF 115-10 (ACTACGAAAT).

Most of the DNA sequences of 3' LTR of all AF 115 isolates were common with those of ALV-B/J. (Figure 3.6). However, scattered transition and transversion substitutions were mostly observed in U3 regions of AF 115-5, AF 115-7 and AF 115-10 isolates. Distinctively, there was a 13-base insertion in the U3 region of AF 115-10 (CTTACACAATAGC). The R region was identical in all AF 115 isolates and ALV-B/J. Several potential transcriptional regulatory sites were found in the U3 region of all AF

115 isolates and ALV-B/J. These included TATA, PAS, Y, PRE, CCAAT, CCAAT related LTR and CArG boxes (Figure 3.6).

A CCAAT box was identified with the consensus sequence (GCAAT) in all AF 115 isolates and ALV-B/J except AF 115-10 (GCTAT). An additional CCAAT enhancer element (ACAAT) was observed in the insertion of AF 115-10 (CTTACACAATAGC). Close by, a CCAAT LTR related enhancer motif (TTAT/CGCAAT) was located in all AF 115 isolates and ALV-B/J. The 5'CArG box had the CC(T/A)TATAAGG sequence while the sequence for the 3' CArG box was CCTTATTAGG. A highly conserved TATA box with a consensus sequence TATTTAA was found in all AF 115 isolates and the naturally occurring recombinant ALV-B/J.

Two highly conserved Y boxes (ATTGG) were situated in two distinct sites in all AF 115 isolates and ALV-B/J. Two closely situated PRE motifs or boxes (GGTGG) were observed in all AF 115 isolates and ALV-B/J. The 5' PRE box sequences overlapped with the 5' Y box sequences. AF 115-7 had two mutations, one in the 5' PRE box (GGTGA) and another in the 3' PRE motif (GATGG). Similarly, AF 115-5 and AF 115-10 had the same mutation in the 3' PRE box as AF 115-7. A key polyadenylation signal (AATAAA) was found in the 3' distal end of U3 of all AF 115 isolates and ALV-B/J.

The 3' UTR and LTR sequences of four AF 115 isolates showed 99.1%-99.6% similarity among each other and high identity ranging from 99.2% to 99.8% to the naturally occurring recombinant ALV-B/J (AF 115-4). Yet, AF 115-5, AF 115-7 and AF 115-10 exhibited 94.2%, 94.2% and 89.6% identity with ALV-B/J respectively. They also showed 89.4%-94.4% identity with the other AF 115 isolates and higher identity among each other (94.2%-96.2%) in the 3' UTR and LTR regions (Table 3.5).

These results were supported by the locations of the different isolates in the cladogram (Figure 3.7). All AF 115 isolates and ALV-B/J were in the same cluster except AF 115-10 due to its sequence mutations and rearrangements in the 3' UTR and LTR. Moreover, the genetic variations noticed in the LTR proviral DNA sequences of AF 115-5 and AF 115-7 placed them in between AF 115-10 and the rest of the AF 115 isolates and the naturally occurring recombinant ALV-B/J.

C. Sequence comparisons of AF 115 isolates and ALV subgroups

1. Comparison of the envelope SU glycoprotein gp85

When the seven AF 115 isolates were compared with representative viruses from different ALV subgroups, the amino acid sequences of gp85 regions of ALV-B/J and all AF 115 isolates except AF 115-5 were found to be very similar to RAV-2 and RSV-SR-B (subgroup B) particularly in the variable and hypervariable regions (Figure 3.8). On the other hand, AF 115-5 shared common sequences with RAV-1 in the vr1, vr2, vr3, hr1 and hr2 clusters. In addition, different ALV subgroups exhibited sequence variations mostly in the five clusters (vr1, vr2, vr3, hr1 and hr2). Subgroup J ALV has the most diverse sequences in these five clusters as well as other regions of gp85 when it was compared with the other ALV subgroups.

The14 conserved cysteine residues in the gp85 of all seven AF 115 isolates and ALV-B/J were found to be conserved in all ALV subgroups except ALV-J. Nine potential N-linked glycosylation sites were conserved in all AF 115 isolates, ALV-B/J and ALV subgroups A-E. However, there were only five conserved potential N-linked glycosylation sites (NXS/T) in all AF 115 isolates, ALV-B/J and all ALV subgroups.

Moreover, there were 14 or 15 potential N-linked glycosylation sites in SU glycoproteins of all AF 115 isolates and ALV-B/J. On the other hand, there were 11 potential sites in RAV-1 (subgroup A), 14 in Pr-RSV-C (subgroup C) and RSV-SR-D (subgroup D), 15 in RAV-2 (subgroup B), 15 in ALV ev-6 (subgroup E) and 18 in HPRS-103 (ALV-J).

The deduced amino acid sequence of the surface (SU) glycoprotein (gp85) of all AF 115 isolates exhibited the highest identity (93.1%-98.8%) to RAV-2 and RSV-SR-B (subgroup B) except AF 115-5 (Table 3.6). The amino acid sequences of gp85 of AF 115-5 showed 89.4% similarity to RAV-1 (subgroup A), 78-78.6% to RAV-2 and RSV-SR-B (subgroup B), 85.2% to Pr-RSV-C (subgroup C), 83.4% to RSV-SR-D (subgroup D), 84.1% to ALV ev-6 (subgroup E) and 35.5% to HPRS-103 (subgroup J).

Six AF 115 isolates and ALV-B/J (AF 115-4) clustered close to RAV-2 and RSV-SR-B (subgroup B) in the phylogenetic tree. However, AF 115-5 was in a separate clade closer to RAV-1, a subgroup A ALV (Figure 3.9). All these results suggested that there are two classes of viruses characterized by different SU domains belonging to two different ALV subgroups.

2. Comparison of the envelope TM glycoprotein gp37

Compared to prototypes of various ALV subgroups, the amino acid sequences of the TM of seven AF 115 isolates and the naturally occurring recombinant ALV-B/J (AF 115-4) were very similar to all ALV subgroups except HPRS-103, a subgroup J ALV (Figure 3.10). Few scattered amino acid substitutions were observed in gp37 of all ALV subgroups compared with AF 115 isolates. HPRS-103 showed the highest divergence in amino acid sequences with the other viruses.

Six of the 9 conserved cysteine residues in the gp37 of all seven AF 115 isolates and ALV-B/J were found to be conserved in all ALV subgroups. Moreover, TM envelope glycoproteins of subgroups A (RS-A), B (RSV-SR-B) and D (RSV-SR-D) contained 9 cysteine residues. On the other hand, there were 7 cysteines in subgroups C (Pr-RSV-C) and J (HPRS-103) and 8 such residues in ALV-E (ALV ev-6). The 3 potential N-linked glycosylation sites (NXS/T) conserved in the gp37 of all AF 115 isolates and ALV-B/J were common to all other ALV subgroups except ALV-J (HPRS-103).

The conserved sequence of the internal fusion peptide (FP) in the TM domain of all AF 115 isolates and ALV-B/J was identical to ev-6 and RSV-SR-D (Figure 3.10). However, there was one amino acid substitution in FP of RS-A, RSV-SR-B and Pr-RSV-C; but four residue changes in HPRS-103. The two cysteines flanking the internal fusion peptide and the central proline were shared by all AF 115 isolates and ALV subgroups.

The TM-encoding amino acid sequences of all seven AF 115 isolates were 93.7%–95.1% identical with RS-A, 94.1%–95.1% with RSV-SR-B, 95.5%–96% with Pr-RSV-C, 94.7%–95.6% with RSV-SR-D, 95.1%–98.5% with ev-6, and 56.9%–57.4% with HPRS-103 (Table 3.7). All seven AF 115 isolates and ALV-B/J (AF 115-4) were grouped closer to subgroup E (Figure 3.11). As expected, subgroup J ALV was in a separate cluster than the rest of the ALV subgroups. Subgroups A, B and D clustered together while ALV-E and ALV-C clustered closer to AF 115 isolates.

3. Comparison of the 3' UTR and LTR

Since ALV-J and sarcoma viruses contain the 3' UTR, sequence alignment of 3' UTR of HPRS-103 and all AF 115 isolates and ALV-B/J was performed. The 3' UTRs of

all AF 115 isolates and ALV-B/J were similar to that of HPRS-103 with some substitutions. They also shared a common DR1 region and a complete E element except AF 115-10, which had a 5-nucleotide deletion and a 6-base insertion (Figure 3.12). Similarly, all AF 115 isolates and ALV-B/J shared an identical CCAAT enhancer (ACAAT) with HPRS-103 at the junction of DR1 and the E element. In addition, AF 115 isolates and ALV-B/J had the same core regions of PU1 and PU2 exhibiting one mutation as that of subgroup J ALV. Sequence analysis of the 3' UTRs of all AF 115 isolates indicated the highest identity (95.6%-97.6%) with the same region of the ALV-J isolate HPRS-103 (Table 3.8). All AF 115 isolates and ALV-B/J were in the same clade as HPRS-103 except AF 115-10 (Figure 3.13).

Most of the proviral DNA sequences of 3' LTR of all AF 115 isolates were most similar to HPRS-103 except for 11-base deletion in the U3 region (Figure 3.14). When compared with HPRS-103, this deletion removed one of the adjacent 11-base enhancer repeat motifs, ATGGTATGATC. On the other hand, HPRS-103 exhibited two direct repeat sequences (GTGGTATGAT and ATGGTATGATC). The R regions of all AF 115 isolates and ALV-B/J were identical to that of subgroup J ALV (HPRS-103), which exhibited variations from other ALV subgroups A, B, C and D (Figure not shown).

The putative transcriptional regulatory sites of all AF 115 isolates and ALV-B/J were similar to those of ALV-J and shared by other exogenous ALVs (Figure not shown). The U3 regions of all AF 115 isolates, ALV-B/J and all exogenous ALV subgroups included two Y boxes, two PRE motifs, CCAAT LTR enhancer and two CArG boxes. On the other hand, a CCAAT-like box was found in all AF 115 isolates, ALV-B/J and ALV subgroups C and J. Moreover, AF 115-10 had one mutation in the CCAAT LTR

enhancer, CCAAT-like box, and 3' PRE motif in addition to an extra CCAAT box in the 13-base insertion. On the other hand, AF 115-5 had a mutated 3' PRE box while AF 115-7 contained two mutated PRE boxes. Some differences in the transcription regulatory elements were observed between exogenous and endogenous ALVs (Figure not shown). Subgroup E ALV (ev-1) had a CCAAT LTR enhancer box downstream of the CCAAT LTR enhancer box of exogenous viruses. Only one CArG, Y and PRE boxes were present in the endogenous ALV. The TATA box and PAS were well conserved in all AF 115 isolates, exogenous and endogenous ALVs.

The DNA sequences of the 3' LTR of the four AF 115 isolates and ALV-B/J showed high identity with HPRS-103 (subgroup J) ranging from 94.8% to 95.6% (Table 3.9). On the other hand, AF 115-5, AF 115-7 and AF 115-10 showed lower sequence identities at 89.2%, 88.8% and 84% respectively. The AF 115 isolates were grouped into two separate clusters (Figure 3.15). Four AF 115 isolates and ALV-B/J (AF 115-4) were clustered close to subgroup J. On the other hand, a separate clade contained AF 115-5, AF 115-7 and AF 115-10, being the most divergent.

D. Sequence comparisons of AF 115 isolates and blasted ALV strains

1. Comparison of the envelope SU glycoprotein gp85

Blast search of the amino acid sequences of the SU domains of all AF 115 isolates revealed high similarity to those of viruses belonging to ALV subgroups A and B. The sequence alignment suggested that the isolates fell into two classes and were very similar to viruses related either to MAV-1 or RAV-2. Six AF 115 isolates were RAV-2-like and AF 115-5 was MAV-1-like based on sequence similarities in the five clusters of variable

(vr1, vr2 and vr3) and hypervariable (hr1 and hr2) regions (Figure 3.16). More specifically, six AF 115 isolates showed sequence similarities mostly to ALV-26, followed by RSV-SR-B and RAV-2 and less to AMAV-2, all of which are subgroup B. On the other hand, AF 115-5 showed most sequence identities to ALV-18, ALV TymS_90, ALV-25 and ALV-48, followed by ALV-1, ALV-10, ALV-20 and less to MAV-1, ALV LR-9 and RSA, all of which are subgroup A.

These results were backed up by the sequence distances data (Table 3.10) with sequence identities between six AF 115 isolates with ALV-26 (94.3% to 97.7%), RSV-SR-B (93.4%-96.8%), RAV-2 (92.8%-96.2%) and AMAV-2 (94.2%-95.1%). On the other hand, AF 115-5 sequence identity with these NCBI published sequences ranged between 78% and 79.9%. Yet, AF 115-5 exhibited sequence identity of 100% with ALV-18, 99.7% with ALV TymS_90 and ALV-25, 99.4% with ALV-48, 99.1% with ALV-1 and ALV-20 and 98.8% with ALV-10.

Phylogenetic tree of these isolates and reference strains from the GenBank was established. Figure 3.17 shows the similarities among AF 115 isolates, MAV-1-like and RAV-2-like isolates already described in graphic format. The phylogenetic analysis showed that AF 115-5 formed a tight cluster with the published sequence for MAV-1-like viruses. The remaining six AF 115 isolates were clustered in the same branch with the published RAV-2-like sequences.

2. Comparison of the envelope TM glycoprotein gp37

NCBI blast search of the amino acid sequences of the TM domains of all AF 115 isolates revealed high similarity to those of viruses belonging to ALV subgroup E. The

sequence alignment suggested that all the isolates were very similar to endogenous viruses such as subgroup E ALVs (ALV-E SD0501 and RAV-0), ev loci (ev-1, ev-3, ev-6 and ev-21) and recombinant ALVs (ADOL 6803A, ADOL 5701A, ALV TymS_90 and ALV PDRC-3249) with subgroup E gp37 (Figure 3.18). Moreover, the fusion peptide sequence was mostly conserved in TM domains of all viruses studied. Based on sequence comparison in the gp37 of these isolates and published sequences, all AF 115 isolates were 97.6%-98.5 with ALV-E SD0501 and ev-6, 97.1%-98% with ev-1, ev-3, ALV TymS_90 and ALV PDRC-3249, 96.6%-97.6% with ADOL 6803A and ev-21, 96.1%-97.1% with ADOL 5701A and 95.6%-96.6% with RAV-0 (Table 3.11). Phylogenetic tree of these isolates and matched viral strains from the GenBank showed that all AF 115 isolates are mostly similar to different viruses containing subgroup E gp37 (Figure 3.19).

3. Comparison of the 3' UTR and LTR

Blast search of proviral DNA sequences of the 3' UTR of all AF 115 isolates revealed high association with 3' UTR of subgroup J ALVs (HPRS-103, UD2, UD-J1, ADOL-7501) and recombinant ALVs with subgroup J 3' UTR (ALV TymS_90 and ADOL 5701A). Six AF 115 isolates had DR1 and E element sequences similar to those of ALV-J isolates and ADOL-5701A, a recombinant ALV-A/J. However, AF 115-10 had similar sequences to ALV TymS_90 based in the rearranged regions (insertion) and putative transcription elements (Figure 3.20). Specifically, the mutated potential transcription factors binding sites (PU1 and PU2) in the 3' UTR were similar in all viruses studied. Interestingly, the 6-base insertion in AF 115-10 3' UTR was identical to

TymS_90 and similar to RSV-NTRE-7 td (recombinant virus between RAV-0 and td RSV-Pr-B) and RSV-td PR2257 (RSV-Pr-C).

The 3' LTR of six AF 115 isolates showed sequence similarities mostly to ALV-J isolates HPRS-103, UD2, UD-J1, ADOL-7501 and recombinant ALV-A/J isolate ADOL-5701A (Figure 3.21). On the other hand, AF 115-10 mostly showed similar sequences in the 3' LTR to ALV TymS_90, a recombinant virus with AEV-like 3' U3. In the U3 regions, the mutations in the enhancer boxes and the 13-base insertion observed in AF 115-10 were identical to those of TymS_90. The 11-base deletion in U3 of all AF 115 isolates was shared with TymS_90, AEV E26, RSV-NTRE-7 td and RSV-td PR2257. The transcription regulatory elements were present in all viruses studied.

The 3' UTR and LTR of six AF 115 isolates exhibited higher sequence identities with those of ALV-J isolates while that of AF 115-10 was more homologous to that of ALV TymS_90 (Table 3.12). Most AF 115 isolates showed sequence identities ranging 92.7%- 95.7% with HPRS-103, 90.4%-93.4% with UD2, 89.7%-92.3% with ADOL-5701A, 89.5%-91.5% with ADOL-7501 and 89.3%-91.4% with UD-J1. On the other hand, AF 115-10 sequence identity with these NCBI published sequences ranged between 87.5% and 90.3%. Moreover, AF 115-10 exhibited sequence identity of 97.2% with TymS_90 while AF 115-5 and AF 115-7 showed higher sequence identity with this isolate (94.3%) than ALV-J isolates (89.3%-93.6%).

Cladogram constructed with the sequences of the 3' UTR and LTR of the seven AF 115 isolates and other NCBI published matching sequences showed that four AF 115 isolates were associated with subgroup J ALVs while AF 115-10 was related to ALV TymS_90 and AF 115-5 and AF 115-7 were in between the two (Figure 3.22).

DISCUSSION

The data presented here document partial molecular structure of provrial DNA of each of the seven AF 115 isolates including the *env* gene and 3' UTR and LTR. The proviral DNA of ALV is usually composed of *gag*, *pol* and *env* genes flanked by LTRs (Bai *et al.*, 1995a). Among different subgroups, the *gag* and *pol* genes are well conserved while the *env* gene is highly diverged and the LTR sequences are relatively variable (Payne, 2001). The major genetic diversity noted among AF 115 isolates were localized in gp85 of *env* of isolate AF 115-5 and 3' UTR and LTR of AF 115-10. This agrees with previous observation that different regions of the genomic proviral DNA can evolve independently (Cui *et al.*, 2003a). The genetic diversity in different regions of the seven AF 115 isolates is most likely to result from mutations in the viral RNA during replication, selection by immune pressure and recombination events among multiple ALV subgroups circulating in chicken flocks. This leads to the generation of novel recombinant ALVs with different genome locations representing different subgroups.

The *env* gene of ALV encodes two functional regions, the surface (SU) and transmembrane (TM) domains, which facilitate entry into the target cell. Moreover, it influences the types of cell targets, and oncogenic spectrum of the virus (Brown and Robinson, 1988; Chesters *et al.*, 2002). For example, subgroups A and B ALVs mostly cause lymphoid leukosis while ALV-J induces myeloid leukosis in chickens (Bai *et al.*, 1995a, Payne *et al.*, 1992b). Recombinant viruses are believed to be more pathogenic than their parental counterparts (Aurigemma *et al.*, 1991; Kogekar *et al.*, 1987).

The SU domain (gp85) of envelope glycoprotein recognizes and binds with specific host cell receptor and determines subgroup specificity (Bai *et al.*, 1995a; Bova *et al.*, 1986 and 1988; Bova-Hill *et al.*, 1991; Dorner and Coffin, 1986; Hunter, 1997; Rong *et al.*, 1997). On the other hand, the TM subunit (gp37) anchors the SU glycoprotein to the viral membrane and mediates fusion of the viral and target cell membranes (Fadly and Payne, 2003; Hernandez *et al.*, 1996). Alignment of the deduced amino acid sequences among AF 115 isolates revealed several substitutions distributed throughout the gp85 subunit and four in the gp37 subunit. However, the importance of these amino acid substitutions is not known.

The SU (gp85) of *env* is known for its considerable genetic variations leading to antigenic diversity and altered phenotype. Compared to ALV-B/J and other AF 115 isolates, AF 115-5 showed the highest diversity in amino acid substitutions in these five variable clusters while AF 115-16 exhibited similar variation only in vr1 region. This genetic divergence displayed by AF 115-5 may reflect changes in host range, subgroup specificity and receptor interaction. Similarly, the *env* gene of NTRE-4 was shown to be a composite of subgroup B *hr*1 region and subgroup E *hr*2, which infected both C/E and T/BD cells (Dorner *et al.*, 1985; Tsichlis *et al.*, 1980). Moreover, subgroup J ALV showed high degree of variability in the gp85 region of *env* among different strains, isolates and subtypes (Fadly and Smith, 1999; Hunt *et al.*, 1999; Silva *et al.*, 2000; Venugopal *et al.*, 1998).

Despite high levels of sequence variability in gp85 SU of AF 115-5, all isolates in addition to ALV-B/J contained 14 and 9 conserved cysteine residues in the SU gp85 and the TM gp37 glycoproteins respectively. Some of the amino acid sequences of ALV

subgroups A-E are very similar containing conserved cysteine residues. These residues are involved in intrachain disulfide bonds and believed to maintain the overall secondary structure of retroviral envelope glycoproteins (Dorner and Coffin, 1986; Linder *et al.*, 1992; Valsesia-Wittmann *et al.*, 1994). These results may reflect the structural stability of envelope glycoproteins of AF 115 isolates.

Like gp85 SU glycoprotein subunit, the gp37 TM glycoprotein subunit exists in the virus particle as a trimer linked to SU through a disulfide bond (Delos and White, 2000; Einfeld and Hunter, 1988; Hernandez and White, 1998; Hunter *et al.*, 1983). The TM glycoprotein of ALSV generally contains a stretch of hydrophobic amino acids at an internal site to the N terminus. This region represents the internal fusion peptide, which is highly conserved among different subgroups and has been implicated in viral fusion activity. Generally, a fusion peptide is a helix of 15 to 25 of relatively hydrophobic amino acids in length, rich in alanine and glycine (White, 1992). A central helix-breaking proline residue usually exists inside the internal fusion peptide. The predicted fusion peptide in the TM domain was conserved in all AF 115 isolates and ALV-B/J. This internal region contained a conserved central proline and was flanked by two conserved cysteines. These cysteines are believed to be involved in forming a disulfide bond at the base of the looped fusion peptide. In addition, these cysteine residues are hypothesized to be essential for proper TM glycoprotein folding, processing, assembly into trimers and fusion activity (Delos and White, 2000).

The envelope glycoproteins are heavily glycosylated especially the SU region (Rong *et al.*, 1997). For example, the SU of the ASLV-A envelope glycoprotein contains 11 potential N-linked glycosylation sites (NXS/T). There were 12 and 3 conserved

potential N-linked glycosylation sites (NXS/T) in SU and TM of all AF 115 isolates as well as ALV-B/J respectively. N-linked glycosylation is important for glycoprotein function through proper folding (Ellgaard *et al.,* 1999; Helenius and Aebi, 2001) and stability (Fenouillet *et al.,* 1990; Imperiali and O'Connor, 1999). Moreover, it has been exploited by viral glycoproteins for immune evasion and receptor usage through direct and indirect protein–protein interactions (Bolmstedt *et al.,* 1996; Kinsey *et al.,* 1996; Reed *et al.,* 1997; Reitter *et al.,* 1998; Schonning *et al.,* 1996; Sjolander *et al.,* 1996; Willey *et al.,* 1996). Two of the gp85 conserved glycosylation sites in these AF 115 isolates were consecutive, but they are not likely to be both utilized at the same time by glycosylation enzymes due to steric interference (Wu *et al.*, 1995). Some of the glycosylation sites may be required for presentation of the adjacent cysteine for proper disulfide bond formation (Delos *et al.*, 2002). Some amino acid substitutions in SU glycoprotein resulted in extra or less glycosylation sites in some of the AF 115 isolates. Whether any of these potential sites are actually glycosylated is not known.

Sequence alignment and distance as well as the phylogenic tree revealed that the SU glycoprotein of AF 115-5 is highly divergent from the rest of AF 115 isolates and the naturally occurring recombinant ALV-B/J. Nonetheless, they showed that the SU amino acid sequences of the rest of the AF 115 isolates were similar to each other and to the ALV-B/J. In addition, sequences encoding the TM (gp37) domain of all AF 115 isolates were highly homologous to each other and ALV-B/J from % identity data and cladogram.

Since 3' UTR and LTR were known to contain sequences important in regulating transcription, translation, splicing, RNA stability, transport and processing; these regions in AF 115 isolates were sequenced. PCR amplification patterns using primers targeted to

different regions of 3' UTR and LTR had suggested that mutations may have occurred in many of these AF 115 isolates. The 3' UTR and LTR sequences of all AF 115 isolates except AF 115-10 were similar to the naturally occurring recombinant ALV-B/J.

The 3' noncoding or untranslated region (3' UTR) between the 3' end of gp37 of *env* and the LTR of ASLV is conserved and essential for efficient replication and viral pathogenesis (Ogert and Beemon, 1998; Ogert *et al.*, 1996; Simpson *et al.*, 1997; Sorge *et al.*, 1983). It has an impact on the development of tumors particularly when it is subjected to mutations and genetic rearrangements (Hue *et al.*, 2006). Moreover, it contains powerful regulatory elements, which influence viral gene expression (Bai *et al.*, 1998a; Robinson *et al.*, 1982; Ruddell, 1995; Zachow and Conklin, 1992). This region is found in some members of ASLV such as RSV and subgroup J ALV (Bai *et al.*, 1995b and 1998a; Hue *et al.*, 2006; Laimins *et al.*, 1984; Tsichlis *et al.*, 1982). The 3' UTR nucleotide sequences can provide information on the phylogenies of the ancestral ALSVs (Onuki *et al.*, 1987). The 3' UTR of ALV J consists of a rTM insert, a direct repeat (DR1) region and an E element (XSR).

Within 3' UTR, the DR 1 was present and almost identical in all AF 115 isolates and ALV-B/J. In fact, the DR1 (or F1) element is found at the 5' end of 3' UTR, as a single copy in ALVs (Bai *et al.*, 1998b; Lupiani *et al.*, 2006; Silva *et al.*, 2000). However, two copies of DR1 are usually flanking the *src* gene in avian sarcoma viruses (Laimins *et al.*, 1984). This element has been associated with efficient accumulation and stability of unspliced RNA in the cytoplasm (Ogert and Beemon, 1998; Ogert *et al.*, 1996), genomic RNA packaging (Aschoff *et al.*, 1999; Sorge *et al.*, 1983), virion production and increased spliced src mRNAs in avian sarcoma viruses (ASVs).

Most AF 115 isolates shared similar E element with ALV-B/J. In general, the E (XSR or F2) element is found as a single copy of a hairpin loop sequence downstream of DR1 in ASVs and ALVs (Lupiani *et al.*, 2006; Payne *et al.*, 1991; Schwartz *et al.*, 1983; Silva *et al.*, 2000; Skalka *et al.*, 1983). The E element may influence the oncogenic spectrum and incidence of some ALSVs through activation of cellular or viral oncogenes (Bai *et al.*, 1995b; Bizub *et al.*, 1984; Laimins *et al.*, 1984; Tsichlis *et al.*, 1982). For example, some ALVs containing the E element caused more frequent tumor expression in certain genetic lines of chickens including recombinant ALV-A/J (Chesters *et al.*, 2006; Lupiani *et al.*, 2003). Similarly, NTRE-7, a recombinant virus between transformation-defective Pr-B and RAV-0, was reported to have inherited E element from the RSV parent (Tsichlis *et al.*, 1982). On the other hand, the E element of AF 115-10 exhibited few rearrangements (5-nucleotide deletion and 6-base insertion). Whether these rearrangements in the E element have profound effects on AF 115-10 replication or oncogenicity in vivo is not known. A putative CCAAT enhancer was observed in all AF 115 isolates and ALV-B/J at the junction of DR1 and the E element. A similar enhancer element (ACAAT) was reported in the U3 region of ALV-J field isolates from different countries (Zavala *et al.*, 2007).

The secondary hairpin stem– loop structure of the E element provides a potential nucleation site for RNA folding and multiple interactions with some host factors. For example, the E element may contain potential enhancer elements (CCAAT) or responsive elements (PU1 and PU2) with the consensus sequence A/C/GTT/GNNGC/A/TAAT/G (Ryden *et al.*, 1993). These elements bind CCAAT/enhancer-binding protein (C/EBP) transcription factors, which regulate viral gene transcription and protein expression

(Baglia *et al.*, 1997; Bowers *et al.*, 1996; Smith *et al.*, 1994). Potential enhancer elements (C/EBP binding sites) were highly conserved in the E element of all AF 115 isolates and ALV-B/J except AF 115-10. The two overlapping core regions of PU1 sites are not likely to be both utilized simultaneously by C/EBP transcription factors due to steric interference. Although the core regions of PU1 and PU2 in all AF 115 isolates contained one nucleotide mismatch, they can still be potentially functional. Similar results were observed in different ALV-J field isolates from France (Hue *et al.*, 2006). In addition, PU1 and PU2 sites from Pr-C RSV exhibited one mismatch compared with the consensus C/EBP binding site and were still functional and able to bind small amounts of C/EBP transcription factors (Ryden *et al.*, 1993).

The 3' LTR of ALV contains a unique 3 (U3) region, a repeat (R) region and a unique 5 (U5) region. Overall, the 3' LTR of AF 115-10 was the least conserved among the isolates and ALV-B/J, followed by AF 115-5 and AF 115-7. The R region was identical in all AF 115 isolates and ALV-B/J. This was expected since this region is very vital for reverse transcription when the reverse transcriptase enzyme recognizes a strong stop signal in R at 5' end of the virus and then jumps to the homologous R at the 3' end. The 5' end of U5 was not sequenced.

The U3 region contains several conserved motifs or transcriptional regulatory elements including promoters and enhancers. Thus, it is believed to play a role in ASLV replication, infection and oncogenesis (Bowers *et al.*, 1994; Ruddell, 1995; Ruddell *et al.*, 1989). Some of the conserved protein binding sequence elements in U3 of ASVs and ALVs include TATA (Laimins *et al.*, 1984), CCAAT (Baglia *et al.*, 1997; Bowers and Ruddell, 1992; Golemis *et al.*, 1990), CArG (Zachow and Conklin, 1992), Y (Ruddell,

1995) boxes and Pentanucleotide repeat elements (PREs). There was a 13-base insertion in the U3 region of AF 115-10 just upstream of the CCAAT enhancer motif. However, the transcriptional regulatory elements typical of avian retroviruses (TATA, PAS, CCAAT, CCAAT LTR enhancer, CArG, PRE and Y boxes) were found in all AF 115 isolates and ALV-B/J.

Toward the 3' end of U3 of all AF 115 isolates and ALV-B/J, there were a highly conserved TATA box and a key polyadenylation signal (PAS). TATA boxes and TATA-related motifs are promoters located at the 3' end of U3 and are involved in initiation of transcription of ASLVs (Cullen *et al.*, 1985). On the other hand, the key polyadenylation signal (AATAAA) corresponds to the AAUAAA polyadenylation signal in the RNA genome of ALSV (Golemis *et al.*, 1990; Guntaka, 1993). CCAAT boxes and CCAAT-related motifs are enhancer elements, to which transcription factors bind leading to enhanced gene transcription (Bowers *et al.*, 1996; Curristin *et al.*, 1997; Ruddell, 1995; Smith *et al.*, 1994). An additional CCAAT LTR enhancer element with the consensus sequence 5'T(T/G)NNG(C/T)AA(T/G)3' is found in the 3' U3 of RSV and a target for the A1/EBP and VBP (bZip) DNA binding factors (Smith *et al.*, 1994; Zachow and Conklin, 1992).

CArG boxes (5'CC(A/T)6GG3') are enhancers and binding sites for enhancer factor III (EFIII) implicated in mediating transactivation of the RSV LTR enhancer (Boulden and Sealy, 1990). In the host, CArG boxes are involved in tissue-specific cellular gene expression (Ruddell, 1995). Y boxes (5'ATTGG3') are inverted 5'CCAAT3' enhancer boxes in ASVs and some ALVs (Laimins *et al.*, 1984). They bind enhancer factor I (EFI) associated with the activation of viral transcription (Greuel *et al.*,

1990; Zachow and Conklin, 1992). Conserved inverse Y boxes (5'ATTGG3') of ALV-J are targets for YB-1/EFI DNA binding factors in chickens (Grant and Deeley, 1993). PRE motifs (5'GGTGG3') may bind early proteins such as the infected cell protein 4 during Marek's disease virus (MDV) infection and lead to transactivation of ALSV LTR (Banders and Coussens, 1994).

All AF 115 isolates and ALV-B/J contained a CCAAT box and a conserved CCAAT LTR enhancer motif, two PRE motifs, and two conserved CArG and Y boxes. The presence of duplicate number of these elements in the LTR of AF 115 isolates is consistent with exogenous viruses such as ALV-J (Zavala *et al.*, 2007). LTRs of endogenous viruses carry weaker or a lower number of enhancers and are thus transcriptionally silent (Ruddell, 1995). However, AF 115-10 contained two mutated CCAAT boxes and 3' PRE motif while both PRE boxes of AF 115-7 had one mutation and AF 115-5 contained a mutated 3' PRE motif. Mutations in PRE boxes have been reported from different ALV-J isolates from France (Hue *et al.*, 2006).

These elements may be targeted by various DNA binding proteins (transcription factors) to enhance transcription, gene expression and *c-myc* activation. Some of the enhancer motifs in the U3 of the LTR of these isolates may act in synergy with the core regions of PU1 and PU2 binding sites of the E element similar to what has been suggested for ALV-J isolates (Hue *et al.*, 2006). Several studies have reported that mutations in some of these enhancers such as CCAAT boxes and CCAAT-related LTR enhancers negatively affected LTR-driven transcription in transient infections in CEF or abolished binding of several transcription factors (Bowers and Ruddell, 1992; Ryden *et al.*, 1993; Smith *et al.*, 1994). Further studies are required to confirm the functional

potential of these sites (wild type and mutated) as enhancers and targets for DNA-binding proteins. The conserved sequences in some of these putative sites can be targeted by primers to improve molecular diagnosis of ALV. The rate of replication and the frequency of tumors (pathogenicity) of different isolates may be variable since these elements were conserved in some but not all the isolates and ALV-B/J.

Since 3' UTR and LTR do not code for structural proteins like the *env*, it can be used to determine virus lineages based on genetic sequences not subject to immune pressure (Zavala *et al.*, 2007). The sequence distances and phylogenies revealed that the 3' UTR and LTR of all AF 115 isolates except AF 115-10 were similar to ALV-B/J. The diversity exhibited by the 3' UTR and LTR of AF 115-10 may have contributed to the moderately distinct genetic lineage of this variant virus.

Sequence variability in SU (gp85) of the envelope is usually clustered in five regions, the variable regions *vr1*, *vr2*, *vr3* as well as the highly variable larger clusters *hr1* and *hr2* (Bova *et al.*, 1986). The *hr1* and *hr2* regions determine receptor binding specificity while the *vr3* region is responsible for receptor recognition (Bova *et al.*, 1988). Sequence comparison based on the five clusters of the SU region (gp85), sequence distances and phylogenetic trees of all AF 115 isolates with known ALV subgroups indicated that they belonged to subgroup B except AF 115-15, which belonged to ALV-A. All AF 115 isolates shared 14 conserved cysteine residues with all ALV subgroups in the gp85. There were 14 conserved N-linked glycosylation sites in the SU proteins of six AF 115 isolates, ALV-B/J and subgroup B ALV. However, the SU of AF 115-5 shared 9 conserved sites with ALV-A.

Similar analyses for the TM portion (gp37) of these isolates suggested they had similar sequences to ALV subgroups A-E. The gp37 sequences of ALV subgroups A-E show high sequence identity with few amino acid substitutions. On the other hand, ALV-J is more diverse and exhibit variable gp37 sequences when compared with the other subgroups (Bai *et al.*, 1995b and 1998b). Moreover, subgroup J ALV contains a nonfunctional redundant TM (rTM), which is a partially duplicated copy of the TM region (Bai *et al.*, 1995b). In fact, all AF 115 isolates shared 6 conserved cysteine residues with all ALV subgroups in the gp37. There were 2 conserved N-linked glycosylation sites in the TM proteins of all AF 115 isolates and ALV subgroups.

Since it was established that all AF 115 isolates like ALV-B/J had a 3' UTR, these sequences were compared to those of ALV-J (HPRS-103). All AF 115 isolates showed similar 3' UTR sequences, % identity, phylogeny and potential enhancer elements to ALV-J including CCAAT box at the junction of DR1 and E element and core regions of PU1 and PU2 in the E element. However, AF 115-10 showed sequence divergence in the E element (5-base deletion and 5-base insertion) compared with ALV-J.

The proviral DNA sequences of 3' LTR of all AF 115 isolates were similar to various exogenous ALV subgroups particularly subgroup J. They shared similar transcription regulatory elements (TATA, PAS, CCAAT and CCAAT related LTR enhancer motifs, two CArG, two Y and two PRE boxes). However, all AF 115 isolates had 11-base deletion compared with HPRS-103, which was responsible for deletion of one of the adjacent repeat elements. These adjacent repeated motifs have been reported to be variable in numbers in different viral strains of ALSV (Bai *et al.*, 1995b; Patschinsky *et al.*, 1986). The recombinant avian carcinoma virus MH2E21 has three adjacent repeats,

the prototype of subgroup J ALV (HPRS-103) and ASV CT10 has two and ASV Y73 has one. The variable numbers of these direct repeats in some viral strains have been hypothesized to be associated with increased virulence or pathogenicity in the host. Similar sequence alignment was observed among the R and U3 regions (11-base deletion) of all AF 115 isolates and HPRS-103 except for a 13-base addition in AF 115-10.

To confirm the relationships between different AF 115 isolates and different ALV subgroups in different genomic region, a blast search was performed using sequences of the envelope and non-coding regions. The sequence comparison of the seven AF 115 isolates with similar NCBI sequences suggested that these viruses are recombinant ALVs. GenBank blast search for sequences similar to AF115 isolates revealed that the SU domains of six of these isolates were RAV-2-like and that of AF 115-5 is MAV-1-like. RAV-2 (subgroup B *env*) and MAV-1 (with an envelope antigenically related to ALV-A) are laboratory propagated strains isolated from stocks of defective RSV or AMV associated with LL, ML and other tumors in chickens (Dougherty, 1987). The gp85 of AF 115-5 showed high association with one (ALV TymS_90) of ten MAV-1-like isolates from commercial layers associated with fowl glioma in Japan (Hatai *et al.*, 2008).

There were also similarities between our isolates and ALV isolates in the SU region from another study. The MAV-1-like isolates (ALV-1, ALV-10, ALV-20, ALV-18, ALV-25, ALV-48) and RAV-2-like isolate (ALV-26) related to our AF 115 isolates were isolated from white leghorn commercial layers in a farm in Canada between 1997 and 1999 (Spencer *et al.*, 2003). It was interesting that our AF 115 isolates came from white leghorn commercial layers from a farm in Canada between 1997 and 1998. Sequence analyses of the related viruses revealed that six isolates contained MAV-1-like

viruses, three had RAV-2-like viruses and one contained a mixture of the two viruses (Spencer *et al.*, 2003).

A blast search demonstrated that the TM domains of all AF 115 isolates were highly homologous to subgroup E ALVs (RAV-0, SD0501, ev-1, ev-3, ev-6 and ev-21). This was similar to results reported on the first naturally occurring recombinant ALV-B/J (AF 115-4) from the same flocks, which had a gp37 belonging to subgroup E ALV (Lupiani *et al.*, 2006). Moreover, our isolates showed similar sequences to two recombinant ALV-A/Js (ADOL 6803A and ADOL 5701A) possessing subgroup E gp37 TM regions, which were isolated from alv6 CEFs infected with subgroup J ALV (Lupiani *et al.*, 2000). The TMs of AF 115 isolates showed high identity to one isolate (ALV TymS_90) associated with fowl glioma in layers in Japan, which also had TM belonging to endogenous viral loci or subgroup E ALV (Hatai *et al.*, 2008). Another virus that was related to our isolates in the gp37 region was ALV PDRC-3249, a recombinant ALV with subgroup A gp85 and subgroup E gp37 and LTR isolated from contaminated commercial Marek's disease vaccines (Barbosa *et al.*, 2008)

Comparisons of 3' UTRs and LTRs of six of the seven AF 115 isolates with published NCBI sequences showed high association with viruses (HPRS-103, UD2, UD-J1 and ADOL-7501) belonging to ALV-J and recombinant ALV-A/J (ADOL 5701A). On the other hand, AF 115-10 showed similar sequences in the 3' UTR and LTR to ALV TymS_90, a recombinant ALV isolated from a case of fowl glioma in layers in Japan. ALV TymS_90 had a MAV-1-like SU domain, an *ev* loci-like TM region, UTR and LTR associated with different exogenous viruses (A, D and J for UTR and AEV E26 for U3) and endogenous viruses in the R and U5 regions (Hatai *et al.*, 2008).

It is clear from sequence comparisons that all seven AF 115 isolates were similar to several ALV subgroups in different parts of the genome. AF 115 isolates are separated from the other ALSVs by a number of recombination events although it is not clear how those events have taken place. This raised the possibility that these isolates might have formed from recombination events among different ALV subgroups in the field or hatchery, where infected chicks were in close proximity with ALV-J positive meat-type chicks. This confirms previous predictions of possible occurrence of natural recombination among different ALV subgroups circulating in chicken flocks (Gingerich *et al.*, 2002; Luinani *et al.*, 2003, Nichol, 1996).

The recombination boundary in all AF 115 isolates is likely to be between gp85 and gp37 sequences. Another recombination hot spot in these isolates is speculated to be between gp37 coding region and 3'UTR sequences upstream of the DR1 region. These recombination hot spots were also predicted in the naturally occurring recombinant ALV with subgroup B SU domain, subgroup E TM region and subgroup J LTR (Lupiani *et al.*, 2006). In the case of AF 115-10, another recombination boundary may be between 3' UTR and 3' LTR sequences among exogenous or endogenous ALVs. For example, ALV TymS_90 was characterized by sequence analysis to be a recombinant virus belonging to different exogenous ALVs (subgroup A, B, C, D and J) and endogenous ALV (ev loci, subgroup E ALV) in different genomic regions (Hatai *et al.*, 2008).

In summary, DNA sequence analysis identified and confirmed that the seven AF 115 isolates were recombinant ALVs with different genomic regions representing different ALV subgroups. This raised the possibility of the presence of potential recombination hotspots or boundaries among different ALV subgroups in these

recombinant ALVs. Moreover, associations between AF 115 isolates and various ALV subgroups and strains were predicted based on rearrangements including deletions and insertions in the 3' UTR and LTR as well as mutations in different genomic regions such as gp85 and LTR regions of recombinant ALVs. Furthermore, the stability of the envelope glycoproteins (SU and TM) were speculated based on the conserved cysteine residues and predicted N-linked glycosylation sites in these sites. In addition, nucleotide sequences in the 3' UTR and LTR of AF 115 isolates of recombinant ALVs revealed the presence of potential transcription regulatory elements, which were mutated in some isolates and may affect efficiency of transcription of these viruses.

In conclusion, our data suggest that there is a big diversity in the seven AF 115 isolates obtained from a single outbreak of ML in commercial chickens. It is a very significant finding since it is the first reported outbreak of recombinant ALVs in field flocks where various forms of recombinant ALVs were isolated and characterized (ALV-A/J and ALV-B/J). This confirms that ALV is mutating and evolving very quickly through recombination, which may have devastating consequences for the poultry industry. Moreover, this will help in the current understanding of ALV epidemiology and retroviral evolution. For example, new highly virulent strains of recombinant ALVs may emerge in the future, causing new disease problems, higher mortality and serious production losses for the poultry industry. In addition to the importance of understanding the molecular structure and relationships among different recombinant strains of ALV in these AF 115 isolates, it is imperative to explore the virus-host interaction and transmission of these viruses in biological systems. Future research experiments should assess the pathogenicity (induction of viremia, shedding, immunity and oncogenicity) of

these isolates in the context of virus (dose and strain) and host (age at inoculation and phenotype) factors.

This huge diversity in ALV isolates may limit the utility of the current diagnostic procedures used for ALV characterization and negatively affects the prevention and control of this economically important virus infection in chickens. Therefore, it is important to incorporate DNA sequence analysis procedures into the current diagnostic testing of chicken flocks and monitoring of breeders for ALV infection and eradication. These DNA sequence data can also help in designing molecular diagnostics such as PCR primers and probes for detection and diagnosis of various ALV isolates in breeders and field infections. Moreover, the possibility that these recombinants have developed in the hatchery reiterate the importance of implementing good management practices during incubation, hatching and rearing of one type of chickens (egg-type or meat-type) from the same source.

These molecular characterization data are valuable since it can contribute to improving the design of expression vectors and vaccines. For example, the transcription regulatory elements in the 3' UTR and LTR such as promoters and enhancer including the mutated forms can be incorporated in the design of expression vectors to study their effect on the efficiency of transcription of cloned foreign genes. Moreover, the efficient cloning of envelope genes into expression vectors will facilitate understanding their expression in cells, their protein structure and function as well as their biological consequences in the host. In addition, the insertion of immunostimulatory genes such as envelope genes in vaccine vectors (recombinant or subunit vaccines) can allow the study

of the induction of cellular and humoral immune responses and protection against ALV infection and tumor development.

Table 3.1. Forward (F) and reverse (R) PCR primers used for amplification of proviral DNA of AF 115 isolates

Primer	Nucleotide Sequence	Annealing Location	ALV Subgroup Specificity
RS-7J (F)[1]	CCCAAAAGGATGAGGTGACTAAGA	*pol* (RT)	All subgroups
ARK 4836 (F)[2,3]	CGAGAGTGGCTCGCGAGATGG	*pol* (RT)	All subgroups
SRB-F (F)[4]	TTACCCCATACGATAGTCCGGCT	*env* (SU, gp85)	Subgroups B and D
SRB-R (R)[3]	AGCCGGACTATCGTATGGGGTAA	*env* (SU, gp85)	Subgroups B and D
2351.3 R8 (R)[4]	CAATGTGGTGGGAGGTAAAATGG	LTR (U5)	Subgroup J

[1] Lupiani *et al.*, 2000
[2] Fadly *et al.*, 2006
[3] Silva *et al.*, 2007
[4] Silva, unpublished data
PCR product size of RS-7J & R8 is 2.5 Kb.
PCR product size of ARK 4836 & SRB-R is 1.0 Kb.
PCR product size of SRB-F & R8 is 2.1 Kb.

Table 3.2. Relevant accession numbers for viral sequences used in analyses

Virus Isolate Designation	ALV Subgroup	GenBank Accession
RAV-1	A	AAA67094
RSA or RS-A	A	NP_040548
RSV-A	A	AAB50878
ALV LR-9	A	AAQ55055
MAV-1	A	AAA46303
ALV-1	A	AF507028
ALV-10	A	AF507029
ALV-18	A	AF507030
ALV-20	A	AF507031
ALV-25	A	AF507032
ALV-48	A	AAN06004
RAV-2	B	AAA87241
RSV-SR-B	B	AAC08989
AMAV-2	B	AAA46306
ALV-26	B	AF507033
AEV E26	B	K03326
Pr-RSV-C	C	1103272A
RSV-td PR2257	C	M21526
RSV-SR-D	D	BAD98245
RAV-0	E	CAA30677
ALV-E SD0501	E	ABO60873
ev-1	E	AAK13201
ev-3	E	AAK13203
ALV ev-6	E	AAK13204
ev-21	E	S23734
HPRS-103	J	Z46390
ALV-J UD-J1	J	AF305091
ALV-J UD2	J	AF307949
ADOL-7501	J	AX027920
ALV PDRC-3249[1]	AE/E	ABX39002
ADOL 5701A[2]	AE/J	AF257655
ADOL 6803A[2]	AE/J	AF257656
RSV-NTRE-7 td[3]	E/B	K03523
ALV TmyS_90[4]	AE/J,AEV,E	AB303223

[1] It is a recombinant ALV with subgroup A gp85 and subgroup E gp37 and LTR.
[2] They are recombinant ALVs with subgroup A gp85, subgroup E gp37 and subgroup J 3' UTR and LTR.
[3] It is a recombinant subgroup E *env* (RAV-0) and subgroup B LTR (RSV-Pr-B).
[4] It is a recombinant ALV with MAV-1 (subgroup A) gp85, subgroup E gp37, subgroup J 3' UTR, AEV E26 U3, subgroup E R and U5.

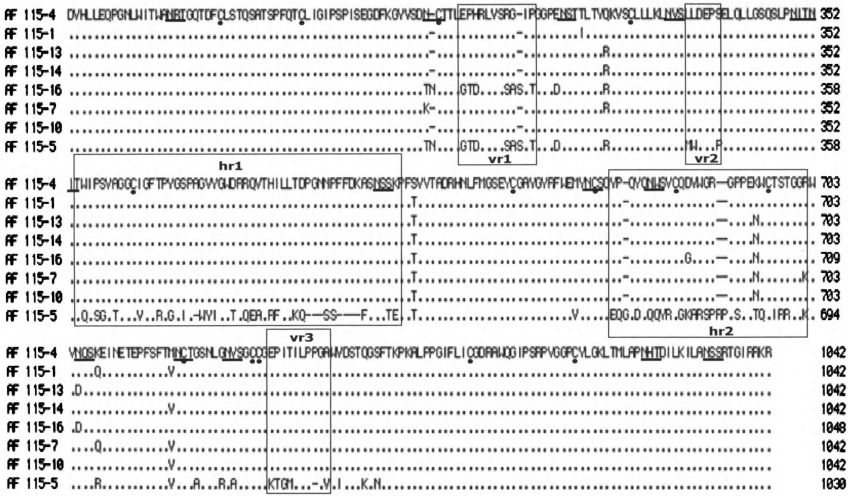


Figure 3.1. Sequence alignment of SU domains of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4): The five clusters of variable and hypervariable regions (vr1, vr2, vr3, hr1 and hr2) are boxed. A dark circle is found below conserved cysteine residues and conserved potential N-linked glycosylation sites are underlined.

Table 3.3. Sequence distances of gp85 amino acids of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4) by ClustalW (slow/accurate, Gonnet)

		Percentage Identity							
		AF 115-4	**AF 115-1**	**AF 115-13**	**AF 115-14**	**AF 115-16**	**AF 115-7**	**AF 115-10**	**AF 115-5**
Percentage Divergence	**AF 115-4**	***	98.9	98.9	98.9	96.3	98	99.1	79.1
	AF 115-1	1.2	***	98.3	98.9	95.7	98.6	99.1	79.4
	AF 115-13	1.2	1.7	***	99.4	97.4	98.6	99.1	79.4
	AF 115-14	1.2	1.2	0.6	***	96.8	99.1	99.7	79.9
	AF 115-16	3.8	4.4	2.6	3.2	***	96.3	96.6	81.8
	AF 115-7	2	1.5	1.5	0.9	3.8	***	98.9	80.2
	AF 115-10	0.9	0.9	0.9	0.3	3.5	1.2	***	79.6
	AF 115-5	24.6	24.2	24.2	23.4	20.9	23	23.8	***

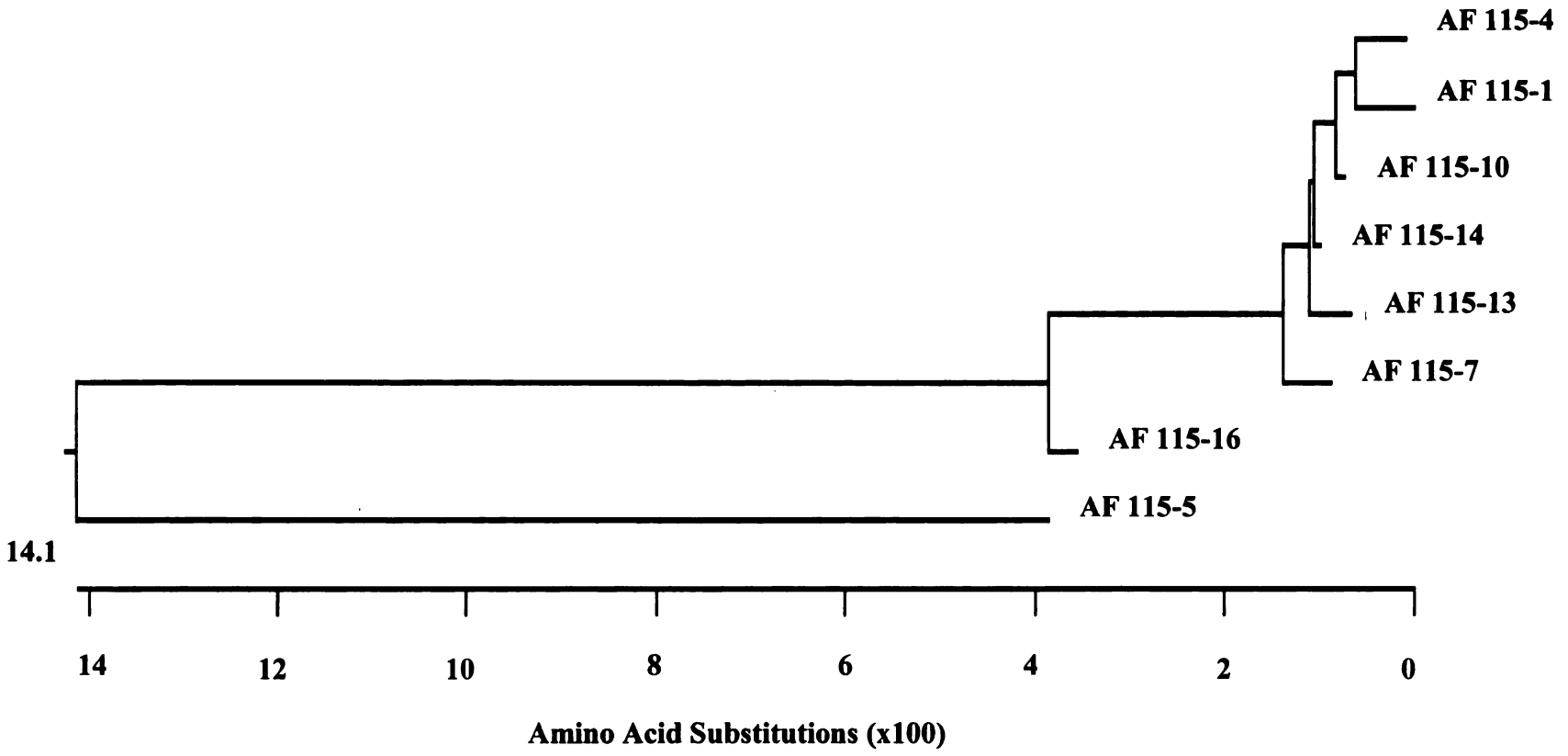


Figure 3.2. Phylogenetic tree of gp85 of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4)



Figure 3.3. Sequence alignment of TM domains of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4): The fusion peptide (FP) sequence is boxed with an open circle is situated under the central poline. A dark circle is found below conserved cysteine residues and conserved potential N-linked glycosylation sites are underlined.

Table 3.4. Sequence identity among gp37 amino acids of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4) by ClustalW (slow/accurate, Gonnet)

		Percentage Identity							
		AF 115-4	**AF 115-1**	**AF 115-13**	**AF 115-14**	**AF 115-16**	**AF 115-7**	**AF 115-10**	**AF 115-5**
Percentage Divergence	**AF 115-4**	***	100	100	99.5	99.5	99.5	99.5	100
	AF 115-1	0	***	100	99.5	99.5	99.5	99.5	100
	AF 115-13	0	0	***	99.5	99.5	99.5	99.5	100
	AF 115-14	0.5	0.5	0.5	***	99	99	99	99.5
	AF 115-16	0.5	0.5	0.5	1	***	99	99	99.5
	AF 115-7	0.5	0.5	0.5	1	1	***	99	99.5
	AF 115-10	0.5	0.5	0.5	1	1	1	***	99.5
	AF 115-5	0	0	0	0.5	0.5	0.5	0.5	***

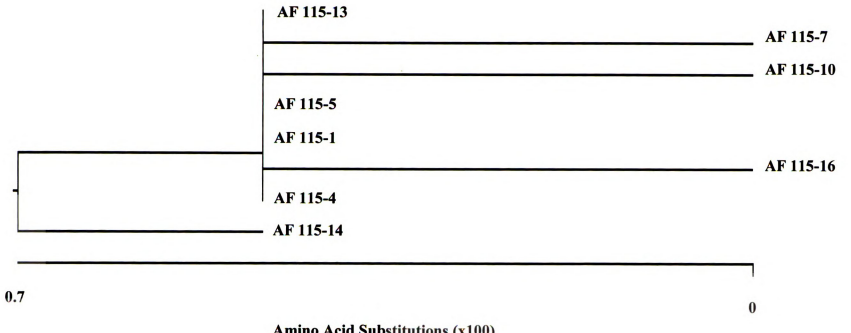


Figure 3.4. Cladogram of gp37 of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4)

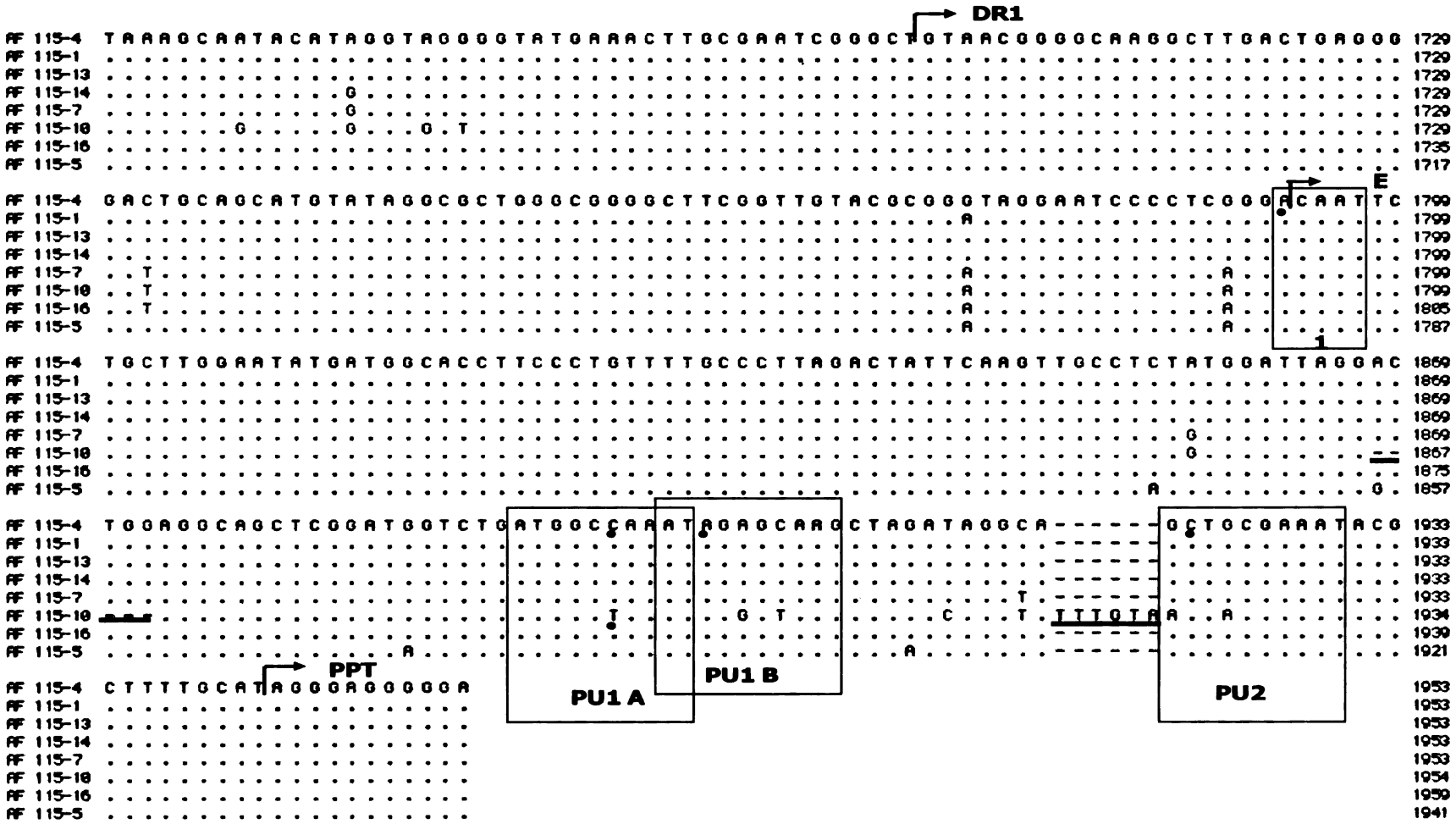


Figure 3.5. Sequence alignment of 3' UTR of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4): Arrows mark the start of DR1, E element and PPT. The first box denotes a potential CCAAT enhancer. PU1 A and PU1 B are two potential overlapping core regions of PU1. PU1 and PU2 are potential enhancer elements. Dark circles are below mutated enhancer residues. Rearrangements (5-base deletion and 6-base insertion) in AF 115-10 are underlined.

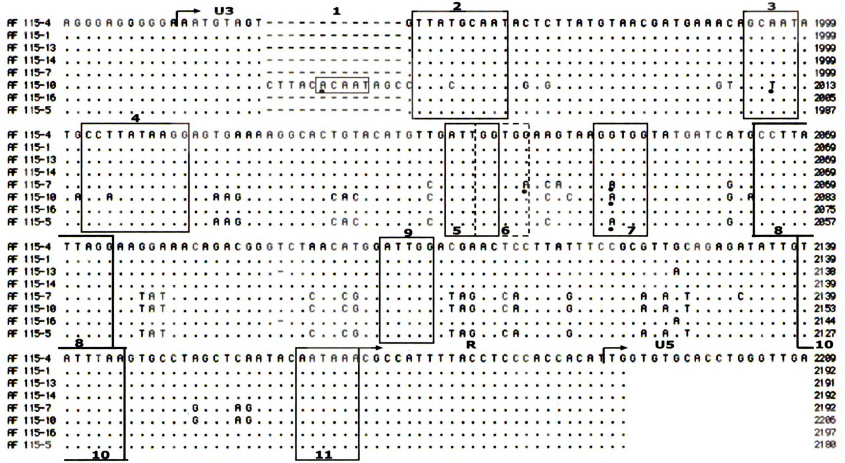


Figure 3.6. Sequence alignment of 3' LTR of seven AF 115 isolates and the recombinant ALV-B/J (AF 115-4): Arrows denote the beginning of U3, R and U5 regions. Potential transcription regulatory elements are boxed. 1and 3 are CCAAT motifs, 2 is CCAAT LTR enhancer, 4 and 8 are GArG boxes, 5 and 9 are Y boxes, 6 and 7 are PRE motifs, 10 is TATA box and 11 is PAS. Dark circles are under mutated residues in enhancer elements

Table 3.5. Percentage Identity and divergence of nucleotide sequences of 3' UTR and LTR of all AF 115 isolates and ALV-B/J (AF 115-4) by ClustalW (slow/accurate, IUB)

		Percentage Identity							
		AF 115-4	**AF 115-1**	**AF 115-13**	**AF 115-14**	**AF 115-7**	**AF 115-10**	**AF 115-16**	**AF 115-5**
Percentage Divergence	**AF 115-4**	***	99.8	99.8	99.8	94.2	89.6	99.2	94.2
	AF 115-1	0.2	***	99.6	99.6	94.4	89.8	99.4	94.4
	AF 115-13	0.2	0.4	***	99.6	94	89.4	99.4	94
	AF 115-14	0.2	0.4	0.4	***	94.4	89.8	99.1	94
	AF 115-7	6.1	5.9	6.3	5.9	***	94.3	94.5	96.2
	AF 115-10	11.4	11.1	11.6	11.1	6	***	89.9	94.1
	AF 115-16	0.8	0.6	0.6	0.9	5.7	10.9	***	94.2
	AF 115-5	6.1	5.9	6.3	6.3	3.9	6.2	6.1	***

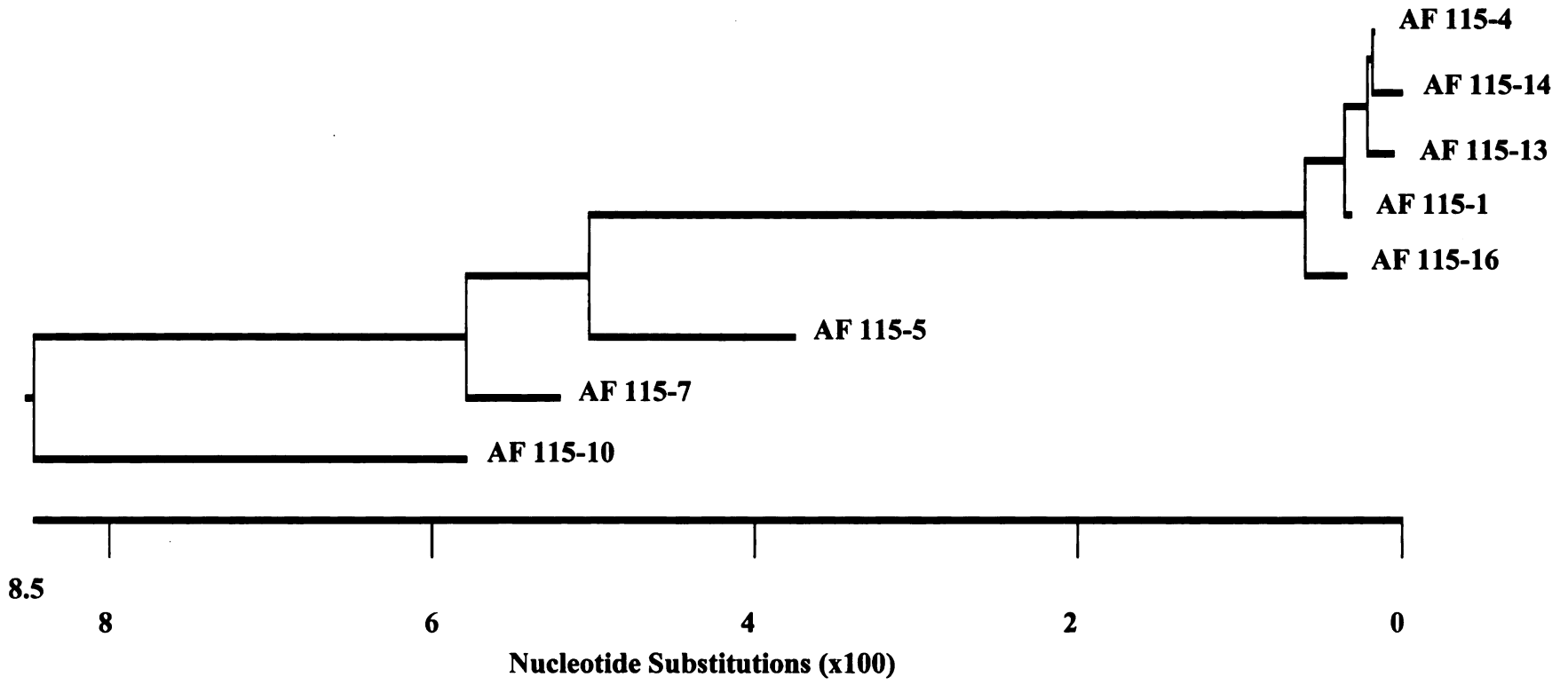


Figure 3.7. Phylogeny relationship among 3' UTR and LTR of seven AF 115 isolates and ALV-B/J (AF 115-4)

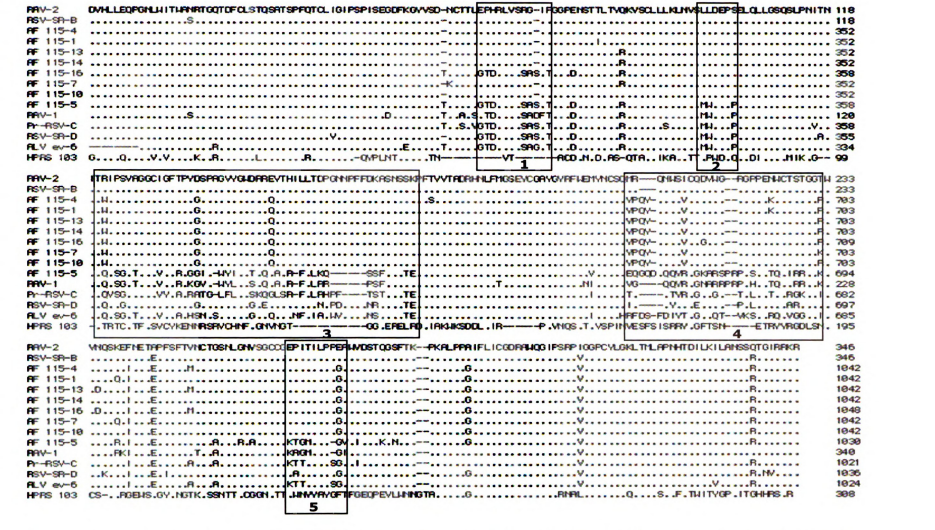


Figure 3.8. Sequence alignment of gp85 of AF 115 isolates and representatives of various ALV subgroups: Boxes 1, 2, 3, 4 and 5 represent the five clusters of variable and hypervariable regions vr1, vr2, hr1, hr2 and vr3 respectively.

Table 3.6. Sequence distances of amino acid sequences of SU domains of AF 115 isolates and representatives of ALV subgroups by ClustalW (slow/accurate, Gonnet)

		Percentage Identity														
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Percentage Divergence	**1**	***	98.8	95.4	95.4	95.4	96	93.1	95.4	96.2	78	78.6	78.5	87.5	79.9	36.6
	2	1.2	***	96	96	96	96.5	93.6	96	96.8	78.6	79.2	79.1	88.1	80.5	36
	3	4.8	4.2	***	98.9	98.9	98.9	96.6	98	99.1	78.8	78.6	79.1	87.5	80.6	36.4
	4	4.8	4.2	1.2	***	98.3	98.9	96	98.6	99.1	79.1	78.9	78.8	87.5	80.3	36.1
	5	4.8	4.2	1.2	1.7	***	99.4	97.7	98.6	99.1	79.1	78.9	79.4	88.1	80.9	36.4
	6	4.2	3.6	1.2	1.2	0.6	***	97.1	99.1	99.7	79.6	79.5	79.6	88.7	81.2	36.4
	7	7.3	6.7	3.5	4.1	2.3	2.9	***	96.3	96.8	81.2	80.2	81.2	89.9	82.5	36.7
	8	4.8	4.2	2	1.5	1.5	0.9	3.8	***	98.9	79.6	79.5	79.1	88.1	80.6	36.1
	9	3.9	3.3	0.9	0.9	0.9	0.3	3.2	1.2	***	79.4	79.2	79.4	88.4	80.9	36.4
	10	26	25.2	25	24.6	24.6	23.8	21.7	23.8	24.2	***	89.4	85.2	83.4	84.1	35.5
	11	25.3	24.5	25.3	24.9	24.9	24.1	23.1	24.1	24.5	11.4	***	83.7	80.7	82.4	36
	12	25.4	24.6	24.6	25	24.2	23.8	21.7	24.6	24.2	16.5	18.4	***	82.9	85.6	36.5
	13	13.7	13	13.7	13.7	13	12.3	10.9	13	12.6	18.8	22.4	19.4	***	84	36.1
	14	23.5	22.7	22.5	22.9	22.1	21.7	20	22.5	22.1	17.9	20.1	16.1	18	***	35.7
	15	125.2	128.1	126.2	127.7	126.2	126.2	124.8	127.7	126.2	130.1	128	125.6	127.6	129.4	***

1: RAV-2; 2: RSV-SR-B; 3: AF 115-4 (ALV-B/J); 4: AF 115-1; 5: AF 115-13; 6: AF 115-14; 7: AF 115-16; 8: AF 115-7; 9: AF 115-10; 10: AF 115-5; 11: RAV-1; 12: Pr-RSV-C; 13: RSV-SR-D; 14: ALV ev-6; 15: HPRS 103.

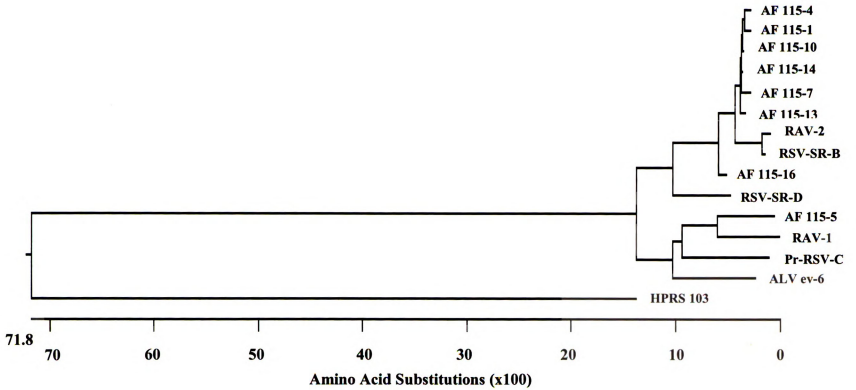


Figure 3.9. Cladogram of gp85 of AF 115 isolates and prototypes of classical ALV subgroups

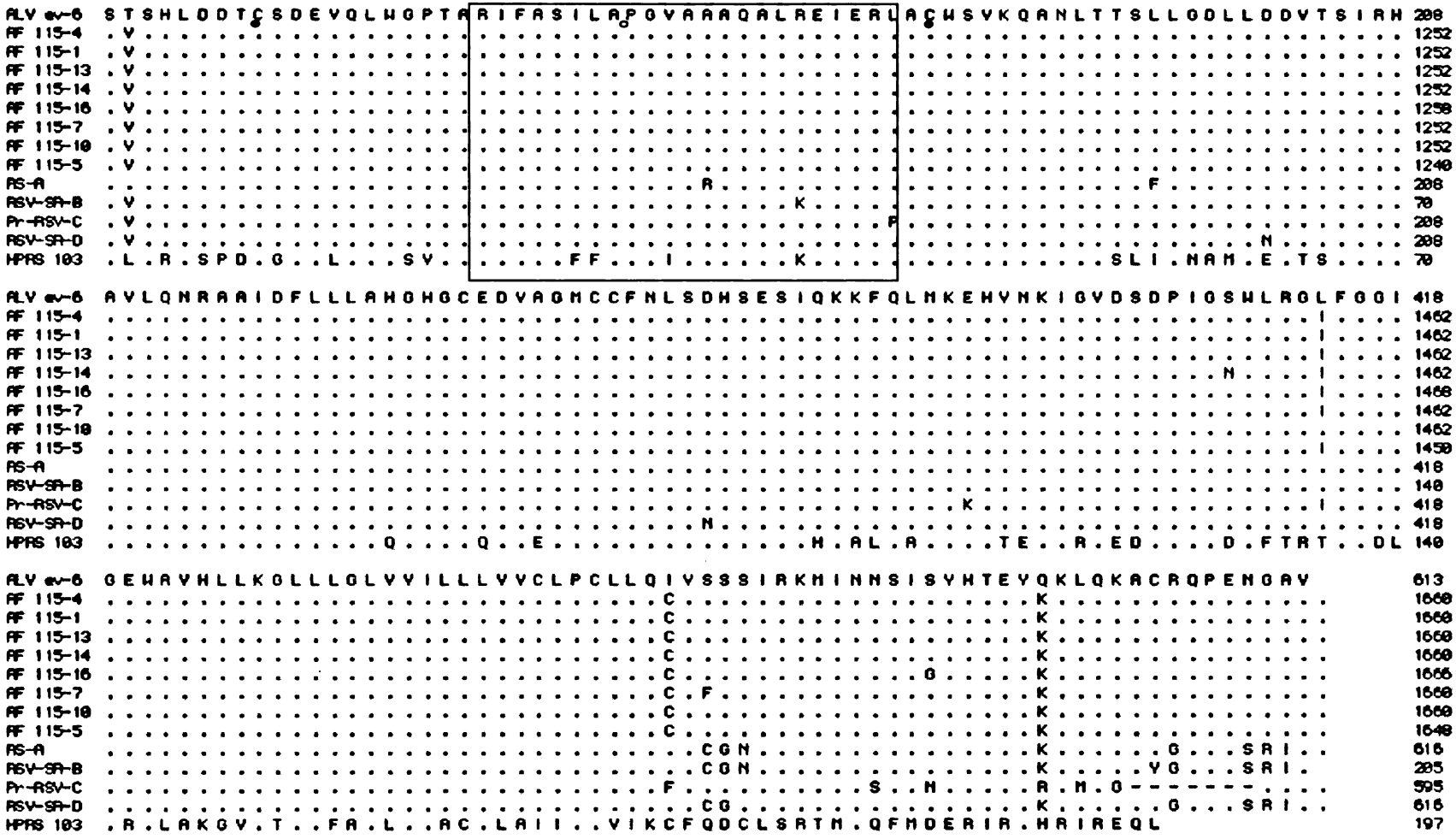


Figure 3.10. Sequence alignment of gp37 of AF 115 isolates and viral prototypes of ALV subgroups: The fusion peptide (FP) sequence is boxed with an open circle is situated under the central proline. A dark circle is found below conserved cysteine residues surrounding the FP.

Table 3.7. Percentage identity and divergence of amino acid sequences of TM domains of AF 115 isolates and different ALV subgroups by ClustalW (slow/accurate, Gonnet)

		Percentage Identity													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Percentage Divergence	**1**	***	98	98	98	97.6	97.6	97.6	98.5	98	95.1	94.6	94.9	95.1	56.9
	2	2	***	100	100	99.5	99.5	99.5	99.5	100	94.2	94.6	96	95.1	57.4
	3	2	0	***	100	99.5	99.5	99.5	99.5	100	94.2	94.6	96	95.1	57.4
	4	2	0	0	***	99.5	99.5	99.5	99.5	100	94.2	94.6	96	95.1	57.4
	5	2.5	0.5	0.5	0.5	***	99	99	99	99.5	93.7	94.1	95.5	94.7	57.4
	6	2.5	0.5	0.5	0.5	1	***	99	99	99.5	93.7	94.1	96	94.7	57.4
	7	2.5	0.5	0.5	0.5	1	1	***	99	99.5	94.2	94.6	95.5	95.1	57.4
	8	1.5	0.5	0.5	0.5	1	1	1	***	99.5	94.7	95.1	95.5	95.6	57.4
	9	2	0	0	0	0.5	0.5	0.5	0.5	***	94.2	94.6	96	95.1	57.4
	10	5.1	6.1	6.1	6.1	6.6	6.6	6.1	5.5	6.1	***	97.6	91.5	97.1	56.3
	11	5.6	5.6	5.6	5.6	6.1	6.1	5.6	5.1	5.6	2.5	***	92.4	97.6	57.4
	12	5.2	4.1	4.1	4.1	4.7	4.1	4.7	4.7	4.1	9.1	8	***	92.5	56.4
	13	5.1	5	5	5	5.5	5.5	5	4.5	5	3	2.5	8	***	56.3
	14	63.2	62.1	62.1	62.1	62.1	62.1	62.1	62.1	62.1	64.4	62.1	64.2	64.4	***

1: ALV ev-6; 2: AF 115-4 (ALV-B/J); 3: AF 115-1; 4: AF 115-13; 5: AF 115-14; 6: AF 115-16; 7: AF 115-7; 8: AF 115-10; 9: AF 115-5; 10: RS-A; 11: RSV-SR-B; 12: Pr-RSV-C; 13: RSV-SR-D; 14: HPRS 103.

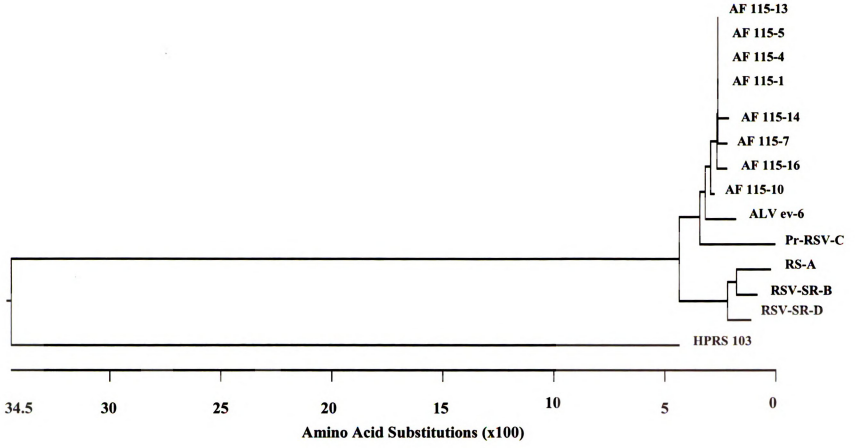


Figure 3.11. Diagram of the phylogenetic relationships among gp37 of AF 115 isolates and different ALV subgroups

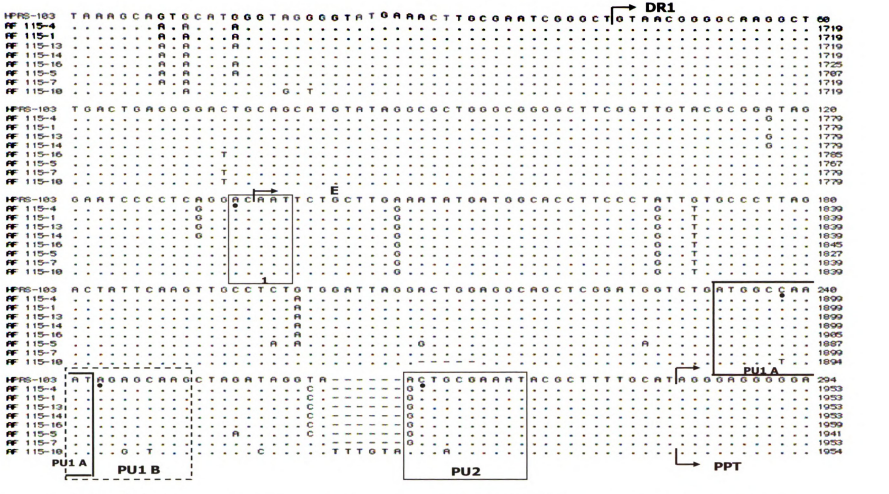


Figure 3.12. Sequence alignment of 3' UTR of AF 115 isolates and HPRS-103 (ALV-J): Arrows mark the start of DR1, E element and PPT. The first box denotes a potential CCAAT enhancer. PU1 A and PU1 B are two potential overlapping core regions of PU1. PU1 and PU2 are potential enhancer elements. Dark circles are under mutated enhancer residues.

Table 3.8. Sequence distances comparison among nucleotide sequences of 3' UTR of all AF 115 isolates and HPRS-103, a subgroup J ALV by ClustalW (slow/accurate, IUB)

		Percentage Identity								
		HPRS-103	**AF 115-4 (ALV-B/J)**	**AF 115-1**	**AF 115-13**	**AF 115-14**	**AF 115-16**	**AF 115-5**	**AF 115-7**	**AF 115-10**
Percentage Divergence	**HPRS-103**	***	96.3	96.6	96.3	96.6	96.6	95.6	97.6	95.8
	AF 115-4 (ALV-B/J)	3.9	***	99.7	100	99.7	99	98	98	94.8
	AF 115-1	3.5	0.3	***	99.7	99.3	99.3	98.3	98.3	95.2
	AF 115-13	3.9	0	0.3	***	99.7	99	98	98	94.8
	AF 115-14	3.5	0.3	0.7	0.3	***	98.6	97.6	98.3	95.2
	AF 115-16	3.5	1	0.7	1	1.4	***	98.3	99	95.8
	AF 115-5	4.6	2.1	1.7	2.1	2.4	1.7	***	97.3	94.5
	AF 115-7	2.4	2.1	1.7	2.1	1.7	1	2.8	***	96.9
	AF 115-10	4.3	5.5	5.1	5.5	5.1	4.3	5.8	3.2	***

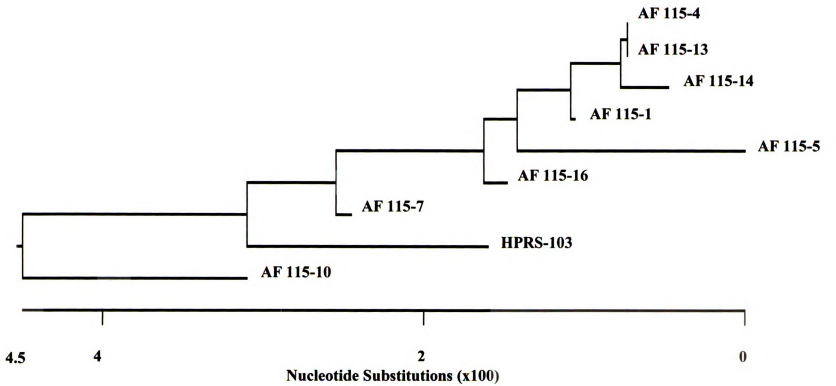


Figure 3.13. Phylogenetic tree of the 3' UTR of all AF 115 isolates and subgroup J ALV isolate HPRS-103

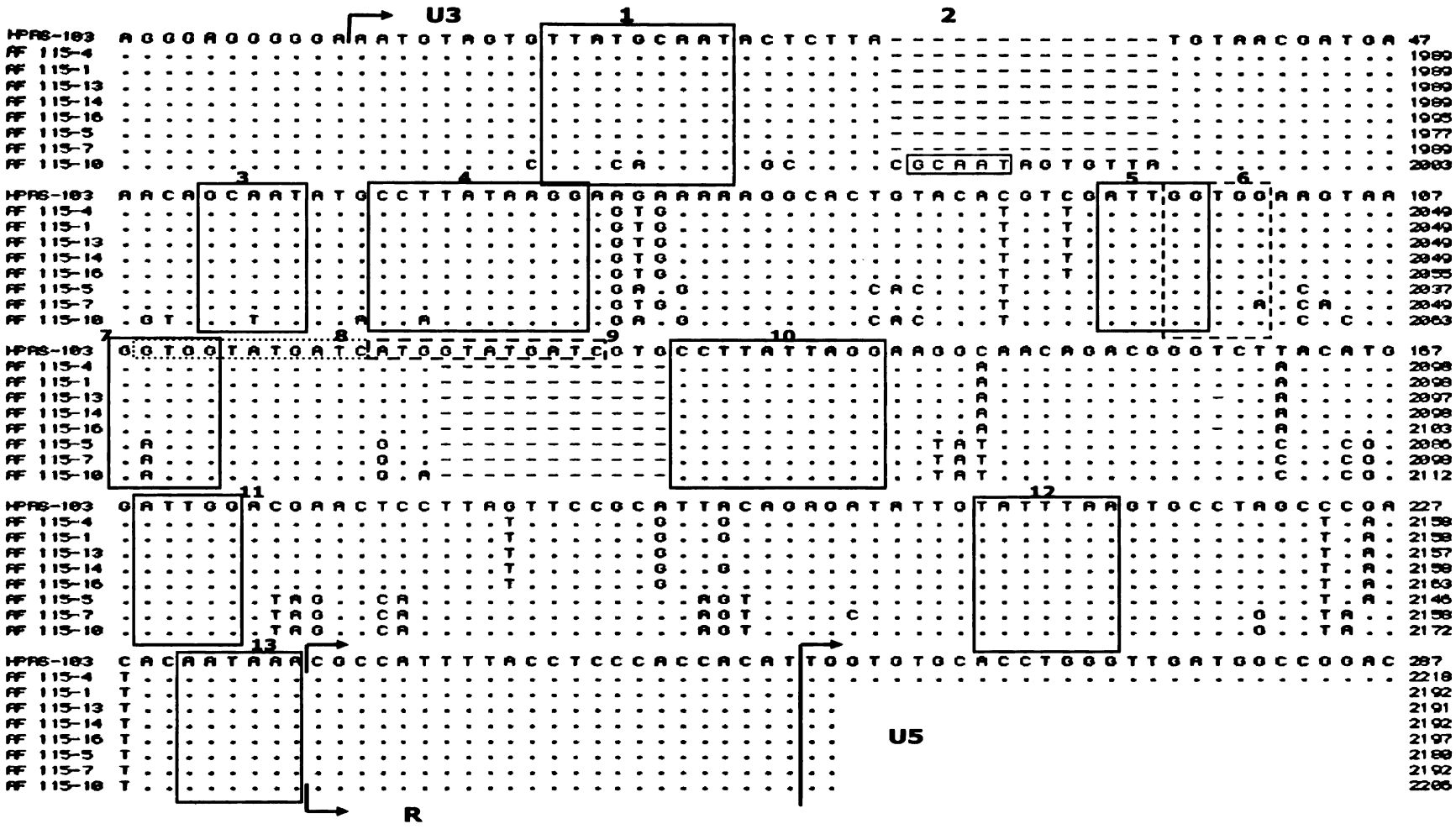


Figure 3.14. Sequence alignment of 3' LTR of all AF 115 isolates with ALV-J HPRS-103: Potential transcription regulatory elements are boxed and include CCAAT LTR enhancer (1), extra CCAAT in AF 115-10 (2), CCAAT (3), GArG (4 and 10), Y (5 and 11) and PRE (6 and 7), adjacent repeated motifs in HPRS-103 (8 and 9), TATA (12) and PAS (13) boxes.

Table 3.9. Comparisons of percentage identity and divergence among nucleotide sequences of 3' LTR of all AF 115 isolates and *HPRS-103* by ClustalW (slow/accurate, IUB)

		Percentage Identity								
		HPRS-103	**AF 115-4 (ALV-B/J)**	**AF 115-1**	**AF 115-13**	**AF 115-14**	**AF 115-16**	**AF 115-5**	**AF 115-7**	**AF 115-10**
Percentage Divergence	**HPRS-103**	***	95.6	94.8	95.2	94.8	95.2	89.2	88.8	84
	AF 115-4 (ALV-B/J)	4.6	***	100	99.6	100	99.6	90	90	84
	AF 115-1	5.4	0	***	99.6	100	99.6	90	90	84
	AF 115-13	5	0.4	0.4	***	99.6	100	89.6	89.6	83.5
	AF 115-14	5.4	0	0	0.4	***	99.6	90	90	84
	AF 115-16	5	0.4	0.4	0	0.4	***	89.6	89.6	83.5
	AF 115-5	11.9	10.8	10.8	11.3	10.8	11.3	***	95.2	94
	AF 115-7	12.3	10.8	10.8	11.3	10.8	11.3	5	***	91.6
	AF 115-10	18.5	18.3	18.3	18.9	18.3	18.9	6.3	9	***

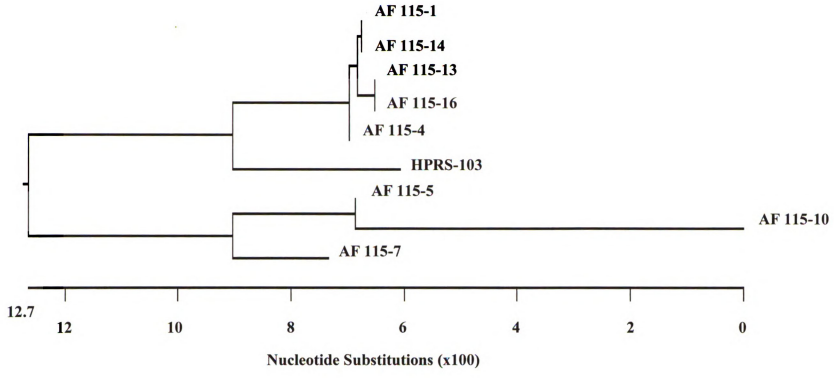


Figure 3.15. Cladogram of the 3' LTR of all AF 115 isolates and subgroup J ALV isolate HPRS-103

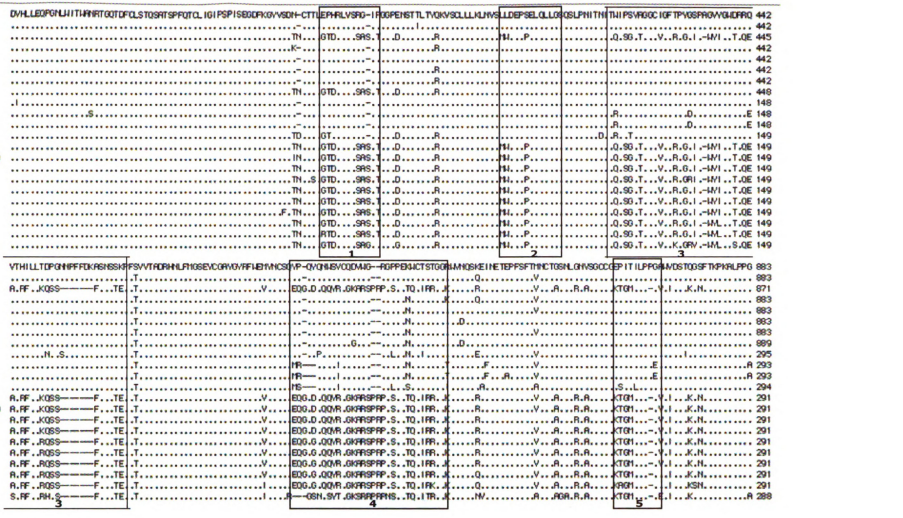


Figure 3.16. Sequence alignment of SU domains of AF 115 isolates and similar blasted viral strains: Boxes 1, 2, 3, 4 and 5 represent the five clusters of variable and hypervariable regions vr1, vr2, hr1, hr2 and vr3 respectively.

Table 3.10. Sequence similarity among amino acids of gp85 regions of AF 115 isolates and associated NCBI published viral strains by ClustalW (slow/accurate, Gonnet)

	Percentage Identity																					
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
A	***	98.9	79.1	98	99.1	98.9	98.9	96.3	96.8	96	95.4	94.5	79.1	79.1	79.4	78.5	78.8	78.8	78.5	79.1	78.8	78
B	1.2	***	79.4	98.6	99.1	98.3	98.9	95.7	97.1	96	95.4	94.2	79.4	79.4	79.1	78.8	79.1	79.1	78.8	79.6	79.4	78
C	24.6	24.2	***	80.2	79.6	79.4	79.9	81.8	79.1	78.6	78	79.9	100	99.7	99.7	99.4	99.1	99.1	98.8	98.3	96.8	90.6
D	2	1.5	23	***	98.9	98.6	99.1	96.3	96.8	96	95.4	94.8	80.2	80.2	79.9	79.6	79.9	79.9	79.6	80.5	80.2	78.9
E	0.9	0.9	23.8	1.2	***	99.1	99.7	96.6	97.7	96.8	96.2	94.8	79.6	79.6	79.4	79.1	79.4	79.4	79.1	79.6	79.4	78.3
F	1.2	1.7	24.2	1.5	0.9	***	99.4	97.4	96.8	96	95.4	94.8	79.4	79.4	79.6	78.8	79.1	79.1	78.8	79.4	79.1	78.3
G	1.2	1.2	23.4	0.9	0.3	0.6	***	96.8	97.4	96.5	96	95.1	79.9	79.9	79.6	79.4	79.6	79.6	79.4	79.9	79.6	78.6
H	3.8	4.4	20.9	3.8	3.5	2.6	3.2	***	94.3	93.4	92.8	94.2	81.8	81.5	82.1	81.2	81.5	81.5	81.2	81.8	81.2	79.6
I	3.2	2.9	24.6	3.2	2.3	3.2	2.6	6	***	94.5	93.9	93.1	79.1	79.1	78.8	78.5	78.8	78.8	78.5	79.1	78.8	78
J	4.2	4.2	25.2	4.2	3.3	4.2	3.6	7	5.7	***	98.8	93.9	78.6	78.6	78.3	78.3	78.3	78.3	78	78.6	78.3	78.5
K	4.8	4.8	26	4.8	3.9	4.8	4.2	7.6	6.3	1.2	***	93.4	78	78	77.7	77.7	77.7	77.7	77.4	78	77.7	77.9
L	5.7	6	23.5	5.4	5.4	5.4	5.1	6	7.3	6.3	7	***	79.9	79.6	79.9	79.3	79.6	79.6	79.3	79.9	79.3	79.5
M	24.6	24.2	0	23	23.8	24.2	23.4	20.9	24.6	25.2	26	23.5	***	99.7	99.7	99.4	99.1	99.1	98.8	98.3	96.8	90.6
N	24.6	24.2	0.3	23	23.8	24.2	23.4	21.3	24.6	25.2	26	23.9	0.3	***	99.4	99.1	98.8	98.8	98.5	98	96.5	90.3
O	24.2	24.6	0.3	23.4	24.2	23.8	23.8	20.5	25	25.6	26.5	23.5	0.3	0.6	***	99.1	98.8	98.8	98.5	98	96.5	90.6
P	25.4	25	0.6	23.8	24.6	25	24.2	21.7	25.4	25.6	26.5	24.3	0.6	0.9	0.9	***	98.5	98.5	98.3	97.7	96.2	90.6
Q	25	24.6	0.9	23.4	24.2	24.6	23.8	21.3	25	25.6	26.5	23.9	0.9	1.2	1.2	1.5	***	100	99.7	98.5	97.1	90.6
R	25	24.6	0.9	23.4	24.2	24.6	23.8	21.3	25	25.6	26.5	23.9	0.9	1.2	1.2	1.5	0	***	99.7	98.5	97.1	90.6
S	25.4	25	1.2	23.8	24.6	25	24.2	21.7	25.4	26	26.9	24.3	1.2	1.5	1.5	1.8	0.3	0.3	***	98.3	96.8	90.3
T	24.6	23.8	1.8	22.6	23.8	24.2	23.4	20.9	24.6	25.2	26	23.5	1.8	2.1	2.1	2.4	1.5	1.5	1.8	***	98	90.9
U	25	24.2	3.3	23	24.2	24.6	23.8	21.7	25	25.6	26.5	24.3	3.3	3.6	3.6	3.9	3	3	3.3	2.1	***	90
V	26	26	10	24.8	25.6	25.6	25.2	23.8	26	25.4	26.2	24.1	10	10.4	10	10	10	10	10.4	9.7	10.7	***
	Percentage Divergence																					

A: AF 115-4 (ALV-B/J); B: AF 115-1; C: AF 115-5; D: AF 115-7; E: AF 115-10; F: AF 115-13; G: AF 115-14; H: AF 115-16; I: ALV-26; J: RSV-SR-B; K: RAV-2; L: AMAV-2; M: ALV-18; N: ALV TymS_90; O: ALV-25; P: ALV-48; Q: ALV-1; R: ALV-20; S: ALV-10; T: MAV-1; U: ALV LR-9; V: RSV-A.

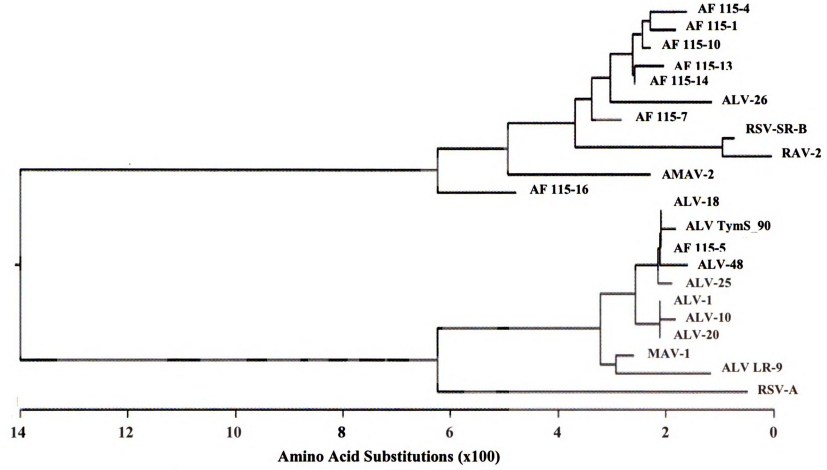


Figure 3.17. Phylogenetic tree of SU domains of AF 115 isolates and related blasted ALV strains from GenBank

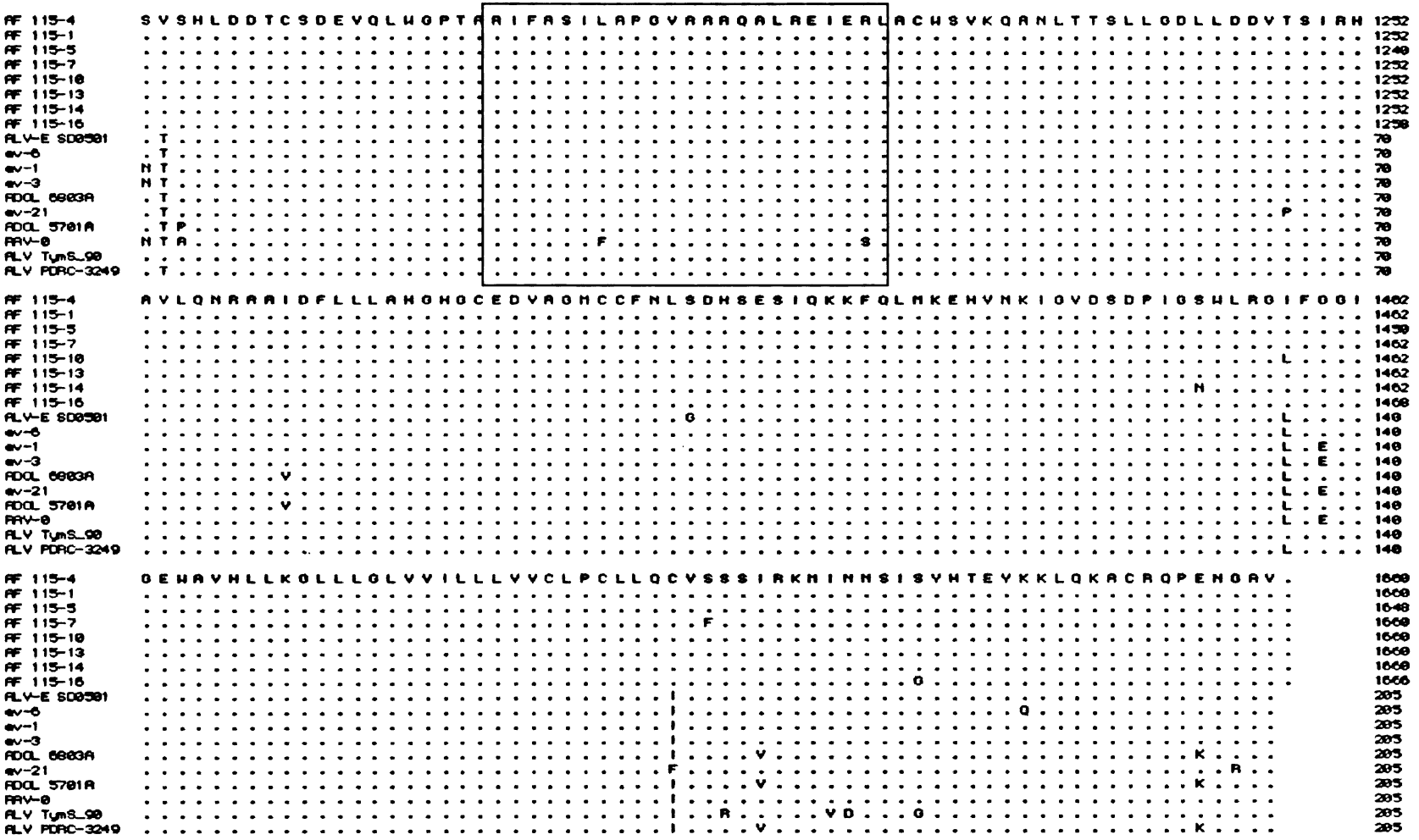


Figure 3.18. Sequence alignment of TM regions of AF 115 isolates and blasted NCBI viral isolates: The fusion peptide (FP) sequence is boxed.

Table 3.11. Comparisons of sequence distances among amino acids of gp37 of AF 115 isolates and highly similar published viruses by ClustalW (slow/accurate, Gonnet)

		Percentage Identity																	
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Percentage Divergence	**1**	***	100	100	99.5	99.5	100	99.5	99.5	98	98	97.6	97.6	97.1	97.1	96.6	96.1	97.6	97.6
	2	0	***	100	99.5	99.5	100	99.5	99.5	98	98	97.6	97.6	97.1	97.1	96.6	96.1	97.6	97.6
	3	0	0	***	99.5	99.5	100	99.5	99.5	98	98	97.6	97.6	97.1	97.1	96.6	96.1	97.6	97.6
	4	0.5	0.5	0.5	***	99	99.5	99	99	97.6	97.6	97.1	97.1	96.6	96.6	96.1	95.6	97.1	97.1
	5	0.5	0.5	0.5	1	***	99.5	99	99	98.5	98.5	98	98	97.6	97.6	97.1	96.6	97.1	98
	6	0	0	0	0.5	0.5	***	99.5	99.5	98	98	97.6	97.6	97.1	97.1	96.6	96.1	97.6	97.6
	7	0.5	0.5	0.5	1	1	0.5	***	99	97.6	97.6	97.1	97.1	96.6	96.6	96.1	95.6	97.1	97.1
	8	0.5	0.5	0.5	1	1	0.5	1	***	97.6	97.6	97.1	97.1	96.6	96.6	96.1	95.6	98	97.1
	9	2	2	2	2.5	1.5	2	2.5	2.5	***	99	98.5	98.5	98	97.6	97.6	97.1	96.6	98.5
	10	2	2	2	2.5	1.5	2	2.5	2.5	1	***	98.5	98.5	98	97.6	97.6	97.1	96.6	98.5
	11	2.5	2.5	2.5	3	2	2.5	3	3	1.5	1.5	***	100	97.6	98	97.1	98.5	96.1	98
	12	2.5	2.5	2.5	3	2	2.5	3	3	1.5	1.5	0	***	97.6	98	97.1	98.5	96.1	98
	13	3	3	3	3.5	2.5	3	3.5	3.5	2	2	2.5	2.5	***	96.6	99.5	96.1	95.6	99.5
	14	3	3	3	3.5	2.5	3	3.5	3.5	2.5	2.5	2	2	3.5	***	96.1	96.6	95.1	97.1
	15	3.5	3.5	3.5	4	3	3.5	4	4	2.5	2.5	3	3	0.5	4	***	96.1	95.1	99
	16	4	4	4	4.5	3.5	4	4.5	4.5	3	3	1.5	1.5	4	3.5	4	***	94.6	96.6
	17	2.5	2.5	2.5	3	3	2.5	3	2	3.5	3.5	4	4	4.5	5.1	5.1	5.6	***	96.1
	18	2.5	2.5	2.5	3	2	2.5	3	3	1.5	1.5	2	2	0.5	3	1	3.5	4	***

1: AF 115-4 (ALV-B/J) ; 2: AF 115-1; 3: AF 115-5; 4: AF 115-7; 5: AF 115-10; 6: AF 115-13; 7: AF 115-14; 8: AF 115-16; 9: ALV-E SD0501; 10: ev-6; 11: ev-1; 12: ev-3; 13: ADOL 6803A; 14: ev-21; 15: ADOL 5701A; 16: RAV-0; 17: ALV TymS_90; 18: ALV PDRC-3249.

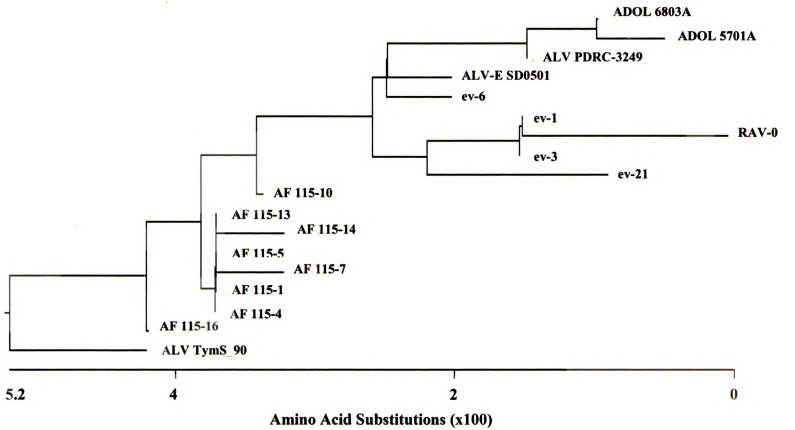


Figure 3.19. Phylogenetic relationship diagram among TM regions of AF 115 isolates and GenBank ALV viral strains

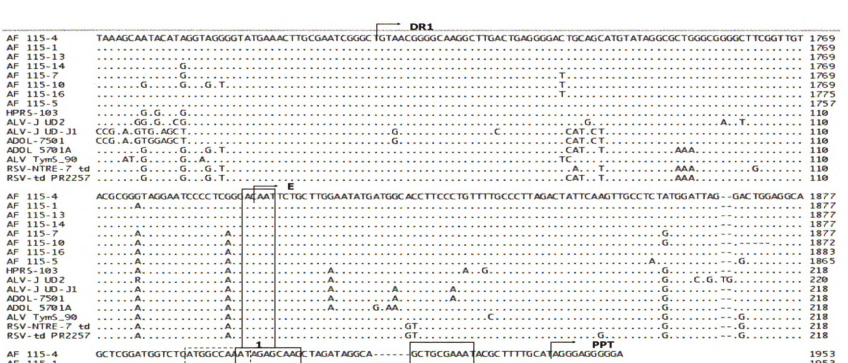


Figure 3.20. Sequence alignment of 3' UTR of AF 115 isolates and blasted viral sequences from NCBI: Arrows mark the start of DR1, E element and PPT. Box 1 represents a potential CCAAT enhancer. PU1 A and PU1 B are two potential overlapping core regions of PU1. PU1 and PU2 are potential enhancer binding elements.

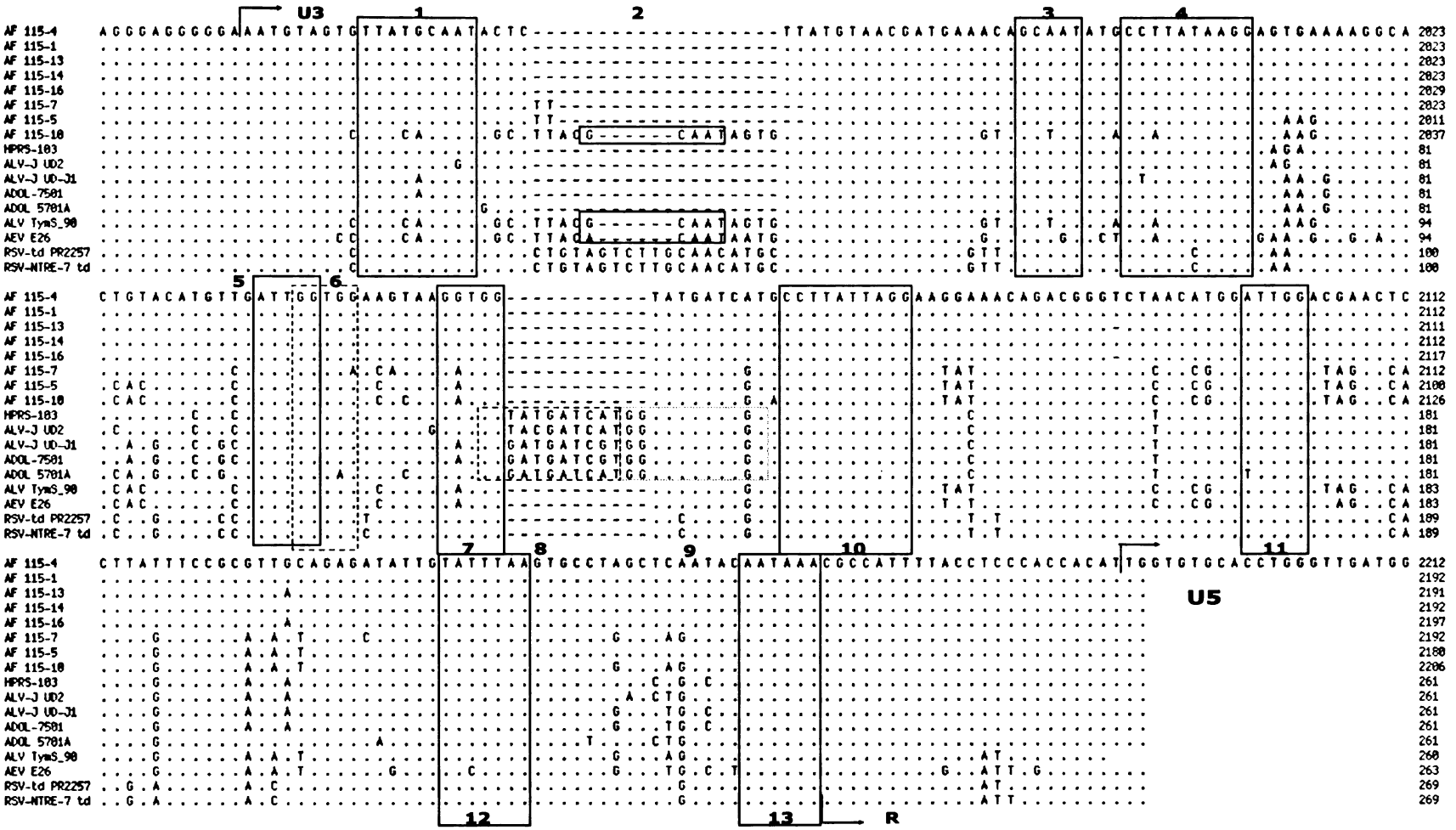


Figure 3.21. Sequence alignment of 3' LTR of all AF 115 isolates with NCBI published viruses: Potential transcription regulatory elements are boxed and include CCAAT LTR enhancer (1), extra CCAAT boxes (2), CCAAT (3), GArG (4 and 10), Y (5 and 11) and PRE (6 and 7), adjacent repeated motifs (8 and 9), TATA (12) and PAS (13) boxes.

Table 3.12. Similarity and divergence among nucleotide sequences of 3' UTR and LTR of all AF 115 isolates and GenBank blasted sequences by ClustalW (slow/accurate, IUB)

		Percentage Identity																
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Percentage Divergence	**1**	***	99.8	94.2	94.2	99.8	99.8	99.2	89.6	95.3	93.2	92.1	91.2	91	89.6	80.9	90.4	90.2
	2	0.2	***	94.4	94.4	99.6	99.6	99.4	89.8	95.5	93.2	92.3	91.4	91.2	89.8	80.9	90.6	90.4
	3	6.1	5.9	***	96.2	94	94	94.2	94.1	92.7	90.4	89.7	89.5	89.3	94.3	86.8	89.5	89.3
	4	6.1	5.9	3.9	***	94	94.4	94.5	94.3	93.6	91.6	89.9	90.2	90.1	94.3	85.9	89.5	89.3
	5	0.2	0.4	6.3	6.3	***	99.6	99.4	89.4	95.5	93.4	91.9	91.4	91.2	89.4	80.5	90.2	90
	6	0.2	0.4	6.3	5.9	0.4	***	99.1	89.8	95.5	93.4	92.3	91.2	91	89.8	80.9	90.6	90.4
	7	0.8	0.6	6.1	5.7	0.6	0.9	***	89.9	95.7	93.4	92.1	91.5	91.4	90	80.5	90.4	90.2
	8	11.4	11.1	6.2	6	11.6	11.1	10.9	***	90.3	88.6	88.6	87.7	87.5	97.2	88.9	87.8	87.6
	9	4.9	4.7	7.8	6.7	4.7	4.7	4.5	10.5	***	96.1	93.6	93.6	93	90.4	82.6	90.8	90.6
	10	7	7	10.3	8.9	6.8	6.8	6.8	12.4	3.8	***	91.5	91.5	91	88.3	82.2	89.3	89.1
	11	8.4	8.2	11.3	11	8.7	8.2	8.4	12.5	6.8	8.9	***	92.6	92.5	87.9	79.9	90.2	90.8
	12	9.5	9.3	11.5	10.6	9.3	9.5	9.1	13.7	6.8	8.9	7.8	***	99.4	88.5	83.2	87.4	88
	13	9.7	9.5	11.7	10.8	9.5	9.7	9.3	13.9	7.4	9.6	8	0.6	***	88.3	82.9	87.2	87.8
	14	11.3	11.1	5.9	5.9	11.6	11.1	10.9	2.8	10.4	12.8	13.4	12.6	12.9	***	90.6	87.6	87.8
	15	22.7	22.7	14.9	16.1	23.2	22.7	23.2	12.3	20.5	21	24.2	19.5	20	10.2	***	83.6	83.3
	16	10.3	10.1	11.4	11.4	10.6	10.1	10.4	13.5	9.9	11.5	10.6	13.9	14.2	13.7	18.9	***	98.9
	17	10.6	10.4	11.7	11.7	10.8	10.4	10.6	13.8	10.1	11.8	9.9	13.3	13.5	13.5	19.3	1.1	***

1: AF 115-4 (ALV-B/J); 2: AF 115-1; 3: AF 115-5; 4: AF 115-7; 5: AF 115-13; 6: AF 115-14; 7: AF 115-16; 8: AF 115-10; 9: ALV HPRS 103; 10: ALV-J UD2; 11: ADOL 5701A; 12: ADOL 7501; 13: ALV-J UD-J1; 14: ALV TymS_90; 15: AEV E26; 16: RSV-NTRE-7 td; 17: RSV-td PR2257.

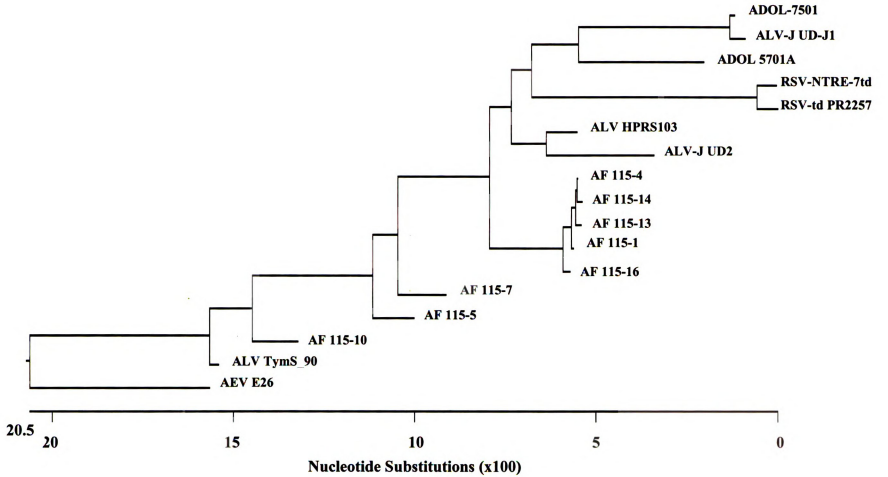


Figure 3.22. Cladogram of 3' UTR and LTR of all AF 115 isolates and similar blasted viral strains from NCBI

CHAPTER 4

Conclusions and Future Studies

INTRODUCTION

Avian leukosis virus (ALV) is one of the most field encountered avian retroviruses associated with different kinds of neoplasms in chickens. ALV is usually classified into six subgroups A, B, C, D, E and J. Infection with ALV constitutes a continuous economical threat to the poultry industry due to the emergence of new mutants and possibly recombinant viral strains. Current efforts for prevention and control of ALV infection have been compromised by significant genetic and antigenic variations in different strains and field isolates. This is due to the fact that the continuous emergence of these new mutants and recombinants hinders current field diagnosis of ALV infection by standard biological and molecular methods. Therefore, regular monitoring and improvement of diagnostic methodology to include new ALV isolates, mutants and recombinants will support the existing efforts for ALV eradication.

Information is currently limited on natural recombination among different ALV subgroups and its impact on virus isolation, diagnosis and pathogenesis in chickens. The research studies reported in this dissertation mainly focused on the biological and molecular characterization of seven new isolates of a naturally occurring recombinant avian leukosis virus (ALV) associated with myeloid leukosis (ML) in commercial layers. In addition, these isolates were compared with the first naturally occurring recombinant ALV-B/J at various levels.

CHAPTER 2 CONCLUSIONS

The overall goal of the research studies in chapter 2 was to characterize seven AF 115 isolates of a naturally occurring recombinant ALV (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) by biological assays and polymerase chain reaction. The most significant finding for this research work was that closely and distinctly related strains of naturally occurring recombinant ALVs were identified using various diagnostic techniques. In fact, the host range and antigenic relationship of the seven AF 115 isolates were comparable with those of the naturally occurring recombinant ALV-BE/J. They indicated that the ALVs present in these isolates were exogenous and most probably subgroup B and not subgroup J.

PCR analysis results revealed that the seven AF 115 isolates contained ALV-B, ALV-J and ALV-B/J sequences. Variable PCR amplification patterns of AF 115 isolates suggested the presence of mutations in different genomic regions of some of these isolates which were confirmed by DNA sequencing data (chapter 3). These conclusions were based on the differences in the annealing locations and sequences of PCR primers used. In addition, similar PCR amplification patterns revealed that AF 115 -1, AF 115-13, AF 115-14 and AF 115- 16 were similar viral strains and closely associated to the naturally occurring recombinant ALV-B/J. On the other hand, different patterns of PCR amplification demonstrated that AF 115-5, AF 115-7 and AF 115-10 were distantly related to the recombinant ALV-B/J (AF 115-4) and the rest of the isolates.

CHAPTER 2 LIMITATIONS

There were limitations encountered during conducting the biological assays in the research studies of chapter 2. Although the host range is considered the most reliable method for subgroup classification, it was not enough by itself to specifically identify the nature of ALVs in our isolates. Analysis of the host range data suggested that ALVs from all isolates belonged either to ALV subgroup A, B or D, but not to J. Moreover, virus neutralization test revealed specific antigenic relationship of our isolates to ALV-B even though it is known to be less reliable than host range assay. Previous unpublished studies used more CEFs and subgroup-specific antisera for the host range and antigenicity assays on AF 115 isolates. Therefore, only reagents (cells and antisera) giving positive results were used in our studies, which may have limited our analysis.

There were advantages and disadvantages for using PCR technology in characterization and establishing relationships among AF 115 isolates. The PCR assay was rapid and effective in confirming ALV detection in our isolates and predicting mutations in different genomic regions. The specificity and utility of the used PCR primers were limited by some of the mutations in our isolates, which may have prevented the primers from annealing to the desired target sequences. Furthermore, some of the primers were shown to anneal to sequences from different ALV subgroups to varying extents through a BLAST search of GenBank. This poses a limitation on the specificity of some of these primers in detection of certain ALV subgroups. On the other hand, the sensitivity of PCR detection of ALV in our isolates was not evaluated, but can be assessed in future studies.

CHAPTER 3 CONCLUSIONS

The main objective of the research studies in chapter 3 was to molecularly characterize seven AF 115 isolates of a naturally occurring recombinant ALV (AF 115-1, AF 115-5, AF 115-7, AF 115-10, AF 115-13, AF 115-14 and AF 115-16) by DNA sequence analysis. The most significant finding from these research experiments was the identification and confirmation of the subgroup designation of the different AF 115 isolates by DNA sequence analysis. All AF 115 isolates are recombinant ALVs with different genomic regions representing different ALV subgroups (Figure 4.1). This was done by comparing the deduced amino acid sequences of gp85 and gp37 and the proviral DNA sequences of 3' UTR and LTR of the seven isolates with the naturally occurring recombinant ALV-B/J, representative ALV subgroups and related ALV strains through NCBI blast. Thus, DNA sequence analysis seems to be the golden method to clearly characterize any ALV isolate in field as well as experimental settings.

Five AF 115 isolates (AF 115-1, AF 115-7, AF 115-13, AF 115-14 & AF 115-16) were closely related to the naturally occurring recombinant ALV-B/J. Similar to ALV-B/J (AF 115-4), these five isolates had a subgroup B gp85, subgroup E gp37 and subgroup J 3' UTR (containing a DR1 and E element) and LTR. On the other hand, AF 115-5 and AF 115-10 displayed sequence variations in the gp85 and 3' UTR and LTR regions respectively. AF 115-5 had a similar genomic organization as the other AF 115 isolates except for a subgroup A gp85. Moreover, the genome of AF 115-10 was similar to the first five AF 115 isolates except for its 3' UTR and LTR, which was highly associated with a recombinant ALV isolate (ALV TymS_90) associated with fowl glioma. These associations were predicted by the unique sequence rearrangements and

mutations in different genomic regions of these recombinant ALVs such as the 3' UTR and LTR of AF 115-10 affecting putative transcription regulatory elements.

Another significant finding was the identification of conserved cysteine residues and potential N-linked glycosylation sites in the SU and TM envelope glycoproteins of all AF 115 isolates. These sequences or sites can affect the envelope glycoproteins stability and functions. Further studies are needed to elucidate the contributions of these conserved sequences to the structure and function of these envelope glycoproteins. Moreover, conserved sequences in the 3' UTR and LTR of AF 115 isolates included putative transcription regulatory elements (CCAAT motif, PU1 and PU2 core regions in the 3' UTR and TATA box, PAS signal, CCAAT motif and CCAAT-related LTR enhancer motif and two boxes of Y, CArG and PRE in the 3' U3 region). In future experiments, PCR primers can be designed to anneal to these conserved sites in the 3' UTR and LTR instead of the variable regions in the *env* and improve molecular diagnosis of ALV.

On the other hand, AF 115-10 contained extra enhancer elements in the rearranged sequences of 3' LTR and had mutations in CCAAT motifs and one of the PRE boxes. The mutation in PRE box of AF 115-10 was shared by AF 115-5 and AF 115-7. Some of these elements showed mutations, which may affect their ability to bind transcription regulatory proteins and thus influence virus transcription. Further studies are needed to evaluate the functional significance of these enhancer elements (wild type and mutated) in transient infections through measuring LTR-driven transcription.

In addition, hotspots for recombination in all AF 115 isolates were predicted based on their sequence analyses and association of their genomic regions with different

ALV subgroups and strains. Taken together, the molecular characterization results suggested that the *env* of all AF 115 isolates may have risen by recombination between exogenous and endogenous ALV sequences. Moreover, the gp37 and the 3' UTR upstream of the DR1 region of all AF 115 isolates as well as 3' UTR and LTR of AF 115-10 may be hotspots for recombination among different ALV subgroups (Figure 4.1).

CHAPTER 3 LIMITATIONS

The ClustalW slow accurate method was used for sequence analyses since it is the most accurate and popular method for typical multiple sequence alignment and their refinement for phylogenetic analysis. Moreover, phylogenetic trees were constructed using the neighbor-joining (NJ) distance-based method utilized in MegAlign. However, the best method for construction of phylogenetic tree depends on the sequence data. Methods such as maximum parsimony, distance-based, and maximum likelihood are generally used to find the optimal tree that best accounts for the sequence data. Each of these methods uses a different type of analysis and has different inherent weaknesses and strengths. Therefore, it is recommended to reanalyze the sequence data by several methods and then compare the results. In addition, the reliability or confidence intervals of the constructed phylogenetic tree can be evaluated by bootstrap analysis procedure. It is a statistical method that estimates the frequency of the appearance of the same branches when sequences are resampled. These methods were not addressed in the analysis, but will be used for further analysis of our AF 115 isolates.

RESULTS COMPARISON

Data from the research experiments in chapter 2 were generally consistent with those in chapter 3. Biological and molecular assays results of most AF 115 isolates were similar to those of the naturally occurring recombinant ALV-B/J. Moreover, the biological (host range and antigenicity) and molecular (PCR and DNA sequencing) results were consistent in most AF 115 isolates. The only exception was AF 115-5, which showed discrepancies in the results by different methods. This can be explained by the possibility of the presence of a mixture of ALVs (A/J and B/J) in this isolate as suggested by data from the different biological and molecular assays. For example, the host range data can be interpreted by presence of a mixture of ALV subgroups in isolate AF 115-5. On the other hand, the virus neutralization and PCR results revealed presence of subgroup B envelope and ALV-B, ALV-J and ALV-B/J like sequences. However, data from sequence analysis of AF 115-5 revealed it is a recombinant ALV-A/J.

Similar results to AF 115-5 isolate were reported in an outbreak of subcutaneous sarcomas in commercial white leghorn layers (Zavala *et al.*, 2006). As with AF 115-5, virus isolation in either DF-1 or *alv6* cells were positive, indicating the presence of exogenous ALVs and probably not antigenically related to ALV-A (Zavala *et al.*, 2006). On the other hand, the same viruses were partially or completely neutralized by antibodies against ALV-A and ALV-B in virus neutralization assays. The authors concluded that there was a possibility of more than one exogenous virus being involved in this natural infection of commercial layers. Furthermore, a field case of mixed infection with MAV-1 (ALV-A) and RAV-2 (ALV-B) was previously reported also in commercial white leghorns (Spencer *et al.*, 2003).

All AF 115 isolates were isolated from commercial layers affected by myeloid leukosis (ML), but, no subgroup J ALV was found in any of the isolates. Moreover, inoculation of the naturally occurring recombinant ALV-B/J (AF 115-4) into susceptible experimental egg-type chickens and different parental lines of commercial white leghorn layers induced predominately LL (Lupiani *et al.*, 2006; Mays *et al.*, 2006). Inoculation of commercial layers with MAV-1 viruses produced exclusively myelocytomas indistinguishable histologically from those induced by ALV-J in meat type chickens (Zavala *et al.*, 2006). It is interesting to speculate that the MAV-1-like virus in AF 115-5 may be responsible for the ML observed in the affected layers.

In short, our data revealed the presence of a huge diversity in the seven AF 115 isolates obtained from a single outbreak of ML in commercial chickens. In fact, this is the first reported outbreak of recombinant ALVs in field flocks where various forms (different variant of ALV-B/J) and combination of recombinant ALVs (ALV-A/J and ALV-B/J) were isolated and characterized (Figure 4.1). In 2000, Lupiani and coworkers isolated and characterized three laboratory generated recombinant ALV-A/J isolates where ADOL 5701A represented a different ALV variant from ADOL 5701AΔ and ADOL 6803A, which sowed nucleotide deletions in their 3' UTRs. Coinfection with genetically divergent strains of HIV-2 and recombination were suspected to occur in some infected individuals (Gao *et al.*, 1994). Moreover, coinfection of puma by two different viral strains of feline immunodeficiency virus was reported (Carpenter *et al.*, 1996). Similarly, coinfection with and recombination by caprine arthritis-encephalitis virus and maedi-visna virus have been documented in naturally infected goats (Pisoni *et*

al., 2007). Lately, dual infection and recombination were believed to be the source of new variation in small-ruminant lentiviruses in goats and sheep (Germain *et al.*, 2008).

This wide diversity of ALV isolates in a single outbreak adds to our current knowledge about ALV evolution and may help in understanding ALV epidemiology. This fast evolution of ALV may have devastating consequences for the poultry industry in the future. It is possible that new highly virulent strains of recombinant ALVs may emerge, causing new disease problems, higher mortality and serious production and economical losses. In addition, the presence of various forms and combinations of these recombinant ALVs in our isolates points to the difficulty in diagnosis and prevention of this economically important pathogen in the field.

RECOMMENDATIONS

The presence of different recombinant ALVs in our AF 115 isolates raises the possibility that these isolates might have formed from recombination events among different ALV subgroups in the field or hatchery. The hatchery involvement in mediating recombination between different ALV subgroups in this outbreak was also suggested previously (Gingerich et al., 2002). Therefore, it is recommended that hatching, processing and rearing of chicks from different sources (meat-type or egg-type) or different breeders be strictly avoided. In the hatchery, stringent biosecurity practices must be implemented including sanitation (cleaning, disinfection and fumigation) and monitoring of hatchery building (including egg storage areas and water lines), equipment (hatchers, incubators and others), eggs and egg transportation equipments, proper handling and optimal storage of eggs and pest control.

In fact, contact transmission of ALV usually occurs among congenitally infected hatchmates, penmates, fomites and the environment. Thus, general management practices in the hatchery and farm must be implemented at all levels of production. These management procedures include using clean eggs and chicks from one source, using disposable chick boxes, rearing one chicken age group, avoiding contact-proximity to other poultry or animals, mechanical vent sexing, vaccination with separate needles, rearing on wire-floored cages, regulating movement of personnel, equipment and vehicles, proper litter and bedding material management and disposal and feed hygiene and transport. In addition, all farm equipment used for incubation, hatching, and brooding of fertilized eggs and chicks should be thoroughly cleaned, disinfected and fumigated after each use.

Various methods for ALV detection should be fast, economical and suitable for large-scale use. ALV characterization in addition to ALV isolation and detection are essential to study ALV epidemiology and evolution and their impact on the poultry industry. ALV infections are diagnosed by virus isolation and identification in commercial, pathogen-free and breeder flocks. Virus isolation is carried out in cell culture using the appropriate chicken embryo fibroblasts with specific host range, followed by p27 ELISA. This biological diagnostic assay remains the standard method for ALV isolation and detection. However, it is recommended that CEFs with different host ranges are used so that the subgroup is identified. For example, (C/O) CEFs are used to detect all ALVs while line 0 CEFs (C/E) and DF-1 (C/E) cell line detect exogenous ALVs. Moreover, different CEFs and cell lines can be utilized to confirm the subgroup of the isolated ALV such as alv6 (C/AE), 7_2 (C/ABDE) and DF-1/J (C/EJ) cell line.

Molecular diagnostic tests seem to be more specific since it detects, identifies and confirms the nature of ALV subgroup including mutants and recombinants in various samples. PCR analysis in conjunction with sequence analysis of ALV isolates can yield important information about ALV diversity. It is recommended that PCR employing generic primers (ARK 4836 & REVSP 6683 or RS-7J & REVSP 6683) are initially used to confirm the presence of ALV. This can be followed by sequence analysis of the isolated PCR product to characterize the different ALV isolates. However, there are no PCR primer pairs currently present, which can amplify the region from beginning of *env* to end of LTR of all ALVs. Therefore, different primers specific for various ALV subgroups can be used to identify the ALV subgroup.

Detection of ALV mutants and recombinants can be possible by using different combinations of PCR primers specific for different ALV subgroups as shown by our current studies. However, since most ALV sequences used for designing these specific PCR primers are located in the *env* and LTR regions, which are prone to mutations and recombination, not all ALV variants will be detected. Therefore, it is recommended that future PCR primers are designed based on less variable sequences in these regions. For example, PCR primers should be designed to anneal to conserved sites (transcription regulatory elements) in the 3' UTR and LTR and less variable *gag* and *pol* regions. This can be followed by sequence analysis of different isolates for ALV epidemiological and evolutionary studies.

FUTURE STUDIES

In addition to the importance of understanding the molecular structure and relationships among different recombinant strains of ALV in these AF 115 isolates, it is imperative to explore the virus-host interaction and transmission of these viruses in biological systems. The oncogenicity of different ALV subgroups is based on differences in *env* or the LTR (Brown and Robinson, 1988; Chesters *et al.*, 2002). AF 115-5 and AF 115-10 exhibited differences in these two regions when compared with the rest of the isolates and the naturally occurring recombinant ALV-B/J (AF 115-4). Future research experiments should assess the pathogenicity (induction of viremia, shedding, immunity and oncogenicity) of these isolates in the context of virus (dose and strain) and host (age at inoculation and phenotype) factors.

Although both subgroup A and B are known to induce lymphoid leukosis in susceptible chickens, AF 115-5 may produce different type of tumors since its gp85 is MAV-1-like. It is expected that AF 115-5 may result in ML when it is inoculated into susceptible chickens compared with AF 115-10 or AF 115-4 which will cause LL. The rate of replication and the frequency of tumors of AF 115-10 may be different from the rest of the isolates due to rearrangements and mutations in the 3' UTR and LTR. Chickens inoculated with AF 115-10 may show faster induction of viremia, more shedding and higher incidence of tumors since AF 115-10 contains an extra CCAAT element, which may increase its transcription and replication potential.

In future animal studies, ALV-susceptible egg-type chickens should be used since meat-type chickens susceptible to ALV-B are hard to find. In previous studies, various parental lines of commercial white leghorn layers (Mays *et al.*, 2006) and experimental ADOL chickens $15I_5 \times 7_1$ (Lupiani *et al.*, 2006) were shown to be susceptible to the

naturally occurring recombinant ALV-B/J (AF 115-4). However, there were no major differences among the different parental lines of commercial chickens and ADOL experimental chickens. Therefore, it is recommended to use the experimental ADOL chickens $15I_5 \times 7_1$ since they are available and accessible. Later, other lines of chickens can be used to evaluate their resistance and immune response to AF 115 isolates representing various forms of naturally occurring recombinant ALVs.

The ALV strains to be used for inoculation of these chickens will include RAV-2 (ALV-B), Hc1 (ALV-J), AF 115-4 (ALV-B/J), AF 115-5 (ALV-A/J and ALV-B/J) and AF 115-10 (ALV-B/J). Each ALV strain will be inoculated into two groups of 30 chickens each either intra-abdominally at hatch or via the yolk sac at 8 days of embryonation with 10^3 tissue culture infectious dose ($TCID_{50}$/ml). Higher doses (10^4 and 10^5 $TCID_{50}$/ml) can be used later to evaluate the effect of dose of the virus on pathogenicity in susceptible chickens. Another 30 age-matched uninoculated chickens will be used as controls. All chickens will be bled at hatch, tested for ALV and maternal antibodies and kept in isolation until termination at 32 weeks of age. At 2, 4, 8, 16 and 32 weeks PI, chickens will be tested for viremia (ALV in serum), shedding (ALV in cloacal swabs) and immune response (ALV neutralizing antibodies in serum) and observed for tumors. Dead and live chickens at the end of the experimental period will be necropsied. Samples of affected tissues will be prepared, appropriately stained and examined for microscopic lesions. The type of tumors induced by these ALV strains will be determined based on characteristic gross and microscopic lesions.

SUMMARY

In summary, these studies demonstrated the importance of using different diagnostic techniques to specifically characterize new emerging ALV isolates such as naturally occurring recombinant ALVs. The findings from this research work will aid poultry industry in diagnosis, prevention and control of new emerging mutant and recombinant ALVs in field flocks and experimental settings. In addition, this research will be useful to scientists studying mutation, recombination, epidemiology and evolution of retroviruses.

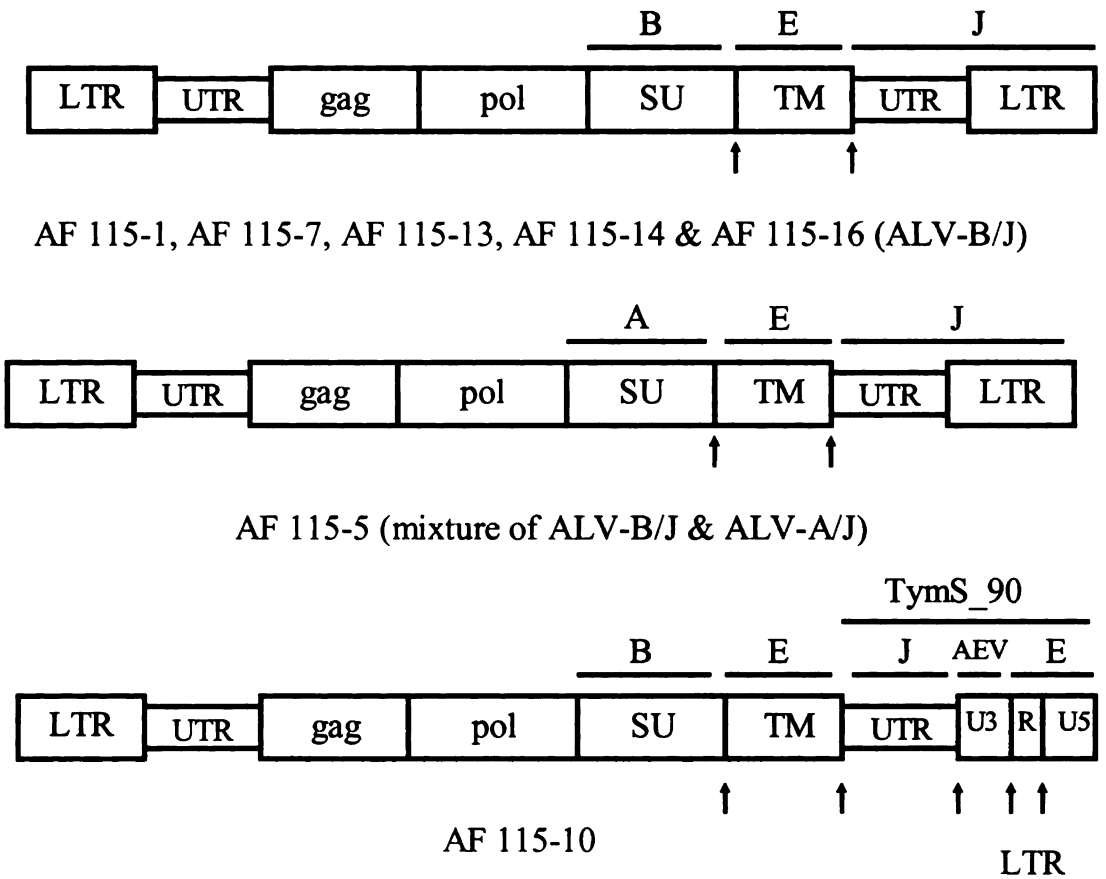


Figure 4.1. Schematic representation of DNA proviruses of AF 115 isolates and homology to known ALV subgroups and strains. Arrows represent potential recombination boundaries or hotspots. LTR: long terminal repeat, UTR: untranslated region; gag: group-specific antigen gene; pol: polymerase gene; SU: surface region; TM: transmembrane region; U3: unique 3' end sequence; R: repeat region; U5: unique 5' end sequence; B: ALV-B, E: ALV-E, J: ALV-J; A: ALV-A; AEV: avian erythroblastosis virus; TymS_90: recombinant ALV strain with MAV-1 (subgroup A) gp85, subgroup E gp37, subgroup J 3' UTR, AEV E26 U3, subgroup E R and U5.

REFERENCES

Adkins, H. B., J. Brojatsch, and J. A. Young. 2000. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. *J Virol* 74:3572-3578.

Afanassieff, M., G. Dambrine, C. Ronfort, F. Lasserre, F. Coudert, and G. Verdier. 1996. Intratesticular inoculation of avian leukosis virus (ALV) in chickens- production of neutralizing antibodies and lack of virus shedding into semen. *Avian Dis* 40:841-852.

Alevy, M. C., and P. K. Vogt. 1978. Ts pol mutants of avian sarcoma viruses: Mapping and demonstration of single cycle recombinants. *Virology* 87:21-33.

Arshad, S. S., K. Howes, G. S. Barron, L. M. Smith, P. H. Russell, and L. N. Payne. 1997. Tissue tropism of the HPRS-103 strain of J subgroup avian leukosis virus and of a derivative acutely transforming virus. *Vet Pathol* 34:127–137.

Aschoff, J. M., D. Foster, and J. M. Coffin. 1999. Point mutations in the avian sarcoma/leukosis virus 3' untranslated region result in a packaging defect. *J Virol* 73:7421–7429.

Astrin, S. M., H. L. Robinson, L. B. Crittenden, E. G. Buss, J. Wyban, and W. S. Haywards. 1979. Ten genetic loci in the chicken that contain structural genes for endogenous avian leukosis viruses. *Cold Spring Harb Symp Quant Biol* 44:1105-1109.

Auclair, B. W., W. M. Collins, R. M. Zsigray, and W. E. Briles. 1995. *B*-complex recombinants and sarcoma regression: Role of *B-L/B-F* region genes. *Poult Sci* 74:434–440.

Aurigemma, R. E., J. L. Torgersen, and R. E. Smith. 1991. Sequences from myeloblastosis-associated virus MAV-2(O) and UR2AV involved in the formation of plaques and the induction of osteopetrosis, anemia, and ataxia. *J Virol* 65:23–30.

Bacon, L. D., R. L. Witter, L. B. Crittenden, A. M. Fadly, and J. Motta. 1981. B-haplotype influence on Marek's disease, Rous sarcoma, and lymphoid leukosis virus-induced tumors in chickens. *Poult Sci* 60:1132-1139.

Bacon, L. D., T. L. Fredericksen, D. G. Gilmour, A. M. Fadly, and L. B. Crittenden. 1985. Test of association of lymphocyte alloantigen genotypes with resistance to viral oncogenesis in chickens. II. Rous sarcoma and lymphoid leukosis in progeny derived from 63 x 15 and 100 x 63 crosses. *Poult Sci* 64:39–47.

Bacon, L. D., L. K. Ch'ng, J. Spencer, A. A. Benedict, A. M. Fadly, R. L. Witter, and L. B. Crittenden. 1986. Tests of association of immunoglobulin allotype genes and viral oncogenesis in chickens. *Immunogenetics* 23: 213–220.

Bacon, L. D., E. J. Smith, L. B. Crittenden, and G. B. Havenstein. 1988. Association of the slow feathering (K) and an endogenous viral (ev 21) gene on the Z chromosome of chickens. *Poult Sci* 67:191-197.

Bacon, L. D., R. L. Witter, and A. M. Fadly. 1989. Augmentation of retrovirus-induced lymphoid leukosis by Marek's disease herpesviruses in White Leghorn chickens. *J Virol* 63:504-512.

Baglia, L. A., W. J. Bowers, and A. Ruddell. 1997. The avian C/EBP gamma gene encodes a highly conserved leucine zipper transcription factor. *Gene* 190:297–302.

Bai, J., K. Howes, L. N. Payne, and M. A. Skinner. 1995a. Sequence of host-range determinants in the env gene of a full-length, infectious proviral clone of exogenous avian leukosis virus HPRS-103 confirms that it represents a new subgroup (designated J). *J Gen Virol* 76:181-187.

Bai, J., L. N. Payne, and M. A. Skinner. 1995b. HPRS-103 (exogenous avian leukosis virus, subgroup J) has an *env* gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. *J Virol* 69:779-784.

Bai, J., L. N. Payne, and M. A. Skinner. 1998a. Long terminal repeat of HPRS-103 containing a mutated EFII site differs functionally from that of RAV-1 and 39 non-coding region contains cis-acting elements. *Avian Pathol* 27:S92.

Bai, J., L. N. Payne, and M. A. Skinner. 1998b. Sequence analysis of an infectious proviral clone of HPRS-103 shows that it represents a new avian retrovirus envelope subgroup (designated J). *Avian Pathol* 27:S92–S93.

Baluda, M. A., B. Perbal, K. E. Rushlow, and T. S. Papas. 1983. Avian myeloblastosis virus: A model for the generation of viral oncogenes from potentially oncogenic cellular genetic elements. *Folia Biologica* 29:18-34.

Banders, U. T. and P. M. Coussens. 1994. Interactions between Marek's disease virus encoded or induced factors and the Rous sarcoma virus long terminal repeat promoter. *Virology* 199:1–10.

Barbosa, T., G. Zavala, and S. Cheng. 2008. Molecular characterization of three recombinant isolates of avian leukosis virus obtained from contaminated Marek's disease vaccines. *Avian Dis* 52:245-252.

Bates, P., J. A. Young, and H. E. Varmus. 1993. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. *Cell* 74:1043-1051.

Bates, P., L. Rong, H. E. Varmus, J. A. T. Young, and L. B. Crittenden. 1998. Genetic mapping of the cloned subgroup A avian sarcoma and leukosis virus receptor gene to the TVA locus. *J Virol* 72:2505-2508.

Bauer, H. 1974. Virion and tumor cell antigens of C-type RNA tumor viruses. *Adv Cancer Res* 20:275.

Bauer, H., R. Kirth, L. Rohrschneider, and H. Gelderblum. 1976. Immune response to oncornaviruses and tumor-associated antigens in the chicken. *Cancer Res* 36:598-602.

Beard, J. W. 1963. Avian virus growths and their etiological agents. *Adv Cancer Res* 7:1.

Bennett, D. D. and S. E. Wright. 1987. Immunization with envelope glycoprotein of an avian RNA tumor virus protects against sarcoma virus tumor induction: Role of subgroup. *Virus Res* 8:73–77.

Benson, S. J., B. L. Ruis, A. M. Fadly, K. F. Conklin. 1998a. The unique envelope gene of the subgroup J avian leukosis virus derives from ev/J proviruses, a novel family of avian endogenous viruses. *J Virol* 72:10157-10164.

Benson, S. J., B. L. Ruis, A. M. Fadly, A. L. Garbers, K. F. Conklin. 1998b. Independent isolates of the emerging subgroup J avian leukosis virus derive from a common ancestor. *J Virol* 72:10301-10304.

Best, S., P. R. Le Tissier, and J. P. Stoye. 1997. Endogenous retroviruses and the evolution of resistance to retroviral infection. *Trends Microbiol* 5:315-318.

Bishop, J. M. and H. Varmus. 1982. Functions and origins of retroviral transforming genes. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.). Molecular Biology of Tumor Viruses: RNA Tumor Viruses, vol. 1. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 999-1108.

Bizub, D., R. A. Katz, and A. M. Skalka. 1984. Nucleotide sequence of noncoding regions in Rous-associated virus-2: Comparisons delineate conserved regions important in replication and oncogenesis. *J Virol* 49:55-65.

Blair, D. G. 1977. Genetic recombination between avian leukosis and sarcoma viruses: Experimental variables and the frequencies of recombination. *Virology* 77:534-544.

Bolmstedt, A., S. Sjolander, J. E. Hansen, L. Akerblom, A. Hemming, S. L. Hu, B. Morein, and S. Olofsson. 1996. Influence of N-linked glycans in V4–V5 region of human immunodeficiency virus type 1 glycoprotein gp160 on induction of a virus-neutralizing humoral response. *J Acquir Immune Defic Syndr Hum Retrovirol* 12:213–220.

Bolognesi, D. P. 1974. Structural components of RNA tumor viruses. *Adv Virus Res* 19:315.

Boral, A. L., S. A. Okenquist, and J. Lenz. 1989. Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. *J Virol* 63:76-84.

Borysenko, L., V. Stepanets, and A. V. Rynditch. 2008. Molecular characterization of full-length MLV-related endogenous retrovirus ChiRV1 from the chicken, Gallus gallus. *Virology* 376:199-204.

Boulden, A., and L. Sealy. 1990. Identification of a third protein factor which binds the Rous sarcoma virus LTR enhancer: possible homology with the serum response factor. *Virology* 174:204–216.

Bova, C. A., J. P. Manfredi, and R. Swanstrom. 1986. *Env* genes of avian retroviruses: Nucleotide sequence and molecular recombinants define host range determinants. *Virology* 152:343-354.

Bova, C. A., J. C. Olsen, and R. Swanstrom. 1988. The avian retrovirus *env* gene family: molecular analysis of host range and antigenic variants. *J Virol* 62:75–83.

Bova-Hill, C., J. C. Olsen, and R. Swanstrom. 1991. Genetic analysis of the Rous sarcoma virus subgroup D *env* gene: mammal tropism correlates with the temperature sensitivity of gp85. *J Virol* 65:2073-2080.

Bowers, W. J. and A. Ruddell. 1992. a1/EBP: a leucine zipper protein that binds CCAAT/enhancer elements in the avian leukosis virus long terminal repeat enhancer. *J Virol* 66:6578–6586.

Bowers, W. J., J. F. Petrosino, C. D. Smith, and A. Ruddell. 1994. Transcriptional regulation of ALV bursal lymphomagenesis. *Leukemia* 8:S211–S213.

Bowers, W. J., L. A. Baglia, and A. Ruddell. 1996. Regulation of avian leukosis virus long terminal repeat-enhanced transcription by C/EBP-Rel interactions. *J Virol* 70:3051–3059.

Boyce-Jacino, M. T., R. Resnick, and A. J. Faras. 1989. Structural and functional characterization of the unusually short long terminal repeats and their adjacent regions of a novel endogenous avian retrovirus. *Virology* 173:157.

Brincat, J. L., J. K. Pfeiffer, and A. Telesnitsky. 2002. RNase H activity is required for high-frequency repeat deletion during Moloney murine leukemia virus replication. *J Virol* 76:88-95.

Brojatsch, J., J. Naughton, M. M. Rolls, Z. Zingler, and J. A. T. Young. 1996. CAR1, a TNFR–related protein, is a cellular receptor for cytopathic avian leukosis–sarcoma viruses and mediates apoptosis. *Cell* 87:845-855.

Brown, M. T. and J. A. Cooper. 1996. Regulation, substrates and functions of src. *Biochem Biophys Acta* 1287:121-149.

Brown, D. W. and H. L. Robinson. 1988. Influence of env and long terminal repeat sequences on the tissue tropism of avian leukosis viruses. *J Virol* 62:4828-4831.

Brown, T. P., N. Stedman, and M. Pantin-Vera. 2000. Mechanisms of decreased growth in broiler chickens congenitally infected with avian leukosis-subgroup J virus. International Symposium on ALV J and other avian retroviruses. Rauischholzhausen, Germany, 63-66.

Burns, D. P., and R. C. Desrosiers. 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J Virol* 65:1843-1854.

Burmester, B. R. 1952. Studies on fowl lymphomatosis. *Ann NY Acad Sci* 54:992.

Burmester, B. R. 1955. Immunity to visceral lymphomatosis in chicks following injection of viruses into dams. *Proc Soc Exp Biol Med* 88:153-155.

Burmester, B. R. 1956. The shedding of the virus of visceral lymphomatosis in the saliva and feces of individual normal and lymphomatous chickens. *Poult Sci* 35:1089-1099.

Burmester, B. R. and R. F. Gentry. 1954. The transmission of avian visceral lymphomatosis by contact. *Cancer Res* 14:34.

Burmester, B. R. and N. M. Nelson. 1945. The effect of castration and sex hormones upon the incidence of lymphomatosis in chickens. *Poult Sci* 24:509-515.

Burmester, B. R. and N. F. Waters. 1956. Variation in the presence of the virus of visceral lymphomatosis in the eggs of the same hens. *Poult Sci* 35:939-944.

Burmester, B. R., M. A. Gross, W. G. Walter, and A. K. Fontes. 1959a. Pathogenicity of a viral strain (RPL 12) causing avian visceral lympho- matosis and related neoplasms. II. Host-virus interrelations affecting response. *J Natl Cancer Inst* 22:103-127.

Burmester, B. R., W. G. Walter, M. A. Gross, and A. K. Fontes. 1959b. The oncogenic spectrum of two "pure" strains of avian leukosis. *J Nat Cancer Inst* 23:277-291.

Burmester, E. R., W. G. Walter, and A. K. Fontes. 1960. Pathogenicity of a viral strain (RPL 12) causing visceral lymphomatosis and related neoplasms. III. Influence of host age and route of inoculation. *J Natl Cancer Inst* 24:1423.

Calnek, B. W. 1968. Lymphoid leukosis virus: A survey of commercial breeding flocks for genetic resistance and incidence of embryo infection. *Avian Dis* 12:104-111.

Campbell, J. G. and E. C. Appleby. 1966. Tumors in young chickens bred for rapid body growth (broiler chickens): A study of 351 cases. *J Pathol Bacteriol* 92:77-90.

Carpenter, M. A., E. W. Brown, M. Culver, W. E. Johnson, J. Pecon-Slattery, D. Brousset and S. J. O'Brien. 1996. Genetic and phylogenetic divergence of feline immunodeficiency virus in puma (Puma concolor). *J Virol* 70:6682-6693.

Carter, J. K. and R. E. Smith. 1983. Rapid induction of hypothyroidism by an avian leukosis virus. *Infect Immun* 40:795-805.

Carter, J. K., J. D. Garlich, W. E. Donaldson, and R. E. Smith. 1983. Influence of diet on a retrovirus-induced obesity and stunting syndrome. *Avian Dis* 27:317-322.

Casci, T. 2000. Not such a variable clock? *Nat Rev Genet* 1:86.

Celander, D., and W. A. Haseltine. 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. *Nature* 312:159-62.

Chai, N. and P. Bates. 2006. Na+/H+ exchanger type 1 is a receptor for pathogenic subgroup J avian leukosis virus. *Proc Natl Acad Sci USA* 103:5531-5536.

Chase, W. B. 1991. Eradication of avian leukosis virus by breeder companies: Reults, pitfalls and cost benefit analysis. In D. Swayne, and D. Zander (eds.). Proceedings of the Avian Tumor Virus Symposium, 5-7.

Chebloune, Y., J. Rulka, F. L. Cosset, S. Valsesia, C. Ronfort, C. Legras, A. Drynda, J. Kuzmak, V. M. Nigon, and G. Verdier. 1991. Immune response and resistance to Rous sarcoma virus challenge of chickens immunized with cell-associated glycoproteins provided with a recombinant avian leukosis virus. *J Virol* 65:5374-5380.

Cheslock, S. R., J. A. Anderson, C. K. Hwang, V. K. Pathak, and W. S. Hu. 2000. Utilization of nonviral sequences for minus-strand DNA transfer and gene reconstitution during retroviral replication. *J Virol* 74:9571–9579.

Chesters, P., K. Howes, J. C. McKay, L. N. Payne, and K. Venugopal. 2001. Acutely transforming avian leukosis virus subgroup J strain 996 defective genome encodes a 72-kDa *gag myc* fusion protein. *J Virol* 75:4219-4225.

Chesters, P. M., K. Howes, L. Petherbridge, S. Evans, L. N. Payne, and K. Venugopal. 2002. The viral envelope is a major determinant for the induction of lymphoid and myeloid tumors by avian leukosis virus subgroups A and J, respectively. *J Gen Virol* 83:2553-2561.

Chesters, P. M., L. P. Smith, and V. Nair. 2006. The E (XSR) element contributes to the oncogenicity of avian leukosis virus (subgroup J). *J Gen Virol* 87:2685–2692.

Chubb, R. C., and P. M. Biggs. 1968. The neutralization of Rous sarcoma virus. *J Gen Virol* 3:87-96.

Clurman, B. E. and W. S. Hayward. 1989. Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: Evidence for stage-specific events. *Mol Cell Biol* 9:2657–2664.

Coffin, J. M. 1979. Structure, replication, and recombination of retrovirus genomes: Some unifying hypotheses. *J Gen Virol* 42:1-26.

Coffin, J. M. 1982. Endogenous viruses. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.). Molecular Biology of Tumor Viruses: RNA Tumor Viruses. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1109-1203.

Coffin, J. M. 1986. Genetic variation in AIDS viruses. *Cell* 46:1-4.

Coffin, J. M. 1992. Structure and classification of retroviruses. In J. Levy (ed.). The Retroviridae, vol. 1. Plenum Press: New York, NY, 19-49.

Coffin, J. M. 1996. Retroviridae: The viruses and their replication. In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.). Fields Virology, 3rd edition. Lippincott-Raven: Philadelphia, 1767-1847.

Coffin, J. M., P. N. Tsichlis, and C. S. Barker. 1980. Variation in avian retrovirus genomes. *Ann N Y Acad Sci* 354:410-425.

Coffin, J. M., S. H. Hughes, and H. E. Varmus. 1997. Retroviruses. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1-25.

Collins, W. M., W. E. Briles, R. M. Zsigray, W. R. Dunlop, A. C. Corbett, K. K. Clark, J. L. Marks, and T. P. McGrail. 1977. The *B* locus (*MHC*) in the chicken: Association with the fate of RSV-induced tumors. *Immunogenetics* 5:333–343.

Cooper, G. M. 1982. Cellular transforming genes. *Science* 217:801-806.

Cooper, M. D., L. N. Payne, P. B. Dent, B. R. Burmester, and R. A. Good. 1968. Pathogenesis of avian lymphoid leukosis. I. Histogenesis. *J Natl Cancer Inst* 41:373-378.

Cooper, M. D., H. G. Purchase, D. E. Bockman, and W. E. Gathings. 1974. Studies on the nature of the abnormality of B cell differentiation in avian lymphoid leukosis: production of heterogeneous IgM by tumor cells. *J Immunol* 113:1210-1222.

Crittenden, L. B. 1968. Observations on the nature of a genetic cellular resistance to avian tumor viruses. *J Natl Cancer Inst* 41:145-153.

Crittenden, L. B. 1975. Two levels of genetic resistance to lymphoid leukosis. *Avian Dis* 19:281-292.

Crittenden, L. B. 1981. Exogenous and endogenous leukosis virus genes: A review. *Avian Pathol* 10:101-112.

Crittenden, L. B. 1983. Recent advances in the genetics of disease resistance: A review. *Avian Pathol* 12:1-8.

Crittenden, L. B. 1991. Retroviral elements in the genome of the chicken: Implications for poultry genetics and breeding. *Crit Rev Poult Biol* 3:73-109.

Crittenden, L. B., and A. M. Fadly. 1985. Responses of chickens lacking or expressing endogenous avian leukosis virus genes to infection with exogenous virus. *Poult Sci* 64:454-463.

Crittenden, L. B. and H. J. Kung. 1983. Mechanisms of induction of lymphoid leukosis and related neoplasms by avian leukosis viruses. In J. M. Goldman and O. Jarret (eds.). Mechanisms of Viral Leukemogenesis. Churchill Livingstone, London, 64-68.

Crittenden, L. B. and J. V. Motta. 1969. A survey of genetic resistance to leukosis-sarcoma viruses in commercial stocks of chickens. *Poult Sci* 48:1751-1757.

Crittenden, L. B. and J. V. Motta. 1975. The role of the tvb locus in genetic resistane to RSV (RAV-0). *Virology* 67:327-334.

Crittenden, L. B. and W. Okazaki. 1966. Genetic influence of the Rs locus on susceptibility to avian tumor viruses. II. Rous sarcoma virus antibody production after strain RPL12 virus inoculation. *J Natl Cancer Inst* 36:299–303.

Crittenden, L. B. and D. W. Salter. 1990. Expression of retroviral genes in transgenic chickens. *J Reprod Fert Suppl* 41:163-171.

Crittenden, L. B. and E. J. Smith. 1984. A comparison of test materials for differentiating avian leukosis virus group-specific antigens of exogenous and endogenous origin. *Avian Dis* 28:1057-1070.

Crittenden, L. B., E. J. Wendel, and J. V. Motta. 1973. Interaction of genes controlling resistance to RSV(RAV-O). *Virology* 52:373–384.

Crittenden, L. B., J. V. Motta, and E. J. Smith. 1977. Genetic control of RAV-0 production in chickens. *Virology* 76:90-97.

Crittenden, L. B., R. L. Witter, and A. M. Fadly. 1979. Low incidence of lymphoid tumors in chickens continuously producing endogenous virus. *Avian Dis* 23:646-653.

Crittenden, L. B., W. S. Hayward, H. Hanafusa, and A. M. Fadly. 1980. Induction of neoplasms by subgroup E recombinants of exogenous and endogenous avian retroviruses (Rous-associated virus type 60). *J Virol* 33:915-919.

Crittenden, L. B., A. M. Fadly, and E. J. Smith. 1982. Effect of endogenous leukosis virus genes on response to infection with avian leukosis and reticuloendotheliosis viruses. *Avian Dis* 26:279-294.

Crittenden, L. B., W. Okazaki, and E. J. Smith. 1983. Incidence of avian leukosis virus infection in broiler stocks and its effect on early growth. *Poult Sci* 62:2382-2386.

Crittenden, L. B., E. J. Smith, and A. M. Fadly. 1984. Influence of endogenous viral (ev) gene expression and strain of exogenous avian leukosis virus (ALV) on mortality and ALV infection and shedding in chickens. *Avian Dis* 28:1037-1056.

Crittenden, L. B., S. McMahon, M. S. Halpern, and A. M. Fadly. 1987. Embryonic infection with the endogenous avian leukosis virus Rous- associated virus-0 alters responses to exogenous avian leukosis virus infection. *J Virol* 61:722-725.

Cui, Z., Y. Du, Z. Zhang, and R. F. Silva. 2003a. Comparison of Chinese field strains of avian leukosis subgroup J viruses with prototype strain HPRS-103 and United States strains. *Avian Dis* 47:1321–1330.

Cui, Z., W. Jianxin, and Z. Zhi. 2003b. Co-infections and interaction of subgroup J avian leukosis viruses and avian reticuloendotheliosis viruses could cause severe immunodepression in broilers. Proceedings of the XIII Congress of the World Veterinary Poultry Association. Denver, CO.

Cullen, B. R., K. Raymond, and G. Ju. 1985. Functional analysis of the transcription control region located within the avian retroviral long terminal repeat. *Mol Cell Biol* 5:438–447.

Cummins, T. J. and R. E. Smith. 1988. Analysis of hematopoietic and lymphopoietic tissue during a regenerative aplastic crisis induced by avian retrovirus MAV-2(O). *Virology* 163:452–461.

Curristin, S. M., K. J. Bird, R. J. Tubbs, and A. Ruddell. 1997. VBP and RelA regulate avian leukosis virus long terminal repeat-enhanced transcription in B cells. *J Virol* 71:5972–5981.

Dang, C. V. 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol* 19:1–11.

Delos, S. E. and J. M. White. 2000. Critical role for the cysteines flanking the internal fusion peptide of avian sarcoma/leukosis virus envelope glycoprotein. *J Virol* 74:9738-9741.

Delos, S. E., M. J. Burdick, and J. M. White. 2002. A single glycosylation site within the receptor binding domain of the avian sarcoma/leukosis virus glycoprotein is critical for receptor binding. *Virology* 294:354–363.

Denesvre, C., C. Soubieux, G. Pin, D. Hue, and G. Dambrine. 2003. Interference between avian endogenous ev/J 4.1 and exogenous ALV J retroviral envelopes. *J Gen Virol* 84:3233-3238.

Dimcheff, D. E., S. V. Drovetski, M. Krishnan, and D. P. Mindell. 2000. Cospeciation and horizontal transmission of avian sarcoma and leukosis virus gag genes in galliform birds. *J Virol* 74:3984-3995.

Distefano, H. S. and R. M. Dougherty. 1966. Mechanims of congenital transmission of avian leukosis virus. *J Natl Cancer Inst* 37:869-883.

Distefano, H. S. and R. M. Dougherty. 1968. Multiplication of avian leukosis virus in the reproductive system of the rooster. *J Natl Cancer Inst* 41:451-464.

Donovan, R. M. and J. F. Fuller. 2004. Retroviridae. In D. C. Hirsh, N. J. Maclachlan, and R. L. Walker (eds.). Veterinary Microbiology, 2nd edition. Blackwell Publishing: Ames, IA, 409-427.

Doolittle, R. F., D. F. Feng, M. S. Johnson, and M. A. McClure. 1989. Origins and evolutionary relationships of retroviruses. *Q Rev Biol* 64:1-30.

Doolittle, R. F., D. F. Feng, M. A. McClure, and M. S. Johnson. 1990. Retrovirus phylogeny and evolution. *Curr Top Microbiol Immunol* 157:1-18.

Dorner, A. J. and J. M. Coffin. 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. *Cell* 45:365-374.

Dorner, A. J., J. P. Stoye, and J. M. Coffin. 1985. Molecular basis of host range variation in avian retroviruses. *J Virol* 53:32-39.

Dougherty, R. M. 1987. A historical review of avian retrovirus research. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 1-27.

Duesberg, P. H., K. Bister, and C. Moscovici. 1980. Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. *Proc Natl Acad Sci USA* 77:5120–5124.

Duprey, S. P. and D. Boettiger. 1985. Developmental regulation of c-myb in normal myeloid progenitor cells. *Proc Natl Acad Sci USA* 82:6937-6941.

Duran-Reynals, F. 1942. The reciprocal infection of ducks and chickens with tumor-inducing viruses. *Cancer Res* 2:343-369.

Eckert, E. A., D. Beard, and J. W. Beard. 1954. Dose-response relations in experimental transmission of avian erythromyeloblastic leukosis. III. Titration of the virus. *J Natl Cancer Inst* 14:1055–1066.

Einfeld, D. and E. Hunter. 1988. Oligomeric structure of a prototype retrovirus glycoprotein. *Proc Natl Acad Sci USA* 85:8688–8692.

Elena, S. F., R. F. Miralles, J. M. Cuevas, P. E. Turner, and A. Moya. 2000. The two faces of mutation: extinction and adaptation in RNA viruses. Biochem *Mol Biol Int* 49:5–9.

Elleder, D., V. Stepanets, D. C. Melder, F. Senigl, J. Geryk, P. Pajer, J. Plachy, J. Hejnar, J. Svoboda, and M. J. Federspiel. 2005. The receptor for the subgroup C avian sarcoma and leukosis viruses, Tvc, is related to mammalian butyrophilins, members of the immunoglobulin superfamily. *J Virol* 79:10408-10419.

Ellgaard, L., M. Molinari, and A. Helenius. 1999. Setting the standards: Quality control in the secretory pathway. *Science* 286:1882–1888.

Enrietto, P. J. and M. J. Hayman. 1987. Structure and virus-associated oncogenes of avian sarcoma and leukemia viruses. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 29-46.

Enrietto, P. J. and J. A. Wyke. 1983. The pathogenesis of oncogenic avian retroviruses. *Adv Cancer Res* 39:269-314.

Fadly, A. M. 1987. Differential diagnosis of lymphoid leukosis. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 197-211.

Fadly, A. M. 1988. Avian leukosis virus (ALV) infection, shedding, and tumors in maternal ALV antibody-positive and -negative chickens exposed to virus at hatching. *Avian Dis* 32:89-95.

Fadly, A. M. 1997. Criteria for the differential diagnosis of viral lymphomas of chickens: A review. In A. M. Fadly, K. A. Schat, and J. L. Spencer (eds.). Proceedings of the Avian Tumor Viruses Symposium. Reno, Nevada, 6-11.

Fadly, A. M. 1998. Subgroup J avian leukosis virus infection in meat-type chickens: A review. Fourth Asian Pacific Poultry Health Conference, AVPA, Melbourne, Australia. Scientific Proceedings, 109-112.

Fadly, A. M. 2000. Isolation and identification of avian leukosis viruses: a review. *Avian Pathol* 29:529–535.

Fadly, A. M. and L. N. Payne. 2003. Leukosis/sarcoma group. In Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne (eds.). Diseases of Poultry, 11th edition. Iowa State University Press: Iowa, 465-516.

Fadly, A. M. and E. J. Smith. 1997. An overview of subgroup J-like avian leukosis virus infection in broiler breeder flocks in the United States. In A. M. Fadly, K. A. Schat, and J. L. Spencer (eds.). Proceedings of the Avian Tumor Viruses Symposium. American Association of Avian Pathologists: Kennett Square, PA, 54-57.

Fadly, A. M. and E. J. Smith. 1999. Isolation and some characteristics of a subgroup J-like avian leukosis virus associated with myeloid leukosis in meat-type chickens in the United States. *Avian Dis* 43:391-400.

Fadly, A. M. and R. L. Witter. 1993. Effects of age at infection with serotype Marek's disease virus on enhancement of avian leukosis virus-induced lymphomas. *Avian Pathol* 22:565-576.

Fadly, A. M. and R. L. Witter. 1998. Oncornaviruses: Leukosis/sarcoma and reticuloendotheliosis, In D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed (eds.). A Laboratory Manual for the Isolation and

Identification of Avian Pathogens. American Association of Avian Pathologists: Kennett Square, PA, 185-196.

Fadly, A. M., W. Okazaki, E. J. Smith, and L. B. Crittenden. 1981a. Relative efficiency of test procedures to detect lymphoid leukosis virus infection. *Poult Sci* 60:2037-2044.

Fadly, A. M., W. Okazaki, and R. L. Witter. 1981b. Hatchery-related contact transmission and short-term small-group-rearing as related to Lymphoid–Leukosis virus-eradication programmes. *Avian Dis* 25:667–677.

Fadly, A. M., T. F. Davison, L. N. Payne, and K. Howes. 1989. Avian leukosis virus infection and shedding in brown leghorn chickens treated with corticosterone or exposure to various stressors. *Avian Pathol* 18:283-298.

Fadly, A. M., R. F. Silva, and L. F. Lee. 2000. Antigenic characterization of selected field isolates of subgroup J avian leukosis virus. In E. F. Kaleta, L. N. Payne, and U. Heffels-Redmann (eds.). Proceedings of the International Symposium on ALV-J and Other Avian Retroviruses. Rauischholzhausen, Germany, 13-22.

Fadly, A. M., R. F. Silva, H. D. Hunt, A. Pandiri, and C. Davis. 2006. Isolation and characterization of an adventitious avian leukosis virus isolated from commercial Marek's disease vaccines. *Avian Dis* 50:380-385.

Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Bal. 2005. Virus Taxonomy: 8th ICTV Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press: London, UK, 1259.

Fenouillet, E., J. C. Gluckman, and E. Bahraoui. 1990. Role of N-linked glycans of envelope glycoproteins in infectivity of human immunodeficiency virus type 1. *J Virol* 64:2841–2848.

Figlerowicz, M., M. Alejska, A. K. ska-Kokorniak, and M. Figlerowicz. 2003. Genetic variability: The key problem in the prevention and therapy of RNA-based virus infection. *Med Res Rev* 23:488-518.

Fleischmann Jr., W. R. 1996. Virology: Viral Genetics. In S. Baron (ed.). Medical Microbiology, 4th edition. The University of Texas Medical Branch: Galveston, TX, chapter 43.

Franklin, R. M. and M. T. Martin. 1980. In ovo tumorigenesis induced by avian osteopetrosis virus. *Virology* 105:245–249.

Fredrickson, T. N., B. R. Burmester, and W. Okazaki. 1965. Transmission of virus from field cases of avian lymphomatosis. II. Development of strains by serial passage in line 15I chickens. *Avian Dis* 9:82-103.

Fulton, J. E., E. L. Thacker, L. D. Bacon, and H. D. Hunt. 1995. Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis in vitro. *Eur J Immunol* 25:2069-2076.

Fung, Y. K., W. G. Lewis, L. B. Crittenden, and H. J. Kung. 1983. Activation of the cellular oncogene c-erbB by LTR insertion: Molecular basis of induction of erythroblastosis by avian leukosis virus. *Cell* 33:357-368.

Gao, F., L. Yue, D. L. Robertson, S. C. Hill, H. Hui, R. J. Biggar, A. E. Neequaye, T. M. Whelan, D. D. Ho, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1994. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *J Virol* 68:7433-7447.

Garcia, M., S. Riblet, N. Ikuta, and V. R. Lunge. 1998. Molecular diagnosis and differentiation of avi- an leukosis virus subgroup J (ALV-J). Proceedings 135th Annual Convention of the American Veterinary Medical Association. Baltimore, MD, 183.

Garcia M., J. El-Attrache, S. M. Riblet, V. R. Lunge, A. S. K. Fonseca, P. Villegas, and N. Ikuta. 2003. Development and application of reverse transcriptase nested polymerase chain reaction test for the detection of exogenous avian leukosis virus. *Avian Dis* 47:41–53.

Gavora, J. S. 1987. Influences of avian leukosis virus infection on production and mortality and the role of genetic selection in the control of lymphoid leukosis. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 241-260.

Gavora, J. S., J. L. Spencer, R. S. Gowe, and D. L. Harris. 1980. Lymphoid leukosis virus infection: Effects on production and mortality and consequences in selection for high egg production. *Poult Sci* 59:2165–2178.

Gavora, J. S., J. L. Spencer, and J. R. Chambers. 1982. Performance of meat-type chickens test-positive and -negative for lymphoid leukosis virus infection. *Avian Pathol* 11:29-38.

Gebriel, G. M., I. Y. Pevzner, and A. W. Nordskog. 1979. Genetic linkage between immune response to GAT and the fate of RSV-induced tumors expression in chickens. *Immunogenetics* 9:327–334.

Germain, K., B. Croise, and S. Valas. 2008. Field evaluation of a gag/env heteroduplex mobility assay for genetic subtyping of small-ruminant lentiviruses. *J Gen Virol* 89:2020-2028.

Geryk, J., O. Machon, R. Hak, K. Trejbalova, J. Hejnar, J. Plachy, I. Karakoz, and J. Svoboda. 1996. Frequent detection of reviremia in ducks persistently infected with avian leukosis retroviruses. *Folia Biol* 42:245-255.

Gifford, R. and M. Tristem. 2003. The evolution, distribution and diversity of endogenous retroviruses. *Virus Genes* 26:291-315.

Gilbert, J. M., L. D. Hernandez, J. W. Balliet, P. Bates, and J. M. White. 1995. Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. *J Virol* 69:7410-7415.

Gilka, F. and J. L. Spencer. 1987. Importance of the medullary macrophage in the replication of lymphoid leukosis virus in the bursa of Fabricius of chickens. *Am J Vet Res* 48:613-620.

Gilka, F. and J. L. Spencer. 1990. Chronic myocarditis and circulatory syndrome in a white leghorn strain induced by an avian leukosis virus: Light and electron microscopic study. *Avian Dis* 34:174-184.

Gingerich, E., R. E. Porter, B. Lupiani, and A. M. Fadly. 2002. Diagnosis of myeloid leukosis induced by a recombinant avian leukosis virus in commercial white leghorn egg laying flocks. *Avian Dis* 46:745-7488.

Goff, S. P. 2007. Retroviridae: The retroviruses and their replication. In D. M. Knipe and P. M. Howley (eds.). Fields Virology, 5th edition. Lippincott Williams & Wilkins: Philadelphia, PA, 2001-2071.

Gojobori, T., and S. Yokoyama. 1985. Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and of its cellular homologues. *Proc Natl Acad Sci USA* 82:4198-41201.

Gojobori, T., and S. Yokoyama. 1987. Molecular evolutionary rates of oncogenes. *J Mol Evol* 26:148-156.

Golay, J., V. Broccoli, G. M. Borleri, E. Erba, M. Faretta, L. Basilico, G. G. Ying, G. Piccinini, L. H. Shapiro, J. Lovric, M. Nawrath, K. Molling, K. Rambaldi, and M. Introna. 1997. Redundant functions of B-Myb and c-Myb in differentiating myeloid cells. *Cell Growth Differ* 8:1305.

Golemis, E. C., N. A. Speck, and N. Hopkins. 1990. Alignment of U3 region sequences of mammalian Type C viruses: Identification of highly conserved motifs and implications for enhancer design. *J Virol* 64:534-542.

Goldfarb, M. P., and R. A. Weinberg. 1981. Generation of novel, biologically active Harvey sarcoma viruses via apparent illegitimate recombination. *J Virol* 38:136–150.

Gordon, H. 1985. Oncogenes. *Mayo Clin Proc* 60:697-713.

Goubin, G., D. S. Goldman, J. Luce, P. E. Neiman, and G. M. Cooper. 1983. Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. *Nature* 302:114-119.

Graevskaya, N. A., G. Heider, S. P. Dementieva, and D. Ebner. 1982. Antibodies to reverse transcriptase of avian oncoviruses in sera of specific-pathogen-free chickens. *Acta Virol* 26:333-339.

Graf, T. and H. Beug. 1978. Avian leukemia viruses. Interaction with their target cells in vivo and in vitro. *Biochem Biophys Acta* 516:269-299.

Grant, C. E. and R. G. Deeley. 1993. Cloning and characterization of chicken YB-1: regulation of expression in the liver. *Mol Cell Biol* 13:4186–4196.

Greuel, B., L. Sealy, and J. E. Majors. 1990. Transcriptional activity of the Rous sarcoma virus long terminal repeat correlates with binding of a factor to an upstream CCAAT box in vitro. *Virology* 177:33–43.

Guntaka, R. 1993. Transcription termination and polyadenylation in retroviruses. *Microbiol Rev* 57:511–521.

Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks. 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. *Science* 232:1548-1553.

Hanafusa, H. 1965. Analysis of the defectiveness of Rous Sarcoma Virus. III. Determining influence of a new helper virus on the host range and susceptibility to interference of RSV. *Virology* 25:248-255.

Harris, D. L., V. A. Garwood, P. C. Lowe, P. Y. Hester, L. B. Crittenden, and A. M. Fadly. 1984. Influence of sex-linked feathering phenotypes of parents and progeny upon lymphoid leukosis virus infection status and egg production. *Poult Sci* 63:401-413.

Hatai, H., K. Ochiai, K. Nagakura, S. Imanishi, A. Ochi, R. Kozakura, M. Ono, M. Goryo, K. Ohashi, and T. Umemura. 2008. A recombinant avian leukosis virus associated with fowl glioma in layer chickens in Japan. *Avian Pathol* 37:127-137.

Hauptli, D., L. Bruckner, and H. P. Ottiger. 1997. Use of reverse transcriptase polymerase chain reaction for detection of vaccine contamination by avian leukosis virus. *J Virol Methods* 66:71–81.

Helenius, A. and M. Aebi. 2001. Intracellular functions of N-linked glycans. *Science* 291:2364–2369.

Hernandez, L. D. and J. M. White. 1998. Mutational analysis of the candidate internal fusion peptide of the avian leukosis and sarcoma virus subgroup A envelope glycoprotein. *J Virol* 72:3259-3267.

Hernandez, L. D., L. R. Hoffman, T. G. Wolfsberg, and J. M. White. 1996. Virus-cell and cell-cell fusion. *Annu Rev Cell Dev Biol* 12:627–661.

Himly, M., D. N. Foster, I. Bottoli, J. S. Iacovoni, and P. K. Vogt. 1998. The DF-1 chicken fibroblast cell line: Transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology* 248:295–304.

Ho, D. D., J. C. Kaplan, I. E. Rackauskas, and M. E. Gumey. 1988. Second conserved domain of gpl20 is important for HIV infectivity and antibody neutralization. *Science* 239:1021-1023.

Hu, W. S., and H. M. Temin. 1990a. Genetic consequences of packaging two RNA genomes in one retroviral particle: Pseudodiploidy and high rate of genetic recombination. *Proc Natl Acad Sci USA* 87:1556-1560.

Hu, W. S., and H. M. Temin. 1990b. Retroviral recombination and reverse transcription. *Science* 250:1227-1233.

Hu, W. S., E. H. Bowman, K. A. Delviks, and V. K. Pathak. 1997. Homologous recombination occurs in a distinct retroviral subpopulation and exhibits high negative interference. *J Virol* 71:6028-6036.

Hudson, B. P., J. C. Wilson, G. Zavala, and J. E. Sander. 2002. Fertility and sperm quality of broiler breeders males infected with subgroup J avian leukosis virus. *Avian Dis* 46:1033-1037.

Hue, D., G. Dambrine, C. Denesvre, S. Laurent, M. Wyers, and D. Rasschaert. 2006. Major rearrangements in the E element and minor variations in the U3 sequences

of the avian leukosis subgroup J provirus isolated from field myelocytomatosis. *Arch Virol* 151:2431–2446.

Hughes, W. F., D. H. Watanabe, and H. Rubin. 1963. The development of a chicken flock apparently free of leukosis virus. *Avian Dis* 7:154-165.

Hunt, H. D., L. F. Lee, D. Foster, R. F. Silva, and A. M. Fadly. 1999. A genetically engineered cell line resistant to subgroup J avian leukosis virus infection (C/J). *Virology* 264:205-210.

Hunt, H. D., B. Lupiani, and A. M. Fadly. 2000. Recombination between ALV-J and endogenous subgroup E viruses. In E. F. Kaleta, L. N. Payne, and U. Heffels-Redmann, U. (eds.). Proceedings of the International Symposium on ALV-J and Other Avian Retroviruses. Rauischholzhausen, Germany, 50-60.

Hunter, E. 1978. The mechanism for genetic recombination in the avian retroviruses. *Curr Top Microbiol Immunol* 79:295-309.

Hunter, E. 1997. Viral entry and receptors. In J. M. Coffin, S. H. Hughes, and H. E. Varmus (eds.). Retroviruses. Cold Spring Harbor Press: Cold Spring Harbor, N.Y, 71-120.

Hunter, E., E. Hill, M. Hardwick, A. Brown, D. E. Schwartz, and R. Tizard. 1983. Complete sequence of the Rous sarcoma virus *env* gene: identification of structural and functional regions of its product. *J Virol* 46:920–936.

Hussain, A. I., V. Shanmugam, W. M. Switzer, S. X. Tsang, A. M. Fadly, D. Thea, R. Helfand, W. Bellini, T. M. Folks, and W. Heneine. 2001. Lack of evidence of endogenous avian leukosis virus and endogenous avian retrovirus transmission to measles, mumps and rubella vaccine recipients. *Emerg Infect Dis* 7:66-72.

Imperiali, B., and S. E. O'Connor. 1999. Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr Opin Chem Biol* 3:643–649.

Introna, M., J. Golay, J. Frampton, T. Nakano, S. A. Ness, and T. Graf. 1990. Mutations in v-myb alter the differentiation of myelomonocytic cells transformed by the oncogene. *Cell* 63:1287–1297.

Introna, M., M. Luchetti, M. Castellano, M. Arsura, and J. Golay. 1994. The myb oncogene family of transcription factors: potent regulators of hematopoietic cell proliferation and differentiation. *Semin Cancer Biol* 5:113.

Jacobs, G. B., M. Nistal, A. Laten, E. J. van Rensburg, A. Rethwilm, W. Preiser, J. Bodem, and S. Engelbrecht. 2008. Molecular Analysis of HIV Type 1 *vif* Sequences from Cape Town, South Africa. *AIDS Res Human Retroviruses* 24:991-994.

Jaffredo, T., R. M. Molina, A. E. al Moustafa, R. Gautier, F. L. Cosset, G. Verdier, and F. Dieterlen-Lievre. 1993. Patterns of integration and expression of retroviral, non-replicative vectors in avian embryos: embryo developmental stage and virus subgroup envelope modulate tissue-tropism. *Cell Adhes Commun* 1:119-132.

Jern, P., G. O. Sperber, and J. Blomberg. 2005. Use of endogenous retroviral sequences (ERVs) and structural markers for retroviral phylogenetic inference and taxonomy. *Retrovirology* 2:50.

Johnson, P. R., T. E. Hamm, S. Goldstein, S. Kitov, and V. M. Hirsch. 1991. The genetic fate of molecularly cloned simian immunodeficiency virus in experimentally infected macaques. *Virology* 185:217-228.

Jordan, F. T. W. 1990. Poultry diseases, 3rd edition. ELBS/Bailliere Tindall: London, 389-414.

Junghans. R. P., L. R. Boone, and A. M. Skalka. 1982. Retroviral DNA H structures: Displacement-assimilation model of recombination. *Cell* 30:53-62.

Kahn, C. A. 2005. The Merck Veterinary Manual, 9th edition. Merck and Co. Inc.: Whitehouse Station, NJ.

Kanter, M. R., R. E. Smith, and W. S. Hayward. 1988. Rapid induction of B-cell lymphomas: Insertional activation of c-myb by avian leukosis virus. *J Virol* 62:1423–1432.

Katz, R. A. and A. M. Skalka. 1990. Generation of diversity in retroviruses. *Annu Rev Genet* 24:409-445.

Kawai, S., and H. Hanafusa. 1972. Genetic recombination with avian tumor viruses. *Virology* 49:37-44.

Kinsey, N. E., M. G. Anderson, T. J. Unangst, S. V. Joag, O. Narayan, M. C. Zink, and J. E. Clements. 1996. Antigenic variation of SIV: Mutations in V4 alter the neutralization profile. *Virology* 221:14–21.

Kogekar, N., T. L. Spurgeon, M. C. Simon, and R. E. Smith. 1987. Proliferative fibromatosis in avian skeletal muscle caused by cloned recombinant avian leukosis viruses. *Cancer Res* 47:2083–2091.

Kris, R. M., I. Lax, W. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin, and J. Schlessinger. 1985. Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and v-erbB protein. *Cell* 40:619–625.

Kumanishi, T., F. Ikuta, K. Nishida, K. Ueki, and T. Yamamoto. 1973. Brain tumors induced in adult monkeys by Schmidt-Ruppin strain of Rous sarcoma virus. *Gann* 64:641-643.

Kurth, R. and H. Bauer. 1972. Cell surface antigens induced by RNA tumor viruses: Detection by cytotoxic microassay. *Virology* 47:426-433.

Kung, H. J. and N. J. Maihle. 1987. Molecular basis of oncogenesis by non-acute avian retroviruses. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 77-99.

Laimins, L. A., P. Tsichlis, and G. Khoury. 1984. Multiple enhancer domains in the 39 terminus of the Prague strain of Rous sarcoma virus. *Nucleic Acids Res* 12:6427–6442.

Landman, W. J. M., J. L. Nieuwenhuisen-van Wilgen, G. Koch, R. M. Dwars, A. Ultee, and E. Gruys. 2001. Avian leukosis virus subtype J *in ovo*-infected specific pathogen free broilers harbour the virus in their feathers and show feather abnormalities. *Avian Pathol* 30:675-684.

Larder, B. A., and S. D. Kemp. 1989. Multiple mutations in HIV-I reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 246:1155-1158.

Lee, C. M. and E. P. Reddy. 1999. The v-myc oncogene. *Oncogene* 18:2997.

Lee, L. F., R. F. Silva, Y. Q. Cheng, E. J. Smith, and L. B. Crittenden. 1986. Characterization of monoclonal antibodies to avian leukosis viruses. *Avian Dis* 30:132-138.

Lee, L. F., P. Wu, and A. M. Fadly. 1998. Recombinant fowlpox virus expressing ALV J envelop protein (East Lansing, USDA ARS Avian Disease and Oncology Laboratory), 105-110.

Lewin, B. 1991. Oncogenic conversion by regulatory changes in transcription factors. *Cell* 64:303-312.

Lewin, B. 1994. Genes. Oxford University Press: NY.

Linder, M., D. Linder, J. Hahnen, H. H. Schott, and S. Stirm. 1992. Localization of the intrachain disulfide bonds of the envelope glycoprotein 71 from Friend murine leukemia virus. *Eur J Biochem* 203:65–73.

Linial, M. and S. Brown. 1979. High-frequency recombination within the *gag* gene of Rous sarcoma virus. *J Virol* 31:257-260.

Linial, M. L., H. Fan, B. Hahn, R. Lwer, J. Neil, S. Quackenbush, A. Rethwilm, P. Sonigo, J. Stoye, and M. Tristem. 2006. Index of Viruses – Retroviridae. In C. Büchen-Osmond (ed.). ICTVdB - The Universal Virus Database, version 4. Columbia University: New York.

Lipsick, J. S. and D. M. Wang. 1999. Transformation by v-Myb. *Oncogene* 18:3047-3055.

Lower, R. 1999. The pathogenic potential of endogenous retroviruses: Facts and fantasies. *Trends Microbiol* 7:350-356.

Luciw, P. A. and N. J. Leung. 1992. Mechanisms of retroviral replication. In J. Levy (ed.). The Retroviridae. Plenum Press: New York, 159-298.

Lupiani, B., H. D. Hunt, R. F. Silva, R.F., and A. M. Fadly. 2000. Identification and characterization of recombinant subgroup J avian leukosis virus (ALV) expressing subgroup A ALV envelope. *Virology* 276:37-43.

Lupiani, B., S. M. Williams, R. F. Silva, H. D. Hunt, and A. M. Fadly. 2003. Pathogenicity of two recombinant avian leukosis viruses. *Avian Dis* 47:425-432.

Lupiani, B., A. R. Pandiri, J. Mays, H. D. Hunt, and A. M. Fadly. 2006. Molecular and biological characterization of a naturally occurring recombinant subgroup B avian leukosis virus with a subroup J-like long terminal repeat. *Avian Dis* 50:572-578.

Maas, R., D. van Zoelen, H. Oei, and I. Claassen. 2006. Replacement of primary chicken embryonic fibroblasts (CEF) by the DF-1 cell line for detection of avian leukosis viruses. *Biologicals* 34:177–181.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York.

Mansky, L. M. 1998. Retrovirus mutation rates and their role in genetic variation. *J Gen Virol* 79:1337–1345.

Martin, J., E. Hernoiu, J. Cook, R. W. O'Neill, and M. Tristem. 1999. Interclass transmission and phyletic host tracking in murine leukemia virus-related retoviruses. *J Virol* 73:2442–2449.

Maynard Smith, J. H. 1998. Evolutionary genetics, 2nd edition. Oxford University Press: Oxford.

Mays, J.K., L. D. Bacon, A. R. Pandiri, and A. M. Fadly. 2005. Response of white leghorn chickens of various B haplotypes to infection at hatch with subgroup J avian leukosis virus. *Avian Dis* 49:214-219.

Mays, J. K., A. R. Pandiri, and A. M. Fadly. 2006. Susceptibility of various parental lines of commercial white leghorn layers to infection with a naturally occurring recombinant avian leukosis virus containing subgroup B envelope and subgroup J long terminal repeat. *Avian Dis* 50:342-347.

McCutchan, F. E. 2000. Understanding the genetic diversity of HIV-1. *AIDS* 14:S31–S44.

McKay, J. C. 1998. A poultry breeder's approach to avian neopplasia. *Avian Pathol* 27:S74-S77.

McKay, J. C. and A. G. Rosales. 2000. Control and eradication of ALV-J. International Symposium on ALV-J and Other Avian Retroviruses. Justus Liebig University: Rauischholzhausen, Germany, 248-255.

Melder, D. C., V. S. Pandkratz, and M. J. Federspiel. 2003. Evolutionary pressure of a receptor competitor selects different subgroup A avian leukosis virus escape variants with altered receptors interactions. *J Virol* 77:10504-10514.

Meyers, P. 1976. Antibody response to related leukosis viruses induced in chickens tolerant to an avian leukosis virus. *J Natl Cancer Inst* 56:381-386.

Mizuno, Y. and S. Itohara. 1986. Enzyme-linked immunosorbent assay to detect subgroup-specific antibodies to avian leukosis viruses. *Am J Vet Res* 47:551-556.

Mladenov, Z., U. Heine, D. Beard, and J. W. Beard. 1967, Strain MC29 avian leukosis virus. Myelocytoma, endothelioma, and renal growths: Pathomorphological and ultrastructural aspects. *J Natl Cancer Inst* 38:251-285.

Monk, R. J., F. G. Malik, D. Stokesberry, and L. H. Evans. 1992. Direct determination of the point mutation rate of a murine retrovirus. *J Virol* 66:3683-3689.

Moscovici, C. and L. Gazzolo. 1987. Virus-cell interactions of avian sarcoma and defective leukemiaviruses. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 151-169.

Motta, J. V., L. B. Crittenden, and W. O. Pollard. 1973. The inheritance of resistance to subgroup C leukosis-sarcoma viruses in New Hampshire chickens. *Poult Sci* 52:578-586.

Motta, J. V., L. B. Crittenden, H. G. Purchase, H. A. Stone, and R. L. Witter. 1975. Low oncogenic potential of avian endogenous RNA tumor virus infection or expression. *J Natl Cancer Inst* 55:685-689.

Nazerian, K. and N. Yanagida. 1995. A recombinant fowlpox virus expressing the envelope antigen of subgroup A avian leukosis/sarcoma virus. *Avian Dis* 39:514-520.

Negroni, M., and H. Buc. 2001. Mechanisms of retroviral recombination. *Annu Rev Genet* 35:275-302.

Nehyba, J., J. Svoboda, I. Karakoz, J. Geryk, and J. Hejnar. 1990. Ducks: A new experimental host system for studying persistent infection with avian leukaemia retroviruses. *J Gen Virol* 71:1937-1945.

Neiman, P. E., L. Jordan, R. A. Weiss, and L. N. Payne. 1980. Malignant lymphoma of the bursa of Fabricius: Analysis of early transformation. *Cold Spring Harb Conf Cell Prolifer* 7:519-528.

Nichol, S. 1996. RNA viruses: Life on the edge of catastrophe [news]. *Nature* 384:218–219.

Nikiforov, M. A. and A. V. Gudkov. 1994. ART-CH: A VL30 in chickens? *J Virol* 68:846-853.

Noteborn, M. H., G. F. de Boer, A. Kant, G. Koch, J. L. Bos, A. Zantema, and A. J. van der Eb. 1990. Expression of avian leukaemia virus env-gp85 in Spodoptera frugiperda cells by use of a baculovirus expression vector. *J Gen Virol* 71:2641-2648.

Ogert, R. A., and K. L. Beemon. 1998. Mutational analysis of the Rous sarcoma virus dr posttranscriptional control element. *J Virol* 72:3407–3411.

Ogert, R. A., L. H. Lee, and K. L. Beemon. 1996. Avian retrovirus RNA element promotes unspliced RNA accumulation in the cytoplasm. *J Virol* 70:3834–3843.

Oh, I. H. and E. P. Reddy. 1999. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* 18:3017-3033.

Okazaki, W., H. G. Purchase, and B. R. Burmester. 1975. Phenotypic mixing test to detect and assay avian leukosis viruses. *Avian Dis* 19:311-317.

Okazaki, W., B. R. Burmester, A. M. Fadly, and W. B. Chase, 1979. An evaluation of methods for eradication of avian leukosis virus from a commercial breeder flock. *Avian Dis* 23:688-697.

Olsen, J. C., and R. Swanstrom. 1985. A new pathway in the generation of defective retrovirus DNA. *J Virol* 56:779-789.

Onuki, Y., A. Ohshima, Y. Kawarabayasi, and T. Takeya. 1987. Comparison of the structural organizations in the 3'-terminal regions of five avian retrovirus strains: RAV 7, RAV 50, B77, PR-B, and SR-B. *Virology* 156:163–166.

O'Rear, J. J. and H. M. Temin. 1982. Spontaneous changes in nucleotide sequence in proviruses of spleen necrosis virus, an avian retrovirus. *Proc Natl Acad Sci USA* 79:1230-1234.

O'Rear, J. J., S. Mizutani, G. Hoffman, M. Flandt, and H. M. Temin. 1980. Infectious and noninfectious recombinant clones of the provirus of SNV differ in cellular DNA and are apparently the same in viral DNA. *Cell* 20:423-430.

Overbaugh, J., L. M. Rudensey, M. D. Papenhausen, R. E. Benveniste, and W. R. Morton. 1991. Variation in simian immunodeficiency virus *env* is confined to V1 and V4 during progression to simian AIDS. *J Virol* 65:7025-7031.

Page, R. D. M., and E. C. Holmes. 1998. Molecular Evolution. A Phylogenetic Approach. Blackwell Science Press: Oxford.

Pani, P. K. 1974. Studies in genetic resistance to RSV (RAV 0): A third type of genetic resistance typified by the W line of fowl and further support of the two loci genetic model. *J Gen Virol* 23:33-40.

Pani, P. K. 1975. Genetic control of resistance of chick embryo cultures to RSV (RAV50). *J Gen Virol* 27:163-172.

Pani, P. K. 1976. Further studies in genetic resistance of fowl to RSV (RAV 0): Evidence for interaction between independently segregating tumour virus B and tumour virus E genes. *J Gen Virol* 32:441-453.

Pathak, V. K. and H. M. Temin. 1990a. Broad spectrum of *in vivo* forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: Substitutions, frameshifts, and hypermutations. *Proc Natl Acad Sci USA* 87:6019-6023.

Pathak, V. K. and H. M. Temin. 1990b. Broad spectrum of *in vivo* forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: Deletions and deletions with insertions. *Proc Natl Acad Sci USA* 87:6024-6028.

Patschinsky T., H. W. Jansen, H. Blocker, R. Frank, and K. Bister. 1986. Structure and transforming function of transduced mutant alleles of the chicken c-myc gene. *J Virol* 59:341–353.

Payne, L. N. 1987. Epizootiology of avian leukosis virus infections. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 47-75.

Payne, L. N. 1992. Biology of avian retroviruses. In J. Levy (ed.). The Retroviridae, vol. 1. Plenum Press: New York, 299-404.

Payne, L. N. 1998. HPRS-103: A retrovirus strikes back. The emergence of subgroup J avian leukosis virus. *Avian Pathol* 27:S36-S45.

Payne, L. N. 2000. History of ALV-J. In E. F. Kaleta, L. N. Payne, and U. Heffels-Redmann (eds.). Proceedings, International Symposium on ALV-J and Other Avian Retroviruses. Rauischholzhausen, Germany, 3-12.

Payne, L. N. 2001. Avian leukosis virus – new mutations: a threat for the upcoming century. *World's Poult Sci J* 57:265-274.

Payne, L. N., and R. C. Chubb. 1968. Studies on the nature and genetic control of an antigen in normal chick embryos which reacts in the COFAL test. *J Gen Virol* 3:379-391.

Payne, L. N. and K. Howes. 1991. Eradication of exogenous avian leukosis virus from commercial layer breeder lines. *Vet Record* 128:8-11.

Payne, L. N. and P. K. Pani. 1971. Evidence for linkage between genetic loci controlling response of fowl to subgroup A and subgroup C sarcoma viruses. *J Gen Virol* 13:253-259.

Payne, L. N. and K. Venugopal. 2000. Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis. *Rev Sci Tech Off Int Epiz* 19:544-564.

Payne, L. N., P. K. Pani, and R. A. Weiss. 1971. A dominant epistatic gene which inhibits cellular susceptibility to RSV (RAV-0). *J Gen Virol* 13:455-462.

Payne, L. N., S. R. Brown, N. Bumstead, K. Howes, J. A. Frazier, and M. E. Thouless. 1991. A novel subgroup of exogenous avian leukosis virus in chickens. *J Gen Virol* 72:801-807.

Payne, L. N., A. M. Gillespie, and K. Howes. 1992a. Myeloid leukaemogenicity and transmission of the HPRS-103 strain of avian leukosis virus. *Leukemia* 6:1167-1176.

Payne, L. N., K. Howes, A. M. Gillespie, and L. M. Smith. 1992b. Host range of Rous sarcoma virus pseudotype RSV (HPRS-103) in 12 avian species: Support for a new avian retrovirus envelope subgroup, designated J. *J Gen Virol* 73:2995-2997.

Payne, L. N., A. M. Gillespie, and K. Howes. 1993. Recovery of acutely transforming viruses from myeloid leukosis induced by the HPRS-103 strain of avian leukosis virus. *Avian Dis* 37:438-450.

Peeters, M., and P. M. Sharp. 2000. Genetic diversity of HIV-1: the moving target. *AIDS* 14:S129–S140.

Perek, M. 1960. An epizootic of histiocytic sarcomas in chickens induced by a cell-free agent. *Avian Dis* 4:85-94.

Perry, L., T. Wight, W. Collins, and W. Dunlop. 1978. Differentiation of progressive versus regressive Rous virus-induced avian sarcomas according to tumor and infiltrating lymphocyte fine structure. *Poult Sci* 57:80-84.

Pham, T. D., J. L. Spencer, and E. S. Johnson. 1999. Detection of avian leukosis virus in albumin of chicken eggs using reverse transcription polymerase chain reaction. *J Virol Meth* 78:1-11.

Piraino, F. 1967. The mechanism of genetic resistance of chick embryo cells to infection by Rous sarcoma virus-Bryan strain (BS-RSV). *Virology* 32:700-707.

Piraino, F., W. Okazaki, B. R. Burmester, and T. N. Fredrickson. 1963. Bioassay of fowl leukosis virus in chickens by the inoculation of 11-day-old embryos. *Virology* 21:396-401.

Pisoni, G., G. Bertoni, M. Puricelli, M. Maccalli, and P. Moroni. 2007. Demonstration of coinfection with and recombination by caprine arthritis-encephalitis virus and maedi-visna virus in naturally infected goats. J Virol 81:4948-4955.

Pizer, E. and E. H. Humphries. 1989. RAV-1 insertional mutagenesis: Disruption of the c-myb locus and development of avian B-cell lymphomas. *J Virol* 63:1630–1640.

Ponten, J. and B. Thorell. 1957. The histogenesis of virus-induced chicken leukemia. *J Natl Cancer Inst* 18:443–453.

Poss, M., H. Ross, A. Rodrigo, J. Terwee, S. VandeWoude, and R. Biek. 2008. The molecular biology and evolution of feline immunodeficiency viruses of cougars. *Vet Immunol Immunopathol* 15: 154-158.

Preston, B. D. and J. P. Dougherty. 1996. Mechanisms of retroviral mutation. *Trends Microbiol* 4:16-21.

Purchase, H. G. 1987. The pathogenesis and pathology of neoplasms caused by avian leukosis viruses. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 171-196.

Purchase, H. G. and N. F. Cheville. 1975. Infection bursal agent of chickens reduces the incidence of lymphoid leukosis. *Avian Pathol* 4:239-245.

Purchase, H. G. and D. G. Gilmour. 1975. Lymphoid leukosis in chickens chemically bursectomized and subsequently inoculated with bursa cells. *J Natl Cancer Inst* 55:851-855.

Purchase, H. G. and J. M. Sharma. 1973. The differential diagnosis of lymphoid leuksois and Marek's disease, slide study set 3. American Association of Avian Pathologists: Kennett Square, PA.

Purchase, H. G., W. Okazaki, and B. R. Burmester. 1972. Long-term field trials with the herpesvirus of turkeys vaccine against Marek's disease. *Avian Dis* 16:57-71.

Purchase, H. G., D. G. Gilmour, C. H. Romero, and W. Okazaki. 1977. Post-infection genetic resistance to avian lymphoid leukosis resides in B target cell. *Nature* 270:61–62.

Qualtiere, L. F. and P. Meyers. 1983. Evidence that avian tumour virus-immune chicken sera recognize only viral structural antigens on the surface of avian tumour virus-infected cells. *J Gen Virol* 64:2387-2397.

Qureshi, M. A. and R. L. Taylor. 1993. Analysis of macrophage functions in Rous sarcoma-induced tumor regressor and progressor 6.B congenic chickens. *Vet Immunol Immunopathol* 37:285-294.

Reddy, E. P., R. K. Reynolds, D. K. Watson, R. A. Schultz, J. Lautenberger, and T. S. Papas. 1983. Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). *Proc Natl Acad Sci USA* 80:2500–2504.

Reed, D. J., X. Lin, T. D. Thomas, C. W. Birks, J. Tang, and R. P. Rother. 1997. Alteration of glycosylation renders HIVsensitive to inactivation by normal human serum. *Immunology* 159:4356–4361.

Rein, A. 1982. Interference grouping of murine leukemia viruses: A distinct receptor for the MCF-recombinant viruses in mouse cells. *Virology* 120:251-257.

Reitter, J. N., R. E. Means, and R. C. Desrosiers. 1998. A role for carbohydrates in immune evasion in AIDS. *Nat Med* 4:679–684.

Rispens, B. H., P. A. Long, W. Okazaki, and B. R. Burmester. 1970. The NP activation test for assay of avian leukosis-sarcoma viruses. *Avian Dis* 14:738-751.

Robertson, J. S., C. Nicolson, A. M. Reily, M. Bentley, G. Dunn, T. Corcoran, G. C. Schild, and P. Minor. 1997. Assessing the significance of reverse transcriptase activity in chick cell-derived vaccines. *Biologicals* 25:403-414.

Robinson, H. L. 1978. Inheritance and expression of chicken genes that are related to avian leukosis sarcoma virus genes. *Curr Top Microbiol Immunol* 83:1-36.

Robinson, H. L. and R. N. Eisenman. 1984. New findings on the congenital transmission of avian leukosis viruses. *Science* 225:417-419.

Robinson, H. L., S. M. Astrin, A. M. Senior, and F. H. Salazar. 1981. Host susceptibility to endogenous viruses: Defective glycoprotein-expressing proviruses interfere with infections. *J Virol* 40:745-751.

Robinson, H. L., B. M. Blais, P. N. Tsichlis, and J. M. Coffin, 1982. At least two regions of the viral genome determine the oncogenic potential of avian leukosis viruses. *Proc Natl Acad Sci USA* 79:1225–1229.

Robinson, H. L., L. Jensen, and J. M. Coffin. 1985. Sequences outside of the long terminal repeat determine the lymphomogenic potential of Rous-associated virus type 1. *J Virol* 55:752-759.

Roizman, B. 1996. Virology: Multiplication. In S. Baron (ed.). Medical Microbiology, 4th edition. The University of Texas Medical Branch: Galveston, TX, chapter 42.

Rong, L., A. Edinger, and P. Bates. 1997. Role of basic residues in the subgroup-determining region of the subgroup A avian sarcoma and leukosis virus envelope in receptor binding and infection. *J Virol* 71:3458–3465.

Roth, R. K., P. Meyers, and R. M. Dougherty. 1971. The presence of avian leukosis virus-group-specific antibodies in chicken sera. *Virology* 45:265-274.

Rous, P., and J. B. Murphy. 1913. Variations in a chicken sarcoma caused by a filterable agent. *J Exp Med* 17:219-231.

Rovigatti, U. G., and S. M. Astrin. 1983. Avian endogenous viral genes. Curr *Top Microbiol Immunol* 103:1-21.

Rubin, H. and P. K. Vogt. 1962. An avian leukosis associated with stocks of Rous sarcoma virus. *Virology* 17:184-194.

Rubin, H., A. Cornelius, and L. Fanshier. 1961. The pattern of congenital transmission of an avian leukosis virus. *Proc Natl Acad Sci USA* 47:1058-1060.

Rubin, H., L. Fanshier, A. Cornelius, and W. F. Hughes. 1962. Tolerance and immunity in chickens after congenital and contact infection with an avian leukosis virus. *Virology* 17:143-156.

Ruddell, A. 1995. Transcription regulatory elements of the avian retroviral long terminal repeat. *Virology* 206:1–7.

Ruddell, A., M. L. Linial, and M. Groudine. 1989. Tissue-specific lability and expression of avian leukosis virus long terminal repeat enhancer-binding proteins. *Mol Cell Biol* 9:5660–5668.

Ruis, B. L., S. L. Benson, K. E. Conklin. 1999. Genome structure and expression of the ev/J family of avian endogenous viruses. *J Virol* 73:5345-5355.

Ryden, T. A., M. de Mars, and K. Beemon. 1993. Mutation of the C/EBP binding sites in the Rous sarcoma virus long terminal repeat and *gag* enhancers. *J Virol* 67:2862–2870.

Sabour, M. P., J. R. Chambers, A. A. Grunder, U. Kuhnlein, and J. S. Gavora. 1992. Endogenous viral gene distribution in populations of meat-type chickens. *Poult Sci* 71:1259-1270.

Sacco, M. A., D. M. J. Flannery, K. Howes, and K. Venugopal. 2000. Avian endogenous retrovirus EAV-HP shares regions of identity with avian leukosis virus subgroup J and the avian retrotransposon ART-CH. *J Virol* 74:1296-1306.

Sacco, M. A., K. Howes, and K. Venugopal. 2001. Intact EAV-HP endogenous retrovirus in Sonnerat's jungle fowl. *J Virol* 75:2029-2032.

Salter, D. W. and L. B. Crittenden. 1991. Insertion of a disease resistance gene into the chicken germline. *Biotechnology* 16:125-131.

Salter, D. W., W. Payne, H. J. Kung, D. Robinson, D. Ewert, W. Olson, L. B. Crittenden, and A. M. Fadly. 1999. Enhancement of expontaneous bursal lymphoma frequency by serotype 2 Marek's disease vaccine, SB-1, in transgenic and non-transgenic line 0 white leghorn chickens. *Avian Pathol* 28:147-154.

Sandelin, K. and T. Estola. 1974. Occurrence of different subgroups of avian leukosis virus in Finnish poultry. *Avian Pathol* 3:159-168.

Sap, J., A. Muñoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennström. 1986. The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature* 324:635–640.

Sawyers, C. L., C. T. Denny, and O. N. Witte. 1991. Leukemia and the disruption of normal hematopoiesis. *Cell* 64:337–350.

Schat, K. 1987. Immunity in Marek's disease and other tumors. In A. Toivanen, and P. Toivanen (eds.). Avian Immunology: Basis and Practice, vol. 2. CRC Press: Boca Raton, FL, 101-128.

Schierman, L. W., D. H. Watanabe, and R. A. McBride. 1977. Genetic control of Rous sarcoma regression in chickens: Linkage with the major histocompatibility complex. *Immunogenetics* 5:325–332.

Schierup, M. H., and J. Hein. 2000a. Consequences of recombination on traditional phylogenetic analysis. *Genetics* 156:879–891.

Schierup, M. H., and J. Hein. 2000b. Recombination and the molecular clock. *Mol Biol Evol* 17:1578–1579.

Schonning, K., B. Jansson, S. Olofsson, and J. E. Hansen. 1996. Rapid selection for an N-linked oligosaccharide by monoclonal antibodies directed against the V3 loop of human immunodeficiency virus type 1. *J Gen Virol* 77:753–758.

Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. *Cell* 32:853–869.

Segura, J. C., J. S. Gavora, J. L. Spencer, R. W. Fairfull, R. S. Gowe, and R. B. Buckland. 1988. Semen traits and fertility of White Leghorn males shown to be positive or negative for lymphoid leukosis virus in semen and feather pulp. *Br Poult Sci* 29:545-553.

Sheiness, D., L. Fanshier, and J. M. Bishop. 1978. Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. *J Virol* 28:600–610.

Sheiness, D. K., S. H. Hughes, H. E. Varmus, E. Stubblefield, and J. M. Bishop. 1980. The vertebrate homolog of the putative transforming gene of avian

myelocytomatosis virus: Characteristics of the DNA locus and its RNA transcript. *Virology* 105:415–424.

Sigel, M. M., P. Meyers, and H. T. Holden. 1971. Resistance to Rous sarcoma elicited by immunization with live virus. *Proc Soc Exp Biol Med* 137:142-146.

Silva, R. F., A. M. Fadly, and H. D. Hunt. 2000. Hypervariability in the envelope genes of subgroup J avian leukosis viruses obtained from different farms in the United States. *Virology* 272:106-111.

Silva, R. F., A. M. Fadly, and S. P. Taylor. 2007. Development of a polymerase chain reaction to differentiate avian leukosis virus (ALV) subgroups: Detection of an ALV contaminant in commercial Marek's disease vaccines. *Avian Dis* 51:663-667.

Simpson, S. B., L. Zhang, R. C. Craven, and C. M. Stoltzfus. 1997. Rous sarcoma virus direct repeat *cis* elements exert effects at several points in the virus life cycle. *J Virol* 71:9150–9156.

Sjolander, S., A. Bolmstedt, L. Akerblom, P. Horal, S. Olofsson, B. Morein, and A. Sjolander. 1996. N-linked glycans in the CD4-binding domain of human immunodeficiency virus type 1 envelope glycoprotein gp160 are essential for the in vivo priming of T cells recognizing an epitope located in their vicinity. *Virology* 15:124–133.

Skalka, A. M., L. Boone, R. Junghans, and D. Luk. 1982. Genetic recombination in avian retroviruses. *J Cell Biochem* 19:293-304.

Skalka, A. M., P. N. Tsichlis, R. Malavarca, B. Cullen, and G. Ju. 1983. Viral sequences determining the oncogenicity of avian leukosis viruses. *Prog Clin Biol Res* 119:105–118.

Skinner, M. A., A. J. Langlois, C. B. McDanal, S. McDougal, D. P. Bolognesi, and T. J. Matthews. 1988. Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. *J Virol* 62:4195-4200.

Smith, E. J. 1987a. Endogenous avian leukemia viruses. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 101-120.

Smith, R. 1987b. Immunology of avian leukosis virus infections. In G. F. de Boer (ed.). Avian Leukosis. Martinus Nijhoff: Boston, MA, 121-130.

Smith, E. J. and A. M. Fadly. 1988. Influence of congenital transmission of endogenous virus-21 on the immune response to avian leukosis virus infection and the incidence of tumors in chickens. *Poult Sci* 67:1674-1679.

Smith, E. J. and A.M. Fadly. 1994. Male-mediated venereal transmission of endogenous avian leukosis virus. *Poult Sci* 73:488-494.

Smith, R. E. and L. J. Van Eldik. 1978. Characterization of the immunosuppression accompanying virus-induced avian osteopetrosis. *Infect Immun* 22:452-461.

Smith, E. J., A. M. Fadly, and W. Okazaki. 1979. An enzyme-linked immunosorbent assay for detecting avian leukosis- sarcoma viruses. *Avian Dis* 23:698-707.

Smith, E. J., U. Neumann, and W. Okazaki. 1980. Immune response to avian leukosis virus infection in chickens: Sequential expression of serum immunoglobulins and viral antibodies. *Comp Immunol Microbiol Infect Dis* 2:519-529.

Smith, L. M., D. W. Salter, R. F. Silva, and L. B. Crittenden. 1986. Selective shedding and congenital transmission of endogenous avian leukosis viruses. *J Virol* 60:1050-1054.

Smith, E. J., A. M. Fadly, I. Levin, and L. B. Crittenden. 1991. The influence of ev6 on the immune response to avian leukosis virus infection in rapid-feathering progeny of slow- and rapid-feathering dams. *Poult Sci* 70:1673-1678.

Smith, C. D., L. A. Baglia, S. M. Curristin, and A. Ruddell. 1994. The VBP and a1/EBP leucine zipper factors bind overlapping subsets of avian retroviral long terminal repeat CCAAT/ enhancer elements. *J Virol* 68:6232–6242.

Smith, E. J., S. M. Williams, and A. M. Fadly. 1998a. Detection of avian leukosis virus subgroup J using the polymerase chain reaction. *Avian Dis* 42:375-380.

Smith, L. M., S. R. Brown, K. Howes, S. McLeod, S. S. Arshad, G. S. Barron, K. Venugopal, J. C. McKay, and L. N. Payne. 1998b. Development and application of polymerase chain reaction tests for the detection of subgroup J avian leukosis virus. *Virus Res* 54:87-98.

Smith, L. M., A. A. Toye, K. Howes, N. Bumstead, L. N. Payne, and K. Venugopal. 1999. Novel endogenous retroviral sequences in the chicken genome closely related to HPRS-103 (subgroup J) avian leukosis virus. *J Gen Virol* 80:261-268.

Solomon, J. J., B. R. Burmester, and T. N. Fredrickson. 1966. Investigationa of lymphoid leukosis infection in genetically similar chicken populations. *Avian Dis* 10:477-484.

Sorge, J., W. Ricci, and S. H. Hughes. 1983. *cis*-Acting packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. *J Virol* 48:667–675.

Spencer, J. L. 1984. Progress towards eradication of lymphoid leukosis viruses: A review. *Avian Pathol* 13:599–619.

Spencer, J. L. 1997. An overview of problems and progress in control of avian leukosis. In A. M. Fadly, K. A. Schat, K.A., and J. L. Spencer (eds.). Proceedings of the Avian Tumor Viruses Symposium. Reno, Nevada, 48-53.

Spencer, J. L. and F. Gilka. 1982. Lymphoid leukosis: detection of group specific viral antigen in chicken spleens by immunofluorescence and complement fixation. *Can J Comp Med* 46:370-375.

Spencer, J. L., L. B. Crittenden, B. R. Burmester, W. Okazaki, and R. L. Witter. 1977. Lymphoid leukosis: interrelations among virus infections in hens, eggs, embryos, and chicks. *Avian Dis* 21:331-345.

Spencer, J. L., F. Gilka, and J. S. Gavora. 1983. Detection of lymphoid leukosis virus infected chickens by testing for group-specific antigen or virus in feather pulp. *Avian Pathol* 12:85-99.

Spencer. J. L., F. Gilka, J. S. Gavora, and P. F. Wright. 1984. Distribution of lymphoid leukosis virus and p 27 group-specific antigen in tissues from laying hens. *Avian Dis* 28:358-373.

Spencer, J. L., B. Benkel, M. Chan, and S. Nadin-Davis. 2003. Evidence for virus closely related to avian myeloblastosis-associated virus type 1 in a commercial stock of chickens. *Avian Pathol* 32:383–390.

Stedman, N. L. and T. P. Brown. 1999. Body weight suppression in broilers naturally infected with avian leukosis virus subgroup J. *Avian Dis* 43:604–610.

Stoye, J. P., and J. M. Coffin. 1985. Endogenous retroviruses. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.). RNA Tumor Viruses, vol. 2. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 357-404.

Stoye, J. P., C. Moroni, and J. M. Coffin. 1991. Virological events leading to spontaneous AKR thymomas. *J Virol* 65:1273–1285.

Stumph, W. E., C. P. Hodgson, M. J. Tsai, and B. W. O'Malley. 1984. Genomic structure and possible retroviral origin of the chicken CR1 repetitive DNA sequence family. *Proc Natl Acad Sci USA* 81:6667-6671.

Svarovskaia, E. S., S. R. Cheslock, W. Zhang, W. Hu, and V. K. Pathak. 2003. Retroviral mutation rates and reverse transcriptase fidelity. *Front Biosci* 8:d117–d134.

Svoboda, J., M. Dvorak, R. Guntaka, and J. Geryk. 1986. Transmission of (LTR, v-src, LTR) without recombination with a helper virus. *Virology* 153:314–317.

Swain, A., and J. M. Coffin. 1992. Mechanism of transduction by retroviruses. *Science* 255:841–845.

Swanstrom, R., R. C. Parker, H. E. Varmus, and J. M. Bishop. 1983. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. *Proc Natl Acad Sci USA* 80:2519–2523.

Taylor Jr., R. L. 2004. Major histocompatibility (*B*) complex control of responses against Rous sarcomas. *Poult Sci* 83:638-649.

Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy. 1982. Pathogenesis of retrovirus-induced disease. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.). RNA Tumor Viruses, vol. 1. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 785-998.

Teich, N., J. Wyke, and P. Kaplan. 1985. Pathogenesis of retroviral-induced disease. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (eds.). RNA Tumor Viruses, vol. 2. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 187-248.

Temin, H. M. 1985. Reverse transcription in the eukaryotic genome: Retroviruses, pararetroviruses, retrotransposons and retrotranscripts. *Mol Biol Evol* 2:455-468.

Temin, H. M. 1992. Origin and general nature of retroviruses. In J. Levy (ed.). The Retroviridae, vol 1. Plenum Press: New York, NY, 1-17.

Tereba, A., L. B. Crittenden, and S. M. Astrin. 1981. Chromosomal localization of three endogenous retroviral loci associated with virus production in white leghorn chickens. *J Virol* 39:282-289.

Thacker, E. L., J. E. Fulton, and H. D. Hunt. 1995. In vitro analysis of a primary, major histocompatibility complex (MHC)-restricted, cytotoxic T-lymphocyte response to avian leukosis virus (ALV), using target cells expressing MHC class I cDNA inserted into a recombinant ALV vector. *J Virol* 69:6439-44.

Trejbalova, K., K. Gebhard, Z. Vernerova, L. Dusek, J. Geryk, J. Hejnar, A. T. Haase, and J. Svoboda. 1999. Proviral load and expression of avian leukosis viruses of subgroup C in long-term persistently infected heterologous hosts (ducks). *Arch Virol* 144:1779-1807.

Tsang, S. X., W. M. Switzer, V. Shanmugam, J. A. Johnson, C. Goldsmith, A. Wright, A. M. Fadly, D. Thea, H. Jaffe, T. M. Folks, and W. Heneine. 1999. Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cell: Investigation of transmission to vaccine recipients. *J Virol* 73:5843-5851.

Tsichlis, P. N., K. F. Conklin, and J. M. Coffin. 1980. Mutant and recombinant avian retroviruses with extended host range. *Proc Natl Acad Sci USA* 77:536–540.

Tsichlis, P. N., L. Donehower, G. Hager, N. Zeller, R. Malavarca, S. Astrin, and A. M. Skalka. 1982. Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its nononcogenic parent: Genetic regions that control growth rate and oncogenic potential. *Mol Cell Biol* 2:1331-1338.

Tsukamoto, K., Y. Kono, K. Arai, H. Kitahara and K. Takahashi. 1985. An enzyme-linked immunosorbent assay for detection of antibodies to exogenous avian leukosis virus. *Avian Dis* 29:1118-1129.

Tsukamoto, K., M. Hasebe, S. Kakita, H. Hihara, and Y. Kono. 1991. Identification and characterization of hens transmitting avian leukosis virus (ALV) to their embryos by ELISAs for detecting infectious ALV, ALV antigens and antibodies to ALV. *J Vet Med Sci* 53:859-864.

Valesia-Wittmann, S., A. Drynda, G. Deleage, M. Aumailley, J. M. Heard, O. Danos, G. Verdier, and F. Cosset. 1994. Modifications in the binding domain of avian retrovirus envelope protein to redirect host range of retroviral vectors. *J Virol* 68:4609-4619.

Vandergon, T. L. and M. Reitman. 1994. Evolution of chicken repeat 1 (CR1) elements: Evidence for ancient subfamilies and multiple progenitors. *Mol Biol Evol* 11:886-898.

Van Woensel, P. A., A. Van Blaaderen, R. J. Moorman, and G. F. de Boer. 1992. Detection of proviral DNA and viral RNA in various tissues early after avian leukosis virus infection. *Leukemia* 6:35S–137S.

Venugopal, K. 1999. Avian leukosis virus subgroup J: A rapidly evolving group of oncogenic retroviruses. *Res Vet Sci* 67:113-119.

Venugopal, K., K. Howes, G. S. Barron, and l. N. Payne. 1997. Recombinant env-gp85 of HPRS-103 (subgroup J) avian leukosis virus: Antigenic characteristics and usefulness as a diagnostic reagent. *Avian Dis* 41:283-288.

Venugopal, K., L. M. Smith, K. Howes, and l. N. Payne. 1998. Antigenic variants of J subgroup avian leukosis virus: Sequence analysis reveals multiple changes in the *env* gene. *J Gen Virol* 79:757-766.

Venugopal, K., K. Howes, D. M. J. Flannery, and L. N. Payne. 2000. Isolation of acutely transforming subgroup J avian leukosis viruses that induce erythroblastosis and myelocytomatosis. *Avian Pathol* 29:327-332.

Vogt, P. K. 1965. Avian tumor viruses. *Adv Virus Res* 11:293-385.

Vogt, P. K. 1971. Genetically stable reassortment of markers during mixed infection with avian tumor viruses. *Virology* 46:947-952.

Vogt, V. M. 1996. Proteolytic processing and particle maturation. *Curr Top Microbiol Immunol* 214:95-131.

Vogt, P. K., and R. R. Friis. 1971. An avian leukosis virus related to RSV (0): Properties and evidence for helper activity. *Virology* 43:223-234.

Vogt, P. K. and R. Ishizaki. 1965. Reciprocal patterns of genetic resistance to avian tumor viruses in two lines of chickens. *Virology* 26:664-672.

Voynow, S.L., and J. M. Coffin. 1985. Evolutionary variants of Rous sarcoma viruses: Large deletion mutants do not result from homologous recombination. *J Virol* 55:67-78.

Wainberg, M. A., B. Beiss, R. Wahi, and E. Israel. 1979. Thymic dependence of cell-mediated immunity to avian sarcomas in chickens. Immunological characterization of a nonvirion antigen in virus-infected cells. *Cell Immunol* 45:344-355.

Wainberg, M. A., B. Beiss, H. Fong, S. Beaupre, and J. Menezes. 1983. Involvement of macrophage-like cells in growth of tumors induced by Avian Sarcoma Viruses. *Cancer Res* 43:1550.

Wainberg, M., S. Beaupre, B. Beiss, and E. Israel. 1987. Differential susceptibility of avian sarcoma cells derived from different periods of tumor growth to natural killer cell activity. *Cancer Res* 43:4774-4780.

Wain-Hobson, S. 1996. Running the gamut of retroviral mutation. *Trends Microbiol* 4:135-141.

Wakenell, P. S. 1997. An overview of problems in diagnosis of neoplastic diseases of poultry. In A. M. Fadly, K. A. Schat, and J. L. Spencer (eds.). Proceedings of the Avian Tumor Viruses Symposium. Reno, Nevada, 1-5.

Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans. 1986. The c-erb-A gene encodes a thyroid hormone receptor. *Nature* 324:641–646.

Weiss, R. A. 2006. The discovery of endogenous retroviruses. *Retrovirology* 3:67.

Weiss, R. A., W. S. Mason, and P. K. Vogt. 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. *Virology* 52:535-552.

Weiss, R., N. Teich, H. Varmus, and J. Coffin. 1985. RNA Tumor Viruses, vol. 2. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Weiss, R. A., P. R. Clapham, J. N. Weber, A. G. Dalgleish, L. A. Lasky, and P. W. Berman. 1986. Variable and conserved neutralization antigens of human immunodeficiency virus. *Nature* 324:572-575.

Weissmahr, R. N., J. Schupbach, and J. Boni. 1997. Reverse transcriptase activity in chicken embryo fibroblast culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. *J Virol* 71:3005-3012.

Weyl, K. G. and R. M. Dougherty. 1977. Contact transmission of avian leukosis virus. *J Natl Cancer Inst* 58:1019-1025.

White, J. M. 1992. Membrane fusion. *Science* 258:917–924.

Willey, R. L., R. Shibata, E. O. Freed, M. W. Cho, and M. A. Martin. 1996. Differential glycosylation, virion incorporation, and sensitivity to neutralizing antibodies of human immunodeficiency virus type 1 envelope produced from infected primary T-lymphocyte and macrophage cultures. *J Virol* 70:6431–6436.

Williams, S. M., S. D. Fitzgerald, W. M. Reed, L. F. Lee, and A. M. Fadly. 2004. Tissue tropism and bursal transformation ability of subgroup J avian leukosis virus in white Leghorn chicken. *Avian Dis* 48:921-927.

Witter, R. W. and A. M. Fadly. 2001. Reduction of horizontal transmission of avian leukosis virus subgroup J in broiler breeder chickens hatched and reared in small groups. *Avian Pathol* 30:641-654.

Witter, R. L., B. W. Calnek, and P. P. Levine. 1966. Influence of naturally occurring parental antibody on the epizootiology of visceral lymphomatosis virus infection in chickens. *Avian Dis* 10:43-56.

Witter, R. L., L. D. Bacon, H. D. Hunt, R. E. Silva, and A. M. Fadly. 2000. Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. *Avian Dis* 44:913-931.

Wu, Z., S. C. Kayman, W. Honnen, K. Revesz, H. Chen, S. Vijh-Warrier, S. A. Tilley, J. McKeating, C. Shotton, and A. Pinter. 1995. Characterization of neutralization epitopes in the V2 region of human immunodeficiency virus type 1 gp120: Role of glycosylation in the correct folding of the V1/V2 domain. *J Virol* 69:2271–2278.

Wyke, J. A. and J. A. Beamand. 1979. Genetic recombination in Rous sarcoma virus: The genesis of recombinants and lack of evidence for linkage between pol, env and src genes in three factor crosses. *J Gen Virol* 43:349-364.

Xu, B., W. Dong, C. Yu, Z. He, Y. Lv, Y. Sun, X. Feng, N. Li, and L. Lee. 2004. Occurrence of avian leukosis virus subgroup J in commercial layer flocks in China. *Avian Pathol* 33:13-17.

Young, J. A., P. Bates, and H. E. Varmus. 1993. Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. *J Virol* 67:1811-1816.

Zachow, K. R. and K. F. Conklin. 1992. CArG, CCAAT, and CCAAT like protein binding sites in avian retrovirus long terminal repeat enhancers. *J Virol* 66:1959–1970.

Zander, D. V., R. G. Raymond, C. F. McClary, and K. Goodwin. 1975. Eradication of subgroups A and B lymphoid leukosis virus from commercial poultry breeding flocks. *Avian Dis* 19:403-423.

Zavala, G., M. W. Jackwood, and D. A. Hilt. 2002. Polymerase chain reaction for detection of avian leukosis virus subgroup J in feather pulp. *Avian Dis* 46:971-978.

Zavala, G., B. Lucio-Martinez, S. Cheng, and T. Barbosa. 2006. Sarcomas and myelocytomas induced by a retrovirus related to myeloblastosis-associated virus type 1 in White Leghorn egg layer chickens. *Avian Dis* 50:201–208.

Zavala, G., S. Cheng, and M. W. Jackwood. 2007. Molecular epidemiology of avian leukosis virus subgroup J and evolutionary history of its untranslated region. *Avian Dis* 51:942-953.

Zhang, J., and Y. Ma. 2001. Evidence for retroviral intramolecular recombinations. *J Virol* 75:6348-6358.

Zhang, J., and H. M. Temin. 1993. Rate and mechanisms of nonhomologous recombination during a single cycle of retroviral replication. *Science* 259:234-238.

Zhang, J., and H. M. Temin. 1994. Retrovirus recombination depends on the length of sequence identity and is not error prone. *J Virol* 68:2409–2414.

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