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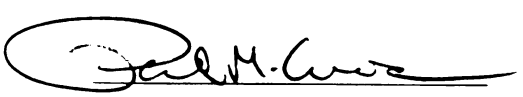
MAREK'S DISEASE VIRUS-MEDIATED ENHANCEMENT OF AVIAN LEUKOSIS VIRUS GENE EXPRESSION AND VIRUS PRODUCTION

presented by

James Thomas Edward-Stephen Pulaski

has been accepted towards fulfillment
of the requirements for

Master's degree in Science



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Date Nov. 13, 1992

MAREK'S DISEASE VIRUS-MEDIATED ENHANCEMENT OF AVIAN LEUKOSIS VIRUS GENE EXPRESSION AND VIRUS PRODUCTION

By

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A THESIS
Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE

Department of Animal Science

1992

ABSTRACT

MAREK'S DISEASE VIRUS-MEDIATED ENHANCEMENT OF AVIAN LEUKOSIS VIRUS GENE EXPRESSION AND VIRUS PRODUCTION

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Direct interaction between two viruses in coinfected cells may promote replication and/or pathogenesis of one or both virus types. In birds, Marek's disease virus (MDV) may be an important cofactor in avian leukosis virus (ALV)-induced disease. Coinfection of susceptible cells with non-oncogenic serotype 2 MDV, an avian herpesvirus, and an oncogenic avian retrovirus, avian leukosis virus (ALV), resulted in enhanced transcription of retroviral genes. Consequently, in vivo assays show increased ALV reverse transcriptase activity and antigen production relative to input concentration of MDV. Interactive laser cytometry was used to detect accumulation of both MDV and ALV antigens within single cells from coinfected cultures. These results suggest a direct role for MDV-encoded or -induced factors in enhancement of ALV gene expression and demonstrate the importance of herpesviruses as cofactors in retrovirus replication and pathogenesis in coinfected cells.

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ACKNOWLEDGMENTS

To my mentor, Dr. Paul M. Coussens, for believing in me even when I found it hard to. To Dr. Crittenden, for guiding me into this field of study. To Matthew/Amy Pulaski and Maria/Bruce Corstange who were always there to support and encourage me. To Mom and Dad, thank you for supporting every decision I made. To all of my friends I grew with in the lab - Ron Southwick, Heidie Camp, Mindy Wilson, Jesse Marcus, Uldis Banders and Virginia Tieber.

Finally, to my committee, for guiding me down the path of science.

THANK YOU!!

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LITERATURE REVIEW

AVIAN LEUKOSIS VIRUS

Retroviruses, viruses of the family retroviridae, are grouped into three subfamilies; oncovirinae ("tumor viruses"), spumavirinae ("foamy viruses"), lentivirinae ("slow viruses"); each having distinctive characteristics described by their latin prefix (Matthews, 1982). Retrovirus proteins have both type specific and group specific determinants. Oncovirinae is further subdivided into genera derived by morphological classification based on electron micrographs of distinctive virus particles. By this particle morphology, oncogenic retroviruses can be distributed into four categories: A-type, B-type, C-type, and D-type particles (Bernhard, Cancer Res. 20; 712).

Avian leukosis virus (ALV) species are retroviruses classified as type C oncoviruses and have been divided into five subgroups, A-E, based upon host range, interference patterns with other subgroups, and serum neutralization tests to define envelope antigen type (Matthews, 1982). Strains of ALV are identified by the pathological lesions produced and their envelope subgroup. They are grouped with sarcoma viruses and given an abbreviated designation based on the neoplasm induced and/or a person or laboratory that studied them. For example, "BH-RSV" is the high titer strain of Bryan, Rous sarcoma virus (Weiss et. al. 1984). Table 1 (Weiss et al., 1984) shows the envelope subgroup classification and abbreviated names for different strains virus such as Rous sarcoma virus (RSV) and avian myoblastosis virus (AMV) listed across from their respective names. In the column marked 'defective' are the transforming viruses that require a 'helper' virus for propagation. These

Table 1. Common Laboratory strains of avian leukosis and sarcoma viruses according to predominant neoplasm induced and virus subgroup

Virus Class According to Neoplasm	Virus Class According to Subgroup					No Subgroup (Defective Virus)
	A	B	C	D	E	
Lymphoid Leukosis virus (LLV)	RAV-1 RIF-1 MAV-1 RPL 12 HPRS-F42	RAV-2 RAV-6 MAV-2	RAV-7 RAV-49	RAV-50 CZAV	RAV-60	
Avian erythroblastosis virus (AEV)						AEV-ES4 AEV-R
Avian Myoblastosis virus (AMV)						AMV-BAI-A E 26
Avian Sarcoma virus (ASV)	SR-RSV-A PR-RSV-A EH-RSV RSV 29	SR-RSV-B PR-RSV-B HA-RSV	B 77 PR-RSV-C	SR-RSV-D CZ-RSV	SR-RSV-E PR-RSV-E	BH-RSV BS-RSV FuSV PRC II PRC IV ESV Y 73 UR1 UR2
Myelocytoma/endothelioma virus						MC 29 MH 2 CM II OK 10
Endogenous virus (EV)					RAV-0 ILV	

(Weiss et. al., 1984)

helper viruses are listed under lymphoid leukosis viruses (LLV). LLV(s) are common field strains of virus that are associated with a variety of oncogenic diseases and substrains can be selected for the prevalence of one kind of disease (Frederickson et al., 1964; Smith and Moscovici, 1969). These viruses are collectively know as LLV, ALV, or transformation-defective viruses (Weiss et al., 1984).

Morphologically, avian retroviruses of the subfamily oncovirus are spherical, enveloped, 80-120 nm in diameter, with two envelope (env) glycoproteins, projecting from the surface. Structurally, retroviruses have four internal group specific antigen (gag) proteins that make up an icosahedral capsid of nonglycosylated structural proteins, and a helical ribonucleoprotein. These core proteins are packaged with reverse transcriptase within the virus envelope (Baur, 1974; Norwinski et. al., 1973).

Subgroup E viruses are known as endogenous avian leukosis viruses (abbreviated EV or *ev*), carried as a complete or defective proviral DNA integrated into different genomic sites of both somatic and germline cells (Crittenden, 1981, Smith, 1987). Thus, they are transmitted genetically and in a Mendelian fashion to progeny (Crittenden et. al., 1977). Phenotypic expression of these loci vary and is not well understood. When the complete *ev* genome is present, subgroup E virus may be produced. Expression of *ev* genes in cells can give positive reactions in enzyme-linked immunosorbent assays (ELISA) for group-specific (gs) antigen, compliment fixation tests for avian leukosis (COFAL), and the chick helper factor (chf) tests (Table 2). Importance of the existence of endogenous virus is not limited to false positives in exogenous virus assays. Crittenden et. al. (1987) demonstrated that an endogenous virus, RAV-0, can cause immune tolerance due to

Table 2.

Names, phenotypes and lines of endogenous avian leukosis viruses (*ev*).

ev	Phenotype	Line or Source[b]
1	gs^- chf^-	Most lines
2	V-E^+	RPRL-7_2
3	gs^+ chf^+	RPRL-6_3
4	gs^- chf^-	SPAFAS
5	gs^- chf^-	SPAFAS
6	gs^- chf^+	RPRL-15I
7	V-E^+	RPRL-15B
8	gs^- chf^-	K-18
9	gs^- chf^+	K-18
10	V-E^+	RPRL-$15I_4$
11	V-E^+	RPRL-$15I_4$
12	V-E^+	RPRL-15I
14	V-E^+	H&N
15(C)	none	K-28 X K-16
16(D)	none	K-28 X K-16
17	gs^- chf^-	RC-P
18	V-E^+	RI
19	V-E^+ (?)[a]	RW
20	V-E^+ (?)[a]	RW
21	V-E^+	Hyline FP

Note: *ev* is associated with the gs^- chf^- phenotype but restriction fragments have not been characterized.
a The presence of five *ev* loci in Reaseheath line W. birds precludes definitive assignment with the V-E^+ phenotype. Definitive association requires further segregation of *ev* genes. Hyline FP birds also carry *ev* 1, *ev* 3, and *ev* 6.
b Not exclusive to line or source. K= Kimber; R= Reaseheath; H&N= Heisdorf and Nelson.

Phenotype	Symbol	*ev* locus
No detectable virus product	gs^- chf^-	1,4,5
Expression of subgroup E envelope antigen	gs^- chf^+	9
Coordinate expression of group specific antigen and envelope antigens	gs^+ chf^+	3
Spontaneous production of subgroup E virus	V-E^+	2

(Smith, 1987)

envelope glycoproteins shared among endogenous and exogenous viruses.

ALV's virion nucleic acid is an inverted dimer of linear positive sense RNA. Basic genetic information for production of infectious virions consists of three genes. In order from 5' to 3': group specific antigen (*gag*) codes for internal nonglycosylated virion proteins; polymerase (*pol*) codes for reverse transcriptase; and envelope (*env*) codes for virion envelope glycoproteins. Redundant sequences, designated long terminal repeats (LTRs), flank each end of the genome and act as promoters for pro-viral DNA transcription initiation and termination (Figure 1). Proviral LTRs can be divided into three distinct regions: U3, R, and U5. They are named so because of their location in the genomic RNA: respectively, *u*niquely at the 3' end, *r*edundant sequence at either end, and *u*niquely at the 5' end (Figure 1). Notice they are referenced in upper case letters in the context of DNA, and lower case for RNA. Other genes for nonstructural components may also be present, but are not necessary for production of infectious virions (Matthews, 1982).

A general outline for retrovirus replication has existed for some time (Temin and Baltimore, 1972). Avian leukosis virus, as with other members of the family retroviridae, are distinguished from other enveloped single-stranded RNA viruses by use of reverse transcriptase and cellular DNA polymerase to produce a DNA intermediate step in replication (Matthews, 1982). After adsorption, penetration, and uncoating, single stranded virion RNA is transcribed into double stranded DNA "provirus" by virion reverse transcriptase. The LTR is formed during reverse transcription (See Figure 1) making retroviral DNA 500 to 1000 base pairs longer than the RNA genome (Fan, 1990). Proviral DNA is transported into the nucleus and integrated into the host genome in a semi-random manner. Cellular RNA polymerase II is responsible for viral RNA transcription from proviral DNA. Initiating in the

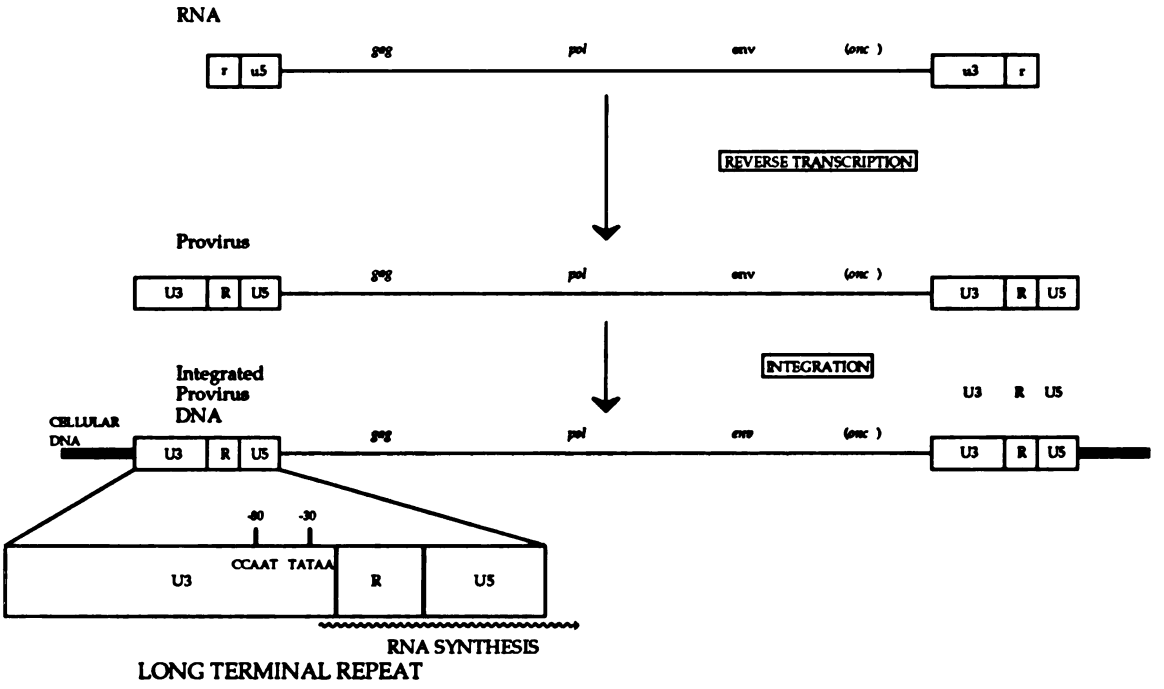


Figure 1 The Retrovirus Genome

upstream LTR at the U3-R border and terminating downstream at the R-U5 border, the proviral DNA is a complete transcription unit. U3 regions of retroviral or proviral LTRs carry proximal and distal promoter elements, enhancer sequences, and sequences that control Pol II initiation sites, thus regulating viral transcription (Dynan and Tijian, 1985). Because LTRs carry all control sequences necessary for initiation of transcription, cleavage, and polyadenylation, the integrated provirus can be expressed as both progeny viral RNA and mRNA (Fan, 1990). These virion mRNAs are processed to resemble host mRNA and are transported to the cytoplasm where they are translated. Resulting structural proteins are assembled into virus particles, in which the progeny RNA, reverse transcriptase, and a cellular tRNA (to be used as a reverse transcriptase primer) is packaged. Envelope transmembrane proteins on the cell surface mark where particles bud,

forming infective virus (Weiss et. al., 1984).

ALV cause a wide variety of neoplasms in chickens and other birds. Although each strain has a characteristic neoplasm, the oncogenic spectrum of different strains may overlap (Beard, 1980). The major clinical disease caused by ALV in the field is called lymphoid leukosis, a B cell lymphoma originating in the bursa of fabricius of mature chickens (Weiss, 1984). Oncogenic patterns are influenced by viral and host factors such as origin (strain) (Fredrickson et. al., 1965), dose (Burmester et. al., 1959), route of infection (Fredrickson et. al., 1964), age (Burmester et. al., 1960), genotype, and sex of host (Crittenden, 1975, Burmester and Nelson, 1945). ALV proviral sequences may insert within the cellular genome resulting in activation of a cellular pro-oncogene. During the normal transcription of the provirus, the 3' or 5' U3 region of the proviral LTR may promote transcription of downstream cellular genes (oncogenes) do to a lack of transcriptional termination at the 5' LTR (Hayward et al., 1981; Payne et. al., 1981).

Because of the extended time before lymphoid leukosis (LL) develops (table 3), ALV is of primary concern to the egg layer industry. No efforts to eradicate the virus, ranging from attempts to produce attenuated strains for vaccines (Okazaki et. al.,1982) to selective breeding for genetic resistance in birds (Crittenden, 1975), have been unsuccessful to date. Exogenous virus has two routes of transmission: vertically from hen to progeny through the egg and horizontally from bird to bird by direct or indirect contact (Rubin et. al., 1961, Ruben et. al., 1962). Because no reliable treatment has yet been devised, eliminating vertical transmission with strict breeding programs and thereby maintaining a virus free flock is the only method of controlling disease.

Table 3.

Comparison of epizootiologic and pathologic features of Marek's disease (MD) and lymphoid leukosis (LL).

Characteristic	MD	LL
Age of onset		
Peak time	2-7 mo.	4-10 mo.
Limits	>1 mo.	>3 mo.
Clinical signs		
Paralysis	Common	Absent
Gross lesions		
Liver	Common	Common
Nerves	Common	Absent
Skin	Common	Rare
Bursa tumor	Rare	Common
Bursa atrophy	Common	Rare
Intestine	Rare	Common
Heart	Common	Rare
Microlesions		
Pleomorphic cells	Yes	No
Uniform blast cells	No	Yes
Bursa tumor	Interfollicular	Intrafollicular
Surface antigens		
MATSA	5-40%	Absent
IgM	<5%	91-99%
B cell	3-25%	91-99%
T cell	60-90%	Rare

(Calnek and Witter, 1991)

MAREK'S DISEASE VIRUS

Viruses of the family herpesviridae are divided into three subfamilies, designated alpha-, beta-, and gammaherpesvirinae based on host range in vitro, cell tropism of latent infection, replication and cytopathology (Table 4 and 5) (Matthews, 1982). An alternative classification scheme, based upon orientation of repeat sequences in the genome has been proposed (Table 6) .

Marek's disease virus is classified as a type E alphaherpesvirus and is further subdivided into three serotypes determined by immunodiffusion and immunoflourescence tests (Roizman et. al., 1992; Bulow and Biggs 1975 a,b). Serotype 1 MDV are mild to very virulent isolates and their attenuated variants. Serotype 2 MDV are naturally occurring nononcogenic isolates, and serotype 3 are isolates of herpesvirus of turkeys (HVT), which is not identical to MDV, but produces similar cytopathic changes in tissue culture and is antigenically related to MDV (Witter et. al., 1970a). Because they are related antigenically to oncogenic serotype 1 MDV and are nonpathogenic, serotype 2 and 3 MDV isolates are used in vaccines (Schat and Calnek, 1978; Zander et. al., 1972).

Four types of virus-cell interactions have been characterized in Marek's infections: Fully productive, semi-productive, non-productive neoplastic, and non-productive latent. In fully productive infections, replication of viral DNA occurs, antigens are synthesized, and fully infectious virus particles are produced resulting in cell death. Fully productive infections have only been found in feather follicle epithelium (Calnek et. al., 1970). Semi-productive infection occurs mainly in lymphoid and parenchymal tissue. This type of infection is exemplified by production of noninfectious naked nuclear virions. Infection is accomplished by cell to cell transmission and also leads

Table 4.

Herpesviruses subfamily division.

Family: Herpesviridae

Subfamilies:

Subfamily 1 (Alphaherpesvirinae)
Host range: In vivo narrow, frequently restricted to the species or genus to which the host belongs. In vitro replicates best in fibroblasts although exceptions exist.
Duration of reproductive cycle: Relatively long.
Cytopathology: Slowly progressive lytic foci in cell culture. The infected frequently become enlarged, (cytomegalia) both in vitro and in vivo. Inclusions containing DNA frequently present in both nuclei and cytoplasm. Carrier cultures easily established.
Latent infection: Possibly in secretory glands, lymphoreticular cells, and kidney and other tissues.

Subfamily 2 (Betaherpesvirinae)
Host range: In vivo narrow, frequently restricted to the species or genus to which the host belongs. In vitro replicates best in fibroblasts although exceptions exist.
Duration of replication cycle: Relatively long.
Cytopathology: Slowly progressing lytic foci in cell culture. The infected cells frequently become enlarged (cytomegalia) both in vitro and in vivo. Inclusions containing DNA frequently present in both nuclei and cytoplasm. Carrier cultures easily established.
Latent infections: Possibly in secretory glands, lymphoreticular cells, kidney and other tissue.

Subfamily 3 (Gammaherpesvirinae)
Host range: In vivo usually limited to the same family or order as the host it naturally infects. In vitro all members of this subfamily replicate in lymphoblastoid cells and some also cause lytic infections in some types of epithelioid and fibroblastoid cells. Viruses in this group are specific for either B or T lymphocytes. In the lymphocyte, infection is frequently arrested either at a prelytic stage with persistence and minimum expression of the viral genome or at a lytic stage, causing cell death without production of complete virions.
Duration of reproductive cycle: Variable.
Cytopathology: Variable.
Latent infection: Latent virus is frequently demonstrated in lymphoid tissue.

(Matthews, 1982)

Table 5.

SELECTED HERPESVIRUSES

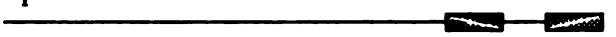
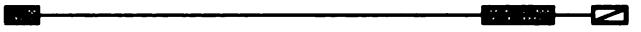
VIRUS NAME	SUBFAMILY	CLASS
HUMAN HERPESVIRUSES		
Herpes simplex virus 1	α	E
Herpes simplex virus 2	α	E
Varicella-Zoster virus	α	D
Epstein-Barr virus	γ	C
Cytomegalovirus	β	E
HERPESVIRUSES OF NONHUMAN PRIMATE		
Ateline herpesvirus 2	γ	B
HERPESVIRUSES OF BONEY FISHES		
Ictalurid herpesvirus 1	α	A
AVIAN HERPESVIRUSES		
Marek's disease virus (Serotype 1)	γ	E
Marek's disease virus (Serotype 2)	γ	E
Turkey herpesvirus (Serotype 3)	γ	E

to cell death (Calnek et al. 1982). Non-productive neoplastic infection, in which the viral genome persists in lymphoid cells with limited tumor and viral antigen production, results in immortalized cells (Witter et al. 1975; Sharma, 1981; Ross, 1985). Non-productive latent infections, in which the viral genome persists in lymphoid cell without production of viral or tumor associated antigens. Virus can be rescued by inoculation of infected cells into chickens or onto cultured cells (Calnek et al., 1981). Observations of latent infections have been restricted to lymphocytes, primarily in T cells (Shek et. al. 1983).

Marek's disease is the most common lymphoproliferative disease of chickens and is characterized by mononuclear infiltration of one or more of

Table 6.

Grouping herpesviruses on the basis of the properties of their genomes

Group	Arrangement of repeated sequences	Number of isometric arrangements[3]
A	A single set repeated at termini in the same orientation 	1
B	Numerous repeats of the same set of sequences at both termini in the same orientation 	1
C	(i) Numerous repeats of the same set of sequences at both termini in the same orientation; (ii) A variable number of tandem repeats of a different sequence internally 	1
D	(i) A single set of sequences from terminus repeated internally; (ii) A subset of terminal sequences repeated at all termini in the same orientation 	2
E	(i) A single set of sequences from both termini repeated in inverted form internally; (ii) A subset of terminal sequences repeated at both termini in the same orientation[1,2] 	4
F	Terminal reiterations in the genome of class F have not yet been described	1

1. Although the genome of Marek's disease virus is characteristic of the E group,it's L and S components do not invert.

2. The presence of a terminal sequence repeated at all termini has yet to be proven in some herpesviruses.

3. Defined by the number of genome populations differing in the location of sequences in the unique regions relative to the termini

(Modified from Matthews, 1982; Koch et al., 1986)

the following: peripheral nerves, gonad, iris, viscera, muscle, and skin (Calnek and Witter, 1991). Symptoms of the disease are variable, and the gross lesions are difficult to distinguish from those of ALV infections (Table 3). Nerve lesions are the most common gross lesions observed in infected birds (Payne,1985) and lead to paralysis of the extremities (Biggs, 1968). T-cell lymphoma is the ultimate response to serotype 1, possibly progressing to tumor development (Calnek and Witter, 1991). Lymphoma composition is complex, consisting of neoplastic, inflammatory, and immunologically active cells (Rouse et. al., 1973). Based on studies of a large number of cell lines, T cells are the usual targets for transformation (Powell et. al., 1974). Neoplastic cells carry MDV DNA, are continuous, and are usually nonproductive in-vivo (Calnek and Witter, 1991).

Virion structure, as revealed by electron micrographs of negative stained preparations, is an enveloped nucleocapsid surrounding a nucleoid varying in shape from spherical to toroid (Nazerian 1974). Virus particles isolated from feather follicle epithelium have envelopes measuring 273-400 nm and appear as irregular, amorphous structures (Calnek et. al. 1970). Negative stained preparations also illustrated a cubic, icosahedral nucleocapsid 150-160 nm (Calnek et. al. 1970) made up of 162 hollow centered capsomeres (Nazerian 1973). The nucleic acid winds around a central structure connecting to two inner capsid poles forming a nucleoid (Nazerian 1974).

As described before, herpesvirus of turkeys (HVT) is a serotype 3 herpesvirus with genomic structure (Cebrian et. al., 1982) and antigenic properties similar to Marek's disease virus (Witter et. al., 1970a). Because of these characteristics, plus it's nononcogenic nature in chickens (Schat and Calnek, 1978), HVT is frequently used as a vaccine against MDV (Zander et.

al., 1972). In addition to similarities in structure and antigenicity, reports have also demonstrated a degree of homology between the two herpesviruses (Igarashi et. al., 1987). Igarashi et. al. have shown the similarities between MDV and HVT genome structure and sites of homology.

MDV DNA is a linear, double stranded molecule with a size between 166 to 184 kilobase pairs (85-110 x 10^6 da MW) (Wilson and Coussens, 1992; Hirai et. al., 1979; Lee et. al., 1971). Both MDV and HVT are "E" type genome structure consisting of a long unique region and a short unique region, each flanked by one internal and one terminal repeat (Cebrian et. al. 1982). All three serotypes differ in homology (Hirai et. al., 1984, Hirai et. al. 1981) and restriction endonuclease digestion patterns (Ross et. al. 1983). Figure 2 compares restriction enzyme maps of serotype 1 and serotype 3 viruses; a map of serotype 2 viruses has not yet been generated.

Transcription of MDV and HVT has a typical α herpesviruses "cascading" pattern ((Fenwick and Owen, 1988; Kato and Harai, 1985). Studies of this cascading cycle are complicated by the fact that MDV and HVT are highly cell associated. Infection is normally cell associated and accomplished by formation of intracellular bridges (Kaleta and Neumann, 1977). To fully study replication, cell free virus must be collected from the feather follicle epithelium (Calnek et. al., 1970) or sonicated cells (Paul M. Coussens, personal communication). The transcription cascade is divided into three distinct temporally regulated phases: Immediate early (IE or α), early (E or β), and late (L or γ) (Maray et. al. 1988). After adsorption and penetration of cell free virus, IE genes begin the transcription cycle. IE gene products regulate E and L gene expression, and do not require prior viral protein production(Kato and Hirai, 1985). Early RNAs, transcribed prior to viral DNA replication but after IE

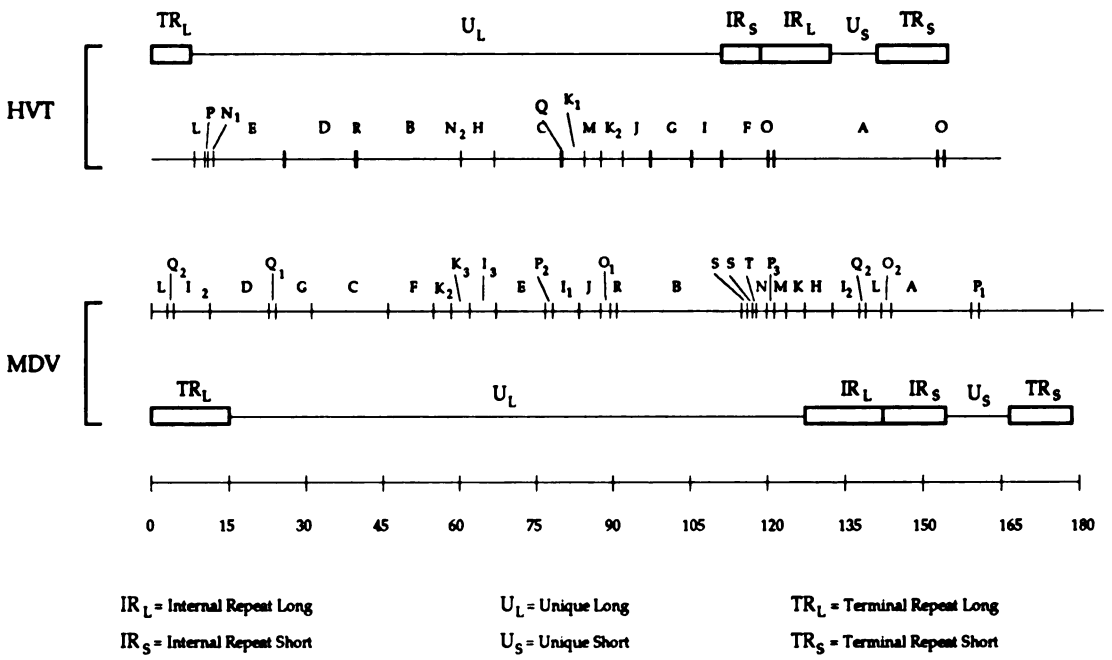


Figure 2 Bam H 1 Maps of HVT and MDV

protein production, specify both nonstructural and structural gene products. Late genes are those that are transcribed only after onset of viral DNA replication (Kato and Hirai, 1985). Temporally, enveloped virions enter the cell by absorption and penetration within an hour of infection (Adldinger and Calnek, 1972). Viral antigens appear in five hours, DNA synthesis in eight hours, nucleocapsid production occurs at 10 hours, and enveloped virion production at 18 hours (Ross, 1985; Hirai et. al., 1980).

MDV is an airborne virus and is spread by direct or indirect contact between chickens and other birds of the genera Gallidae (Biggs and Payne, 1967; Colwel and Schmittle 1968; Cho and Kenzy, 1975). Although the exact route of infection is uncertain, the respiratory tract is the most effective natural means of infection (Calnek and Hitchner, 1969). MDV's infection route and the short incubation period between infection and onset of

symptoms (3-8 weeks) makes MDV a concern to intensive egg layer and broiler breeder industries where turnover time of birds extends beyond 8 weeks (Purchase, 1985; Calnek and Witter, 1991). Mortality nearly equals morbidity and, prior to vaccines, losses in affected flocks range from 25 to 60% (Purchase, 1985). Similar to ALV, there is no effective treatment for Marek's disease. However, three classes of prophylactic vaccines do exist: attenuated serotype 1 MDV (Churchill et. al., 1969), HVT (Okazaki et. al., 1970), and avirulent isolates of serotype 2 (Schat and Calnek, 1978). Vaccination programs based on attenuated MDV, HVT and serotype 2 MDV have reduced bird losses to less than 5% (Purchase, 1985).

RETROVIRUS-HERPESVIRUS INTERACTION

In chickens, increased tumors associated with avian leukosis virus has been observed in hosts coinfected with MDV (Bacon et. al., 1989; Frankel et. al., 1974). Immune-compromising effects of either virus type may be responsible for the observed augmentation during dual infections (Calnek et. al., 1975, Purchase et. al., 1968). However, in-vitro studies demonstrate that herpesviruses and retroviruses interact directly resulting in enhanced expression of each other (Casareale, et al., 1989; Petersen et al., 1990; Dworkin and Drew, 1990; Resnick et al., 1990). In addition, infectious endogenous ALV particles have been isolated from kidney tumors of MDV-infected specific pathogen free (SPF) birds having nonproductive endogenous ALV loci (Campbell and Frankel, 1979). Both cases demonstrate activation of nonprolific ALV endogenous virus by MDV. Studies have also demonstrated birds containing ALV endogenous loci are more susceptible to exogenous

ALV infection (Crittenden et. al., 1987). This suggests *ev* may play a major roll in MDV-enhanced ALV expression in field cases.

Enhanced virus production as a result of direct interaction between retroviruses and herpesvirus has never been proven in a natural system. However, interaction between the viruses has been suggested in several studies. Coinfection of cells with human herpesviruses and HIV may augment herpesvirus-related gene expression and virus production. Clinical studies show a direct correlation of increased replication and pathogenesis associated with herpesviruses like varicella-zoster virus, cytomegalovirus (hCMV), and Epstein-Barr virus, when the subject is coinfected with human immunodeficiency virus (HIV) (Petersen et al., 1990; Dworkin and Drew, 1990; Resnick et al., 1990). Casareale et al. demonstrated hCMV-enhanced lysis of a T4+ T lymphoblastoid cell line (CR-10) persistently infected with the human immunodeficiency virus (HIV) (Casareale, et al., 1989). In similar studies by Skolnik et al. on HIV/hCMV-coinfected H9 cells (an HIV CD4+ human lymphoblastoid cell line), HIV-1-enhanced productive hCMV infection and HIV-1 replication, indicating coinfection is beneficial to both viruses (Skolnik et al., 1988).

Transient assays using LTR reporter gene constructs are common in establishing transactivation of a retrovirus by a herpesvirus (Barry et. al. 1990; Biegalke et. al. 1991; Mosca et. al. 1987; Tieber et al. 1990). Immediate early genes (IE) of herpes simplex virus 1 activate promoters of HIV LTRs in LTR-chloramphenicol acetyltransferase (CAT) gene constructs (Mosca et al., 1987). Biegalke et. al. (1991) demonstrated that two hCMV IE genes are also capable of activating promoters in HIV-1's LTR in experiments where the LTR was linked to a lac Z reporter gene. Similar to those HIV-1 studies, ALV-LTR-

CAT reporter gene constructs have been used to demonstrate MDV-mediated activation of LTR-controlled expression of CAT (Tieber et. al. 1990).

Although these studies do not provide direct evidence that herpesvirus/retrovirus coinfection can lead to enhanced production of either virus type, they do suggest that interaction between the viruses does occur. The major goal of the work presented in the following manuscript was to establish a link between ALV/MDV coinfection and increased production of infectious ALV.

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THESIS BODY

INTRODUCTION

Herpesviruses and retroviruses are associated with a variety of diseases in both mammalian and avian species (Weiss et al., 1984; Fields et al., 1991). Although the individual pathogenesis of each virus has been studied extensively, few investigations have focused on potential herpesvirus-retrovirus interactions in coinfected cells and hosts. Most species carry latent herpesvirus in cells which are also susceptible to retrovirus infection. Thus, coinfection does not require that both viruses enter a host organism coincidentally. Infection with human immunodeficiency virus (HIV) augments replication and pathogenesis associated with latent varacella-zoster virus, cytomegalovirus (hCMV), and Epstein-Barr virus (Petersen et al., 1990; Dworkin and Drew, 1990; Resnick et al., 1990). Conversely, increased retroviral associated tumor incidence is correlated with retrovirus-herpesvirus coinfection in birds (Bacon et al., 1989). In this case, infection with a herpesvirus may activate an otherwise benign endogenous retrovirus. Dual infection often has devastating effects on host organisms, complicating prevention, diagnosis, and treatment. Synergism between herpesviruses and retroviruses has been primarily attributed to opportunistic infection due to the immune compromising effects of one or both virus types. However, coinfection of cells with hCMV and HIV may augment HIV gene expression and virus production (Casareale, et al., 1989; Ho et al., 1990; Skolnik et al., 1988). Factors encoded by various herpesviruses are capable of

transactivating retroviral long terminal repeat (LTR) promoters linked to reporter genes in cultured cells (Tieber et al., 1990; Gendelman et al., 1986; Kenney et al., 1988; Ostrove et al., 1987; Davis et al., 1987). However, increased retroviral gene expression or virus production due directly to herpesvirus-retrovirus interactions in cells coinfected with intact viruses has not been convincingly demonstrated in any system.

To elucidate the nature and potential effects of direct herpesvirus-retrovirus interactions in coinfected cells, we have examined interactions between Marek's disease virus (MDV), an oncogenic avian herpesvirus, and avian leukosis virus (ALV), an avian retrovirus. The MDV/ALV system is an ideal model since both viruses have a similar cell tropism in-vitro and in-vivo (Weiss et al., 1984; Hirumi et al., 1974). Thus, results of in-vitro experiments using MDV and ALV are readily testable in vivo. In addition, interactions between MDV and ALV in coinfected hosts have been suggested (Campbell and Frankel, 1979; Campbell et al., 1978; Jakovleva and Mazuranko, 1979; Witter et al., 1985; Peters, 1973). Initially, it was proposed that manifestation of Marek's disease (MD) required exposure to both MDV and ALV (Peters, 1973). However, there is no direct correlation between expression of retroviral genomes and induction of MD tumors (Calnek and Payne, 1976). Although no evidence of direct MDV-ALV interaction in coinfected cells exists, MDV is capable of transactivating Rous sarcoma virus (RSV) LTR promoters linked to reporter genes (Tieber et al., 1990). Non-oncogenic serotype 2 MDV, used in many MDV vaccine preparations, transactivate RSV LTR promoters more efficiently than oncogenic (serotype 1) viruses (Tieber et al., 1990). Augmentation of ALV induced disease by MDV follows a similar serotype dependent pattern (Bacon

et al., 1989). As with HIV and human herpesviruses, immune suppression by MDV may be at least partially responsible for increased susceptibility to retrovirus infection and pathogenesis. Birds which express endogenous ALV loci are significantly more susceptible to MDV-mediated increases in ALV infection than birds which contain no endogenous loci (Bacon et al., 1989; Crittenden et al., 1987; Ignjatovic and Bagust, 1985). Thus, immune tolerance to ALV proteins, constitutively expressed from endogenous loci genes or gene fragments may be an important factor in MDV-induced augmentation of ALV infection and pathogenesis.

In the present report, we have examined the effect of MDV/ALV coinfection on ALV gene expression and virus production. Our results demonstrate that factors encoded or induced by MDV are capable of activating transcription of ALV genes in coinfected cells. Ultimately, MDV-mediated transactivation of ALV gene transcription results in increased production of ALV proteins and virus.

MATERIALS AND METHODS

Cells and Viruses

Primary Line 0 chick embryo fibroblast (CEF) cells were prepared as described by Glaubiger et al. (1983). CEF cells (1×10^6) were plated on 60mm culture dishes and incubated at 37°C in an atmosphere of 95% air, 5% CO_2. Secondary line 0 CEF cells are susceptible to infection by all serotypes of MDV and all subgroups of ALV. In addition, line 0 CEF cells do not contain endogenous retroviral (ev) loci, making them an ideal cell system in which to study possible MDV/ALV interactions. Infection of line 0 CEF cells with

MDV results in visible "plaques" of enlarged or syncytial type cells that are used to quantitate virus titer expressed in plaque forming units (PFU). ALV virus titer is quantitated by enzyme linked immunosorbant assay (ELISA) and is therefore reported in infectious units (IU) rather than plaque forming units. Propagation and storage of RAV-2 ALV (generous gift of A. Fadly, USDA-ADOL, East Lansing MI) was essentially as described by Bacon et al. (1989). Growth and maintenance of serotype 2 MDV, strain SB-1 has been described previously (Tieber et al.,1990). Cells were infected with RAV-2 ALV alone, serotype 2 MDV, strain SB-1 alone, or with both viruses at titers indicated in the text and figure legends.

Analysis of ALV RNA in MDV/ALV coinfected cells.

Total cellular RNA was isolated from infected cells and control, uninfected cells at 105 hours post-infection by the proteinase K/SDS method (Sambrook et al., 1989). Cultures containing MDV were monitored for MDV-induced plaque formation by light microscopy. At 105 hours post-infection, cell monolayers innoculated with 5×10^5 or 1×10^5 of MDV were fully involved. Total RNA (15 ug) was electrophoresed through denaturing agarose gels (1.2 %) containing 2.2 M formaldehyde as described (Sambrook et al., 1989). Separated RNA was transferred to supported nitrocellulose membranes (Optibind, Schleicher & Schuell, Keene N.H.), prehybridized and hybridized as described (Sambrook et al., 1989). Radiolabeled probes specific for ALV transcripts were prepared from a 3.9 kb Xho I fragment of plasmid RCAS (Hughes et al., 1987), containing an intact non-permuted ALV provirus (generously provided by S. Hughes, NCI-FCRF, Frederick, MD) RNA bands hybridized to ALV probe on northern blots were visualized by autoradiography using Dupont Cronex lightening-plus

intensifying screens. Approximate RNA band sizes were determined by comparison to an ethidium-bromide stained lane of RNA standards (Bethesda Research Laboratories, Inc., Bethesda MD) run on the same gel.

For slot-blot analysis of total ALV RNA levels, RNA (6.0 ug) from coinfected and control cultures (uninfected, ALV infected, and MDV infected) was denatured in 2.2 M formaldehyde and spotted unto supported nitrocellulose. All samples were analyzed in triplicate (n=3). Total ALV RNA was detected by hybridization to a 3.9 kb ALV-specific probe as described for northern blots. Following autoradiography, RAV-2 RNA hybridized to radiolabeled ALV probe DNA was quantitated by scanning densitometry.

Nuclear run-off transcription assays. Nuclei were collected 105 hours post-infection from control uninfected CEF, CEF infected with 5 x 105 IU of RAV-2 ALV, CEF infected with 5 x 10^5 PFU of strain SB-1 MDV, and CEF infected with 5 x 10^5 PFU of strain SB-1 MDV plus 1 x 10^5 IU of RAV-2 ALV as described by Stewart et al. (1987). Run-off transcription and hybridization of radiolabeled transcription products with strips of supported nitrocellulose membranes was performed essentially as described (Stewart et al.,1987). Each nitrocellulose strip contained 5 ug each of: plasmid RCAS (cloned ALV genome, Hughes et al., 1987), p19MDA2.35 (Coussens and Velicer, 1988), pBR322, and pA1 (chicken beta-actin, Cleveland et al., 1980). All plasmid DNA was linearized with appropriate restriction enzymes prior to immobilization on membranes.

Assay for ALV gs antigen and reverse transcriptase activity.

Quantities of gs antigen in 0.1 ml of cell culture lysate were measured by ELISA essentially as described (Smith et al., 1979). Control cultures included uninfected CEF cells, CEF infected with 1 x 10^5 IU of ALV alone, and CEF

infected with 1 x 10^5 PFU of strain SB-1 MDV alone. In each assay, quadruplicate samples of each group were analyzed (n=4). Results were considered positive if absorbance readings (490nm) were 0.2 above negative controls (uninfected cells). Concentrations of gs antigen were quantitated by comparison to a standard absorbance curve prepared with each assay using a known concentration of RSV subgroup C gs antigen (generous gift of Dr. Eugene Smith, USDA-ADOL, East Lansing, MI).

Reverse Transcriptase Assay for ALV Production

RT activity associated with retroviral particles purified from culture media of MDV/RAV-2 coinfected cells was measured essentially as described (Tereba and Murti, 1977). Quadruplicate samples of each control and infected group were analyzed (n=4). All coinfected cultures contained 1 x 10^5 IU of RAV-2 and the amount of SB-1 MDV indicated in the text and figure legends. Control cultures used in RT assays were as detailed for gs antigen ELISA.

Interactive Laser Cytometry of MDV/ALV Coinfected Cells.

Double immune fluorescence assays were quantitated by the adherent cell analysis and sorting (ACAS) cytometer (Meridian Instruments, Okemos, MI). Secondary Line 0 CEF cells were infected with 1 x 10^5 IU of RAV-2 and 5 x 10^5 PFU of SB-1 MDV, diluted 1:10 and transferred to tissue culture chamber slides (1 ml per chamber) (Nunc, Inc., Naperville, IL). Control cells infected with 1 x 10^5 IU of ALV alone, 1 x 10^5 PFU of MDV alone, or uninfected were also plated in chamber slides. Infected and control cells were incubated at 37°C in an atmosphere of 95% air, 5% CO_2 for 50 hours. In preparation for immune fluorescence, cells were fixed with 5% acetic acid in methanol for 30 minutes and rinsed 3 times with PBS. Chamber slides containing permeablized and fixed cells were coated with 3% bovine serum

albumin (BSA) and 0.1% Tween 20 for 45 minutes. Rabbit anti-p27 antibodies (SPAFAS, Inc., Storrs, CT) were employed as primary antibody against ALV. A mouse monoclonal antibody, Y-5 (generous gift of Dr. Lucy Lee, USDA-ADOL), specific for serotype 2 MDV (Lee et al., 1983), was used as primary antibody to detect MDV.

Secondary antibody for detection of ALV was goat anti-rabbit conjugated to phycoerythrin. Secondary antibody for MDV detection was goat anti-mouse conjugated to fluorescein isothiocyanate (FITC). All antibodies were diluted 1:10 in PBS, prior to incubation with fixed cell preparations. Culture chambers were coated for 1 hour with primary antibody, rinsed extensively with PBS, and incubated an additional hour with 50 ul of diluted (1:10) secondary antibody solution. Cells were rinsed 3 times in PBS prior to visualization. Different combinations of antibody solution were used as controls to calculate background fluorescence and interference. Fluorescence intensity was measured using an ACAS 470 interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI). Units set to color values are photons measured per unit time. Fluorescent labels were excited with an argon laser at 488nm. Emissions of FITC (detector 1, MDV) at 520 nm and phycoerythrin (detector 2, ALV) between 570 nm and 580 nm were measured by two photomultiplier tubes (PMT) bound by detection limits of 515 nm and 675 nm, respectively. PMT 1 detected fluorescent light passed through a 575 nm dichroic mirror then a 530 nm band pass filter, thereby detecting emissions between 515 nm and 545 nm (FITC, serotype 2 MDV). PMT 2 detected all fluorescence emissions between 575 nm and 675 nm reflected from the dichroic mirror (Phycoerythrin, ALV gs antigen). Data were visualized as a digitized image of detector output from a scanned field.

DATA ANALYSIS

All data were analized using standard error of the mean and graphed at 99% confidence level. The calculations were performed using Sigmaplot (Jandel Scientific, Courte Madera, CA) on a Zenith Model ZDC-1217 personal computer.

RESULTS

Elevated levels of ALV RNA in MDV/ALV coinfected cells.To examine the effect of MDV on retroviral gene expression in MDV/ALV coinfected cells, 1 x 106 Line 0 CEF cells were coinfected with 1 x 105 IU RAV-2 ALV and 5 x 105 PFU of MDV, strain SB-1. Total cellular RNA was extracted 105 hours post-infection and analyzed for the presence of ALV specific RNA. Uninfected CEF cells did not contain any loci that produced RNA capable of hybridization to ALV specific probes (Figure 3, lane 1). In RAV-2 infected CEF cells, two RNA species of 8.6 and 4.0kb (corresponding to full-length genomic ALV and spliced envelope-specific messages, respectively, Payne et al., 1981) hybridized to ALV probes (Figure 3, Lane 2). Cells coinfected with MDV and RAV-2 contained at least 5-fold more ALV specific RNA than cells infected with RAV-2 alone (Figure 3, Lane 4). A third RNA species of 2.2 kb, present in MDV/RAV-2 coinfected cells, hybridized to ALV probes (Figure 3, Lane 4). Following prolonged exposure of film to the northern blot membrane, hybridization to a similar 2.2 kb RNA species in cells infected with RAV-2 alone was detected (data not shown). Subgenomic messages similar to the 2.2

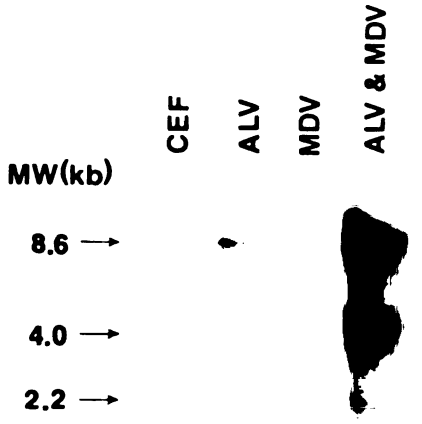

FIGURE 3: Analysis of ALV RNA in MDV/ALV coinfected cells.
Primary Line 0 CEF cells were prepared and maintained as described in Materials and Methods. Cells (1×10^6) were infected with 1×10^5 IU of RAV-2 ALV alone (Lane 2), 1×10^5 PFU of serotype 2 MDV, strain SB-1 alone (Lane 3), or 1×10^5 PFU and IU of MDV and RAV-2, respectively (Lane 4). Total cellular RNA was isolated from infected cells and control, uninfected cells (Lane 1) at 105 hours post-infection by the proteinase K/SDS method (Sambrook et al., 1987) and electrophoresed through denaturing agarose gels (1.2 %) containing 2.2 M formaldehyde. Separated RNA was transferred to supported nitrocellulose membranes (Optibind, Schleicher & Schuell, Inc., Keene N.H.), prehybridized and hybridized to ALV-specific probes as described in Materials and Methods. RNA bands hybridized to ALV probe were visualized by autoradiography using Dupont Cronex lightening-plus intensifying screens. Approximate RNA band sizes were determined by comparison to an ethidium-bromide stained lane of RNA standards (Bethesda Research Laboratories, Inc., Bethesda MD) run on the same gel.

kb species observed in MDV/RAV-2 coinfected cells (Figure 3, Lane 4) have been observed previously in northern blots of ALV infected cells (Payne et al., 1981). RNA isolated from CEF cells infected with strain SB-1 MDV alone did not hybridize to ALV specific probes (Figure 3, Lane 3). Thus, increases in RAV-2-associated RNA in MDV/RAV-2 coinfected cells were not a product of cross reactive MDV or cellular RNA species. These results demonstrate that coinfection with MDV and ALV augments accumulation of retroviral RNA.

Levels of total ALV RNA in MDV/ALV coinfected cells are directly related to input MDV titers. To define more precisely the effect of MDV on RAV-2 RNA levels in coinfected cells, CEF cells were infected with a constant amount of RAV-2 (1 x 10^5 IU) and decreasing amounts of MDV. Total RNA was extracted at 105 hours post-infection and subjected to slot-blot analysis using an ALV specific probe. Consistent with results of northern blot analysis (Figure 3), coinfection of CEF with 1 x 10^5 PFU of MDV and 1 x 105 IU of RAV-2 increased total ALV RNA expression by approximately 5-fold, relative to cells infected with RAV-2 alone (Figure 4). As the amount of input MDV was reduced, total RAV-2 RNA concentration decreased to levels observed in cells infected with ALV alone (Figure 4). Presumably, decreasing the amount of input MDV reduces the number of cells coinfected with MDV and RAV-2. Alternatively, reduced levels of MDV may limit the quantity of MDV encoded or induced factors available for interaction with RAV-2. In either case, our results demonstrate a direct relationship between MDV infection and ALV RNA levels in co-infected cells.

MDV-mediated enhancement of ALV gene transcription in MDV/ALV coinfected cells. Though transactivation of ALV LTR promoters by factors encoded or induced by MDV was the most likely explanation for increases in

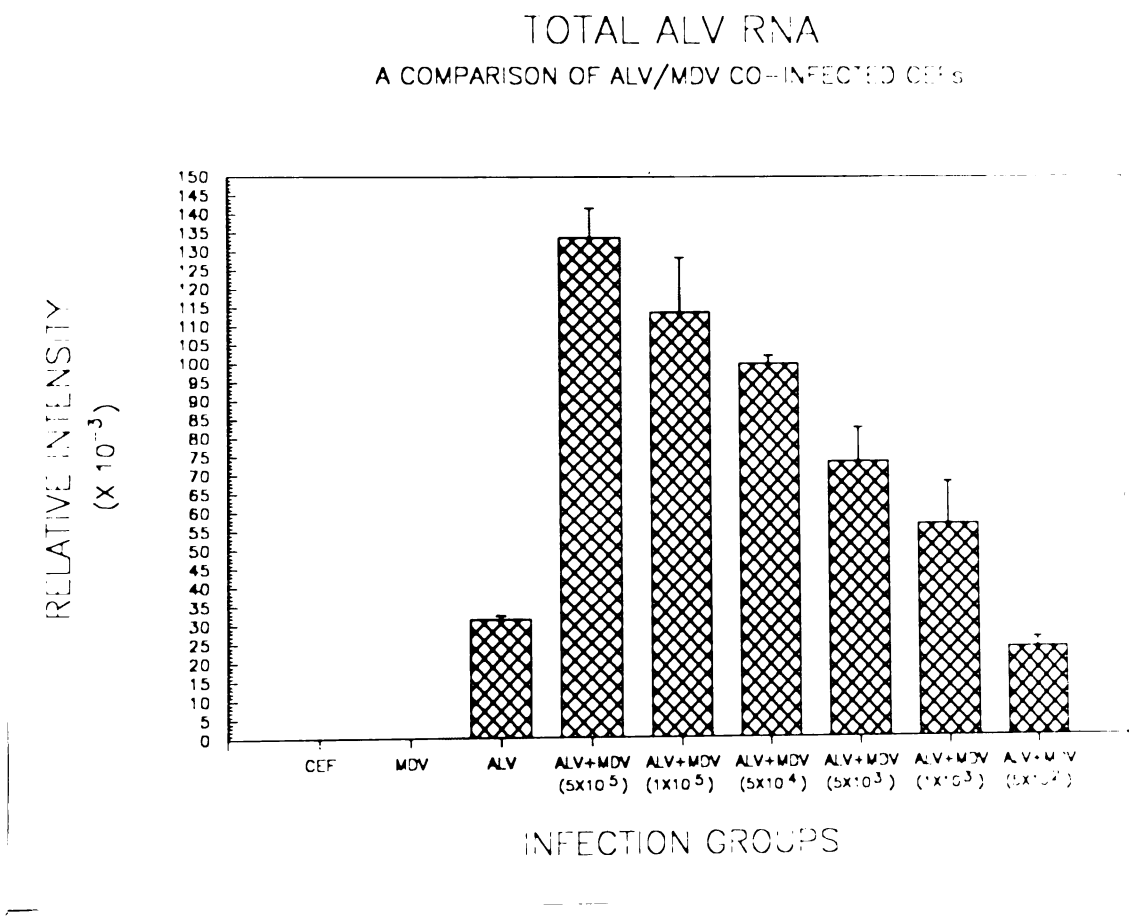

FIGURE 4: Effect of input MDV titer on levels of ALV RNA in coinfected cells.
Total ALV RNA levels in Line 0 CEF cells, coinfected with 1 x 10^5 IU of RAV-2 and various amounts of MDV, was isolated as described (Sambrook et al., 1987, Materials and Methods). PFU of MDV added to each plate of culture sets is denoted in parentheses below the appropriate x-axis position. RNA from coinfected cultures as well as control cultures (uninfected, ALV infected, and MDV infected) was denatured in 2.2 M formaldehyde and spotted unto supported nitrocellulose. All samples were analyzed in triplicate (n=3). Total ALV RNA was detected by hybridization to a 3.9 kb ALV-specific probe as described for Figure 3. Following auto- radiography, RAV-2 RNA hybridized to ALV probe DNA was quantitated by scanning densitometry.

ALV specific RNA following coinfection with MDV, it was possible that MDV increased RAV-2 RNA stability. To distinguish between these possibilities, nuclear run-off transcription assays were performed. Equivalent amounts of radiolabeled nuclear run-off transcription products were used to probe linearized and denatured ALV (Hughes et al., 1987), cloned serotype 1 MDV gp57-65 gene (Coussens and Velicer, 1988), chicken beta-actin (Cleveland et al., 1980), and pBR322 control DNA immobilized on supported nitrocellulose. As expected, run-off transcription of chicken beta-actin remained relatively constant throughout all treatment groups (Figure 5). In contrast, run-off transcription of ALV-specific RNA in nuclei of MDV/RAV-2 coinfected cells wasover 3-fold more efficient than that observed in nuclei from cells infected with RAV-2 alone (Figure 5). Consistent with data from northern blot hybridizations, run-off transcripts from uninfected CEF or CEF infected with MDV alone did not hybridize to ALV DNA. Though we cannot completely rule out the possibility that MDV increases RAV-2 RNA stability, our results clearly demonstrate that factors encoded or induced by MDV infection can activate transcription of ALV RNA in coinfected cells.

MDV-mediated activation of ALV gene transcription results in increased ALV protein expression and virus production. Herpesvirus-mediated enhancement of retroviral transcription may lead to augmentation of retroviral pathogenesis and virus production, provided that ALV specific RNA is efficiently translated in coinfected cells. To determine the effect of MDV-mediated transactivation on retrovirus protein expression incoinfected cells, extracts of coinfected cells and media were analyzed for RAV-2 group-specific (gs) antigen. Cells coinfected with 5×10^5 PFU of MDV and 1×10^5 IU of RAV-2 contained approximately 10-fold more gs antigen than those

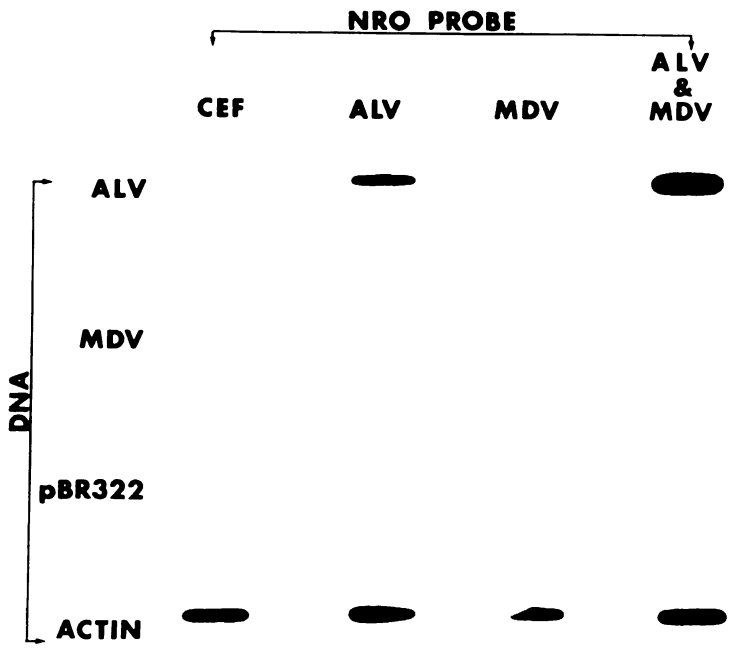

Figure 5: Nuclear run-off transcription assay of MDV/ALV coinfected CEF.
Nuclei were collected 105 hours post-infection from: control uninfected CEF (CEF), CEF infected with 5 x 10^5 IU of RAV-2 (ALV), CEF infected with 5 x 10^5 PFU of strain SB-1 MDV (MDV), and CEF infected with 5 x 10^5 PFU of strain SB-1 MDV plus 1 x 10^5 IU of RAV-2 (ALV & MDV). Run-off transcription reactions and hybridization of radiolabeled transcripts to supported nitrocel- lulose membranes were performed essentially as described by Stewart et al. (1987). Each nitrocellulose strip contained 5 ug each of: plasmid RCAS (cloned ALV genome, Hughes et al., 1987) (ALV, row 1), p19MDA2.35 (Coussens and Velicer, 1987) (MDV, row 2), pBR322 (row 3), and pA1 (chicken beta-actin, Cleveland et al., 1980) (actin, row 4). All plasmid DNA was linearized with appropriate restriction enzymes prior to immobilization on membranes. Due to the low homology between serotype 1 MDV and serotype 2 MDV at the locus represented by p19MDA2.35, hybridization of run-off transcripts to serotype 1 DNA could only be detected following prolonged exposure of membranes to film (data not shown).

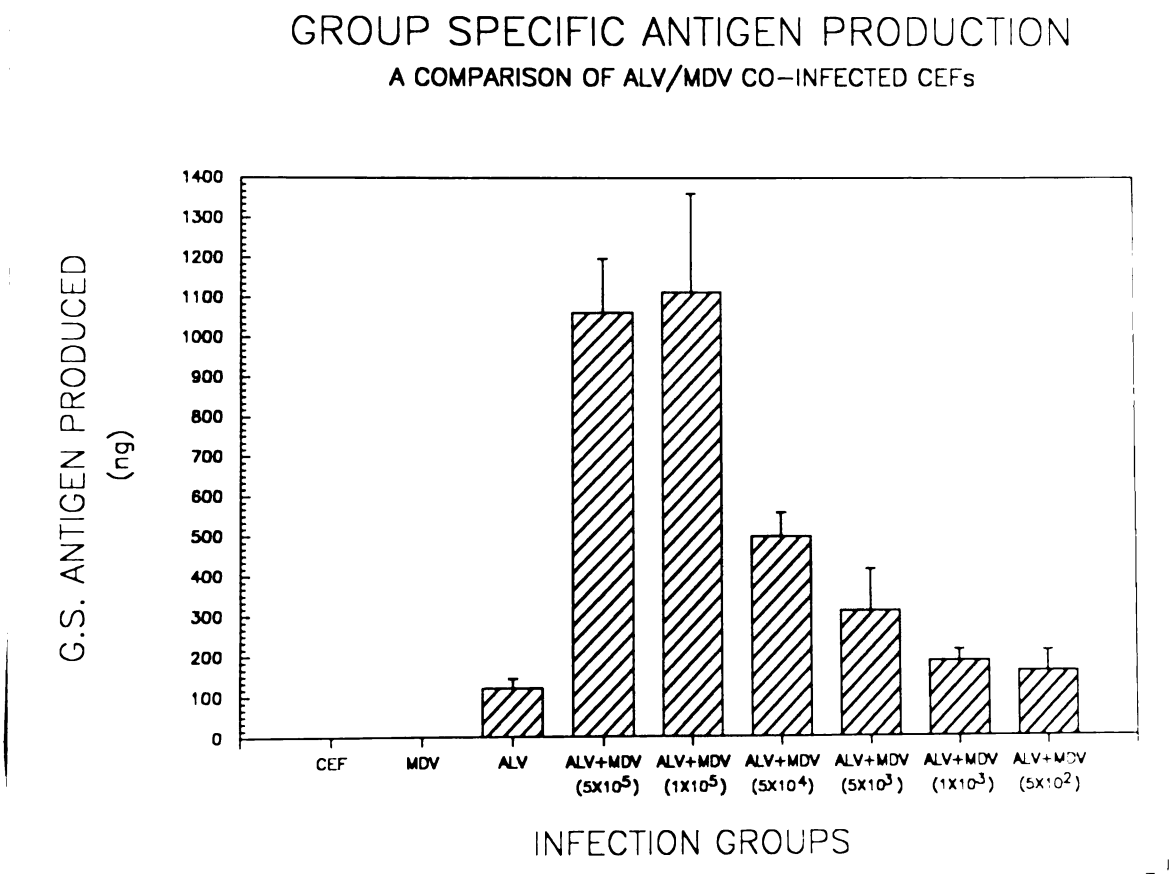

FIGURE 6: Effect of input MDV titer on levels of ALV gs antigen in coinfected cells. Quantities of gs antigen in cell culture lysate were measured by ELISA as described in Materials and Methods. Control cultures included uninfected CEF cells (CEF), CEF infected with 1 x 10^5 IU of ALV alone (ALV), and CEF infected with 1 x 10^5 PFU of MDV alone (MDV). Coinfected groups (ALV & MDV) are labeled with the appropriate input PFU of MDV in parentheses below the x-axis. All coinfected plates contained 1 x 10^5 IU of RAV-2 ALV. Quadruplicate samples of each group were analyzed (n=4). Concentrations of gs antigen were quantitated by comparison to a standard absorbance curve prepared using a known concentration of RSV subgroup C gs antigen (generous gift of Dr. Eugene Smith, USDA-ADOL, East Lansing, MI).

infected with RAV-2 alone (Figure 6). As with RAV-2 RNA levels, gs antigen production in coinfected cells was directly related to the amount of input MDV, falling to near basal levels at MDV concentrations of 5×10^2 PFU per 10^6 CEF cells (Figure 6). Thus, MDV-mediated enhancement of retroviral RNA levels leads to increased production of retroviral proteins in coinfected cells, relative to cells infected with ALV alone.

Retrovirus production requires proper packaging of genomic RNA, assembly and packaging of retrovirus proteins, and budding of mature viral particles from the plasma membrane. To determine if MDV-mediated increases in ALV RNA and protein expression could lead to augmentation of ALV production in coinfected cells, RAV-2 viral particles were isolated from media of CEF cultures infected with RAV-2, MDV, or RAV-2 plus MDV, as well as control uninfected cultures. Virus particles were pelleted by ultracentrifugation, resuspended in a constant volume, and disrupted for assay of reverse transcriptase (RT) activity. RT is carried in the capsid of mature retroviral particles and is thus an indirect measure of retrovirus production. In contrast to gs antigen production and RAV-2 RNA expression, RT activity did not exhibit a direct relationship with respect to amount of input MDV (Figure 7). The highest concentrations of RT activity (approximately 3-fold over RAV-2 alone) were associated with lower amounts (5×10^3 to 1×10^3 PFU) of input MDV. Increasing input MDV to 5×10^5 PFU reduced RT activity to near basal levels (Figure 7), suggesting that higher concentrations of MDV may interfere with RAV-2 virus production, perhaps by competition for host factors involved in virus release. Alternatively, higher concentrations of input MDV may cause extensive cell death, thereby reducing the number of viable cells capable of releasing

matureretroviral particles. Nevertheless, our results clearly indicate that coinfection of cells with MDV and ALV augments retrovirus production. Accumulation of MDV and ALV antigens in single cells. Presumably, direct virus-virus interactions would require both virus types to be resident in a single cell. Alternatively, factors encoded or induced by one virus would need to accumulate within a cell infected with another virus type. To determine if MDV and ALV proteins were capable of accumulating within a single cell, cultured CEF cells coinfected with 1×10^5 PFU of SB-1 MDV and 1×10^5 IU of RAV-2 were permeablized by acetone fixation on glass multi-chambered slides. Fixed cells were treated with anti-ALV and anti-MDV antibodies followed by appropriate second antibodies conjugated to either phycoerythrin (RAV-2) or fluorescein isothiocyanate (FITC) (MDV). Following extensive washing, cells were visualized using an interactive laser cytometer (Meridian Instruments, Okemos, MI). Fluorescence due to both phycoerythrin and FITC was visible in coinfected cells (Figure 8), indicating that both MDV and RAV-2 antigens were present in single cells. Cells containing antigens of only one virus type were also visible in the same field (Figure 8). Control uninfected cells exhibited no detectable fluorescence. Cultures infected with MDV alone or with ALV alone displayed fluorescence due only to FITC or phycoerythrin, respectively (data not shown). In selected fields, between 28% and 88% of cells contained ALV antigens only, while 5% to 28% of cells contained only MDV antigens and 5% to 28% of cells contained antigens of both viruses. Results of interactive laser cytometry suggest that MDV and ALV antigens are able to accumulate in the same cell, thus supporting a direct interaction between the two viruses.

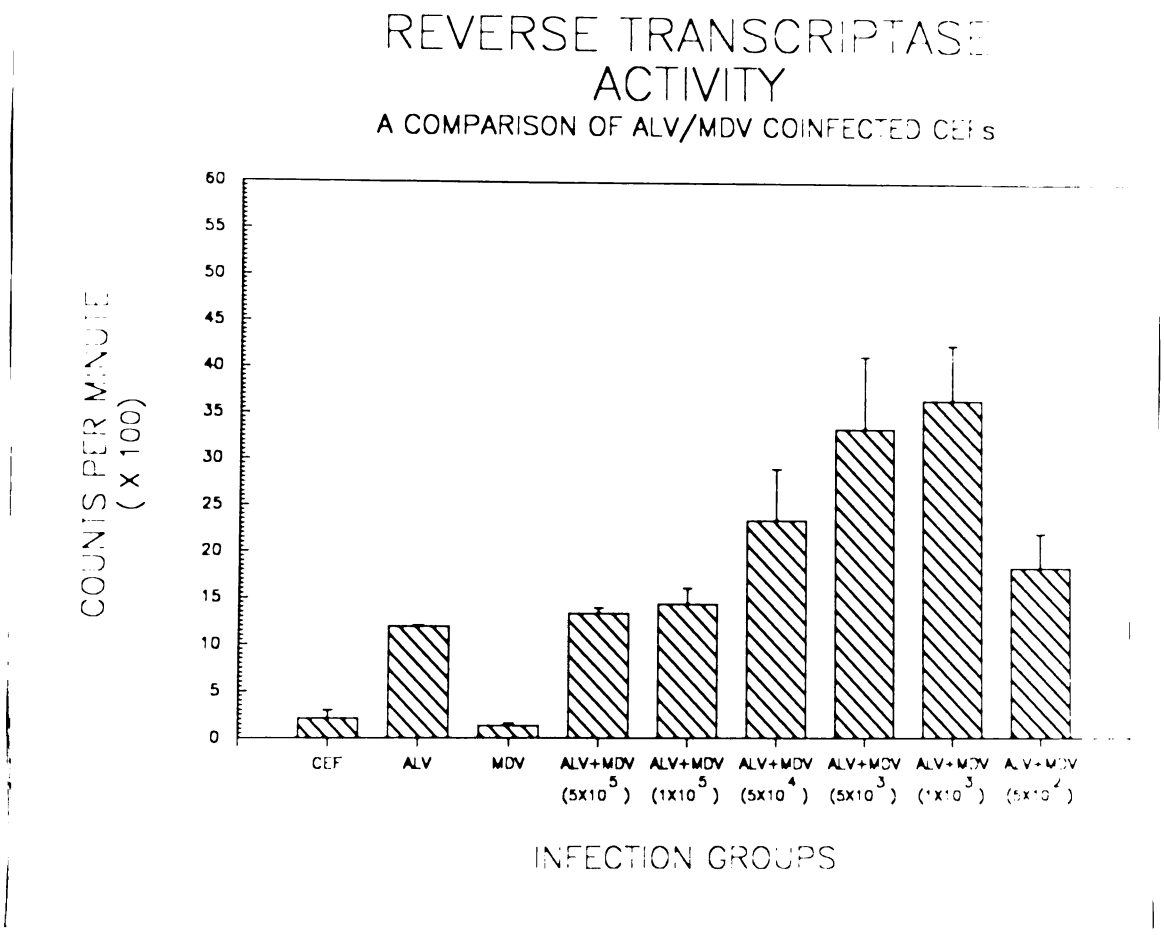

FIGURE 7: Effect of input MDV titer on ALV associated reverse transcriptase activity in media of coinfected cells.
RT activity associated with retroviral particles purified from culture media of MDV/RAV-2 coinfected cells was measured as described in Materials and Methods. Quadruplicate samples of each control and infected group were analyzed (n=4). Controls and infected groups are as detailed for Figures 3 and 4. All coinfected cultures contained 1 x 10^5 IU of RAV-2 and the amount of SB-1 MDV indicated in parenthesis below the appropriate x-axis position.

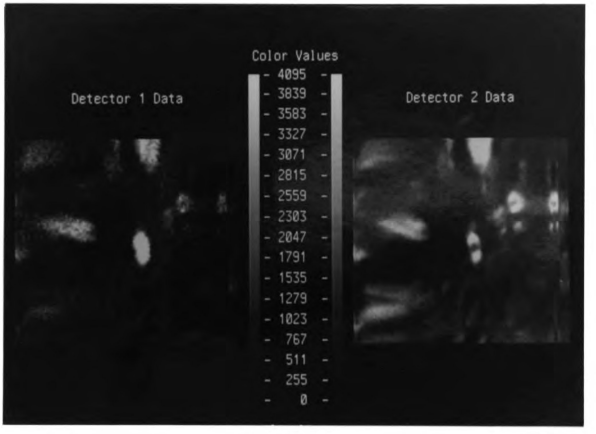

Figure 8: Interactive laser cytometry of MDV/ALV coinfected cells.
Double immune fluorescence assays quantitated by the adherent cell analysis and sorting (ACAS) cytometer (Meridian Instruments, Okemos, MI). Secondary Line 0 CEF cells , were infected with 1 x 10^5 IU of RAV-2 and 5 x 10^5 PFU of SB-1 MDV, diluted 1:10 and transferred to tissue culture chamber slides (1 ml per chamber) (Nunc, Inc., Naperville, IL). Control cells infected with 1 x 10^5 IU of ALV alone, 1 x 10^5 PFU of MDV alone, or uninfected were also plated in chamber slides. Cells were maintained and fixed with acetic acid/acetone prior to antibody incubations as described in Materials and Methods. Rabbit anti-p27 antibodies (SPAFAS, Inc., Storrs, CT) were employed as primary antibody against ALV. A mouse monoclonal antibody, Y-5 (generous gift of Dr. Lucy Lee, USDA-ADOL, East Lansing, MI), specific for serotype 2 MDV (Lee et al., 1983), was used as primary antibody to detect MDV. Secondary antibody for detection of ALV was goat anti-rabbit conjugated to phycoerythrin. Secondary antibody for MDV detection was goat anti-mouse conjugated to fluorescein isothiocyanate (FITC). Different combinations of antibody solution were used as controls to calculate background fluorescence and interference. Fluorescence intensity was measured using an ACAS 470 interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI). Units set to color values are photons measured per unit time. Fluorescent labels were excited with an argon laser at 488nm. Emissions of FITC (detector 1, MDV) at 520 nm and phycoerythrin (detector 2, ALV) between 570 nm and 580 nm were measured by two photomultiplier tubes (PMT) bound by detection limits of 515 nm and 675 nm, respectively. Figure 8 represents a digitized image of detector output.

DISCUSSION

Herpesviruses produce transcription factors capable of transactivating retroviral LTR promoters in a promiscuous fashion (Casarele et al., 1989; Ho et al., 1990; Skolnik et al., 1988; Tieber et al., 1990). Herpesvirus-mediated augmentation of retroviral induced disease by coinfection of susceptible hosts (Bacon et al., 1989), further suggests that herpesviruses may be important cofactors in some retroviral-induced diseases. We have presented evidence which clearly indicates that herpesvirus and retrovirus antigens can accumulate in the same cell, consistent with results of Nelson et al. (1988) which indicate that hCMV and HIV may infect the same cells in AIDS patients. In the case of MDV and RAV-2, MDV-encoded or -induced factors transactivate RAV-2 gene transcription, ultimately leading to increased production of infectious retrovirus from coinfected cells, relative to cells infected with ALV alone. Enhanced expression of RAV-2 RNA and gs antigen is dependent upon the quantity of input MDV. These results suggest that MDV is directly responsible for enhanced ALV gene expression. Results presented in this report do not distinguish between cellular transactivating factors induced by MDV infection and those encoded by MDV. However, subgenomic fragments of MDV are capable of efficiently transactivating the RSV LTR promoter (Tieber et al., 1990; Coussens et al., Manuscript in preparation), suggesting that MDV may encode factors responsible for augmentation of ALV gene expression in coinfected cells. Expression of retrovirus genes occurs following integration of proviral DNA into the host cell genome (review, Weiss et al., 1984). Thus, it seems likely that increased levels of ALV RNA and gs antigen observed in this report result from

MDV-mediated transactivation of integrated provirus LTR promoters. Experiments to determine if MDV is capable of transactivating the LTR promoters of endogenous retroviral loci are in progress. It is possible that MDV-mediated transactivation of endogenous retroviral loci may augment expression of cellular genes adjacent to the provirus insertion site. In the case of ALV insertion near the myc proto-oncogene locus, immortalization and/or oncogenic transformation may result. In this scenario, a non-oncogenic herpesvirus and a benign retrovirus (i.e. RAV-0) may combine to produce a lethal neoplasm. In the case of HIV and CMV, activation of HIV gene transcription increases production of the tat protein (Sklonik et al., 1988; Ho et al., 1990). Subsequent tat-mediated activation of CMV gene expression results in a truly synergistic coinfection. In the case of MDV and ALV, however, increased levels of ALV gene expression and virus production result in reduced titers of MDV, relative to cells infected with MDV alone (Frankel and Groupe, 1971). Evidence from our laboratory suggests that ALV proviruses integrate into MDV genomes in coinfected cells. In many cases, ALV integration produces a lethal mutation, thus reducing MDV titers (Wilson and Pulaski, unpublished observations). Interactions between ALV and MDV thus resemble a parasitic, rather than a synergistic relationship.

ACKNOWLEDGMENTS

We thank S. Conrad, H. Roehl, L.P. Provencher, M. Flesverb, and J. Sell for helpful advice and assistance, A. Fadly for helpful discussions and for providing RAV-2 ALV, and L. Lee for providing Y-5 monoclonal antibody. We also thank H.A. Tucker and J.J. Ireland for helpful suggestions and critical review of the manuscript. This work was supported, in part, by grants # 88-37266-3983 and 90-34116-5329 awarded to P.M. Coussens under the Competitive Research and Special Research Grants Programs, respectively, administered by the U.S. Department of Agriculture, by the Michigan Agricultural Experiment Station, and the Research Excellence Fund, State of Michigan.

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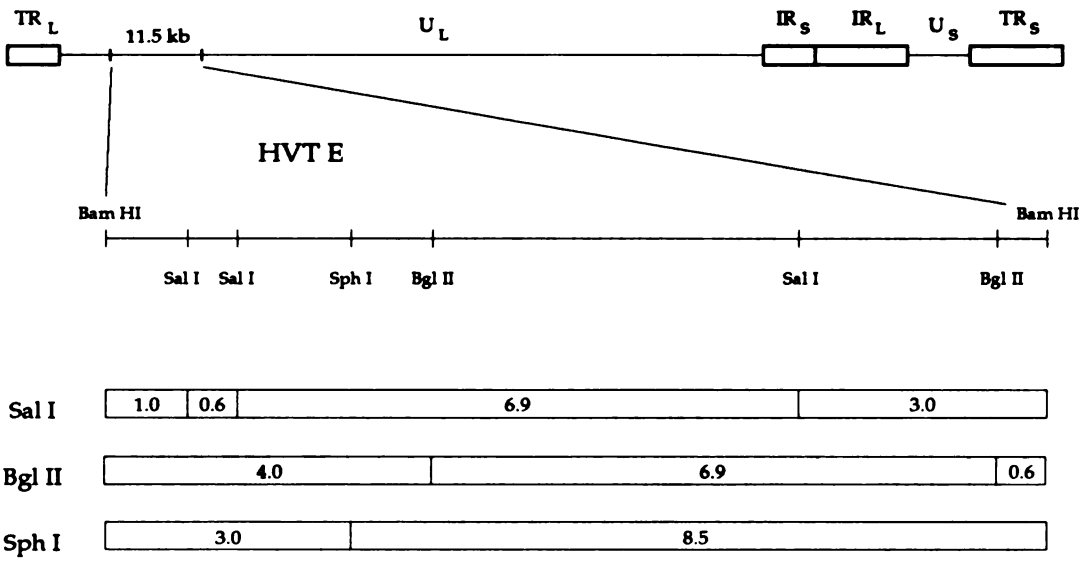
CONTINUED RESEARCH

As described before, herpes virus of turkeys (HVT) not only is similar to MDV in genomic structure (Cebrian et. al., 1982) and antigenic properties (Witter et. al., 1970a), but also has displayed homology in hybridization studies (Igarashi et. al., 1987). Because it also had demonstrated the ability to enhance CAT expression in RSV-CAT constructs (Tieber et. al. 1990), it is a viable alternative to MDV for characterization of transactivating genes.

CAT assays were used by Dr. Paul Coussens to characterize the site in HVT that expresses the gene responsible for enhancing gene expression. Transient assays using an RSV LTR-CAT construct were performed on different HVT Bam HI restriction fragment clones. Clones were separated into five groups according to where they mapped on the HVT genome. Cotransfection with RSV-CAT established the HVT Bam HI E clone to be responsible for the highest level of CAT enhancement (Personal communication, Dr. Paul Coussens). HVT E is approximately 11.5 Kbp in length and is cloned into a 6.4 Kbp vector, pHC 79 (Fukuchi et. al. 1984). It lies on the right hand side of the U_L region of HVT's genome (Figure 9) and has exhibited homology to Bam H1 restriction fragments Q_1 and G in southern blot analysis of MDV serotype 1 GA strain (Igarashi et. al. 1987). To further ascertain the enhancer expression site on the HVT E fragment, a restriction endonuclease map was made (Figure 9). Subclones were later to be constructed and employed in transient assays with RSV-CAT.

To construct a restriction endonuclease map, purified HVT E clone and HVT E fragment were digested with Bam HI, Bgl II, Eco RI, Sal I, and Sph I in different combinations. The digests were subjected to electrophoresis on a



Sizes in kilobase pairs (Kb)

Figure 9 HVT Bam H1 E Restriction Enzyme Map

0.8% agarose gel at 50 V next to a λ Hind III ladder. Ethidium bromide stained gels were then studied for fragment sizes and restriction patterns.

Sal I digests formed four fragments ranging in size from 0.6 to 6.9 Kbp. Three fragments of 0.6, 4, and 6.9 Kbp were generated by Bgl II digestion. Cutting with Sph I yielded two fragments 3.0 and 8.5 Kbp in length. Because Eco RI did not cut the E fragment it was used in double digests of the Bam HI E clone to position other restriction sites. See figure 9 for the restriction enzyme map.

Used in CAT assays, the subclones and restriction enzyme map will help localize the gene(s) responsible for ALV enhancement. This manner of limiting the number of base pairs to sequence and study will accelerate further research.

SUGGESTED RESEARCH

Continued research should begin with investigation of Marek's disease virus infection in avian leukosis virus subgroup E infected cells. It could be of great interest to the poultry industry if vaccine strains of MDV were activating latent ALV. ELISA, reverse transcriptase assays, RNA quantitation, and nuclear run-off transcription assays could all be used to detect any changes in endogenous virus activity.

Research investigating the transactivator gene down to as small an area as possible. Because ALV enhancer expression has already been located in the HVT E. Using chloramphenicol acetyl-transferase (CAT) assays, we can test for transactivator gene expression in HVT E fragment subclones. When the site of expression is found, it should be sequenced. Once open reading frames are identified, they can be compared to other known herpes virus sequences for similarity.

To further characterize the transactivating protein, running SDS-PAGE on HVT E transfected whole cell extracts could determine it's size and number of subunits it contains. Comparison with similar extracts run on a non-denaturing gel (PAGE) could partially determine mechanism of enhancement if the transactivating protein binds to other cellular proteins. Protein sequence data derived from nucleotide sequence should also be compared to other protein sequences for similarity.

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