

THESIS



#### This is to certify that the

dissertation entitled Involvement of cellular on cogenea in Avian leukeria vina indused Neoplastic diseases : Lymphaid leukeria and Engtherkenhemia presented by

YUEN-KAI T. Fung

has been accepted towards fulfillment of the requirements for

ph. D degree in Biochemistry

Br. Ku

Date 1/24/8>

MSU is an Affirmative Action/Equal Opportunity Institution

0-12771





#### RETURNING MATERIALS:

Place in book drop to remove this checkout from your record. <u>FINES</u> will be charged if book is returned after the date stamped below.

MAB 1 5 2082	

# INVOLVEMENT OF CELLULAR ONCOGENES IN AVIAN LEUKOSIS VIRUS INDUCED NEOPLASTIC DISEASES: LYMPHOID LEUKOSIS AND ERYTHROLEUKEMIA

By

Yuen-Kai T. Fung

### A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

### ABSTRACT

### INVOLVEMENT OF CELLULAR ONCOGENES IN AVIAN LEUKOSIS VIRUS INDUCED NEOPLASTIC DISEASES: LYMPHOID LEUKOSIS AND ERYTHROLEUKIMIA

By

Yuen-Kai T. Fung

Avian leukosis viruses (ALVs) are a group of chronic RNA tumor viruses that do not encode an oncogene in their viral genome, yet can induce a variety of neoplasia in chickens after a long latent period. It has been proposed that ALV induces neoplasia in chicken by integrating upstream from cellular oncogenic sequences, thereby enhancing their expression with the promotor sequence in the 'C' region. In this thesis it is shown that in ALV induced lymphoid leukosis, the ALV provirus has integrated next to the cellular sequence, c-myc, homologous to the oncogene of MC 29 (avian myelocytomatosis virus). Similarly, in ALV induced erythroleukemia, many of the clonal tumor erythroblasts were found to have tumor specific fragments comprised of ALV and c-erb, the cellular counterpart of the oncogene of avian erythroblastosis virus (AEV). It has been proposed that the erb sequence in AEV is a hybrid of two gene loci, A and B, transduced from the cell genome. Previous studies using a temperature sensitive mutant and a non-conditional mutant of AEV have implied a role of the A gene in the transformation of erythroblasts. In the present study, most tumor specific fragments were found to be a hybrid of ALV and the c-erb sequence corresponding to the B

. 

In one case, both A and B were shown to be in the same tumor specific fragment associated with some ALV sequences. The erb gene expression was found to be elevated in some tumors to a level compatible with leukemic tissue from AEV infected birds. On the other hand, other tumors do not show enhancement of erb gene expression although they are pathologically similar. The implication of these findings on the transformation mechanism is discussed.

### ACKNOWLEDGEMENTS

I wish to thank Mr. Leonard Provencher, Miss Deborah Eagen, Dr. Aly Fadly and Dr. Lyman Crittenden of the U.S. Department of Agriculture Science & Education Administration, Agriculture Research, Regional Poultry Research laboratory, East Lansing, Michigan, for their valuable help and education throughout the course of my study. I also wish to thank Dr. C. Sweeley for his understanding and tolerance in setting me on the path of scientific research during my early years.

The helpful and stimulating discussion and valuable advice of Professor Jerry Dodgson, Edward Fritsch and John Wang are deeply appreciated.

I wish to also thank Dr. Sweeley for the stipend support during the first three years and last three months of my stay here, and Dr. Kung for the stipend support in between.

Dr. Kung has truly been an inspiration to me. He has forged the right laboratory environment in which a student can be as creative as he/she desires with frequent rewarding results. During my stay in his laboratory, I have enjoyed unlimited freedom in setting research goals, research planning and execution. I also thank him for putting at my

ii

disposal all laboratory equipment and chemicals.

It has been quite enjoyable working with my lab colleagues, especially Mr. Robert Swift for his late night discussions and companionship in the laboratory.

## TABLE OF CONTENTS

----

		Page					
LIST OF FIGURES	•	v					
LITERATURE REVIEW	•	1					
Introduction	•	1					
I. The Architecture of the Virion	•	2					
Viral RNA	•	2 6					
II. Replication of Retroviruses	•	7					
III. Pathology of Retroviruses	•	8					
Acute Viruses	•	8					
Chronic Viruses	•	25					
REFERENCES	•	30					
ARTICLE - On the Mechanism of Retrovirus-Induced Avian Erythr leukemia: Alteration of Cellular Erb Gene Structur and Expression. Yuen-Kai T. Fung and Hsing-Jien Ku (1981) (to be submitted to <u>Cell</u> ).	o- e ing,						
APPENDIX - On the Mechanism of Retrovirus-Induced Avian Lymphoid							

APPENDIX - On the Mechanism of Retrovirus-Induced Avian Lymphoid Leukosis: Deletion and Integration of the Proviruses. (1981) Yuen-Kai T. Fung, Aly M. Fadly, Lyman B. Crittenden and Hsing-Jien Kung, Proc. Natl. Acad. Sci. USA <u>78</u>, 3418-3422.

# LIST OF FIGURES

Figures	;	Page
LITERAT	URE REVIEW	
1	Architecture of the retrovirus virion	3
2	Schematic representation of the replication of retroviruses, using the prototype ASV for illustration	4-5
3	Schematic of retroviruses	10-1
4	Expression of the genome of AEV, adapted from published data (69,70)	17
ARTICLE	1	
1	Kinetics of development of chicken erythroleukemia	25
2	Blood smear slides of AEV and RAV-1 infected birds	27
3	Analysis of DNA from tumor and normal tissues for the presence or absence of AEV proviruses	29
4	Analysis of EcoRI and BamHI digestion patterns of genomic DNA samples from infected and uninfected chickens	31
5	Analysis of genomic DNA with DNA probes specific for ALV and erb	35
6	Kinetic of development of disease in chicken 4	38
7	Dot blot analysis of erb gene expression in avian erythroleukemia	40
8	Restriction maps of tumor specific fragments from chicken 4	42

### LITERATURE REVIEW

### Introduction

The retroviruses are a group of RNA-containing animal viruses that have been found in virtually all species of vertebrates. Many different retroviruses exist, and, as our knowledge about them has increased over the years since their early identification as disease causing agents, an extensive system of taxonomy has been developed for their classification. Retroviruses differ from each other in the size and number of virion proteins, morphology, host range, genomes, and pathogenicity.

Among all retroviruses, the avian RNA tumor viruses are the best characterized with respect to their biochemical and genetic make-up as well as possible mechanisms of replication and transformation. The following review is thus limited mostly to the avian tumor viruses. This includes a brief review of the architecture of these viruses, their mode of replication, and, in greater detail, their pathogenicity. Readers interested in biochemical archeology are referred to the excellent review by Gross (1).

### I. The architecture of the virion

Figure 1 shows the architecture of a typical virion. The outer envelope of the virion is a lipid bilayer derived from the plasma membrane of the host cell during the virion budding process. Protruding from the exterior of the viral envelope are glycoprotein(s) encoded in the viral genome (env). These glycoproteins are involved in the recognition of host receptors, essential to the virus's ability to penetrate cells. Moreover, these glycoproteins may elicit an immune response in the host animal.

Enclosed inside the envelope is a protein core, the center of which is the ribonucleoprotein consisting of the diploid RNA genome, RNA binding protein and reverse transcriptase (pol).

### Viral RNA

Two single stranded RNA molecules of ca 5-10kb, each of which contains the entire genetic information of the virus, are held together near their 5' ends by a base-paired structure. A haploid subunit of the retrovirus genome is illustrated in figure 2. The RNA genome exhibits many features of eukaryotic mRNA. Thus the genome is bound at the 5' end by a cap structure  $5'-m^7$ GpppGm and has a poly adenylic acid (Poly A) tail at the 3' end with a low level of internal methylation. In ASV, a molecule of tRNA<sup>Trp</sup> serves as the primer for the initiation of DNA synthesis by binding at a site ((-)PB) 101 nucleotides from the 5' end of the genome.



Figure 1. Architecture of the retrovirus virion





Schematic representation of the replication of retroviruses, using the prototype ASV for illustration. Figure 2.

R is defined as a 16- to 21- nucleotide terminal repeat (2-4). (-)PB, minus strand initiation site, is the tRNAT<sup>P</sup> binding site for the primer of the first (-) strand of viral DNA (5,6).

 $(\star)Bs,$  plus strand initiation site. Us and ull are duplicated to generate the US and U3 are the 5' and 3' sequences in the NNA genome that repeat in the DNA.





### Viral Genes

Three viral genes are essential for the replication. Gag codes for the structural protein of the viral core. Pol codes for RNA-dependent DNA polymerase which copies the RNA genome into DNA. Env codes for the viral envelope glycoproteins. A fourth viral gene, src or onc, is not essential for the survival of the virus and is found only in viruses with the ability to rapidly transform cells to the oncogenic state.

In addition to these coding sequences, other portions of the genome serve important functions in the life cycle of the virus. The terminal repeats provide "sticky ends" for circularization of the viral RNA genome whereby reverse transcription can proceed through the 5' terminus and reinitiate at the 3' end of the RNA (7).

The 5' and 3' sequences that are duplicated during replication are termed U5 and U3, respectively. The U5 and U3 together (and R) form the large terminal repeat (LTR) of the viral DNA. The LTR sequence may serve regulatory functions in the synthesis and processing of viral RNA (8). Recent DNA sequencing data on the LTR and related neighboring sequences (9-11) has yielded the following information about the U3 region of the genome.

- Judging by the presence of stop codons in all three reading frames, this region probably does not code for a protein.
- (2) Two possible promotors for transcription by RNA polymerase II exist, one resembles the promotors for -globin and SV40; the other is a stretch rich in A+T.
- (3) There is a poly A addition signal in this part of the genome.
- (4) A structural feature of the U3 5' end, may account for the high frequency of deletion of the non-essential oncogene.



- (5) The presence of a direct repeat in the U3 and U5 region may help circularization of the linear viral <u>DNA</u> into covalently closed circles.
- II. Replication of Retroviruses

Figure 2 shows a schematic representation of the replication of retroviruses (12), using the ASV as a prototype. The replication of the retrovirus begins with the infection of the virus into the host cell. The viral RNA is transcribed into DNA by the reverse transcriptase associated with the ASV genome. Synthesis starts from a site 101 nucleotides from the 5' end with an existing cellular tRNA as a primer. The short direct repeat (solid square labelled "R") at both ends of the genome is used to facilitate the necessary transfer of reverse transcriptase from the 5' end of the genome to the 3' end.

Using this first (minus) strand of viral DNA as a template, the second strand (plus) of viral DNA is synthesized (13). The resulting product is a linear duplex DNA molecule containing long terminal repeats at both ends (figure 3). DNA sequencing data indicates that the LTR's conclude with short and often imperfect inverted repeats. Notice that the viral DNA bears strong structural resemblance to transposable elements, for example Tn9. In both elements, large direct repeats embrace gene coding domains. The direct repeats themselves, in turn, end with short inverted repeats.

The linear DNA can then migrate to the nucleus where some of it is converted to a closed circular species (14) possibly by several different mechanisms (15-17), generating circles with one or both copies of the LTR.



The viral DNA integrates, apparently randomly, into the host genome. The proviral (integrated) DNA is colinear with the linear viral DNA (18,19). However, a few (generally two) nucleotide pairs present at the ends of the linear viral DNA appear to be missing in the proviral DNA. Reminiscent of the integration site of transposable elements, direct repeats of a few cellular base pairs, which are present only once in unoccupied integration sites, are found at the ends of the integrated proviral DNA.

This proviral DNA can then serve as a template for transcription. Genomic RNA is produced for packaging into new virions. Subgenomic RNAs are produced with the 5' leader sequence spliced onto different parts of the RNA genome. Proteins produced are then available for packaging. Readers interested in the processing of viral proteins are referred to the recent review by J.M. Bishop (20).

### III. Pathology of Retroviruses

RNA tumor viruses can be classified into the acute and the chronic viruses according to their pathology.

<u>Acute Viruses</u>. The acute viruses can induce neoplastic diseases in vivo rapidly and efficiently. Moreover they can usually induce cell transformation in tissue culture. This ability of acute viruses to transform efficiently is due to the presence of specific oncogenes in the viral genome. The genome of Rous sarcoma virus for example, includes the usual viral genes (gag, pol, env) essential for the replication of the virus. In addition, as was shown in figure 2, the virus carries a 1.5 kilobase "sarcoma gene", src, responsible for transformation. It has been shown that subgenomic fragments of RSV containing src are capable of



transforming NIH 3T3 cells in culture (21), indicating that src by itself is capable of inducing transformation. Since RSV carries the entire set of viral genes needed for replication, it is replication-competent (see figure 3b). However, many acute viruses are replication-defective.

In these replication-defective viruses, an essential portion of the RNA genome is replaced by the oncogene. Figure 3A shows a schematic representation of the genome of a typical acute defective virus. The extent and precise site of deletions of viral genes in the genome of defective acute viruses vary among different viruses. Many different viruses carrying specific oncogenes have been identified. For example, the sarcoma gene found in Fujinami sarcoma virus (FSV) (22,23) bears no sequence homology with the src gene of ASV or any other known avian oncogenes (22). The viral genome of FSV is a 4-5 kb RNA (the smallest known RNA tumor viral genome) containing a 5' gag gene-related sequence of 1 kb, an internal specific 3 kb oncogene, and a 3'-terminal sequence of about 0.5 kb related to the C region of avian tumor viruses. Like other replication-defective acute viruses, FSV requires a helper virus to replicate, in this case FAV (Fujinami associated virus). Another recently isolated strain of avian sarcoma virus Y73 (24) has a genomic architecture similar to that of FSV. Y73 encodes a 90 k protein which, like pp60 src has kinase activity for tyrosine residue. FSV also encodes a 140k protein with tyrosine protein kinase associated activity (29,30). Again, Y73 is replication-defective and requires a helper virus for its propagation. In contrast ASVs can replicate on their own. Nevertheless, both FSV and Y73 induce sarcomas in chickens and transform chicken fibroblasts in culture as efficiently as ASV. This overlapping of the oncogenic spectrum of different sarcomagenic RNA subgroups is not unique



Figure 3. Schematic of retroviruses.

- Replication-defective acute virus e.g. AEV (avian erythroblastosis virus). Replication-competent acute virus e.g. AEV (avian arrowan virus). Replication-competant chronic virus e.g. ALV (avian leukosis virus). Replication-competent nononogenic virus e.g. ARV-0 (endogenous virus).
- DC.B.A.



-,

Figure 3



to the avian system. Parallel examples can be found in the replicationdefective retroviruses in mammals such as the Harvey-Kirsten sarcoma viruses (25,26). It now appears that the Harvey and Kirsten strains of sarcoma virus encode enzymatically and serologically related src proteins. The sarcoma genes in each virus, however, show only a small region of homology (32).

Another group of replication-defective viruses that can induce neoplasia rapidly in vivo and transform appropriate target cells in vitro are the acute leukemia viruses. These include the Friend (27) and Abelson (28) viruses of mice and three groups of leukemia virus (AEV, MC 29, and AMV) in chicken. An excellent review on the molecular biology of Friend virus has appeared recently (31). The Friend virus complex consists of two components, a replication-defective rapidly-transforming virus (the spleen focus-forming virus, SFFV) and a replication-competent type-C helper virus which helps the transmission of SFFV (37). SFFV can rapidly transform erythroid precursor cells in spleen of adult mice. The helper virus on the other hand can cause a lymphoid leukemia after a latent interval of up to 6 months (33-36). However, formal proof that SFFV can transform erythroid target cells in the absence of co-infection with a replication competent murine leukemia virus (MuLV) does not exist. Circumstantial evidence has been provided by the studies of Hankins et al (38) who show that in vitro infection of bone marrow cells with Friend virus complex resulted in a marked increase in the number of erythroid burst-forming units five days after initiation of the culture, whereas these were not observed with F-MuLV infection alone. Moreover, SFFV rescued from nonproducer cells with thymic leukemia-inducing Moloney



MuLV causes erythroleukemia and not thymic leukemia following inoculation into adult mice. Thus SFFV may indeed be capable of inducing erythroleukemia by itself. While F-MuLV most often induces lymphoid leukemia, studies done in Scolnick's laboratory have shown that several isolates of F-MuLV have the capacity to induce a rapid spleenic leukemia in newborn mice after a latent interval of only 4 to 6 weeks (39). In contrast to SFFV these clonal isolates of F-MuLV are not rapidly leukemogenic for adult mice. McCarry et al. (40) have isolated an F-MuLV strain that induces myeloid leukemia. It seems likely that the genome of F-MuLV may have been modified by passage in rats or mice, generating the different strains of viruses observed. One such example is the F-MCF virus (Friend-Murine Mink cell focus-inducing virus) an env gene recombinant of ecotropic F-MuLV and endogenous xenotropic virus isolated from the leukemic spleens of Swiss mice inoculated with cloned F-MuLV (41). The genome of F-MuLV is typical of other type C viruses (figure 3c). Three-fourths of the genome of SFFV are identical to that of F-MuLV, the remaining one-fourth is derived from the env gene of murine xenotropic viruses. Interestingly, this SFFV specific env gene sequence shares extensive homology with the env gene of the MCF mentioned above. In fact with the exception that F-MCF is replication competent while SFFV is replication defective, F-MCF and SFFV are quite similar. This has led to the proposal that both F-MCF and SFFV are recombinants of F-MuLV and the env gene of murine xenotropic virus and that further deletion of F-MCF gives rise to SFFV. However it should be noted that MCF viruses derived in other murine leukemia virus systems are known to cause lymphoid and not erythroid leukemia (42). The transformation of erythroid cells by SFFV may therefore reside in a portion of the SFFV genome other than or



in addition to the env gene common to both SFFV and MCF. As yet there is no formal genetic proof that SFFV encodes for a gene product responsible for leukemic transformation of erythroid cells.

The Abelson murine leukemia virus, A-MuLV, on the other hand has been shown to encode for a specific oncogene responsible for transformation of the target cells (43,47). A-MuLV is a replication-defective virus derived during passage of M-MuLV in mice (28). It can rapidly induce leukemia in vivo as well as transform bone marrow cells in vitro (28,44,45). A number of A-MuLV strains have been isolated (46); each encodes a protein corresponding to a fusion between the gag gene product of MuLV and a polypeptide encoded by a 3.6 kb A-MuLV specific gene sequence derived from normal mouse genome (48-50). Moreover, this oncogenic sequence was found to be present in rat, hamster, human and chicken cells (48). The A-MuLV specifically transforms B-type lymphoblasts and fibroblasts of mice.

It should be emphasized that the genome of A-MuLV resembles more closely the prototype structure shown in figure 3 than does SFFV. SFFV might simply be a recombinant of ecotropic and xenotropic MuLV sequences without actually carrying a cellular oncogene like other acute RNA tumor viruses do.

In the avian system, more than ten strains of defective leukemia viruses have been discovered. Recent studies have provided the biochemical (51,52) and genetic (53,54) basis for classification of these viruses into three groups. They are: AEV (avian erythroblastosis virus)-type consisting of strains R and ES4 (55,56), which are probably identical; MC29 (avian myelocytomatosis virus)-type consisting of MC 29, CM11, OK10


and MH2 strains; and AMV (avian myeloblastosis virus)-type strains consisting of the BA1/A strain of AMV and of E 26 (57).

AEV: The AEV-type strains can induce an acute erythroleukemia and anemia one to two weeks after inoculation into chickens. If the inoculation is intramuscular, most of the strains can also induce sarcomas at the site of injection. The target cells transformed by AEV have been found to display distinct phenotypes of differentiation (51,52). Cells transformed by AEV bear striking similarities to precursor cells of erythrocytes as revealed by their expression of high levels of histone H5 (found only in erythroid cells of nonmammalian species) and erythroblast cell surface antigen. Moreover, markers for mature erythrocytes, for example heme, globin, carbonic anhydrase and erythrocyte cell surface antigen, are expressed at low levels. The expression of these erythroblastic molecular markers in transformed bone marrow cell cultures is found to be identical to those of the transformed cells in vivo. Moreover, cells transformed by the ts 34 mutant (58) express the same erythroblast molecular markers at the permissive temperature but show an increase in the expression of hemoglobin, carbonic anhydrase and erythrocyte cell surface antigen when shifted to the nonpermissive temperature. More recently the characteristics of the specific target cells transformable by AEV have been determined by a combination of physical and immunological methods (60). Specific antisera have been developed which can distinguish between the several erythrocyte precursors at different stages of differentiation (61), viz. CFU-M (colony forming units in marrow), BFU-EC (burst forming units - erythrocytic), CFU-E (colony forming units - erythrocytic), erythroblasts and erythrocytes, in that order (62,63). It was found that the BFU-E are target



cells for infection by AEV. Since transformation by AEV either <u>in vivo</u> or <u>in vitro</u> gives rise to erythroblast-like cells it is possible that the initially transformed BFU-E can undergo some maturation to the CFU-E or erythroblast stage. Further work is needed to demonstrate this hypothesis in vivo.

In addition to transformation of hematopoietic cells, AEV can transform cloned chicken embryo fibroblasts (in this case, cell cloning was essential to eliminate hematopoietic target cells that were present in chicken embryo cell cultures). AEV-transformed fibroblasts are similar to RSV transformed fibroblasts in many ways (59). Morphologically, both transformed cells show the disappearance of actin cable, a decrease in Large External Transformation Specific (LETS) protein and an increase in the microvilli at their surface. Biochemically, they are agglutinable by lectin and show an increase in the rate of hexose uptake. These cells are capable of anchorage independent growth and inducing sarcomas in vivo.

The structure of the AEV genome has recently been elucidated by molecular cloning (64). The AEV genome is about 5.1 kb long (carrying one LTR). At least 50% of the sequence, flanked by about 1 kb of gag sequence and 0.4 kb of env sequence, is the AEV specific oncogene erb. Transfection of the cloned DNA ligated to the DNA of RAV-1 (a helper virus) leads to the production of AEV virus capable of transforming both fibroblasts and bone marrow cells.

Erb, the AEV specific gene sequence, is defined by the absence of homology with the genomes of other avian retroviruses like src, myc (MC 29) or myb (AMV). Several lines of evidence indicate that the erb gene in AEV may be composed of two functional domains: (see Fig. 4)





Figure 4. Expression of the genome of AEV, adapted from published data (69,70).



- (1) Two AEV-specific proteins, one of 75,000 (p75) the other of about 40,000 (p40) molecular weight, have been identified by <u>in vitro</u> translation of AEV viral RNAs (65,66,68). The p75 is a fusion protein containing gag-specific and AEV specific (erb) domains. This p75 appears to be identical to the p75 immunoprecipitated with anti-gag serum from nonproducer cells transformed by AEV (67). The second protein, p40, is encoded entirely by the 3' half of the AEV specific sequence, erb. Tryptic peptide mapping indicates that p75 and p40 do not share sequence homology (with the possible exception of one of five identifiable p40 peptides).
- It may be argued that the 20S RNA that gives rise to p40 is a (2) degradation product of the 28S AEV viral RNA that codes for p75. However, recent studies have shown that AEV transformed fibroblast as well as erythroblasts contain two AEV-specific mRNA's (69) of 5.3 and 3.5 kb (28-308) and 22-248 (70). Hybridizations of these mRNA's with DNA probes from different regions of the AEV genome have revealed that the 5.3 kb represents the entire AEV genome. The 3.5 kb mRNA hybridizes to most parts of the genome except for the gag sequence and the 5' region of erb. This suggests that the 3.5 kb RNA is derived from the 5.3 kb RNA via a splicing mechanism. As mentioned above, the erb region of the AEV genome is about 3 kb; this corresponds to a potential coding capacity of 100,000. The p75 translated from the 5.3 kb mRNA contains the p19 region of gag and this would leave about 55,000 daltons of this protein coded for by the erb region. Based on this consideration, it was



suggested that only the 5' half of the erb was translated into p75 while the 3' half of erb was translated into p40. Together p75 and p40 account for the full coding capacity of the erb sequence.

The erb gene has its cellular homologue in normal uninfected cells. Normal fibroblasts appear to contain 1 to 2 copies per cell of erb sequences in the genome(53). Liquid hybridization studies have suggested that both domains of the erb are expressed (71). Using northern hybridization four distinct polyadenylated RNAs that anneal to cDNA erb were discovered. The larger two RNAs anneal only with probes from one half of erb while the two smaller RNAs anneal to the other half of erb (69,72). Moreover, molecular cloning of the cellular erb sequences indicated that the two domains of erb are separated from and bear no sequence homology to each other (73). These observations thus support the hypothesis that the erb sequence in AEV comes from a recombination of two cellular genes.

(3) A third line of evidence that the AEV erb sequence contains two functional domains comes from the AEV mutants isolated by T. Graf's group (58,74-76). One of these is a nonconditional mutant designated td 359 AEV which has lost its ability to transform erythroblasts <u>in vitro</u> and <u>in vivo</u> but is still capable of transforming fibroblasts <u>in vitro</u> and of inducing sarcomas <u>in vivo</u> (77). td 359 AEV has recently been shown to synthesize a gag-gene related protein ( p75) which has a 1000 dalton deletion from p75. The mutant lacks 3 out of the 53 lysine-arginine tryptic peptides resolved in p75 but contains an extra peptide. The deletion has been located in the erb



region of the molecule. Moreover, no change in the size of p40 was observed. This data thus suggested a role of p75 in the transformation of erythroblasts but not fibroblasts.

The selective disruption of transformation ability again argues for the presence of two functional domains in the erb sequence of AEV. However, it is premature to assign a role of erythroblast transformation to the p75 and fibroblast transformation to p40. As yet no enzyme activity associated with these proteins have been detected.

Another mutant is a temperature sensitive mutant of AEV designated ts 34 AEV (58,75) isolated from bone marrow cultures infected with mutagenized AEV. ts 34 shows a decrease in transformation of bone marrow cells in vitro and reduced leukaemogenicity in vivo as compared to wild type AEV. The in vivo effect is manifested by the decreased incidence of leukemic death (46% as compared to 100% in control animals injected with 100 times fewer colony-forming units of wild-type AEV). Moreover, there is a delay of about 1 week in the onset of the disease and in the death of the chickens. Spontaneous regression of erythroblastosis was observed in some of the chicks. The blood cells isolated exhibit temperature sensitivity for haemoglobin expression just like the bone marrow cells infected with ts 34 in vitro. This result is taken as evidence to support the hypothesis that AEV exerts its leukemogenic potential by blocking a step(s) in the differentiation of its target cell. Interestingly ts 34 is only temperature sensitive for some of the transformation parameters of fibroblasts such as the production of plasminogen activator protease, rate of hexose uptake and in vivo sarcomagenicity. However, temperature has no effect on focus formation and growth of colonies in vitro. In addition, the morphology of the transformed cells as well



as the disarrangement of actin cables and loss of LETS protein are not affected by a shift of temperature. This result suggests that the same gene required for the transformation of erythroblast may also be required for the transformation of fibroblasts. This is somewhat in contrast to the mutation expressed by td 359. It would be necessary to compare the genetic structure of these two mutants before one can conclusively determine the relationship of the two erb domains to the multiple transformation ability of AEV.

MC 29: The MC 29 group of defective tumor viruses induces a large variety of neoplasias in chickens. MC 29 induces myelocytomatosis or related myeloid leukemia, carcinoma, mesotheliomas and endotheliomas (57). <u>In vitro</u>, it is capable of inducing the proliferation of macrophage-like transformed bone marrow cells. In addition, it can induce morphological transformation of fibroblasts and epithelial cells such as chicken embryo kidney cells (77) or liver cells in culture. This ability of MC 29 to transform cells of the epitheloid linkage in culture probably reflects its ability to induce carcinomas such as hepatocarcinoma and adenocarcinoma in vivo.

The target cells for MC 29 transformation have been found to display distinct phenotypes characteristic of early stages of differentiation within the myeloid lineage. Bone marrow cells transformed by MC 29 <u>in</u> <u>vitro</u> or <u>in vivo</u> are ameboid in shape and are only slightly adherent. Pilot experiments done in Graf's laboratory have failed to show any parameters of lymphoid differentiation (as detected by fluorescent staining with anti-B or anti-T serum) in MC 29 transformed bone marrow cells. However, recent studies (78) have implicated the c-myc, the cellular equivalent of the MC 29 oncogene v-myc in lymphoid leukosis. I



shall return to this point later in the chronic leukemia viruses section. On the other hand. MC 29 transformed bone marrow cells definitely include cells of myeloid lineage (51). These cells are positive for Fc receptors, are phagocytic and exhibit macrophage antigen on their surface. However, they have a quite low ATPase activity and are negative for myeloblast antigens. All these differentiation characteristics are typical of normal cultured macrophages. However, while this target cell of MC 29 is guite mature in the differentiation pathway it is still slightly less mature than the fully differentiated macrophage. This is shown by the fact that MC 29-transformed cells are less adherent and divide more rapidly, and that they can spontaneously differentiate into macrophages. On the other hand Gazzels et al. (52) have reported that tertiary macrophage cultures obtained from the peripheral blood of adult (3-4 month old) chickens are still sensitive to the transforming effect of MC 29. The genome of MC 29 has recently been elucidated by molecular cloning (79). The MC 29 specific gene sequence (myc oncogen) is about 1.5 kb and is flanked by gag and env sequences. The entire genome is about 5.5 kb. The cloned DNA apparently represents an authentic copy of the MC 29 genome as transfection of the DNA leads to the production of transforming MC 29 virus.

MC 29-transformed cells contain a gag gene-related protein of 110,000 molecular weight (80). Tryptic peptide mapping has shown that P110 is a fusion protein containing products of gag gene-derived sequences and myc gene-derived sequences. Direct involvement of P110 in transformation has been suggested by the isolation of MC 29 mutants (81) which synthesize smaller gag gene-related proteins. These mutants have a 100 fold decreased efficiency in transformation of haematopoietic cells.



Moreover, the transformed cell colonies were smaller and difficult to grow. On the other hand the transformation of fibroblasts is not affected. This is reminiscent of td 359 AEV described before. However, in contrast to AEV, MC 29 infected cells generate only one mRNA species (69) of 5.4 kb detectable with MC 29 myc sequence. It remains to be seen whether there are domains within the P110 that are required for transformation of macrophages but not fibroblasts.

The p75 AEV and P110 of MC 29 have recently been shown to serve as substrates for phosphorylation in vivo (82). Moreover, the P110 of MC 29 was also phosphorylated in vitro by a kinase activity associated with immunocomplexes. Whether it does have intrinsic kinase activity remains to be seen.

AMV: The myeloblastosis virus AMV causes an acute myeloblastosis within a few weeks after infection of chickens (57). AMV is capable of transforming hematopoietic cells of the myeloid lineage in vitro. However, attempts to transform fibroblasts have been unsuccessful. Bone marrow cells transformed by AMV resemble myeloblasts and express myeloid markers. The studies of Gazzolo et al. (83) have suggested that AMV is capable of transforming immature (myeloblasts) and mature myeloid clels (macrophages). As was mentioned before, tertiary culture of peripheral blood from adult chickens were still sensitive to the transformation effect of MC 29 (52). The same is also true for AMV. One obvious objection of this conclusion could be that there are present a small number of immature myeloid cells in the adult chicken blood culture which are transformed by AMV and MC 29. Recent studies (84) however have shown that AMV is indeed capable of infecting and transforming mature macrophages in vitro.



The genome of AMV is about 7.2 kb (85) and it carries the entire gag sequence and part of the pol gene but lacks most (or all) of the env gene. A partial restriction map of an integrated provirus of AMV has been obtained recently (86).

Two RNA species have been detected in the AMV virion as well as in cells transformed by AMV (85). The 7.2 kb mRNA corresponds to the genome of AMV. The 2.3 kb mRNA carries only the 5' and 3' termini of the genome but not gag-, pol-, or env- sequences, and it may be a subgenomic mRNA. That this small mRNA may encode the product of the AMV transforming gene is suggested by the fact that the mRNA carries AMV-specific sequences. Almost nothing is known about the transformation protein of AMV.

Possible mechanisms of transformation by these three groups of defective viruses have been proposed by Graf et al. (57). Based on the observations of ts 34 AEV, td 359 AEV and the mutant of MC 29 which synthesizes a smaller fusion protein, it was concluded that the continuous expression of the oncogene in each virus is necessary for the induction and maintenance of transformation of their target cells. It was proposed that these viral oncogene products represent modified version of their cellular counterparts which are themselves proteins required for normal differentiation of the target cell. The viral oncogene products compete with these normal differentiation proteins in the binding of hypothetical differentiation-required components. The net result is a block in the normal differentiation of the target cells.

Such a model explains well the lineage specificity of defective leukemia viruses (87). Moreover it has been shown that this target cell



specificity is not due to the ability of the different strains to infect only certain types of hematopoietic cells. AEV, for example, was found to replicate in macrophages and express p75 but does not transform the macrophages. MC 29 is able to replicate in erythroblasts as well as myeloblasts and to express the p110 oncogene protein without transforming these cells. These findings thus support the above model of blocking differentiation as the basis of transformation.

However, it should be pointed out that the AEV and MC 29 mutants can transform fibroblasts in addition to their hematopoietic target cells. The above model cannot readily account for these facts. Moreover, since AMV can transform mature differentiated macrophages to produce cells of the less mature type, this model is not sufficient to account for this apparent reversal of differentiation phenotype.

## Chronic Viruses

The genome of a typical chronic virus is shown in figure 3c. These viruses do not seem to carry an oncogene like the defective viruses. Nonetheless chronic viruses induce most types of neoplasia observed with defective viruses albeit at a much slower rate of development. In the murine system, accumulating evidence points to the formation of recombinants in the env gene as a crucial event in virus-induced leukemogenesis. It has been shown that (88) in M-MuLV induced leukemogenesis a certain recombinant structure is a prerequisite for the onset of neoplasia. This recombinant structure shows close structural similarities to the previously described MCF-type viruses. No common integration sites can be detected for these infecting M-MuLV (89)





suggesting the activation of endogeneous oncogene may not be the mechanism for leukemogenesis. Rather, the finding of MCF type structures associated with the appearance of tumors suggests that the expression of a dualtropic glycoprotein on the cell surface is sufficient to stimulate transformation. Given the paucity of data, many other models are still possible.

In the avian system, tremendous progress has been made in the past two years in elucidating the role of the chronic virus ALV induced tumorigenesis. The genome of a typical ALV (avian leukosis virus) is shown in figure 3c. These viruses are replication competent, they induce neoplasia after a long latent period (several months), and with lower efficiency than the defective leukemia viruses. Moreover, they have never been observed to transform cells in vitro, neither fibroblasts nor their in vivo target cells. In vivo, ALV can cause a whole spectrum of neoplasia (90). These include the neoplasia induced by the three defective viruses such as myeloblastosis (AMV) myelocytomatosis (MC 29), hepatocarcinoma (MC 29) and erythroblastosia (AEV) and other neoplasias not known to be induced by the defective viruses such as osteopetrosis, hemangioma, and lymphoid leukosis. Moreover, ALV has been shown (91) to induce sarcoma in vivo by recombination with endogenous sarcoma gene sequences to generate sarcoma virus.

The fact that end-point-diluted ALV can still cause most of the above mentioned diseases indicates that a single viral entity is responsible. More recently, ALV recovered from cells transfected with molecularly cloned ALV DNA has been observed to induce a number of the above mentioned neoplasia (unpublished observations).



It is known that the breed of chickens used has a determining effect on the diseases inducible. Thus the inbred chicken line 15I is quite susceptible to the development of lymphoid leukosis whereas line  $6_3$  is resistant (92). Moreover, the resistance has been shown to reside in the B target cell (93). Lymphoid leukosis is the most common neoplasia observed in many other strains of chickens. Erythroblastosis is the main neoplasm in the inbred line 15<sub>1</sub> and 15B but is only infrequently induced in other lines of chickens.

The mechanism whereby ALV can induce these diseases has been a mystery for many years and is the main theme of this thesis. The remainder of this literature review will be devoted to an examination of recent developments in the elucidation of the mechanism of ALV induction of neoplasia culminating to an introduction of the main body of this thesis.

One of the model of the possible mechanism comes from the observation that there exists an ALV of endogenous origin, designated RAV-O. RAV-O produced from chickens has not been shown to induce neoplasms in chickens susceptible to the exogenous ALV (94,98). Moreover, the very low level of lymphoid leukosis observed in some cases is most likely due to spontaneous development of the disease instead of induction of RAV-O (95).

The genome of RAV-O is similar to that of exogenous ALV with two exceptions, the env gene and the C region. Exogenous ALV env gene codes for subgroup-A,-B,-C,-D anitgens, RAV-O env gene codes for subgroup E. Exogenous ALV has a C region,  $C^{x}$ , different from that of RAV-O,  $C^{n}$ . The studies of Robinson and her colleagues (96) have shown that recombinant





viruses which carry subgroup E env and C<sup>X</sup> (C region of exogenous) induce a similar incidence of diseases just like exogenous ALV. This effectively rules out a role of env in the transformation process. Evidence that  $C^{X}$  may play a role has been indirect; it was noticed that  $C^{X}$  confers to the virus a much higher growth rate than  $C^n$  does. As was metioned in the beginning of this review, the C region is contained in part of the LTR of The LTR is repeated at both ends of the integrated the provirus. proviral DNA and contains promotor like sequences. It is thus reasonable to suggest that the promotor has enhanced the transcription of the provirus thereby generating more of the viral RNA. It should be noted that the level of viremia does not correlate with the appearance of disease (96). This argues against a role of virus growth rate in the induction of the diseases. That the LTR can enhance transcription of cellular sequences downstream from the integration site of the provirus has been demonstrated in many incidences (99). Based on these observations it was proposed (97) that ALV exerts its oncogenic effect by enhancing transcription of cellular oncogenes. The simialrity in the disease spectra between the defective leukemia virus and ALV suggests that the same set of oncogenes are involved in the induction of these The detection of the cellular counterpart of these oncogenes diseases. further points to the possibility that ALV may promote the transcription of these cellular oncogenes. Neel et al. (78) were the first to test this hypothesis and subsequently found that (100) in LL tumors the LLV proviruses are integrated next to the c-myc gene and that enhanced expression of this gene is observed. This finding was confirmed by myself (see appendix) and by Payne et al. (101). ALV can induce erythroblastosis in susceptible chickens. It is thus logical to suggest





that the cerb gene (AEV) is activated by ALV integration. This is indeed found to be true and is the main theme of this thesis. It should be noted that while ALV can enhance the expression of the adjacent cellular sequences, it does not need to do so strictly through its promotor. In fact, the structure of ALV provirus is so similar to that of transposable elements that any number of effects observed for transposable elements can be equally applicable to the provirus. Payne et al. (102) has observed integration of proviral DNA downstream or upstream as well as upstream in an orientaiton opposite to that of c-myc transcription. This argues that mechanisms in addition to promotor-insertion may be involved.



REFERENCES



## REFERENCES

- Gross, L. (1970). Oncogenic Viruses, 2nd ed. Pergamon Press, Oxford pp. 1-99.
- Shine, J., Czernilofsky, A.P., Friedrich, R., Goodman, H.M., and Bishop, J.M. (1977) Proc. Natl. Acad. Sci. USA 74, 1473-1477.
- 3. Haseltine, W.A., Maxam, A., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 989-993.
- 4. Schwartz, D.E., Zamecnik, P.C., and Weith, H.L. (1977) Proc. Natl. Acad. Sci. USA 74, 994-998.
- 5. Harada, F., Sawyer, R.C. & Dahlberg, J.D. (1975) J. Biol. Chem. 250, 3487-3497.
- 6. Taylor, J.M. and Illmensee, R. (1975) J. Virol. 16, 553-558.
- 7. Junghans, R.P., Hu, S., Knight, C.A., and Davidson, N. (1977) Proc. Natl. Acad. Sci. USA 74, 447-481.
- 8. Coffin, J.M. (1979) J. Gen. Virol. 42, 1-26.
- 9. Yamamoto, T., Jay, G., and Pastan, I. (1980) Proc. Natl. Acad. Sci. USA 77, 176-180.
- 10. Yamamoto, T., Tyagi, J.S., Fagan, J.B., Jay, G., deCrombrugghe, B., and Pastan, I. (1980) J. Virol. 35, 436-443.
- 11. Swanstrom, R., DeLorbe, W.J., Bishop, J.M., and Varmus, H.E. (1981) Proc. Natl. Acad. Sci. USA 78, 124-128.
- Majors, J.E., Swanstrom, R., DeLorbe, W.J., Payne, G.S., Hughes, S.H., Ortiz, S., Quintrell, N., Bishop, J.M., and Varmus, H.E. (1981) Cold Spring Harbors Symp. Quant. Biol. vol. 45 in press.
- 13. Kung, H.J., Fung, Y.K., Majors, J.E., Bishop, J.M., and Varmus, H.E. (1980) J. Virol. 37, 127-138.

- 14. Shank, P.R. and Varmus, H.E. (1978) J. Virol. 25, 104-114.
- 15. Shank, P.R., Cohen, J.C., Varmus, H.E., Yamamoto, K.R., and Ringold, G.M. (1978) Proc. Nat. Acad. Sci. USA 75, 2112-2116.
- 16. Shank, P.R., Hughes, S.H., Kung, H.-J., Majors, J.E., Quintrell, N., Guntaka, R.V., Bishop, J.M., and Varmus, H.E. (1978) Cell 15, 1383-1395.
- 17. Hsu, T.W., Sabran, J.L., Mark, G.E., Guntaka, E.V., and Taylor, J.W. (1978) J. Virol. 28, 810-818.
- Hughes, S.H., Shank, P.R., Spector, D.H., Kung, H.-J., Bishop, J.M., Varmus, H.E., Vogt, P.K., and Breitman, M.L. (1978). Cell 15, 1397-1410.
- 19. Sabran, J.L., Hsu, T.W., Yeater, C., Kaji, A., Mason, W.S., and Taylor, J.M. (1979) J. Virol. 29, 170-178.
- 20. Bishop, J.M. (1978) Annu. Rev. Biochem. 47, 35-88.
- 21. Copeland, N.G., Zelentz, A.D., and Cooper, G.M. (1980) Cell 19, 863-870.
- 22. Lee, W.H., Bister, K., Pawson, A., Robbins, T., Moscovici, C., and Duesberg, P.H. (1980) Proc. Natl. Acad. Sci. USA 77, 2018-2022.
- Hanafusa, T., Wang, L.-H., Anderson, S.M., Karess, R.E., Hayward, W.S., and Hanafusa, H. (1980) Proc. Natl. Acad. Sci. USA 77, 3009-3013.
- 24. Kawai, S., Yoshida, M., Segawa, K., Suigiyama, H., Ishizaki, R., and Toyoshima, K. (1980) Proc. Natl. Acad. Sci. USA 77, 6199-6203.
- Fischinger, P.J. (1980) Type C RNA transforming viruses. In Molecular Biology of RNA Tumor Viruses. Stephenson, J.R., ed. Academic Press, NY pp. 163-198.
- Shih, T.Y. and Scolnick, E.M. (1980) Molecular Biology of Mammalian Sarcoma Viruses in Viral Oncology G. Klein, ed. Raven Press, NY pp. 135-160.
- 27. Friend, C. (1957) J. Exp. Med. 105, 307-318.
- 28. Abelson, H.T., and Rabstein, L.S. (1970) Cancer Res. 30, 2213-2222.
- 29. Patschinsky, T. and Sefton, B.M. (1981) J. Virol. 39, 104-114.
- Bister, K., Lee, W.-H., and Duesberg, P.H. (1980) J. Virol. 36, 617-621.


- 31. Troxler, D.H., Ruscetti, S.K., and Scolnick, E.M. (1980) Biochim. Biophys. Acta 605, 305-324.
- 32. Ellis, R.W., DeFeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R., and Scolnick, E.M. (1981) Nature 292, 506-511.
- 33. Dawson, P.J., Tacke, R.B., and Fieldsteel, A.H. (1968) Br. J. Cancer 22, 569-576.
- 34. Rowson, K.E. and Parr, I.B. (1970) Int. J. Cancer 5, 96-102.
- 35. Steeves, R.A., Eckner, R.J., Bennett, M., Mirand, E.A., and Trudel, P.J. (1971) J. Nat. Cancer Inst. 46, 1209-1217.
- 36. Dawson, P.J., Tacke, R.B., and Fieldsteel, A.H. (1966) Br. J. Cancer 20, 114-121.
- 37. Steeves, R.A. (1975) J. Nat. Cancer Inst. 54, 289-297.
- 38. Hankins, W.D., Kust, T.A., Koury, M.J., and Krantz, S.B. (1978) Nature 276, 506-508.
- 39. Troxler, D.H. and Scolnick, E.M. (1978) Virology 85, 17-27.
- 40. McGarry, M.P., Steeves, R.A., Eckner, R.J., Mirand, E.A., and Trudel, P.J. (1974) Int. J. Cancer 13, 867-878.
- 41. Troxler, D.H., Yuan, E., Linemeyer, D., Ruscetti, S., and Scolnick, E. (1978) J. Exp. Med. 148, 639-653.
- 42. Hartley, J.W., Wolford, N.K., Old, L.J., and Rowe, W.P. (1977) Proc. Natl. Scad. sci. USA 74, 789-792.
- 43. Witte, O.N., Goff, S., Rosenberg, N., and Baltimore, D. (1980) Proc. Natl. Acad. Sci. USA 77, 4993-4997.
- 44. Scher, C.D. and Siegler, R. (1975) Nature (London) 253, 729-731.
- 45. Rosenberg, N., Baltimore, D., and Scher, C.D. (1975) Proc. Natl. Acad. Sci. USA 72, 1932-1936.
- 46. Rosenberg, N. and Witte, O.N. (1980) J. Virol. 33, 340-348.
- 47. Reynolds, F.H., van de Ven, W.J.M., and Stepehnson, J.R. (1980) J. Virol 36, 374-386.
- 48. Dale, B. and Ozanne, B. (1981) Molecular and Cellular Biology 1, 731-742.



- Baltimore, D., Shields, A., Otto, G., Goff, S., Besmer, P., Witte, O., and Rosenberg, N. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, 849-854.
- 50. Goff, S.P., Gilboa, E., Witte, O.N., and Baltimore, D. (1980) Cell 22, 777-785.
- 51. Beug. H., von Kirchbach, A., Doderlein, G., Conscience, J.-F., and Graf, T. (1979) Cell 18, 375-390.
- 52. Gazzolo, L., Moscovivi, C., Moscovici, M.G., and Samarut, J. (1979) Cell 16, 627-638.
- 53. Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T., and Stehelin, D. (1979) Nature 281, 452-455.
- 54. Stehelin, D. and Graf, T. (1978) Cell 13, 745-750.
- 55. Graf, T., Royer-Pokora, B., Schubert, G.E., and Beug, H. (1976) Virology 71, 423-433.
- 56. Hayman, M.J., Royer-Pokora, B., and Graf, T. (1979) Virology 92, 31-45.
- 57. Graf, T. and Beug, H. (1978) Biochim. Biophys. Acta 516, 269-300.
- 58. Graf, T., Ade, N., and Beug. H. (1978) Nature 275, 496-501.
- 59. Royer-Pokora, B., Beug, H., Claviez, M., Winkhardt, H.-J., Friis, R.R., and Graf, T. Cell 13, 751-760.
- 60. Gazzolo, L., Samarut, J., Bouabdelli, M., and Blanchet, J.P. (1980) Cell 22, 683-691.
- 61. Samarut, J., Blanchet, J.P., and Nigon, V. (1979) Dev. Biol. 72, 155-166.
- 62. Samarut, J. and Nigon, V. (1975) J. Embryol. Exp. Morph. 33, 259-278.
- 63. Samarut, J. and Bouabdelli, M. (1980) J. Cell Physiol., in press.
- 64. Veenstrom, B., Fanshier, L., Moscovici, C., and Bishop, J.M. (1980) J. Virol. 36, 575-585.
- 65. Lai, M.M., Neil, J.C., and Vogt, P.K. (1980) Virology 100, 475-487.
- 66. Yoshida, M. and Toyoshima, K. (1980) Virology 100 484-487.
- 67. Hayman, M.J., Royer-Pokora, B., and Graf, T. (1979) Virology 92, 31-45.



- 68. Pawson, T. and Martin, G.S. (1980) J. Virol. 34 280-284.
- 69. Sheiness, D., Vennstrom, B., and Bishop, J.M. (1981) Cell 23, 291-300.
- 70. Anderson, S.M., Hayward, W.S., Neel, B.G., and Hanafusa, H. (1980) J. Virol. 36, 676-683.
- 71. Saule, S., Roussel, M., Lagrou, C., and Stehelin, D. (1981) J. Virol. 38, 409-419.
- 72. Vennstrom, B., et al. in preparation.
- 73. Varmus, H. (personal communication).
- 74. Beug, H. and Graf T. (1980) Virology 100, 348-356.
- 75. Beug, H., Kitchener, G., Doderlein, G., Graf, T., and Hayman, M. (1980) Proc. Natl. Acad. Sci. USA 77, 6683-6686.
- 76. Royer-Pokora, B., Grieser, S., Beug, H., and Graf, T. (1979) Nature 282, 750-752.
- 77. Bonar, R.A., and Paulson, D.F. (1974) J. Natl. Cancer Inst. 53, 711-718.
- 78. Neel, B.G., Hayward, W.S., Robinson, H.L., Fang, J., and Astrin, S.M. (1981) Cell 23, 323-334.
- 79. Vennstrom, B., Moscovici, C., Goodman, H.M., and Bishop, J.M. (1981) J. Virol. 39, 625-631.
- 80. Bister, K., Hayman, M.J., and Vogt, P.K. (1977) Virology 82, 431-448.
- 81. Ramsay, G., Graf, T., and Hayman, M.J. (1980) Nature 288, 170-172.
- 82. Bister, K., Lee, W.-H., and Duesberg, (1980) J. Virol. 36, 617-621.
- Gazzolo, L., Samarut, J., Moscovici, C., Moscovici, M.G., and Quash, G. (1977) in Avian RNA Tumor Viruses (Marlati, S. and de Giuli, C., eds.), pp. 35-44, Piccin editore, Padua, Italy.
- 84. Durban, E.M. and Bettiger, D. (1981) J. Virol. 37, 488-492.
- Gonda, T.J., Sheiness, D.K., Fanshier, L., Bishop, J.M., Muscovici,
  C., and Moscovici, M.G. (1981) Cell 23, 279-290.
- 86. Souza, L.M. and Baluda, M.A. (1980) J. Virol. 36, 317-324.
- 87. Graf, T., Beug, H., and Hayman, M.J. (1980) Proc. Natl. Acad. Sci. USA 77, 389-393.



- 88. van der Putten, H., Quing, W., van Raaij, J., Maandag, E.R., Verma, I.M., and Berns, A. (1981) Cell 24, 729-739.
- 89. Jaehner, D., Stuhlman, H., and Jaenisch, R. (1980) Virol. 101, 111-123.
- 90. Purchase, H.R. and Burmeister, B.R. (1978) In Diseases of Poultry M.S. Hofsad, B. W. Calnek, C.F. Helmboit, W.M. Reid and H.M. Yoder, Jr., eds. (Ames, Iowa: Iowa State University Press) pp. 418-468.
- 91. Hanafusa, H., Halpern, C.C., Buchhagen, D.L., and Kawai, S. (1977) J. Exp. Med. 146, 1735-1747.
- 92. Crittenden, L.B. (1975) Avian Diseases 19, 281-292.
- 93. Purchase, H.G., Gilmour, D.G., Romero, C.H., and Okazaki, W. (1975) Nature 270, 61-62.
- 94. Crittenden, L.B., Witter, R.L., and Fadly, A.M. (1979) Avian Diseases 23, 646-653.
- 95. Crittenden, L.B., Witter, R.L., Okazaki, W., and Nelman, P.E. (1979) J. Natl. Cancer Inst. 63, 191-200.
- 96. Robinson, H.L., Pearson, M.N., DeSimone, D.W., Tsichlis, P.N., and Coffin, J.M. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, 1133-1142.
- 97. Tsichlis, P.N. and Coffin, J.M. (1980) J. Virol. 33, 238-249.
- 98. Motta, J.V., Crittenden, L.B., Purchase, H.G., Stone, H.A., and Witter, P.L. (1975) J. Nat. Cancer Inst. 55, 685-689.
- 99. Quintrell, N., Hughes, S.H., Varmus, H.E., and Bishop, J.M. (1980) J. Mol. Biol. 143, 363-393.
- 100. Hayward, W., Neel, B.G., and Astrin, S.M. (1981) Nature (London) 290, 475-480.
- 101. Payne, G.S., Courtneidge, S.A., Crittenden, L.B., Fadly, A.M., Bishop, J.M., and Varmus, H.E. (1981) Cell 23, 311-322.
- 102. Payne, G.S., Bishop, J.M., Varmus, H.E. (1981) Nature, in press.



ARTICLE I

•



# On the Mechanism of Retrovirus-Induced Avian Erythroleukemia: Integration of the Proviruses and Alteration of Cellular erb Gene Structure

Yuen Kai T. Fung<sup>\*†</sup>, and Hsing-Jien Kung<sup>\*</sup>

- \* Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824
- <sup>†</sup> Present address: Department of Microbiology and Immunology, University of California, San Francisco, California 94143



### INTRODUCTION

Retroviruses can be classified according to the pathology they elicit either as acute or chronic tumor viruses (Graf and Beug, 1978). In the avian system, recent studies have provided the biochemical and genetic basis (Beug <u>et al.</u>, 1979; Jazzolo <u>et al.</u>, 1979; Roussel <u>et al.</u>, 1979; Stehelin <u>et a</u>l., 1978) for classifying the acute leukemia viruses into three types (Graf <u>et al.</u>, 1980; Stehelin <u>et al.</u>, 1980). They are: (1) avian erythroblastosis virus (AEV), (2) avian myelocytomatosis-type virus (MC29, CMII, OK10, MH2), and (3) avian myeloblastosis-type viruses (AMV and E26). Each of these three groups of viruses have their own specific oncogenic sequences in the viral genome and they differ in the type of neoplasm incuded. AEV induces sarcomas and erythroblastosis; MCV induces sarcomas, myelocytomas and carcinomas, whereas AMV induces myeloblastosis. Injection of these viruses into day old chickens results in rapid transformation of their respective target cells and death of the chickens.

The AEV group of acute leukemia viruses, for example, can induce an acute erythroleukemia and anemia in chickens one to two weeks after inoculation. The target cells transformed by AEV have been found to display distinct phenotypes of differentiation (Graf and Beug, 1978; Jazzolo, 1979). Cells transformed by AEV bear striking similarities to precursor cells of erythrocytes as revealed by their expression of high levels of histone H5 and erythroblast cell surface antigen (Beug <u>et al</u>., 1979). By using specific antisera which can distinguish between the several erythrocyte precursors at different stages of differentiation, it



has been suggested that the target cells for infection by AEV are the burst forming unit-erythrocytic cells (BFU-E). Jazzolo et <u>al.</u>, 1980).

Based on observations of the differentiation phenotype of the transformed target cells and studies of a temperature sensitive mutant of AEV (ts34) (Graf et al., 1978a) it was proposed that AEV can induce an arrest of differentiation in their target cells which leads to leukemic transformation (Graf and Beug, 1978). Specifically, the transforming protein of AEV is thought to competitively inhibit the activity of a lineage-specific homologous cellular protein required for normal differentiation of the target cell. This AEV transforming protein has been shown to be a fusion product of part of the gag sequence and part of the 5' domain of the AEV oncogene, v-erb. Proof that this is the transforming protein comes from studies of the non-conditional mutant td359 (Royer-Pokora et al., 1979). This mutant is defective for the transformation of bone marrow cells in vitro and fails to induce erythroblastosis in vivo. Examination of the protein products revealed a deletion of 1000 daltons in the gag-gene fusion protein P45. Tryptic peptide mapping has indicated a deletion in the v-erb gene region suggesting that erb does play a role in the transformation process (Berg et al., 1980).

td359 is still capable of transforming fibroblasts <u>in vitro</u> and induce sarcomas <u>in vivo</u>. This has lead to the proposal that there may be two functional domains in the v-erb gene.

The structure of the AEV genome has recently been elucidated by molecular cloning (Vennstrom <u>et al</u>., 1980). The genome is about 5.1 kb long (with one LTR). v-erb is at least 2.5 kb of the AEV genome, flanked by about 1 kb of gag sequence and 0.4 kb of env sequence. Several lines of evidence have suggested the existence of two functionally different

-3-



v-erb gene loci in the AEV genome: (1) Two AEV-specific proteins, which bear little or no sequence homology, P75 and P40, have been identified by in vitro translation of AEV viral RNAs (Lai et al., 1980; Yoshida et al., 1980; Pawson et al., 1980; and Anderson et al., 1980). (2) Two AEV-specific mRNAs of 5.3 and 3.5 kb (Sheiness et al., 1981; Anderson et al., 1980) have been identified in AEV transformed cells. Hybridization of these mRNAs with DNA probes from different regions of the AEV genome have revealed that the 5.3 kb represents the entire genome. The 3.5 kb mRNA hybridizes to all but the 5' region of the v-erb domain and the gag region. Since P75 carries gag sequences it was thus proposed that the 5.3 kb mRNA codes for P75 while the 3.5 kb mRNA codes for P40. Four distinct mRNA's of c-erb have been discovered in uninfected cells (Sheiness et al., 1981). Two of these hybridize to one half of erb while the smaller two species hybridize to the other half. This implies the existence of two c-erb domains in the cellular genome. (3) The AEV mutant, td 359 (Royer-Pokora et al., 1979) mentioned above, which has lost its ability to transform erythroblasts, has been shown to synthesize a gag gene related protein ( $\Delta P75$ ) which has a 1000 dalton deletion from P75 (Beug et al. 1980). No change was observed in the size of P40. This data suggested a role of P75, containing the 5' domain of the v-erb sequence, in the transformation of erythroblasts. For convenience of discussion, we shall designate this 5' v-erb domain as 'A' locus and the 3' v-erb domain as 'B' locus.

While AEV carries an oncogene and can thus rapidly transform, it lacks the genes needed for its own replication and must therefore rely on the presence of a helper virus for its propagation.

-4-



The helpers, avian leukosis viruses (ALV), are not known to encode any oncogene in their genomes. They have never been observed to transform cells <u>in vitro</u>. Nonetheless, they can induce the same type of neoplasms of defective viruses albeit at a much slower pace (Purchase <u>et</u> <u>al</u>., 1978). These neoplasia include myeloblastosis, myelocytomatosis, hepatocarcinoma and erythroblastosis. They can also induce other neoplasia not known to be induced by the defective viruses such as osteopetrosis, hemangioma and lymphoid leukosis. ALV has been shown also to induce sarcoma <u>in vivo</u> by recombination with endogenous sarcoma gene sequences to generate sarcoma virus (Hanafusa <u>et al</u>., 1977).

The breed of the chickens used has a determining effect on the disease inducible. Thus the inbred chicken line 15I is quite susceptible to the development of lymphoid leukosis whereas line  $6_3$  is resistant (Crittenden, 1975). Moreover, the resistance has been shown to reside in the B target cell (Purchase <u>et al.</u>, 1975). Lymphoid leukosis is the most common neoplasia observed in many other strains of chickens. Erythroblastosis is the main neoplasm in the inbred line 15I and 15B but is only infrequently induced in the other line of chickens. Sometimes a chicken infected with ALV can simultaneously develop lymphoid leukosis and erythroblastosis.

The mechanism whereby ALV can induce these diseases has been a subject of intense investigation (Neiman <u>et al.</u>, 1980; Neel <u>et al.</u>, 1981; Payne <u>et al.</u>, 1981a; Hayward <u>et al.</u>, 1981; Fung <u>et al.</u>, 1981). It has been proposed that (Tsichlis and Coffin, 1980) the C region of the ALV provirus exerts its oncogenic effect by enhancing transcription of downstream cellular sequences. Subsequently, this putative cellular Oncogenic sequence has been shown, in the case of lymphoid leukosis, to

-5-

be the cellular homolog of the transforming gene of MC29 (Hayward <u>et al.</u>, 1981). This integration of ALV next to the c-myc gene in lymphoid leukotic tumors has subsequently been confirmed (Payne <u>et al.</u>, 1981a; Fung <u>et al.</u>, 1981). The location and orientation of integration of the ALV suggests that a mechanism other than or in addition to promotor insertion may be involved (Payne, 1981).

In the present paper, we report the involvement of c-erb in ALV induced erythroblastosis in susceptible chickens.

#### RESULTS

#### Strategy

The inbred chicken line  $15_1$  was choosen because it routinely shows a higher incidence of erythroblastosis than other lines when inoculated with ALV. Two other lines,  $15_15x7_2$  and  $15_8x0$ , have also been studied. End point dilution cloned RAV-1 virus was inoculated either into chick embryos or into day-old chicks (intraparitonally or intravenously). In addition, uninfected chickens as well as chickens infected with the acute erythroblastosis virus AEV were included as controls.

To monitor the disease, blood samples were drawn from the chickens at regular intervals for hematocrit and blood smear slide analysis. Chickens showing signs of the disease were sacrificed and their nucleic acids analyzed.

# Kinetics of development of the diseases and morphologic pathology

Typical examples of crude quantitation of the erythroblasts and the mature erythrocytes at various times post inoculation is shown in Fig. 1. Two forms of erythroblastosis were observed in the chickens injected with RAV-1, the anemic (0 - 0) and the proliferative form (0 - 0). In both cases, marked anemia was observed. However, relatively few immature erythroblasts were observed in the anemic form in contrast to the presence of abundant erythroblasts in the proliferative form. The buffy coat cells form the top layer of white colored blood cells in a hematocrit: it consists of erythroblasts and various white blood cells.

Chickens injected with AEV (---- dotted line) show an abrupt elevation of the percentage of buffy coat cells within 1-2 weeks post

-7-

inoculated (Therwath et al., 1978). In contrast, chickens inoculated with RAV-1 did not show any sign of change until about 10-14 weeks, at which time both the percentage of erythrocytes dropped and the percentage of buffy coat cells escalated abruptly. Similar to those in AEV inoculated birds, these two events (development of anemia and proliferation of blast cells) accelerated to their peak in 2-3 days, then the birds died.

As shown in Fig. 2a,c blood smears from birds inoculated with AEV or RAV-1 at the preleukemic stages were indistinguishable from uninfected controls (not shown). At the terminal stages of the disease (Fig. 2b,d), blood smears from either RAV-1 or AEV infected birds showed an abundance of erythroblasts. This increase in the concentration of the erythroblasts accounts for the increase in the percentage of buffy coat cells. The erythroblasts from RAV-1 inoculated birds are indistinguishable from those inoculated with AEV by our staining procedure.

In the proliferative form, diffuse enlargement of the liver and the spleen were observed. These organs were usually cherry red in color. This alteration in the visceral organs in the proliferative form has been ascribed to a hemostasis resulting in an extensive accumulation of erythroblasts in the blood sinusoids and capillaries (Purchase <u>et al</u>., 1978). Thus, the morphologic pathology and the observed short burst of blast formation prior to death of the birds are identical in both RAV-1 and AEV injected birds. The major difference lies in the time of onset of the disease symptoms.

#### Alteration in c-erb sequence in RAV-1 infected birds

Despite the significant difference between the latency of erythroblastosis induced by RAV-1 and that by AEV, it is crucial to rule out the

-8-

possible contamination of the RAV-1 virus with AEV. We examined the tumor tissues by restriction enzyme digestion analysis for the presence or absence of AEV provirus. The restriction endonuclease cleavage map of cloned AEV DNA has been reported (Vennstrom et al., 1980). We took advantage of the fact that BamHI cuts the AEV genome several times, generating, among others, a 2.6 kb fragment which carries a major part of the AEV-specific region. As shown in Fig. 3, in addition to the endogenous c-erb BamHI fragments, AEV infected birds, lanes a and b, show a 2.6 kb fragment hybridizable to  $erb_T$ , a cDNA probe that carries the majority of the erb domain of AEV. No such fragments can be detected in either of the RAV-1 infected leukemic birds (lanes c and d), or the uninfected control (lanes e and f). Moreover, birds at the preleukotic stage infected with AEV also show minimal or no 2.6 kb fragments corresponding to infecting AEV provirus. As would be expected from the genomic maps shown in Fig. 3, cDNA probes corresponding to the gag region or the 5' end region of erb all hybridized to the 2.6 kb fragment. No hybridization to this 2.6 kbp fragment was detected with a DNA probe corresponding to the 3' portion of the v-erb domain  $(erb_R)$ . Moreover, there is a direct correlation between the concentration of buffy coat cells and the intensity of the 2.6 kb fragment of a particular tumor. The 2.6 kb fragment can also be detected in the liver of the leukemic bird due to hemostasis of erythroblasts.

Because of polymorphisms in the c-erb gene, restriction endonuclease digestion patterns sometimes vary even between members of the same inbred chicken line. To unambiguously identify any potential alteration in the c-erb genes, most blood samples were separated into the buffy coat cell fraction, which was enriched in the immature erythroblast, and the mature

-9-

erythrocyte fraction (see Experimental Procedure) or in the cases when whole blood was used comparison was made with blood from the same chicken at preleukemic stage. For each chicken, DNA was extracted from the two blood fractions as well as from the liver and the spleen. The restriction enzyme digestion patterns of EcoRI and BamHI of these DNA samples after hybridization to  $erb_T$  are shown in Fig. 4. Fig. 4A lanes 1-4 show the digestion patterns of blood of an uninfected chicken. The buffy coat (lane 1) and erythrocyte fraction (lane 2) yield the same pattern indicating there is no primary structural differences between the DNA with respect to EcoRI (lanes 1,2) or BamHI (lanes 3,4) digestion. Figure 4A lanes 5-8 show that there is no alteration in the primary structure of the erb gene when the chicken, infected with RAV-1, showed no sign of developed erythroblastosis and eventually died of L.L.

Figure 4B shows the EcoRI and BamHI digestion patterns of AEV infected chickens at the preleukotic stage (lanes 1 and 3) and at the leukemic stage (lanes 2 and 4). Evidence for the existence of the AEV provirus is shown by the presence of the 2.6 kb BamHI fragment characteristic of the infecting AEV. Since EcoRI cuts once in the infecting AEV proviral DNA, the absence of erb fragments (lane 2), in addition to the background (lane 1), is indicative of the nonclonal origin of the tumor. The infecting AEV provirus therefore appears to have integrated randomly in the erythroblasts.

Figure 4C shows the EcoRI and BamHI restriction digestion pattern of RAV-1 infected chickens positively diagnosed as having developed erythroblastosis. Using EcoRI digestion, erb fragments (marked by ) in addition to the background (cf. also Fig. 4A) are observed in the buffy coat fraction (EB) but not in the erythrocyte (EC) fraction of the same

-10-

chicken. Extra fragments are sometimes observed in the liver and the spleen (sp) samples. To determine the absence of AEV provius contamination in these tumor samples the BamHI digestion of these samples are included for comparison.

In contrast to the AEV induced erythroblastosis samples, none of these RAV-1 induced erythroblastosis samples show a 2.6 kb BamHI fragment characteristic of AEV proviruses, indicating that the tumor specific fragments are not due to infecting AEV provirus. The presence of tumor specific fragments (marked by ) is in contrast to the DNA samples from AEV infected birds, and points to a clonal origin of these tumors.

Not all RAV-1 induced erythroblastosis samples are of clonal origin; we have also observed apparently nonclonal tumors in some of the birds. Fig. 4D, lanes 3 and 4 show that no specific fragments can be detected using either BamHI or EcoRI in these birds (and a few other enzymes, data not shown). The absence of a BamHI 2.6 kb fragment is taken as evidence for the absence of v-erb in these tumors. We have also encountered RAV-1 injected chickens that have developed the anemia form of erythroleukemia. Analysis of the buffy coat cells and erythrocytes revealed no obvious differences in the restriction digestion patterns when hybridized to  $erb_T$  (Fig. 4E).

#### Nature of the tumor specific fragments

# A. with respect to ALV sequences

One obvious possible origin of the tumor specific fragments observed is the integration of ALV near the endogenous erb sequence in the cell genome. As shown in the schematic restriction map of ALV in Figure 5, there is an EcoRI site in the LTR of ALV. Integration of ALV upstream or

-11-

downstream from an erb sequence would result in a new restriction fragment if there is no EcoRI site between the c-erb sequence and the LTR. Such a sequence would be detectable with DNA probes from either the LTR or the corresponding erb sequence. On the other hand, if ALV integrates into the cell genome to form a recombinant structure in the fashion of the exogenous AEV genome (see Figure 5 for the schematic of AEV exogenous provirus) one would expect the tumor specific fragments to be detected with either the erb sequence or the ALV gag sequence. Fig. 5 shows such analysis of chickens 1-4. Hybridization with probes specific for the ALV LTR (lanes 7 and 8) shows specific fragments corresponding to the one detected with the  $erb_T$  probe (lanes 1,2). These are chicken 1: 4.2 kb; chicken 2: 5.3 kb; chicken 3: 3.8 kb and chicken 4: 7.1 kb. No such fragments are detected with gag hybridization (lanes 9 and 10). Instead a 2.45 kb fragment appears in gag hybridization indicating this part of the ALV genome is intact.

# B. with respect to the AEV sequence

Having established this relationship of ALV integration, we next wish to address the question of the structure of erb in the tumor specific fragments. As was mentioned before, there appear to be two distinct c-erb domains 'A' and 'B' in the cellular genome. We were interested in determining which of these two apparently structurally and functionally different cellular erb sequences is involved in the generation of tumor specific fragments. We took advantage of the observation (Sheiness <u>et al</u>., 1981) that a 0.5 kb PstI fragment of the AEV genome can specifically hybridize to the 5' domain of v-erb (5.3 kb mRNA). Hybridization of the chicken genomic DNA samples with this probe,  $erb_1$  (lanes



3,4), revealed an EcoRI fragment in normal chickens at 24 kb which was also seen in the hybridization with  $erb_T$  probe. All except chicken 4 tumor DNA failed to show any extra fragments. Chicken 4 shows a T.S. fragment of 7.1 kb, corresponding to the T.S. fragment seen when using the LTR or the  $erb_T$  probe. It is thus apparent that the LTR is linked to the c-erb 'A' locus in this sample.

To investigate the possible involvement of the other c-erb gene. locus 'B', the sequence corresponding to most of the 3' half of the v-erb genome was used for preparing a cDNA probe  $erb_{R}$  (see Figure 5). The sequence of this part of the genome is well within the 3.5 kb mRNA and would therefore be free of sequence homology with the 'A' locus. Hybridization of EcoRI digested genomic DNA with this probe shows most of the fragments detected in a normal chicken genome with  $erb_T$ . Fragments not detected include the 24 kb 5' c-erb gene fragment and a few other fragments presumably corresponding to the 5' region of the 'B' locus. Figure 5 shows that the tumor specific fragments detected with  $erb_T$  in chickens 3 and 4 show up again with this probe. This indicates that the 'B' locus is probably involved in the T.S. specific fragment of these two chickens. No such hybridization can be detected in chicken number 1. This may mean that ALV has integrated at the 5' end of the 'B' gene. It has recently been confirmed by hybridization with a DNA probe corresponding to the 5' end of the B gene (not shown). Interestingly, chicken number 4 shows hybridization to both the 'A' and 'B' gene. We have further characterized the structure of this tumor specific fragment by molecular cloning of the EcoRI and BamHI restriction fragments and demonstrated the presence of both A and B loci sequences in the fragment (see below).



# Kinetics of the development of disease

Figure 6 shows the kinetics of the development of the disease. Blood samples taken at 4 and 8 weeks post infection are shown in lanes 1,2,4,5,7,8,10 and 11. It can be seen that at the terminal stage, a tumor specific fragment detectable with ALV LTR and AEV can be detected with either Sac I or Eco RI digestion. (Using other enzymes, i.e., Bam HI, Pvu II, Pst I gave the same results.) Thus, the virus appeared to have integrated randomly in the genomes of different cells. At the later stage selective growth advantage of the tumor clone resulted in the detection of the tumor specific fragments.

# Enhanced expression of erb in erythroblastosis tumor

We have analyzed the expression of the erb sequences in various tissues. Figure 7 shows the dot blot of the RNA, from control uninfected birds (lanes 1,2) and RNA from a RAV-1 infected bird that did not develop erythroblastosis but died eventually of L.L. (lanes 3,4). No enhanced expression of the erb gene can be observed. Fig. 7 lanes 5-8 are the RNA from the blood and liver of AEV infected birds. It can be seen that birds (lanes 5,6) at the preleukemic stage show little enhancement in the expression of erb; presumably the AEV virus has not spread. At the terminal stage of erythroblastosis, lanes 7 and 8, both the liver and the blood show enhanced (>100 fold) expression of v-erb.

Figure 7, lanes 9-18 are the RNA from the different tissues of RAV-1 infected leukemic birds. Lanes 9-14 are from birds with T.S. fragments and lanes 15-18 are from birds that show no T.S. fragments. It can be seen that erb gene expression in chickens 2,3 and 5 is enhanced to about the same extent as that of the AEV infected leukemic bird. We take this

-14-



to mean that c-erb expression is enhanced by the ALV integration event. Notice that not all birds have 100 fold enhancement of expression. Chickens 1 and 6 have about 1-10 fold enhancement (lanes 9,10,17 and 18). This could be due to the fact that only a small portion of the samples are tumor cells. This is apparently the case with chicken 1 as judging from the intensity of the tumor specific fragment as compared to that of the endogenous erb loci in Southern blots.

# Molecular cloning of tumor specific fragments

The tumor specific fragments of chicken 4 are particularly intriguing in that both the 'A' and 'B' loci are found to associate with the LTR, but not gag region, of the ALV. Therefore, we cloned the 7.1 kb EcoRI fragment (shown in Fig. 4C, lane 16) into  $\lambda$ gtWES. We have also cloned the 6.6 kb BamHI fragment (shown in Fig. 4C, lane 18) into  $\lambda$ Ch28. Not surprisingly, these two clones overlapped to a large extent. A restriction map of the clones is shown in Fig. 8. To the left of the clone is a stretch of cellular sequence that does not hybridize to any of the erb or ALV probes used. erb<sub>L</sub> and erb<sub>R</sub> hybridize almost equally well to a large portion of the clones spanning about 4.5 kb. The LTR is located to the right of most of the erb sequence (see Fig. 8). Interestingly, a stretch of erb specific fragment that will hybridize only to the erb<sub>R</sub> probe is situated just beyond the LTR.

The sizes of the fragments hybridizable to the erb probes, added together, are much larger than the 2.5 kb erb domain of AEV. Moreover, the restriction map of the erb portion of the clone does not conform to that of AEV. For example, there are two SacI sites in AEV, but there are none in the clone (the cloned fragments in the cellular genome are



apparently flanked by two SacI sites 13.9 kb apart). Therefore, we tentatively conclude that the tumor specific fragment contains c-erb sequences. Experiments are in progress to further analyze the nature of these tumor specific fragments.



### DISCUSSION

#### AEV and RAV-1 induce similar erythroleukemia in chickens

The disease symptoms of erythroleukemia in chickens infected with either RAV-1 alone or together with AEV are remarkably similar. In both cases an abrupt elevation in the buffy coat cell concentration are observed prior to the death of the chicken. Upon autopsy, the bird has cherry red liver and an enlarged spleen. The blood appears to be swamped with immature erythroblast-like cells. Despite these similarities, which prompted us to suggest that the two viruses induce the same type of erythroleukemia in chicken, the kinetics of development of the disease induced by RAV-1 is quite different from that of AEV.

In DNA samples from AEV infected leukemic birds BamHI digestion gives rise to a 2.6 kb internal fragment hybridizable to erb<sub>T</sub>. This is characteristic of the infecting AEV provirus. The absence of tumor specific fragments upon EcoRI digestion of AEV induced tumor DNA indicates the nonclonal origin of the tumor. EcoRI which cuts once close to the left end of the AEV provirus, also does not give rise to any DNA fragments which would be indicative of unintegrated AEV provirus, linear or circular. We thus conclude that the burst of erythroblasts observed in AEV-infected birds represents the proliferation of erythroblasts harboring integrated AEV provirus(es), rather than a massive spread of the AEV virus. The rapidity of this abrupt increase in erythroblasts is also observed in RAV-1 infected birds. Blood samples obtained from birds at the preleukemic stage do not have elevated levels of erythroblasts and show no tumor specific bands. Molecular hybridization analysis indicates that tumor specific bands are detected in the DNA from erythroblast


enriched fractions but not from the erythrocyte fractions (faint T.S. bands observed in the erythrocyte fraction are probably due to residual erythroblasts left in the fractionation procedure). Thus the burst of erythroblasts at the terminal stage of the disease is the result of the exponential growth of cells harboring the tumor specific sequences detected. The absence of the 2.6 kb fragment characteristic of AEV proviruses suggests that the tumor observed is not due to contaminating AEV in the RAV-1 stock used.

## Tumor specific fragments are heterogenous in size

If a tumor specific fragment is generated by the integration of ALV proviruses near an oncogene one would expect it to hybridize to probes of the ALV provirus. EcoRI cuts once in the ALV LTR separating it from the main body of the ALV. This would thus eliminate size variation of tumor specific fragments due to the possible alteration in the proviral structure. Hybridization of EcoRI digested tumor DNA with the LTR probe revealed a heterogenous population of tumor specific fragments. None of these fragments hybridize to probes made from oncogenes like myc, myb or src (data not shown). However, they all hybridize to erb. This points to the involvement of the erb gene in the transformation event which leads to a burst of erythroblast proliferation. Moreover, the tumor specific BamHI fragments are different sizes than the 2.6 kb erb fragment of v-erb indicating that AEV is not involved in these tumors. The only possible origin of the erb sequences in these tumor specific fragments are thus from the endogenous c-erb gene.



## Altered c-erb structure in RAV-1 induced erythroleukemia

Previous studies have suggested the possible existence of two different functional domains of v-erb which bear no sequence homology to each other (Sheiness <u>et al</u>., 1981). It has been shown that at least four distinct (poly A+) mRNAs are present, each of which hybridizes with only one of the v-erb domains (Vennstrom, in preparation). This implies an indication that c-erb, the cellular locus that gives rise to v-erb, is divided into two functional domains, possibly corresponding to the two domains of v-erb.

In our studies, analysis of DNA from uninfected birds with  $erb_{L}$ and  $erb_{R}$  shows no cross hybridization of these two probes to any given restriction fragment. We take this to mean that the two independent functional domains of c-erb are probably physically separated as well. The studies of AEV mutants (Royal-Pokora <u>et al.</u>, 1979; Beug <u>et al.</u>, 1980) have suggested a role of the 'A' domain in the transformation of erythroblasts. In the present study, we have encountered cases where only the 'B' locus is involved (e.g., chicken 3) as well as cases where both 'A' and 'B' loci are involved (e.g., chicken 4). There are also chickens (e.g. chicken 5, 6) that show no tumor specific fragments at all. One possible explanation would be the existence of restriction enzyme sites between the ALV and the erb sequence which separate the two sequences when that restriction enzyme is used.

# Alteration in the expression of c-erb

Employing the dot blot technique (Thomas, 1980) for RNA, we have detected a 100 fold or more elevation in the expression of v-erb in AEV infected cells at the leukemic stage. In chickens 2, 3 and 5 infected

-19-



control, no elevation in the expression of sarcoma genes was observed in any of these samples.

#### Promoter insertion or insertion mutagenesis?

The enhancement of c-myc transcription in lymphoid leukosis induced by ALV has been previously reported (Hayward <u>et al.</u>, 1981; Payne <u>et al.</u>, 1981). The mechanism by which this enhancement occurs is not understood. It has been suggested that the enhancement of transcription is due to the insertion of the LTR promotor upstream from an oncogene (Tsichlis and Coffin, 1980) and in this case the c-myc gene (Hayward <u>et al.</u>, 1981). Studies by Payne <u>et al</u>. have shown that ALV proviruses can assume several different configurations with respect to c-myc downstream or upstream in either orientation of transcription with respect to that of c-myc. Interestingly, all of these configurations resulted in an enhancement of the transcription efficiency of the c-myc. It therefore appears that the LTR can exert its influence by mechanisms other than or in addition to promotor insertion, for example, insertion mutagenesis (Payne <u>et al.</u>, 1981; Varmus et al., 1981).

Whatever the mechanism may be, these findings that the expression of c-myc was elevated in the majority of the tumors support the proposal (Hayward, 1981) that ALV exerts it oncogenic effects in lymphoid leukosis by increasing the expression of c-myc.

In our present study, we have observed an elevated level of transcription of erb sequences in many of our tumors. However, we have also observed tumors that apparently have no enhancement of erb expression. Nevertheless, exponential proliferation of erythroblasts was observed. Moreover, it has been observed that there are tumors in lymphoid leukosis

-20-





## EXPERIMENTAL PRODUCURES

## Viruses and route of infection

The RAV-1 virus stock used has been described (Fung <u>et al.</u>, 1981). The AEV virus stock ES4 was propagated in Qt6 cells.  $10^4$  infectious units of RAV-1 were introduced into either the yolk sac of embryos or i.p. into day-old chicks of the inbred line 15I.

#### Monitoring the progress of the disease

Beginning the fourth week after injection, chickens were monitored twice weekly for symptoms of disease. Blood was drawn from the wing veins for hematocrit and preparation of blood smear slides. Blood smear slides were prepared and stained with May-Grunwald stain as previously described (Lucas et al., 1961). Chickens diagnosed as having developed proliferative leukemia and/or anemia were sacrificed.

# Fractionation of cells and extraction of nucleic acids

The blood samples were fractionated by centrifugation in a table top centrifuge at 750 rpm for 10 minutes. Most of the supernatant was removed. The white layer of buffy coat cells on top was stirred and sucked up slowly with a pasteur pipette using a circular motion. The remaining erythrocyte fraction was resuspended in PBS (phosphate buffered saline) and the spinning repeated once more. The combined top layer of buffy coat was washed once before use.



## Extraction of nucleic acids

Nucleic acids were extracted as described (Fung <u>et al</u>., 1981). Total RNA was purified from contaminating DNA by repeated centrifugation using a cushion of 5.7 M CsCl in a SW41 rotor at 35 k for 24 hrs at 20°C. The RNA sample pelleted to the bottom and the DNA remained on the CsCl cushion. The RNA sample was further purified by digestion with DNAse I (Worthington) and ethanol precipitated before use.

# Enzyme digestion, electrophoresis, dot blot and hybridization

Restriction enzyme digestion, electrophoresis in 0.8% agarose gel and hybridization were performed as described (Fung <u>et al.</u>, 1981). Dot blots of RNA were essentially as described (Thomas, 1980).

# Preparation of 32P-DNA

 $32p-cDNA_{5}$  was prepared as previously described (Fung <u>et</u> <u>al.</u>, 1981). 32p-DNA gag,  $32P-DNA_{LTR}$ ,  $32p-DNA_{src}$ ,  $32p-DNA_{erb_T}$ ,  $32p-DNA_{erb_L}$ , and  $32p-DNA_{erb_R}$  were prepared by nick translation of cloned DNA. Briefly, 1 µg of gel purified DNA was mixed with 25 µCi each of 32p-dGTP, TTP, dATP and dCTP at 800 Ci/mmole (New England Nuclear) in 50 mM Tris pH 7.5, 10 mM MgSO<sub>4</sub>, 1 mM DTT and 50 µg/ml BSA. The reaction was started by adding 10 units of <u>E. coli</u> DNA polymerase I (New England Nuclear) and 200 pg of Sigma DNAase I. Nick translation was allowed to proceed at 14-16°C for 2 hours. The nick translated DNA was fractionated on a 10 ml G-50 column, and precipitated with two volumes of ethanol before use.



All the cloned DNA were derived from recombinant plasmid generously supplied to us by Drs. D. Sheiness, W. DeLorb and J.M. Bishop. DNAgag was obtained from a subcloned 1.35 k bp BamHI DNA fragment derived from the gag gene of ASV DNA as described (DeLorb <u>et al.</u>, 1980). DNA<sub>Src</sub> was obtained from a subcloned 0.8 k bp Pvu II DNA fragment derived from the src gene of ASV DNA.  $cDNA_{LTR}$  was obtained from a subcloned 0.32 k bp EcoRI DNA fragment derived from the LTR region of ASV DNA.  $DNA_{erb_{T}}$  was obtained from a subcloned 2.5 k bp PvuII DNA fragment (Sheiness <u>et al.</u>, 1981).  $DNA_{erb_{L}}$  was obtained from a PstI digest of the above mentioned PvuII subclone of AEV oncogene. This fragment corresponds to the onc<sub>AEV</sub> fragment A described by Sheiness <u>et al.</u>, 1981) and the  $DNA_{erb_{R}}$  corresponds to the onc<sub>AEV</sub> fragments C and D.



Figure 1. Kinetics of development of chicken erythroleukemia. The percentage measured by hematocrit at regular intervals post inoculation. AEV of erythrocyte (EC) (  $\Longrightarrow$  ) and buffy coat cells (EB) (----) were infected bird (---); RAV-1 infected birds: proliferative form (....); anemic form (0.0.0).

-25-







Figure 2. Blood smear slides of AEV and RAV-1 infected birds.

- (A) AEV infected bird at preleukemic stage;
- AEV infected bird at leukemic stage ( 5% buffy coat cells); (B)
- (C) RAV-1 infected bird at preleukemic stage;
- (D) RAV-1 infected bird at leukemic stage ( 10% buffy coat cells);
- (EC) erythrocyte; (EB); erythroblast.





- Figure 3. Analysis of DNA from tumor and normal tissues for the presence or Digestion hybridized to  $erb_T$ , a cDNA probe carrying most of the erb domain of chicken genomic DNA carrying an AEV provirus would generate a absence of AEV provirus. DNA samples were digested with BamHI, electrophoresed (0.8% agarose), Southern transferred, and of AEV. A schematic of the AEV proviral DNA is shown. signature fragment of 2.6 kb hybridizable to  $erb_{T^{\star}}$ 
  - Lanes a,b. Tumor tissue from AEV infected birds at the leukemic stage.
- c,d. Tumor tissue from RAV-1 infected birds at the leukemic stage.
- e,f. Normal tissue from uninfected birds of the same age.





- Genomic DNA samples buffy coat cells; (EC) eryuthrocyte; (PL) blood samples obtained at were digested with EcoRI or BamHI, electrophoresed (0.8% agarose preleukemic stage; (L) blood samples obtained at leukemic stage; Analysis of EcoRI and BamHI digestion patterns of genomic DNA (EB) gel), transferred and hybridized to a cDNA probe, erb<sub>T</sub>. (sp) spleen; (Bu) bursa; (♣) tumor specific fragment. samples from infected and uninfected chickens. Figure 4.
- A: (Lanes 1-4) uninfected chicken; (lanes 5-8) RAV-1 infected chicken that subsequently died of lymphoid leukosis with no sign of erythroblastosis.
- (Lanes 1,3) AEV infected chickens at preleukemic stage; (lanes 2,4) at terminal stage of erythroleukemia. н. В
- (lanes 1-6) chicken 1; (lanes 7-10) chicken 2; (lanes 11-14) RAV-1 infected chickens with proliferative erythroleukemia chicken 3; (lanes 15-18) chicken 4. ပ်
- D: RAV-1 infected chickens with proliferative erythroleukemia (lanes 1-4) chicken 5; (lanes 5-8) chicken 6.
- E: RAV-1 infected chicken that shows anemia, but no apparent proliferation of erythroblasts (see also Figure 1).











Ū.

Figure 5. Analysis of genomic DNA with cDNA probes specific for ALV and erb. electrophoresed (0.8% agarose gel), transferred to nitrocellulose DNA samples were analyzed by restriction digestion with EcoRI, paper, and hybridized to different probes:

erb<sub>T</sub>, a nick translated CDNA of the 2.5 kb AEV specific region that encompasses most of the v-erb domain of AEV (see Experimental Procedure Section).

domain that encodes the 5.3 and 6.1 kb mRNA reported by Sheiness et erbL, a nick translated cDNA of a 0.5 kb pstI restriction fragment. This corresponds to the unique sequence of the erb al. (1981).

this 0.9 kb region corresponds to the right end of the other erb restriction fragment and 0.4 kb BamHI/pvuII fragment. Together erbg, a mixture of nick translatedcDNA of 0.5 kb BamHI doma i n.

from a subclone of SRA-2 DNA corresponding to the right half of the LTR, a nick translated cDNa of a EcoRI restriction fragment gag region in SRA-2.

EC, erythrocyte; EB, erythroblast; kb, molecular weight in kilobases; N.D., not determined; , tumor specific fragments.

-35-





tion; 8, 8 weeks post inoculation, T, terminal stage, kb, molecular Figure 6. Kinetic of development of disease in chicken 4. Blood samples at restriction enzyme digestion analysis. 4, 4 weeks post inoculapreleukemic and leukemic stages were taken for EcoRI and sacI nucleotide of ALV LTR;  $\texttt{erb}_L$  and  $\texttt{erb}_T,$  see legend of Figure 5. weight in kilobases; 5',  $cDNA_5'$  corresponding to the 101

۰.


details. The chickens involved here are the same ones presented in cellular RNA was extracted from the tissues, purified to remove DNA Figure 7. Dot blot analysis of erb gene expression in avian erythroleukemia. contamination and spotted onto nitrocellulose paper according to the method of P. Thomas, 1978. See Experimental Procedures for EC, erythrocytes; EB, buffy coat cells; ck, chicken. Total previous figures.

-40-





cloned in the BamHI site of Ch28. These two clones overlapped to a site of Agtwes. The 6.6 kb BamHI fragment (Figure 4C, lane 18) was large extent and their restriction maps were combined into the one 7.1 kb EcoRI fragment (Figure 4C, lane 16) was cloned in the EcoRI Restriction maps of tumor specific fragments from chicken 4. The shown here, expressed in kilobases. Figure 8.

= portion of clone hybridizable to the LTR of ALV. = portion of the clone hybridizable to the  $erb_R$ Erbg LTR

portion of the 'B' loci only.

 $Erb_{L}$  and  $Erb_{R}$  = portion of the clone hybridizable to both

Erbl and Erbg.

(see Figure 5 for definition of probe used).



## REFERENCES

- Anderson, S.M., Hayward, W.S., Neel, B.G. and Hanafusa, H. (1980) Avian erythroblastosis virus produces two mRNA's. J. Virol. 36, 676-683.
- Beug, H. and Graf, T. (1980) Transformation parameters of chicken embryo fibroblasts infected with the ts34 mutant of avian erythroblastosis virus. Virology 100, 348-356.
- Beug, H., Kitchener, G., Doderlein, G., Graf, T. and Hayman, M. (1980) Mutant of avian erythroblastosis virus defective for erythroblast transformation; deletion of the erb portion of P75 AEV suggests function of the protein in leukemogenesis. <u>Proc. Natl. Acad. Sci.</u> <u>USA 77</u>, 6683-6686.
- Crittenden, L.B. (1975) Two levels of genetic resistance to lymphoid leukossi. <u>Avian Diseases</u> 19, 281-292.
- DeLorbe, W.J., Luciw, P.A., Varmus, H.E., Bishop, J.M. and Goodman, H.M. (1980) Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. <u>J. Virol.</u> <u>36</u>, 50-61.
- Fung, Y.K., Fadly, A., Crittenden, L.B., Kung, H.J. (1981) On the mechanism of retrovirus-induced avian lymphoid leukosis: deletion and integration of the proviruses. <u>Proc. Natl. Acad. Sci. USA</u> 78, 3418-3422.
- Gazzolo, L., Moscovici, C., Moscovici, M.G. and Samarut, J. (1979) Response of hemopoietic cells to avian acute leukemia viruses: effects on the differentiation of the target cells. <u>Cell</u> <u>16</u>, 627-638.
- Gazzolo, L., Samarut, J., Bouabdelli, M. and Blanchet, J.P. (1980) Early precursors in the erythroid lineage are the specific target cells of avian erythroblastosis virus in vitro. Cell 22, 683-691.
- Graf, T. and Beug, H. (1978) Avian leukemia virses: interaction with their target cells <u>in vivo</u> and <u>in vitro</u>. <u>Biochim. Biophys. Acta</u> <u>516</u>, 269-299.
- Graf, T., Ade, N. and Beug, H. (1978a) Temperature-sensitive mutant of avian erythroblastosis virus suggests a block of differentiation as mechanism of leukemogenesis. Nature 275, 496-501.
- Graf, T., Beug, H., von Kirchbach, A. and Hayman, M.J. (1980) Three new types of viral oncogenes in defective avian leukemia viruses. II. Biological, genetic, and immunochemical evidence. Cold Spring Harbor Quant. Biol., Vol. <u>44</u>, 1225-1234.
- Hanafusa, H.C., Halpern, C.C., Buchhagen, D.L. and Kawai, S. (1977) Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. 146, 1735.

- Hayward, W.S., Neel, B.G. and Astrin, S.M. (1981) ALV-induced lymphoid leukosis: activation of a cellular onc gene by promoter insertion. <u>Nature</u> (London) 290, 475-480.
- Lai, M.M.C., Neil, J.C. and Vogt, P.K. (1980) Cell-free translation of avian erythroblastosis virus RNA yields two specific and distinct proteins with molecular weights of 75,000 and 40,000. <u>Virology 100</u>, 475-483.
- Lucas, A.M. and Jamroz, C. (1961) <u>In</u> Atlas of Avian Hematology. <u>Agri.</u> Monograph 25, U.S. Department of Agriculture.
- Neel, B.G., Hayward, W.S., Robinson, H.L., Fang, J. and Astrin, S.M. (1981) Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. <u>Cell</u> 23, 323-334.
- Neiman, P., Payne, L.M. and Weiss, R.A. (1980) Viral DNA in bursal lymphomas induced by avian leukosis viruses. J. Virol. 34, 178-186.
- Pawson, T. and Martin, G.S. (1980) Cell-free translation of avian erythroblastosis virus RNA. J. Virol. 34, 280-284.
- Payne, G.S., Bishop, J.M. and Varmus, H.E. (1981) Multiple arrangements of viral DNA and an activated host oncogen (c-myc) in bursal lymphomas. Nature, in press.
- Payne, G.S., Courtneidge, S.A., Crittenden, L.B., Fadly, A.M., Bishop, J.M. and Varmus, H.E. (1981) Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell 23, 311-322.
- Purchase, H.R. and Burmeister, B.R. (1978) Neoplastic diseases. Leukosis/sarcoma group. In Diseases of Poultry, M.S. Hosfad, B.W. Calnek, C.F. Helmboit, W.M. Reid and H.M. Yoder, Jr., eds. (Ames, Iowa: Iowa State University Press), pp. 418-468.
- Purchase, H.E., Gilmour, D.G., Romero, C.H. and Okazaki, W. (1975) <u>Nature</u> 270, 61-62.
- Roussel, M.S., Saule, C., Lagrou, C., Rommens, C., Beug, H., Graf, T. and Stehelin, D. (1979) Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. <u>Nature</u> 281, 425-455.
- Royer-Pokora, B., Griesen, S., Beug, H. and Graf, T. (1979) Mutant avian erythroblastosis virus with restricted target cell specificity. <u>Nature</u> (London) <u>282</u>, 750-752.
- Sheiness, D., Vennstrom, B. and Bishop, J.M. (1981) Virus-specific RNAs in cells infected by avian myelocytomatosis virus and avian erythroblastosis virus: modes of oncogene expression by defective leukemia viruses. <u>Cell 23</u>, 291-300.

- Stehelin, D. and Graf, T. (1978) Avian myelocytomatosis and erythroblastosis viruses lack and transforming gene src of ASVs. <u>Cell</u> 13, 745-750.
- Stehelin, D., Saule, S., Roussel, M., Sergeant, A., Lagrou, C., Rommens, C. and Raes, M.B. (1980) Three new types of viral oncogenes in defectrive avian leukemia viruses. I. Specific nucleotide sequences of cellular origin correlate with specific transformation. Cold Spring Harbor Quant. Biol., Vol. 44, 1215-1224.
- Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. <u>Proc. Natl. Acad. Sci. USA</u> 77, 5201-5205.
- Therwath, A. and Scherrer, K. (1978) Post-transcriptional suppression of globin gene expression in cells transformed by avian erythroblastosis virus. Proc. Natl. Acad. Sci. USA 75, 3776-3780.
- Tsichlis, P.N. and Coffin, J.M. (1980) Recombinants between endogenous and exogenous avian tumor viruses: Role of the C region and other portions of the genome in the control of replication and transformation. J. Virol. 33, 238-249.
- Varmus, H.E., Quintrell, N. and Ortiz, S. (1981) Retroviruses as mutagens: Insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. Cell <u>25</u>, 23-36.
- Vennstrom, B., Fanshier, L., Moscovici, C. and Bishop, J.M. (1980) Molecular cloning of the avian erythroblastosis virus genome, and recovery of oncogenic virus by transfection of chicken cells. J. Virol. 36, 575-585.
- Yoshida, M. and Toyoshima, K. (1980) <u>In vitro</u> translation of avian erythroblastosis virus RNA: identification of two major polypeptides. Virology 100, 484-487.



# On the mechanism of retrovirus-induced avian lymphoid leukosis: Deletion and integration of the proviruses

(RNA tumor virus/B-lymphocyte tumor/proviral DNA/MC-29 oncogene)

YUEN-KAI T. FUNG<sup>\*</sup>, ALY M. FADLY<sup>†</sup>, LYMAN B. CRITTENDEN<sup>†</sup>, AND HSING-JIEN KUNG<sup>\*‡</sup>

\*Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824; and <sup>†</sup>U.S. Department of Agriculture, Science and Education Administration, Agriculture Research, Regional Poultry Research Lab, East Lansing, Michigan 48823

Communicated by Norman Davidson, March 2, 1981

ABSTRACT There is considerable evidence that infection by avian lymphoid leukosis viruses can lead to tumor development in the target organ of the host. The mechanism by which virus-induced oncogenic transformation occurs, however, is not clearly understood. As a first step toward deciphering this process, we have characterized the proviruses of the lymphoid leukosis viruses in DNAs extracted from the leukotic and metastatic tumors by using restriction enzyme digestion and filter hybridization analysis with radioactive probes specific for the infecting genome. Our results indicate (i) that lymphoid leukosis tumors are clonal in origin; (ii) that there are multiple sites in the cellular genome of the target tissue where the virus DNA can integrate and that, in the majority of the tumors, at least one such site of each tumor is adjacent to a cellular sequence related to the oncogene of MC-29 virus; and (iii) that deletions and other structural alterations in the proviral DNA may facilitate tumorigenesis.

The oncogenic retroviruses can be separated into at least two classes that appear to induce neoplasms by different molecular mechanisms. The more extensively characterized group includes viruses that induce rapid neoplasms, encode genes for cell transformation (probably of host origin), and are often defective, requiring a helper virus for infectivity or replication (1, 2). The second group induces neoplasms that have long latent periods, have no known genes coding directly for cell transformation, and are not defective in replication. Among these, some appear to have the potential for inducing several types of neoplasms (1, 2). The first class of viruses, although of basic interest in studies of in vitro cell transformation, are probably laboratory products, while the second class of viruses is likely to be responsible for the majority of naturally occurring retrovirus-induced neoplasms. Viral induction of avian lymphoid leukosis (LL) is an excellent model of neoplasm by a virus of the second group. The steps leading to mortality with LL include the infection of the target cell in the bursa of Fabricius, the transformation of the target cells not earlier than 3 to 4 weeks of age, the development of the grossly visible bursal tumor at 10-16 weeks of age, and the metastasis to visceral organs leading to massive lymphoid tumors and death, usually after 16 weeks of age (3).

The present studies are aimed at characterizing the newly integrated exogenous proviruses in LL tumor cell DNA to provide insight into the molecular events that lead to the development of LL.

### MATERIALS AND METHODS

Cell Culture, Viruses, and Biochemicals. A RAV-1 virus stock, purified by three cycles of propagation at limiting dilu-

tions, was used. Infection of chicken embryo fibroblast cultures was carried out at a multiplicity of 0.1, and the infected cells were passaged at least four times before DNA extraction. The media of such cultures contained a high level of reverse-transcriptase activity (4). For the synthesis of cDNA probes, concentrated Prague C virus, purified by repeated banding in sucrose gradients, was used (5). DNA polymerase I, DNase I, and restriction endonucleases were purchased from commercial sources, and  $[\alpha^{-32}P]dCTP$  was from ICN.

Induction of Lymphoid Leukosis. Day-old chickens of a cross between RPRL (Regional Poultry Research Lab) lines  $15I_5$  and  $7_2$  were inoculated by the intra-abdominal route with  $10^5$  infectious units of RAV-1. The chickens were free of common avian pathogens and reared in plastic canopy isolators to 12 weeks of age and then moved to semi-isolated cages. From 120 through 150 days, the birds were palpated for bursal enlargment twice weekly. Sixteen birds were taken for DNA extraction. All tissue samples were immediately transferred to vessels containing liquid nitrogen and then stored at  $-70^{\circ}$ C until use. For experiments to study the provirus in bursal tissue at preneoplastic stage, a portion of the bursa was surgically removed 4 weeks after virus inoculation.

DNA Extraction and Enzyme Digestions. Frozen tissues were homogenized in a glass barrel with a loose Teflon pestle in 40 vol of 10 mM Tris·HC1, pH 7.5/5 mM EDTA. Protease K (25  $\mu$ g/ml) and NaDodSO<sub>4</sub> (1%) were added to the homogenate. After incubation at 37°C for 2 hr, the solution was adjusted to 0.1 M NaCl and extracted with phenol/chloroform. The DNA samples were concentrated by EtoH precipitation. Digestions of DNA with restriction endonucleases were conducted at 37°C for 2 hr. The digested DNAs were analyzed on 0.8% agarose gels and then transferred to nitrocellulose paper and hybridized with appropriate radioactive probes as described (6).

Hybridization Reagents. The radiolabeled nucleotides in all of the following probes were derived from  $[\alpha^{-32}P]dCTP$ . (*i*) cDNA<sub>3'</sub>, which carries the 3'-terminal sequences ( $\leq 200$  nucleotides) of the viral genome, was synthesized by using the avian myeloblastosis virus polymerase on  $\leq 8S$  poly(A) containing RNA and oligo(dT)<sub>12-18</sub> (P-L Biochemicals) as primer. Oligo(dT)-primed cDNA<sub>3'</sub> was then purified by chromatography twice on oligo(dT)-cellulose after hybridizing to poly(A) (6). (*ii*) cDNA<sub>5'</sub>, which represents the 5'-terminal 101 nucleotides of the viral genome, was synthesized by using detergent-activated virion as described (7) and purified by isolation of the 101-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: LL, lymphoid leukosis; LLV, lymphoid leukosis virus; MDal, megadalton(s); LTR, long terminal repeat; ev, endogenous viral; TS, tumor specific; CSV, chicken syncitial virus. <sup>‡</sup> To whom reprint requests should be addressed.

#### Biochemistry: Fung et al.

mer from a 10% polyacrylamide gel (7). (iii) cDNA<sub>rep\*</sub> was synthesized in the same way as cDNAs, except that the gel-purification step was omitted. This probe, enriched in cDNAs, carries ≥80% sequences of the entire genome. It is capable of detecting all three Sac I-derived endogenous virus fragments corresponding to the major loci as described by Astrin et al. (8). In addition, cDNAren\* also detects a 2.5-megadalton (MDal) end fragment (see Fig. 1B, lanes 1 and 2), which preferentially hybridizes to cDNAs, (iv) DNA probes specific for the oncogene of MC-29 (avian myelocytomatosis virus strain 29) (1, 9) were prepared by nick translation (10) of a plasmid clone, pMyc-Pst, supplied to us by D. Sheiness and I. M. Bishop (University of California, San Francisco). pMyc-Pst, which carries principally the putative oncogene, was derived by subcloning a Pst fragment of a DNA clone carrying the entire MC-29 genomic sequence.

### RESULTS AND DISCUSSION

Viral Etiology and Development of Lymphoid Leukosis. Twenty-nine day-old (154, ×7, 2) chickens were innoculated with avian lymphoid leukosis virus (LLV), RAV-1. All birds either died of or were killed bearing lymphomas by 253 days of age. Tissues were taken from 16 of the birds for DNA extractions and histopathological examinations. Among these 16, all except 1 contained lesions in the bursa of Pabricus, 53 add oeveloped secondary spleen or liver tumors. Thus, in our experimental system, a near 100% incidence of bursal lymphoma was obtained after virus inoculation. Such a high lymphoma incidence, together with the presence of RAV-1 provinues in all the tumor samples (see below), is consistent with a viral etiology for this disease.

Strategies for the Identification of Exogenous Provirus. The studies described here are principally based on digestion analyses with Sac I and EcoRI and hybridization with the sequencespecific probes ONA-epre- ONA-are, and CDA-2, CDA-uprise sequences representing the entire RAV-1 viral genome. (e)DNA<sub>3</sub> and CDNA<sub>5</sub>, on the other hand, are specific for the 3' and 5' terminal sequences of the viral RAS apome (see Materials and Methods). The sequences contained in cDNA<sub>3</sub> and CDNA<sub>4</sub> (shown in Fig. 1 As aboved 3 and 5) together comprise the long terminal repeat (LTR) present at both ends of the provirus. As the 3'-terminal region (=200 nucleotides) of the RAV-1 genome does not share much homology with any endogenous viral (ev) sequences. I chromosome (11, 12), we have used cDNA<sub>3</sub>, extensively to distinguish the infecting RAV-1 DNA from ev sequences.

Most chickens of a  $(15I_5 \times 7_9)$  cross have three ev loci, ev 6, ev 1, and ev 2.§ We have used Sac I digestion to document the presence of exogenous proviruses in tumor DNAs and to identify their integration patterns. This is based on the following considerations: First, Sac I has a single cleavage site in RAV-I proviral DNA, and the fragment sizes are determined not only by the location of this site in the viral genome but also by the nearest enzyme cleavage site in the flanking cellular sequence (Fig. 1A). Therefore, Sac I digestion can provide information concerning the integration site of exogenous proviral DNA. Second, as shown by Astrin and coworkers (8, 13), Sac I digestion of normal chicken DNAs gives a relatively simple fragmentation pattern of the ev sequences; additional bands corresponding to the newly integrated exogenous provirus in the tumor DNA can be readily identified. On cleavage of the genomic DNA with Sac I and hybridization with cDNArep\*, the ev sequences are shown as four bands of Mr 13, 5.9, 3.7, and



FIG. 1. Restriction enzyme cleavage maps of a colinearly integrated RAV-1 provirus DNA and identification of tumor-specific (TS) proviral DNA. Mr in MDal. (A) Cleavage maps of EcoRI and Sac I. Open triangles indicate Eco RI sites not present in the ev sequences. represents the LTR, which is located at both termini of the viral DNA and carries the 3'- and 5'-terminal sequences of the RNA genome. The wavy line denotes the flanking cellular sequences. The bars indicate the EcoRI fragments detectable by CDNA5. (B) Restriction enzyme digestion analysis of proviral DNA. The DNA samples were extracted from bursa tumor 10 (lanes 2 and 4), from the nontumorous thymus (lanes 1 and 3) of the same bird, from the in vitro RAV-1-infected (lane 6) or uninfected (lane 5) chicken embryo fibroblasts of line (15Is × 7a). and from the bursal tissues of a bird inoculated with RAV-1 4 weeks earlier (lane 8) and of an uninoculated bird (lane 7). They were digested with Sac I or EcoRI and analyzed on 0.8% agarose gels and by Southern blotting hybridizations with cDNArep, and cDNA3.

2.5 MDal. In the example shown in Fig. 1B, both nontumor (lame 1) and tumor tissue (lane 2) DNA display these four bands. DNA from the tumor displays two additional bands (M, 8 and 4.0 MDal), which we refer to as tumor specific or TS bands. The evogenous origin of the TS bands was established by hybridization with cDNA<sub>3</sub>, which detects only RAV-1 DNA. The specficitivy of this probe is shown by the complete absence of er-related fragments in the DNA from nontumor tissue (lane 3). Hybridization of the tumor DNA with cDNA<sub>3</sub> (lane 4) shows two distinct bands with size identical to the TS bands detected by cDNA<sub>3</sub>,...

In contrist to Sac 1, there are several cleavage sites for EcoRIin the viral genome, which therefore allows us to analyze the internal structural arrangement of the exogenous provinal DNA (Fig. 1A). More important, ev sequences lack the two outer EcoRI sites (indicated by open triangles), which are found onlyin the exogenous provinal DNA. Consequently, either the 1.4or the 0.7-MDI argument specifically indicates the presence ofev sequences in cellular DNA, as seen by comparing the DNApattern of a RAV-1 infected culture of chicken embryo fibroblasts with that of an uninfected culture (lanes 5 and 6). The 1.4-MDaI framemer (indicated by triangle) is present only in the

<sup>§</sup> Among the 16 characterized birds, numbers 1–13 carry all three ev loci. Numbers 14–16 lack ev 2.



FIG. 2. TS proviral DNA as identified by Sac I digestion. (A) cDNA<sub>3</sub>, hybridization with the DNA samples isolated from bursal or liver (L) tumors. Lane C (control) represents the normal thymus DNA of bird 1. (B) cDNArep. hybridization with the DNA samples from bursal nodules 1 (B1) and 2 (B2) and normal thymus (T) of bird 10. Dots indicate the TS bands-i.e., fragments detected in the tumor

infected sample (lane 6). Indeed, this specific exogenous viral marker enabled us to demonstrate that, in >90% of the RAV-1 inoculated birds, extensive infection of the bursa tissue had occurred as early as 4 weeks after inoculation; a typical example is shown in lane 8, where the 1.4-MDal fragment can be seen in the 4-week bursal DNA of the inoculated bird. This band. however, is absent in the bursal DNA of an uninoculated control (lane 7)

Newly Acquired Provirus in Tumor DNA and Clonality of the Tumors. As discussed above, Sac I digestion in conjunction with cDNA37 hybridization provides a sensitive means for identification of the integration pattern of the newly acquired proviruses. A survey of DNA of all bursal tumors by this analysis shows that each tumor DNA displays at least one TS band (Fig. 2A), providing strong evidence that all tumors acquired at least all or a portion of one exogenous provirus.

It is noteworthy that DNA samples taken from bursal tissues of birds at preneoplastic stages, when assaved by the same method, do not have any TS band, although extensive infection of the target tissue by exogenous viruses can be documented (Fig. 1B; unpublished results). These data suggest that the initial infection of the target tissue by RAV-1 results in the integration of proviral DNA at many sites in the cellular genome of a large number of cells. The fact that TS bands can be identified in all tumors at the terminal stage indicates that each tumor results from selective growth of a homogeneous population of cells (which are characterized by a common proviral DNA structure). The origin of the tumors, therefore, is probably clonal. This conclusion is further supported by the observation that DNAs isolated from multiple tumor nodules located on the same bursa display TS bands distinct from one another, indicating that these different tumor nodules are derived from independently infected and transformed cells. An example is given in Fig. 2B; the two bursal tumor nodules (B1 and B2) of bird 10 have entirely different Sac I-TS band (indicated by dots) patterns when compared with each other or with the normal thymus tissue control (lane T). These observations are consistent with the results of others (14-16), which also indicated that

LL tumors are consequences of clonal growths of transformed cells.

The data in Fig. 2 also show the size variation of TS bands in different tumors, suggesting that integration in a number of sites can lead to the development of a tumor. However, another equally plausible, but not mutually exclusive, possibility is that deletion within the proviral DNA contributes to size variation.

Frequent Deletion of the Provirus in Tumor DNA. Evidence for the deletion of viral sequences from some of these proviruses was provided by experiments in which EcoRIcleaved tumor DNA was hybridized with cDNA5' probe. Fig. 1A shows that cDNA5' can specifically detect the 1.4-MDal EcoRI fragment near the left end, which carries the entire gag (group-specific-antigen) sequence. As discussed above (Fig. 1B), the 1.4-MDal gag-containing fragment can be readily detected in the undeleted RAV-1 provirus found both in in vitro infected cells and in the bursal tissue of inoculated birds at preleukosis stages. By contrast, in many tumor DNAs (e.g., 2, 3, 5, 9L, and 12 in Fig. 3A), the 1.4-MDal fragment (triangle) is completely absent. A similar conclusion was reached from hybridizations with cDNAnn or probes specific for the gag sequences and from Sac I digestion analysis (data not shown). These data thus demonstrate that some of the RAV-1 provirus in the LL tumors have undergone extensive structural alteration.

Multiple Integration Sites of the Proviruses in Tumor DNA. Hybridization of EcoRI-cleaved tumor DNA with cDNAz- also detects the right-end viral-cell junction fragment and provides reliable information concerning the integration site of proviral DNA (Fig. 1A), because the  $M_r$  of such fragments cannot be influenced by the potentially extensive deletion(s) in the viral genome. To identify the junction fragments, individual tumor DNAs were compared with DNAs from normal tissues (e.g., thymus or muscle) of the same animals. The representative samples of normal tissue DNAs shown in lanes C1 and C2 of Fig. 3A serve as controls for tumor DNA samples in lanes 1-13 and 14-16, respectively. In both controls, only the fragments corresponding to the endogenous viruses were detected: there are seven EcoRI-ev fragments in C1 DNA, including the very faint



FIG. 3. Deletion and integration of the proviruses as analyzed by EcoRI digestion. (A) cDNA5 hybridization with DNA samples of bursal or liver (L) tumors developed in birds 1-16. Normal thymus controls, C1 and C2, are from birds 9 and 16. (B) pMvc-Pst hybridization with representative tumor DNA samples. Triangles indicate the 1.4-MDal EcoRI fragments and stars represent the right-end viralcell junction fragments, M, in MDal.

Table 1. Identification of fragments

Bird	Sample	M <sub>r</sub> of right- end cell-viral junction, MDal	EcoRI 1.4-MDal fragment
1.	Bursa*	1.8,† 1.3, 0.9	+, Δ
2.	Bursa <sup>‡</sup>		Δ
3.	Bursa	$2.3, 1.7^{+}$	Δ
4.	Bursa	1.8 <sup>+</sup>	+
5.	Bursa	$2.8, 1.8^{+}$	Δ
6.	Bursa	2.0+	+
7.	Bursa	2.0 <sup>+</sup>	+
8.	Liver	2.8, 2.6, 1.85 <sup>+</sup>	+
9.	Bursa	1.7 <sup>+</sup>	+
	Liver 1	2.0,† 1.7	Δ
	Liver 2	$2.0,^{+}1.7$	Δ
	Liver 3	2.0, + 1.7	Δ
	Liver 4	2.0,† 1.7	Δ
10.	Bursa 1	ND	ND
	Bursa 2	$2.4,^{\dagger}1.7, 1.5$	Δ
11.	Bursa	1.7*	+
	Liver	1.7+	Δ
	Spleen	1.7*	Δ
12.	Bursa	1.75 <sup>+</sup>	Δ
13.	Bursa <sup>‡</sup>		+
14.	Bursa	1.8 <sup>†</sup>	+
15.	Bursa	2.4,* 1.8,* 1.7*	+
16.	Bursa	1.8+	+

Right-end cell-viral junction fragments were identified by cDNA<sub>5</sub>. \* Bird 1 carries three proviruses; two of them carry deletion in the gag gene, and the other appears to carry an intact *EcoRI* 1.4-MDal fragment.

<sup>+</sup> Also detectable by pMyc-Pst.

\* Although the detections of the right-end junction fragments by CDNAs, in birds 2 and 13 are not obvious, TS Fragments hybridizable to pMy-Pst are present in these tissues. Birds 2 and 13 carry encyc containing TS fragments of 1.5 and 2.4 MDal, respectively. +, The left-end internal EcoRI 1.4-MDal fragment is present; a, the EcoRI 1.4-MDal fragment is absent: ND. not determined.

1.7-MDal band, which is weakly detectable by cDNA<sub>5'</sub>. C2 DNA has a similar EcoRI cleavage pattern, except that the two small fragments (1.9 and 1.7 MDal) of ev 2 are missing. When the tumor DNAs were compared with these controls, new fragments of different sizes appeared. Those fragments, indicated by stars, were identified as right-end cell-viral junction fragments<sup>¶</sup> and their sizes are given in Table 1. (Identification of some of the new fragments that migrate at positions close to the ev fragments-e.g., the 1.7-MDal band-was aided by the significantly higher intensity of that band seen in tumor tissue over the corresponding ev fragment observed in normal tissue DNA of the same bird.) The size heterogeneity of the end fragments indicates multiple integration sites. However, it appears that the right-end junction fragments in the size range 1.7-2.5 MDal are more common than others. It is also noteworthy that, in several cases, the tumor DNA carries more than one TS end fragment and, hence, more than one provirus. These multiple RAV-1 proviruses possibly resulted from multiple virus infections of the progenitor cell of a monoclonal tumor. Alternatively, these samples may represent semiclonal tumors in which several tumor clones coalesced together, as has been suggested for certain terminal LL tumors, based on histopathological evidence (17).

Linkage of the RAV-1 Provirus with the MC-29 Related Endogenous Sequences, Recent studies by Hayward et al. (18) strongly implicate a cellular sequence related to the oncogene of the acute leukemia virus, MC-29, in LL virus leukemogenesis. The progenitor sequence of MC-29 oncogene (designated as c-myc) has been shown to be highly conserved and present in the genomes of all vertebrates (9). We wished to determine whether the infecting RAV-1 DNA is physically linked to the c-myc in the LL tumors characterized in this study. To examine this possibility, a cloned DNA pMvc-Pst that specifically carries the MC-29 oncogene sequence was used as a molecular hybridization probe. Representative samples for pMyc-Pst hybridizations to EcoRI-cleaved tumor DNAs are shown in Fig. 3B. In normal tissue (lane C), only one high M, band corresponding to the *c-muc* locus is detected; in the tumor tissues (lanes 7, 8, etc.), additional bands (indicated by stars) are also observed. The sizes of these additional bands are primarily in the 1.7-2.5 MDal range and match well with the corresponding viral-cell junction fragments assigned by hybridization with cDNA<sub>37</sub> in Fig. 3A. These results indicate that, in these LL tumor DNAs, the c-muc gene (on one of the two chromosomes) is joined with the RAV-1 provirus. Based on this analysis, we could demonstrate that, in all tumors in which the right-end junction fragment can be clearly detected by cDNA5, linkage between the RAV-1 provirus and the c-mvc sequence exist (see Table 1). In most of the samples in which multiple RAV-1 proviruses are present, a single one is linked to the c-myc sequence. In one case (i.e., bird 15, Table 1), all three proviruses are linked to the c-mvc. We take the most straightforward interpretation and suggest that bird 15 bursal tumor consists of three coalescing tumor clones and each carries a RAV-1 provirus integrating next to the c-muc gene, but at a slightly different position.

On the Mechanisms of Oncogenic Transformation. The mechanism by which LLV induces oncogenic transformation is especially intriguing because there is no evidence indicating that LLV codes for an oncogenic product. It has been postulated that specific integration of the LLV DNA into a site near a host oncogene might promote the expression of the oncogene (19). This possibility is particularly attractive in view of the fact that the two LTRs flanking the viral genome contain characteristics of promoters for oukaryoit transcription (20, 21) and that the sequence in the left-end LTR participates in the genesis of viral mRNAs (22, 23). Similarly, the rejeties in the genesis of viral identification of novel mRNAs species in LLV induced tumors, which earry both LTR-related sequences and sequences possibly of host origin supports this hypothesis (15, 16, 18).

The relationship of specific integrations to oncogenic transformation. Hayward et al. (18) have recently reported that, in the LL tumors, LLV proviruses are integrated next to the c-myc genes and that enhanced expression of MC-29 sequences are observed (18). These authors have suggested that insertion of the LLV provirus promotes the expression of the c-muc gene, thereby triggering the oncogenic transformation. Our data confirm some of their observations. We find that, in most of the LL tumors described here, at least one RAV-1 provirus of each tumor is covalently joined to the endogenous myc locus; however, as seen by the various sizes of the RAV-1-oncMCV joining fragments, the exact integration sites of RAV-1 proviruses are not always identical in individual tumors. These results suggest that integration of RAV-1 at one of several sites near the c-myc gene is conducive to transformation. Recently, we have extended this analysis to the LL-like tumors induced by chicken syncitial vi-

For those samples which carried deletions in the 1.4-MDaf fragment, it is important to rule out the possibility that these new bands of novel sizes are derived from the gag containing 1.4 MDaI internal fragment by structural alterations. This was accomplished by further hybridization of these bands with DNA probes specific for gag region. All of the right-end fragments assigned above failed to hybridize to such a probe.

ruses (CSV). We have previously shown that CSV, a member of the reticuloendotheliosis virus that bears no genetic relationship to LLV, is capable of inducing LL with similar latency and pathology (25). In this case too, we have been able to demonstrate linkage between the c-myc the CSV provirus in all tumors characterized (unpublished results). As CSV DNA and RAV-1 DNA, including their LTRs, share very little sequence homology with each other (26, 27), the finding that they are both integrated at positions next to the *c-myc* gene in LL tumors strongly implicates this gene and, possibly, adjacent sequences in the transformation of lymphocytes. The detailed mechanisms whereby the integration of either RAV-1 or CSV promotes the expression of the *c-myc* gene have yet to be elucidated.

The significance of the viral deletions to oncogenic transformation. One striking finding is the detection of extensive deletions of proviral DNA in at least 40% of the tumors analyzed. It is possible that deletions of the viral genome that disrupt the transcriptional program of viral RNA facilitate the transcription of the downstream cellular sequences. Perhaps the transcription of viral RNA from the left LTR extending into the right LTR may affect the initiation at the right LTR. A disruption of the transcriptional program caused by a deletion in the proviral DNA may expose the right LTR and allow efficient transcription of the downstream putative oncogene. The following observations are consistent with the importance of the LTR in the transformation process: (i) all tumor tissues analyzed in this study contain at least one LTR sequence (identified by cDNA<sub>3'</sub> and  $cDNA_{5'}$  probes) and (*ii*) one tumor (5) harbors extensively deleted proviruses which possess very little, if any, viral sequences other than the LTRs (unpublished data).

Alternatively, the deletion of viral sequences may play a role in the selective growth of the tumor clones. Those cells in which the expression of viral antigens is eliminated by deletion may therefore be rendered less immunogenic and able to escape the host immune response. Histopathological examination shows that, at the onset of the disease, there are many microscopically observed enlarged bursal follicles (considered to be the transformed cell clones) (28, 29). Immune selection may account for the finding that only a limited number develop into tumors.

Irrespective of the role of deletion of provirus in the tumorigenic process, our data show that the presence of a complete provirus is not required at the terminal stage of the tumor. This finding lends further support to the hypothesis that the oncogene(s) involved in the maintenance of cells in the transformed and tumorous state is of cellular rather than of viral origin.

We are grateful to Drs. D. Sheiness and J. M. Bishop for providing the pMyc-Pst DNA clone. We thank Drs. G. Payne and H. E. Varmus for communicating data before publication, Mr. Lenny Provencher for excellent technical assistance, and Dr. S. Dube for helpful discussions. We also thank Drs. M. Fluck, E. Fritsch, and J. Dodgson for helpful comments and Ms. S. Uselton for assistance in manuscript preparation. This work was supported by a grant from the Michigan State University Foundation to H.-J.K. and in part by Interagency Agreement 1-cp-40214 with the Division of Cancer Cause and Prevention, National Cancer Institute.

- Graf, T. & Beug, H. (1978) Biochim. Biophys. Acta Rev. Cancer 516, 229–259.
- 2. Robinson, H. (1978) Curr. Topics Microbiol. Immunol. 83, 1-36.
- Crittenden, L. B. (1980) in Viruses in Naturally Occuring Cancers (Cold Spring Harbor Laboratories Cold Spring Harbor, NY), pp. 529-546.
- Robinson, H., Swanson, C. A., Hruska, J. F. & Crittenden, L. B. (1976) Virology 69, 63-74.
- Kung, H. J., Bailey, J. M., Davidson, N., Vogt, P. K., Nicolson, M. O. & McAllister, R. M. (1974) Cold Spring Harbor Symp. Quant. Biol. 39, 827-834.
- Shank, P. R., Hughes, S., Kung, H. J., Guntaka, R. V., Bishop, J. M. & Varmus, H. E. (1978) Cell 15, 1383-1395.
- Friedrick, R., Kung, H. J., Baker, B., Varmus, H. E., Goodman, H. M. & Bishop, J. M. (1977) Virology 79, 198–215.
- Astrin, S. M., Robinson, H. L., Crittenden, L. B., Bluss, E. G., Wyban, J. & Hayward, W. S. (1979) Cold Spring Harbor Symp. Quant. Biol. 44, in press.
- Sheiness, D. K., Hughes, S. H., Varmus, H. E., Stubblefield, E. & Bishop, J. M. (1980) Virology 105, 415–424.
- Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) Proc. Natl. Acad. Sci. USA 72, 1184–1188.
- 11. Neiman, P. E., Das, S., MacDonnel, D. & McMillin-Helsel, C. (1977) Cell 11, 321–329.
- 12. Coffin, J. M., Champion, M. & Chabot, F. (1978) J. Virol. 28, 972-991.
- 13. Tereba, A. & Astrin, S. M. (1980) J. Virol. 35, 888-894.
- 14. Neiman, P., Payne, L. N. & Weiss, R. A. (1980) J. Virol. 34,
- 178-186.
  15. Neel, B. C., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. A. (1981) Cell 23, 323-334.
- Payne, G. S., Courtneidge, S. A., Crittenden, L. B., Fadly, A. M., Bishop, J. M. & Varmus, H. E. (1981) Cell 23, 311-322.
- 17. Purchase, H. G. & Burmester, B. R. (1978) in *Diseases of Poultry* (Iowa State Univ. Press, Ames, IA), 7th Ed., Chapt. 15, pp. 437-438.
- Hayward, W., Neel, B. G. & Astrin, S. M. (1981) Nature (London) 290, 475–480.
- 19. Tsichlis, P. N. & Coffin, J. M. (1980) J. Virol. 33, 238-249.
- Yamamoto, T., Jay, G. & Pastan, I. (1980) Proc. Natl. Acad. Sci. USA 77, 176–180.
- 21. Yamamoto, T., de Crombrugghe, B. & Pastan, I. (1980) Cell 22, 787-797.
- 22. Weiss, S. R., Varmus, H. E. & Bishop, J. M. (1977) Cell 12, 983-992.
- 23. Melon, P. & Duesberg, P. H. (1977) Nature (London) 270, 631-634.
- Quintrell, N., Hughes, S., Varmus, H. E. & Bishop, J. M. (1980) J. Mol. Biol. 143, 363–393.
- 25. Witter, R. L. & Crittenden, L. B. (1979) Int. J. Cancer 23, 673-678.
- Shimotohono, K., Mizutani, S. & Temin, H. M. (1980) Nature (London) 285, 550-554.
- 27. Kang, C. & Temin, H. M. (1973) J. Virol. 12, 1314-1324.
- Cooper, M. D., Payne, L. N., Dent, P. B., Burmester, B. R. & Good, P. A. (1968) J. Natl. Cancer Inst. 41, 373–389.
- Neiman, P., Payne, L. N. & Weiss, R. A. (1980) in Viruses in Naturally Occuring Cancers (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 519–528.







