MOLECULAR ASPECTS OF TURKEY HERPESVIRUS LATENCY IN CHICKENS: IDENTIFICATION AND LOCALIZATION OF LATENT INFECTIONS

By

Margo Steele Holland

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1994

ABSTRACT

MOLECULAR ASPECTS OF TURKEY HERPESVIRUS LATENCY IN CHICKENS: IDENTIFICATION AND LOCALIZATION OF LATENT INFECTIONS

By

Margo Steele Holland

Marek's disease virus (MDV) is composed of three serotypes. Marek's disease virus serotype 3 is also known as turkey herpesvirus (HVT) because it was originally isolated from turkeys. All 3 MDV serotypes induce productive and latent infections. Productive infections result in extensive viral antigen expression while latent infections have limited or no viral antigen expression in the presence of the viral genome. During a productive infection, structural viral proteins such as glycoprotein B are produced. Based on indirect immunofluorescence assays (IFA) and co-cultivation assays, expression of HVT gB was detected in lymphoid tissue, the spleen, thymus and bursa. Criteria for a latent infection was the presence of HVT genome in the absence of HVT gB expression. In situ hybridization detected HVT RNA while IFA was used to demonstrate gB was not expressed. Latent infections were found in the spleen, thymus, bursa, sciatic plexus, brachial plexus and feather tips. In lymphoid tissue, spleen, thymus and bursa, latent HVT RNA was present in CD4+ and CD8+ lymphocytes. *In vivo* studies indicate that a latent HVT

infection occurs in the bursa but the infection is subsequently cleared from the bursa. In the brachial plexus and sciatic plexus, satellite cells have latent HVT, however, the cell type harboring latent HVT in the feather tips could not be identified. To investigate the reduction in transcription during latency, probes representing 80% of the HVT genome were used to identify the region of the genome that is transcriptionally active during latency. Only the repeat regions flanking the Unique long region, the junction between the inverted repeats flanking the unique long and unique short regions and a small portion of the unique region of the latent HVT genome was transcribed. Limited transcription during latency may be involved in controlling either the establishment, maintenance or reactivation from the latent state.

To my husband, Robert E. Holland
without whose love, support,
encouragement and occasional tirade
this dissertation would not have been completed.
and
to my beautiful daughter, Ashley N. Holland
who gave me the inspiration
to finish.

A special dedication to
my father, John T. Steele
and mother, Gwendolyn G. Boutte.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to the members of my graduate committee. Special thanks to my research advisor, Dr. Robert Silva for allowing me to grow professionally and for his encouragement, expertise, and infinite patience. I would like to thank Dr. Bull for his constant support, advice, and helpful discussions. To Dr. Mackenzie, thank you for your belief in my abilities, guidance, and unwavering support. I would also like to thank Dr. Witter for his constructive suggestions and giving me the opportunity to use the facilities at the Avian Disease and Oncology Laboratories. Last, but not least, I thank Dr. Velicer for his helpful comments and suggestions.

I would like to thank my Tuskegee family here at Michigan State: Dr. Sheila Grimes for her constant encouragement, friendship and keeping me from being ABD. Also, thanks to Dr. Eric Kufour-Mensah for his friendship and moral support. A special thanks to Mrs. Pat Lowrie for always being there when I needed her.

I gratefully acknowledge all the individuals at the Avian Disease and Oncology Laboratory who assisted me by providing technical assistance and expertise. There are no words that can adequately express my gratitude to my Aunt,

Laura Adams, brother, John Steele, and stepfather, Robert Boutte.

I appreciate the financial support given to me by the Equal Opportunity Program Programmatic Fund which allowed me to concentrate solely on my graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

Chapter II:

Chapter III:

Chapter IV:

Chapter V:

Chapter VI:

LIST OF FIGURES

Chapter I:

Chapter II:

Chapter IV:

LIST OF ABBREVIATIONS

AG876	EBV cell line
B95	EBV cell line
BJAB	EBV cell line
BL	Burkitt's lymphoma
BP	Basepair
BHV	Bovine herpesvirus
BVDU	(E)-5-(2-bromovinyl)-2'deoxyuridine
BZLF-1	leftward open reading frame from the BamHI-Z fragment of EBV genome
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
C15	Burkitt's lymphoma cell line
CALLA	Burkitt's lymphoma marker
CD	Cluster designation
CEF	Chick embryo fibroblasts
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
DPI	Days post infection
DS	Dyad symmetry elements
EBER	Epstein barr encoded small RNA
EBNA	Epstein barr nuclear antigen
FC126	Strain of HVT

FFE	Feather follicle epithelium
FP	BamHI-F promoter
FR	Family of repeat elements
G:C	Guanine:cytosine
G/M	Grams per mole
GA-5	Serotype 1 MDV strain
HRS	Hodgkin Reed Sternberg
HSV	Herpes simplex virus
HVT	Turkey herpesvirus
IBR	Infectious bovine rhinotracheitis
ICAM	Intracellular adhesion molecule
ICP	Infected cell protein
IETU-1	Immediate early transcription unit 1
INF-alpha	Interferon-alpha
IR_L	Inverted repeat long
ISH	In situ hybridization
JC-5	EBV cell line
JM-10	Serotype 1 MDV strain
KB	Kilobase
KDa	Kilodaltons
LAT	Latency associated transcript
LCL	Lymphoblastoid cell line
LFA	Leukocyte function associated
LMF	Latency media factor
LMP	Latent membrane protein
LPBF	Latent promoter binding factor
MCMV	Murine cytomegalovirus

MD	Marek's disease
MDCC-BO1	Serotype 1 MDV and HVT infected lymphoid cell line
MDV	Marek's disease virus
MHC	Major histocompatibility complex
MKT-1	Serotype 1 MDV and HVT infected lymphoid cell line
mRNA	messenger ribonucleic acid
N-linked	Amino
N-line	Strain of chickens
NPC	Nasopharyngeal carcinoma
NM	Nanometer
ORF	Open reading frame
P-line	Strain of chickens
PBMC	Peripheral blood mononuclear cells
PFU	Plaque forming unit
POLY A+	Polyadenylated
PRV	Pseudorabies
RAE-1	Burkitt's lymphoma cell line
Raji	EBV cell line
RNA	Ribonucleic acid
S3	Guanine or cytosine in codon 3
SB-1	Serotype 2 MDV strain
SV40	Simian virus 40
SVV	Simian varicella virus
T10	Thoracic vertebrae 10
TK	Thymidine kinase
TPC	Thyroid papillary carcinoma cell line
TR_L	Terminal repeat long

TS	Temperature sensitive
VIA	Viral internal antigens
VP16	Viral protein 16
VZV	Varicella zoster virus
X50-7	EBV cell line
ZEBRA	BamHI-Z EBV replication activator

INTRODUCTION

Herpesviruses are of economic importance in both mammalian and avian species. Marek's disease (MD) is an avian lymphoproliferative neoplastic disorder caused by a herpesvirus. Marek's disease virus (MDV) is divided into 3 serotypes. Serotype 1 consist of oncogenic viruses and their attenuated forms while serotype 2 are nononcogenic viruses. Serotype 3 are naturally occurring nononcogenic viruses isolated originally from turkeys (HVT). Turkey herpesvirus is the predominant vaccine used worldwide to prevent the occurrence of tumors induced by MDV serotype 1. However, the vaccine is not 100% effective and tumors still develop especially with very virulent strains of MDV serotype 1. Even though the vaccine inhibits tumor formation, it does not prevent infection of chickens with MDV serotype 1 nor the spread of the virus to other chickens. The losses to the poultry industry resulting from MD are substantial even after vaccination.

Marek's disease virus has properties' characteristic of both alphaherpesvirus and gammaherpesvirus. Originally, MDV was classified as a gammaherpesvirus based on its biological properties. Similar to Epstein Barr virus (EBV), a member of the gammaherpesvirus family, MDV infects lymphocytes and

causes lymphoid neoplasia. However, the genomic structure of MDV more closely resembles alphaherpesviruses, specifically herpes simplex virus (HSV). The herpesvirus study group has determined that neither MDV serotype 1 nor 2 correspond with any of the subfamilies, HVT remains classified in the gammaherpesvirus family (Roizman, 1992).

A characteristic common to all herpesviruses is the ability to induce both productive and latent infections. Latent infections perpetuate the spread of herpesviruses in the host population and prevent the eradication of the virus. Latency is defined as the presence of the viral genome with a simultaneous restriction of lytic gene transcription within a specific cell type. Restriction of viral antigen expression could prevent the host from mounting an effective immune response that would in turn inhibit clearance of the virus.

There are only a few published reports on latent infections in MDV. Most of the publications on MDV latent infections are limited to <u>in vitro</u> studies on MDV serotype 1 latently infected cell lines. Also, the focus of MDV latent infections have concentrated exclusively on reactivated infections in cell cultures, ignoring other features of latency such as its establishment and maintenance. Furthermore, <u>in vitro</u> studies may not present a true representation of <u>in vivo</u> latent infections. The more efficient means to study latency initially, may be to identify features of latency <u>in vivo</u> and later study these

features *in vitro*. Research on MDV latency has concentrated solely on MDV serotype 1. Similar to EBV, MDV serotype 1 has both transforming and latent capabilities. Both EBV and MDV serotype 1 transform infected cells concurrent with the establishment of latent infection. This feature provides problems in studying latency. The major complication is the difficulty in differentiating characteristics of latency from transformation. For example, limited viral transcription, replication and antigen expression occur in latent and transformed cells. Therefore, it is difficult to differentiate between results attributable to latency from those results generated by transformation. To eliminate this problem, we chose to study latency in HVT because HVT has numerous advantages over other herpesviruses:

1. Any feature present during limited transcription and DNA replication is related to latency not transformation because HVT is a nontransforming virus.
2. The genome of HVT is homologous to MDV serotype 1 suggesting that certain characteristics of HVT latency may be similar in MDV serotype 1 latency.
3. There are inherent problems in the study of herpesviruses in animal models. These problems do not occur with HVT since HVT is studied in avian species. For example, HSV latency is studied in 3 different animal models, guinea pig, rabbit, and mouse. The results obtained depend on the animal model used.
4. A drawback with studies of EBV latency that extensively

use cell cultures is that there may be substantial differences from *in vivo* latent infections.

5. The inexpensive cost of care and upkeep of poultry makes avian herpesviruses an ideal model to study latency.

The control of MD will occur only through the exploration of mechanisms such as latency that are involved in the pathogenesis and dissemination of disease. However, knowledge concerning MDV latency is limited. Due to the limited research on latency in avian herpesviruses, a heavy emphasis in this literature review is on latency in mammalian herpesviruses. It is unknown whether MDV latency resembles alpha, beta or gammaherpesviruses. Therefore, we need to examine all aspects of latency in alpha, beta, and gammaherpesviruses to determine which, latency in MDV, resembles.

Biological Characteristics of Marek's Disease Virus

In 1907, a Hungarian pathologist named Josef Marek identified a neuronal paralytic condition occurring in chickens (Payne, 1985). In 1967, the etiological agent was identified as a cell associated herpesvirus. Difficulty in isolating the virus caused the 50 year lapse between discovery of the disease and the etiological agent (Solomon et al., 1968; Churchill and Biggs, 1967). The successful growth of MDV in cell culture facilitated the identification of many biological characteristics. The structure of MDV is similar to other herpesviruses. Marek's disease virus

consists of an outer envelope, the tegument, the capsid and the inner core that contains the DNA. The enveloped particles measure 150 to 160 nm in diameter. In most infected cells, the envelope is associated with the inner nuclear lamellae. By comparison, the fully enveloped virus particle measures 273-400 nm in FFE. Using electron microscopy, naked hexagonal nucleocapsids are seen in the nucleus of non-productively infected cells. These nucleocapsids are 95 to 100 nm and contain 162 capsomeres (Nazerian et al., 1968).

On the basis of indirect immunofluorescence and agar gel precipitin assays, MDV is divided into 3 serotypes (Bulow and Biggs, 1975a; Bulow and Biggs, 1975b). Marek's disease virus serotype 1 are the oncogenic viruses and their attenuated variants. Oncogenic strains are further subdivided into 3 groups: very virulent, virulent and low virulent. The pathogenic classifications are based on the prevention of MD by the use of HVT vaccines. Very virulent strains induce visceral and neural tumors in birds vaccinated with HVT. Two examples of very virulent strains are MD-5 and MD-11 (Witter et al., 1984). Virulent strains do not induce tumors in HVT vaccinated chickens. However virulent strains cause tumors in susceptible, but not resistant, lines of chickens. Strain's GA and JM are examples of virulent MDV (Eidson and Schmittle, 1968; Purchase and Biggs, 1967). A low virulence strain, such as

CU-2, causes tumors only rarely in susceptible lines of chickens (Smith and Calnek, 1973).

Marek's disease virus serotype 2 and HVT require no further modification before use as a vaccine. However, MDV serotype 1 requires appropriate attenuation for use as a vaccine because of its' oncogenic characteristic. Repeated passage of MDV serotype 1 in cell culture results in attenuation of the virus (Nazerian, 1971). Attenuated serotype 1 is nononcogenic and it is used as a vaccine. A low virulent strain, Rispens, has been attenuated and used as a cell-associated vaccine predominately in the Netherlands (Rispens at al., 1972). This vaccine spreads readily by contact, but the spread does not occur rapidly enough to induce immunity in cagemates. Immunity is necessary to neutralize exposure to virulent virus in early life (Rispens et al., 1972). Research is being performed to allow use of Rispens as a vaccine in the U.S. (Witter, 1987 and Witter, 1991).

Marek's disease virus serotype 2 are nononcogenic, but strains such as SB-1, cause cytolytic infections in lymphoid organs (Schat et al., 1978; Calnek et al., 1979). In 1970, Witter et al. isolated a nononcogenic herpesvirus from turkeys and designated it turkey herpesvirus or HVT. On the basis of its antigenic relationship to MDV serotype 1, HVT was classified as serotype 3. HVT is highly effective as a cell-associated or cell free vaccine in preventing MD (Okazaki et al., 1970; Witter et al., 1970; Calnek et al.,

1970b; Purchase et al., 1972a; Purchase et al., 1972b). The ability of HVT to protect against MD is the basis for the differentiation between very virulent and virulent serotype 1 strains. Bivalent vaccines, that include both serotype 2 and serotype 3 in their formulation, also prevent MD. Thus, MDV is important as a model for the prevention of viral induced cancer.

Clinical and Pathological Characteristics of MDV

Marek's Disease Virus is spread by horizontal transmission. The virus survives in moulted feathers and dander for prolonged periods at low temperatures. The moulted feathers and dander are inhaled by uninfected chickens and the virus subsequently infects the respiratory tract. The infection is disseminated from the respiratory tract to other areas of the body. Consequently, moulted feathers and dander are the major source of MDV contamination (Calnek et al., 1970a; Beasley et al., 1970). Even though HVT spreads easily among turkeys, minimal contact spread occurs in chickens (Cho et al., 1971; Cho, 1975).

With a MDV serotype 1 infection, the most consistent clinical sign is spastic or flaccid paresis. Flaccid paresis progresses to complete paralysis of the limbs. Transient paralysis is a less severe sign. It occurs six to ten weeks after exposure to the virus and may last for one to two days (Settnes, 1982; Calnek and Witter, 1991).

Blindness, a result of ocular opacity is another clinical sign of MD. Nonspecific clinical signs include: dilation of the crop, gasping, vertical or lateral recumbency and ruffled feathers.

The most common gross pathological change is enlargement of peripheral nerves. Autonomic nerves, especially the celiac, brachial and sciatic plexuses, are usually affected (Goodchild, 1969). The nerves appear gray to yellow in color with a loss of cross striations. Another gross change is the presence of lymphoid tumors in various organs. Nonspecific gross lesions include muscular wasting, atherosclerosis and iridocyclitis (Pappenheimer et al., 1929).

On histopathological examination, peripheral nerve lesions are grouped into 3 types: types A, B, and C. Type A lesions consist of neoplastic cell accumulations. Additionally, type A lesions are characterized by infiltration of nerves with lymphoblastic cells, demyelination and Schwann cell proliferation. Inflammation, including infiltration of nerves with few lymphocytes and plasma cells are indicative of type B lesions. Lastly, type C lesions are a mild version of type B (Payne and Biggs, 1967). Serotype 1 infections of parenchymatous organs induce inflammation that can progress to neoplasia. HVT induces a transient lymphocytic proliferation in nerves that lasts 3-4 days. Subsequently, no microscopic lesions are present. The pathogenesis of serotype 1 is well

established. Cytolysis occurs 3-6 days post infection (dpi) followed by the formation of intranuclear inclusion bodies. A productive serotype 1 infection consists of two types, fully productive and restrictive productive. Fully productive infections are present only in the feather follicle epithelium (FFE). The virus in fully productive infections is enveloped, cell-free, and infectious (Calnek et al., 1970b). A restrictive productive infection occurs in most MDV-infected cells. This type of infection contains non-enveloped virus that is non-infectious (Calnek and Witter, 1991).

Another characteristic of serotype 1 infection is transformation of infected cells, specifically T lymphocytes. MDV-infected T-lymphocytes contain 5-15 copies of MDV genome. There is limited MDV genomic expression in transformed cells (Ross, 1985). Methylation of the genome may be responsible for the limited genomic expression (Kanamori et al., 1987). Non-productive infections in transformed cells may also account for limited gene expression.

Molecular Biology of MDV

The MDV genome is composed of linear, double-stranded DNA with a 46% G:C ratio (Cebrian et al., 1982). The molecular weight of MDV DNA is 120 x 10^6 daltons while the size is 160 kb. The density of MDV DNA is 1.706 g/m, similar to chicken DNA. This feature makes the separation

of MDV DNA from chicken genomic DNA extremely difficult. The genomic structure is composed of a unique long region and unique short region each flanked by terminal and inverted repeats. The unique regions flanked by repeat regions are characteristic of group E genomes (Cebrian et al., 1982).

The standard group E genomic herpesviruses has similar replication and transcription patterns. Replication and transcription have been well characterized in HSV and presumably nonproductive MDV infections follow this same pattern. The virus attaches to the target cell and initiates fusion between the viral envelope and cell membrane. Next, the virus gains entrance into the cell's cytoplasm, and once in the cytoplasm, transcriptional transactivator viral protein 16 (VP16) and virion host shutoff (VHS) proteins are released from the viral tegument. These proteins are then transported to the nucleus to perform their specific functions. The capsid migrates to the nucleus and fuses with the nuclear membrane with the subsequent release of linear viral DNA. The DNA circularizes upon release from the capsid. VP16 initiates transcription of the immediate early (IE) genes followed by a cascade of temporally regulated genes. IE gene products turn on early genes which in turn initiate transcription from late genes. Viral replication occurs between early and late gene transcription by a rolling circle mechanism

generating head to tail concatamers (Roizman and Sears, 1990).

The analysis of RNA from cell lines and cell culture provides the basis for MDV transcriptional patterns. In MDV-infected cell cultures, Maray et al. (1988) identified 8 immediate early, 2 early and 11 late gene transcripts. The total number of transcripts ranges from 29 to 42 depending on the MDV strain infecting the cell culture. Transcription occurred from approximately 60% of the genome with the transcripts ranging in size from 0.8 to 13 kb (Maray et al., 1988). In contrast, other researchers report transcriptional activity from 12% to 45% of the MDV genome (Silver et al., 1979). The reason for the differences in transcriptional activity is unknown. The difference may result from the use of different viral strains in the two studies. Maray et al. (1988) used a strain of virus that produces an active infection so transcription is high. In comparison, Silver et al. (1979) used a non-producing cell line that has a very low level of transcription. As previously mentioned, MDV strains that induce tumor formation have limited transcription. Early results demonstrate that 12% to 14% of MDV is transcribed in a non-productive lymphoblastoid cell line. Treatment of cells with iododeoxyuridine increases transcription by 42% (Silver et al., 1979).

Repeated cell passage of MDV serotype 1 results in a loss of tumorigenicity and amplification of a 132 bp repeat

(Silva and Witter, 1985). BamHI-D and -H contain the expansion that is associated with loss of tumorigenicity. Multiple forms of BamHI-D and -H exist in pathogenic and attenuated MDV serotype 1. Comparisons of the amplified region revealed transcription of a 1.8 kb mRNA in oncogenic serotype 1 that was replaced by a 0.4 kb truncated transcript in attenuated variants (Bradley et al., 1989). Both transcripts are transcribed in a rightward direction. By comparison, Chen and Velicier (1991) demonstrated that initiation and termination of transcription from the amplified region occurred at several sites in the rightward or leftward direction. The difference in results between Bradley's and Chen's research could be explained by the use of two different techniques to analyze the transcripts. Chen and Velicier (1991) used cDNA sequence analysis linked with S1 nuclease protection analysis to consistently demonstrate multiple transcription initiations and terminations. Bradley et al. (1989) generated probes from single BamHI -D and -H fragments that cannot determine direction or boundaries of transcription.

The protection afforded by HVT against MDV serotype 1 induced neoplasia appears to have an immunological basis. This immunological protection has stimulated an interest in the antigens common to both serotype 1 and HVT. Identification of proteins encoded by MDV transcripts is incomplete. Forty-six polypeptides, that were similar in serotype 1 and HVT, were detected by imunoprecipitation

(Ikuta et al., 1981; VanZaane et al., 1982). Using agar gel precipitation, three common antigens have been identified in cell culture. These antigens were designated antigens A, B, and C. The Protein Nomenclature Committee of the 3rd International Marek's disease Symposium, Osaka Japan, 1988, recommended that Antigen A and B be designated gp57-65 and gp100/60/49, respectively. The homology between HSV glycoproteins and MDV antigens A and B, have given rise to the designations of glycoprotein C (gC) homologue for antigen A and glycoprotein B (gB) homologue for antigen B (Coussens et al., 1990; Ross et al., 1989b). An HSV homologue for antigen C has not yet been identified.

gC is present in both cell extracts and culture fluids. This result establishes gC as a secreted glycoprotein. The molecular weight of gC is 57 to 65 kDa. A precursor protein with a molecular weight of 44 kDa is seen with pulse-chase and cell-free translation assays. In addition, gC is a primary antigen recognized by MDV antiserum, however, its function is unknown (Isfort et al., 1987). Attenuation of MDV serotype 1 results in a loss of tumorigenicity and gC expression, but the relationship between the two is unknown (Churchill et al., 1969). A 4.6 kb segment, located in the BamHI-B fragment, encodes the precursor protein (Fig. 1) (Isfort et al., 1987). A 2.35 kb DNA segment transcribes the 1.8 kb mRNA which in turn encodes the precursor protein. Analysis of the complete nucleotide sequence reveals an open reading frame (ORF) of 1,515 nucleotides. The ORF encodes a

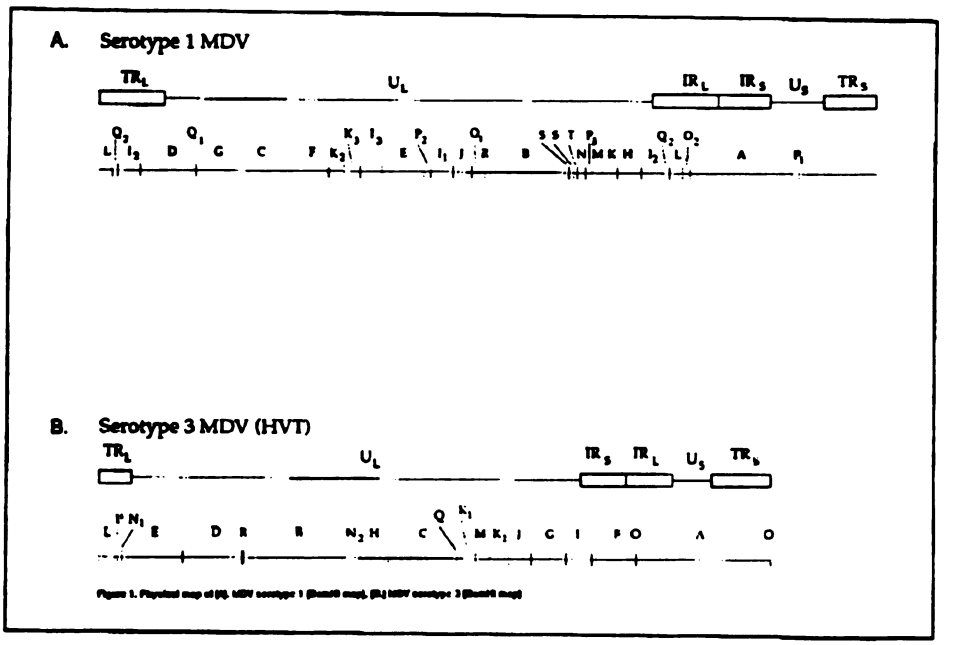
putative 505 amino acid polypeptide. The predicted sequence indicates features common to other glycoproteins. These features include a hydrophobic amino terminal region that functions as part of the signal peptide and also N-linked glycosylation. Although gC has a carboxyl-terminal membrane anchor sequence, the secretory nature of gC indicates that this sequence is inadequate in anchoring the protein in the cell membrane (Coussens and Velicier, 1988).

The gene encoding HVT gC was found by stringently hybridizing a HVT genomic library to the serotype 1 gC gene. HVT gC gene mapped to BamHI-K_1 and M fragments and is co-linear to serotype 1 gC. Total G+C content of HVT gC is 46% compared to a G+C content of 41% for serotype 1 (Coussens and Velicer, 1988). The 5' nucleotide sequences of HVT and serotype 1 are conserved while their 3' sequences are divergent. Translation of sequence data reveals a single ORF.

The ORF could encode a 523 amino acid polypeptide with a molecular weight of 58,587. Six sites of N-linked glycosylation exist in this putative polypeptide. Using amino acid sequence data, HVT and serotype 1 gC proteins are 73% homologous. Searches of the Protein Information Resource (PIR) data bank reveal significant homology between HVT, MDV serotype 1 and herpes simplex virus (HSV) gC. In addition, HVT and MDV serotype 1 gC share homology with gC-like glycoproteins from other herpesviruses including

varicella-zoster, pseudorabies and equine herpes virus (Binn and Ross, 1989).

Mutants unable to express gC can infect and replicate in cell culture. This result indicates that gC is dispensable for viral replication in cell culture (Coussens et al., 1990). In addition, gC mutants require a longer incubation time to infect cell cultures (Schat et al., 1985). Therefore, gC may enable a virus to readily infect cells but has no role in viral replication in cell culture. However, the importance of gC in viral infectivity and replication <u>in vivo</u> is unknown.



The B antigen is a late gene antigen composed of a complex of three glycoproteins that are 100, 60, and 49 kDa in size. As previously mentioned, the MDV B antigen is a HSV glycoprotein B (gB) homologue. MDV gB has a 50% amino acid identity to gB of alphaherpesviruses. The pattern of immunoprecipitated proteins identified the close relationship that exists between serotype 1 and HVT gB (Silva and Lee, 1984). The gB gene was cloned, sequenced and localized to the BamHI-I_3 and -K_3 fragments (Fig. 1). This region transcribes a 2.7 kb mRNA that encodes a putative 844 amino acid long polypeptide. Using hydrophobicity data, hydrophobic amino acids were located near the N terminus and showed similarity to the signal sequence of HSV gB. Additionally, hydrophobic amino acids are close to the C terminus and may represent an anchor sequence (Ross et al., 1989a). On the basis of its immunogenicity, gB may play a significant role in vaccine protection. gB induces humoral and cell-mediated immunity against HSV (Cantin et al., 1987) and pseudorabies virus (PRV) infections (Marchioli et al., 1987). To explore immunity induced by MDV gB, Yanagida et al. (1992) constructed a recombinant fowlpox vector expressing MDV serotype 1 gB under the control of a vaccinia virus promoter. Immunofluorescence and immunoprecipitation assays were used to confirm gB expression from the fowlpox recombinant (Yanagida et al., 1992). The recombinant induced neutralizing antibodies and protected chickens

against challenge with very virulent strains of serotype 1 (Nazerian et al., 1992).

Latent MDV infections

Latent infections are nonproductive with viral replication being either limited or nonexistent. The most extensive examination of MDV latency was in serotype 1 infected cell lines. Most lymphoblastoid cell lines are nonproductive, hence MDV infections in these cell lines are considered latent. MKT-1 is a nonproductive cell line, that was established from a MDV serotype 1-infected chicken kidney tumor. MKT-1 contains 15 MDV genomes per cell, that exist as closed circular plasmids (Tanaka et al., 1978). Another non-producer cell line, MDCC-BO1, is quite different from MKT-1. MDCC-BO1 contains both MDV serotype 1 and HVT genomes (Kitamoto et al., 1980) at a genome equivalent of 3.8 and 1.8 copies per cell, respectively (Hirai et al., 1981). In MDCC-BO1, both MDV serotype 1 and HVT have nucleosomal patterns and exist as closed circular DNA (Hirai et al., 1981). Co-cultivation assays of MDCC-BO1 with chicken embryo fibroblasts (CEF) yield HVT but not MDV serotype 1 DNA. Possibly, MDV serotype 1 has deletion of sequences necessary for reactivation or its' DNA is non-infectious. However, a shift in temperature from 41^0C to 37^0C can induce viral replication thus increasing genome equivalents. Phosphonoacetic acid inhibits productive MDV

DNA synthesis but has no effect on latent MDV DNA (Nazerian et al., 1977; Hirai et al., 1981).

Calnek et al. (1981) characterized latent MDV infections using a cell culture assay. Tissues, from chickens infected with MDV, were examined for the expression of viral internal antigens (VIA). In most instances, tissues required co-cultivation with CEF before VIA could be detected. According to Calnek, the detection of VIA in these cell cultures indicates reactivation of latent MDV. On the basis VIA expression, T-lymphocytes were the target cells for latent MDV serotype 1 in the spleen (Calnek et al., 1981).

Various strains of MDV serotype 1, 2 and 3, were used to infect both N-line and P-line chickens. VIA was not detected in spleen samples before in vitro cultivation. However, VIA was present 42-78 hours after co-cultivation of spleens with CEF. MDV serotype 1-infected chickens had the largest number of cells expressing VIA. These cells were detected 3 to 5 days after infection but prior to co-cultivation (Calnek et al., 1981). Using dual fluorescence and cell fractionation studies, latent MDV serotype 1 was present in Ia-bearing T cells. In comparison, a small percentage of B-cells contained latent MDV serotype 1. MDV genomes were detected in T-cell fractions by dot blot hybridization but not by in situ hybridization (ISH) (Calnek et al., 1984).

Immunosuppression prolongs cytolytic infections by preventing the switch to latency (Buscaglia et al., 1988). Spleen cells produced latency maintenance factor (LMF), after being stimulated with concanavalin A. LMF inhibits the expression of VIA; removal of LMF subsequently leads to the expression of VIA. LMF is considered a cytokine although its mechanism of action is still unknown (Buscaglia and Calnek, 1988). Another study examined whether immunosupression caused by reticuloendotheliosis virus (REV) had any effect on MDV latency. Surprisingly, REV failed to reactivate MDV from latency. However, REV did reduce both cytolytic and latent MDV infections (Buscaglia et al., 1989).

Unfortunately, information on HVT latent infection is scant. The main reason is the criterion for latency set forth by Calnek et al. (1981) has not been demonstrated in HVT infections. Even though co-cultivation assays demonstrated the levels of HVT genomic copies were comparable to MDV serotype 1, HVT rarely expressed VIA. Therefore, another method besides reactivation of latent infections is needed to characterize HVT latent infections.

The ability of viruses to perpetuate their existence in nature by the mechanism of latency is an important characteristic from a disease control standpoint. Herpesviruses have the capability of persisting in the host in the presence of both natural and vaccinal immunity. It is interesting to reflect on the outcome of a herpesvirus

that loses the ability to undergo latency. First, would the virus be able to sustain its existence in the host? Secondly, in the case of oncogenic herpesviruses, would they be able to transform cells in the absence of latency? Additionally, what is the role of host immunity in latent infections? Lastly, would disease control inhibit the latent phase of infection? Study of the mechanism of latency will allow a better understanding of the persistence of herpesviruses in the host population.

There are three main components to a latent infection: establishment, maintenance and reactivation. An understanding of the molecular features responsible for these properties may allow the prevention of latency and transmission to susceptible hosts. A classical example of latent viruses is herpesviruses. Herpesviruses consist of three families: alpha herpesvirus, beta herpesvirus, and gamma herpesvirus families.

ALPHAHERPESVIRUS FAMILY

The alpha herpesvirus family has a wide host range, short replication cycle, spreads rapidly in cell culture and causes cytolysis. In addition, alphaherpesviruses are neurotropic, thus latent infections are present in nervous tissue. Members of this family include herpes simplex virus, varicella zoster virus, equine herpesvirus, bovine herpesvirus, and pseudorabies virus.

HERPES SIMPLEX VIRUS

Biological characteristics of Latency

Herpes Simplex Virus (HSV) is the classic prototypical alphaherpesvirus and is well characterized. Primary infections occur at epithelial lined orifices with the subsequent replication of HSV in susceptible cells. HSV gains entry into the nervous system by retrograde axonal flow. Once HSV reaches the nervous system, the virus can establish latency.

Segmental innervation of the vertebrae trunk, in the form of overlapping dermatomes, was used to monitor the spread of HSV from skin to the sensory ganglia. HSV was followed from nerves innervating the site of inoculation to ganglia that innervate neighboring parts of the flank. Mice were infected in the Thoracic (T) 10 dermatome that overlaps T9 and T11. Anatomical distribution of productive infection was located in T8 to T12 of the thoracic and lumbar vertebrae. By comparison, latent infection had a wider

distribution, from T6 to L1. T7 and T13 ganglia became latently infected despite the absence of detectable gene expression. The distribution of infectious virus and cells expressing viral genes during a primary infection, correlate with the expected pattern of innervation of the inoculation site. Therefore, the molecular pathways of productive and latent infection with a virulent strain of HSV-1 can diverge from a very early stage. This means that virulent HSV can establish latency without initiating the pathway associated with productive infection (Speck and Simmon, 1991). The lack of cytolysis in the nervous system during latency is due to the absence of a prior productive infection.

The site of latent HSV infection is dependent on the route of inoculation. Within the nervous system, latent HSV is present predominately in neurons of the trigeminal ganglia of humans, mice, rabbits and guinea pigs (Croen et al., 1987; Spivak and Fraser, 1988; Stevens et al., 1988; Rock et al., 1987; Stroop et al., 1984). Conversely, latent HSV was also reported in non-neuronal cells of the trigeminal ganglia (Tenser et al., 1991). Inoculation of a rabbit's eye can result in latent HSV infection in the ipsilateral and contralateral cornea and geniculate ganglia (Furuta et al., 1992).

Molecular Biology of HSV

HSV is composed of two serotypes, HSV-1 and HSV-2. The genomes of the two serotypes are approximately 152 kb in

length. On the basis of the sequence arrangement which is analogous to MDV, HSV also has a group E genome. Group E genomes form 4 different isomeric structures. Association of latent HSV DNA with nucleosomes may limit gene expression by impeding access of RNA polymerase to the genome (Deshmane and Fraser, 1989). In gamma herpesvirus infections, extensive methylation of the latent genome limits gene expression. However, latent HSV DNA is not extensively methylated (Dressler et al., 1987). Restricted gene expression results in limited transcription during latency.

Latent Transcription

Latent HSV transcripts could play an important role in the establishment, maintenance, or reactivation of latent HSV. The region of the genome encoding ICP0 is also transcriptionally active during latency (Deatly et al., 1987). Identification of three latency associated transcripts (LAT) resulted in an extensive attempt to characterize the transcripts. LAT are antisense and transcribed in the opposite direction from the 3' end of ICP0. The LATs measure approximately 2.0, 1.45, and 1.4 kb in size (Wagner et al., 1988a; Weschler et al., 1988; Spivak and Fraser, 1988).

Analysis of the 2 kb LAT reveals: 1. A TATA box is located 650 nucleotides upstream of the 5' end of LAT (Weschler et al., 1989). 2. The LATs are poorly polyadenylated or not polyadenylated at all (Spivak and

Fraser, 1987; Wagner et al., 1988b). 3. The LATs are located in the nucleus of the cell (Deatley et al., 1987; Deatley et al., 1988; Stevens et al., 1987; Stroop et al., 1984). 4. The 5' end of LAT has a consensus sequence with similarities to vertebrate splice donor sites while a splice acceptor site is located 1.95 kb downstream of the 5' end (Spivak et al., 1991; Wagner et al., 1988b; Weschler et al., 1988). 5. Sequence data revealed the presence of 2 potential ORFs (Weschler et al., 1989).

The ORFs potentially encode for proteins that are 12 kd and 36 kd in size. The 12 kd ORF is included in an exon situated within the 36 kd ORF but with a shift in the reading frame. The 36 kd ORF has an intron containing an in-frame termination signal. The termination of the message would yield a 33 kd protein as suggested previously (Wagner et al., 1988b; Weschler et al., 1988). The presence of these ORFs imply that at least one LAT is a functional mRNA.

Sequence data shows LAT transcription begins 28 nucleotides from the consensus TATA box sequence and extends to a polyadenylation site 8.3 kb downstream (Dobson et al., 1989). A 8.3 kb LAT has been found in lytic but not latent infections. The relevance of LAT is complicated by the presence of LAT production during the lytic phase of infection albeit at a lower level than in latency (Spivak and Fraser, 1988). This result weakens the contention of a role for LAT during the establishment, maintenance or reactivation of latency. However, LAT functions as a potent

antisense inhibitor of ICP0 gene expression (Farrell et al., 1991).

A strong possibility exists that LAT is actually an intron and not mRNA. Splicing introns from transcripts promotes the movement of mRNA from the nuclear to the cytoplasmic compartment (Buchman and Berg, 1988). However, LAT localizes to the nuclear portion of the cell. a feature that is not characteristic of mRNA. Generally, introns are presumed destroyed since they do not normally accumulate within cells. A vector containing LAT was cloned into a B-galactosidase gene and transfected into COS cells. Using Northern blot hybridizations, a transcript was isolated. This transcript was the size of B-galactosidase RNA and could only have been obtained after splicing (Farrell et al., 1991). This result provides further evidence that LAT is an intron.

Initiation of LAT gene transcription occurs after binding of RNA polymerase to the LAT promoter. The LAT promoter is over 660 nucleotides upstream of the 5' end of the 2 kb LAT. Using transient CAT assays in cell lines originating from neurons, LAT promoter activity was shown to have neuronal specificity (Zwaagstra et al., 1990; Batchelor and O'Hare, 1990). Since LAT is the only RNA transcribed during latency in neurons, regulation of LAT promoter is probably different from regulation of other HSV promoters (Zwaagstra et al., 1990).

Positive and negative functional elements exert control over the LAT promoter. A LAT promoter binding factor (LPBF) binds to a palindromic sequence within the promoter region. Additionally, LPBF plays a major role in the regulation of the LAT promoter (Zwaagstra et al., 1991).

Functional Analyses of Latency through Mutations

Although transcripts from lytic genes are absent during latency, this does not preclude lytic gene proteins from functioning during latency. To understand the role these proteins play during latency, mutants deleted for the genes encoding lytic proteins were examined for their ability to establish, maintain or reactivate latent infections. All of the mutants examined were able to establish and maintain latent infections. This suggests that the genes examined are not required for establishing or maintaining latency. However, the major change observed was the decrease or inability of the HSV mutants to reactivate from a latent infection.

Various HSV mutants demonstrate the role that immediate early genes (IE) play in reactivation of HSV. A genetically engineered variant with a deletion in the ICP4 gene possesses the ability to reactivate from latency (Dobson et al., 1990). However, another deletion mutant for ICP4 did not replicate nor reactivate from a latent infection (Leib et al., 1989).

An ICP27 deletion mutant did not replicate nor reactivate from latency. Mutants deleted for ICP0 could replicate but varied in their ability to reactivate (Leib et al., 1989). Also, ICP0 independent of other HSV gene products reactivates latent HSV-2 (Zhu et al., 1990). Mutations in the gene encoding for ICP0 affect viral growth and plaque formation but do not change the virus' ability to reactivate from latency. ICP0 is required for reactivation of latent HSV (Russell et al., 1987). Mutants with deletion in the VP16 gene show that VP16 is dispensable for the establishment and reactivation of HSV latent infections (Steiner et al., 1990). A deletion mutant lacking most of the gene encoding for ribonucleotide reductase is impaired in its ability to replicate and failed to reactivate from latency (Jacobson et al., 1989). The late gene, gC is nonessential for the establishment and reactivation of latency.

Mutants deleted for thymidine kinase (TK) establish latency in ganglia of mice but can not replicate nor reactivate from latency (Leist et al., 1989; Coen et al., 1989; Tenser et al., 1989). In contrast, mutants that produce low levels of a truncated TK establish reactivatable latent infections (Coen et al., 1989) and mutants failing to express TK can reactivate from latency in rabbits (Caudill et al., 1986). These conflicting results may be due to the different animal models used, i.e. TK mutants do not reactivate in mice or guinea pigs but they do in rabbits,

while production of even small amounts of TK can result in reactivation of HSV in the guinea pig and mouse model. Also, Tenser et al. (1989) demonstrated that TK- mutants were able to express LAT.

LAT deletion mutants provide a better understanding of reactivation and the role LAT plays in this mechanism. LAT deletion mutants disrupted in both copies of the LAT gene become latent in tissue culture. The mutant is unable to replicate but can establish latency after corneal inoculation and explantation assays. These assays show the mutant's ability to reactivate from latency (Block et al., 1990). However, a different LAT mutant can replicate in the rabbit cornea and establish latent infections in the trigeminal ganglia but does not reactivate efficiently (Hill et al., 1990; Leib et al., 1989; Steiner et al., 1989). Additionally, LAT deletion mutants establish a latent infection in mice and have the ability to reactivate from the latent state (Izumi et al., 1989; Ho et al., 1989; Sedarati et al., 1989; Javier et al., 1988). Therefore, some, but not all LAT mutants reactivate less efficiently than wild-type HSV, thus implying that LAT has a major function in reactivation. This information also underscores the importance of selecting an appropriate animal model because various animal models will yield different results.

One method used to assess reactivation capabilities of latent HSV is the manipulation of incubation temperature. Temperature sensitive (ts) mutants can establish latency, be

maintained at the nonpermissive temperature and reactivate by decreasing the temperature to a permissive level. Some ts mutants have a neuron-specific, temperature sensitive, replication defect. Changing the incubation temperature from 38.5^0 to 31^{0}C corrected the defect (Cook et al., 1986).

In Vitro Model for Assessment of Latency

An in vitro model to study the establishment, maintenance and reactivation of latency has been developed. HFL-F cells were treated with (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and interferon-alpha. The cells were then infected with HSV-1. After removal of BVDU and INF-alpha, increasing the incubation temperature from 37^0 to 40.5^{0}C maintained latency in HFL-F cells. Decreasing the incubation temperature to 37^{0}C or superinfecting with human cytomegalovirus caused reactivation of HSV. Reactivation of HSV by this method, allowed the detection of viral DNA but not virus specific transcripts (Scheck et al., 1989). Variations of this in vitro model included the treatment of infected cells with cycloheximide prior to a temperature shift from 37^0 to 40.5^{0}C (Shiraki and Rapp, 1986)

Reactivation of Latency

Examining factors that inhibit the establishment, maintenance or reactivation of latent HSV may aid in preventing the spread of latent infections within a population. There is a correlative relationship between the

ability of HSV to replicate and establish a latent infection. This correlation can be abolished by chemotherapy, interferon or passive immunization. These factors have little effect on viral replication but reduce ganglionic latent infections (Eis and Schneweis, 1986).

Putative factors that prompt reactivation of latent HSV include stress, neurectomy, fever, immunosuppression, trauma such as irritation of epithelial surfaces (Tenser et al., 1988) and ultraviolet light (Perna et al., 1987). Smoking tobacco causes irritation to the oral mucosa that results in tar-condensate-induced-viral-reactivation whereas snuff extract does not (Park et al., 1987). All these factors have one common characteristic, the ability to influence the levels of intracellular c-AMP through messengers such as catecholamines and arachadonic acid. Increased levels of c-AMP cause increases in viral reactivation from latency. Decreases in c-AMP and elevated c-GMP levels inhibit viral replication from latency (Leib et al., 1991).

Animal Models for study of HSV Latency

Numerous animal models have been established to examine the mechanism of HSV latency. The lack of spontaneous reactivation in the mouse makes it the most popular animal model to study establishment and maintenance of latency but not reactivation. In contrast, the guinea pig is not considered a good animal model because of spontaneous reactivations. Furthermore, sterile lesions develop in HSV-

infected guinea pigs that yield minute amounts of virus. The presence of sterile lesions leads one to speculate if the reactivations are real or the result of the host immune response. Spontaneous reactivation of HSV also occurs in the rabbit, albeit at a much lower level than the guinea pig. Latent HSV was identified in trigeminal ganglia, brain stem and cornea from rabbits using slot blot hybridization. However, no infectious virus was demonstrated by explantation assays (Kudelova et al., 1991). On the basis of the study of latent HSV in rabbit cornea, the ocular model is an efficient method to study HSV-1 reactivation (Gordon et al., 1990; Gordon et al., 1986). Intrastromal injection of deionized water induced HSV reactivation. Similarly, a beta-1 and beta-2 blocker, timolol, reactivated latent HSV in this eye model (Hardwick et al., 1987).

Another animal model recently reported for the study of HSV latency is the tree shrew. The tree shrew latency model demonstrates that virulent wild-type HSV strains colonize in ganglia. Conversely, avirulent strains and LAT deletion mutants only persist in the spleen. In addition, a 1.5 kb transcript was found in latently infected ganglia but this transcript was missing or a different size in latently infected spleen (Rosen et al., 1989). Marmosets have also been investigated as a possible animal model. Although marmosets are highly susceptible and require small doses of virus, mortality rates are high (Roizman and Sears, 1987).

The use of animal models have provided many of the results regarding HSV latency. Nonetheless, no animal system matches the circumstances surrounding human HSV latency exactly. Consequently, caution must be taken in the interpretation of these results. Examination of herpesvirus latency in the natural host is a logical step and therefore, a review of the literature on herpesvirus latency in animals, such as bovine and swine is appropriate.

BOVINE HERPESVIRUS

Biological and Molecular Characteristics

Bovine herpes virus (BHV) is the causative agent of Infectious Bovine Rhinotracheitis (IBR). The clinical signs of IBR are rhinotracheitis, conjunctivitis, genital infections, and meningoencephalitis (Kahrs, 1981). Like other members of the alphaherpesvirus family, latent BHV infections are in the autonomic and sensory nerve ganglia (Narita et al., 1978; Homan and Easterday, 1980; Homan and Easterday, 1983). Fine mapping of the BHV genome indicates that latent transcription is restricted to the TR_L and IR_L region of the genome. These regions overlap an IE gene encoded on the opposite strand of the latent transcription unit. Latently infected bovine trigeminal ganglia contain latency-related RNA (LR-RNA) that range in size from 0.77 to 1.16 kb (Kutish et al., 1999).

Transcription

To understand the significance of the LR gene, it becomes important to examine factors that regulate the gene's expression. LR-RNA include putative RNA polymerase II promoter sequences and a polyadenylation signal (Kutish et al., 1990). The LR promoter was 10 fold more active in cultures of sensory neurons when compared to simian virus 40 (SV40) promoter and enhancer sequences (Jones et al., 1990). In contrast, the SV40 promoter was more efficient in bovine, rodent, and monkey cells when compared to the LR promoter.

LR-RNA are not expressed until late in the lytic cycle of infected bovine cells. A 146 bp sequence located in the LR promoter exerts negative regulation on transcription. The 146 bp regulatory element has a moderate enhancing ability in bovine sensory ganglionic neurons, rodent and monkey cells. A 258 bp region of the promoter was found to cis-activate HSV-1 TK promoter in neuronal cells but did not cis-activate the HSV-1 TK promoter activity in other cell types. This 258 bp region did not have promoter activity. In addition, nerve growth factor stimulated transcription in neuronal cells but not other cell types (Bratanich and Jones, 1992).

Removal of LR promoter sequences enhanced the ability of immediate-early transcription unit 1 (IEtu-1). However, the addition of LR promoter sequences repressed IEtu-1 trans-acting ability. Therefore, LR-RNA can downregulate IEtu-1 activity (Bratanich et al., 1992).

Sequence data of LR-RNA have shown the presence of 2 ORF. LR-ORF-1 began with a translational start site and was out of frame when compared to LR-ORF-2. Interestingly, LR-ORF-2 had a high degree of alternating purine-pyrimidine bases that implied the presence of Z-DNA formation. The positional base preference analysis of Staden suggests with a high probability that portions of both LR-ORF-1 and LR-ORF-2 encode for proteins. However, no proteins associated with latency have been identified yet (Kutish et al., 1990).

Reactivation of Latent Infections

Reactivation studies demonstrated that BHV replication can occur after the administration of dexamethasone, a glucocorticoid (Davies and Duncan, 1974; Kahrs, 1981; Rock et al., 1992; Sheffy and Davies, 1972). Comparable to other alphaherpesviruses, BHV multiplied at the initial site of infection after reactivation (Guy and Potgieter, 1985). A 15 hour delay of viral transcription and focal neuronal cell death were present after dexamethasone induced reactivation (Rock et al., 1992).

Humoral immunity has been used to differentiate between a primary BHV-1 infection and reactivated BHV-1 in bovine. The presence of both IgG1 and IgG2 in a reactivated infection differs from a primary infection, where only IgG1 is produced; thus the two stages of infection can be differentiated based on distribution of antibody activity between the immunoglobulin isotypes (Guy and Potgieter, 1985).

Rabbit Model

On the basis of the expense and difficulty in obtaining large numbers of experimental bovine, rabbits were used in a majority of latent BHV infection studies (Rock et al., 1987). Nevertheless, the same problems found in using of animal models in HSV latency studies, apply to using rabbits as animal models for BHV latency. Another animal that is infected with an alphaherpesvirus is swine. Pseudorabies is

a naturally occurring herpesvirus infection of swine and latency in this infection will now be reviewed.

PSEUDORABIES

Biological Characteristics

Pseudorabies virus (PRV) has a wide host range and causes a fatal disease in most animal species except swine. Older swine can survive PRV and become latently infected. Latently infected swine serve as a source of transmission of PRV to susceptible animals (McFerran et al., 1984). Vaccination of swine does not prevent infection or the establishment of latency by PRV but it does reduce shedding after reactivation (Mock et al., 1980; Wittman et al., 1983; Schoenbaum et al., 1990).

Molecular Characteristics

Latency occurred in swine 7 weeks post infection (Rhiza et al., 1984). The use of co-cultivation assays demonstrated latent PRV in trigeminal ganglia, olfactory and optic nerves, cerebral cortex, medulla oblongata, gasserian ganglia, and tonsils (Gutekunst, 1980; Rhiza et al., 1984; Beran et al., 1980; McFarlane and Thawley, 1985; Sabo and Rajcani, 1976). Even though latent PRV is present in a number of different ganglia, latent PRV is most frequently found in trigeminal ganglia, identical to latent HSV (Gutekunst, 1979; Gutekunst et al., 1980; van Oirschot and Gielkens, 1984; Rhiza et al., 1984). At least 30 genomic copies of PRV were estimated in latently infected cells, a lower number than seen with other alphaherpesviruses. The physical state of latent PRV DNA was predominately a linear,

unit-length molecule unlike latent HSV DNA that is circular. Similar to latent HSV, latent PRV was not methylated (Rhiza et al., 1986). Further analysis of the physical state of PRV nucleic acid may clarify the activity of the genome during latency.

Transcription

A limited region of the genome is transcriptionally active during latency. This region corresponds to the IE gene and extends from map units 0.65 to 0.75 (Lokensgard et al., 1990). However, Priola et al., (1990) identified a slightly larger region of the latent genome that is transcribed, from 0.64 to 0.82 map units. The total transcription unit for LAT is no larger than 12.6 kb (Priola and Stevens, 1991). The role this transcription unit plays in the establishment, maintenance and reactivation from latency is unknown. However, latent transcripts have been identified that correspond to this region.

Two latency associated transcripts (LAT) were detected in latently infected trigeminal ganglia (Cheung, 1989). The LAT transcripts were in the cytoplasm of latently infected neurons unlike latent HSV that is present in the nucleus (Priola et al., 1990). The RNA were 2.0 and 0.95 kb in size and polyadenylated. The 5' limit of LAT was localized to U_L region while the 3' limit was present in the IR_L (Priola and Stevens, 1991). As seen with other alphaherpesviruses, these LATs are partially co-linear and antisense to an IE

gene of PRV, IE180. In contrast, Priola et al., (1990) found Poly(A)- transcripts that ranged in size from 4.5 to 5.5 kb.

Cheung (1991) devised a unique method to identify LATs. A series of overlapping cDNA clones identified a large LAT approximately 8.5 kb in size. The large LAT had 4.6 kb of intervening sequences and was not present in productively infected cultures. The role of LAT in latency may be elucidated through examination of LAT mutants.

Another alphaherpesvirus that has the ability to undergo latency is the varicella zoster virus (VZV) which infects monkeys and humans.

VARICELLA-ZOSTER VIRUS

Biological Characteristics

Varicella virus causes infections in both simian and humans. In humans, VZV causes chickenpox and shingles. Primary varicella infection occurs predominately during childhood. However, VZV reactivates as clinical herpes zoster and commonly occurs in the geriatric or immunosuppressed population. VZV usually reactivates from latency no more than once (Croen et al., 1988; Meier and Straus, 1992). VZV has a propensity for thoracic (T3-T12) and lumbar (L1-L2) nerves (Hope-Simpson, 1965; Mazur and Dolin, 1978).

Latency characteristics

Latent VZV are in both neuronal cells and nonneuronal cells in the ganglia (Croen et al., 1988; Hyman et al., 1983; Gilden et al., 1987). Using Southern blot hybridization, approximately one copy of latent VZV DNA per cell was demonstrated in the trigeminal ganglia (Gilden et al., 1983). More specifically, polymerase chain reaction (PCR) was used to show that six to thirty-one latent VZV copies are present per 100,000 ganglion cells (Mahalingam et al., 1993). In contrast to other members of alphaherpesvirus family, latent VZV transcripts occur from the U_L and IR_S regions of the genome (Croen et al., 1988).

Considerable effort has been made to identify animal models for human VZV. However, no clinical signs or

pathological changes have been seen in animals infected with VZV (Myers et al., 1980; Myers et al., 1985).

Animal Model for Latency Studies

Primates were suggested as likely candidates for an animal model for VZV latency in humans. Like VZV infections in humans, the simian varicella virus (SVV) infects predominately dorsal root ganglia (Mahalingam et al., 1991). SVV establishes latency without the clinical signs of varicella. PCR was used to amplify latent SVV in the thoracic, trigeminal and cervical ganglia. Latent SVV was not detected in the brain of infected monkeys. Reactivation of SVV from latency has been unsuccessful (Mahalingam et al., 1992). SVV interacts with cytomegalovirus and this interaction will be examined further in the next section.

BETAHERPESVIRUS FAMILY

Cytomegaloviruses (CMV) are the major viruses belonging to the beta herpesvirus family. CMV are species-specific, replicate slowly in cell culture and cause disease to develop slowly in the host. Betaherpesviruses also differ from alphaherpesviruses by sequence complexity through gene acquisition and sequence divergence. CMV have a predilection for crossing the placental barrier unlike members of the alphaherpesviruses.

CYTOMEGALOVIRUS

High numbers of latent cytomegalovirus (CMV) have been detected in multiple organs of individuals acquiring CMV subsequent to organ transplants, blood transfusions, and in ovarian teratocarcinoma cells (Ho et al., 1975; Musiani et al., 1984). Immunosuppression of the host was the most probable cause of latent CMV in multiple organs.

In Vitro Latency Model

An in vitro latency model system was developed in human thyroid papillary carcinoma cell line (TPC-1). TPC-1 cultures were heated to 40^0C for 48 hours, infected with CMV and held at 40.5^0C for 30 days without detecting infectious virus. Reactivation was accomplished by decreasing the temperature to 37^0C (Tanaka et al., 1987). However, prostaglandin synthesis inhibitors such as indomethacin and

tetracaine prevent reactivation of CMV in the TP1 latency model (Tanaka et al, 1988).

Another in vitro latency model was generated using CMV infected human embryo lung fibroblast treated with interferon-alpha and acyclovir and incubated at 40.5^{0}C. No infectious virus was detected until removal of acyclovir and lowering of the temperature to 37^{0}C (Cockley and Rapp, 1986). Latent cytomegalovirus was present in supposedly "specific pathogen free" monkeys. Reactivation occurred following immunosuppression and exposure of the monkeys to SVV (Ohtaki et al., 1988).

Latent Mouse Cytomegalovirus Localization

Mouse cytomegalovirus (MCMV) is a useful model which corresponds to cytomegalovirus in humans. MCMV was recovered from latently infected macrophages stimulated with thioglycollate. As was expected, recovery of latent MCMV from unstimulated macrophages was low when compared to stimulated macrophages (85% vs. 10%) (Brautigam et al., 1979). Using co-cultivation assays, latent MCMV was detected in spleen cells, salivary glands and reproductive tissue but not in the brain, thymus, liver, or kidney (Olding et al., 1976; Dutko and Oldstone, 1979). More recently, latent MCMV was demonstrated in the salivary gland, spleen, and kidney using PCR (Klotman et al., 1990). In addition, latent MCMV was detected in the sciatic nerve (Abols-Mantyh et al., 1987). Latent MCMV was recovered from

enriched stromal cell fractions using co-cultivation, however, MCMV was not detected in the spleen by *in situ* hybridization assays (Mercer et al., 1988). Hayashi et al. (1985) first suggested latent MCMV in ocular tissue but limitations of his experiments did not allow differentiation of reactivated latent infection from chronic persistent infection. Subsequent research by Rabinovitch et al. (1990) clearly indicates ocular tissue as a site for latent MCMV.

Similar to Epstein Barr Virus, CMV have been found in human tumors (Giraldo et al., 1980; Huang and Paogano, 1978). However, it is unknown whether CMV is a carcinogen. Since MDV does induce tumors, an analysis of latency in a known oncogenic herpesvirus is justified.

GAMMAHERPESVIRUS FAMILY

EPSTEIN BARR VIRUS

Biological Features

An extensive review of a herpesvirus with similar biological characteristics to MDV may aid in understanding MDV latency. MDV and Epstein Barr Virus (EBV) have numerous characteristics in common; for example, both infect lymphocytes. Additionally, MDV serotype 1 and EBV transform lymphocytes albeit with different selectively, MDV serotype 1 transforms T-cells while EBV transforms B-cells. The similarities between MDV and EBV make it necessary to review in detail aspects of latency in EBV to determine if MDV latency more closely resembles alphaherpesviruses or gammaherpesviruses. Future experiments can then be designed that parallel latency in alphaherpesviruses or gammaherpesviruses.

EBV can persist in the host in a latent form. Latent EBV is found in Burkitt's lymphoma, nasopharyngeal carcinoma (NPC) (Raab-Traub et al., 1983), rheumatoid arthritis, infectious mononucleosis, and Hodgkin's disease (Sandvej et al., 1993). In Hodgkin's disease, the EBV genome is present in Hodgkin and Reed-Sternberg cells (HRS) (Weiss et al., 1989; Herbst et al., 1991).

Transcription

Similar to alphaherpesviruses, transcription of latent EBV is restricted when compared to transcription during a

lytic infection (Rickinson et al., 1987). However, unlike alphaherpesviruses, approximately 10 different proteins have been identified during latency. The proteins are designated: EBNA-1, -2, -3A, -3B, -3C, -LP, all which are present in the nucleus. Two integral membrane proteins, LMP-1 and -2 and two EBV encoded small RNAs (EBERs) that are also present during the various types of latency (Kieff and Liebowitz, 1990).

EBV establishes 3 types of latency (Rowe et al., 1992). Type I latency is found in Burkitt's lymphoma. In type I latency, EBV encodes for EBNA-1 only (Gregory et al., 1990; Rowe et al., 1992). Type II latency is present in nasopharyngeal carcinomas. Similar to type I latency, EBV produces EBNA-1 during type II latency however, LMP-1 and LMP-2 are also detected (Young et al., 1988; Fahraeus et al., 1988; Smith and Griffin, 1991; Brooks et al., 1992, Busson et al., 1992). Type III latency is unique because continued passage of Burkitt's lymphoma (BL) results in a conversion from type I to type III latency. In addition, type III latency is found in immunoblastic B-cell lymphomas and EBV transformed lymphoblastoid cell lines (LCL). Latent EBV expresses EBNA-1, -2, -3A, -3B, -3C and LP, LMP-1, LMP-2 and EBERs during type III latency (Lear et al., 1992). More recently, additional transcripts from the BamHI-A region have been identified in all types of latency. The putative promoter is in the BamHI-I region of the genome (Brooks et al., 1993).

Type I Latency

In type I latency, the promoter closest to the BamHI-F and -Q border (Fp) expresses EBNA-1. All other latent promoters are inactive during type I latency (Sample et al., 1991; Schaefer et al., 1991; Sample et al., 1992). Expression from the Fp promoter yields a distinct EBNA-1 transcript that is a splice product (Sample et al., 1991). Fp promoter contains two transactivators of gene expression. These transactivators are uncharacteristically located downstream of the transcriptional start site. One element exerts positive regulation while the other is a trans-acting negative regulator of Fp promoter activity. Negative regulation of Fp is mediated through the binding of EBNA-1 to the EBNA-1 DNA binding domain III (Sample et al., 1992).

Epstein Barr Nuclear Antigen-1

EBNA-1 is a DNA binding protein that recognizes a palindrome as its binding site. The carboxy-terminal one-third of EBNA-1 binds to DNA. (Rawlins et al., 1985; Jones et al., 1989). There are three EBNA-1 binding sites present in the EBV genome. Two sites are located in the latent origin of replication (Ori-P) while the third site is in the BamHI-Q fragment of EBV.

The binding of EBNA-1 to Ori-P allows the linear genome to circularize. Linear EBV genomes change to circular forms 16 to 20 hours after infection. This conversion requires the host cell to be in the G1 stage of the cell cycle. The

circular forms are characteristic of latency. Subsequently, the circular EBV persist as episomes in latently infected cells (Reisman et al., 1985; Yates et al., 1984; Yates et al., 1985; Adams, 1987).

Even though neither cellular proliferation nor stimulation of the activation marker CD23 are needed for latency, only cells expressing CD23 contain circular episomes. Thus, CD23 expression is not required for proliferation or immortalization. However, circularization is necessary for the establishment and maintenance of latency (Katz et al., 1989).

EBNA-1 activates Ori-P for one viral replicative cycle per host cell cycle (Adams, 1987; Yates and Guan, 1991). The region of Ori-P binding to EBNA-1 consists of 2 elements, a family of repeats, (FR) and dyad symmetry elements (DS). FR contain 20 EBNA-1 binding sites which initiate replication of the latent EBV episome. FR function by transcriptional activation of various promoters. Also, FR induce latent episomes to enter the daughter cells following cell division (Reisman et al., 1986; Sugden and Warren, 1989). The second element, DS, has only 4 EBNA-1 binding sites. Additionally, DS are responsible for initiation of replication (Wysokenski and Yates, 1989; Gahn and Schildkraut, 1989). FR are the first element bound by dimeric EBNA-1 followed by binding to DS. DS and FR are 1 kb apart, so they rely on a mechanism known as "DNA looping" to allow them to be brought into close proximity of one

another. DNA looping allows enhancer or repressor sequences to interact with sequences at a distance (Goldsmith et al., 1993).

As indicated previously, the ability of latent EBV to replicate is dependent on EBNA-1. EBNA-1 is composed of 641 amino acids which are high in proline content. The size of EBNA-1 ranges between 65 and 85 kDa. The variation in size of EBNA-1 in the various EBV isolates, depends on the number of codons in the simple triplet nucleotide internal repeat array (IR3) (Heller et al., 1981). IR3 encodes a glycine-alanine copolymer domain which is an immunogenic part of EBNA-1 (Hennessy and Kieff, 1983). In contrast, Yates et al. (1985) have demonstrated that this copolymer domain is a nonessential site for EBNA-1 functions.

IR3 encodes a portion of the 2.0 kb exon contained in the 3.7 kb transcript that encodes EBNA-1 (Hennessy et al., 1983). The transcript is present in low copy numbers. The protein encoded by this transcript is present in much higher quantities than would be expected from such few mRNAs. This 3.7 kb transcript is located in the U3-IR2 region and is polycistronic (Fig. 2). Long range splicing generates this transcript and the exons map to IR1-U2, U2, and U3 regions (Speck and Strominger, 1985).

Some cell lines differ in the region of the genome that is transcribed and interactions between proteins and latent EBV genome. The Raji cell line was established from an African Burkitt tumor and contains 50 copies of latent EBV

in an episomal circular form that can be induced to transcribe early genes. Also, Raji cells express an EBNA 1 that migrates faster than EBNA-1 of other cell lines due to a shorter IR3 repeat region (Rowe et al., 1987a).

EBV in lymphoblastoid tumors grown in nude mice have a restricted transcription pattern. The U2-IR2 and U4 region are transcriptionally active, but the majority of mRNA is transcribed from the U2-IR2 (Fig. 2). Conversely, the U4 region is not heavily transcribed nor are polyA+ transcripts demonstrated in epithelial tumors from nude mice (King et al., 1982).

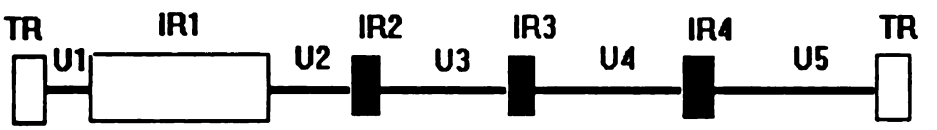


Figure 2. Genomic structure of Epstein Barr Virus

Methylation

Methylated DNA sequences are usually not expressed. DNA sequences of EBV are deficient in CpG but contain an excess of TpG and CpA. This is a diagnostic trait of higher eucaryotic DNA sequences. The eucaryotic DNA sequences have methylated cytosines in CpG doublets that result in

increased mutations to TpG. Similarly, EBNA-1 gene is methylated in Rael, an EBV latently infected lymphoblastoid cell line and C15, developed from an EBV positive NPC co-transplanted into nude mice. In both Rael and C15, EBNA-1 is expressed. CpG pairs in Ori-P were hypomethylated. This suggests a specific function for methylation in the regulation of latency that is not seen with HSV (Ernberg et al., 1989). As stated previously, Burkitt's lymphoma expresses only EBNA-1 (Rowe et al., 1987b) but a demethylating agent, 5-azacytidine can upregulate the expression of EBNA-2, -3A, -3B, -3C, -LP and LMP. This indicates that DNA methylation plays an active role in the regulation of latent EBV genome (Masucci et al., 1989). Inhibition of DNA methylation during type I latency by 5-azacytidine caused the activation of Wp and Cp promoters with the expression of proteins observed in type III latency. This suggests that methylation may play a role in Fp inactivity (Masucci et al., 1989; Schaefer et al., 1991). In addition, methylation of Wp during type I latency inhibits its activity. This result provides further evidence that methylation controls promoter activity by negative regulation (Jansson et al., 1992).

Type III Latency

The instability of type I latency yields a conversion to type III latency by continued passage of Burkitt's lymphoma cells (Gregory and Rickinson, 1990). Anti-IgG

induced reactivation of latent EBV genome in a BL cell line. This induction is mediated through a calcium/calmodulin pathway. Interestingly, the reactivation of EBV by anti-IgG is blocked by cAMP while the reactivation of HSV is induced by cAMP (Daibata et al., 1990). Whether the calcium pathway is involved in the conversion from type I to type III latency is unknown. Type III latency also occurs in healthy individuals which are persistently infected with EBV.

In type III latency, the FP promoter is inactive. Promoters Cp or Wp located in BamHI-C and -W regions are responsible for expression of EBNA-1, -2, -3A, -3B, and -3C proteins. The bi-directional LMP promoters transcribe LMP proteins (Lear et al., 1992; Woisetschlaeger et al., 1990). LCL carry multiple extrachromosomal copies of EBV as an episome. In EBV infected lymphomas, restriction fragments indicative of fused termini demonstrate circular, episomal, latent genomes (Katz et al., 1988).

Nuclear Proteins

The nuclear proteins expressed during type III latency have been well characterized. Immunofluorescence microscopy identified the subnuclear location of the latent EBV nuclear proteins. Metaphase chromosomes bind EBNA-1 while EBNA-2, -3A, -3C and -LP are localized to subnuclear granules. EBNA-1 differs also in its lack of nuclear matrix association while EBNA-2, -3A, -3C, and -LP are associated to the nuclear matrix and chromatin fractions (Petti et al.,

1988). All three types of EBV latency express EBNA-1. In addition, EBV encoded small RNAs (EBERs) are also present although their function is unknown.

EBV encoded small RNAs (EBERs)

The EBERs are nonpolyadenylated. EBERs are the most abundant latent RNA with approximately 10^7 transcripts present per latently infected cell (Howe and Shu, 1989). EBER genes are inhibited by alpha-amanitin which is consistent with RNA polymerase III transcription. The localization of EBER to the nucleus is in association with an unknown cellular protein (Howe and Steitz, 1986; Howe and Shu, 1989).

Sequence analysis reveals similarity between EBERs and adenovirus VA1 and VA2; both have similar secondary structures. A potential role of EBERs is in nuclear RNA splicing. The small size of EBERs correlates with other small nuclear ribonucleoproteins which function in processing mRNA to their mature functional form (Kieff and Leibowitz, 1990.

Epstein Barr Nuclear Antigen-Leader Protein (EBNA-LP)

Epstein Barr nuclear antigen-leader protein (EBNA-LP) has some remarkable features. EBNA-LP is transcribed from the leader sequence of each of the EBNA mRNAs. The spliced product of the 1st and 2nd exons of all EBNAs forms the EBNA-LP initiation codon. EBNA-LP consists of alternating

22 or 44 amino acid repeat domains. Phosphorylation of EBNA-LP occurs at multiple serine sites. EBNA-LP is present in the nucleus of cells (Kieff and Leibowitz, 1990). Transcription from IRI and U2 which encodes EBNA-LP and -2 occurs earlier and more abundantly when compared to domains encoding EBNA-3A, -3B, -3C or -1. Similar to EBNA-1 and -3, the size of EBNA-LP varies depending on the EBV strain (Finke et al., 1987).

Epstein Barr Nuclear Antigen-3 (EBNA-3)

Genes encoding EBNA-3 have a common origin. These genes are located in tandem in the EBV genome. They are designated EBNA-3A, EBNA-3B and EBNA-3C (Bodescot et al., 1987; Rickinson et al., 1988). EBNA-3 is associated with latent infection based on its presence in IB4 and Namalwa cell lines. IB4 cells were derived by transforming human placental lymphocytes with EBV strain B95-8 while Namalwa is a nonpermissive Burkitt tumor cell line. These cell lines do not express proteins from early or late genes (Hennessy and Kieff, 1985).

As mentioned previously, the size of the protein varies depending on the EBV isolate. The EBNA-3 size variation indicates genetic polymorphism that exists between cells. Human sera detected a 140 kDa protein in X50-7 and Raji cells but not JC-5 cells (Hennessy and Kieff, 1985). X50-7 cell are human umbilical cord lymphocytes (HUCL) immortalized by B95-8 EBV. JC-5 was produced by

transforming HUCL with Burkitt's lymphoma cells. Sera from patients with rheumatoid arthritis reacted with the 140 kDa protein. B95-8 cells, a marmoset B lymphoid cell line, encode an 87 kDa protein while AG876 cells, a virus producing B lymphoid cell line, encode an EBNA-3 that is 78-80 kDa (Wang et al., 1990).

A human sera reacts with EBNA 2 and EBNA 3 but not EBNA 1. WC serum is specific for EBNA 3 in P3HR-1 cells because the serum does not react with EBNA-1 and EBNA-2 is not expressed in these cells (Hennessy and Kieff, 1985). Functionally, EBNA-3 induces B-lymphocyte activation antigens CD21 but not CD23.

Epstein Barr Nuclear Antigen-2 (EBNA-2)

EBNA-2 has important roles in: the processing of B-lymphocyte activation to the lymphoblastoid phenotype (Abbot et al., 1990), the lowering of rodent fibroblast serum dependence (Dambaugh et al., 1986) and the induction of B-cell activation (Cordier et al., 1990).

The IR1-U2 domain of the EBV genome transcribes a 3 kb mRNA that encodes EBNA-2. The transcript is spliced and only 3 copies of the transcript are present per cell in IB4 cell lines. IB4 is a latently infected B-cell line established by infection of human umbilical cord lymphocytes with EBV (van Santen et al., 1983). The principal exon was identified in IR1 region by DNA sequencing. However, this

exon does not hybridize to the 3 kb transcript (Cheung et al., 1982).

The 3 kb transcript that encodes EBNA-2 has exons totaling 2 kb. These exons are unique in their organization of complex splicing which originate in the IR1 domain and extend into the U2 domain (Dambaugh et al., 1986). The first splice site creates an AUG. The importance of this distinctive type of splicing is unknown. EBNA-2 mRNA has other unique features. The translation initiation site is over 1 kb from the RNA cap site and the ORF terminates 138 bases before the start site of translation. EBNA-2 is encoded by an ORF present entirely in the U2 region (Sample et al., 1986). Additionally, a biscistronic mRNA translates EBNA-LP from its 5' ORF while translating a portion of EBNA-2 from its 3' ORF (Wang et al., 1987). EBNA-2 is composed of 491 acidic amino acids (Kieff and Liebowitz, 1990).

EBNA-2 is necessary for EBV induced B cell growth transformation. An EBV mutant, P3HR-1, has a deletion encompassing the entire IR1-U2 region which encodes EBNA-2. This mutant can infect lymphocytes but is unable to produce transformation which indicates a role for EBNA-2 in transformation (Hennesey and Kieff, 1985). However, since other genes are present in this region, a role in transformation can not be discounted.

The expression of latent membrane protein (LMP) occurs through transactivation of LMP promoters by EBNA-2 (Ghosh and Kieff, 1990; Fahreus et al., 1988; Wang et al., 1990;

Zimber-Strohl et al., 1991). During low passage of BL cells, EBNA-2 and LMP expression is low. Continued passage of BL cells results in an increase in EBNA-2 and LMP expression (Rowe et al., 1987a). Additionally, the expression of LMP at high levels was shown to be toxic to B lymphoid cell lines (Hammerschmidt and Sugden, 1989).

Type II Latency-Latent Membrane Protein (LMP)

RNA polymerase II is responsible for transcription of EBNAs and LMP (Howe and Shu, 1989). Interestingly, transcription from LMP promoter is lower than EBNA transcription. However, LMP mRNA is the most abundant of the latency mRNA besides EBERs. This result indicates either LMP mRNA is more efficiently processed or it has a longer half-life (Sample and Kieff, 1990). The genes expressing EBNA-2 and LMP were examined for their degree of methylation in various cell lines. In Rael and C15, these genes were extensively methylated but not in lymphoblastoid cell line (LCL).

LMP expression occurs from promoters located in BamHI-N region of the genome during Type II latency (Brooks et al., 1992; Busson et al., 1992; Kerr et al., 1992; Smith et al., 1991; Smith et al., 1992). The bidirectional LMP promoters are 3 kb apart and drive the LMP1 transcript leftward while driving LMP2A and 2B rightward (Laux et al., 1989; Sample et al., 1986). LMP1 functions as an oncogene in rodent fibroblasts (Baichwal and Sugden, 1988), induces B-

lymphocyte activation (Wang et al., 1990), and induces cellular adhesion molecules in EBV-negative Burkitt's lymphoma, Louckes. Expression of LMP occurs only if termini are fused as in latency and only rarely from integrated genomes (Hurley et al., 1991; Laux et al., 1988).

Latent EBV interacts with cellular genes to activate B-lymphocyte proliferation. However the mechanism responsible for this activation is unknown. Cellular vimentin expression is abolished by mutagenesis of LMP. However, the restoration of LMP expression increases vimentin levels (Birkenbach et al., 1989). Vimentin usually localizes with LMP on the plasma membrane. This localization forms a bridge between LMP and the cytoskeleton (Leibowitz et al., 1987). Deletion of the amino-terminus of LMP abolishes the ability of LMP to associate with the cytosketon and prevents localization of LMP at the cell periphery (Wang et al., 1988a). The amino terminus of LMP stimulates EBV-induced cytotoxic T-lymphocyte (CTL) (Murray et al., 1988). More specifically, a sequence of 10 amino acids from LMP can induce a CTL response. This result indicates the presence of a LMP antigenic determinant (Thorley-Lawson et al., 1985).

LMP expression results in the loss of contact inhibition and anchorage independence in rodent fibroblasts. When the amino terminus and first four transmembrane domains of LMP are deleted, transformation of rodent fibroblasts does not occur (Wang et al., 1988b). LMP1 expression

induces adhesion molecules ICAM-1, LFA-1 and LFA-3 in various cell lines. Additionally, LMP1 expression downregulates the Burkitt's lymphoma marker, CALLA. The induction of LFA-3 and ICAM-1 leads to an increased adhesion to T-lymphocytes resulting in more effective T-cell immune surveillance (Gregory et al., 1988). LMP1 and EBNA-2 coexpression enhanced CD23 surface expression as opposed to either alone (Wang et al., 1990). Cells positive for CD23 and infected with EBV undergo immortalization (Thorley-Lawson and Mann, 1985). In HRS cells, there is no induction of LFA-1, ICAM-1 and LFA-3 expression by LMP-1 nor enhancement of CD23 expression (Sandvej et al., 1993).

Recent evidence also implicated EBV in diseases of maturation defects in myelopoiesis. This information suggests a role for EBV during hematopoiesis maturation (Revoltella et al., 1989). LMP1 may play a role in impeding differentiation of hematopoietic cells along the neutrophil lineage in favor of the monocyte/macrophage lineage. Therefore, LMP1 may inhibit the production of mature neutrophils (Fairbairn et al., 1993).

LMP2B is smaller than LMP2A because of a lack of 119 amino-terminal amino acids found in LMP2A. The fused terminal repeats of the latent EBV genome encode both LMP2A and LMP2B. LMP2A is a 54 kDa protein encoded by a 2.3 kb transcript. In contrast, a 2.0 kb transcript encodes LMP2B. LMP2B is 40 kDa in size (Busson et al., 1992). The expression of LMP2A and 2B was tested using antiserum

against fusion proteins. Using immunofluorescence, these proteins were shown to co-localize with LMP1 (Longnecker and Kieff, 1990).

LMP2A interacts with tyrosine kinase and is involved in serine and tyrosine phosphorylation (Longnecker et al., 1991). LMP2A also inhibits calcium mobilization by cross-linking with agents which normally stimulate calcium release such as IgM, CD19, and MHC class II. Furthermore, LMP1 can reverse the inhibition of calcium caused by LMP2A resulting in a return to normal calcium levels (Miller et al., 1993).

As mentioned previously, type II latency occurs in NPC. Only EBNA-1 is expressed regularly in NPC while LMP is detected on 50% of NPC (Allday et al., 1989). Transfection of the gene that encodes LMP, one of only two proteins expressed in NPC, into a epithelial cell line resulted in a change in epithelial cell surface phenotype. The change resembled the surface changes seen with NPC. The ability of LMP to prevent terminal differentiation could lead to answers in the quest to determine the pathogenesis of NPC (Dawson et al., 1990).

Data indicate that regions of the genome that are transcribed in latently infected lymphoblastoid cell lines are also transcribed in nasopharyngeal tissue. However, other regions of the genome are also transcriptionally active in nasopharyngeal carcinoma specifically BamHI-B, -K, -Y, -B1, I1, and -A fragments as well as EcoRI F and -G fragments (Raub-Traub et al., 1983). Antibodies to EBNA-1

were detected in individuals with BL, NPC or rheumatoid arthritis while antibodies to other EBNAs were observed in only a small proportion of sera tested (Rowe et al., 1987a).

Codon Usage of Genes Expressed During Latency

Eight different genes are expressed during latent EBV infection. These genes display codon usage that are different from genes expressed during a lytic infection. The percentage of S3 (G or C in codon 3) is lower in latent genes than those expressed in a lytic infection. In addition, the largest difference in amino acid usage between latent and lytic genes is between codon types SSN and WWN (W is A or T while N is any nucleotide). There are two possible explanations for the difference in codon usage between latent and lytic infections. First, the difference in codon usage may prevent the harmful effects that latent viral gene expression could have on the host such as competition between virus and host for limited host resources. Secondly, latent genes could have been obtained by the viral genome during evolution of the virus (Karlin et al., 1990).

Reactivation

Epstein Barr virus is the only herpesvirus in which a protein responsible for reactivation has been identified. The WZhet fragment contains a 2.7 kb fragment designated BZLF1 (Rooney et al., 1988; Grogan et al., 1987). BZLF1

gene encodes a 43 kDa protein, BamHI fragment Z Epstein-Barr replication activator (ZEBRA). ZEBRA is necessary for the EBV latent genome to switch to active replication (Miller, 1990; Taylor et al., 1989; Countryman et al., 1987). During latency, ZEBRA expression is repressed. Rearrangements in the WZhet region induce the expression of ZEBRA (Rooney et al., 1988). Agents such as phorbol esters, butyrate and serum factors also induce the activation of ZEBRA gene (Taylor et al., 1989). ZEBRA is a site specific DNA binding protein that probably functions as a transcriptional transactivator (Miller, 1990). Whereas ZEBRA increases the activity of two early promoters, it decreases the activity of three latent promoters; BamHI-C, and BamHI-W EBNA promoters and the bidirectional LMP promoter (Kenney et al., 1989). Inhibition of signals produced by the glucocorticoid receptor represses Cp promoter. The inhibition of signals is mediated through activation of c-fos by ZEBRA. ZEBRA also transactivates its own promoters (Sinclair et al., 1991).

The literature review reflects the fundamental problems involved in the various latency models that are currently used. In this dissertation, a simple system allowing manipulation of latent herpesvirus infections is explored. My research centers on analysis of productive and latent infections at the molecular level. Research focus was aimed at identifying a means to detect a productive infection and latent infection and being able to differentiate between the two. I have analyzed latent HVT infections. The objectives of this study were:

1. To determine if gB is expressed <u>in vivo</u> and whether gB expression can be used to monitor a productive infection.

2. To determine temporal aspects of the termination of a productive HVT infection in peripheral blood mononuclear cells (PBMC) of chickens.

3. To identify termination of a productive HVT infection in lymphoid organs; bursa, thymus and spleen.

4. To demonstrate the presence of latent HVT in various tissues: PBMC, bursa, thymus, spleen, sciatic plexus, brachial plexus, feather follicles, cerebrum, cerebellum, pons, medulla oblongata, dorsal root ganglia from cervical vertebrae 12-14 and lumbosacral vertebrae.

5. To locate the presence of latent HVT in T cell subsets.

6. To detect the region of the genome that is transcriptionally active during latency.

Comparative Study of Tissue Preparation and Conditions for Combined Immunofluorescence and *in situ* Hybridization

Margo S. Holland[1,2], Charles D. Mackenzie[1], Robert W. Bull[1], and Robert F. Silva[2]

Department of Pathology, Michigan State University[1], USDA-Agricultural Research Service, Avian Disease and Oncology Laboratory[2], East Lansing, MI 48823

ABSTRACT

The objective of this study was to identify a single set of conditions that would be optimal for both indirect immunofluorescence assays (IFA) and in situ hybridization (ISH). Various fixatives were evaluated using a system of IFA to detect turkey herpesvirus glycoprotein B (gB) and ISH to identify HVT mRNA. A precipitating fixative (acetone) was compared to cross-linking fixatives [buffered glutaraldehyde-picric acid (BGPA), formalin, and 4% paraformaldehyde] in both IFA and ISH using spleen, thymus, bursa, sciatic plexus, brachial plexus, and feather tips of 28 day-old chickens. The cross-linking fixative, 4% paraformaldehyde, was the optimal fixative for preserving all tissues examined and could be used for both IFA and ISH. Glass slide preparation, incubation temperatures, and tissue processing were individually evaluated for ISH and IFA. Silylated slides provided the best retention of tissue sections for ISH and IFA. In the IFA, 37 C was the ideal incubation temperature while the optimal incubation temperature in ISH was 47 C. Blocking agents decreased background fluorescence. Of the tested blocking agents, Evans blue dye prevented background fluorescence to a greater extent than either calf serum or bovine serum albumin. This study provides the first published account comparing cross-linking fixatives to preserve tissues in order to detect viral proteins and nucleic acids in lymphoid and nervous tissue.

Introduction

Indirect immunofluorescence (IFA) and *in situ* hybridization (ISH) can be used to detect proteins and nucleic acids. The difficulty in using these two techniques in the same tissue results from the basic fact that established protocols for these two techniques usually incorporate different tissue processes. One of the most critical components of tissue processing is tissue fixation.

Successful fixation of tissues includes: minimal extraction of membrane proteins, prevention of dissolution of tissues during storage and adequate morphological preservation (Elias, 1990). Fixatives accomplish these objectives in different ways. Precipitating fixatives preserve tissues by coagulating proteins. Precipitating fixatives act by turning protein chains outwards thus breaking the hydrogen bonds and salt links. This action reveals to a varying extent end groups of side chains which interact with the precipitating fixative (Pearse, 1968). Cross-linking fixatives preserve tissues by forming bonds with protein end groups. Cross-linking fixatives react with free amino groups present on lysine residues, or arginine and asparagine side chains (Kitamoto and Maeda, 1980). Both precipitating and cross-linking fixatives cause alterations in the tertiary structure of tissue antigens. These alterations modify accessibility of the antigen thereby

interfering with both the penetration and binding of a marker antibody (Elias, 1990).

Immunohistochemistry requires a balance between maintaining the immunogenicity of tissue antigens and preserving morphological detail. Precipitating fixatives such as acetone, methanol or ethanol are used to fix tissues for IFA because they do not form additive complexes, nor block antigenic determinants (Culling et al., 1980; Elias et al., 1989). Precipitating fixatives typically are used for the detection of antigens localized to the cytoplasm and plasma membrane (Culling et al., 1980; Farmilio and Stead, 1990). Precipitating fixatives enhance the localization of a large group of tissue antigens when compared to certain cross-linking fixatives (Mitchell et al., 1985). In addition, precipitating fixatives allow the antibody sufficient accessibility to the antigen (Farmilio and Stead, 1990). Even though precipitating fixatives have been used in IFA, they still present some problems. Precipitating fixatives may not penetrate the tissue sufficiently and also produce shrinking of cells (Farmilio and Stead, 1990).

The inability of most antibodies to penetrate the bond formed by cross-linking fixatives has limited the use of cross-linking fixatives in IFA. The use of cross-linking fixatives has therefore been restricted to the detection of antigens present on the cell surface (Farmilio and Stead, 1990).

Fixation of tissues for ISH must involve adequate immobilization of nucleic acids, prevention of degradation of nucleic acids by enzymes and preservation of tissues for long storage periods. As with all histological techniques, ISH requires maximal morphologic preservation of tissues. Perfusion of tissues with select fixatives achieves these objectives. Cross-linking fixatives penetrate tissues and preserve cell morphology at a level superior to precipitating fixatives. This is the main reason that cross-linking fixatives are used to preserve tissues for ISH. Tissues used for ISH are fixed primarily with 4% paraformaldehyde. Buffered glutaraldehyde-picric acid (BGPA) has not been evaluated for its use in detecting viral antigens and nucleic acids by IFA and ISH at the light microscopic level. A study of the comparisons of conditions in IFA and ISH is also lacking in the literature.

In this report, we compare various conditions for IFA and ISH to identify optimal conditions for the concurrent use of both techniques in the same tissue. The comparisons included cross-linking and precipitating fixatives for the preservation of a variety of tissues. The model system used in this study was turkey herpesvirus (HVT). The various procedures were applied to the detection of viral glycoprotein and RNA in HVT infected cells.

MATERIALS AND METHODS

Birds and holding facilities. One day old chickens (N=12) of an F_1 cross between $15I_5$ males and 7_1 females were obtained from an unvaccinated breeder flock. The chickens were inoculated intra-peritoneally with 2000 pfu of HVT, strain FC126. HVT is Marek's disease virus (MDV) serotype 3. Separate Horsfall-Bauer isolators housed HVT-infected and uninoculated control chickens under similar conditions.

Tissue isolation. At 28 days postinfection, birds were anesthetized with halothane. The skin, musculature and peritoneum were incised and reflected to expose the abdominal organs. The ribs were separated at the costochondral junction to expose the heart. A 20 gauge catheter was placed in the left ventricle and 5ml/kg of body weight of physiological buffered saline solution [(PBS) 120 mM NaCl, 2.7 mM KCL, and 10 mM PO_4 buffer, pH 7.4] was perfused into the heart. After infusion of PBS, the right auricle was incised to allow drainage of PBS and blood. Each of the individual fixatives (4% paraformaldehyde, 10% formalin, BGPA) was perfused into different chickens at 5ml/kg of body weight by gravity. The spleen, bursa, thymus, sciatic plexus, brachial plexus and feathers were each removed and cut into 2 cm x 4 mm sections. The tissues were placed into tissue cassettes and immersed in 25 ml of the fixative for 24 hours. The tissues were then processed for sectioning by standard paraffin embedding techniques.

Preservation of tissues. Tissues were preserved by either freezing or fixation. For preservation by freezing, tissues were immersed in 30% sucrose, a cryoprotectant that prevents the formation of ice crystal artifacts. Next, tissues were quick frozen by submerging them in a container of liquid nitrogen. Isopentane, a mounting media, was applied to a small section of tissue and the tissue was adhered to a round cork for sectioning and stored at -70 C. The alternate method was to fix tissues by perfusion with either 10% formalin, 4% paraformaldehyde or BGPA for 24-72 hours.

Peripheral Blood Mononuclear Cells. Two ml of blood were collected from each bird into vacuum tubes that contained the anticoagulant, heparin. Approximately 0.25 ml of blood was drawn into a capillary tube and two drops were placed on a slide and smeared. These smears were fixed with either 4% paraformaldehyde, acetone or 10% formalin (Table I). Twenty of the blood smears were not chemically fixed (Table II). Buffy coat cells were removed from blood samples by centrifugation (IEC model HN-SII, Damon/IEC Division, Needham Hts, Mass.) for 20 minutes at 500 rpm. Buffy coat cells were collected and cytocentrifuged onto gelatin coated, poly-l-lysine coated, or uncoated slides (Table III and IV).

Gelatin Coated Slides. Slides were soaked in sulfuric acid diluted 2:1, rinsed with running water, and then soaked overnight in hot water with detergent followed by four changes of distilled water. The gelatin solution contained 0.1% calf skin gelatin and 0.01% chromium potassium sulfate that was filtered through Whatman #1 filter paper. The slides were dipped twice in gelatin. Next the slides were dried at 37 C overnight and stored at room temperature in a desiccant.

Poly-l-lysine Coated Slides. Slides were immersed in Triton-X for 2 hours, followed by a wash in running water for 1 hour. Slides were soaked in 2 changes of 100% ethanol for 10 minutes and allowed to air dry. Slides were dipped in poly-l-lysine ([L]-polylysine, 50 ug/ml in 10 mM Tris-HCL, pH 8.0) for 30 minutes. Poly-l-lysine coated slides were air-dried and stored at room temperature with silica gel as the desiccant.

Blocking Agents. Nonspecific blocking agents were used to reduce nonspecific staining (Table I, III, IV, V, VI and VII). All sections were treated with 3% hydrogen peroxide for 5 minutes and washed in saline solution. The sections were then immersed in either 1% Evans blue dye at 37 C for 30 minutes, 3% Bovine serum albumin at 37 C for 1 hour, or 10% calf serum at 37 C for 1 hour.

Indirect Immunofluorescence Assay. Six micron thick sections of spleen, bursa, thymus, sciatic plexus, brachial plexus, and feather tips were placed on either poly-l-lysine, gelatin, or silylate coated slides (Onascoe, Houston, TX). Monoclonal antibodies L78 and IAN.86 were used as primary antibodies. L78 is specific for HVT gB while IAN.86, cross reacts with HVT and MDV serotype 1 gB. Monoclonal antibodies were added to the slides at dilutions ranging from 1:10 down to 1:1000 (Table II and VIII).

Two hundred and fifty uls of each Mab were added to each slide that contained either mononuclear cells or tissue sections. The slides were incubated for 30 minutes at 37 C, room temperature, or on ice. The slides were washed 3 times with deionized water followed by 3 washes with PBS. Two hundred and fifty uls of goat anti-mouse IgG conjugated to isothiocyanate were added to the slides and the slides were incubated at 37 C, room temperature or on ice for 30 minutes. The slides were washed 3 times with deionized water followed by 3 washes with PBS. Coverslips were applied to the glass slides and examined with an ultra-violet microscope (Leitz-Wetzlar, Germany).

In situ hybridization. The ISH procedure described by Brahic and Haase (1978) was modified for use in this study. The BamHI F fragment of HVT was subcloned into pBluescript vector (pKS-). An HVT ^{32}P labeled DNA probe was made using random primers DNA labeling system (BRL Life Technologies,

Inc., Gaithersburg, MD) and purified using a G-50 sephadex column. Tissue sections were incubated with proteinase K (1 ug/ml) for 30 minutes in a humidified chamber at 37 C followed by a 1 minute wash in deionized water. The sections were placed in 0.1 M triethanolamine, pH 8.0 for 1 minute. The next step was a 10 minute incubation in 0.1 M triethanolamine, and 0.25% acetic anhydride at room temperature. The sections were washed in 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate, pH 7), dehydrated through graded ethanol washes and air-dried. Probes were denatured at 100 C for 5 minutes and added to 50 ul of hybridization buffer (50% deionized formamide, 3x SSC, 10% dextran sulfate, 50mM Na_2PO_4, 1x Denhardt's, 100 ug/ml yeast tRNA, 100 ug/ml denatured salmon sperm DNA, and 100 mM dithiothreitol). The concentration of the probe ranged from 6 x 10^7 to 1 x 10^9 counts per minute/ug of DNA. Approximately 10 ng of probe DNA was added to each tissue section. The sections were covered with wax film cut slightly larger than the section to prevent evaporation of the hybridization buffer. Hybridization of the sections was performed in a humidified chamber to prevent drying of the tissue sections. To determine the optimal hybridization temperature, sections were hybridized at either 37 C, 40 C, 45 C or 47 C for 24 hours.

Posthybridization consisted of 5 washes: 1. 2x SSC for 30 minutes at room temperature, 2. 1x SSC for 30 minutes at room temperature, 3. 0.2x SSC for 15 minutes at room

tem

or

The

dri

emu

The

tig

pho

ove

7,

(Ea

The

her

Eva

cri

aft

of

bou

wit

dis

jud

temperature, 4. 0.2x SSC for 60 minutes at 37 C, 40 C, 45 C or 47 C, and 5. 0.2x SSC for 30 minutes at room temperature. The sections were dehydrated in graded ethanol and air dried.

For autoradiography, slides were dipped in Kodak NTB emulsion 2 (Eastman Kodak, Rochester, NY) in total darkness. The slides were placed in slide holders and then in a light tight adjustable cut film developing tank (Yankee photography, Phoenix, AZ.). The slides were allowed to dry overnight at room temperature and then incubated for 2, 4, 7, or 10 days at 4 C. The slides were developed in D-19 (Eastman Kodak, Rochester, NY) and fixed in Kodak rapid fix. The slides were washed in water and the tissues stained with hematoxylin and eosin.

Evaluation criteria. Cellular morphology was used as the criterion to compare the quality of fixation both before and after IFA and ISH. "Excellent" morphology was the presence of clear cellular outlines, nuclear and cytoplasmic boundaries and ability to differentiate cell types, while with "good" morphology the cellular outlines were less distinct. The inability to distinguish cell margins were judged as "poor" morphology.

RESULTS

Fixation. After fixation of tissues, the sections were evaluated for morphological preservation. The cellular morphology of formalin fixed tissue was good before further processing. However, after processing tissues for IFA, cellular morphology was poor in formalin fixed tissues. On the basis of these results, formalin was regarded as an unsuitable fixative for tissues used for IFA. Cellular morphology was excellent after performing ISH on formalin fixed tissues. BGPA and 4% paraformaldehyde fixed tissues had excellent cellular morphology prior to IFA and ISH. In addition, quality of morphology was regarded as excellent after treatment of tissues for ISH and IFA. The precipitating fixative, acetone provided poor fixation in relation to cellular morphology before and after treatment of tissues. 4% paraformaldehyde provided excellent fixation of both cytocentrifuged peripheral blood mononuclear cells (PBMC) and blood smears. Acetone and formalin provided good morphology of PBMC and blood smears. Therefore, comparisons between BGPA, 4% paraformaldehyde, formalin and acetone demonstrated that 4% paraformaldehyde was superior for fixing tissues and PBMC for the use in IFA and ISH.

Immunocytochemistry. Evans blue dye decreased background fluorescence more efficiently than either bovine serum albumin or calf serum (Table I, III, IV, V, VI, and VII).

The fixatives, BGPA and 4% paraformaldehyde allowed staining of tissues with monoclonal antibodies. Tissues incubated at 37 C had fluorescing cells more consistently than tissues incubated at room temperature or ice. Under optimal conditions for IFA, results such as those in Figure 2 were obtained.

After performing IFA on avian blood, nonspecific fluorescence of the nucleated red blood cells by isothiocyanate fluorescein was seen. To decrease the background fluorescence, various blocking agents were tested (Table I, III, IV and VII). Neither bovine serum albumin nor calf serum reduced background fluorescence. However, the addition of Evans blue dye changed the nonspecific fluorescence of RBC to a dull yellow color. The color change allowed differentiation of RBC from positive fluorescing cells.

A comparison between blood smears and cytocentrifugation determined which method to use for preparing cells for subsequent IFA (Tables I, II, III, IV, and VII). With the blood smears, RBC obscured the mononuclear cells. Nonspecific fluorescence of RBC compounded the problem of identifying positive fluorescing mononuclear cells in IFA. Separation of mononuclear cells from RBC followed by cytocentrifugation concentrated mononuclear cells. This allowed the examination of a larger number of mononuclear cells for IFA. Mononuclear cells

affixed to glass slides by cytocentrifugation were positive for fluorescence in the IFA.

Background fluorescence was higher with unfixed cells and thus made this approach unsuitable for IFA (Table II and VII). Comparisons of fixatives demonstrated the best results were obtained using 4% paraformaldehyde (Table I, III, and IV). With 4% paraformaldehyde as the fixative, the fluorescence was bright green and background fluorescence was low (Figure 1). In contrast, acetone fixed positive cells had a less intense green color and slightly higher background fluorescence. Positive cells fixed with formalin had patchy yellow green fluorescence and background levels were high.

The antibodies compared were IAN.86, a monoclonal antibody that reacts with HVT and MDV serotype 1 gB and L78 that reacts with HVT gB only. L78 gave superior results, based on samples being consistently positive (Table III and IV). Few positive cells were seen after treatment with IAN.86 and results were not consistent from sample to sample. However, the conditions that proved optimal for detection of gB by L78 also were optimal for IAN.86. The examination of various dilutions of L78 determined which gave positive fluorescence with the lowest background levels (Table II). The background level of fluorescein staining was high with very concentrated dilutions such as 1:10. In comparison, dilute preparations such as 1:1000 had low background but few cells stained positively. A 1:100

dilution of L78 gave the best results when compared to dilutions ranging from 1:10 to 1:1000. The cells were bright green and the background fluorescence was low. Incubating cells and antibodies at a temperature of 37 C gave consistently positive fluorescing cells (Table I, III, IV, VI, and VII). However, incubation of cells for IFA at room temperature had fewer positive fluorescing cells and cells were not consistently positive between samples. In addition, incubating cells on ice gave negative results. The comparison of incubation temperatures demonstrated that 37 C was the ideal temperature compared to room temperature and incubation on ice.

Coating of slides.

Retention of tissue sections on slides treated with various coatings are represented in Table IX. Tissue sections did not readily adhere to uncoated slides. Poly-l-lysine and gelatin had loss of tissue sections. However, silylated slides produced the best results of all the coatings tested; sections were not lost after performing either ISH or IFA from silylated slides.

In situ hybridization.

Table IX and X represent the results obtained when comparing parameters for optimal ISH. While acetone fixed tissues had positive foci, the cellular morphology was so poor that cellular outlines could not be identified. The cellular

morphology with all the cross-linking fixatives was excellent after ISH. However, formalin fixed tissues had a small number of foci in tissue sections that were positive. BGPA and 4% paraformaldehyde gave comparable results. However, the number of foci and tissue sections positive was higher with 4% paraformaldehyde fixed tissues. Figures 3A, B, and C demonstrate the grain density of positive foci with BGPA, 4% paraformaldehyde and formalin fixed tissues respectively. Tissues incubated at 47 C had the most positive foci. Also, tissues immersed in emulsion for 4 days at 4 C had the lowest background grains. The conditions demonstrated with lymphoid tissues worked equally well with nervous tissues.

DISCUSSION

We identified a single set of conditions optimal for performing both IFA and ISH on sections from the same tissue sample in lymphoid and nervous tissues. ISH and IFA are intricate procedures whose results depend on the interrelationship between a number of factors such as: fixation, immunological reagents and their concentrations, probes, blocking agents, coating of slides, incubation temperature and tissue type.

Fixation of tissues is one of the most important components of IFA and ISH. The delivery of the fixative is the first important consideration. Fixation by block immersion yields inconsistent results because some tissue section areas are fixed more uniformly than others (Feldman et al., 1983). Fixation begins at the periphery of the tissue and proceeds inward, where the speed of the inward progress is critical and in some cases allows postmortem autolysis to occur in the center of the specimen (Preece, 1972). By comparison, perfusion fixation yields a more homogenous preservation and uniform staining of cells in tissues. Therefore, perfusion fixation allows better staining patterns than immersion fixation (Feldman et al., 1983).

We provide the first published account using BGPA to fix tissues for the subsequent use in the detection of viral nucleic acids by ISH. Even though BGPA provided excellent

resu

cons

cho

che

pen

sta

ISH

be

was

or

we

DN

pe

th

f

c

G

t

i

results, its carcinogenicity is a factor that must be considered prior to its usage.

Formalin is believed by some to be the fixative of choice for histochemistry because it is easy to prepare, cheap, provides consistent morphological preservation, penetrates tissues swiftly and it is suitable for most stains (Elias, 1990). Formalin was unsuitable for IFA and ISH in our studies because positive fluorescence could not be differentiated from background fluorescence. This effect was independent of the blocking agent, concentration of Mab, or temperature of incubation.

ISH results were negative when formalin fixed tissues were examined. Formalin reduces the amount of accessible DNA and RNA 10 to 35% and this could account for its poor performance in ISH assays (Singer et al., 1986). Based on this information, the poor results obtained from IFA on formalin fixed tissues may have resulted from extensive cross-linking of proteins thus inhibiting immunoreactivity. Greater accessibility of the probe and antibody to their target is required before formalin fixed tissues can be used in ISH and IFA.

The widespread use of formalin in anatomic pathology makes the development of monoclonal antibodies that can be used on these tissues important. Monoclonal antibodies that can circumvent problems encountered when using formalin fixed tissues in IFA would be of diagnostic use in

retrospective studies (Antel et al., 1985; Vente et al., 1987; West et al., 1986).

We chose a monoclonal antibody over a polyclonal antibody because of greater specificity. Antibodies chosen for IFA should balance sensitivity and specificity since an increase in one result in a decrease in the other (Mackenzie, 1992). Comparison of dilutions of the monoclonal antibody L78 revealed that dilutions around the 1:100 range had low background staining and the highest number of cells with positive fluorescence.

IFA is sometimes performed on frozen tissue sections because of the conformational changes in the structure of proteins caused by fixation. After fixation, the change in tertiary structure of the antigen may hinder accessibility of the antigenic determinant thus making frozen sectioning a viable option (Elias, 1990).

Blocking agents decreased the nonspecific background fluorescence observed with avian nucleated RBC. Fixatives insert free aldehyde groups on proteins that can nonspecifically bind to fluorescein conjugated antibodies, thereby increasing background fluorescence (Eldred et al., 1983; Weber et al., 1978). Only Evans blue dye decreased the background staining to any appreciable degree. Evans blue dye changed the staining of the RBC from a green color seen with positive fluorescence to a dull yellowish color that allows differentiation from positively staining cells.

Due to the cost of performing IFA and ISH, retention of the tissue sections to the slide is of the utmost importance in optimizing conditions. Silylated slides provided the best results in our hands.

The tissue type did not influence the results obtained with ISH to any appreciable extent. However, lymphoid tissue had more positive foci than nervous tissue. This could be a result of virus predilection for lymphocytes rather than an actual consequence of tissue type.

In conclusion, this study is one of the few comprehensive studies that examines presence of viral proteins and nucleic acids in the same tissues. We compare various parameters for the simultaneous use of IFA and ISH in lymphoid and nervous tissues. Other investigators have performed immunohistochemistry (IHC) and ISH in tissues. However, the majority of these studies examined cellular proteins and nucleic acids. The fixatives and tissues they preserved varies between studies. The differences in fixation of the following studies demonstrates an absence of standards between common procedures. Brain cultures were fixed with a mixture of formaldehyde, glutaraldehyde and picric acid for use in IFA compared to fixation of cultures with 4% paraformaldehyde for use in ISH (Zeller et al., 1985). However, another study (Chollat-Namy et al., 1993) fixed rat brain with 4% paraformaldehyde for IHC and used unfixed brain for ISH. Ichikawa and Ajiki (1992) fixed tissues with 4% paraformaldehyde and 0.4% glutaraldehyde for

use in ISH while tissues used for IHC were frozen. Bouin's fluid and 10% formalin have also been used as fixatives in IFA and ISH (Larsson and Hougaard, 1992). One study used 4% paraformaldehyde to fix tissues for ISH and IFA. While viral nucleic acids were detected, cellular proteins were identified (Sellon et al., 1992). These results reflect the differences in procedures for IHC and ISH found in the literature. Our study attempts to standardize some of the variables for IFA and ISH. The standardization provides a guideline for individuals to follow when choosing fixatives and other parameters involved in IFA and ISH.

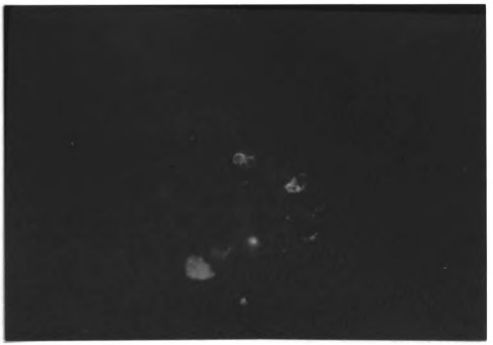


Figure 1. Demonstration of peripheral blood mononuclear cells (PBMC) fixed with 4% paraformaldehyde. The PBMC were blocked with Evans Blue Dye and stained with L78 at a dilution of 1:100 (450x).

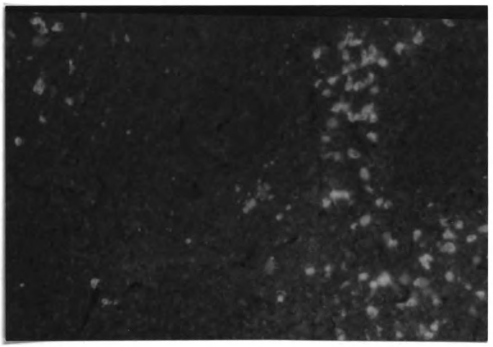


Figure 2. Photomicrograph of a section of thymus from a bird infected with HVT after immunostaining for HVT gB. The conditions for indirect immunofluorescence assay included: fixation using 4% paraformaldehyde, blocking the tissue with Evans blue dye, staining with L78 at a 1:100 dilution and a incubation temperature of 37 C. Notice the green fluorescence of cells stained with L78 while the red blood cells nonspecifically stain a dull yellow-green color (450x).

Fig

int

hyb

BGP

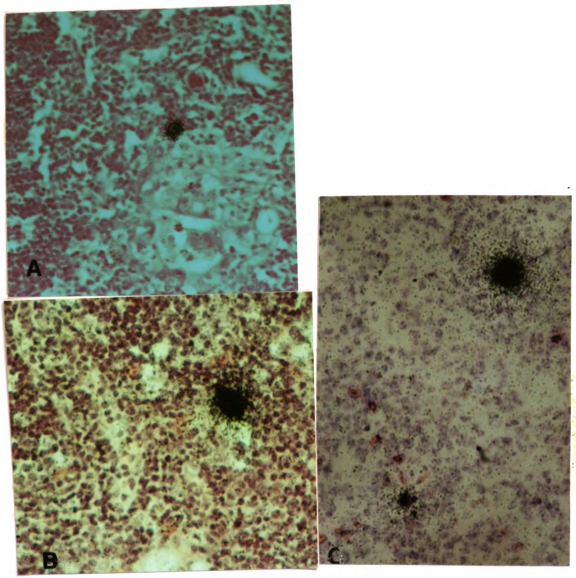


Figure 3. Photomicrograph of sections of thymus from birds infected with HVT. The thymuses were processed for <u>in situ</u> hybridization as described in Materials and Methods. (A) BGPA (B) 4% paraformaldehyde (C) formalin (450x).

Table I

Comparison of conditions using L78 or IAN.86 monoclonal antibodies for IFA in fixed blood smears

		4% Para[a]	Acetone	Formalin
Blocking Agents	Evans Blue Dye	+	-/+	-/+
	Bovine Serum Albumin	+	-	-
	Calf Serum	+	-	-
Slide Coatings	Poly-l-lysine	+	+	-/+
	Gelatin	+	+	-
	No Coating	-/+	-	-
Incubation Temperature	37 C	+	+	-/+
	Room Temperature	-/+	-	-
	Ice	-	-	-

[a]Fixative-4% Paraformaldehyde.

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table II

Comparisons of concentrations of L78 or IAN.86 monoclonal antibodies for IFA in unfixed cytocentrifuged PBMC and unfixed blood smears

	Blood Smears	PBMC
MoAb-L78[a]		
1:10[c]	-	-
1:20	-	-
1:50	-/+	-/+
1:100	-/+	+
1:500	-	-
1:1000	-	-
MoAb-IAN.86[b]		
1:10[c]	-	-
1:20	-	-
1:50	-/+	-/+
1:100	-/+	+
1:500	-	-
1:1000	-	-

[a]Monoclonal antibody against turkey herpesvirus (HVT) glycoprotein B (gB).

[b]Monoclonal antibody against Marek's disease virus serotype 1 and HVT gB.

Table II (cont.)

[c]**Dilution of antibodies tested.**

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table III

Comparison of conditions for IFA using L78 for the detection of gB expression in fixed cytocentrifuged PBMC

		4% Para[a]	Acetone	Formalin
Blocking Agents	Evans Blue Dye	+++	+	+
	Bovine Serum Albumin	++	+	+
	Calf Serum	++	+	+
Slide Coatings	Poly-l-lysine	+++	+	+
	Gelatin	++	+	+
	No Coating	+	+	+
Incubation Temperature	37 C	+++	+	+
	Room Temperature	-	-	-
	Ice	-/+	-	-

[a]Fixative-4% Paraformaldehyde.

+++ =highest number of positive cells; lower background fluorescent level-barely noticeable; best results of parameters compare; excellent cell morphology

++ =intermediate level of positive cells; background level-noticeable; good cell morphology.

+ =low level of positive cells; background level-high; cellular outline not distinct.

Table III (cont.)

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table IV

Comparison of conditions for IFA using IAN.86 for the detection of gB expression in fixed cytocentrifuged PBMC

		4% Para[a]	Acetone	Formalin
Blocking Agents	Evans Blue Dye	+	+	-/+
	Bovine Serum Albumin	-/+	-/+	-
	Calf Serum	-/+	-/+	-/+
Slide Coatings	Poly-l-lysine	+	-/+	-/+
	Gelatin	-/+	-/+	-
	No Coating	-	-	-
Incubation Temperature	37 C	+	-/+	-/+
	Room Temperature	-	-	-
	Ice	-/+	-	-

[a]Fixative-4% Paraformaldehyde.

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table V

Optimum conditions for IFA using L78 as the monoclonal antibody in tissues

		4% Para[a]	BGPA[b]	Formalin
Blocking Agents	Evans Blue Dye	+++	+++	+
	Bovine Serum Albumin	++	++	+
	Calf Serum	+	+	+
Slide Coatings	Silylated	+++	+++	+++
	Poly-l-lysine	++	++	++
	Gelatin	+	+	+
	No Coating	-/+	-/+	-/+
Incubation Temperature	37 C	+++	+++	+
	Room Temperature	-	-	-
	Ice	-	-	-

[a]Fixative-4% Paraformaldehyde.

[b]Fixative-Buffered Glutaraldehyde-Picric Acid.

+++ =highest number of positive cells; low background fluorescent level; best overall results of parameters

Table V (cont.)

compared; excellent cell morphology.

++ =intermediate level of positive cells; background level-noticeable; good cell morphology.

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table VI

Optimum conditions for IFA in tissues using IAN.86 as the monoclonal antibody

		4% Para[a]	BGPA[b]	Formalin
Blocking Agents	Evans Blue Dye	+	+	-
	Bovine Serum Albumin	-/+	-/+	-
	Calf Serum	-/+	-/+	-
Slide Coatings	Silylated	+	+	-
	Poly-l-lysine	-/+	-/+	-
	Gelatin	-/+	-/+	-
	No Coating	-	-	-
Incubation Temperature	37 C	+	+	-
	Room Temperature	-	-	-
	Ice	-	-	-

[a]Fixative-4% Paraformaldehyde.

[b]Fixative-Buffered Glutaraldehyde-Picric Acid.

+ =intermediate level of positive cells; background level-noticeable; good cell morphology.

Table VI (cont.)

-/+ =low level of positive cells; background level-high; cellular outline not distinct.

- =no positive cells distinguishable from background level; high background fluorescence.

Table VII

Comparison of conditions using L78 or IAN.86 monoclonal antibodies for IFA in unfixed PBMC and blood smears

		Blood Smears	PBMC
Blocking Agents	Evans Blue Dye	-/+	+
	Bovine Serum Albumin	-	-/+
	Calf Serum	-	-/+
Slide Coatings	Poly-l-lysine	-/+	+
	Gelatin	-/+	-/+
	No Coating	-	-
Incubation Temperature	37 C	-/+	+
	Room Temperature	-	-/+
	Ice	-	-

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table VIII

Determination of optimal concentrations of monoclonal antibodies used in IFA for the detection of HVT gB in tissues and PBMC

	4% Para[a]	BGPA[b]	Formalin
MoAb-L78[c]			
1:10[e]	+	+	+
1:20	+	+	+
1:50	++	++	+
1:100	+++	+++	++
1:500	+	+	+
1:1000	+	+	-
MoAb-IAN.86[d]			
1:10[e]	-/+	-/+	-
1:20	-/+	-/+	-
1:50	-/+	-/+	-
1:100	+	+	-
1:500	-/+	-/+	-
1:1000	-/+	-/+	-

[a]Fixative-4% paraformaldehyde

[b]Fixative-buffered glutaraldehyde-picric acid

[c]Monoclonal antibody against turkey herpesvirus (HVT) glycoprotein B (gB).

Table VIII (cont.)

[d]Monoclonal antibody against Marek's disease virus and HVT gB.

[e]Dilutions of L78 and IAN.86

+++ =highest number of positive cells; low background fluorescent level; best overall results of parameters compared; excellent cell morphology

++ =intermediate level of positive cells; background level-noticeable; good cell morphology.

+ =low level of positive cells; background level-high; cellular outline not distinct.

-/+ =few positive cells.

- =no positive cells distinguishable from background level; high background fluorescence.

Table IX

Comparison of fixatives and slide coatings for retention of positive tissue sections in IFA and ISH

		Number of positive sections retained/Total sections
Fixatives	4% paraformaldehyde	25/25
	BGPA	23/25
	Formaldehyde	5/25
	Acetone	7/25*
Slide Coatings	Poly-l-lysine	19/25
	Silylated	25/25
	Gelatin	5/25
	None	5/25

*- Cellular morphology was very poor with this fixative.

Table X

Optimum conditions for ISH in tissues

		4% Para[a]	BGPA[b]	Formalin
Slide Coatings	Silylated	+++	+++	+++
	Poly-l-lysine	++	++	++
	Gelatin	+	+	+
	No Coating	-	-	-
Incubation Temperature	37 C	+	+	+
	40 C	+	+	+
	45 C	+++	++	+
	47 C	+++	+++	+
Days of Incubation in Emulsion	2	+	+	+
	4	+++	+++	+
	7	+	+	+
	10	+	+	+

[a]Fixative-4% paraformaldehyde.

[b]Fixative-buffered glutaraldehyde-picric acid.

+++ =Positive foci scattered throughout tissue; Excellent cellular morphology.

++ =Positive foci localized to limited area of tissue; Good cellular morphology.

Table X (cont.)

+ =Very few positive foci; Cellular morphology ranges from good to poor.

- =No cells adhered to slides.

Chapter III

Identification and Localization of gB Expression in Lymphoid Tissues of Chickens Infected with Turkey Herpesvirus

M. S. Holland,[ABC] R. F. Silva,[B] C. D. Mackenzie,[A] R. W. Bull,[A] and R. L. Witter[B]

[A]Department of Pathology, College of Veterinary Medicine, Michigan State University, East Fee Hall, East Lansing, Michigan 48824

[B]Avian Disease and Oncology Laboratory, USDA/Agricultural Research Service, 3606 E. Mount Hope, East Lansing, Michigan 48823

[C]Corresponding author

Accepted for publication by Avian Diseases 1/94

SUMMARY. One-day-old chickens were inoculated with turkey herpesvirus (HVT). Using an indirect immunofluorescence assay with a monoclonal antibody against HVT glycoprotein B (gB), we determined the course of productive HVT infection in peripheral blood mononuclear cells (PBMCs), spleen, thymus, and bursa. PBMCs were examined from days 4 through 35 postinfection (PI). The spleen, thymus, and bursa were examined from 21 through 70 days PI. Although productive infection in PBMCs was detected at 4 to 12 days PI, it ended by 14 days PI. Splenic cells expressed gB at 21, 28, 35, and 70 days PI, whereas the thymus was positive for gB expression at 21 and 35 days PI. The bursa was never positive for gB expression. At 21, 28, 35, and 70 days PI, plaque formation after co-cultivation of PBMCs with chicken embryo fibroblasts indicated the presence of HVT in infected chickens by co-cultivation assays. On the basis of indirect immunofluorescence assay, gB expression in the spleen and thymus indicates a productive HVT infection in chickens.

INTRODUCTION

Turkey herpesvirus (HVT) is a non-oncogenic serotype 3 Marek's disease virus (MDV) that shares considerable DNA homology with the oncogenic serotype 1 (Gibbs et al., 1983). HVT and MDV serotype 1 are antigenically related (Ikuta et al., 1982; Ikuta et al., 1983; Silva and Lee, 1984). The antigenic relatedness is presumed responsible for the success of HVT as a vaccine. Following HVT vaccination, chickens are protected against lymphomas caused by MDV serotype 1 (Okazaki et al., 1970; Purchase et al., 1972). The immunological mechanism of HVT-induced protection is unknown. Particularly, the HVT antigens responsible for this protection are unknown.

Numerous cross-reactive antigens common to HVT and MDV serotype 1 have been identified (Ikuta et al., 1982; Ikuta et al., 1983; Ikuta et al., 1984a; Ikuta et al., 1984b; Lee et al., 1983; Silva and Lee, 1984). A complex of three MDV glycoproteins induces an immune response in infected chickens (Ono et al., 1984; Silva and Lee, 1984; Silva and Lee, 1985). The complex of glycoproteins is designated glycoprotein B (gB). This designation is based on the homology between the genes encoding MDV gB and herpes simplex virus (HSV) gB. Sequence analysis demonstrates conservation of the gene encoding gB among the various herpesviruses (Snowden et al., 1985). In addition, researchers have sequenced the MDV serotype 1 gB gene and

expressed it in a fowlpox vector (Nazerian et al., 1992; Ross et al., 1990; Yanagida et al., 1992). The sequence data further demonstrates the homology between MDV and HSV gB.

Similar to other herpesviruses, MDV infection results in productive and latent infections (Adldinger and Calnek, 1973; Calnek and Hitchner, 1969; Calnek et al., 1970; Calnek et al., 1984). It is unknown whether these phases occur separately or simultaneously.

The expression of viral antigens in the presence of infectious virus is characteristic of a productive infection. Also, viral DNA is replicated and viral particles are produced during a productive infection. Electron microscopy did not identify viral particles in lymphoid tumor cells from MDV-infected chickens (Calnek et al., 1970; Nazerian et al., 1971). However, electron microscopy did demonstrate that replication of enveloped infectious MDV occurs in feather follicle epithelium (FFE). This information identifies FFE as the source for contact transmission of MDV (Calnek et al., 1970). The identification of virus in the FFE provided confirmation that complete MDV replication occurs in chickens. Thus, FFE is the only known source of cell-free MDV (Calnek et al., 1970). Cell-free MDV can produce cytopathic effects in cell culture and in MD in chickens.

The data demonstrating productive infection in lymphoid tissue from HVT-infected chickens is indirect. Calnek and

co-workers (1979) confirmed the presence of HVT in lymphoid and other tissues by co-cultivation assays. However, they were unable to demonstrate HVT antigen expression directly in these tissues. Circumstantial evidence indicates that HVT productive infection occurs in FFE. The evidence is the infrequent occurrence of contact transmission of HVT in chickens (Cho, 1974; Cho and Kenzy, 1975; Cho et al., 1971). In addition, inoculation of 8-week-old cagemates with HVT resulted in horizontal transmission (Cho, 1974; Cho and Kenzy, 1975; Cho et al., 1971).

Other investigators have identified infections as latent by co-cultivation of infected cells with a permissive cell culture followed by the identification of viral internal antigens (Calnek et al., 1984). Latent MDV serotype 1 infections are present in the T-cell population (Shek et al., 1982; Shek et al., 1983). During the latent phase of MD, initial examination of infected cells revealed that no viral internal antigens or viral particles are present. However, subsequent co-cultivation of infected cells with susceptible chicken embryo fibroblasts (CEFs) leads to expression of viral internal antigens and viral particles (Calnek et al., 1984; Shek et al., 1982). This method of latency detection is dependent upon reactivation of a latent infection with the subsequent demonstration of viral internal antigens. The mechanism of latency in HVT infections probably is comparable to that seen with MDV serotype 1 infections. The basis of this assumption is the

genomic homology that exists between the two serotypes. However, the proof for HVT latency is not as strong as with serotype 1.

Conclusive evidence demonstrating HVT antigen expression in lymphoid and other tissues is lacking. We initiated this research to examine more carefully HVT antigen expression in lymphoid organs. We used a monoclonal antibody specific for HVT and applied improved histochemical techniques. In this communication, we describe the expression of a late HVT gene product *in vivo*. Also, we identify viral replication occurring in various tissues at different times, based on viral antigen expression.

MATERIALS AND METHODS

Animals. One-day-old chickens (N = 30) of the F_1 progeny of Avian Disease and Oncology Laboratory $15I_5$ males and 7_1- females were obtained from an unvaccinated breeder flock and were negative for MDV serotype 1 antibodies. Presumably progeny were negative for HVT and antibodies against HVT. The $15I_5$ x 7_1- F_1 progeny were inoculated intra-abdominally with 2000 plaque forming units of an HVT strain, FC126, passage 10 (Witter et al., 1970). Separate Horsfall-Bauer isolators housed the HVT-infected and uninoculated control chickens (N=30) under similar conditions.

Fixation of peripheral blood mononuclear cells. Approximately one to five mls of blood was removed from HVT-infected chickens divided into groups of 4 to 12 chickens and groups of 5 to 10 uninoculated chickens. Four to twelve chickens from each group were bled on days 4, 6, 7, 10, 12, 14, 21, 28, 35, and 70 PI. The blood was collected into tubes containing the anticoagulant, heparin. Blood elements were separated by centrifugation (IEC model HN-SII, Damon/IEC Division, Needham Heights, Mass.) at 500 rpm for 20 minutes. Buffy coat cells were resuspended in Leibowitz-McCoy media supplemented with 4% calf serum and other blood elements were discarded. The buffy coat cells were either assayed for virus (see section on Co-cultivation assays) or processed for subsequent use in indirect immunofluorescence

assays. Buffy coat cells for use in indirect immunofluorescence assays (IFA) were initially adhered to poly-l-lysine coated slides by cytocentrifugation (Cytospin II, Shandon Southern Instruments, Sewickley, PA.) at 250 rpm for 5 minutes. Three groups of five to fifteen cytospins from each chicken were prepared. Each group of cytospins contained a total of 3000 to 5000 peripheral blood mononuclear cells (PBMCs). To optimize the IFA, PBMCs were treated with different fixatives. Initially, we compared cross-linking fixatives (4% paraformaldehyde and 10% formalin) with a precipitating fixative (100% acetone). The PBMCs were preserved by immersion fixation. One group of cytospins was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at room temperature for 30 minutes. The second group of cytospins was fixed with 10% neutral buffered formalin at room temperature, for 30 minutes. The third group of cytospins was fixed with ice-cold 100% acetone for 30 minutes at 4 C. All cytospins were dipped in PBS at room temperature for 5 minutes. Cytospins were stored in 70% ethanol at 4 C until ready for use in IFA.

Co-cultivation assays. These assays have been described previously (Purchase, 1969). Briefly, 1 x 10^6 buffy coat cells were added to primary chicken embryo fibroblasts (CEFs) cultures. Cultures of CEFs and buffy coat cells were maintained in Leibowitz-McCoy medium supplemented with 1%

calf serum (Witter et al., 1970) in petri dishes at 37 C in 5% CO_2. Cultures were examined daily for the appearance of plaques beginning 4 days after the initial seeding of buffy coat cells on CEFs. If the cultures were negative for plaques 7 days after the initial seeding, we examined a maximum of three subcultures before assigning a final negative result. Each subculture consisted of the infected monolayer being treated with trypsin and co-cultivated on a fresh monolayer of secondary CEFs.

Fixation of lymphoid tissues. Three groups of two to four HVT-infected chickens at 21, 28, 35, and 70 days PI were anesthetized with halothane administered through a nose cone. Also, three groups of 2 uninfected chickens were anesthetized with halothane at the same time intervals as the infected chickens. Once the chickens were anesthetized, 70% ethanol was applied to the ventral aspect of the chickens. A transverse incision through the skin was made above the cloaca. At each end of the transverse incision, vertical incisions were made that extended into the cervical region and the skin was reflected. The same type of incisions were made through the musculature, peritoneal, and thoracic cavities. The keel was dislodged toward the head of the chicken. An incision was made in the pericardial sac and the heart was exposed. A 20 gauge catheter was placed in the left ventricle and heparinized saline was infused by gravity flow, at a volume of 5ml/kg of body weight. Once

the heart began to swell, an incision was made in the right auricle to allow outflow of the blood and saline. A continuous flow of fluid was maintained throughout the saline infusion.

Previously, we determined that cross-linking fixative gave more consistent results in IFA than precipitating fixatives. Therefore, we compared three cross-linking fixatives: 4% paraformaldehyde, buffered glutaraldehyde-picric acid (BGPA), and formalin. The fixatives were perfused into the chicken's heart after complete infusion of the heparinized saline solution. The fixatives were perfused by gravity flow at a volume of 5 ml of fixative/kg of body weight. One group of chickens was perfused with 4% paraformaldehyde, one group (group 2) was perfused with BGPA (2 ml 50% glutaraldehyde, 15 ml saturated aqueous picric acid, and 83 ml 0.1 M Sorenson's phosphate buffer) and group 3 chickens were perfused with 10% formalin. After perfusion, the spleen, thymus, and bursa were removed for preparation of tissue blocks cut into 2 cm x 4 cm sections. The blocks of tissues were placed in tissue cassettes which are perforated capsules that allow the fixative access to the tissue while preventing loss of tissue blocks. Blocks of tissues were placed in a 50 ml conical tube containing the perfusing fixative at 10 to 20 times the tissue volume. The tissue blocks remained in the fixatives for 18 to 24 hours at room temperature. The tissue blocks were prepared for paraffin embedding by routine procedures and then were

cut to 6 micron thick sections. The tissue sections were mounted onto poly-l-lysine coated frosted-end slides. The tissue sections were stored in a slide box at room temperature until IFA was performed on the sections.

Negative and positive control PBMCs and tissues. Uninfected PBMCs, spleen, thymus, and bursa served as negative controls, HVT-infected PBMCs, thymus, and spleen served as positive controls. The various examination periods of PBMCs included infected PBMCs from all preceding test periods as positive controls. The positive tissue controls were determined after performing preliminary experiments that demonstrated sections of thymus and spleen that expressed gB. These tissue sections were used as the positive controls. Both uninfected and infected PBMCs and tissues were examined together.

Morphometry. To standardize the variations in tissue section sizes, image analysis was performed. We used a Nikon/Joyce-Loebel image analyzer system for quantitative evaluation of tissue sections by light microscopy. The Nikon/Joyce-Loebel image analyzer system consists of a MTI series 68 color video camera, a PC Vision Plus Frame Grabber (Imaging Technology), a Sony analog RGB monitor, and a Numonics digitizing tablet connected to an IBM-compatible computer (Compu-Add 325). The software package used with the image analyzer was the Jandel Video Analysis system.

The images measured were the boundaries of tissue sections counterstained with .01% Evans blue dye. A monitor interfaced to a computer projected video images of the tissue sections. A crosshair pointing device of a digitizing tablet traced the boundaries of the tissue section.

Indirect immunofluorescence assay. The immunofluorescence technique has previously been described (Purchase, 1969). Briefly, PBMCs were washed three times with deionized water followed by three washes with PBS. Fifty microliters of a monoclonal antibody specific for HVT gB (L78) were then added to each tissue section (Lee et al., 1983); the monoclonal antibody (L78) was provided by L. F. Lee (USDA-ARS, Avian Disease and Oncology Laboratory, East Lansing, Mich.). The tissue sections were incubated at 37 C for 30 minutes. After incubation, the tissue sections were rinsed three times with deionized water. The final wash consisted of rinsing the tissue sections three times with PBS. Fifty microliters of fluorescein-conjugated rabbit anti-mouse immunoglobulin was added to the slides for 30 minutes at 37 C. The PBMCs and tissues were washed with PBS. Coverslips were placed over the cells and tissues before fluorescence microscopic examination. After performing IFA, four individuals examined different groups of control and experimental slides from different time periods. To standardize the variations in tissue section sizes, both the

average and range of positive cells were calculated per square mm of tissue. The calculation was: Number of positive cells/average size of tissue section.

Pathology. All chickens were necropsied. Gross examination of visceral organs was performed *in situ*. Hematoxylin-and-eosin-stained tissue sections were examined microscopically.

Statistical analysis. Viremia levels were analyzed using a Fischer's exact test for 2 x 2 tables. The 1-tailed value for $P < 0.05$ was based on comparisons between the pooled total of observations before and including 12 days PI and pooled total of observations after 12 days PI.

RESULTS

Comparison of fixatives in PBMC. PBMCs fixed with formalin were negative in the IFA. PBMCs fixed with acetone had high background fluorescence, and positive fluorescence was not consistent between samples. Cells fixed with acetone were a dull, low-intensity green color. When 4% paraformaldehyde was used as a fixative, the fluorescence was bright green. In addition, more cells were positive. Further, the pattern of fluorescence was more consistent in color and distribution compared with fluorescence from tissues fixed with BGPA or formalin. The fluorescence pattern of cells fixed with 4% paraformaldehyde was granular and located on the cell surface. On the basis of these results, we selected 4% paraformaldehyde to study the expression of gB in PBMCs.

Expression of gB in PBMC. Expression of gB in PBMCs was detected on days 4 to 12 PI, but not on days 14 through 35 PI. The cells staining positive for gB were identified as lymphocytes based on their morphological characteristics. In addition, cells expressing gB were 1% to 21% of the total PBMCs present in a sample. All chickens examined from 4 to 12 days PI had cells expressing gB. At 14 days PI, the number of chickens having cells expressing gB dropped to zero. Analysis of pooled data indicates that the decline in the numbers of birds expressing gB was significant

($P < 0.00001$). Negative control-PBMCs from uninfected chickens did not express gB.

Comparisons of fixatives with lymphoid tissue. Either formalin-fixed tissues were negative for gB expression, or gB was expressed at very low levels. Comparison of 4% paraformaldehyde and BGPA-fixed tissues showed the same number of cells positive for gB (data not shown). Fluorescing cells appeared bright green in tissues fixed with 4% paraformaldehyde, whereas BGPA-fixed tissues had fluorescing cells that were slightly duller green. In tissues fixed with 4% paraformaldehyde, either fluorescence encompassed the entire cell, or small multifocal segments of an individual cell fluoresced. Background fluorescence of erythrocytes was yellowish-green and located cytoplasmically. Results demonstrated that 4% paraformaldehyde was a better choice for fixative than formalin. BGPA was comparable to 4% paraformaldehyde in terms of fixation, intensity, and distribution of fluorescence. BGPA-fixed cells fluoresced a slightly duller color than cells fixed with 4% paraformaldehyde. The difference in color between cells preserved in the two fixatives was small. In addition, the color difference did not interfere with assigning cells as either positive or negative for fluorescence. However, we selected 4% paraformaldehyde over BGPA mainly because of the highly carcinogenic nature of BGPA.

Expression of gB in lymphoid tissues. Lymphoid organs from HVT-infected chickens were examined using IFA at 21, 28, 35, and 70 days PI (Table 2). Splenic cells stained positive for gB at each time interval and were confined to the white pulp. In thymic tissue, cells expressing gB were detected at 21 and 35 days PI but not at 70 days PI and they were present in the medulla. The cells expressing gB appeared to be small lymphocytes based on their morphological characteristics. At 21 days PI, the spleen had 463 positive cells per square mm of tissue, whereas the thymus had 200 positive cells (Table 2). The number of positive cells decreased over 1-2 weeks in both the spleen and thymus. The bursa was negative for gB expression at all time intervals.

Presence of HVT in PBMCs. Co-cultivation of PBMCs with CEFs demonstrated the presence of HVT in inoculated chickens. Expression of gB was detected at the same times as HVT virions (Table 1). PBMCs taken 4 to 12 days PI developed plaques 4 to 7 days after co-cultivation with CEFs. In some instances, PBMCs from chickens sampled 14 to 70 days PI, required propagation times of 14 to 21 days with CEFs before plaques appeared. The proportion of chickens that demonstrated HVT infections by co-cultivation assays decreased to 50% by 35 days PI and 25% by 70 days PI.

DISCUSSION

In this paper, we demonstrate with consistency HVT-infected cells in lymphoid tissue that express gB. Spleen cells expressing gB were restricted to the marginal zone and periarterial lymphoid sheaths of the white pulp. These locations in the spleen contain predominantly T-cells. Productively infected cells were localized to the medullary region of the thymus. Morphologically, the cells expressing gB in the thymus appear to be small lymphocytes. Lymphocytes, epithelial cells, macrophages, and Hassall's corpuscles are present in the medulla of the thymus. The epithelial cells are believed to be intimately involved in the maturation of T-cells that accumulate in the medulla. Lymphocytes in the cortex mature and migrate to the medulla. Small lymphocytes predominate in the medulla. Only 1% of T-cells leave the thymus via the peripheral blood. The other 99% are destroyed in the thymus. The majority of cells that populate lymphoid organs circulate through the peripheral blood (Raviola, 1975; Tizard, 1977).

Our inability to detect gB expression in the bursa raises some questions regarding the presence of HVT in B-cells. It has been shown that serotype 1 initially induces a productive infection in B cells and later also in T-cells (Calnek et al., 1984; Shek et al., 1982). The bursa contains predominantly B-lymphocytes (Tizard, 1977). In addition, the follicular region of the spleen contains

predominantly B-lymphocytes. We were unable to detect gB expression in B-cells. Conclusive proof on the presence of gB expression in B-cells requires the demonstration of B-cell markers. Additionally, it is possible that HVT induces an active infection in B-cells at an early time. Since our earliest examination time was 21 days PI, the virus may have been cleared by this time.

There is a marked difference between gB expression in lymphoid organs versus PBMCs. The PBMCs expressed gB at 4 to 12 days PI and disappeared by 14 days PI whereas the spleen and thymus both had cells expressing gB at 21 days PI. The absence of gB expression in the PBMCs beginning at 14 days PI and extending to 35 days PI, marks the termination of productive HVT infection in PBMCs. The spleen and thymus may still contain some productively infected cells, even though PBMCs did not contain a productive HVT infection at later times.

The demonstration of virus is an important component of productive infections. The appearance of plaques on co-cultivation assays demonstrates the presence of HVT in PBMCs taken 4 to 12 days PI. We detected gB expression during this period as well. In contrast, we found that the frequency of virus isolation decreased at the same time that cells expressing gB decreased. With the passage of time, it becomes more difficult to isolate virus using co-cultivation assays for reactivation of virus. We showed that occasional increased propagation times are required for viral

reactivation. One reason for the difficulty in isolating virus may be an inability to reactivate infectious virus as time passes. Viremia in chickens infected with HVT peaks in titer at 8 to 15 days PI and gradually declines (Schat et el., 1978). Purchase et al. (1972) demonstrated that HVT was isolated from 86% of chickens 70 days PI but from only 58% of the chickens by 532 days PI. These results are consistent with our findings that reactivation of infectious virus decreases with time.

Previous research has failed to demonstrate that a productive HVT infection occurs in the same manner as serotype 1 (Calnek et al., 1979; Fabricant et al., 1982). There are a number of explanations for our ability to demonstrate antigen-expressing cells in lymphoid tissues which is indicative of a productive infection. The major reason is improvements in technology. Our results demonstrate that the type of fixative used to preserve the tissue is an important determining factor in demonstrating antigen expression in vivo. Others used convalescent sera previously (Calnek et al., 1979; Churchill et al., 1973; Fabricant et al., 1982), whereas we used monoclonal antibodies to detect viral antigens, which are more specific and sensitive than convalescent sera. Also, the strain of chickens used may play a role in our ability to demonstrate HVT antigens. Calnek et al. (1970) have previously shown that chickens with genetic differences had variations in the number of positive tissues and number of viral antigen

positive cells within these tissues. This is the first report that infected $15I_5 \times 7_1$- chickens with HVT and looked for gB expression *in vivo*. This strain of chickens may express HVT antigens at a higher level than other strains. Other researchers have used different strains of chickens in their attempts to detect HVT antigen expression (Calnek et al., 1979; Churchill et al., 1973; Fabricant et al., 1982; Phillips and Biggs, 1972; Shek et al., 1982). These strains of chickens may have less HVT antigen positive cells than $15I_5 \times 7_1$- chickens.

Shek *et al.* (1982) have looked specifically for viral internal antigen of chickens inoculated with HVT and found very few cells positive. The low gB expression levels in the present study suggest that HVT viral antigens are difficult to demonstrate. These results indicate that very sensitive techniques are required for detection.

A sensitive assay was developed to detect a productive HVT infection in various tissues. The use of fixatives and monoclonal antibodies to identify gB expression may be valuable for detecting other MDV or HVT antigens.

Table 1. Immunofluorescent detection of HVT gB in peripheral blood mononuclear cells of chickens infected at 1 day of age with HVT.

Days PI[A]	% gB Positive Cells[B]		No of positive samples/ No of chickens tested[C]	Viral Presence[D]
	Mean	Range		
4	3.2	2-5	5/5	5/5
6	7.3	3-21	6/6	6/6
7	2.7	2-4	7/7	7/7
10	2.6	1-5	9/9	9/9
12	3.0	2-6	5/5	5/5
14	0	0	0/10	7/10
21	0	0	0/10	6/10
28	0	0	0/12	8/12
35	0	0	0/10	5/10
70	ND[E]	ND	ND	1/5

Table 1 (cont.)

[A] Days post intra-abdominal inoculation of one-day old chickens with 2000 pfu of turkey herpesvirus.

[B] Cytocentrifugation was used to place peripheral blood mononuclear cells (PBMC) onto poly-l-lysine coated slides. PBMC were then fixed with 4% paraformaldehyde. A total of 3000 to 5000 PBMC were examined. The indirect immunofluorescence assay included L78 monoclonal antibody against gB antigen at each time interval.

[C] Five to 15 cytospins were examined for each bird tested.

[D] Co-cultivation assays determined the presence of virus in the buffy coat cells.

[E] Not Done

Table 2. Immunofluorescent detection of HVT gB in lymphoid tissues from chickens infected at one day of age with HVT.

Tissue	Days PI[A]	No. of positive cells		Ave. size of tissue section[B] (cm^2)	No. gB positive cells/sq. mm tissue		No. birds with gB positive cells/ total birds
		Ave.	Range		Ave.	Range	
Spleen	21	205	17-587	.4	463	38-1334	2/2
Spleen	28	208	21-701	.5	416	42-1402	2/2
Spleen	35	19	0-71	.7	26	0-97	4/4
Spleen	70	8	0-21	1.2	6	0-17	4/4
Thymus	21	61	21-90	.3	200	106-300	2/2
Thymus	35	5	0-21	.2	19	0-84	4/4
Thymus	70	0	0	.2	0	0	0/4

Table 2 (cont.)

Bursa	21	0	0	.7	0	0	0/2
Bursa	28	0	0	.7	0	0	0/2
Bursa	35	0	0	.8	0	0	0/4
Bursa	70	0	0	.8	0	0	0/4

[A] days post infection. Chickens were perfused with 4% paraformaldehyde. The tissues were removed and placed in fresh 4% paraformaldehyde at the various time intervals. The tissues were cut to 6um thick sections and placed on poly-l-lysine coated slides and prepared for indirect immunofluorescence assay (IFA) as described in Materials and Methods. A monoclonal antibody against HVT gB, L78 was used in IFA

[B] Five to thirty tissue sections were examined per bird at each time interval. Morphometry was used to measure the size of the tissue sections.

Chapter IV

Latent Turkey Herpesvirus Infection in Lymphoid, Nervous and Feather Tissues

Margo S. Holland, Robert F. Silva, Charles D. Mackenzie, Robert W. Bull

ABSTRACT

We compared turkey herpesvirus (HVT) glycoprotein B (gB) expression with the presence of HVT-RNA in lymphoid, nervous, and feather tissues. Indirect immunofluorescence assays (IFA) detected gB expression while *in situ* hybridization (ISH) detected HVT-RNA. The expression of gB can be used to differentiate a productive from a latent infection. Progression of a productive infection to a latent stage of infection resulted in the loss of gB expression. HVT-RNA was present in both productive and latent infections.

Termination of gB expression varied in time of occurrence between tissues. In the bursa, spleen and thymus, gB expression was detected at 3 weeks post infection (PI). However, only 2 lymphoid organs, the spleen and thymus, had gB expression at 4 and 5 weeks PI. Subsequent examinations revealed gB expression only in the spleen at 10 weeks PI. Cessation of gB expression occurred in the spleen at 15 weeks PI. We did not detect gB expression in the sciatic plexus, brachial plexus, nor feather tips at 3 or 4 weeks PI. Using ISH, HVT-RNA was found in all tissues at all time intervals except for the bursa. In the bursa, HVT-RNA was found at 3 and 4 weeks PI but not at 5, 10 or 15 weeks PI. Our results suggest that the absence of gB expression and presence of HVT-RNA provide a simple method to identify latent infections in a variety of tissues.

INTRODUCTION

Herpesviruses are a model for the study of latent viral infections. The importance of latency is a direct result of the ability of viruses to persist in nature. Latent viruses have been demonstrated in a number of herpesvirus systems (Baichwal and Sugden, 1988; Roizman and Sears, 1990). A prototypic gammaherpesvirus, Epstein Barr Virus (EBV), induces latent infections in lymphoid and epithelial tissues. Latent EBV is most commonly found in Burkitt's lymphomas and nasopharyngeal carcinomas (Klein, 1989). Herpes simplex virus (HSV), which is a prototypical alphaherpesviruses, establishes latent infections in nervous tissue (Rock, 1993).

Turkey herpesvirus (HVT) is a gammaherpesvirus based on its biological properties but its genomic structure resembles that of an alphaherpesvirus. Antigenically, HVT is a serotype 3 Marek's disease virus (MDV). HVT has both genomic and antigenic properties similar to MDV serotype 1 and 2 (Gibbs et al., 1983; Silva and Lee, 1985; Witter et al., 1970). Unlike, MDV serotype 1, HVT, which was originally isolated from turkeys, is nononcogenic (Witter et al., 1970). The major economic importance of HVT is its use as a vaccine to prevent the development of lymphoid neoplasms caused by MDV serotype 1 (Okazaki et al., 1970).

All MDV serotypes can induce both productive and latent infections. Viral DNA replication and antigen synthesis are characteristics of a productive infection. Productive

infections occur predominately in feather follicle epithelium and to a lesser extent in lymphocytes (Calnek et al., 1970a). Productive MDV serotype 1 infections are cytolytic and result in inclusion body formation. Productive HVT infections are not reported to cause cell damage but do cause inclusion body formation.

Latent MDV serotype 1 infections are characterized by the presence of the viral genome with no viral antigen production (Calnek et al., 1984). Calnek et al. (1981) identify latent MDV serotype 1 infections by the appearance of viral internal antigens (VIA) following short term culture of MDV infected cells with chick embryo fibroblasts (CEF). The assay developed by Calnek et al. (1981) detected MDV serotype 1 but not HVT latent infections. Latent MDV serotype 1 infections were predominately found in lymphocytes (Shek et al., 1983; Calnek et al., 1984). Since HVT latent infections have not been identified, a method that allows the detection of HVT latent infections needs to be developed.

Previously, we identified a productive HVT infection in lymphoid tissues. We detected a late gene protein, glycoprotein B (gB) using indirect immunofluorescence (IFA) while simultaneously detecting HVT by co-cultivation assays (Holland et al., 1993). The absence of gB expression should serve as an indicator for latency. In this report, we examined various tissues to identify the time that gB expression was absent by IFA. To ensure that HVT was

present even if gB was not expressed, we used ISH to detect HVT RNA.

MATERIALS AND METHODS

Chickens and housing. Strain $15I_5$ x 7_1- chickens were from a flock that was negative for serotype 1 and not vaccinated for HVT. The resulting offspring do not have antibodies against HVT or serotype 1. This strain of birds were inoculated at one day of age with 2000 plaque forming units of HVT, strain FC126, passage 10 (Witter et al., 1970). Uninoculated, control chickens were kept separate in Horsfall-Bauer cages.

Explant cocultivation. Two to five mls of blood were removed from the heart. Buffy coat cells were separated from other blood elements by centrifugation (IEC model HN-SII, Damon/IEC Division, Needham Hts, Mass) at 500 rpm for 20 minutes and resuspended in Leibowitz McCoy media. Buffy coat cells were co-cultivated with secondary CEF. Co-cultivation's were passaged at 5 to 7 day time intervals for a 3 to 4 week period, if cytopathic effects were absent. If cytopathic effects developed, plaques were examined and then co-cultivation assays were discarded.

In situ hybridization (ISH). Chickens were anesthetized and then perfused with saline followed by 4% paraformaldehyde. The spleen, thymus, bursa, sciatic plexus, brachial plexus, feather tips, cerebrum, cerebellum, pons, and dorsal root ganglia from the region of cervical vertebrae 12-14 (C12-14) and lumbosacral vertebrae (LS) were removed and placed in

fresh 4% paraformaldehyde. Tissues were embedded in paraffin, and sectioned at 5 microns. Sections were affixed to silylated slides. The ISH were performed as described previously (Holland et al., 1993). Uninfected tissue sections served as controls. In addition, infected tissue sections examined for HVT-RNA were treated with RNase A (100 ug/ml in 2x SSC) for 30 minutes at 37 C, postfixed in 4% paraformaldehyde for 2 hours, and dehydrated in graded ethanol.

Indirect immunofluorescence assay. Tissue sections were incubated with the primary antibody, L78, for 30 minutes at 37 C. L78 is a monoclonal antibody specific for HVT gB. Next, tissues were washed 3 times with deionized water, followed by 3 washes with physiological buffered saline (PBS). Goat anti-mouse fluorescein conjugate was added to tissue sections and incubated at 37 C for 30 minutes. Following the washes, a mixture of glycerin:PBS at a ratio of 2:1 was placed on tissue sections. Coverslips were overlaid and tissue sections were examined using ultraviolet light microscopy.

RESULTS

Identification of latently infected tissues. Detection of gB expression varied temporally in the spleen, thymus, and bursa. In the spleen, thymus and bursa, gB expression and HVT-RNA were detected at 3 weeks PI (Table I). However, only the spleen and thymus expressed gB at 4 and 5 weeks PI. Subsequent IFA revealed gB expression in the spleen at 10 weeks PI. Termination of gB expression occurred at 15 weeks PI in the spleen. While HVT-RNA was present in the spleen and thymus at all examination periods, the bursa had HVT-RNA only at 3 and 4 weeks PI. We did not detect gB expression in the sciatic plexus, brachial plexus or feather tips at 3 or 4 weeks PI (Table II); however, ISH successfully demonstrated HVT-RNA in these tissues at all time intervals. Areas of the feather tissue were not examined because of shattering of the tissue upon sectioning. When the feather shatters, only the base remains because of the composition of the feather. Only feathers that did not shatter were included in the study.

The cerebellum, cerebrum, pons, dorsal root ganglia of cervical vertebrae 12-14 and lumbosacral vertebrae were negative for gB expression at 15 weeks PI. Even though these tissues had background levels of foci after performing ISH, they could not be designated either negative or positive for latent HVT. Therefore, the results for brain and spinal cord were inconclusive.

Localization of latent HVT. In situ hybridization was used to demonstrate HVT-RNA in various tissues. Latent HVT was present in periarteriolar sheaths of the cortical region of the spleen (Figure 1). We never detected latent HVT in splenic follicles. The density of the grains prevented the visualization of the exact cell type that contained HVT-RNA in the majority of splenic sections.

In contrast to the spleen, latent HVT concentrated in the medullary region of the thymus (Figure 2). Latent HVT was found in epithelial cells and lymphocytes of the thymus. Positive foci were also detected in Hassall's corpuscles.

Latent HVT was in the medullary region of the bursa (Figure 3). As with the spleen, the density of the grains inhibited the determination of the exact cell type containing latent HVT in the bursa. The bursa was the only tissue that latent HVT was detected at only one examination period.

The sciatic plexus and brachial plexus had latent HVT dispersed throughout the tissue sections (Figure 4). Latent HVT was found in epithelioid cells, fibroblasts and collagen fibers. However, the density of grains prevented us from determining if any other cell types contained latent HVT. The feather tips had latent HVT scattered throughout the entire tissue. Positive foci were seen in the feather follicle epithelium (Figure 5), dermal papillae, feather sheath, inner sheath cells, ramogenous zone (Figure 6) and

feather pulp. Only the regions containing latent HVT were identified, not the cell type.

Detection of latently infected chickens by co-cultivation. Co-cultivation of CEF with peripheral blood mononuclear cells (PBMC) rescued HVT from infected PBMC. Using this method, 100% of the infected birds contained HVT from 4 days post infection (PI) to 12 days PI. At 14 days PI, HVT was shown in only 7 of 10 infected birds by co-cultivation assays (Table III). The number of chickens positive for HVT continued to decline from 14 to 70 days PI.

DISCUSSION

For the first time, we have identified latent HVT infections in the spleen, thymus, bursa, brachial plexus, sciatic plexus, and feather tips of chickens. Previous studies were unable to conclusively detect latent HVT in infected chickens, although latent MDV serotype 1 infections have been identified (Shek et al., 1983).

The absence of gB expression in the presence of HVT-RNA suggests the infection is latent. The time of detection of a latent HVT infection varied in lymphoid tissues. HVT-infected spleen fit this criteria for latency at approximately 15 weeks PI. The positive foci representative of HVT-RNA were in the cortical region of the spleen. Even though the latent HVT infection was identified in the spleen at 15 weeks PI, latent HVT may have been present in individual cells before 15 weeks PI. The thymus contained a latent HVT infection in the medulla at 10 weeks PI. Lymphocytes, macrophages and epithelial cells contained the latent HVT.

The bursa had very different results from the spleen and thymus. gB expression was not detected in the bursa at 4 weeks PI but HVT-RNA was present. Beginning at 5 weeks PI and continuing through 15 weeks PI, neither gB nor HVT-RNA was detected in the bursa. These results suggest that a latent HVT infection occurs at 4 weeks PI and the infection is cleared from the bursa by 5 weeks PI.

Nervous tissue, sciatic plexus, brachial plexus, and feather tips have latent HVT infections at 3 week PI. However, since we never detected gB expression in the sciatic plexus, brachial plexus or feather tips, gB was probably expressed prior to 3 weeks PI. The importance of the temporal difference in the establishment of latent infections is unknown.

Using ISH, examination of brain, cerebrum, cerebellum, pons, spinal cord, and dorsal root ganglia for latent HVT showed reactions on both infected and uninfected tissues. Clusters of grains were present in both infected and uninfected brain and spinal cord tissue. The grain density was similar to that seen in positive lymphoid, nervous and feather tissues. This observation suggests nonspecific hybridization occurred in the brain and spinal cord tissue.

One explanation for the results seen in the brain and spinal tissue is the occurrence of false positives. Electrostatic interactions of probes with tissue sections and cross hybridization of probes with nucleic acids other than those of interest are just two examples of non-specific signals that can occur. Additionally, the brain has been found to have a high incidence of non-specific signals with ISH.

We speculate that cellular factors indigenous to specific tissues may play a role in the establishment of latency. An example of a cellular factor involved in the initial stages of a productive HSV infection is the cellular

protein octamer (OTF-1). OTF-1 is a vital component in the regulation of HSV transcription. OTF-1 forms a complex with a HSV protein, virion protein 16 (VP16) (Preston et al., 1988; O'Hare and Goding, 1988; O'Hare et al., 1988). OTF-1 binds to HSV DNA and serves as a bridge for VP16 to induce immediate-early gene transcription (Gerster and Roeder, 1988; McKnight et al., 1987; O'Hare et al., 1988).

In comparison, a lymphocyte-neuron specific octamer binding protein (OCT-2) has been identified that binds to a motif present in the immediate early (IE) gene promoter and effectively inhibits the activity of IE genes (Kemp et al., 1990). One report from an in vitro latency model suggests that OCT-2 protein suppression of IE genes allows HSV to establish a latent infection as opposed to an active or productive infection (Lillycrop et al., 1991). It is currently unknown whether a protein similar to OCT-2 exists in HVT infections. However, the similarities that exist between HSV and HVT suggest that the establishment of HVT latency may occur by a mechanism similar to that of HSV.

Latent alphaherpesvirus is found in nervous tissue while gammaherpesvirus is present in lymphoid tissue. We have demonstrated that latent HVT is found in both nervous and lymphoid tissues. This result suggests that HVT does not fit neatly into alphaherpesvirus or gammaherpesvirus but has properties of both families. A resolution to this dilemma may be achieved by determining the specific cell type harboring the latent HVT genome.

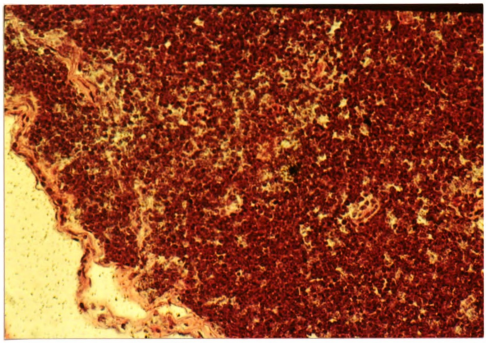


Figure 1. Photomicrograph of a spleen section from a chicken latently infected with turkey herpesvirus (HVT). Notice the cortical region of the spleen which contains a single foci of grains. The cluster of grains is indicative of *in situ* hybridization between a HVT DNA probe radiolabeled with ^{32}P and target HVT-RNA present in lymphocytes (H & E stain, 200x).

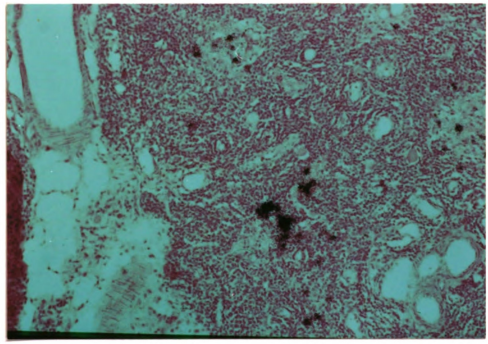


Figure 2. Photomicrograph of an <u>in situ</u> hybridization performed in the thymus from a chicken infected with HVT 105 days previously. Notice the multitude of black positive foci that represent the presence of latent HVT in the medullary region of the thymus (H & E stain, 100x).

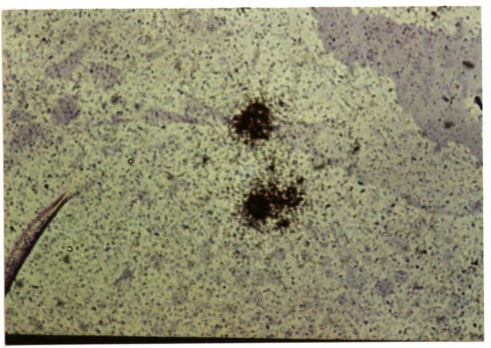


Figure 3. Bursal section of a 28-day-old chicken infected with HVT at one day of age. In situ hybridization was used to detect latent HVT. The 2 clusters of black grains are positive hybridization between an ^{32}P-labeled HVT DNA probe and HVT-RNA in lymphocytes in the medullary region of the bursa (H & E stain, 200x).

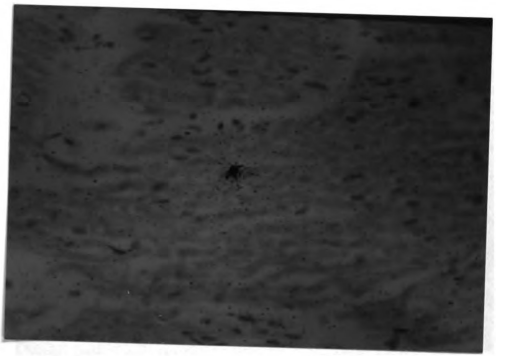


Figure 4. Sciatic plexus from a 35-day-old chicken infected with HVT at 1-day of age. Notice the cluster of black grains present within a satellite cell after *in situ* hybridization between HVT-DNA and target HVT-RNA. These positive foci were seen throughout the sciatic plexus and brachial plexus (H & E stain, 200x).

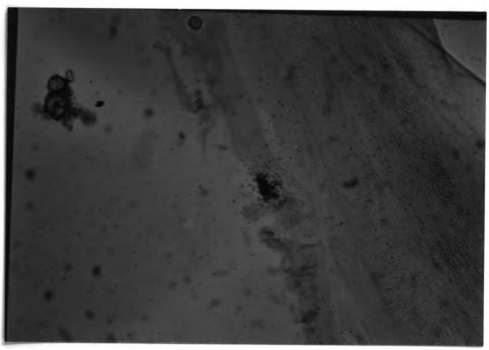


Figure 5. Feather tips from a 28-day-old chicken infected with HVT at 1-day-old. In situ hybridization between a ^{32}P HVT DNA and HVT-RNA within the feather. Notice the single cluster of grains in the ramogenous zone of the feather (H & E stain, 450x).

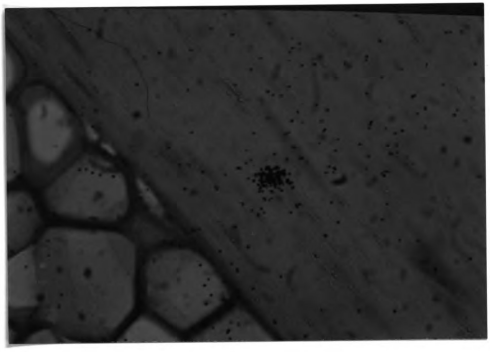


Figure 6. Photomicrograph of a section of feather from a 28-day-old chicken infected with HVT at 1-day-old. In situ hybridization was performed on these tissues to detect latent HVT. Note a large positive foci located in the feather follicle epithelium (H & E stain, 200x).

Table I

Identification of lymphoid tissues expressing HVT gB

Tissue	DPI[1]	ISH[2]	# gB positive cells[3]
Spleen	21	+	463
Spleen	28	+	416
Spleen	35	+	26
Spleen	70	+	6
Spleen	105	+	0
Thymus	21	+	200
Thymus	28	+	120
Thymus	35	+	19
Thymus	70	+	0
Thymus	105	+	0
Bursa	21	+	10
Bursa	28	+	0
Bursa	35	-	0
Bursa	70	-	0
Bursa	105	-	0

[1] Days post infection. One-day old chickens were inoculated with 2000 pfu of HVT. Chickens were perfused with 4% paraformaldehyde at the various time intervals. The tissues

Table I (cont.)

were sectioned and placed on silylated slides **and either** *in situ* **hybridization** (ISH) or indirect **immunofluorescence assay** (IFA) were performed as described in **Materials and Methods.**

[2] **ISH was used to detect** HVT using **Bam HI-F fragment as the probe.**

[3] **The average number of** HVT gB **positive cells per square millimeter of tissue.** The gB positive cells were identified **using IFA with** L78, a monoclonal antibody against HVT gB.

Table II

Identification of tissues containing latent HVT

Tissues	DPI[1]	ISH[2]	IFA[3]
Sciatic p.	21	+	-
Sciatic p.	28	+	-
Sciatic p.	35	+	-
Brachial p.	21	+	-
Brachial p.	28	+	-
Brachial p.	35	+	-
Feather tips	21	+	-
Feather tips	28	+	-
Cerebellum	105	*	-
Cerebrum	105	*	-
C12	105	*	-
C13	105	*	-
C14	105	*	-
LS	105	*	-
Pons	105	*	-

Table II (cont.)

Tissues	DPI[1]	ISH[2]	IFA[3]
Spleen	105	+	-
Thymus	70	+	-
Thymus	105	+	-
Bursa	21	+	+
Bursa	28	+	-
Bursa	35	-	-
Bursa	70	-	-
Bursa	105	-	-

[1] Days post inoculation. One-day old chickens were inoculated with 2000 pfu of HVT. Chickens were perfused with 4% paraformaldehyde at the various time intervals. The tissues were sectioned and placed on silylated slides and either *in situ* hybridization (ISH) or indirect immunofluorescence assay (IFA) were performed as described in Materials and Methods.

[2] ISH was used to detect latent HVT using Bam HI-F fragment as the probe.

[3] IFA using L78 monoclonal antibody to determine that gB was not expressed in these tissues.

p. = plexus

* = foci was not above background levels.

Table III

Detection of HVT in peripheral blood mononuclear cells

DPI[1]	Presence of virus[2]
4	5/5
6	6/6
7	7/7
10	9/9
12	5/5
14	7/10
21	6/10
28	8/12
35	5/10
70	1/4

[1] Days post infection. One-day old chickens were inoculated with 2000 pfu of HVT. At the indicated time periods, between 1 to 5 ml of blood was removed. The peripheral blood mononuclear cells (PBMC) were separated from other blood cells by centrifugation.

[2] Using co-cultivation assays with infected PBMC overlaid onto a chick embryo fibroblast monolayer, the assays were examined for the appearance of plaques which is indicative of presence of virus

CHAPTER V

DETECTION OF LATENT TURKEY HERPESVIRUS IN CHICKEN T-CELL SUBSETS

M.S. Holland[1,2] , R.F. Silva[2], C.D. Mackenzie[1], and R.W. Bull[1]

[1]Dept. of Pathology, Michigan State University, East Lansing Michigan, 48824, U.S.A. [2]U.S. Department of Agriculture, Agricultural Research Service, Avian Disease and Oncology Laboratory, 3606 East Mount Hope Rd., East Lansing, Michigan 48823, U.S.A.

ABSTRACT

Chicken spleen and thymus were sites of latent turkey herpesvirus (HVT) infections. At 15 weeks post infection, the spleen and thymus were examined using indirect immunofluorescence assays (IFA) to confirm that HVT glycoprotein B was not expressed. The absence of gB expression ensures that the HVT infection is latent. Combined IFA and in situ hybridization performed on alternating serial sections (ISH) identified T-lymphocyte subsets as the cell types that contain latent HVT in the spleen and thymus. Monoclonal antibodies recognized CD4+ and CD8+ on the 1st tissue section while HVT probes identified latent HVT in the same cells on adjacent tissue sections. Therefore, we conclude that latent HVT is in T-lymphocytes that express either CD4 or CD8 markers.

INTRODUCTION

Marek's disease virus (MDV) induces both productive and latent infections. Shek et al. (1983) reported that these two types of infections occur in distinct lymphocyte populations. Productive infections are cytolytic and produce intranuclear inclusion bodies and cell loss (Calnek, 1986). Two types of productive infections exist: a fully productive and a productive-restrictive. A fully productive infection occurs in feather follicle epithelium and results in fully infectious virions (Calnek et al., 1970a). In contrast, productive-restrictive infections are in lymphocytes, epithelial cells and cultured cells. Most virions in a productive-restrictive infection are non-enveloped and non-infectious (Calnek and Witter, 1991). Productive-restrictive infections are present in B-lymphocytes.

Previous studies identified latent MDV serotype 1 infections in the spleen. However, identification of cells containing latent virus has been difficult at the molecular level. The use of reactivation studies has determined the cell type latently infected with MDV serotype 1 is T-lymphocytes (Shek et al., 1983; Calnek et al., 1981). The T-lymphocyte is also the target of transformation by MDV serotype 1.

We have chosen to study latency in HVT because it is nononcogenic and that eliminates the difficulty in differentiating between transformation and latency.

Previously, we demonstrated that the absence of glycoprotein B (gB) expression can be used as an indicator of latency (Holland et al., 1993). HVT latently infects the spleen, thymus, bursa, sciatic plexus, brachial plexus and feather tips (Holland et al., 1993).

The present study attempts to identify the cell type harboring latent HVT in lymphoid tissue. The use of markers will aid in identifying whether T-helper or T-suppresser lymphocytes contain latent HVT. In situ hybridization (ISH) and indirect immunofluorescence assays (IFA) were performed on serial sections to identify cells from lymphoid tissues that contain latent HVT.

MATERIALS AND METHODS

Animals, Experimental Infection and Tissue Preparation. Four one-day-old $15I_5$- x 7_1- chickens were inoculated with 2000 pfu of FC126, a strain of HVT. The parental strain of $15I_5$- x 7_1- chickens was negative for MDV serotype 1 antibodies. The parental strain of chickens had not been vaccinated for HVT. Four uninfected chickens were housed in separate isolators and used as negative controls.

Reactivation Studies. Fifteen weeks post infection (PI), 5 mls of blood was drawn from each chicken into tubes containing EDTA. The blood components were separated by centrifugation at 500 rpm for 20 minutes (IEC model HN-SII, Damon/IEC Division, Needham Hts, Mass.) Buffy coat cells from infected and uninoculated chickens were co-cultivated with chicken embryo fibroblasts (CEF) in cell culture. Plaques were counted 5-7 days after plating.

Indirect Immunofluorescence Assay. Fifteen weeks PI, chickens were anesthetized with halothane. Next, chickens were perfused with saline at 5 ml/kg of body weight. The saline perfusion was followed by perfusion with 4% paraformaldehyde at a rate of 5ml/kg of body weight. Thymus, bursa, and spleen were excised and placed in fresh 4% paraformaldehyde. The tissues were embedded in paraffin and serial 3 micron sections or 6 micron sections were cut with a microtome. Monoclonal antibodies (L78) against a

late gene HVT antigen, glycoprotein B (gB) were used to detect gB expression. L78 was provided by L.F. Lee (ADOL, USDA-ARS, East Lansing, MI). The second 3 micron serial section from the thymus, bursa or spleen was used in IFA. Monoclonal antibodies against chicken lymphocyte markers CD4 and CD8 (Dr. Lillyhoj, USDA-ARS, Beltsville, MD) were applied to tissue sections in IFA. The development and characterization of the antibodies have been reported (Lee et al., 1983, Silva et al., 1984 and Lillehoj et al., 1988). The IFA procedure has previously been described (Holland et al., 1993).

In Situ Hybridization. The in situ hybridization (ISH) assay used in this study has been described previously (Brahic and Haase, 1978). ISH were performed on alternating serial sections cut 3 microns thick. A ^{32}P-labeled probe was prepared from the HVT BamHI-F fragment by random priming (BRL Life Technologies, Inc., Gaithersburg, MD). The probe mixture was poured over a sephadex column so that unincorporated nucleotides passed through the column while the probe bound to the sephadex beads. After elution from the column, the probe was denatured by boiling for 5 minutes at 100 C and added to hybridization buffer. Approximately 10 ng of probe at a range of 6×10^7 to 1×10^9 cpm/ug of DNA was added per tissue section. The sections were hybridized for 12-18 hours in a shaking incubator at 47 C with 50 ul of probe mixture per tissue section. The slides

were washed with SSC, dehydrated with graded ethanol and air dried. For autoradiography, slides were immersed in Kodak NTB 2 emulsion (Eastman Kodak, Rochester, NY). The coated tissue sections were allowed to dry at room temperature in total darkness for 12 hours after which they were incubated at 4 C for 4 days. The sections were fixed, developed and stained with H & E.

RESULTS

In vitro reactivation of latent HVT from infected chickens. At 15 weeks PI, CEFs were co-cultivated with PBMC. After 5-7 days, co-cultivation assays were examined for plaque formation. In the absence of plaque formation, the cells were passaged at least twice before a negative result was made. Plaque formation developed from only 1 of 4 HVT latently infected PBMC. The PBMC from the other 3 chickens did not induce plaques, even after 2 passages of the co-cultivations. Infectious HVT was not detected in any PBMC from uninoculated chickens.

Identification of cells containing latent HVT. HVT gB expression was not detected in the thymus, spleen nor bursa by IFA (Table I). ISH was used to identify latent HVT in all infected chickens. Serial sections of the thymus, bursa, and spleen allowed the detection of HVT RNA on the 1st serial section by ISH. IFA was performed on the 2nd serial section using either a monoclonal antibody specific for CD4 or CD8 surface antigen. The thymus and spleen contained latent HVT RNA at 15 weeks PI but the bursa did not. Latent HVT was localized to T-lymphocyte subsets in the thymus and spleen (Table I). All infected chickens had CD4+ and CD8+ cells that contained latent HVT. CD4+ and CD8+ cells containing latent HVT ranged from 0 to 3 per field depending on the tissue section. The latently infected cells were primarily in the medullary region of the

thymus. However, the spleen had latent HVT confined to the cortex (Figure 1). Uninfected control tissues were negative for HVT infections.

DISCUSSION

Previous studies identified T-lymphocytes as the target for latent MDV serotype 1 infections (Calnek et al., 1981). However, the target for latent HVT had not been reported. The primary objective of this study was to determine if CD4+ or CD8+ cells contain latent HVT.

Serial sections of thymus, spleen, and bursa at different levels of the tissues were examined for CD4 and CD8 markers using IFA. An absence of gB expression in the presence of HVT indicated a latent infection. Adjacent sections were examined for latent HVT by ISH. Serial sections allowed the examination of the same cell on adjacent sections. Latent HVT was detected in all chickens examined. gB expression was not detected in any of the infected or uninfected chickens.

This study demonstrated that both CD4+ and CD8+ cells contain latent HVT. CD4 and CD8 are markers found on mature T lymphocytes (Suter, 1982). Approximately 80% of the mononuclear cells in the spleen are T-lymphocytes (Chi et al., 1981). CD4+ cells comprise 60% of mature T lymphocytes while CD8+ cells constitute 30% of T-lymphocytes (Cotran et al., 1989). T-lymphocytes acquire these cell surface markers in the thymus while undergoing maturation. CD4 markers are present on T-helper cells and some monocytes. In contrast, CD8 is found on cytotoxic and suppressor T-lymphocytes (Cotran et al., 1989). These markers accommodate interactions between T-lymphocytes and other

cells. CD4 markers interact with MHC class II molecules whereas CD8 markers bind to MHC class I molecules (Suter, 1982). The CD4+ and CD8+ cells that contain latent HVT were present in periarteriolar lymphoid sheath (PALS) region in the spleen. PALS contain predominately T-lymphocytes (Sharma and Tizard, 1984). CD4+ and CD8+ cells in the medullary region of the thymus contained latent HVT. The significance of the presence of latent HVT in CD4+ and CD8+ cells is unknown.

Latent MDV serotype 1 is present in T-lymphocytes (Calnek et al., 1981). In addition, MDV serotype 1 is immunosuppressive, which may be related to its presence in T-lymphocytes. To date, no reports have been published concerning immunosuppression caused by HVT. Latent HVT may interact with the host's immune system in a manner different from MDV serotype 1.

In this study, we showed latent HVT in CD4+ and CD8+ cells as well as in cells that did not carry these markers. The importance of latent HVT in CD4+ and CD8+ cells is unknown.

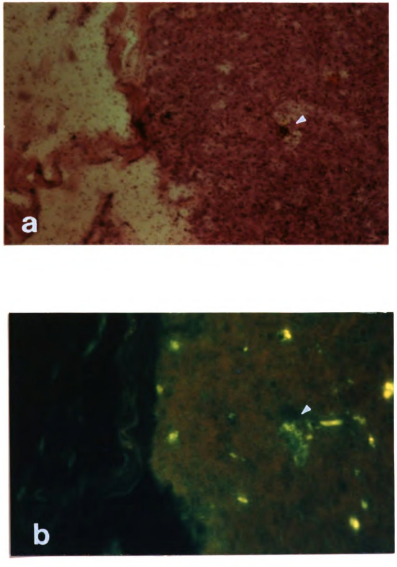


Figure 1. Detection of latent HVT in CD4+ cells. (a) The arrow indicates a positive focus that represents latent HVT by ISH. A HVT Bam-HI F fragment was ^{32}P labeled and used as the probe (15 weeks post infection); (b) The arrow points to a fluorescing cell that is the same cell shown in Fig. 1A. The cell is positive for CD4 antigen as indicated by IFA. Not all CD4+ cells contained latent HVT. (Magnification x400)

Table I

Detection of latent HVT by *in vitro* reactivation studies, ISH, and IFA

Chicken No.	Reactivation[a]	Tissue	ISH[b] gB[c]	IFA of CD4/CD8[d]	IFA of
		thymus	+	-	+
1	+	spleen	+	-	+
		bursa	-	-	-
		thymus	+	-	+
2	-	spleen	+	-	+
		bursa	-	-	-
		thymus	+	-	+
3	-	spleen	+	-	+
		bursa	-	-	-
		thymus	+	-	+
4	-	spleen	+	-	+
		bursa	-	-	-

[a] Five ml of blood was removed from 4 chickens 105 days post infection. Buffy coat cells were separated from other blood elements using centrifugation and co-cultivated with chicken embryo fibroblasts. The co-cultivations assays were examined for plaques 5 to 7 days later. The appearance of

Table I (cont.)

plaques indicated that latent HVT was reactivated.

[b] *in situ* hybridization (ISH) was performed as described in Materials and Methods. The probe used in the ISH was the BamHI-F fragment cloned into pBluescript (KS-).

[c] Indirect immunofluorescence assay (IFA) included a monoclonal antibody, L78, for HVT glycoprotein gB detection. An absence of gB expression but the presence of HVT-RNA indicates that HVT is latent.

[d] Monoclonal antibodies identified lymphocyte surface markers CD4 and CD8 in an IFA assay. A + indicates that latent HVT was present in cells with either CD4+ or CD8+ markers.

Chapter VI.

Transcriptional Activity of Latent Turkey Herpesvirus Infections in Chickens Occurs From Multiple Regions

M.S. Holland[1,2*], R.F. Silva[2], C.D. Mackenzie[1], and R.F. Bull[1]

[1]Dept. of Pathology, CVM, Michigan State University, East Lansing, MI; [2]ADOL, USDA-Agricultural Research Service, East Lansing, MI

ABSTRACT

One-day-old chickens were inoculated with turkey herpesvirus (HVT) and 105 days post inoculation (PI), the spleen, thymus, bursa, cerebrum, cerebellum, pons, and medulla oblongata were removed and examined by *in situ* hybridization (ISH) and indirect immunofluorescence assay (IFA). Using IFA, the absence of gB expression in the above tissues indicated that the infections were latent. Transcriptional activity of latent HVT genome was identified using ISH. HVT BamHI-B, -C, -E, -F, -G, -I, -O, and -P clones were radiolabeled and used as probes in the ISH. BamHI-B, -C, -E, -G, and -P are located in the unique long (U_L) region of the HVT genome. BamHI-F represents the repeat regions flanking the U_L region (TR_L and IR_L) and BamHI-O straddles the junction between the IR_L and short inverted repeat (IR_S). The BamHI-I clone is located at the U_L region adjacent to the IR_L. Transcriptional activity was detected from the BamHI-F, -I, -G, and -O regions in the spleen and thymus. No activity was seen with probes from the BamHI-B, -C, -E, and -P region. However, BamHI-B, -C, -E, and -P probes hybridized to their corresponding HVT fragments in Southern blot hybridizations. Treatment of tissue sections with RNase prior to addition of probes in ISH abolished signal.

Latent HVT transcripts were not detected in the bursa at 105 days PI using ISH. Results were inconclusive for the presence of latent HVT in the cerebrum, cerebellum, pons,

and medulla oblongata using ISH. In conclusion, multiple regions of the latent HVT genome express transcripts. These transcripts may have a significant role in the establishment, maintenance, or reactivation of latent infections by HVT.

INTRODUCTION

Herpesviruses infect a significant number of species ranging from chickens to man. Herpesviruses are divided into alphaherpesvirus, betaherpesvirus, and gammaherpesvirus based on their biological properties. Marek's disease virus (MDV) serotype 1 and Epstein Barr virus (EBV) have been classified as gammaherpesviruses based on their tropism for lymphocytes (Roizman and Sears, 1990). Another biological characteristic is their ability to induce lymphoid tumors.

At the molecular level, the structure of MDV more closely resembles alphaherpesviruses such as herpes simplex virus (HSV) rather than gammaherpesviruses (Cebrian et al., 1982). Both MDV and HSV have a type E genomic structure (Buckmester et al., 1988). Type E genomes consist of a unique long (U_L) and unique short (U_S) region, each flanked by terminal and inverted internal repeats (TR_L, IR_L, TR_S, and IR_S) (Wadsworth et al., 1975). Another MDV serotype, MDV serotype 3 also has a type E genome. Marek's disease virus serotype 3 was originally isolated from turkeys hence the name, turkey herpesvirus (HVT) (Witter et al., 1970). HVT is antigenically related to MDV serotype 1 (Silva and Lee, 1985). Thus, HVT and MDV serotype 1 are serologically related to one another, biologically similar to gammaherpesviruses and analogous to alphaherpesviruses at the molecular level.

One characteristic, common to all herpesviruses, is the ability to establish latent infections. Latency is the

presence of the genome with limited viral gene expression. However, differences exist between alphaherpesvirus and gammaherpesvirus latency. In alphaherpesviruses, latency associated transcription is limited to the TR_L and IR_L. Latent transcription is anti-sense and overlaps immediate early genes (Croen et al., 1987). In comparison, gammaherpesviruses have a more expanded transcription during latency. Latent transcription is seen from internal repeats 1, 2 and 3 as well as the terminal repeat, and unique regions 1, 2, 3, and 5 of EBV.

While alphaherpesviruses are found in nervous tissue during latency, latent gammaherpesviruses are isolated from lymphoid tissue. Latent HVT is present in lymphoid, nervous and feather tissues (Holland et al., 1993). This feature shows that latent HVT has a characteristic similar to both alphaherpesvirus and gammaherpesvirus.

A lymphoblastoid cell line, MKT-1, is thought to contain latent MDV serotype 1. The virus in MKT-1 is considered latent because it is non-productive even though MKT-1 contains 15 copies of the virus per cell. Non-productive cells lines do not produce virus nor viral antigens (Tanaka et al., 1978). Some transforming viruses are non-productive. The MDV serotype 1 present in MKT-1 is also a transforming virus.

Using <u>in situ</u> hybridization (ISH), we studied latent HVT transcription in the thymus, spleen, bursa, cerebrum, cerebellum, pons and medulla oblongata of HVT

infected chickens. We show that multiple regions of the HVT genome are transcribed during latency.

MATERIALS AND METHODS

Experimental Animals

(i) Infection. Five one-day-old chickens were inoculated intraperitoneally with 2000 pfu of HVT, strain FC126, passage 10. Another five chickens were uninfected and used as negative controls.

One hundred and five days post infection (PI), all infected and uninfected chickens were euthanized by exsanguination and 4% paraformaldehyde was perfused throughout the body. The thymus, spleen, bursa, cerebrum, cerebellum, pons and medulla oblongata were removed and examined using ISH as detailed below.

(ii) Co-cultivation. Peripheral blood mononuclear cells (PBMC) were isolated 105 days PI for virus recovery using co-cultivation assays. The PBMCs were separated from other blood elements by centrifugation at 500 rpm for 20 minutes and washed twice with Leibowitz-McCoy solution supplemented with 10% calf serum. The PBMCs were counted with an hemocytometer and seeded at 10^6 cells onto a confluent monolayer of chick embryo fibroblasts (CEF). The co-cultivation assay served as a detector of reactivated virus. Co-cultivation assays were examined at 5-7 day time intervals and if no cytopathic effects occurred, the cells were passaged after trypsinization.

(iii) Tissues Preparation. The thymus, spleen, bursa, cerebrum, cerebellum, pons and medulla oblongata were taken from HVT-infected chickens and placed in fresh 4%

paraformaldehyde for 24 hours. The tissues were placed in a Fischer Histomatic (Model 166) and processed under vacuum for 1 hour in each of the following chemicals; 65% ethanol, 2 times in 80% ethanol and 95% ethanol each, 3 times in 100% ethanol, 3 times in xylene, and 3 times in paraffin. The tissues were cut to 5 micron thick sections and placed on silylated slides (Onascoe, Houston, TX).

Indirect immunofluorescence (IFA). Monoclonal antibody against HVT specific glycoprotein B (gB) (L78) was used for the detection of gB expression. The IFA procedure was previously described (Holland et al., 1993). The expression of gB was examined to determine if the HVT infection was truly latent.

Probes. BamHI-B, -C, -E, -F, -G, -I, -O, and -P clones were digested with BamHI. The BamHI fragments were electrophoresed using a minigel apparatus through a 0.7% agarose gel in TBE buffer (89mM boric acid, 2mM EDTA, 89mM Tris-borate [pH8.0]) at 50 V for 4 hours. Gels were stained for 30 minutes in 0.5 ug of Ethidium bromide per ml and visualized using a 254 nm ultraviolet transilluminator. The BamHI fragments were cut out of the gel and the DNA eluted. The recovered DNA was ligated to pBluescript (KS-) and cloned into Escherichia coli. Positive clones were identified by plating on X-Gal (Sigma) minimal agar plates. The resulting clones were radiolabeled with ^{32}P using a

random primer kit (BRL Life Technologies, Gaithersburg, MD). The unincoporated nucleotides were separated from the probe by gel filtration chromatography on Sephadex G-50. The final probe products had specific activity ranging from 6.9 x 10^7 to 1 x 10^9 cpm per ug of DNA.

In situ hybridization. The probes were used to detect HVT RNA using ISH. The ISH procedure has previously been described (Brahic and Haase, 1978). Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol solutions and digested with Proteinase K. DNase-free RNase was boiled for 15 minutes to remove any residual DNase that may have been present. Sections probed for viral RNA were treated with RNase A (40 ug/ml of 2x SSC) for 30 minutes at 37 C. The slides were refixed in 4% paraformaldehyde. Sections were hybridized with 10 ng of individual ^{32}P-labeled Bam HI fragments per tissue section for 24 hours at 47 C in 2x SSC-50% formamide-10% dextran sulfate-0.01 M Tris (pH 7.4)-0.001M EDTA-0.02% bovine serum albumin-0.02% polyvinylpyrrolidone. As a control, the same plasmid containing no viral insert was used as a probe under identical ISH conditions. Another control consisted of omitting the probe from the hybridization conditions. Uninfected thymus, spleen, bursa, cerebrum, cerebellum, pons, and medulla oblongata were negative controls while acutely infected thymus and spleen were positive controls. After hybridization, slides were washed in varying

concentrations of SSC. After dehydration in graded ethanol solutions, the slides were coated with NTB-2 emulsion (Kodak, Rochester, NY), exposed for 4 days at 4 C developed and stained with hematoxylin and eosin. Backgrounds were relatively free of nonspecific autoradiographic grains. Cells were considered positive when they exhibited clusters of grains too numerous to count.

Results

BamHI Probes Hybridizing to RNA in Latently Infected Thymus. ^{32}P-labeled BamHI cloned fragments representing approximately 80% of the HVT genome were individually hybridized to sections of thymus, spleen, bursa, cerebrum, cerebellum, pons and medulla oblongata taken from chickens latently infected with HVT. The BamHI restriction fragments-B, -C, -E, -F, -G, -I, -O, and -P (Fig. 1) were used as probes to determine whether the transcripts present in the various tissues were homologous to these regions of the HVT genome.

Treatment of latently infected tissues with RNase A resulted in the loss of detectable HVT hybridization. The RNase A was boiled to denature any residual DNase activity. The RNase A was not tested on undegraded RNA, so a small possibility exists that the target detected in the ISH was HVT DNA. However, this is highly unlikely because the tissue sections were not heated, therefore the HVT DNA present was double stranded and unavailable for hybridization with the probes. Thus, the target detected was HVT-RNA. pBluescript (KS-) with no viral insert was included as a probe in ISH. This probe did not hybridize to HVT-infected or uninfected tissues indicating specificity of the HVT probe. When probe was not added to the ISH no positive foci was seen in infected or uninfected tissues. None of the HVT BamHI clones hybridized to uninfected spleen, thymus, or bursa.

Transcription of the latent HVT genome in the thymus was detected using Bam HI-F, -I, and -O probes (Fig. 2). The only probe that hybridized to tissue sections from every infected chicken was the HVT BamHI-F fragment (Table I). Most of BamHI-F fragment is within the TR_L and IR_L. BamHI-I and -O probes hybridized to at least 7 out of 10 thymus sections per chicken. BamHI-I straddles the junction of the U_L and IR_L. BamHI-O is at the junction between the IR_L and IR_S. Thymus sections probed with BamHI-I and O fragments had twice as many positive foci per high powered field when compared with sections probed with BamHI-F fragment. There was no detectable hybridization with Bam HI-B, -C, -E, -G, and -P probes in HVT-infected thymus. These probes are within the U_L region exclusively.

Similar to results obtained from thymus, latent HVT RNA in the spleen hybridized to BamHI-F, -G, -I, and -O probes (Table II). Four out of ten spleen sections from one chicken hybridized to BamHI-G probe but, BamHI-G did not hybridize with spleen sections from the other 4 infected chickens or uninfected chickens.

The positive foci detected on infected cerebrum, cerebellum, pons, and medulla oblongata was also seen with uninfected cerebrum, cerebellum, pons, and medulla oblongata, after hybridization with BamHI-B, -C, -E, -F, -G, -I, -O, and -P probes. Therefore, the results were inconclusive in regards to the presence of latent HVT in the cerebrum, cerebellum, pons, and medulla oblongata. In

addition, latent HVT was not detected in the bursa at 105 days PI.

DISCUSSION

We described previously the criteria for HVT latency and the site of latent HVT infections. The criteria for HVT latency was the presence of HVT RNA in the absence of gB expression. In addition, both lymphoid and nervous tissue harbor latent HVT (Holland et al., in press). Based on these results, 105 days PI thymus and spleen were examined to determine the region of the genome transcriptionally active during latency.

No hybridization was detected using probes covering >80% of the U_L region of the HVT genome. Probes specific for the TR_L, and IR_L and adjacent regions hybridized to latently HVT-infected thymus and spleen. The findings of this study indicate that latent HVT transcription is limited to the TR_L and IR_L as well as adjacent areas.

Latent HSV transcription is confined to the TR_L and IR_L (Deatly et al., 1987; Rock et al., 1987). The region of the genome transcriptionally active during HSV latency encodes ICP0. However, the transcripts present during latency are antisense to ICP0. It is unknown whether an ICP0 homologue exists in HVT. The transcripts present during HSV latency appear localized to the nuclei of latently infected cells. The location of transcripts in latently infected cells during HVT latency is still unknown.

The function and significance of latency-associated transcription from the HVT genome is unknown. The BamHI-B fragment contains the gene that encodes glycoprotein B (gB).

gB is a late gene glycoprotein that is highly conserved among the herpesviruses (Ross et al., 1989). An absence of gB expression indicates a state of latency in HVT infections. Thus, an absence of gB expression correlates well with an absence of transcription from BamHI-B fragment.

The results of latent transcription from HVT, MDV serotype 1 and HSV suggest that the repeat regions and adjacent sequences play an important role in the transcription of HVT and HSV. Whether this role is involved in the production of proteins for the establishment, maintenance or reactivation of latency is unknown. However, no latent proteins from HVT, MDV serotype 1 or HSV have been identified.

Transcription during HSV latency is not essential for the establishment or maintenance of latency (Javier et al., 1988; Leib et al., 1989; Steiner et al., 1989). In contrast, studies demonstrated that LAT- mutants reactivate more slowly from latency (Leib et al., 1989). In addition, some LAT- mutants failed to reactivate from latency (Hill et al., 1990). This result indicates that LAT may produce a protein product that has a part in reactivation. However, no protein product has been identified in association with LAT. By analogy to HSV, HVT latency may have similar properties.

The HVT BamHI-G probe hybridized to spleen sections from only one chicken. Whether this result was due to nonspecific hybridization is unknown. However, in HSV

latency, cross hybridization of HSV nucleotide sequences with either mouse or human DNA occurs. Also, HSV nucleotides cross hybridize with 28S ribosomal DNA or RNA (Peden et al., 1981; Stevens et al., 1987). Jones et al., (1987) demonstrated this cross hybridization occurred between a 375 bp fragment present in the TR_L and IR_L of HSV.

ISH signals may represent reactivation rather than latency signals. The absence of gB expression in the tissues examined, provides strong evidence in favor of latent HVT transcription. The manner that the chickens and their tissues were handled prior to IFA and ISH provides evidence that the transcription detected was due to latency as opposed to reactivation. In studies that have long intervals between death and fixation of tissues, viral reactivation becomes a serious concern (Croen et al., 1987). However, we exsanguinated the chickens under anesthesia and fixed the tissues in situ. This procedure should minimize the possibility of reactivation of HVT.

The spleen and thymus that hybridized to BamHI-I and -O probes, appeared to have a larger number of positive foci than sections hybridizing to a BamHI-F probe. This result suggests that BamHI-I and -O regions are more actively transcribed during latency than BamHI-F region. However, the BamHI-F probe hybridized more consistently than the BamHI-I or -O probe. Whether this observation has any relevancy is unknown. Quantitative ISH could determine if

BamHI-I and -O region produce more transcripts than BamHI-F region, as suggested by our results.

In conclusion, we identified regions of the HVT genome that are transcriptionally active during latency. Future studies should identify and characterize transcripts present during latency.

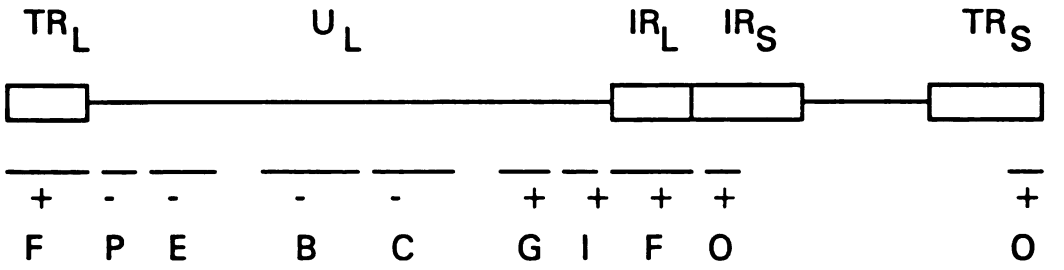


Figure 1. Transcription from the Latent HVT Genome.

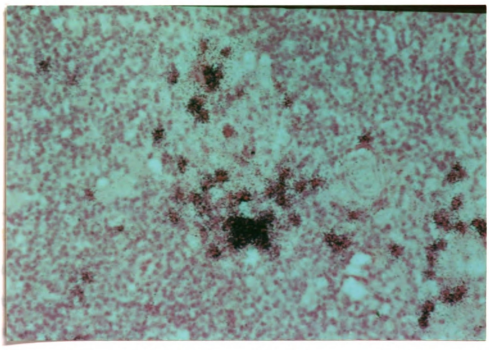


Figure 2. Photomicrograph of a thymus section from chicken infected at 1-day-old. At 105 days after infection, in situ hybridization was performed on thymic sections using a ^{32}P HVT-DNA probe to detect latent HVT-RNA in thymus (H & E stain, 200x).

Table I

Detection of HVT-RNA in thymus

Chick No.	BamHI B[b]	BamHI C	BamHI E	BamHI F	BamHI G	BamHI I	BamHI O	BamHI P
1	0/10[a]	0/10	0/10	10/10	0/10	9/10	10/10	0/10
2	0/10	0/10	0/10	10/10	0/10	9/10	10/10	0/10
3	0/10	0/10	0/10	10/10	0/10	7/10	9/10	0/10
4	0/10	0/10	0/10	10/10	0/10	8/10	9/10	0/10
5	0/10	0/10	0/10	10/10	0/10	9/10	10/10	0/10

[a] Results represent positive tissue sections/total tissue sections examined

[b] BamHI fragments were the probes used in in situ hybridization.

Table II

Detection of HVT RNA in the Spleen[a,b]

Chick No.	BamHI B[b]	BamHI C	BamHI E	BamHI F	BamHI G	BamHI I	BamHI O	BamHI P
1	0/10[a]	0/10	0/10	10/10	4/10	8/10	7/10	0/10
2	0/10	0/10	0/10	10/10	0/10	9/10	8/10	0/10
3	0/10	0/10	0/10	10/10	0/10	10/10	7/10	0/10
4	0/10	0/10	0/10	10/10	0/10	10/10	9/10	0/10
5	0/10	0/10	0/10	10/10	0/10	8/10	8/10	0/10

[a] Results are number of tissue sections with positive foci/total tissue sections examined.

[b] BamHI fragments represent the probes used in the *in situ* hybridization.

Chapter VII.

Summary and Conclusions

Productive and latent phases are two different pathways that a herpesvirus infection can follow. Productive infections are well characterized in herpes simplex virus (HSV). The similarity between HSV and HVT suggest that mechanisms controlling a productive infection are alike. Productive infections produce viral antigen expression. Cascade synthesis of HSV proteins results in the initial production of immediate early (IE) proteins. Immediate early proteins have regulatory functions and are necessary for the synthesis of subsequent proteins. Following IE proteins, early proteins are expressed and are responsible for viral DNA synthesis. The expression of early proteins occurs prior to late proteins. Late proteins are structural proteins and include glycoproteins that are found in the membrane of infected cells and envelopes of mature virus particles.

During a productive infection, HSV expresses approximately twelve glycoproteins. One of the best characterized glycoproteins is glycoprotein B (gB). Glycoprotein B promotes ingress of the virus into a target cell by first binding to heparin sulfate. Subsequent to this attachment, the virus gains entry to the cell by penetration and fusion of gB in the viral envelope with the cellular membrane. Another feature of gB is that it elicits a potent immunogenic response from the host. In addition,

gB is the most highly conserved glycoprotein among the alpha, beta, and gammaherpesvirus families.

To identify a productive HVT infection in chickens, gB expression was analyzed in PBMC and lymphoid tissues. Indirect immunofluorescence assay revealed gB expression in PBMC, spleen, thymus, and bursa. Splenic cells in the cortex expressed gB while cells in the medulla of the thymus and bursa express gB. The expression of gB was temporally regulated in PBMC and lymphoid tissue. The PBMC expressed gB at 4 to 12 days PI. Even though the spleen, thymus and bursa exhibited gB expression at 21 days PI, only the spleen and thymus revealed gB expression at 28 and 35 days PI. By 70 days PI, only the spleen had detectable gB expression. The termination of productive HVT infections occurred in the PBMC prior to termination in lymphoid tissue. At the end of the productive phase of infection, gB can no longer be detected *in vivo*. After gB expression ends, the latent phase of infection begins.

Latency is the presence of HVT genome with no or limited antigen expression. The antigen(s) expressed during latency are unknown but it is known that gB is not produced during latency. The absence of gB expression can be used to define a latent infection. The termination of gB expression occurred at different time intervals in different tissues. Latency was detected initially in PBMC at 14 days PI. The bursa had latent HVT at 28 days PI but latent HVT was not detected in the thymus until after 35 days PI. However, the

spleen did not contain a latent HVT infection until 105 days PI. These results do not preclude individual cells from containing latent HVT prior to identifying the tissue as being latently infected.

The presence of latent HVT in lymphoid tissue corresponds with classifying HVT as a gammaherpesvirus since members of this family also latently infect lymphoid tissue. Since HVT has genomic characteristics similar to alphaherpesviruses, it stands to reason that latent HVT may be found in the same location as latent alphaherpesviruses. HSV is a classical prototype of alphaherpesvirus and latent HSV is found in nervous tissue. Similar to latent HSV, latent HVT was also found in nervous tissue. Latent HVT was present in sciatic plexus and brachial plexus. In addition, feather tissue contained latent HVT. These results demonstrate that HVT can not be easily classified as a member of either alphaherpesvirus or gammaherpesvirus family.

In addition to identifying the tissues that harbor latent HVT, <u>in situ</u> hybridization revealed the cell type containing the latent virus. Within lymphoid tissue, predominately lymphocytes contained latent HVT. Using a combination of IFA and ISH on alternating serial sections, CD4+ and CD8+ cells were shown to contain latent HVT in the spleen and thymus. Since the bursa is composed of predominately lymphocytes, the latent infection in the bursa was probably in B-lymphocytes. Latent HVT in the bursa was

detected at only one time interval, 28 days PI. After this time, the latent infection was not evident in the bursa. This result indicates that possibly B-lymphocytes can not sustain a latent infection. Further support to this argument come from the study of MDV serotype 1 latent infections. While productive MDV serotype 1 infections occur in B-lymphocytes, only T-lymphocytes appear to support latent MDV serotype 1 infections.

The presence of latent HVT in nervous tissue led us to expect an infiltration of latently infected lymphocytes. On the contrary, latent HVT was present predominately in satellite cells. The only member of alphaherpesviruses that latently infects satellite cells is varicella zoster virus. All the other members infect neurons during latency. Latent HVT was not detected in neurons. Even though feathers contained latent HVT, the cells infected could not be identified.

Another prominent characteristic of latent HVT is limited transcriptional activity. Transcription of latent HVT mapped to the BamHI-F, -G, -I, and -O fragments. These fragments are located in the TR_L and IR_L and adjacent regions. Transcription of latent HSV also localized to TR_L and IR_L of the viral genome. The TR_L and IR_L encode for an immediate early protein ICP0 during lytic infections, but LAT is transcribed from this region during latent HSV infections. However, homologues for ICP0 in HVT have not been described in published literature. In addition, no

transcript that corresponds to LAT has been reported in latent HVT infections. Therefore, the significance of transcription from the TR_L and IR_L region of HVT latent genome is unknown.

The transcriptional pattern of both latent HVT and HSV are extremely limited when compared to lytic gene transcription. However, latent HVT transcription is more expanded than HSV but less than EBV. To date, no proteins associated with latency have been identified in either HVT or HSV *in vivo* but latent proteins are expressed from EBV. Further studies are necessary to investigate latent HVT proteins and the transcripts encoding them to determine if they function in the regulation of latency.

Results obtained from this research provide a beginning for the investigation into HVT latency. One goal of latency studies is the identification of a protein product involved in either the establishment, maintenance or reactivation of latent HVT.

Based on ISH, regions of the HVT genome transcribed during latency are the BamHI-F, -G, -I, and -O fragments. Since these BamHI fragments range in size from 2.0 kb to 14 kb, the next step is to fine map the LR-genes. Northern blot analysis, ISH and partial sequence analysis will allow characterization and fine mapping of the LR genes. The use of strand specific probes in Northern blots and ISH will reveal the transcriptional direction of the LR genes. After identifying the transcriptional direction of the LR genes,

characterization of the transcripts can occur. Northern blot analysis, ISH, primer extension and S1 nuclease mapping can be used to analyze LR transcripts. The characteristics that can be determined using these techniques are: the 5' and 3' ends of LR-RNA, the number of LR-RNA, the size of LR-RNA and the location of LR-RNA within cells and tissues. Nucleotide sequence analysis of LR-RNA will clarify splice donor and acceptor sites, potential ORF, and identify the putative promoter. Sequence analysis could be accomplished by producing a cDNA library and sequencing the resulting cDNA.

Sequence analysis would identify potential ORF(s) which could be translated to demonstrate amino acid sequences. Computer analysis of translation products from the ORF would reveal any amino acid sequence similarity to known protein sequences. Once an ORF that encodes a potential latency-related protein is identified, fusion protein products can be produced to analyze the function of latency related proteins. These LR proteins could play a role in the establishment, maintenance, or reactivation of latency.

Analysis of a putative LR promoter could lead to a number of interesting discoveries. Latent transcripts are present in both lymphoid and nervous tissue. By using chloramphenicol acetyltransferase assays (CAT) in neuron derived cell lines and lymphoid cell lines, cellular specificity could be determined. In today's technology, avian neuronal cell lines have not been developed. Using

CAT assays, the minimal promoter could be mapped to determine the constitutive activity in neuronal and lymphoid cells. Cellular factors that interact with positive or negative functional elements in or near the LR promoter could be detected using gel-shift competition experiments and DNase I footprint analysis.

Since viral latency lasts indefinitely, a LR promoter could possibly function throughout latency. Based on this premise, a LR promoter could be inserted into a vaccine vector and express the gene of interest as long as a latent infection is maintained and the infection is not reactivated. Presumably, a highly immunogenic gene product that is expressed from a LR promoter would substantially improve the efficacy of a vaccine. This gene product could induce an effective immune response in the host thereby, allowing clearance of the viral infection. Thus, continued research on latency could fulfill two goals. First, determining the viral proteins responsible for inducing latent infections could ultimately result in preventing the establishment, maintenance, and reactivation of latent virus. In addition, the viral proteins involved in latent infections could be cloned into expression vectors for improved recombinant vaccines.

LIST OF REFERENCES

Abbot, S. D., M. Rowe, K. Cadwallader, A. Ricksten, J. Gordon, F. Wang, L. Rymo, and A. B. Rickinson. (1990). Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. J. Virol. 64:2126-2134.

Abols-Mantyh, I., L. Siegel, M. A. Verity, H. Londe, and M. C. (1987). Latency of mouse cytomegalovirus in sciatic nerve. Neur. 37:1809-1812.

Adams, A. (1987). Replication of latent Epstein-Barr virus genomes in Raji cells. J. Virol. 61:1743-1746.

Adldinger, H. K., and B. W. Calnek. (1973). Pathogenesis of Marek's disease: early distribution of virus and viral antigens in infected chickens. J. Natl. Cancer Inst. 50:1287-1298.

Allday, M. J., D. H. Crawford and B. E. Griffin. (1989). Epstein-Barr virus latent gene expression during initiation of B cell immortalization. J. Gen. Virol. 70:1755-1764.

Antel, J. P., J. Kuchibhotla, and K. Stefansson. (1985). Generation of monoclonal antibodies recognizing neuronal elements in formalin-fixed, paraffin-embedded human tissues. J. Neuropathol. Exp. Neurol. 44:533-540.

Baichwal, V. R., and B. Sugden. (1988). Latency comes of age for herpesviruses. Cell 52:787-789.

Batchelor, A. H., and P. O'Hare. (1990). Regulation and cell type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. J. Virol. 64:3269-3279.

Beasley, J. N., L. T. Patterson, and D. H. McWade. (1970). Transmission of Marek's disease by poultry house dust and chicken dander. Am. J. Vet. Res. 31:339-344.

Beran, G. W., E. B. Davies, P. V. Arambulo, C. A. Will, H. T. Hill, and D. L. Rock. (1980). Persistence of pseudorabies virus in infected swine. J. Am. Vet. Med. Assoc. 176:998-1000.

Binn, M. M. and L. J. N. Ross. (1989). Nucleotide sequence of the Marek's disease virus (MDV) B-1B A-antigen and the identification of the MDV A antigen as the herpes simplex virus-1 glycoprotein C homologue. Virus Research. 12:371-382.

Birkenbach, M., D. Liebowitz, F. Wang, J. Sample, and E. Kieff. (1989). Epstein-Barr virus infection or the latent infection membrane protein induces vimentin expression. J. Virol. 63:4079-4084.

Block, T. M., J. G. Spivak, I. Steiner, S. Deshmane, M. T. McIntosh, R. P. Lirette, and N. W. Fraser. (1990). A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. J. Virol. 64:3417-3426.

Bodescot, M., M. Perricaudet, and P. Farrell. (1987). A promoter for the highly spliced EBNA family of RNAs of Epstein-Barr virus. J. Virol. 61:3424-3430.

Bradley, G., M. Hayashi, G. Lancz, A. Tanaka, and M. Nonoyama. (1989). Structure of the MDV BamHI-H gene family: A gene potentially important for tumor induction. *In* Advances in Marek's Disease Research. (Kato, S. T. Horiuchi, T. Mikami, and K. Hirai, eds.). Japanese Association on Marek's Disease, Osaka.

Brahic, M., and A. T. Haase. (1978). Detection of viral sequences of low reiteration frequency by in situ hybridization. Proc. Natl. Acad. Sci. USA 75:514-517.

Bratanich, A. C., and C. J. Jones. (1992). Localization of *cis*-acting sequences in the latency-related promoter of bovine herpesvirus 1 which are regulated by neuronal cell type factors and immediate-early genes. J. Virol. 66:6099-6106.

Bratanich, A. C., N. D. Hanson, and C. J. Jones. (1992). The latency-related gene of bovine herpesvirus 1 inhibits the activity of immediate-early transcription unit 1. Virology 191:988-991.

Brautigam, A. R., F. J. Dutko, L. B. Olding, and M. B. A. Oldstone. (1979). Pathogenesis of murine cytomegalovirus infection: the macrophage as a permissive cell for cytomegalovirus infection, replication, and latency. J. Gen. Virol. 44:349-359.

Brooks, L., Q. Y. Yao, A. B. Rickinson, and L. S. Young. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J. Virol. 66:2689-2697.

Brooks, L. A., A. L. Lear, L. S. Young, and A. B. Rickinson. (1993). Transcripts from the Epstein-Barr virus BamHI A fragment are detectable in all three forms of virus latency. J. Virol. 67:3182-3190.

Buchman, A. R., and P. Berg. (1988). Comparison of intron-dependent and intron-independent gene expression. Mol. Cell. Biol. 8:4395-4405.

Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M. E. G. Boursnell, L. J. N. Ross, and M. M. Binns. (1988). Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: Implications for herpesvirus classification. J. Gen. Virol. 69:2033-2042.

Bulow, V. V., and P. M. Biggs. (1975a). Differentiation between strains of Marek's disease virus and turkey herpesvirus by immunofluorescence assays. Avian Pathol. 4:133-146.

Bulow, V. V. and P. M. Biggs. (1975b). Precipitating antigens associated with Marek's disease viruses and a herpesvirus of turkeys. Avian Pathol. 4:147-162.

Buscaglia, C., and B. W. Calnek. (1988). Maintenance of Marek's disease herpesvirus latency in vitro by a factor found in conditioned medium. J. Gen. Virol. 69:2809-2818.

Buscaglia, C., B. W. Calnek, and K. A. Schat. (1988). Effect of immunocompetence on the establishment and maintenance of latency with Marek's disease herpesvirus. J. Gen. Virol. 69:1067-1077.

Buscaglia, C., B. W. Calnek, and K. A. Schat. (1989). Effect of reticuloendotheliosis virus and infectious bursal disease virus on Marek's disease herpesvirus latency. Avian Pathol. 18:265-281.

Busson, P., R. McCoy, R. Sadler, K. Gilligan, T. Tursz, and N. Raab-Traub. (1992). Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. J. Virol. 66:3257-3262.

Calnek, B. W., and S. B. Hitchner. (1969). Localization of viral antigen in chickens infected with Marek's disease herpesvirus. J. Natl. Cancer Inst. 43:935-949.

Calnek, B. W., H. K. Adldinger, and D. E. Kahn. (1970a). Feather follicle epithelium: A source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. 14:219-233.

Calnek, B. W., S. B. Hitchner, and H. K. Adldinger. (1970b). Lyophilization of cell-free Marek's disease herpesvirus and a herpesvirus from turkeys. Appl. Microbiol. 20:723-726.

Calnek, B. W., T. Ubertini, and H. K. Adldinger. (1970c). Viral antigen, virus particles, and infectivity of tissues with Marek's disease. J. Natl. Cancer Inst. 45:341-351.

Calnek, B. W., J. C. Carlisle, J. Fabricant, K. K. Murthy, and K. A. Schat. (1979). Comparative pathogenesis studies with oncogenic and nononcogenic Marek's disease viruses and turkey herpesvirus. Am. J. Vet. Res. 40:541-548.

Calnek, B. W., W. R. Shek, and K. A. Schat. (1981). Latent infections with Marek's disease virus and turkey herpesvirus. J. Natl. Cancer Inst. 66:585-590.

Calnek, B. W., K. A. Schat, L. J. N. Ross, W. R. Shek, and C.-L. H. Chen. (1984). Further characterization of Marek's disease virus-infected lymphocytes. I. in vivo infection. Int. J. Cancer 33:289-298.

Calnek, B. W. (1986). Marek's disease-a model for herpesvirus oncology. CRC Crit. Rev. Microbiol. 12:293-320.

Calnek, B. W., and Witter, R. L. (1991). Marek's disease. *In* Diseases of Poultry. (Calnek, B. W., H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, eds.) Iowa State University, Iowa.

Cantin, E., R. Eberle, J. Baldick, B. Moss, D. Willey, A. Notkins, and H. Openshaw. (1987). Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. Proc. Natl. Acad. Sci. USA 84:5908-5912.

Caudill, J. W., E. Romanowski, T. Araullo-Cruz, and Y. J. Gordon. (1986). Recovery of a latent HSV-1 thymidine kinase negative strain following iontophoresis and co-cultivation in the ocularly-infected rabbit model. Curr. Eye Res. 5:41-45.

Cebrian, J., C. Kaschka-Dierich, N. Berthelot, and P. Sheldrick. (1982). Inverted repeat nucleotide sequences in the genomes of Marek's disease virus and the herpesvirus of the turkey. Proc. Natl. Acad. Sci. USA 79:555-558.

Chen, X., and L. F. Velicer. (1991). Multiple bidirectional initiations and terminations of transcriptions in the Marek's disease virus long repeat regions. J. Virol. 65:2443-2451.

Cheung, A., and E. Kieff. (1982). Long internal direct repeat in Epstein-Barr virus DNAs. J. Virol. 44:286-294.

Cheung, A. (1989). Detection of pseudorabies virus transcripts in trigeminal ganglia of latently infected swine. J. Virol. 63:2908-2913.

Cheung, A. (1991). Cloning of the latency gene and the early protein 0 gene of pseudorabies virus. J. Virol. 65:5260-5271.

Chi, D. S., J. E. Galton, and G. J. Thorbecke. (1981). Role of T-cells in immune responses of the chicken. *In* Avian Immunol. (Rose, M. E., L. N. Payne and B. M. Freeman, eds.). British Poultry Science Ltd. Edinburgh.

Cho, B. R., S. G. Kenzy, and S. A. Haider. (1971). Horizontal transmission of turkey herpesvirus to chickens. 1. Preliminary observation. Poult. Sci. 50:881-887.

Cho, B. R. (1974). Horizontal transmission of turkey herpesvirus to chickens: IV. Viral maturation in the feather follicle epithelium. Avian Dis. 19:170-176.

Cho, B. R. (1975). Horizontal transmission of turkey herpesvirus to chickens. V. Viral maturation in the feather follicle epithelium. Avian Dis. 22:136-141.

Chollat-Namy, A., I. S. Delamanche, and C. Bouchaud. (1993). Variation in the expression of c-*fos* after intoxication by soman. Comparative study using in situ hybridization and immunohistochemistry. Brain Res. 603:32-37.

Churchill, A. E., and P. M. Biggs. (1967). Agent of Marek's disease in tissue culture. Nature 215:528-530.

Churchill, A. E., R. C. Chubb, and W. Baxendale. (1969). The attenuation with loss of oncogenicity of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J. Gen. Virol. 4:557-564.

Churchill, A. E., W. Baxendale, and G. Carrington. (1973). Viraemia and antibody development in chicks following the administration of turkey herpesvirus. Vet. Rec. 31:327-334.

Cockley, K. D., and F. Rapp. (1986). Analysis of long term human cytomegalovirus latency in vitro. Intervirol. 26:129-139.

Coen, D. M., M. Kosz-Vnenchak, J. G. Jacobson, D. A. Leib, C. L. Bogard, P. A. Schaffer, K. L. Tyler, and D. M. Knipe. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc. Natl. Acad. Sci. USA 86:4736-4740.

Cook, M. L., T. L. Thompson, and J. G. Stevens. (1986). A herpes simplex virus mutant is temperature sensitive for reactivation from the latent state: evidence for selective restriction in neuronal cells. Virology 155:293-296.

Cordier, R., A. Calender, M. Billaud, U. Zimber, G. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm, and G. M. Lenoir. (1990). Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR-1 genome induces expression of B-cell activation molecules CD21 and CD23. J. Virol. 64:1002-1013.

Cotran, R. S., V. Kumar, and S. L. Robbins. (1989). Diseases of Immunity. *In* Robbins Pathologic basis of disease. (W. B. Saunder Staff, ed.). W. B. Saunders Co., Philadelphia, PA.

Countryman, J. U., H. Jenson, R. Seibl, H. Wolf, and G. Miller. (1987). Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J. Virol. 12:3672-3679.

Coussens, P. M., and L.F. Velicer. (1988). Structure and complete nucleotide sequence of the Marek's disease herpesvirus gp 57-65 gene. J. Virol. 62:2373-2379.

Coussens, P. M., M. R. Wilson, H. Camp, H. Roehl, R. J. Isfort, and L. F. Velicer. (1990). Characterization of the gene encoding herpesvirus of turkeys gp57-65: Comparison to Marek's disease virus gp 57-65 and Herpes simplex virus glycoprotein C. Virus Genes 3:291-307.

Croen, K. D., J. M. Ostrove, L. J. Dragovic, J. E. Smialek, and S. E. Straus. (1987). Latency herpes simplex virus in human trigeminal ganglia. N. Eng. J. Med. 317:1427-1432.

Croen, K. D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus. (1988). Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex viruses. Proc. Natl. Acad. Sci. USA 85:9773-9777.

Culling, C. F., P. E. Reid, and N. M. Sinott. (1980). The effect of various fixatives and trypsin digestion upon the staining of routine paraffin-embedded sections by the peroxidase-antiperoxidase and immunofluorescent technique. J. Histotechnol. 3:10-22.

Daibata, M., R. E. Humphreys, K. Takada, and T. Sairenji. (1990). Activation of latent EBV via anti-IgG-triggered, second messenger pathways in the Burkitt's lymphoma cell line AKATA. J. Immunol. 144:4788-4793.

Dambaugh, T., K. Hennessy, S. Fennewald, and E. Kieff. (1986). The EBV genome and its expression in latent infection. *In* The Epstein-Barr virus: recent advances. (Epstein, M. A. and B. G. Achong, eds.) William Heinemann Medical Books Ltd., London.

Davies, D. H., and J. R. Duncan. (1974). The pathogenesis of recurrent infections with infectious bovine rhinotracheitis virus induced in calves by treatment with corticosteroids. Cornell Vet. 64:340-366.

Dawson, C. W., A. B. Rickinson, and L. S. Young. (1990). Epstein-Barr virus latent membrane protein inhibits human epithelial cell differentiation. Nature (London) 344:777-780.

Deatley, A. M., J. G. Spivack, E. Lavi, and N. W. Fraser. (1987). RNA from an immediate early region of the HSV-1 genome is present in the trigeminal ganglia of latently infected mice. Proc. Natl. Acad. Sci. USA 84:3204-3208.

Deatley, A. M., J. G. Spivack, E. Lavi, D. R. O'Boyle II, and N. W. Fraser. (1988). Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissue of mice map to similar regions of the viral genome. J. Virol. 62:749-756.

Deshmane, S. L., and N. W. Fraser. (1989). During latency herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. 63:943-947.

Dobson, A. T., R. Sederati, G. Devi-Rao, W. M. Flanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman. (1989) Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus J. Virol. 63:3844-3851.

Dobson, A. T., T. P. Margolis, F. Sedarati, J. G. Stevens, and L. T. Feldman. (1990). A latent, nonpathogenic HSV-1 derived vector stably expresses beta-galactosidase in mouse neurons. Neuron 5:353-360.

Dressler, G. R., D. L. Rock, and N. W. Fraser. (1987). Latent herpes simplex virus type 1 DNA is not extensively methylated in vivo. J. Gen. Virol. 68:1761-1765.

Dutko, F. J., and M. B. A. Oldstone. (1979). Murine cytomegalovirus infects spermatogenic cells. Proc. Natl. Acad. Sci. USA 76:2988-2991.

Eidson, C. S., and S. C. Schmittle. (1968). Studies on acute Marek's disease. I. Characteristics of isolate GA in chickens. Avian Dis. 12:467-476.

Eis, A. M. and Schneweis, K. E. (1986). Pathogenesis of genital herpes simplex virus infection in mice. IV. Quantitative aspects of viral latency. Med. Microbiol. Immunol. Berl. 175:281-292.

Eldred, W. D., C. Zuckerman and H. J. Karten. (1983). Comparison of fixation and penetration enhancement techniques for use in ultrastructural immunocytochemistry. J. Histochem. Cytochem. 31:285-292.

Elias, J. M., A. Gown, and E. Jaffe. (1989). Quality control in immunocytochemistry: Report on a workshop sponsored by the Biological Stain Commision. Am. J. Clin. Pathol. 92:836-846.

Elias, J. M. (1990). Immunohistochemical Methods. *In* Immunohistopathology: A practical approach to diagnosis. (Elias, J. M., ed.) American Society of Clinical Pathologists, Chicago.

Ernberg, I., K. Falk, J. Minarovits, M. G. Masucci, and G. Klein. (1989). The role of methylation in the phenotype dependent modulation of Epstein Barr nuclear antigen 2 and latent membrane protein genes in cells latently infected with EBV. J. Gen. Virol. 70:2989-3002.

Fabricant, J., B. Calnek, and K. Schat. (1982). The early pathogenesis of turkey herpesvirus infection in chickens and turkeys. Avian Dis. 26:257-264.

Fahraeus, R., H. Li-Fu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nisson, M. Yadaf, P. Busson, T. Tursz, and B. Kallin. (1988). Expression of the Epstein-Barr virus genome in nasopharyngeal carcinoma. Int. J. Cancer 43:329-338.

Fairbairn, L. J., J. P. Stewart, I. N. Hampson, J. R. Arrand, and T.M. Dexter. (1993). Expression of Epstein-Barr virus latent membrane protein influences self-renewal and differentiation in a multipotential murine hematopoietic 'stem cell' line. J. Gen. Virol. 74:247-254.

Farmilo, A. J., and R. H. Stead. (1990). Fixation in immunochemistry *In* Handbook: Immunochemical Staining Methods (Naish, S. J., ed), Dako Corp. 1989.

Farrell, M. J., A. T. Dobson, and L. T. Feldman. (1991). Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. USA 88:790-794.

Feldman, G., M. Maurice, and D. Bernuau. (1983). Penetration of enzyme-labeled antibodies in tissues and cells: A review of the difficulties. *In* Immunoenzymatic Techniques. (Avrameas, S., P. Drueet, and R. Masseyeff, eds.) Elsevier Science Publishers, New York.

Finke, J., M. Rowe, and B. Kallin. (1987). Monoclonal and polyclonal antibodies against Epstein-Barr virus nuclear antigen 5 (EBNA-5) detect multiple protein species in

Burkitt's lymphoma and lymphoblastoid cell lines. J. Virol. 61:3870-3878.

Furuta, Y., T. Takasu, K. C. Sato, S. Fukuda, Y. Inuyama, and K. Nagashima. (1992). Latent herpes simplex virus type 1 in human geniculate ganglia. Acta. Neuropathol. 84:39-44.

Gahn, T. A., and C. L. Schildkraut. (1989). The Epstein-Barr virus origin of plasmid replication, *oriP*, contains both the initiation and termination sites of DNA replication. Cell 58:527-535.

Gerster, T., and R. G. Roeder. (1988). A herpesvirus *trans*-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA 85:6247-6251.

Ghosh, D., and E. Kieff. (1990). *cis*-Acting regulatory elements near the Epstein-Barr virus latent-infection membrane protein transcriptional start site. J. Virol. 64:1855-1858.

Gibbs, C. P., K. Nazerian, L. Velicer, and H.-J Kung. (1983). Extensive homology exists between Marek's disease herpesvirus and its vaccine virus, herpesvirus of turkeys. Proc. Natl. Acad. Sci. USA 81:3365-3369.

Gilden, D. H., A. Vafai, Y. Shram, Y. Becker, M. Devlin, and M. Wellish. (1983). Varicella-zoster virus DNA in human sensory ganglia. Nature 306:478-480.

Gilden, D. H., A. R. Hayward, J. Krupp, M. Hunter-Laszlo, J. C. Huff, and A. Vafai. (1987). Varicella-zoster virus infection of human mononuclear cells. Virus Res. 7:117-129.

Giraldo, G., E. Beth, and E.-S, Huang. (1980). Kaposi's sarcoma and its relationship to cytomegalovirus (CMV). III. CMV DNA and CMV early antigens in Kaposi's sarcoma. Int. J. Cancer 26:23-29.

Goldsmith, K., L. Bendell, and L. Frapper. (1993). Identification of EBNA1 amino acid sequences required for the interaction of the functional elements of the Epstein-Barr virus latent origin of DNA replication. J. Virol. 67:3418-3426.

Goodchild, W. M. (1969). Some observations on Marek's disease (fowl paralysis). Vet. Rec. 84:87-89.

Gordon, Y. J., T. P. Arullo-Cruz, and E. Romanowski. (1986). The development of an improved murine iontophoresis reactivation model for the study of HSV-1 latency. Invest. Opthalmol. Vis. Sci. 27:1230-1234.

Gordon, Y. J., J. L. McKnight, C. Ostrove, E. Romanowski, and T. Arullo-Cruz. (1990). Host species and strain differences affect the ability of an HSV-1 ICP0 deletion mutant to establish latency and spontaneously reactivate in vivo. Virology 178:469-477.

Gregory, C., R. Murray, C. Edwards, and A. Rickinson. (1988). Down regulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underline tumor cell escape from virus-specific T-cell surveillance. J. Exp. Med. 167:1811-1824.

Gregory, C., M. Rowe, and A. Rickinson. (1990). Different Epstein-Barr virus (EBV)B-cell interactions in phenotypically distinct clones of a Burkitt lymphoma cell line. J. Gen. Virol. 71:1481-1495.

Grogan, E. A., H. Jenson, J. Countryman, L. Heston, L. Gradoville, and G. Miller. (1987). Transfection of a rearranged viral DNA fragment WZ het, stably converts latent EBV infection to productive infection in lymphoid cells. Proc. Natl. Acad. Sci. USA 84:1332-1337.

Gutenkunst, D. E. (1979). Latent pseudorabies virus infection in swine detected by RNA-DNA hybridization. Am. J. Vet. Res. 40:1568-1572.

Gutekunst, D. E., E. Pirtle, L. D. Miller, and W. C. Stewart. (1980). Isolation of pseudorabies virus from trigeminal ganglia of a latently infected sow. Am. J. Vet. Res. 41:1315-1316.

Guy, J. S., and L. N. D. Potgieter. (1985). Kinetics of antibody formation after the reactivation of bovine herpesvirus-1 infection in cattle. Am. J. Vet. Res. 46:899-901.

Hammerschmidt, W., and B. Sugden. (1988). Characterization of *oriLyt*, a lytic origin of DNA replication of Epstein-Barr virus. Cell 55:427-433.

Hardwick, J., E. Romanowski, T. Arullo-Cruz, and Y. J. Gordon. (1987). Timolol promotes reactivation of latent HSV-1 in the mouse iontophoresis model. Invest. Opthalmol. Vis. Sci. 28:580-584.

Hayashi, K., I. Kurihara, and Y. Uchida. (1985). Studies of ocular murine cytomegalovirus infection. Invest. Ophthalmol. Vis. Sci. 26:486-493.

Heller, M., T. Dambaugh, and E. Kieff. (1981). IX. Variation among viral DNAs from producer and nonproducer infected cells. J. Virol. 38:632-648.

Hennessy, K., and E. Kieff. (1983). One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. Proc. Natl. Acad. Sci. USA 80:5665-5669.

Hennessy, K., and E. Kieff. (1985). A second nuclear protein is encoded by EBV in latent infection. Science 220:1396-1398.

Hennessy, K., S. Fennewald, and E. Kieff. (1985). A third virus nuclear protein in lymphoblasts immortalized by Epstein-Barr virus. Proc. Natl. Acad. Sci USA 82:5944-5948.

Herbst, H., F. Dallenbach, M. Hummel, G. Neidobitek, S. Pileri, N. Mueller-Lantzsch, and H. Stein. (1991). Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc. Natl. Acad. Sci. USA 88:4766-4770.

Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G Stevens. (1990). Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology 174:117-125.

Hirai, K., K. Ikuta, N. Kitamoto, and S. Kato. (1981). Latency of herpesvirus of turkey and Marek's disease virus genomes in a chicken T-lymphoblastoid cell line. J. Gen. Virol. 53:133-143.

Ho, D. Y., and E. S. Mocarski. (1989). Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA 86:7596-7600.

Ho, M., S. Suwansirikul, J. N. Dowling, L. A. Youngblood, and J .A. Armstrong. (1975). The transplanted kidney as a source of cytomegalovirus infection. N. Engl. J. Med. 293:1109-1112.

Holland, M. S., R. F. Silva, C. D. Mackenzie, R. W. Bull, and R. L. Witter. (1993). Identification of and localization of gB expression in lymphoid tissues of chickens infected with turkey herpesvirus. Avian Dis. (Accepted for Publication).

Homan, E. J., and B. C. Easterday. (1980). Isolation of bovine herpesvirus-1 from trigeminal ganglia of clinically normal cattle. Am. J. Vet. Res. 41:1212-1213.

Homan, E. J., and B. C. Easterday. (1983). Experimental latent and recrudescent bovine herpesvirus-1 infections in calves. Am. J. Vet. Res. 44:309-313.

Hope-Simpson, R. E. (1965). The nature of herpes zoster: a long term study and a new hypothesis. Proc. R. Soc. Med. 58:9-20.

Howe, J. G., and J. A. Steitz. (1986). Localization of Epstein-Barr virus encoded small RNAs by in situ hybridization. Proc. Natl. Acad. Sci. USA 83:9006-9010.

Howe, J. G., and M. Shu. (1989). Epstein-Barr virus small RNA (EBER) genes: unique transcription units that combine RNA polymerase II and III promoter elements. Cell 57:825-834.

Huang, E-S., and J. S. Paogano. (1978). Cytomegalovirus DNA and adenocarcinoma of the colon: evidence for latent viral infection. Lancet. 1:957-960.

Hurley, E. A., L. D. Klaman, S. Agger, J. B. Lawrence, and D. A. Thorley-Lawson. (1991). The prototypical Epstein-Barr virus-transformed lymphoblastoid cell line IB4 is an unusual variant containing integrated but no episomal viral DNA. J. Virol. 65:3598-3963.

Hyashi, K., I. Kurihara, and Y. Uchida. (1985). Studies of ocular murine cytomegalovirus infection. Invest. Opthalmol. Vis. Sci. 26:486-492.

Hyman, R. W., J. R. Ecker, and R. B. Tenser. (1983). Varicella-zoster virus RNA in human trigeminal ganglia. Lancet. 6:814-816.

Ichikawa, T., and K. Ajiki. (1992). Development of an in situ hybridization histochemistry for choline acetyltransferase mRNA with RNA probes. Zoo. Sci. 9:305-314.

Ikuta, D., Y. Nishi, S. Kato, and K. Hirai. (1981). Immunoprecipitation of Marek's disease virus-specific polypeptides with chicken antibodies purified by affinity chromatography. Virology 114:277-281.

Ikuta, K., K. Nakahima, S. Ueda, D. Kato, and K. Hirai. (1984a). Studies on the serological cross-reaction between Marek's disease virus and herpesvirus of turkeys using monoclonal antibodies to major virus-specific polypeptides. Arch. Virol. 81:337-343.

Ikuta, K., S. Ueda, S. Kato, and K. Hirai. (1984b). Processing of glycoprotein gB related to neutralization of Marek's disease virus and herpesvirus of turkeys. Microbiol. Immunol. 28:923-933.

Isfort, R. J., I. Sithole, H-J. Kung, and L. F. Velicer. (1986). Molecular characterization of the Marek's disease herpesvirus B antigen. J. Virol. 59:411-419.

Isfort, R. J., and L. F. Velicer. (1987). Identification of the gene encoding Marek's disease herpesvirus A antigen. J. Virol. 61:2614-2620.

Izumi, K. M., A. B. McKelvey, G. B. Devi-Rao, E. K. Wagner, and J. G. Stevens. (1989). Molecular and biological characterization of a type 1 herpes simplex virus (HSV-1) specifically deleted for expression of the latency-associated transcript (LAT). Microbiol Path. 7:121-130.

Jacobson, J. G., S. L. Martin, D. M. Coen. (1989). A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. 63:1839-1843.

Jansson, A., M. Masùcci, and L. Rymo. (1992). Methylation of discrete sites within the enhancer region regulates the activity of the Epstein-Barr virus BamHI W promoter in Burkitt lymphoma lines. J. Virol. 66:62-69.

Javier, R. T., J. G. Stevens, V. B. Dissette, and E. K. Wagner. (1988). A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 166:254-257.

Jones, C., G. Delhon, A. Bratanich, G. Kutish, and D. Rock. (1990). Analysis of transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. J. Virol. 64:1164-1170.

Jones, H., S. D. Hayward, and D. Rawlins. (1989). Interaction of the lymphocyte-derived Epstein-Barr virus nuclear antigen EBNA-1 with its DNA-binding sites. J. Virol. 63:101-110.

Jones, T. R., C. L. Parks, and D. J. Spector. (1987). Hybridization of herpes simplex virus DNA and human ribosomal DNA and RNA. Virology 144:384-397.

Kahrs, R. F. (1981). Viral diseases of cattle, (Kahrs, R.F., ed.) Iowa State University Press, Iowa.

Kanamori, A. K., K. Ikuta, S. Ueda, S. Kato, and K. Hirai. (1987). Methylation of Marek's disease virus DNA in T-lymphoblastoid cell lines. J. Gen. Virol. 68:1485-1490.

Karlin, S., B. E. Blaisdell, and G. A. Schachtel. (1990). Contrasts in codon usage of latent versus productive genes of Epstein-Barr virus: data and hypotheses. J. Virol. 64:4264-4273.

Katz, B. Z., J. C. Niederman, B. A. Olson, and G. Miller. (1988). Fragment length polymorphisms among independent

isolates of Epstein-Barr virus from immunocompromised and normal hosts. J. Infect. Dis. 157:299-308.

Kemp, L. M., C. L. Dent, and D. S. Latchman. (1990). Octamer motif mediates transcriptional repression of HSV immediate-early genes and octamer-containing cellular promoters in neuronal cells. Neuron 4:215-222.

Kenney, S., J. Kamine, E. Holley-Guthrie, J. Lin, and J. Pagano. (1989). The Epstein-Barr virus (EBV) BZLF-1 immediate-early gene product differentially affects latent versus productive EBV promoters. J. Virol. 63:1729-1736.

Kerr, B. M., A. L. Lear, M. Rowe, D. Broom-Carter, L. S. Young, S. M. Rookes, P. H. Gallimore and A. B. Rickinson. (1992). Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. Virology 187:189-201.

Kieff, E., and D. Liebowitz. (1990). Epstein-Barr virus and its replication *In* Field's Virology, (Fields, B., D. Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, and B. Roizman, eds.), Raven Press Ltd., New York.

King, W., T. Dambaugh, M. Heller, J. Dowling and E. Kieff. (1982). Epstein-Barr virus DNA. XII. A variable region of the EBV genome is included in the P3HR-1 deletion. J. Virol. 43:979-986.

Kitamoto, N., K. Ikuta, and S. Kato. (1980). Persistence of genomes of both herpesvirus of turkeys and Marek's disease virus in a chicken T-lymphoblastoid cell line. Biken J. 23:1-8.

Kitamoto, Y., and H. Maeda. (1980). Reevaluation of the reaction of formaldehyde at low concentration with amino acid. J. Biochem. 87:1519-1525.

Klein, G. (1989). Viral latency and transformation: The strategy of Epstein-Barr virus. Cell 58:5-8.

Klotman, M. E., S. C. Henry, R. C. Greene, P. C. Brazy, P. E. Klotman, and J. D. Hamilton. (1990). Detection of mouse cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification. J. Infect. Dis. 161:220-225.

Kudelova, M., M. Kostal, L. Cervenakova, J. Rajcani, and H. C. Kaerner. (1991). Pathogenicity and latency competence for rabbits of the herpes simplex virus type 1 ANGpath gC and gE defective mutants. Acta. Virol. 35:438-449.

Kutish, G., T. Mainprize, and D. Rock. (1990). Characterization of the latency-related transcriptionally

active region of the bovine herpesvirus 1 genome. J. Virol. 64:5730-5737.

Larsson, L. -I., and D. M. Hougaard. (1992). Detection of gastrin and its messenger RNA in Zollinger-Ellison tumors by non-radioactive in situ hybridization and immunocytochemistry. Histochem. 97:105-110.

Laux, G., M. Perricaudet, and P. J. Farrell. (1988). A spliced Epstein-Barr virus gene expressed in latently transformed lymphocytes is created by circularization of the linear viral genome. EMBO J. 7:769-774.

Laux, G., A. Economy, and P. J. Farrell. (1989). The terminal protein gene 2 of Epstein-Barr virus is transcribed from a bidirectional latent promoter region. J. Gen. Virol. 70:3079-3084.

Lear, A. L., M. Rowe, M. F. Kurilla, S. Lee, S. Henderson, E. Kieff, and A. B. Rickinson. (1992). The Epstein-Barr virus (EBV) nuclear antigen 1 BamHI F promoter is activated on entry of EBV-transformed B cells into the lytic cycle. J. Virol. 66:7461-7468.

Lee, L. F., X. Liu, and R. L. Witter. (1983). Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. J. Immunol. 130:1003-1006.

Leib, D. A., C. L. Bogard, M. Koszpvnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer. (1989). A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. 63:2893-2900.

Leib, D. A., K. C. Nadeau, S. A. Rundle, and P. A. Schaffer. (1991). The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: Role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc. Natl. Acad. Sci. USA 88:48-52.

Liebowitz, D., R. Kopan, E. Fuchs, J. Sample, and E. Kieff. (1987). An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. Mol. Cell. Biol. 7:2299-2308.

Leist, T. P., R. M. Sandri-Goldin, and J. G. Stevens. (1989). Latent infections in spinal ganglia with thymidine kinase-deficient herpes simplex virus. J. Virol. 63:4976-4978.

Lillycrop, K. A., C. L. Dent, S. C. Wheatley, M. N. Beech, N. N. Ninkina, J. N. Wood, and D. S. Latchman. (1991). The

octamer-binding protein Oct-2 represses HSV immediate-early genes in cell lines derived from latently infectable sensory neurons. Neuron 7:381-390.

Lillyhoj, H. S., E. P. Lillehoj, D. Weinstock, and K.A. Schat. (1988). Functional and biochemical characterization of avian T-lymphocyte antigens identified by monoclonal antibodies. Eur. J. Immunol. 18:2059-2065.

Lokensgard, J. R., E. G. Thawley, and T. W. Molitor. (1990). Pseudorabies virus latency: restricted transcription. Arch. Virol. 110:129-136.

Longnecker, R. L., and E. Kieff. (1990). A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and co-localizes with LMP1. J. Virol. 64:2319-2326.

Longnecker, R. L., B. Druker, T. M. Roberts, and E. Kieff. (1991). An Epstein-Barr virus protein associated with cell growth transformation interacts with a tyrosine kinase. J. Virol. 65:3681-3692.

Mahalingam, R., D. Smith, M. Wellish, W. Wolf, S. N. Dueland, R. Cohrs, K. Soike, and D. Gilden. (1991). Simian varicella virus DNA in dorsal root ganglia. Proc. Natl. Acad. Sci. USA 88:2750-2752.

Mahalingam, R., P. Clarke, M. Wellish, A. N. Dueland, K. F. Soike, D. H. Gilden, and R. Cohrs. (1992). Prevalence and distribution of latent simian varicella virus DNA in monkey ganglia. Virology 188:193-197.

Mahalingam, R., M. Wellish, D. Lederer, B. Forghani, R. Cohrs, and D. Gilden. (1993). Quantitation of latent varicella-zoster virus DNA in human trigeminal ganglia by polymerase chain reaction. J. Virol. 67:2381-2384.

Marchioli, Y. R, E. Petrovskis, J. Timmins, and L. Post. (1987). Evaluation of pseudorabies virus glycoprotein gp50 as a vaccine for Aujeszky's disease in mice and swine: Expression by vaccinia virus and Chinese hamster ovary cells. J. Virol. 61:3977-3982.

Maray, T., M. Malkinson, and Y. Becker. (1988). RNA transcripts of Marek's disease virus (MDV) serotype-1 infected and transformed cells. Virus Genes 2:49-68.

Masucci, M., B. Contreras-Salazar, E. Ragnar, K. Falk, J. Minarovits, I. Ernberg, and G. Klein. (1989). 5-Azacytidine up-regulates the expression of Epstein-Barr virus nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt's lymphoma line Rael. J. Virol. 63:3135-3141.

Mazur, M. H., and R. Dolin. (1978). Herpes zoster at the NIH: A 20 year experience. Am J. Med. 65:738-344.

McFarlane, R. G., and D. G. Thawley. (1985). DNA hybridization procedure to detect pseudorabies virus DNA in swine tissue. Am. J. Vet. Res. 46:1133-1136.

McFerran, J. B., R. M. McCracken, and C. Dow. (1984). The role of the carrier pig in the epidemiology of Aujeszky's disease. Curr. Top. Vet. Med. Anim. Sci. 27:403-415.

McKnight, J. L. C., T. M. Kristie, and B. Roizman. (1987). The binding of the virion protein mediating alpha gene induction in herpes simplex virus 1 infected cells to its *cis* site requires cellular proteins. Proc. Natl. Acad. Sci. USA 84:7061-7065.

Meier, J. L., and S. E. Straus. (1992). Comparative biology of latent varicella-zoster virus and herpes simples virus infections. J. Inf. Dis. 166:S13-23.

Mercer, J. A., C. A. Wiley, and D. H. Spector. (1988). Pathogenesis of murine cytomegalovirus infection: identification of infected cells in the spleen during acute and latent infections. J. Virol. 62:987-997.

Miller, C. L., R. Longnecker, and E. Kieff. (1993). Epstein-Barr virus latent membrane protein 2A blocks calcium mobilization in B lymphocytes. J. Virol. 67:3087-3094.

Miller, G. (1990). Epstein-Barr virus. *In* Virology, (Fields, B. N., D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.), Raven Press, New York.

Mitchell, D., S. Ibrihim, and B. A. Guterson. (1985). Improved immunohistochemical localization of tissue antigens using modified methacarn fixation. J. Histochem. Cytochem. 33:491-498.

Mock, R. E., R. A. Crandell, and G. M. Mesfin. (1980). Induced latency in pseudorabies vaccinated pigs. Canad. J. Comp. Med. 45:56-59.

Murray, R. J., L. S. Young, and A. Calendar. (1988). Different patterns of Epstein-Barr virus gene expression and of cytotoxic T-cell recognition in B-cell lines infected with transforming (B95-8) or nontransforming (P3HR1) virus strains. J. Virol. 62:894-901.

Musiani, M., M. Zerbini, C. Capri, M. Plazzi, G. Sermasi, D. Belletti, and R. Sacchi. (1984). Serological screening for

the prevention of transfusion-acquired cytomegalovirus infection. J. Infect. 9:148-152.

Myers, M. G., H. L. Duer, and C. K. Hausler. (1980). Experimental infection of guinea pigs with varicella-zoster virus. J. Infect. Dis. 142:414-420.

Myers, M. G., L. R. Stanberry, and B. J. Edmond. (1985). Varicella-zoster virus infection of strain 2 guinea pigs. J. Infect. Dis. 151:106-113.

Narita, M., S. Inui, K. Namba, and Y. Shimizu. (1978). Neural changes in calves after intraconjunctival inoculation with infectious bovine rhinotracheitis virus in calves treated with dexamethasone. Am. J. Vet. Res. 39:1399-1403.

Nazerian, K. N., J. J. Solomon, R. L. Witter, and B. R. Burmester. (1968). Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. 127:177-182.

Nazerian, K. (1971). Further studies on the replication of Marek's disease virus in the chicken and in cell culture. J. Natl. Cancer Inst. 47:207-217.

Nazerian, K., L. Lee, R. Witter, and B. Burmester. (1972). Ultrastructural studies of a herpesvirus of turkeys antigenically related to Marek's disease virus. Virology 43:442-452.

Nazerian, K. N. (1974). DNA configuration in the core of Marek's disease virus. J. Virol. 13:1148-1150.

Nazerian, K. N., E. A. Stephens, J. M. Sharma, L. F. Lee, M. Gailitis, and R. L. Witter. (1977). A nonproducer T-lymphoblastoid cell line from Marek's disease transplantable tumor (JMV). Avian Dis. 21:69-76.

Nazerian, K. N., L. Lee, N. Yanagida, and R. Ogawa. (1992). Protection against Marek's disease by a fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus. J. Virol. 66:1409-1413.

O'Hare, P., and C. R. Goding. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell 52:435-445.

O'Hare, P., C. R. Goding, and A. Haigh. (1988). Direct combinatorial interaction between a herpes simplex regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J. 7:4231-4240.

Ohtaki, S., R. Hondo, H. Kodama, and T. Kurata. (1988). Experimental activation of latent cytomegalovirus infection of the captive red (F1) cynomologus monkeys by live or killed varicella-zoster virus. Acta. Pathol.-Jpn. 38:967-978

Okazaki, W., H. G. Purchase, and B. R. Burmester. (1970). Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14:413-429.

Olding, L. B., D. T. Kingsbury, and M. B. A. Oldstone. (1976). Pathogenesis of cytomegalovirus infection. Distribution of viral products, immune complexes and auto immunity during latent murine infection. J. Gen. Virol. 33:267-280.

Pappenheimer, A. M., L. C. Dunn, and V. Cone. (1929). A study of fowl paralysis (neuro-lymphomatosis gallinarum). Storrs Agric. Exp. Stn. Bull. 143:187-290.

Park, N. H., E. G. Herbosa, and J. P. Sapp. (1987). Effect of tar condensate from smoking tobacco and water-extract of snuff on the oral mucosa of mice with latent herpes simplex virus. Arch. Oral Biol. 32:47-53.

Payne, L. N., and P. M. Biggs. (1967). Studies on Marek's disease. II. Pathogenesis. J. Natl. Cancer Inst. 39:281-302.

Payne, L. N. (1985). Historical review. *In* Marek's Disease, (Payne, L. N., ed.). Martin Nijhoff, Boston.

Pearse, A. G. E. (1968). The chemistry of fixation. In Histochemistry: Theoretical and applied. (Pearse, A.G.E., ed.) Williams and Wilkins, Co. Baltimore.

Peden, K., P. Mounts, and G. S. Hayward. (1982). Homology between mammalian cell DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high-complexity probe. Cell 31:71-80.

Perna, J. J., M. L. Mannix, J. F. Rooney, A. L. Notkins, and S. E. Straus. (1987). Reactivation of latent herpes simplex virus infection by ultraviolet light: a human model. J. Am. Acad. Dermatol. 17:473-478

Petti, L., J. Sample, F. Wang and E. Kieff. (1988). A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. J. Virol. 62:1330-1338.

Phillips, P. A., and P. Biggs. (1972). Course of infection in tissues of susceptible chickens after exposure to strains of Marek's disease virus and turkey herpesvirus. J. Natl. Cancer Inst. 49:1367-1373.

Preece, A. (1972). Fixation of tissues. *In* A manual for histologic technicians (Terzakis, J., M.E. Long, and H.J. Ioachim, eds.) Little, Brown and Co. Boston.

Preston, C. M., M. C. Frame, and M. E. M. Campbell. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early regulatory DNA sequence. Cell 52:425-430.

Priola, S. A., D. P. Gustafson, E. K. Wagner, and J. G. Stevens. (1990). A major portion of the latent pseudorabies virus genome is transcribed in trigeminal ganglia of pigs. J. Virol. 64:4755-4760.

Priola, S. A., and J. G. Stevens. (1991). The 5' and 3' limits of transcription in the pseudorabies virus latency associated transcription unit. Virology 182:852-856.

Purchase, H. G., and P. M. Biggs. (1967). Characterization of five isolates of Marek's disease. Res. Vet. Sci. 8:440-449.

Purchase, H. G. (1969). Immunofluorescence in the study of Marek's disease. I. Detection of antigen in cell culture and an antigenic comparison of eight isolates. J. Virol. 3:557-565.

Purchase, H. G., W. Okazaki, and B. R. Burmester. (1972a). Long term field trials with the herpesvirus of turkeys vaccine against Marek's disease. Avian Dis. 16:57-71.

Purchase, H. G., W. Okazaki, and B. R. Burmester. (1972b). The minimum protective dose of the herpesvirus turkeys vaccine against Marek's disease. Vet. Rec. 91:79-84.

Rabinovitch, T., J. O. Oh, and P. Minasi. (1990). In vivo reactivation of latent murine cytomegalovirus in the eye by immunosuppressive treatment. Inv. Ophthalmol Vis. Sci. 31:657-663.

Raub-Traub, N., R. Hood, C. S. Yang, B. Henry, and J. S. Pagano. (1983) Epstein-Barr virus transcription in nasopharyngeal carcinoma. J. Virol. 48:580-590.

Raviola, E., (1975). Thymus. *In* A textbook of histology, (Bloom, F., D. W. Fawcett). W. B. Saunders Co., Pennsylvania.

Rawlins, D., G. Milman, S. D. Hayward, and G. Hayward. (1985). Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell 42:859-868.

Reisman, D., J. Yates, and B. Sugden. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol. 5:1822-1832.

Reisman, D., and B. Sugden. (1986). *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1 Mol. Cell. Biol. 6:3838-3846.

Revoltella, R. P., E. Vignetti, A. Fruscalzo, M. Park, G. Ragona, G. Rocchi, and E. Calef. (1989). Epstein-Barr virus DNA sequences in precursor monocyte-macrophage cell lines established from the bone marrow of children with maturation defects of haematopoiesis. J. Gen. Virol. 70:1203-1215.

Rhiza, H. -J., T. C., Mettenleiter, and G. Wittman. (1984). Occurrence of pseudorabies virus DNA in latently infected pigs. Curr. Top. Vet. Med. Anim. Sci. 27:429-444.

Rhiza, H. -J., T. C. Mettenleiter, V. Ohlinger, and G. Wittman. (1986). Herpesvirus (pseudorabies virus) latency in swine: Occurrence and physical state of viral DNA in neural tissues. Virology 155:600-613.

Rickinson, A. B., L. S. Young, and M. Rowe. (1987). Influence of the Epstein-Barr virus nuclear antigen EBNA-2 on the growth phenotype of virus-transformed B-cells. J. Virol. 61:1310-1317.

Rickinson, A.B., B. Kallin, and H. Alexander. (1988). BamHI-E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. Proc. Natl. Acad. Sci. USA 85:995-999.

Rispens, B. H., H. J. Van Vloten, N. Mastenbriek, H. J. L. Maas, and K. A. Schat. (1972). Control of Marek's disease in the Netherlands. I Isolation of an avirulent Marek's disease virus (strain CVI988) and its use in laboratory vaccination trials. Avian Dis. 16:108-125.

Rock, D. L., S. L. Beam, and J. E. Mayfield. (1987). Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. J. Virol. 61:3827-3831.

Rock, D., J. Lodensgard, T. Lewis, and G. Kutish. (1992). Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. J. Virol. 66:2484-2490.

Rock, D. L., (1993). The molecular basis of latent infections by alphaherpesviruses. Virology 4:157-165.

Roizman, B., and A. E. Sears. (1987). An inquiry into the mechanisms of herpes simplex virus latency. Annu. Rev. Microbiol. 41:543-571.

Roizman, B., and A. E. Sears. (1990) Herpes simplex viruses and their replication. *In* Virology, (Roizman, B., and D.M. Knipe, eds.) Raven Press Ltd., New York.

Roizman, B. (1992). The family Herpesviridae: an update. Arch. Virol. 123:425-449.

Rooney, C., N. Taylor, J. Countryman, H. Jenson, J. Kolman, and G. Miller. (1988). Genome rearrangements activate the Epstein-Barr virus gene whose product disrupts latency. Proc. Natl. Acad. Sci. USA 85:9801-9805.

Rosen-Wolff, A., J. Scholz, and G. Darai. (1989). Organotropism of latent herpes simplex virus type 1 is correlated to the presence of a 1.5 kb RNA transcript mapped within the BamHI DNA fragment B (0.738 to 0.809 map units). Virus Res. 12:43-51.

Ross, L. J. N. (1985). Molecular biology of the virus. *In* Marek's disease. (Payne, L. N., ed.) Nijhof, Boston.

Ross, L. J. N., and B. Milne. (1989). Manipulation of the genomes of MDV and HVT. *In* Advances in Marek's Disease Research. (Kato, S., T. Horiuvhi, T. Mikami, and K. Hirai, Eds.), Japanese Association on Marek's Disease, Osaka.

Ross, L. J. N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. (1989). Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70:1789-1804.

Ross, L. J. N., M. Kramer, W. Willenbacher, U. Wieland, E. Lorentzen, and R. Braun. (1990). Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. J. Virol. 64:2491-2497.

Rowe, M., D. T. Rowe, and C. D. Gregory. (1987a). Differences in B-cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO.J. 6:2743-2751.

Rowe, M., H. Evans, L. Young, K Hennessy, and E. Kieff. (1987b). Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen. Virol. 68:1575-1586.

Rowe, M., A. Lear, D. Croom-Carter, A. Davies, and A. Rickinson. (1992). Three pathways of Epstein-Barr virus gene

activation from EBNA-1 positive latency in B lymphocytes. J. Viro. 66:122-131.

Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston. (1987). Herpes simplex virus genes involved in latency in vitro. J. Gen. Virol. 68:3009-3018.

Sabo, A., and J. Rajcani. (1976). Latent pseudorabies virus infection of pigs. Acta. Virol. 15:87-94.

Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff. (1986). Nucleotide sequences of messenger RNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA 83:5096-5100.

Sample, J., D. Liebowitz, and E. Kieff. (1989). Two related Epstein-Barr virus membrane proteins are encoded by separate genes. J. Virol. 63:933-937.

Sample, J., and E. Kieff. (1990). Transcription of the Epstein-Barr virus genome during latency in growth-transformed lymphocytes. J. Virol. 64:1667-1674.

Sample, J., L. Brooks, C. Sample, L. Young, M. Rowe, C. Gregory, A. Rickinson, and E. Kieff. (1991). Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. Proc. Natl. Acad. Sci. USA 88:6343-6347.

Sample, J., E. B. D. Henson, and C. Sample. (1992). The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. J. Virol. 66:4654-4661.

Sandvej, K. B., S. J. Hamilton-Dutoit, and G. Pallesen. (1993). Influence of Epstein-Barr virus encoded latent membrane protein 1 on the expression of CD23 antigen, ICAM-1 and LFA-3 in Hodgkin and Reed-Sternberg cells. A morphometric analysis. Leuk. Lymph. 9:95-101.

Schaefer, B., M. Woisetschlaeger, J. Strominger, and S. Speck. (1991). Exclusive expression of Epstein-Barr virus nuclear antigen 1 Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA 88:6550-6554.

Schat, K. A., and B. W. Calnek. (1978). Characteristics of an apparently nononcogenic Marek's disease virus. J. Natl. Cancer Inst. 60:1075-1082.

Schat, K. A., R. D. Schultz, and B. W. Calnek. (1978). Marek's disease: Effect of virus pathogenicity and genetic

susceptibility on response of peripheral blood lymphocytes to concanavalin A. *In* Advances Comparative Leukosis Research (Bentvelzen, P., J. Hilgers, and D. S. Yohn, eds.) Elsevier, Amsterdam.

Schat, K. A., B. W. Calnek, and J. Fabricant. (1981). Influence of oncogenicity of Marek's disease virus on evaluation of genetic resistance. Poult. Sci. 60:2559-2566.

Schat, K. A., B. W. Calnek, J. Fabricant, and D. L. Grahm. (1985). Pathogenesis of infection with attenuated Marek's disease virus strains. Avian Pathol. 14:127-146.

Scheck, A. C., B. Wigdahl, and F. Rapp. (1989). Transcriptional activity of the herpes simplex virus genome during establishment, maintenance, and reactivation of in vitro virus latency. Intervirol. 30:121-136.

Schoenbaum, M. A., G. W. Beran, D. P. Murphy. (1990). Pseudorabies virus latency and reactivation in vaccinated swine. Am. J. Vet. Res. 51:334-338.

Sedarati, F., K. M. Izumi, E. K. Wagner, and J. G. Stevens. (1989). Herpes simplex virus type 1 DNA polymerase accounts for the capacity of an intertypic recombinant to spread from eye to central nervous system. J. Virol. 63:4455-4460.

Sellon, D. C., S. T. Perry, L. Coggins, and F. G. Fuller. (1992). Wild-type equine infectious anemia virus replicates in vivo predominately in tissue macrophages, not in peripheral blood monocytes. J. Virol. 66:5906-5913.

Settnes, O. P. (1982). Marek's disease-a common naturally herpesvirus-induced lymphoma of the chicken. Nord. Veterinaermed. Suppl.:11-132.

Sharma, J. M., and I. Tizard. (1984). Avian cellular immune effector mechanisms-A review. Avian Pathol. 13:357-376.

Sheffy, B. E., and D. H. Davies. (1972). Reactivation of a bovine herpesvirus after corticosteroid treatment. Proc. Soc. Exp. Biol. Med. 140:974-976.

Shek, W. R., K. Schat, and B. Calnek. (1982). Characterization of non-oncogenic Marek's disease virus-infected and turkey herpesvirus-infected lymphocytes. J. Gen. Virol. 63:333-341.

Shek, W. R., B. W. Calnek, K. A. Schat, and C. -L. H. Chen. (1983). Characterization of Marek's disease virus-infected lymphocytes: Discrimination between cytolytically and latently infected cells. J. Nat. Cancer Inst. 70:485-491.

Shiraki, K., and F. Rapp. (1986). Establishment of herpes simplex virus latency in vitro with cycloheximide. J. Gen. Virol. 67:2497-2500.

Silva, R. F., and L. F. Lee. (1984). Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. Virology 136:307-320.

Silva, R. F., and L. F. Lee. (1985). Isolation and partial characterization of three glycoproteins common to Marek's disease virus and turkey herpesvirus-infected cells. *In* Proc. Int. Symp. on Marek's Disease. (Calnek, B. W. and J. L. Spencer, eds.) Am. Assoc. Avian Pathol., Kennett Square, PA.

Silva, R. F., and R. L. Witter. (1985). Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J. Virol. 54:690-696.

Silver, S., A. Tanaka, and M. Nonoyama. (1979). Transcription of the Marek's disease virus genome in a nonproductive chicken lymphoblastoid cell line. Virology 93:127-133.

Sinclair, A. J., M. Brimmell, F. Shanahan, and P. J. Farrell. (1991). Pathways of activation of the Epstein-Barr virus productive cycle. J. Virol. 65:2237-2244.

Singer, R. H., J. B. Lawrence, and C. Villnave. (1986). Optimization of in situ hybridization using isotopic and nonisotopic detection methods. Biotechniques 4:230-232.

Smith, M. W., and B. W. Calnek. (1973). Effect of viral pathogenicity on antibody production in Marek's disease. Avian Dis. 17:727-736.

Smith, P., and B. Griffin. (1991). Differential expression of Epstein-Barr viral transcripts for two proteins (TP1 and LMP) in lymphocyte and epithelial cells. Nucleic Acids Res. 19:2435-2440.

Smith, P., and B. Griffin. (1992). Transcription of the Epstein-Barr virus gene EBNA-1 from different promoters in nasopharyngeal carcinoma and B-lymphoblastoid cells. J. Virol. 66:706-714.

Solomon, J. J., R. L. Witter, K. N. Nazerian, and B. R. Burmester. (1968). Studies on the etiology of Marek's disease. I. Propagation of the agent in cell culture. Proc. Soc. Exp. Biol. Med. 127:173-177.

Snowden, B. W., P. Kinchington, K. Powell, and I. Halliburton. (1985). Antigenic and biochemical analysis of

gB of herpes simplex type 1 and type 2 and of cross reacting glycoproteins induced by bovine mammillitis virus and equine herpesvirus type 1. J. Gen. Virol. 66:231-247.

Speck, S., and J. Strominger. (1985). Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen 1: a potentially polycistronic message generated by long range splicing of several exons. Proc. Natl. Acad. Sci. USA 82:8305-8309.

Speck, P. G., and A. Simmons. (1991). Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1. J. Virol. 72:4001-4005.

Spivak, J. G., and N. W. Fraser. (1987). Detection of herpes simplex type 1 transcripts during latent infection in mice. J. Virol. 61:3841-3847.

Spivak, J. G., and N. W. Fraser. (1988). Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. 62:1479-1485.

Spivak, J. G., G. M. Woods, and N. W. Fraser. (1991). Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0 kb latency-associated transcript (LAT) translation inhibition of LAT open reading frames by the intron within the 2.0 kb LAT. J. Virol. 65:6800-6810.

Steiner, I., J. G. Spivack, P. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser. (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. 8:505-511.

Steiner, I., J. G. Spivack, S. L. Deshmane, C. I. Ace, C. M. Preston, and N. W. Fraser. (1990). A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J. Virol. 64:1630-1638.

Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science 235:1056-1059.

Stevens, J. G., L. Haar, D. D. Porter, M. L. Cook, and E. K. Wagner. (1988). Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. J. Infect. Dis. 158:117-123.

Stroop, W. G., D. L. Rock, and N. W. Fraser. (1984). Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by in situ hybridization. Lab. Invest. 51:27-38.

Sugden, B., and N. Warren. (1989). A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. 63:2644-2649.

Suter, M. M., (1982). Immunopathology. *In* Mechanisms of disease: A textbook of comparative pathology. (Pine, J. W., L. Napora, and A. K. Schwartz, eds.) Williams and Wilkins, Maryland.

Tanaka, A., S. Silver, and M. Nonoyama. (1978). Biochemical evidence of the nonintegrated status of Marek's disease virus DNA in virus-transformed lymphoblastoid cells of chickens. Virology 88:19-24.

Tanaka, J., T. Ogura, J. Sato, and M. Hatano. (1987). Establishment and biological characterization of an in vitro human cytomegalovirus latency model. Virology 161:62-72.

Tanaka, J., T. Ogura, H. Iida, J. Sato, and M. Hatano. (1988). Inhibition of prostaglandin synthesis inhibits growth of human cytomegalovirus and reactivation of latent virus in a productively and latently infected human cell line. Virology 163:205-208.

Taylor, N., J. Countryman, C. Rooney, D. Katz, and G. Miller. (1989). Expression of the BZLF-1 latency-disrupting gene differs in standard and defective Epstein-Barr viruses. J. Virol. 63:1721-1728.

Tenser, R. B., W. A. Edris, and K. A. Hay. (1988). Herpes simplex virus latent infection: reactivation and elimination of latency after neurectomy. Virology. 167:302-305.

Tenser, R. B., D. A. Hay, and W. A. Edris. (1989). Latency-associated transcript but not reactivatable virus is present in sensory ganglion neurons after inoculation of thymidine kinase-negative mutants of herpes simplex virus type 1. J. Virol. 63:2861-2865.

Tenser, R. B., W. A. Edris, K. A. Hay, and B. E. De Galan. (1991). Expression of Herpes simplex virus type 2 latency-associated transcript in neurons and nonneurons. J. Virol. 65:2745-2750

Thorley-Lawson, D. A., and K. P. Mann. (1985). Early events in Epstein-Barr virus infection provide a model for B cell activation. J. Exp. Med. 162:45-59.

van Oirschot, J. T. and A. L. J. Gielkens. (1984). In vivo and in vitro reactivation of latent pseudorabies virus in pigs born to vaccinated sows. Am. J. Vet. Res. 45:567-571.

van Santen, V., A. Cheung, M. Hummel, and E. Kieff. (1983). RNA encoded by the IR1-U2 region of Epstein-Barr virus DNA in latently infected growth transformed cells. J. Virol. 46:424-433.

Van Zaane, D., J. M. A. Brinkhof, F. Westenbrink, and A. L. J. Gielkens. (1982). Molecular-biological characterization of Marek's disease virus. I. Identification of virus specific polypeptides in infected cells. Virology 121:116-132.

Vente, J., H. W. M. Steinbusch, and J. Schipper. (1987). A new approach to immunocytochemistry of 3',5'-cyclic guanosine monophosphate: Preparation, specificity, and initial application of a new antiserum against formaldehyde-fixed 3',5'-cyclic guanosine monophosphate. Neuroscience 22:361-370.

Wadsworth, S., R. J. Jacob, and B. Roizman. (1975). Anatomy of herpes simplex virus DNA: location of replication recognition signals within defective virus genomes. Proc. Natl. Acad. Sci. 15:1487-1497.

Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. F. Shang, E. M. Flanagan, and J. G. Stevens. (1988a). Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. 62:1194-1202.

Wagner, E. K., W. M. Flanagan, G. Devi-Rao, Y. -F Zhang, J. M. Hill, K. P. Anderson, and J. G. Stevens. (1988b). The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. 62:4577-4585.

Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff. (1988a). Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. J. Virol. 62:4173-4184.

Wang, D., D. Liebowitz, and E. Kieff. (1988b). The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform rodent fibroblasts. J. Virol. 62:2337-2346.

Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff. (1987). Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA 84:3452-3456.

Weber, K., P. C. Rathke, and M. Osburn. (1978). Cytoplasmic microtubular images in glutaraldehyde fixed tissue culture cells by electron microscopy and by immunofluorescence. Proc. Natl. Acad. Sci. USA 75:1820- 1827.

Weiss, L. M., L. A. Movahed, R. A. Warnke, and J. Sklar. (1989). Detection of Epstein-Barr virus viral genomes in Reed-Sternberg cells of Hodgkin's disease. N. Engl. J. Med. 320:502-506.

Weschler, S. L., A. B. Nesburn, R. Watson, S. M. Slanina, and H. Ghiasa. (1988). Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. J. Virol. 62:4051-4058.

Weschler, S. L., A. B. Nesburn, J. Zwaagstra, and H. Ghiasi. (1989). Sequence of the latency related gene of herpes simplex virus type 1. Virology 168:168-172.

West, K. P., A. Warford, and L. Fray. (1986). Detection of B cell, T cell and myeloid antigens in paraffin sections. J. Pathol. 148:123A.

Witter, R. L., K. N. Nazerian, H. G. Purchase, and G. H. Burgoyne. (1970). Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am. J. Vet. Res. 31:525-538.

Witter, R. L., J. M. Sharma, L. F. Lee, J. M. Opitz, and C. W. Henry. (1984). Field trials to test efficacy of polyvalent Marek's disease vaccines in broilers. Avian Dis. 28:44-60.

Witter, R. L. (1985). Vaccines and vaccination against Marek's disease. *In* Proc. Int. Symp. Marek's Dis. (Calnek, B. W., and J. L. Spencer, eds.) Am Assoc. Avian Pathol, Kennett Square, PA.

Witter, R. L. (1987). New serotype 2 and attenuated serotype 1 Marek's disease vaccine viruses: Comparative efficacy. Avian Dis. 31:752-765.

Witter, R. L. (1991). Attenuated revertant serotype 1 Marek's disease viruses: safety and protective efficacy. Avian Dis. 35:877-891.

Wittmann, G., V. Ohlinger, and H. -J. Wang. (1983). Occurrence and reactivation of latent Aujeszky's disease following challenge in previously vaccinated pigs. Arch. Virol. 75:29-41.

Woisetschlaeger, M., C. Yandava, L. Furmanski, J. Strominger, and S. Speck. (1990). Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc. Natl. Acad. Sci. USA 87:1725-1729).

Wu, T. -C., R. B. Mann. P. Charache, S. D. Hayward, S. Staal, B. C. Lambe, and R. F. Ambinder. (1990). Detection of EBV gene expression in Reed-Sternberg cells of Hodgkins disease. Int. J. Cancer 46:801-804.

Wysokenski, D., and J. Yates. (1989). Multiple EBNA-1 binding sites are required to form an EBNA-1 dependent enhancer and to activate a minimal replicative origin within *oriP* of Epstein-Barr virus. J. Virol. 63:2657-2666.

Yanagida, N., R. Ogawa, L. Lee, and K. Nazerian. (1992). Recombinant fowlpox viruses expressing the glycoprotein B homologue and the pp38 gene of Marek's disease virus. J. Virol. 66:1402-1408.

Yates, J., N. Warren, D. Reisman, and B. Sugden. (1984). A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA 81:3806-3810.

Yates, J., N. Warren, and B. Sugden. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature (London) 313:812-815.

Yates, J., and N. Guan. (1991). Epstein-Barr virus-derived plasmids replicated only once per cell cycle and are not amplified after entry into cells. J. Virol. 65:483-488.

Young, L., C. Dawson, D. Clark, H. Rupani, P. Busson, T. Tursz, A. Johnson, and A. Rickinson. (1988). Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J. Gen. Virol. 69:1051-1065.

Zeller, N. K., T. N. Behar, M. E. Dubois-Dalcq, and R. A. Lazzarini. (1985). The timely expression of myelin basic protein gene in cultured rat brain oligodendrocytes is independent of continuous neuronal influences. J. Neurosci. 5:2955-2962.

Zhu, X., J. Chen, C. S. H. Young, and S. Silverstein. (1990). Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products. J. Virol. 64:4489-4498.

Zimber-Strobl, U., K. -O. Suentzenich, G. Laux, D. Eick, M. Cordier, A. Calender, M. Billaud, G. M. Lenoir, and G. W. Bornkamm. (1991) Epstein-Barr virus nuclear antigen 2 activated transcription of the terminal protein gene. J. Virol. 65:415-423.

Zwaagstra, J. H. Ghaisi, A. B. Nesburn, and S. L. Weschler. (1989). In vitro promoter activity associated with the latency-associated transcript gene of herpes simplex virus type 1 J. Gen. Virol. 70:2163-2169.

Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel, and S. L Weschler. (1990). Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. J. Virol. 64:5019-5028.

Zwaagstra, J. C., H. Ghiasi, A. B. Nesburn, and S. Weschler. (1991). Identification of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simplex virus type 1 (HSV-1). Virology 182:287-297.