1
1001

This is to certify that the

thesis entitled

TOWARD ISOLATION OF LATENT
TURKEY HERPESVIRUS TRANSCRIPTS

presented by

Tim D. Tesmer

has been accepted towards fulfillment
of the requirements for

M.S. degree in Microbiology

Major professor

Date 8/23/2001

TOWARD ISOLATION OF LATENT TURKEY HERPESVIRUS TRANSCRIPTS

By

Tim D. Tesmer

AN ABSTRACT OF A THESIS

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

MASTER OF SCIENCE

Department of Microbiology

2001

Dr. Robert Silva

ABSTRACT

TOWARD ISOLATION OF LATENT TURKEY HERPESVIRUS TRANSCRIPTS

By

Tim D. Tesmer

A common feature of all herpesviruses is the ability to produce a latent infection in which viral antigens are not detectable on herpesvirus infected cells. Latent turkey herpesvirus (HVT) infections have been characterized by the presence of the HVT genome with the absence of glycoprotein B expression. The purpose of this study was to isolate and characterize HVT transcripts present during latency (latency associated transcripts). To accomplish this goal, a cDNA library was obtained which had been constructed from messenger ribonucleic acid (mRNA) isolated from chicken spleen latently infected with HVT. Initially, standard colony lift procedures were used to screen the library, but a high nonspecific background was encountered. A polymerase chain reaction (PCR) method also met with high background difficulties and we were unable to isolate the latency associated transcripts (LAT) clone(s) of interest.

To Mom, Dad, and Tony

ACKNOWLEDGEMENTS

I would like to thank my major advisor, Margo Holland for her support and guidance. I would also like to thank my committee members: John Fyfe, Richard Schwartz, Bob Silva, and the late Lee Velicer for their expertise, help, and patience with this thesis and my research. Special thanks to Dr. Silva for his advice and input on this thesis. Finally, I want to thank my family and friends for their encouragement and support throughout my time in graduate school.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES
(continued)

Page

LIST OF TABLES

CHAPTER 1:

LITERATURE REVIEW

Marek's Disease Virus:

Marek's Disease was first reported in the literature by Josef Marek (Marek, 1907). Marek's disease is a lymphoproliferative disease of chickens characterized by peripheral nerve demyelination and the formation of T cell lymphomas in chickens. Report of the first experimental transmission of the disease in 1962/63 preceded the propagation of the highly cell-associated herpesvirus in cell cultures in 1967/68 (Sevoian and Chamberlain, 1962; Biggs and Payne, 1963; Churchill and Biggs, 1967; Nazerian and Burmester, 1968; Nazerian, *et al.*, 1968; Solomon, *et al.*, 1968, Witter, *et al.*, 1969). Infectious, cell-free MDV was isolated from feather follicle epithelium, but not from bursal or gonadal tissues, by grinding, sonication, centrifugation, then filtration, and used to induce Marek's disease in susceptible chickens (Calnek, *et al.*, 1970). Enveloped herpesviruses were observed by electron microscopy in these preparations (Calnek, *et al.*, 1970).

The natural route of MDV transmission is via sloughing of feather follicle epithelium containing infectious, enveloped MDV. It is believed that the virus is inhaled by a chicken, and travels to the lymphoid organs by some as yet unknown route within 24-36 hours of exposure (Schat and Xing, 2000). The virus then infects B cells through splenic ellipsoid-associated reticulum cells which line the blood vessels (Jeurissen, *et al.*, 1992). It is thought that during a response to the infection by activated T cells that they become infected with MDV, and by another unknown mechanism, the virus becomes latent (Calnek, 1986).

The pathogenicity of this lymphoproliferative disease is affected by many factors such as strain, dose, route of exposure, host genotype, age at infection, sex, passive antibody, and environmental factors such as stress (Calnek and Witter, 1984). For many years, outbreaks of MDV caused significant chicken mortality until the development of an attenuated vaccine in 1969 (Churchill, *et al.*, 1969a; Churchill, *et al.*, 1969b). Since then, MDV has been classified into three different serotypes based upon oncogenic potential, agar gel precipitation and indirect fluorescent antibody assays (Von Bulow and Biggs, 1975a, 1975b; Schat and Calnek, 1978). MDV1 are mild to very virulent oncogenic strains of MDV and their attenuated derivatives. MDV2 are naturally occurring, nononcogenic strains of MDV. Turkey herpesvirus (HVT) was originally isolated in turkeys, and is nononcogenic in chickens.

Attenuated MDV1, and HVT vaccines were the first successful vaccines used worldwide to prevent tumors in any species (Calnek and Witter, 1984; Purchase, 1985). Bivalent and trivalent MDV vaccines contain different combinations of HVT, MDV2 and attenuated MDV1 strains. Despite the development of many new vaccines, the threat of MDV to the poultry industry persists due to the emergence of increasingly virulent MDV strains. (Witter, 1997; Witter, 1998)

MDV was originally classified as a gammaherpesvirus due to its lymphoproliferative nature, but was later reclassified as an alphaherpesvirus due to its genomic structure. The genome of MDV is double stranded DNA, and was originally estimated at 150-180 Kb (Lee, *et al.*, 1971; Wilson and Coussens, 1991). Recently, the sequence of two MDV1 strains were published by separate labs (Lee, *et al.*, 2000; Tulman, *et al.*, 2000). The size of MDV1, strain MD5 was 177, 874bp, with 103

predicted proteins (Tulman, *et al.*, 2000). With both the GA and MD5 strains, 55 genes in the unique long region were found to be homologous to herpes simplex virus-1 genes, but several putative genes remain uncharacterized, or have unknown function (Lee, *et al.*, 2000; Tulman, *et al.*, 2000). The unique long region of the HVT genome, strain FC-126, with its terminal repeats was sequenced by one lab, and simultaneously, the entire genome was published separately by another lab (Kingham, *et al.*, 2001; Alfonso, *et al.*, 2001). Of significance in the putative unique genes is a *Bcl-2* homolog, a gene implicated in hindering apoptosis in other systems, in the IR_S/TR_S of HVT. HVT also lacks the putative oncoprotein Meq, CxC chemokine and the phosphoprotein pp24 found in MDV1 (Alfonso, *et al.*, 2001).

MDV1, MDV2, and HVT share a similar genome structure with a unique long (U_L) region flanked by inverted repeats (TR_L, IR_L); and a unique short (U_S) region, also flanked by inverted repeats (IR_S, TR_S) (Figure 1-1) (Cebrian, *et al.*, 1982, Ono, *et al.*, 1992). All MDV serotypes share a collinear relationship. HVT and MDV1 have 76 conserved genes, many throughout the unique long and unique short regions (Gibbs, *et al.*, 1984; Igarashi, *et al.*, 1987; Lee, *et al.*, 2000; Tulman, *et al.*, 2000; Alfonso, *et al.*, 2001; Kingham, *et al.*, 2001; Izumiya, *et al.*, 2001). HVT also has thirteen genes which have no homologs within MDV1, many of which are located within the repeat regions of the virus, including a *Bcl-2* homolog found in HVT, but not reported in any other alphaherpesvirus (Alfonso, *et al.*, 2001). Transcriptional regulation of MDV genes proceeds in a cascade-type fashion with transcription of immediate early (IE) genes first, followed by early (E) genes, and then late (L) gene transcription. MDV IE genes, which do not require prior viral protein synthesis, activate expression of E and L genes. E genes

are transcribed prior to viral DNA replication, and L genes are transcribed only after viral DNA replication (Maray, *et al.*, 1988).

Other Herpesviruses:

Herpes Simplex Virus type 1 (HSV-1) is an alphaherpesvirus which infects human ganglia productively, can establish a latent infection to persist within the cell, and reactivate from latently infected cells. There is a family of HSV-1 latency associated transcript(s) (LAT) of 1.5, 2, 8.5, and 10.4 kb, which map antisense to the immediate-early viral genes ICP0 and ICP34.5 in the long inverted repeats (Croen, *et al.*, 1987; Rock, *et al.*, 1987; Spivack and Fraser, 1987; Steiner, *et al.*, 1988; Stevens, *et al.*, 1987; Gordon, *et al.*, 1988). The two smaller LAT are spliced from the two larger LAT, and the smaller LAT are more abundant during latency of HSV-1. HSV-1 LAT are necessary for efficient reactivation of HSV-1 from latency, but are not required for the establishment or maintenance of latency (Rock, *et al.*, 1987; Stevens, *et al.*, 1987; Perng, *et al.*, 1994; Javier, *et al.*, 1988; Sedarati, *et al.*, 1989). In addition to a role in reactivation, HSV-1 LAT may aid in the establishment of latency by inhibition of apoptosis. Apoptosis occurred in trigeminal ganglia infected with a LAT negative HSV-1, but to a lesser extent with a LAT positive HSV-1. *In vitro*, a plasmid expressing HSV-1 LAT blocked apoptosis in primary human lung cells, monkey kidney cells, and murine neuroblastoma cells (Perng, *et al.*, 2000). The ability to protect more neurons from apoptotic death during HSV-1 infection may allow HSV-1 to establish latency in more neurons (Perng, *et al.*, 2000). An additional factor which may affect HSV-1 latency is the stability of a putative stem-loop hairpin mapping to the 3' end of the 2kb LAT intron. Mutagenesis of

this region confirmed the importance of this stem-loop for stability of the LAT. A similar hairpin-loop structure was predicted for the 2.3kb LAT of HSV-2 (Krummenacher, et al., 1997).

Herpes Simplex Virus type 2 (HSV-2) is an alphaherpesvirus with many similarities to HSV-1. The upstream untranscribed promoter regions of the LAT are highly conserved between HSV-1 and HSV-2 (Krause, *et al.*, 1991; McGeoch, *et al.*, 1991). A family of HSV-2 LAT of 2.2 and 8-9 kb in length have been identified and, like HSV-1, map antisense to the ICP0 gene in the long repeat regions (Suzuki and Martin, 1989; Mitchell, *et al.*, 1990; Croen, *et al.*, 1991; Krause, *et al.*, 1991). ICP0, an immediate early viral protein, positively and negatively transregulates viral gene transcription. Like HSV-1, the LAT for HSV-2 appear to be involved in reactivation from latency (Krause, *et al.*, 1995). Replacing the HSV-2 LAT region with that of the HSV-1 LAT region caused the recombinant virus to have reactivation characteristics like that for HSV-1 (an increase in ocular and facial/oral lesions). Reversion of that mutant back to the wild type HSV-2 restored native HSV-2 reactivation characteristics (recurrent genital lesions), suggesting that the pattern of reactivation from latency for each virus was LAT specific (Yoshikawa, *et al.*, 1996).

Varicella-zoster virus (VZV) is an alphaherpesvirus which primarily infects humans. Unlike other alphaherpesviruses, VZV latency is maintained in the satellite cells next to ganglia and has several regions which are transcriptionally active during a latent infection (Croen, *et al.*, 1988). Another unique feature of VZV latency is the lack of any HSV LAT homolog within its genome. Instead, VZV genes 21, 29, 62, and 63 are expressed in latent human trigeminal ganglia (Cohrs, *et al.*, 1996). VZV gene 62 is an

immediate early phosphoprotein which enhances the infectivity of transfected VZV DNA, probably by activating transcription of viral proteins (Cohrs, *et al.*, 1996). VZV gene 63 is an immediate-early protein which has both transactivation and repression characteristics: it activates transcription of early viral proteins and represses the transcription of VZV gene 63 (Cohrs, *et al.*, 1996). VZV gene 29 is an early protein which can bind single-stranded DNA, and VZV gene 21 function is unknown , but may encode a nucleocapsid protein (Cohrs, *et al.*, 1996; Xia and Straus, 1999). A putative latent protein (open reading frame 63) was detected in the cytoplasm of VZV latently infected human ganglia (Mahalingam, *et al.*, 1996).

Cytomegalovirus (CMV) is a betaherpesvirus which infects humans and can exist in a latent state within bone marrow-derived hematopoietic cells. CMV encodes latent transcripts that are spliced (sense) and unspliced (antisense) to the *ie*1/*ie*2 (Kondo and Mocarski, 1995). The sense latent CMV transcripts use novel start sites compared to the productive transcripts, thus differing from them significantly, and may encode a novel protein of 94 amino acids (aa) (Kondo, *et al.*, 1996). The antisense latent CMV transcripts may encode novel proteins of 154 and 152 aa (Kondo, *et al.*, 1996). Finally, antibodies to the 94aa and 152aa predicted CMV proteins were detected in the serum of CMV seropositive individuals, providing evidence for the expression of these latent proteins *in vivo* (Kondo, *et al.*, 1996).

Epstein-Barr virus (EBV) is a gammaherpesvirus which has extensive transcription of the genome during latency. Viral genes expressed during EBV latency include 6 nuclear proteins: Epstein Barr nuclear antigen (EBNA) –1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-LP, and latent membrane protein (LMP). Also expressed are two

polyadenylated transcripts thought to encode membrane proteins LMP-2A and LMP-2B, and two highly expressed abundant small nuclear RNA-like EBV RNAs (EBERS) (Bornkamm and Hammerschmidt, 2001). The genomic structure of these herpesviruses are compared in figure 1-3.

MDV Latency:

Similar to other alphaherpesviruses, MDV can infect their host productively and persist in a latent state, but fully infectious virions are only produced within the chicken feather follicle epithelium (Calnek and Witter, 1984). It is unclear if latency occurs concurrently, or is a prerequisite to transformation in cells infected by MDV1 (Wen, *et al.*, 1988; Calnek and Witter, 1991). LAT appear to map to the short and long repeat regions in both MDV1 and HVT (Sugaya, *et al.*, 1990; Holland and Silva, 1999). A putative MDV1 LAT antisense to ICP4 has been identified (Cantello, *et al.*, 1994; Li, *et al.*, 1994). Two small MDV1 RNAs (nonpolyadenylated) and a ~10kb RNA (mostly polyadenylated) are spliced, and appear to overlap the putative start site for ICP4 (Cantello, *et al.*, 1997). Cyclohexamide was used in experiments to show that expression of the ~10.6kb LAT RNA required protein synthesis, suggesting it may be categorized as a late protein of MDV1 (Li, *et al.*, 1998).

Turkey Herpesvirus Latency:

HVT latency has been defined as the presence of the viral genome in the absence of HVT late gene expression (Holland, *et al.*, 1996; Holland, *et al.*, 1998). HVT latency was assessed at 21, 28, 35, 70, and 105 days post infection (Holland, *et al.*, 1998). At

105 days post HVT infection, a late gene antigen, glycoprotein B (gB), was not detected in the chicken spleen or thymus using indirect immunofluorescence, but the HVT genome was detected in the same cells by *in situ* hybridization (Holland, *et al.*, 1998). Transcription of the latent HVT genome was also studied and mapped to the *Bam*HI F, G, I, and O fragments (TR_L, IR_L, IR_S, TR_S, and U_L regions) (Holland and Silva, 1999) (Figure 1-2).

Summary:

Despite the availability of vaccines against Marek's disease virus, it remains a significant pathogen in the poultry industry. HVT is a substantial part of vaccine defense against oncogenic MDV strains, although the gene(s) responsible for that protection still remain elusive. Latency plays an important role in the life cycle of other herpesviruses, but little is known about the role of latency in either MDV1 or HVT. Since latency occurs simultaneously with transformation in MDV1, it is difficult to ascertain the function of MDV1 LAT transcripts isolated thus far. The MDV1 LAT are found clustered in a single region, similar to HSV-1 and HSV-2. In contrast, evidence thus far suggests that HVT LAT are transcribed from several different regions, similar to EBV. It will be interesting to find out if these different patterns of latent transcription between MDV1 and HVT are in any way linked to the ability of MDV1 to transform and produce T cell lymphomas, while HVT lacks these characteristics. The goal of this research was to allow isolation and characterization of LAT transcripts which may be involved in HVT latency.

APPENDIX:
Figures for Chapter 1

Figure 1-1: MDV genome by serotype. MDV1: strain MD5 (Tulman, *et al*, 2000); MDV2: strain HPRS24 (Izumiya, *et al*, 2001); HVT: strain FC-126 (Alfonzo, *et al.*, 2001, Kingham, *et al.*, 2001). Scale: 1cm = 10kilobases.

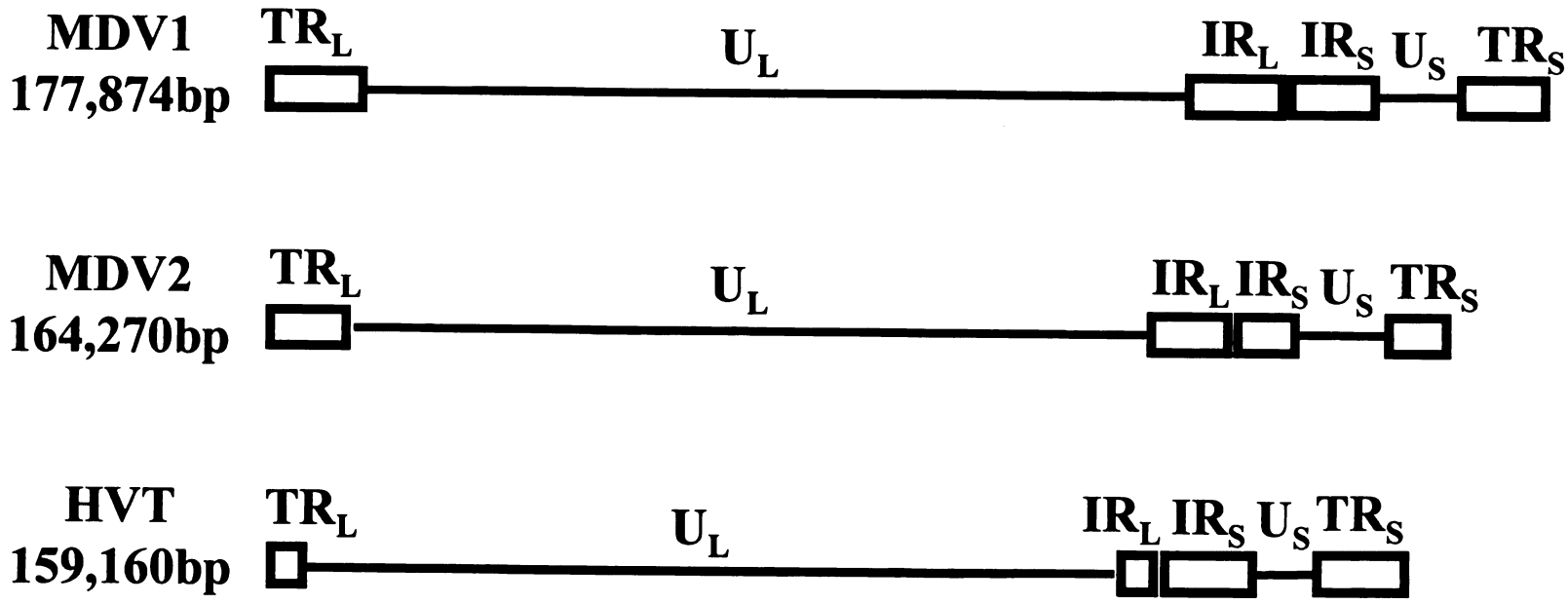
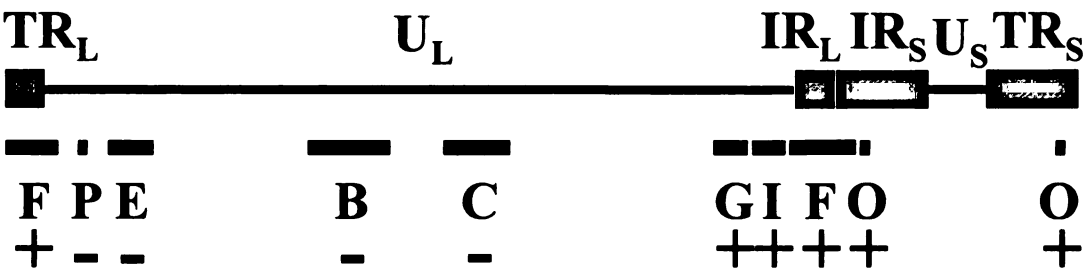

MDV1
177,874bp
TRL
UL
IRL
IRS
US
TRS
MDV2
164,270bp
TRL
UL
IRL
IRS
US
TRS
HVT
159,160bp
TRL
UL
IRL
IRS
US
TRS

Figure 1-2: Transcriptional activity of latent HVT genome as determined by *in situ* hybridization. Fragments underlining the genome are HVT *Bam*HI digestion products (Holland, *et al*, 1998; Holland, Silva, 1999; Holland, unpublished data).



+ = Hybridized to RNA in HVT latently infected tissues

- = Did not hybridize to RNA in HVT latently infected tissues

Figure 1-3: Comparison of the generalized genomic structure of Marek's disease virus, Herpes Simplex virus-1, Varicella-Zoster virus, Cytomegalovirus, and Epstein-Barr virus. (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome; Fields, *et al.*, 1996)

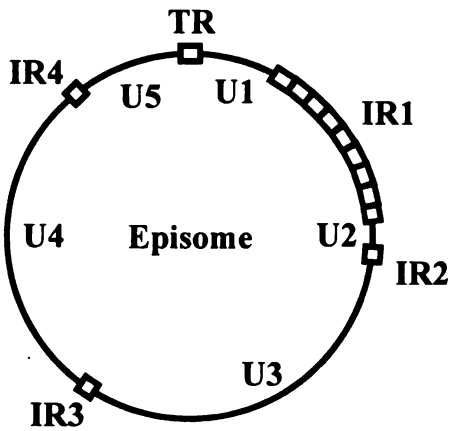
MDV1: 177,874bp

HSV-1: 152,261bp

VZV: 124,884bp

CMV: 229,354bp

EBV: 172,281bp



REFERENCES:

Alfonso, C.L., E.R. Tulman, Z. Lu, L. Zsak, D.L. Rock, G.F. Kutish. The genome of turkey herpesvirus. J. Virology, **75**(2):971-978. 2001.

Biggs, P.M., L.N. Payne. Transmission experiments with Marek's disease (fowl paralysis). Letters to the Editor, Vet Rec, **75**:177-79. 1963.

Bornkamm, G.W., W. Hammerschmidt. Molecular virology of Epstein-Barr virus. Phil Trans R Soc Lond B, p. 437-459. 2001.

Calnek, B.W., H.K. Adldinger, D.E. Kahn. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Disease, **24**: 219-233. 1970.

Calnek, B.W., R.L. Witter. Marek's Disease. p. 325-360. In M.F. Hofstad, H.J. Barns, B.W. Calnek, W.M. Reid, and H.W. Yoder, Jr (ed.) Diseases of Poultry, 8th ed. Iowa State University Press, Ames. 1984.

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

Calnek, B.W., R.L. Witter. Marek's Disease. p. 342-385. In B.W. Calnek, H.J. Barnes, C.W. Reid, H.W. Yoder, Jr (ed.) Diseases of Poultry, 9th ed. Iowa State University Press, Ames. 1991.

Cantello, J.L., A.S. Anderson, R.W. Morgan. Identification of latency-associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. J. Virology, **68** (10):6280-90. 1994.

Cantello, J.L., M.S. Parcells, A.S. Anderson, R.W. Morgan. Marek's disease virus latency-associated transcripts belong to a family of spliced RNAs that are antisense to the ICP4 homolog gene. J. Virology, **71**(2):1351-61. 1997.

Cebrian, J., C. Kaschka-Dierich, N. Berthelot, P. Sheldrick. Inverted repeat nucleotide sequences in the genomes of Marek's disease virus and the herpesvirus of the turkey. Proc Natl Acad Sci, USA, **79**(2):555-8. 1982.

Churchill, A.E., P.M. Biggs. Agent of Marek's disease in tissue culture. Nature, **215**:528-30. 1967.

Churchill, A.E., R.C. Chubb, W. Baxendale. 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 Virology, **4**:557-564. 1969a.

Churchill, A.E., L.N. Payne, R.C. Chubb. Immunization against Marek's Disease using a live attenuated virus. Nature, **221**:744-47. 1969b.

Cohrs, R.J., K. Sprock, M.B. Barbour, G. Owens, R. Mahalingham, M.E. Devlin, M. Wellish, D.H. Gilden. Varicella-Zoster virus (VZV) transcription during latency in human ganglia: construction of a cDNA library from latently infected human trigeminal ganglia and detection of a VZV transcript. J. Virology, **68**(12):7900-08. 1994.

Cohrs R.J., M. Barbour, D.H. Gilden. Varicella-zoster virus (VZV) transcription during latency in human ganglia: detection of transcripts mapping to genes 21, 29, 62, and 63 in a cDNA library enriched for VZV RNA. J. Virology, **70**(5):2789-96. 1996.

Croen K.D., J.M. Ostrove, L.J. Dragovic, J.E. Smialek, S.E. Straus. Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene "anti-sense" transcript by in situ hybridization. N Engl J. Med, **317**(23):1427-32. 1987.

Croen K.D., J.M. Ostrove, L.J. Dragovic, S.E. Straus. 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**(24):9773-7. 1988.

Croen, K.D., J.M. Ostrove, L. Dragovic. Characterization of herpes simplex virus type 2 latency-associated transcription in human sacral ganglia and in cell culture. J. Infect Dis, **163**:23-28. 1991.

Fields, B.N., D.M. Knipe, P.M. Howley. Fields virology. p. 2221-2585. In R.M. Chanock, J.L. Melnick, T.P. Monath, B. Roizman, S.E. Straus (ed.) Fields virology, v. 2, third edition. Lippincott-Raven, Philadelphia. 1996.

Gibbs, C.P., K. Nazerian, L.F. Velicer, H.J. Kung. Extensive homology exists between Marek disease herpesvirus and its vaccine virus, herpesvirus of turkeys. Proc Natl Acad Sci, USA, **81**(11):3365-9. 1984.

Gordon Y.J., B. Johnson, E. Romanowski, T. Araullo-Cruz. RNA complementary to herpes simplex virus type 1 ICP0 gene demonstrated in neurons of human trigeminal ganglia. J. Virology, **62**(5):1832-5. 1988.

Holland, M.S., C.D. Mackenzie, R.W. Bull, R.F. Silva. A comparative study of histological conditions suitable for both immunofluorescence and *in situ* hybridization in the detection of herpesvirus and its antigens in chicken tissues. J. Histochem Cytochem, **44**(3):259-265. 1996.

Holland, M.S., C.D. Mackenzie, R.W. Bull, R.F. Silva. Latent turkey herpesvirus infection in lymphoid, nervous and feather tissues of chickens. Avian Diseases, **42**:292-9. 1998.

Holland, M.S., R.F. Silva. Tissue-specific restriction of latent turkey HVT transcription. Acta Virol, **43**(2-3):148-51. 1999.

Igarashi, T., M. Takahashi, J. Donovan, J. Jesip, M. Smith, K. Hirai, A. Tanaka, M. Nonoyama. Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. Virology, **157**:351-8. 1987.

Izumiya, Y., H.-K. Jang, M. Ono, T. Mikami. A complete genomic DNA sequence of Marek's disease virus type 2, strain HPRS24. Curr Top Microbiol Immunol, **255**:191-221. 2001.

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

Jeurissen, S.H.M., E.M. Janse, F. Wagenaar, G.F. de Boer. The role of splenic ellipsoid-associated reticulum cells in the pathogenesis of Marek's disease. p. 211-15. In: de Boer, G.F., S.H.M. Jeurissen (ed.) Proceedings of the 4th international symposium on Marek's disease. Ponsen and Looijen, Wadeningen. 1992.

Kingham, B.F., V. Zelnik, J. Kopacek, V. Majerciak, E. Ney, C. Schmidt. The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses. J. Gen Virology, **82**:1123-1135. 2001.

Kondo, K., E.S. Mocarski. Cytomegalovirus latency and latency-specific transcription in hematopoietic progenitors. Scand J. Infect Dis Suppl, **99**:63-67. 1995.

Kondo, K., J. Xu., E.S. Mocarski. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proc Natl Acad Sci, USA, **93**:11137-11142. 1996.

Krause P.R., J.M. Ostrove, S.E. Straus. The nucleotide sequence, 5' end, promoter domain, and kinetics of expression of the gene encoding the herpes simplex virus type 2 latency-associated transcript. J. Virology, **65**(10):5619-23. 1991.

Krause, P.R., L.R. Stanberry, N. Bourne, B. Connelly, J.F. Kurawadwala, A. Patel, S.E. Straus. Expression of the herpes simplex virus type 2 latency-associated transcript enhances spontaneous reactivation of genital herpes in latently infected guinea pigs. J. Exp Med, **181**:297-306. 1995.

Krummenacher, C., J.M. Zabolotny, N.W. Fraser. Selection of a nonconsensus branch point is influenced by an RNA stem-loop structure and is important to confer stability to the herpes simplex virus 2-kilobase latency-associated transcript. J. Virology, **71**(8):5849-5860. 1997.

Lee, L.F., E.D. Kieff, S.L. Bachenheimer, B. Roizman, P.G. Spear, B.R. Burmester, K. Nazerian. Size and composition of Marek's Disease Virus Deoxyribonucleic Acid. J. Virology, 7:289-94. 1971.

Lee, L.F., P. Wu, D. Sui, D. Ren, J. Kamil, H.J. Kung, R.L. Witter. The complete unique long sequence and the overall genomic organization of GA strain of Marek's disease virus. Proc Natl Acad Sci, USA, **97**(11):6091-96. 2000.

Li, D.S., J. Pastorek, V. Zelnik, G.D. Smith, L.J. Ross. Identification of novel transcripts complementary to the Marek's disease virus homologue of the ICP4 gene of herpes simplex virus. J. Gen Virology, **75**(7):1713-22. 1994.

Li, D., G. O'Sullivan, L. Greenall, G. Smith, C. Jiang, N. Ross. Further characterization of the latency-associated transcription unit of Marek's disease virus. Arch Virology, **143**(2):295-311. 1998.

Mahalingam R., M. Wellish, R. Cohrs, S. Debrus, J. Piette, B. Rentier, D.H. Gilden. Expression of protein encoded by varicella-zoster virus open reading frame 63 in latently infected human ganglionic neurons. Proc Natl Acad Sci, USA, **93**(5):2122-4. 1996.

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

Marek, J. Multiple Nervenentzundung (Polyneutitis) bei Huhnern. Dtsch Tieraerztl Wochenschr, **15**:417-421. 1907.

McGeoch, D.J., S.L. Cunningham, G. McIntyre, A. Dolan. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. J. Gen Virology, **72**:3057-3075. 1991.

Mitchell, W.J., S.L. Deshmane, A. Dolan, D.J. McGeoch, N.W. Fraser. Characterization of herpes simplex virus type 2 transcription during latent infection of mouse trigeminal ganglia. J. Virology, **64**(11):5342-8. 1990.

Nazerian, K., B.R. Burmester. Electron microscopy of a herpesvirus associated with the agent of Marek's disease in cell culture. Cancer Res, **28**:2454-62. 1968.

Nazerian, K., J.J. Solomon, R.L. Witter. Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc Soc Exptl Biol Med, **127**:177-182. 1968.

Ono, M., R. Katsuragi-Iwanaga, T. Kitazawa, N. Kamiya, T. Horimoto, M. Niikura, C. Kai, K. Hirai, T. Mikami. The restriction endonuclease map of Marek's disease virus (MDV) serotype 2 and collinear relationship among three serotypes of MDV. Virology, **191**(1):459-63. 1992.

Perng, G.C., E.C. Dunkel, P.A. Geary, S.M. Slanina, H. Ghiasi, R. Kaiwar, A.B. Nesburn, S.L. Wechsler. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient *in vivo* spontaneous reactivation of HSV-1 from latency. J. Virology, **68**(12):8045-55. 1994.

Perng, G.C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S.M. Slanina, F.M. Hofman, H. Ghiasi, A.B. Nesburn, S.L. Wechsler. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science, **287**:1500-1503. 2000.

Purchase, H.G. Clinical disease and its economic impact. p. 17-42. In LN Payne (ed.) Marek's Disease (Scientific basis and methods of control). Martinus Nijhoff Publishing, Boston. 1985.

Rock, D.L., A.B. Nesburn, H. Ghiasi, J. Ong, T.L. Lewis, J.R. Lokensgard, S.L. Wechsler. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virology, **61**(12):3820-6. 1987.

Schat, K.A., B.W. Calnek. Characterization of an apparently nononcogenic Marek's disease virus. J. Natl Cancer Inst, **60**(5):1075-1082. 1978.

Schat, K.A., Z. Xing. Specific and nonspecific immune responses to Marek's disease virus. Developmental and Comparative Immunology, **24**:201-221. 2000.

Sedarati, F., K.M. Izumi, E.K. Wagner, J.G. Stevens. Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. J. Virology, **63**:4455-4458. 1989.

Sevoian, M., D.M. Chamberlain. Experimental reproduction of the ocular form. Vet Med, **57**:608-9. 1962.

Solomon, J.J., R.L. Witter, K. Nazerian, B.R. Burmester. Studies on the etiology of Marek's disease. Proc Soc Exp Biol Med, **127**:173-77. 1968.

Spivack, J.G., N.W. Fraser. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virology, **61**(12):3841-7. 1987.

Steiner, I., J.G. Spivack, D.R. O'Boyle II, E. Lavi, N.W. Fraser. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virology, **62**:3493-96. 1988.

Sugaya, K., G. Bradley, M. Nonoyama, A. Tanaka. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virology, **64**(12):5773-82. 1990.

Suzuki S., J.R. Martin. Herpes simplex virus type 2 transcripts in trigeminal ganglia during acute and latent infection in mice. J. Neurol Sci, **93**(2-3):239-51. 1989.

Tulman, E.R., C.L. Afonso, Z. Lu, L. Zsak, D.L. Rock, G.F. Kutish. The genome of a very virulent Marek's disease virus. J. Virology, **74**(17):7980-7988. 2000.

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

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

Wen, L.T., A. Tanaka, M. Nonoyama. Identification of Marek's disease virus nuclear antigen in latently infected lymphoblastoid cells. J. Virology, **62**(10):3764-71. 1988.

Wilson, M.R., P.M. Coussens. Purification and characterization of infectious Marek's Disease virus genomes using pulsed field electrophoresis. Virology, **185**:673-80. 1991.

Witter, R.L., J.J. Solomon, G.H. Burgoyne. Cell culture techniques for primary isolation of Marek's disease-associated herpesvirus. Avian Diseases, **1**:101-118, 1969.

Witter, R.L. Increased virulence of Marek's disease virus field isolates. Avian Diseases, **41**(1): 149-63. 1997.

Witter, R.L. Control strategies for Marek's disease: a perspective for the future. Poultry Science, **77**(8):1197-203. 1998.

www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome

Xia, D., S.E. Straus. Transcript mapping and transregulatory behavior of varicella -zoster virus gene 21, a latency-associated gene. Virology, **258**(2):304-13. 1999.

Yoshikawa, T., J.M. Hill, L.R. Stanberry, N. Bourne, J.F. Kurawadwala, P.R. Krause. The characteristic site-specific reactivation phenotypes of HSV-1 and HSV-2 depend upon the latency-associated transcript region. J. Exp Med, **184**:659-664. 1996.

CHAPTER 2

TOWARD ISOLATION OF LATENT TURKEY HERPESVIRUS TRANSCRIPTS

Abstract:

A common feature of all herpesviruses is the ability to produce a latent infection in which viral antigens are not detectable on herpesvirus infected cells. Latent turkey herpesvirus (HVT) infections have been characterized by the presence of the HVT genome with the absence of late gene expression, such as glycoprotein B (gB). Previous studies had shown that latent HVT transcripts could be detected in latent HVT chicken spleen at 105 days post-inoculation (dpi) by *in situ* hybridization, but gB could not be detected by immunohistochemistry in those same lymphoid cells (Holland, *et al.*, 1998). We hypothesized that HVT latency associated transcripts (LAT) could be isolated from multiple regions of the HVT genome for characterization from a cDNA phage library constructed from a HVT latently infected spleen at 105 dpi. To accomplish this goal, a cDNA library was constructed from messenger ribonucleic acid (mRNA) isolated from chicken spleen latently infected with HVT. With standard phage plaque colony lift procedures, initial attempts to isolate HVT LAT clones from the library encountered a high background of clones that were not homologous to HVT. A polymerase chain reaction (PCR) method was then used with HVT-homologous and either M13 forward or reverse oligonucleotides to amplify HVT transcripts from the latent cDNA library. The PCR products were screened by Southern blot using HVT genomic probes. Several putative LAT clones were isolated from latent cDNA sublibraries, but Southern analysis suggested they were not HVT LAT clones.

Materials and Methods

Gel electrophoresis: Standard electrophoresis techniques were used (Sambrook, *et al.*, 1989).

Southern blot: After separation of the DNA samples by gel electrophoresis, the upper left corner of the gel was removed for blot orientation, and the DNA was denatured by soaking the gel twice in 0.4N NaOH for 15 minutes. Hybond:N membrane (Amersham, Piscataway, NJ) and 3mm Whatman paper were cut to the size of the gel and soaked in 0.4N NaOH for 15 minutes. Following standard Southern capillary transfer methods for at least 12 hours, the membrane was dried at 70C for 15 minutes, and the DNA was cross-linked to the membrane with the autocrosslink function of a Stratalinker 1800 UV crosslinker (Stratagene, LaJolla, CA). After Southern blotting, the gel was stained with ethidium bromide to confirm the DNA had transferred out of the gel.

Southern hybridization: Each Southern membrane was prehybridized for one hour at 65C in hybridization solution [1mM EDTA, 0.5M Sodium Phosphate, 100ug/ml sheared salmon sperm DNA, and 7% SDS] to barely cover the membrane in a Techne hybridizer HB-1D rollerbottle oven. Following at least 12 hours of hybridization in the hybridization solution + denatured probe at 65C, the membrane was washed twice for 15 minutes at 65C in wash solution A [1mM EDTA, 40mM Sodium Phosphate (pH7.2), 5% SDS] followed by three 15 minute washes at 65C in wash solution B [1mM EDTA, 40mM Sodium Phosphate, (pH7.2), 1% SDS].

Probe labeling: The standard protocols for the NEBlot^{R} phototope kit [New England Biolabs, Beverly (NEB)] were followed. 500ng of template DNA was denatured at 100C for 5 minutes, followed by immediate quenching on ice. The kit labeling mix, dNTPs, and 3' to 5' exonuclease-negative Klenow enzyme were added to the denatured template DNA and placed at 37C for 6 hours. The reaction was terminated with 5ul of 0.2M EDTA (pH 8.0) and the probe was precipitated by adding 5ul 4M LiCl, 150ul ethanol, and incubating at –70C for 30 minutes. The probe was spun at 14,000 RPM in a microcentrifuge, washed with 70% ethanol, respun, briefly dried in a speedvac, resuspended in 80ul of sterile water at approximately 20ng/ul concentration, and stored at –20C.

Fluorescent Southern development: The standard protocol for the NEB Phototope^{R}-Star detection kit were followed (New England Biolabs, Beverly, MA). Immediately following the Southern membrane washing, the membrane was covered in approximately 1cm of blocking solution [5% SDS, 25mM Sodium Phosphate, (pH 7.2)] and shaken for 5 minutes. All steps of the development were performed at room temperature with extensive shaking. Streptavidin was bound to the biotinylated probe DNA in blocking solution at a concentration of 10ug/ml, and the membrane + solution was shaken for 5 minutes. The membrane was washed twice for 5 minutes in 200ml of 1:10 diluted (in water) blocking solution. Biotinylated alkaline phosphatase was bound to the biotinylated probe DNA in 1 ml blocking solution/cm^2 of membrane at a concentration of 0.5ug/ml and the membrane + solution was shaken for 5 minutes. The membrane was then washed twice in 200ml 1:10 diluted (in water) wash solution II (100mM Tris-HCL,

100mM NaCl, 10mM $MgCl_2$, pH 9.5). The CDP-Star assay buffer was diluted 1:25 in water and 2.5ml/100cm^2 was added to the membrane in a sealed bag with 25ul CDP-Star/100cm^2 and shaken for 5 minutes. The solution and all air was removed, and the bag resealed for exposure to Kodak scientific imaging film.

Northern blot: The method of Hodge (1994) was followed. In short, 1.3g of agarose was dissolved into 95ml of 1x MOPS [0.02M 3-(N-morpholino) propanesulfonic acid sodium salt, 9mM sodium acetate, 1mM EDTA disodium salt, pH 7.0]. The solution was cooled to 50C, 5ml 37% formaldehyde solution was added, and the gel was poured. Total RNA (10ug) was thawed on ice and 4 volumes of RNA sample buffer (500ul formamide, 100ul 10x MOPS, 150ul 37% formaldehyde, 100ul glycerol, 100ul 1% bromophenol blue) were added. The sample was placed at 65C for 10 minutes, ice quenched, and spun briefly in a microcentrifuge. The gel was prepared for sample loading by adding 1x MOPS as running buffer and electrophoresed at 50V, to allow 75% migration of the bromophenol blue dye. The gel was then photographed, soaked in 0.05M NaOH, 2x SSC for 10 minutes, and soaked twice in 10x SSC for 20 minutes. A standard northern transfer was set up with 10x SSC as transfer buffer and Hybond:N serving as membrane. After transfer, the membrane was allowed to air dry. Cross-linking, prehybridization, hybridization, and detection methods were the same as those described for Southern blots. Note: all solutions were prepared using DEPC-treated water.

Plating cDNA library: Stratagene (La Jolla, CA) ZAP Express™ cDNA synthesis kit protocols were followed. *E coli*, strain XL1-Blue MRF' cells were grown in SM buffer [0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 50mM Tris-HCl (pH 7.5), 0.01% gelatin] to an OD_{600} of 0.5. The desired number of phage to be plated on a 150mm plate (100 to 5 x 10^4) were gently mixed with 200ul of the XL1-Blue MRF' cells and incubated for 15 minutes. To this mixture, 5ml of 48C NZY top agarose (0.8%) was added, swirled, and immediately poured onto a prewarmed (37C) NZY plate (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% NZ amine, 1.5% agar, pH 7.5) to overlay the entire plate. After cooling at room temperature for 10 minutes, the plates were incubated at 37C for 12-16 hours.

Plaque and Colony Lifts: Double colony lifts were performed, and three small identical notches were removed from both membranes for orientation. After a one minute of absorption for the first lift (2 minutes for the second), the membrane was soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 2 minutes. The membrane was transferred to neutralization solution [1.5M NaCl, 0.5M Tris-HCl (pH 7.5)] for one minute, and then into fresh neutralization solution for another minute. After rinsing the membrane for one minute in 2X SSC, the membrane was placed in 1M ammonium acetate, 1X SSC for one minute. The membrane was paper clipped to whatman paper to prevent curling and dried for 10 minutes at 70C. The DNA was cross-linked to the membrane with the autocrosslink function of the Stratagene 1800 UV crosslinker (Stratagene, La Jolla, CA). The membrane was then shaken in Proteinase K solution (2X SSC, 20mg/ml Proteinase K) at 55C for one hour. The Proteinase K solution was neutralized with PMSF solution [2X SSC, 1mM phenylmethylsulfonyl fluoride (PMSF)]

for 15 minutes at room temperature, followed by two 2X SSC rinses for 15 minutes. This Proteinase K treatment was recommended in the nonradioactive Southern blot hybridization protocols (NEB, Beverly, MA), presumably to remove clone host proteins and make the target DNA more accessible to the nonradioactive probe. Southern blot hybridization protocols, as described previously, were then followed.

DNA isolation: For tissues, a small ($0.5cm^2$)frozen section was placed into a sterile petri dish with 2ml digestion buffer [100mM NaCl, 10mM Tris (pH 8.0), 25mM EDTA (pH 8.0), 0.5% SDS] and minced using a sterile razor. Note, all transfers of the DNA sample were performed using a wide-bored pipette tip to minimize DNA shearing. The resulting slurry was transferred into two 1.5ml eppendorf tubes. For tissue culture, the cells (up to 5×10^8) were pelleted and suspended in 2 ml digestion buffer. Proteinase K was added to a final concentration of 50ug/ml and the sample was placed at 50C for 12 hours with occasional gentle shaking by inversion. After digestion, the sample was split into two eppendorf tubes, and extracted twice with phenol:chloroform:isoamyl alcohol (50:49:1), and once with chloroform: isoamyl alcohol (49:1), with mixing for the extraction by tube inversion for 30 seconds. The DNA was precipitated with 2 volumes ethanol at room temperature for 30 minutes, with occasional gentle mixing by inversion. Following pelleting of the DNA by centrifugation at 14K RPM in a microcentrifuge, the sample was gently resuspended in 500ul water, and RNase A was added to a final concentration of 0.2mg/ml. The sample was incubated at 37C for 1 hour and re-extracted as above. The DNA was re-precipitated, spun, dried in a speedvac, and resuspended in

100ul water. The DNA was dissolved into solution by incubation at 65C for at least 30 minutes and quantified by spectrophotometry.

cDNA library DNA isolation: Following overnight growth of the library sample of interest, digestion buffer was added to 700ul of sample to the final concentrations: 10mM Tris (pH 8.0), 5mM EDTA, 0.5% SDS, and 200ug/ml proteinase K, and then incubated 12-16 hours at 56C. The sample was phenol:chloroform:isoamyl alcohol extracted as described for DNA isolation, the DNA was precipitated with 2 volumes of ethanol at –80C for 20 minutes, spun 14K RPM in a microcentrifuge, 70% ethanol washed, respun, dried in a speedvac, resuspended in 200ul water, and quantified by spectrophotometry.

RNA isolation: Life Technologies (Gaithersburg, MD) TRIzol™ LS reagent and protocol were used for total RNA isolation.

Polymerase chain reaction (PCR): To amplify a desired stretch of DNA from a certain template, two opposing oligonucleotides (12.5pmol) were used. The total reaction volume of 25ul contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.1% gelatin, 20pM dNTPs, 1.25 units Sigma (St. Louis) *Taq* DNA Polymerase, and 100ng template DNA. The general amplification cycle employed (with variations in annealing temperature) was 95C for 3 minutes, 30 X [95C, 1 minute; 65C, 1 minute; 72C, 2 minutes], 72C for 10 minutes, and storage at 4C prior to gel electrophoresis.

RT-PCR: Clontech (Palo Alto) 1st-strand™ cDNA synthesis kit was used to synthesize cDNA from total RNA, prior to PCR as previously described.

Chicken embryo fibroblast (CEF) preparation: Twenty fertilized $15I_5$ x 7_I chicken eggs (11 days old) were obtained and sprayed liberally with 70% ethanol. The remainder of the procedure was carried out within a sterile hood environment. Each egg was re-sprayed with 70% ethanol, and a pair of sterile scissors was used to remove the top 1cm of the egg. A second pair of sterile scissors was used to remove the embryo from the egg and decapitate it. The body was placed into a sterile decanter flask containing a sterile stirbar and 150ml 37C phosphate buffered saline (PBS). Both pairs of scissors were re-sterilized in a beaker containing 70% ethanol and cheesecloth to remove any fragments. The scissors were then placed into a beaker with sterile PBS to remove the ethanol. Once all the eggs were processed, the decanter flask was closed and placed onto a stirplate for 5 minutes. The chicken embryos were washed 2-4 times with fresh PBS. Next, 150ml 37C Leibovitz-McCoy media (LM) with 0.05% trypsin was added to the flask. After one minute on the stirplate, the LM + trypsin was decanted in the hood. Then 150ml 37C LM + 0.05% trypsin was added, and the flask was placed onto a stirplate for 5 minutes. The LM + trypsin was similarly decanted into a sterile 50ml conical tube containing an equal volume of sterile LM + 10% calf serum (CS) and placed on ice. More LM + trypsin was added to the flask and this process was repeated until a gelatinous mass remained. Following trypsinization, the CEF were pelleted by centrifugation at 2300 x g for 5 minutes. The pellet was resuspended in 25ml LM + 10% CS, and then gently pipetted and squeezed through 4-6 sterile layers of cheesecloth. This

process was repeated through fresh, sterile cheesecloth. Both cheesecloths were rinsed with additional LM + 10% CS. The CEF were repelleted, resuspended in LM + 10%CS, quantified by microscopy, and placed at 4C.

Results

cDNA library screening:

To isolate latent HVT transcripts, a cDNA library had been created previously in the lab. It had been constructed using mRNA isolated from a chicken spleen latently infected with HVT. The spleen had been removed from a chicken at 105 dpi with HVT and quick frozen in liquid nitrogen. The Zap Express cDNA synthesis and cloning kit was used to make the cDNA library (Stratagene, LaJolla, CA). Briefly, the mRNA was converted into cDNA using 5-methyl dCTP to protect against *Xho* I digestion during directional cloning into a phagemid vector immediately prior to packaging and amplification of the phage library. (Figure 2-1). A cDNA library from CEF productively infected with HVT was also similarly constructed at this time. This productive HVT library would be used to determine if any putative LAT isolated from the latent HVT spleen library appear to be unique to latent spleen infections, or if they can also be isolated from productive HVT infections.

To isolate a phage with the clone of interest from this latent HVT spleen library, the primary cDNA library was titred and plated onto 20 NZY plates at 5×10^4 phage/150mm plate. Single plaque lifts were performed, and the HVT *Bam*HI F, G, I and O genomic regions were used as simultaneous probes in plaque hybridizations to isolate latent HVT transcripts (Figure 2-2). Twenty-one phage plaques were selected and cored from the primary plates. The phage titre was determined for each sample and plated at 5×10^2 phage/150mm plate for secondary screening by colony hybridization. Positive phage plaques were again selected, cored, titred, and plated at 50 phage/100 millimeter

plate for tertiary screening by colony hybridization. Seven isolated phage plaques were cored and converted into phagemid clones. DNA was isolated, analyzed by restriction enzyme analysis, and Southern blot was performed with the same HVT *Bam*HI F, G, I, and O genomic probes as before. Clones ranging in size from approximately 750 to 2400 base pairs mapped to the HVT TR_L and IR_L by colony hybridization. Sequence analysis, however, revealed that the clones were not homologous to HVT, and that the plaque hybridization screening process had a high background problem.

The HVT *Bam*HI F genomic region was selected for use in subsequent colony hybridizations of the latent HVT spleen cDNA library. This part of the HVT genome was chosen as a target area because HVT-infected chicken tissues had more latent transcripts as detected by *in situ* hybridization from this region than the other positive regions (Figure 1-2) (Margo Holland, personal communication). Use of the entire *Bam*HI F genomic region as a probe for plaque hybridizations was not successful in isolating clones homologous to HVT from the latent HVT spleen cDNA library. To reduce the size of the probes used in Southern analysis and to focus on a smaller target area, the HVT *Bam*HI F genomic region was digested with the restriction enzyme *Sma* I and subcloned. These smaller probes were used in Southern analysis of PCR screening methods described below.

The number of unique clones of the cDNA library was estimated at 4×10^7 during construction (Margo Holland, personal communication). If one cell in one hundred were latently infected with HVT in the spleen, the background of other cDNA clones in our library would be significant. Since the ineffectiveness of traditional colony hybridization

methods could be due to the rarity of LAT clone(s) within the cDNA library, a more sensitive method was sought.

PCR screening method for the cDNA library:

Oligonucleotides were designed throughout the HVT *Bam*HI F region for PCR screening of the cDNA library (Figure 2-3). These oligonucleotides were also used in automated sequencing to walk along the HVT *Bam*HI F region and design additional oligonucleotides. This sequence data would also allow us to identify any HVT genes which might exist antisense to LAT, as found in other herpesviruses. Prior to the completion of the sequence of this region, the entire HVT sequence was submitted for publication and made available for our use (Alfonso, *et al.*, 2001; Kingham, *et al.*, 2001).

In a PCR reaction, a single oligonucleotide complementary to the HVT *Bam*HI F genomic region was used in conjunction with a oligonucleotide homologous to the library phagemid vector (m13 forward or reverse primer), with the latent HVT cDNA phagemid library serving as template (Figure 2-4) (Cohrs, *et al.*, 1994). A similarly constructed productive HVT cDNA library and CEF genomic DNA served as negative controls. To assess the sensitivity and determine the possible ability of this PCR method to detect a single clone in our cDNA library, a serial dilution was performed with target gB DNA into a Lambda DNA background. The gB PCR amplification products were visible by ethidium bromide staining of the electrophoresed products in the 10fg dilution lane (Figure 2-5), which corresponded to approximately 1200 gB molecules. The 100ng of lambda DNA in that lane corresponded to approximately 1.9×10^9 lambda molecules. Since we were able to detect an approximate 1.5×10^6 dilution of gB into the lambda, our

clone of interest in our latent spleen cDNA library of ~4 x 10^7 unique clones would need to be present at greater than 27 copies to be visually detected by this method.

The oligonucleotide designated H12 was used in conjunction with the m13 forward oligonucleotide in PCR, and the amplification products were analyzed by Southern blot with the 731bp *Sma* I subfragment as probe. A fragment was amplified from our latent HVT cDNA library and hybridized to the Southern probe, but was not seen in either our productive cDNA library, or from CEF genomic DNA (Figure 2-6). The PCR amplification product from the latent HVT cDNA template was cloned and sequenced to determine that the product was 405bp and identical to the HVT *Bam*HI genomic region (Figure 2-7).

To enrich for our clone of interest, 1 x 10^7 phage were divided into 20 tubes. After 4 hours of partial amplification, samples from each tube were removed and frozen at –80C in multiple aliquots for future isolations. Following overnight growth of the remaining culture, DNA was isolated from the samples, and PCR with the H12 and m13 forward primers was used to identify the sublibrary pool(s) containing the LAT clone(s) of interest (Figure 2-8). Southern blot analysis with the *Bam*HI F 731base pair (bp) *Sma* I subclone as probe of the primary (Figure 2-9) and secondary sublibraries (Figure 2-10) identified the cDNA library pools with the clone(s) of interest. Following enrichment of the LAT clone of interest to a background of about 2000 unique clones, traditional double colony lift methods were used to isolate several clones. The pure clones were converted into phagemid and screened by Southern analysis as before. Although the clone of interest was hybridized by the probe in Southern analysis of the double colony lifts, it was not homologous in a Southern blot of the restriction digested phagemid clone DNA.

Of concern was an amplification product from CEF genomic DNA sample (Figure 2-10; Figure 2-11). Several unsuccessful attempts were made to re-amplify the product from CEF genomic DNA, under the same PCR conditions, to allow cloning and analysis.

It was thought at this time that perhaps the m13 forward oligonucleotide might not be specific enough to isolate the HVT LAT clone, and the H40, H41 and H42 oligonucleotides were designed. Nested PCR was pursued to obtain a PCR-amplifiable product visible to the naked eye following gel electrophoresis without the need for Southern analysis. Figure 2-11 shows the product of nested PCR with the H41/H42 oligonucleotides following the initial PCR amplification with the H12/H40 oligonucleotides. Unfortunately, nested PCR did not increase the specificity of the amplification, and nonspecific products were amplified from all DNA templates. This nonspecific amplification will be pondered in the discussion section.

Chicken experiment:

To allow confirmation *in vivo* of any LAT clone isolated from our cDNA library, thirty-nine chickens, line $15I_5 \times 7_I$ Ab-, were obtained. Twenty-two birds were infected with HVT by intraperitoneal injection at 1 day old with 2000 plaque forming units of HVT FC-126, and placed in Horsfall-Bauer isolators. Seventeen chickens were similarly injected with PBS, and placed in a separate isolators to serve as a negative control. Infection of all HVT-infected chickens, and absence of infection in uninfected chickens was confirmed by cocultivation assay of peripheral blood lymphocytes over CEF. Five chickens from the uninfected and HVT-infected groups were euthanized at day 5, with three chickens from the HVT-infected, and two chickens from the uninfected group at 32,

56, 92, and 119 days post-inoculation (dpi). Immediately following euthanization, one half of the bursa, spleen, thymus, brachial nerve, sciatic nerve, kidneys, heart, and liver were harvested, quick frozen in an ethanol dry ice bath, and stored at –80C to allow isolation of RNA and DNA for use in molecular analysis. The remainder of those same tissues were fixed in 4% paraformaldehyde for 24-48 hours, switched to 70% ethanol, and embedded in paraffin to allow indirect immunohistochemistry.

Analysis of chicken tissues:

To determine if any LAT were expressed *in vivo*, RT-PCR was performed on latent HVT chicken tissue total RNA with the candidate HVT oligonucleotides. To optimize our parameters for RT-PCR, total RNA was isolated from CEF and CEF productively infected with HVT (CEF/HVT). RT-PCR with gB oligonucleotides (Figure 2-12) produced an amplification product in the CEF/HVT RNA sample (no reverse transcriptase), suggesting the presence of DNA contamination. The RNA isolation procedure was modified slightly to avoid DNA contamination (less aqueous sample was removed to avoid disturbance of the interface which could contain DNA). A PCR of this RNA with gB oligonucleotides failed to amplify any DNA contamination (Figure 2-13). RT-PCR with GAPDH oligonucleotides (Figure 2-13) failed to amplify any target DNA in the samples lacking reverse transcriptase, but did amplify in the CEF DNA, U19 with reverse transcriptase (RT), and H19 with RT samples as expected. A similar PCR analysis using GAPDH oligonucleotides with new total RNA sample preparations, however, demonstrated a continuing DNA contamination problem (Figure 2-14). The

use of Rnase-free Dnase I enzyme was pursued as a means of eliminating this DNA contamination, but the process was not completed prior to the end of the project.

Discussion

In hindsight, false paths taken during this research project have become apparent. Double colony lifts must always be performed with as short and specific a probe that is feasible in order to avoid isolation of nonspecific clones. The decision to search sublibrary 11 (figure 2-10) based upon the faint band visible on that blot may have been hasty, and the Southern should have been repeated to confirm the results prior to pursuit of sublibraries containing the clone of interest. It is also important to avoid repetitive sequences within both oligonucleotides and probes to limit nonspecific binding and isolation of nonspecific clones. A possible explanation for the amplification product from CEF genomic DNA in figure 2-10 and 2-11 was formulated when it was determined that the H40 oligonucleotide contained a repetitive sequence. If the H40 oligonucletide can bind to a repetitive sequence within the chicken genome, all that would remain would be for the m13 forward primer to bind nonspecifically within a few thousand base pairs downstream during one of the initial cycles of PCR and a nonspecific PCR product would result. That same repetitive sequence within that amplified product would be homologous to the repetitive sequence present within the HVT probe from that region, resulting in the isolation of nonspecific clones from the cDNA library that were not homologous to HVT. Avoidance of such repetitive sequences should be easy, now that the genomic sequence is available for strains from all three seroptypes of MDV. It yet remains undetermined whether any LAT clone(s) exist within our latent HVT spleen

cDNA library, but future steps should be taken to enrich any such library to the extent possible for HVT-specific clones prior to extensive screening measures. This will probably require the synthesis of additional libraries, but the time, effort, and materials saved should be well worth the investment.

Our current definition of HVT latency consists of detection of the viral genome by *in situ* hybridization with the absence of gB expression in the same cell, as detectable by indirect immunohistochemistry. The sensitivity of these molecular tools may not be the same. PCR of the genome and RT-PCR to detect gB transcription may be more sensitive methods, but there are benefits and difficulties with this approach. While the use of RT-PCR should be more sensitive than indirect immunohistochemistry, RT-PCR would not differentiate a few cells reactivating from latency from the background of latently infected cells. In contrast, a possible benefit of using RT-PCR is that it may allow detection of a few cells reactivating from latency. Also, RT-PCR would not reveal whether that transcript would be successfully translated and the gB product expressed on the HVT-infected cell surface. For the time being, in situ hybridization used in conjunction with indirect immunohistochemistry remains a good way do detect the viral genome without detectable gB expression within a single cell latently infected with HVT.

Even though an HVT LAT has not been isolated at this time, several significant things have been accomplished. *Sma* I subclones of the HVT *Bam*HI F genomic region are available for use as probes to focus our ability to detect the region(s) to which HVT LAT are homologous. Oligonucleotides are available for analysis of the entire HVT *Bam*HI F region of the genome. An enrichment process for cDNA clones of interest has been developed and proven effective for isolation of specific clones from a significant

background population. Several latent HVT cDNA sublibraries are available for screening and isolation of clones of interest. Thymus, spleen, liver, kidneys, bursa, brachial and sciatic nerves are available from an HVT infection time course between 6 and 119 dpi for immediate molecular and immunohistochemical analysis. Once HVT LAT or other genes of interest have been identified, these tissues will prove invaluable for rapid *in vivo* detection of their level, distribution, and time course of expression.

Several future avenues for isolating HVT LAT are possible, and the methods believed most likely to succeed will be presented here first. To search for HVT LAT, RT-PCR can be used with oligonucleotides designed throughout the HVT *Bam*HI F region of the genome in combination with fresh total RNA prepared from the 119dpi thymus and spleen tissues latently infected with HVT. An alternative method for isolation of total RNA, such as traditional guanidium thiocyanate preparations, should eliminate the DNA contamination described here with the Trizol preparations. (Sambrook, *et al.*, 1989; Life Technologies, Gaithersburg, MD). Following amplification of (a) putative LAT, Northern blot analysis and *in situ* hybridization can be used to further characterize the size and tissue localization of the transcripts. Although northern blot analysis may not be sensitive enough to detect (a) latent transcript(s) from a single HVT-infected cell within a background of uninfected cells, it is worth pursuing. *In situ* hybridization has already proved successful in identifying regions of the HVT genome active during a latent infection.

Primer extension could be used to identify the 5' end of a LAT transcript. This would be accomplished using reverse transcriptase to make a cDNA to the end of the transcript from a radiolabeled oligonucleotide. To determine the size of the product, the

end-labeled cDNA would be run on a denaturing polyacrylamide gel in a lane next to a sequencing reaction of the HVT genome with the same oligonucleotide. Once the 5' end of the transcript has been identified, an oligonucleotide can be designed to the 5'end and used in conjunction with an oligo(dT) in RT-PCR to amplify the transcript. The PCR product can then be cloned and sequenced for analysis.

Alternatively, to reduce the number of background clones encountered in the experiments presented here, a new cDNA library enriched for HVT-specific sequences could be created and screened using the PCR method previously described here (Cohrs, *et al.*, 1994). Briefly, repetitive sequences would be removed from the HVT IR_S/TR_S genomic DNA and the DNA would be biotinylated. The biotinylated DNA would be bound to avidin-coated beads and total RNA from latently infected spleen would be passed over the beads to allow specific binding to occur. Extensive washing would remove any nonspecific RNA and the specific RNA would be eluted for use as template to create a cDNA library (Cohrs, *et al.*, 1996). A productive HVT spleen cDNA library of similar construction should also be considered for use as a control for the presence of gB clones and to determine if the LAT clone(s) are present. This biotinylation method permits positive selection for clones of interest, rather than using a subtractive library where the goal is to remove unwanted RNA, but not the clones of interest.

Another possible method would involve *in situ* hybridization using the *Sma* I subclones of the HVT *Bam*HI F region of the genome as probes, to further narrow the region(s) of the genome active during HVT latency. Subcloning of the *Sma* I clones may be warranted here to avoid using any of the repetitive sequences within the *Bam*HI F region as a probe. The HVT *Bam*HI G, I, and O regions of the HVT genome could also

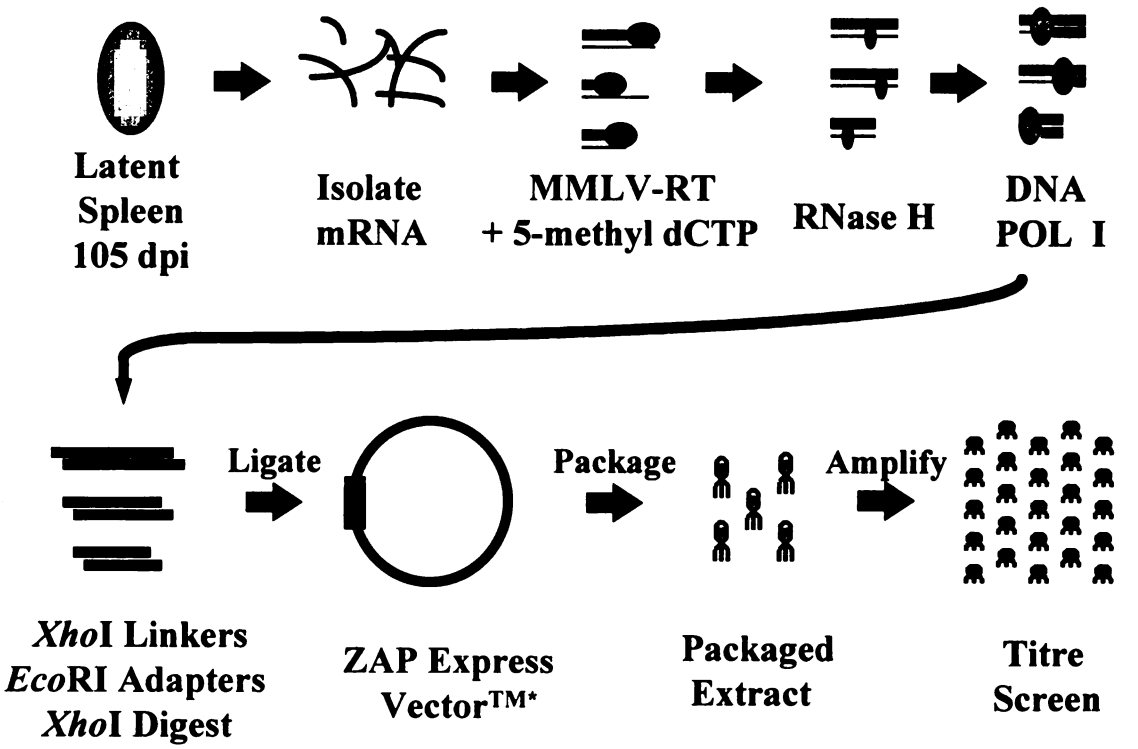
be subcloned and used as probes for *in situ* hybridizations. The 119 dpi latent HVT thymus and spleen tissue samples embedded in paraffin would serve as the target for these experiments vs. uninfected samples as negative controls.

APPENDIX:
Tables and Figures for Chapter 2

Table 2-1: Sequences of frequently used oligonucleotides.

Oligonucleotide	Sequence	Homologous to:
H12	5' AAATACAGGGCGTGTGTGAA	HVT *Bam* HI F
H38	5' CCGTTACCTCTTTCCACTTTGCC	HVT gB
H39	5' AGGCATACAGCTATCCGAAGACG	HVT gB
H40	5' AAACCTAACCACGACTTTTCCCG	HVT *Bam* HI F

Figure 2-1: Methods used to create the cDNA library obtained for isolation of HVT LAT.


Latent
Spleen
105 dpi
Isolate
mRNA
MMLV-RT
+ 5-methyl dCTP
RNase H
DNA
POL I
Ligate
Package
Amplify
XhoI Linkers
EcoRI Adapters
XhoI Digest
ZAP Express
Vector™*
Packaged
Extract
Titre
Screen

* = Stratagene

Figure 2-2: Process used to screen the HVT latent spleen cDNA library.

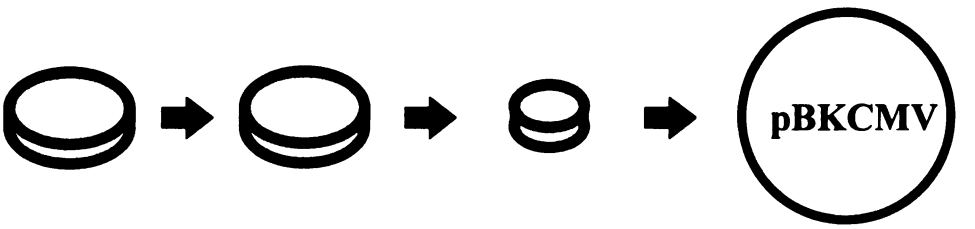
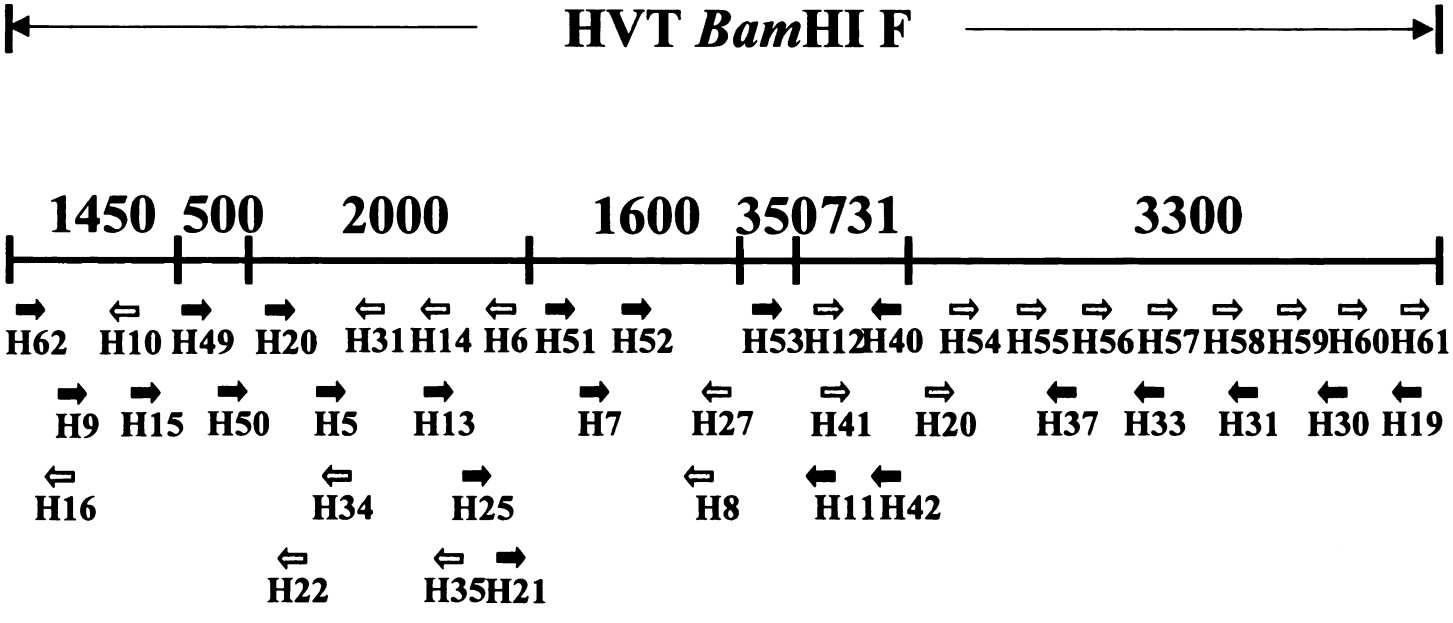

pBKCMV
1° Screening
50,000
Plaques
2° Screening
500
Plaques
3° Screening
50
Plaques
Create
Phagemid
Using
Helper Phage

Figure 2-3: Map of oligonucleotides complementary to the HVT *Bam*HI F genomic region.



| = Sma I restriction site

⬅ = Same strand as the m13 forward oligonucleotide

⇨ = Same strand as the m13 reverse oligonucleotide

Figure 2-4: PCR method used to facilitate isolation of HVT LAT from the cDNA library.

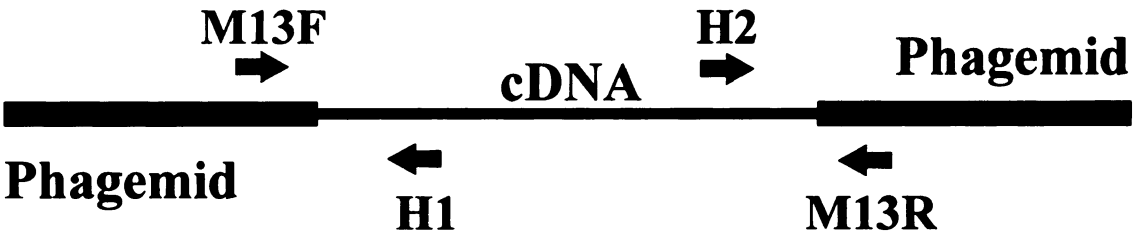

M13F
H2
Phagemid
cDNA
Phagemid
H1
M13R

Figure 2-5: Negative image of PCR products using H38/H39 (gB oligonucleotides) with a template of gB (pBR322 plasmid containing the MDV glycoprotein B clone), diluted in Lambda DNA to assess sensitivity of detection. PCR conditions were: 1x 94C for 3 minutes, 30x (94C for 1 minute, 63C for 1 minute, 72C for 2 minute), 72C for 10 minutes.

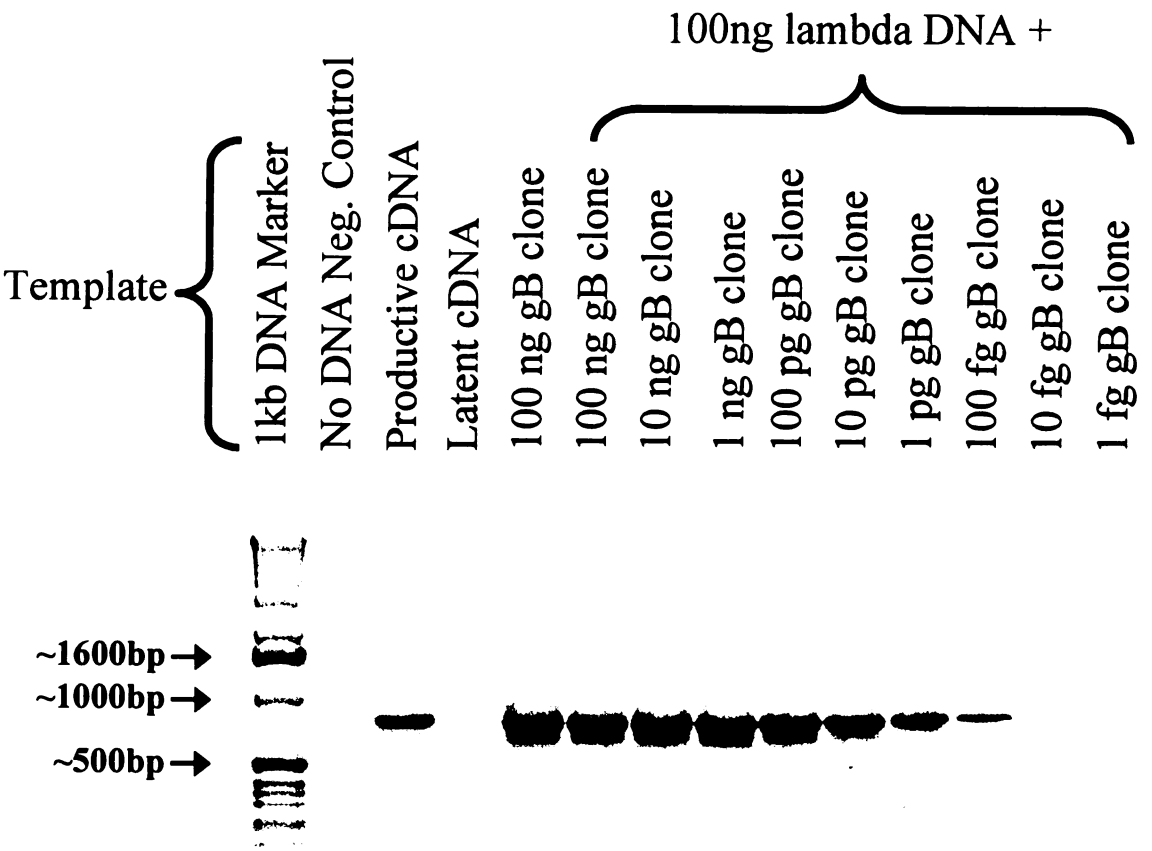
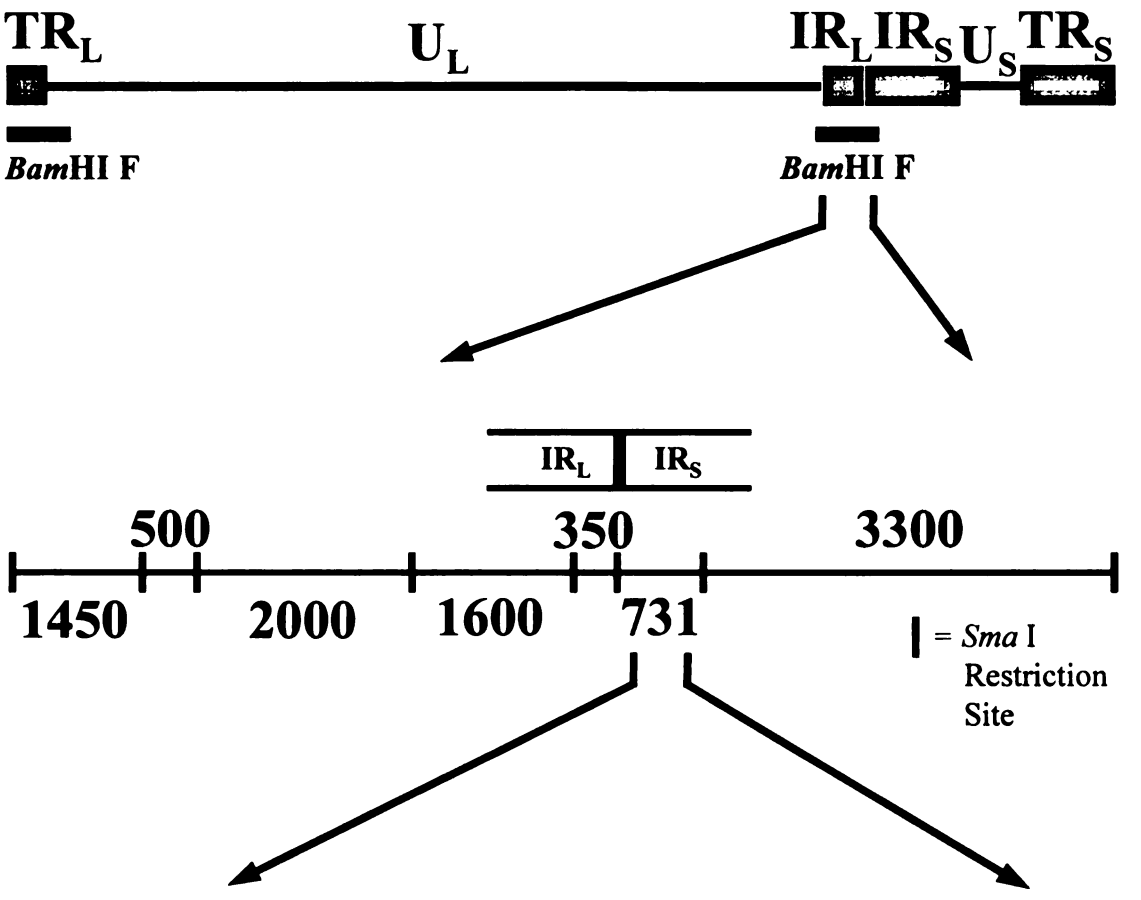

Template
1kb DNA Marker
No DNA Neg. Control
Productive cDNA
Latent cDNA
100 ng gB clone
100ng lambda DNA +
100 ng gB clone
10 ng gB clone
1 ng gB clone
100 pg gB clone
10 pg gB clone
1 pg gB clone
100 fg gB clone
10 fg gB clone
1 fg gB clone
~1600bp
~1000bp
~500bp

Figure 2-6: Southern analysis of PCR products. The m13 forward and H12 oligonucleotides were used with the latent HVT cDNA library as template; PCR conditions were:1x 94C for 2 minutes, 30x (94C for 30 seconds, 55C for 1 minute, 72C for 1 minute), 72C for 10 minutes. Southern conditions were: 65C, overnight with the 731bp *Sma* I subclone of the HVT *Bam*HI F region. Blot wash and development conditions were as previously described, with a 13 hour exposure.

Positive Control
Blank
CEF genomic DNA template
Productive HVT cDNA template
Latent HVT cDNA template
4000bp
3000bp
2000bp
1600bp
1000bp
500bp
300bp

Figure 2-7: Map and sequence for the 405 bp PCR amplification product of H12/m13 forward oligonucleotides with latent HVT cDNA library as template.




```
1   ACTAGTGATTAAATACAGGGCGTGT GTGAAGAGAAGAAGATTTCGCGGAG

51  CCTGACACTGGCCGAGGGAAAACAG GTCACTGGAGGTTTCGGGATGAAGC

101 GACCGAGATTTCTCAGGTTCGTTTC TCGCCGGCTCTCTGATCGGCCCTCT

151 ATGTGAGCCGACATTCTTATATTTC TCTCTGGTCGCAATGTTTTTGTTGT

201 GATGATTACCCGTGGGCACCTGACA AAGCAAAATAAACACCACTGAATTG

251 TCACGAAGTCTCCTTGTTTGTCTGT GCGTACCATGCAATGGGAAGGGGGC

301 CATCGGTTTGTTTGGGGGGAGCGGT GTGTACTTTTCTGCAATGCGCGGCC

351 GGGAAAAGTCGTGGTTAGGTTTAAG GGTTTGAAATAAGACGGGTACCTGT

401 TAACC
```

Figure 2-8: Enrichment procedure for isolation of LAT clones from the latent HVT cDNA library.

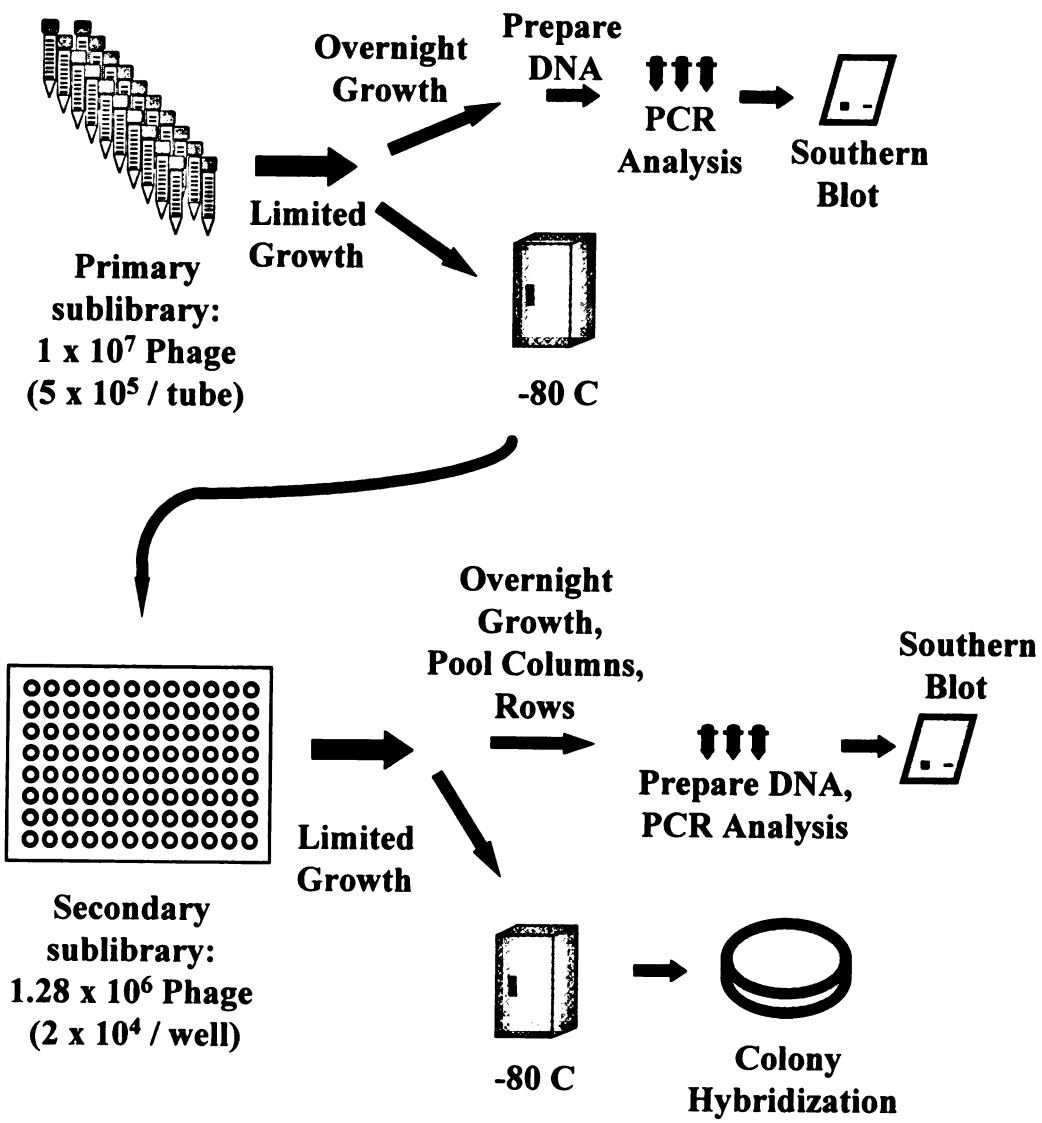

Overnight Growth
Prepare DNA
PCR Analysis
Southern Blot
Limited Growth
Primary sublibrary: 1 x 10^7 Phage (5 x 10^5 / tube)
-80 C
Overnight Growth, Pool Columns, Rows
Southern Blot
Prepare DNA, PCR Analysis
Limited Growth
Secondary sublibrary: 1.28 x 10^6 Phage (2 x 10^4 / well)
-80 C
Colony Hybridization

Figure 2-9: Southern analysis of PCR products. The m13 forward and H12 oligonucleotides were used with the primary sublibrary as template. PCR conditions were: 1x 94C for 2 minutes, 30x (94C for 30 seconds, 55C for 1 minute, 72C for 1 minute), 72C for 10 minutes. Southern conditions were: 65C, overnight with the 731bp *Sma* I subclone of the HVT *Bam*HI F region as probe. Blot wash and development conditions were as previously described, with a 13 hour exposure.

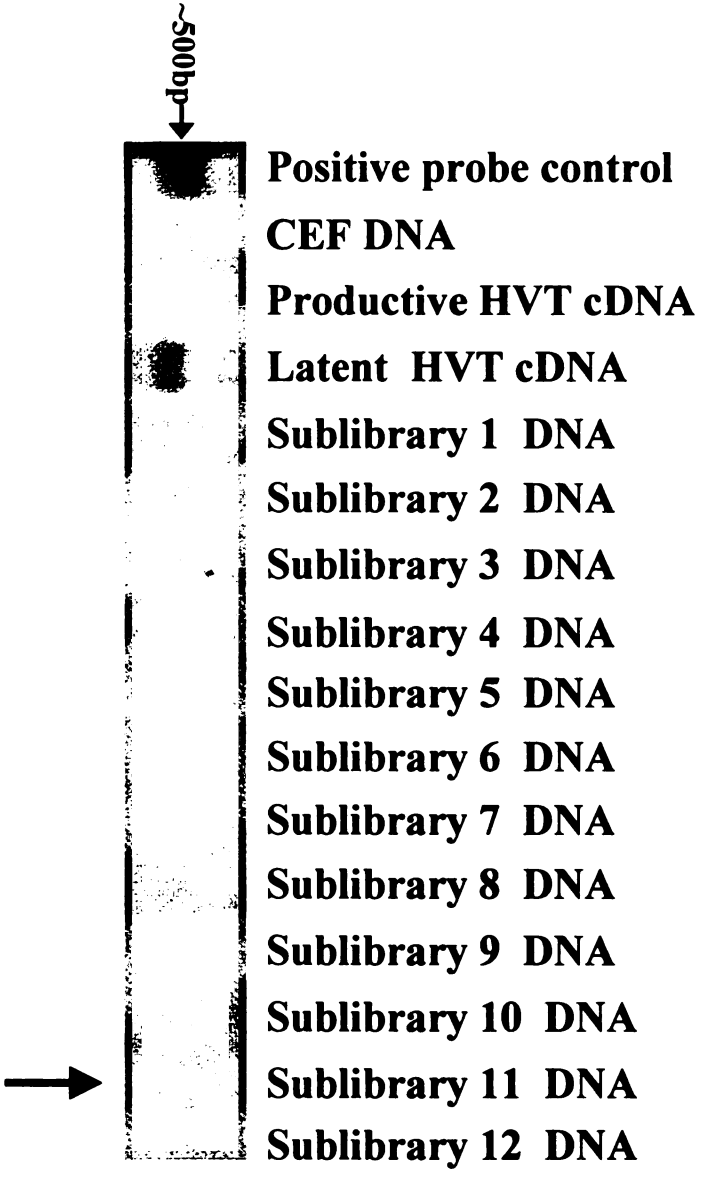
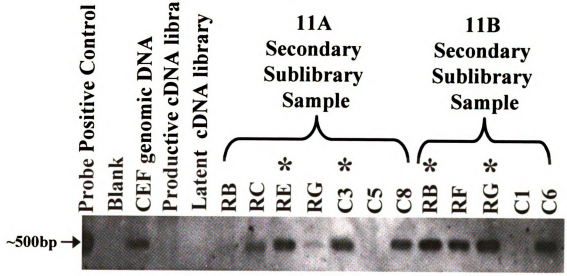

~500bp
Positive probe control
CEF DNA
Productive HVT cDNA
Latent HVT cDNA
Sublibrary 1 DNA
Sublibrary 2 DNA
Sublibrary 3 DNA
Sublibrary 4 DNA
Sublibrary 5 DNA
Sublibrary 6 DNA
Sublibrary 7 DNA
Sublibrary 8 DNA
Sublibrary 9 DNA
Sublibrary 10 DNA
Sublibrary 11 DNA
Sublibrary 12 DNA

Figure 2-10: Southern analysis of PCR products. The m13 forward and H12 oligonucleotides were used with the latent HVT cDNA 11A and 11B secondary sublibraries as template. PCR conditions were:1x 94C for 2 minutes, 30x (94C for 30 seconds, 55C for 1 minute, 72C for 1 minute), 72C for 10 minutes. Southern conditions were: 65C, overnight with the 731bp *Sma* I subclone of the HVT *Bam*HI F region. Blot wash and development conditions were as previously described, with a 50 minute exposure.



* = Chosen for double colony lifts

Figure 2-11: Negative image of nested PCR using H12/H40 followed by H41/H42 oligonucleotides. PCR conditions were: 1x 95C for 3 minutes, 30x (95C for 1 minute, 63C for 1 minute, 72C for 2 minutes), 72C for 10 minutes for the primary and nested PCR. The results of the H41/H42 nested PCR are shown here.

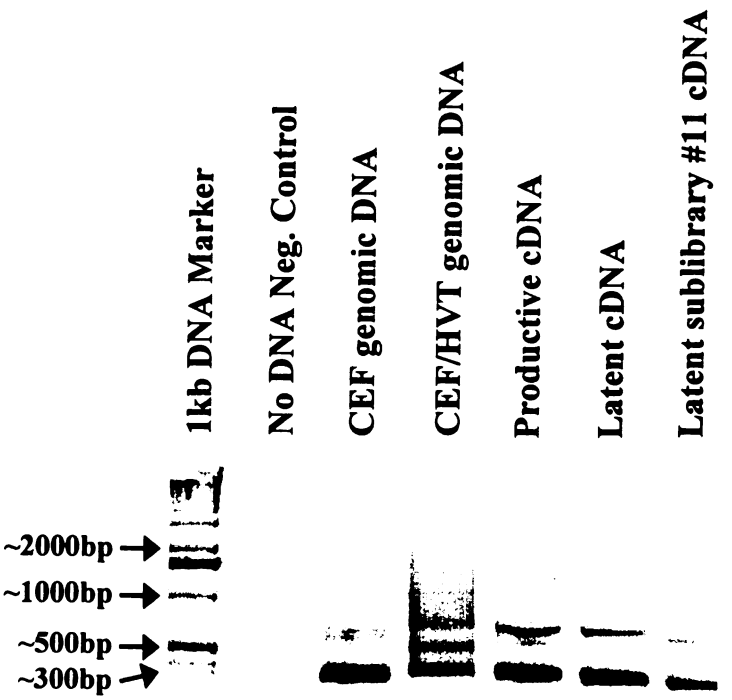

~2000bp
~1000bp
~500bp
~300bp
1kb DNA Marker
No DNA Neg. Control
CEF genomic DNA
CEF/HVT genomic DNA
Productive cDNA
Latent cDNA
Latent sublibrary #11 cDNA

Figure 2-12: Negative image of PCR using gB oligonucleotides. PCR conditions were: 1x 95C for 3 minutes, 30x (95C for 1 minute, 60C for 1 minute, 72C for 2 minutes), 72C for 10 minutes. Photo obtained using a Kodak digital camera imaging system.

~1600bp
~1000bp
~500bp
1kb Marker
No DNA
CEF RNA
CEF/HVT RNA
CEF cDNA
CEF/HVT cDNA
CEF Genomic DNA
CEF/HVT Genomic DNA

Figure 2-13: Negative image of PCR using gB oligonucleotides. PCR conditions were: 1x 95C for 3 minutes, 30x (95C for 1 minute, 60C for 1 minute, 72C for 2 minutes), 72C for 10 minutes.

1kb Marker
No DNA
CEF DNA
CEF/HVT DNA
U19 RNA - No RT
U19 RNA - RT
H19 RNA - No RT
H19 RNA - RT
~1600bp
~1000bp
~500bp
~300bp

Figure 2-14: Negative image of PCR of CEF and spleen total RNA using GAPDH oligonucleotides to asses DNA contamination. PCR conditions were: 1x 95C for 3 minutes, 30x (95C for 1 minute, 55C for 1 minute, 72C for 2 minutes), 72C for 10 minutes. H1, 6, 10, 13, 16, 19 = HVT-infected chickens, U1, 19 = uninfected chickens.

~1000bp
~500bp
~300bp
1kb Marker
No DNA
CEF DNA #1
CEF DNA #2
H1 Spleen RNA
H6 Spleen RNA
H10 Spleen RNA
H13 Spleen RNA
H16 Spleen RNA
H19 Spleen RNA
U1 Spleen RNA
U19 Spleen RNA

Literature Cited

Cohrs, R.J., K. Sprock, M.B. Barbour, G. Owens, R. Mahalingham, M.E. Devlin, M. Wellish, D.H. Gilden. Varicella-Zoster virus (VZV) transcription during latency in human ganglia: construction of a cDNA library from latently infected human trigeminal ganglia and detection of a VZV transcript. J. Virology, **68**(12):7900-08. 1994.

Cohrs R.J., M. Barbour, D.H. Gilden. Varicella-zoster virus (VZV) transcription during latency in human ganglia: detection of transcripts mapping to genes 21, 29, 62, and 63 in a cDNA library enriched for VZV RNA. J. Virology, **70**(5):2789-96. 1996.

Fields, B.N., D.M. Knipe, P.M. Howley. Fields virology. p. 2221-2585. In R.M. Chanock, J.L. Melnick, T.P. Monath, B. Roizman, S.E. Straus (ed.) Fields virology, v. 2, third edition. Lippincott-Raven, Philadelphia. 1996.

Hodge, R. Preparation of RNA gel blots. P. 49-53. In PG Isaac (ed.), Methods in Molecular Biology, Vol. 28: Protocols for Nucleic Acid Analysis by Nonradioactive Probes. Humana Press, Totowa. 1994.

Holland, M.S., C.D. Mackenzie, R.W. Bull, R.F. Silva. Latent turkey herpesvirus infection in lymphoid, nervous and feather tissues of chickens. Avian Diseases, **42**:292-9. 1998.

Holland, M.S., R.F. Silva. Tissue-specific restriction of latent turkey HVT transcription. Acta Virol, Apr-Jun, **43**(2-3):148-51. 1999.

Sambrook, J., E.F. Fritsch, T. Maniatis. Molecular cloning 2nd edition. In N. Ford, C. Nolan, M. Ferguson (ed.), Cold Springs Harbor Laboratory Press, Plainview. 1989.

www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome