Marek's Disease Virus Latency

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1 Introduction

Marek's disease (MD) is one of the most fascinating herpetic disorders because infection of susceptible chickens results in the rapid formation of T-cell lymphomas that are preventable by vaccination (Calnek and Witter 1997). For almost half a century, the commercial poultry industry has been dependent on effective MD vaccines. Because Marek's disease virus (MDV) is a relatively difficult herpesvirus to study, information regarding it has lagged behind that for some other herpes-

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viruses. Latency is no exception to this general statement. MDV latency appears somehow connected to transformation, although the relationship between these two states is by no means clear. For example, it is not known whether latency is a precursor to transformation or whether it is a parallel manifestation of disease. In this review, I will attempt to define latency for MDV, describe systems in which it can be studied, review what is known about transcription during latency, and point out gaps in our existing understanding of this mode of infection.

1.1 Definition of Latency

Herpesvirus latency is defined as the presence of the viral genome without production of infectious virus except during episodes of reactivation (FELDMAN 1991; FRASER et al. 1992; GARCIA-BLANCO and CULLEN 1991; STEVENS 1989; WAGNER 1991). Latency is distinguished from both abortive and nonpermissive infections in that it is reversible. Maintenance of a reservoir of latently infected cells within an immunocompetent host is critical to viral persistence. The molecular details of latency vary among herpesviruses and are intimately related to the nature of the host cell that is latently infected. Herpesviruses that latently infect neurons, such as herpes simplex virus type 1 (HSV-1), exhibit very little viral gene expression during latency since the neuron is a nondividing cell. On the other hand, herpesviruses that latently infect lymphocytes, such as Epstein-Barr virus, exhibit more viral gene expression during latency. These lymphotropic herpesviruses must replicate to the extent that they ensure their continued presence in dividing lymphocytes.

MDV assumes a latent posture in T cells (CALNEK and WITTER 1997; CALNEK et al. 1981, 1984) and can be reactivated by cell culture propagation of T cells isolated from infected chickens (CALNEK et al. 1981a, 1984). In transformed T cells, whether tumors or lymphoblastoid cells, MDV is in some form of latent state. However, it is not clear that latency in transformed cells precisely mirrors latency in non-transformed T cells. In addition, it seems reasonable that various states of latency exist certainly among lymphoblastoid cell lines, perhaps among tumors, and even perhaps among non-transformed latently infected cells.

1.2 Systems for Studying MDV Latency

Systems for studying MDV latency can be divided into those that involve non-transformed cells and those that use transformed cells. One system that falls in the former class is simply the use of non-transformed T cells isolated from MDV-infected chickens (CALNEK et al. 1981a). Latency is difficult to study in non-transformed T cells because of problems with achieving long-term culture of these cells. Maintaining latency in T-cell cultures derived from MDV-infected chickens is even more difficult since the virus readily reactivates once the cells are placed in culture. This system has been most useful for defining factors that

maintain latency of T cells (discussed in Sect. 5) (Buscaglia and Calnek 1988). In addition, herpesvirus of turkeys (HVT) has been used to study latency of chicken herpesviruses outside the context of transforming infections (see Sect. 2) (Holland et al. 1996, 1998, 1999).

Most studies regarding latency have been done on lymphoblastoid cell lines. These cell lines are derived from MDV-induced tumors and many have been characterized to varying degrees (Nazerian 1987). In lymphoblastoid cell lines, MDV gene expression appears to be carefully regulated with the majority of transcription being derived from the repeat regions of the viral genome. Most lytic genes are not expressed in these cell lines, but treatment with iododeoxyuridine and other nucleoside analogs induces expression of lytic genes and production of viral antigens (Dunn and Nazerian 1977; Calnek et al. 1981).

Recent work involving lymphoblastoid cell lines derived from tumors induced by recombinant MDV strains indicates that marker genes expressed from a lytic promoter (SV40 early) inserted into the MDV genome are not efficiently expressed in lymphoblastoid cells, indicating that the genome in these cells is maintained in a latent state. However, marker gene expression is efficient in MDV that has been chemically reactivated from these cells, regardless of whether the marker gene is *lacZ* (Parcells et al. 1999) or *gfp* (Dienglewicz and Parcells 1999). Regulation of gene expression in these recombinant virus-derived cell lines does not appear to be mediated by methylation. Since they contain a marked virus, these cell lines provide a very useful system for studying MDV latency and reactivation.

Another model system for studying MDV latency is the fibroblastic OCL cell line system. These cell lines are derived from OU2 cells, chemically transformed chicken embryo fibroblasts (CEF) (Ogura and Fujiwara 1987). Two of the cell lines, OU2.1 and OU2.2, harbor MDV in a latent state when the cells are sub-confluent (Abujoub and Coussens 1995, 1996, 1997). When these MDV-infected cell lines reach confluency, MDV is reactivated. Related cell lines have been described that harbor serotype 2 MDV (SB1-OCL) and serotype 3 HVT (HVT-OCL) also in the latent state until the cells reach confluency (Abujoub and Coussens 1999). Although these cell lines have not been widely accepted as CEF substitutes for MDV vaccine production, they do offer a model system that has yet to be fully exploited for studying latency and reactivation of all three serotypes.

Avian leukosis virus (ALV)-induced B cell tumors can harbor latent serotype 2 MDV. In one report, latent SB-1 was discovered in B-cell lines established from ALV-induced tumors present in MDV-vaccinated chickens (Fynan et al. 1993). Fewer than 3% of these cells express MDV antigens unless 5-azacytidine is present during culture to prevent methylation. A similar finding has been reported for LSCC-BK3 clone A (BK3A), an ALV-induced B-cell line that harbors a serotype 2 MDV designated 2H (Hihara et al. 1998). About 40% of BK3A cells express a serotype 2 MDV antigen, but another cell line established from the same ALV-infected chicken (LSCC-BK3 clone 2C) does not express serotype 2 MDV antigens.

2 The MDV Genome During Latency

Ross pointed out that latently infected, non-transformed T cells contain very few copies of the MDV genome. In particular, MDV DNA is not detectable in these cells by in situ hybridization, with the limit of detection being five genome copies/cell. These cells do appear to be latently infected, however, for when cultured, viral antigens are produced and viral DNA is detectable by in situ hybridization (Ross 1985).

Latently infected, transformed cells from both lymphoblastoid cell lines and tumors harbor greater numbers of the MDV genome (Ross et al. 1981). For lymphoblastoid cell lines, the number of MDV genome copies present reflects the nature of the cell line. In particular, MDCC-HPRS1 and MKT1 have been reported to harbor 8 to 15 genome copies (Kaschka-Dierich et al. 1979; Tanaka et al. 1978), but MSB1 cells can harbor from 60 to more than 100 genome copies (Kaschka-Dierich et al. 1979; Nazerian and Lee 1976).

Viral DNA in lymphoblastoid cells and in tumor cells appears to be generally intact (Ross 1985). In lymphoblastoid cells, the viral genome exists in both episomal and integrated forms depending on the cell line (Kaschka-Dierich et al. 1979; Tanaka et al. 1978). Using Southern hybridization, Gardella gel electrophoresis, and in situ hybridization of metaphase and interphase chromosomes, it has been demonstrated that integration of viral DNA into the host chromosome occurs readily (6/6 cell lines examined) (Delecluse and Hammerschmidt 1993). The preferred integration sites vary among integration events but generally lie near the telomeres of large- and mid-sized chromosomes or on the minichromosomes. Covalently closed circular DNA is occasionally seen (1/6 cell lines examined), and most of the cell lines (4/6) have a small population of cells undergoing lytic infection and producing linear MDV genomes. When similar techniques were applied to the analysis of the MDV genome in lymphomas, episomal forms of the viral DNA were not found, viral DNA was randomly integrated at multiple chromosomal sites, and clonality of tumors was obvious from the integration patterns (Delecluse et al. 1993).

3 Transcription During Latency

3.1 General Patterns of Transcription in Latently Infected Cells

3.1.1 MDV

An early report indicated that 12–14% of the MDV genome is transcribed in lymphoblastoid cells (Silver et al. 1979). Northern analysis on polyA+ RNA purified from three lymphoblastoid cell lines (MDCC-MSB1, MDCC-RP1, and MDCC-LS1) indicated that the majority of transcripts hybridize to the repeats

flanking the unique long (UL) region of the genome (HIRAI et al. 1988). Another report indicated that only a few transcripts (< 10) are detectable in lymphoblastoid cell lines [MDCC-HP1 (non-producer) and MDCC-CU41 (non-expression)], and these are derived from immediate-early genes that map in the unique short (US) region and in the repeat sequences flanking the UL (IRL) and US (IRS) regions (SCHAT et al. 1989). Others have reported that 29–32 transcripts are derived from many sites on the genome (MARAY et al. 1988). Using polyA+ RNA and probing Northern blots with sequences representing about 95% of the MDV genome, SUGAYA et al. (1990) reported that 29 viral transcripts are present in kidney lymphomas and 32 in MKT-1 cells. These transcripts hybridize to only 20% of the genome, the relevant regions being the IRL, IRS, and the adjacent unique sequences (SUGAYA et al. 1990).

Given the difficulties of the MDV system, and the lack of genomic sequence information at the time of these studies, they are remarkably consistent. Minor differences could reflect the nature and passage history of the lymphoblastoid cell lines or the state of latency/reactivation of the tumors at the time of harvest for RNA purification. Taken together, these results indicate that transcription of the MDV genome in MDV-transformed lymphoblastoid cells and tumors is more extensive than that of the latent HSV-1 genome, but less extensive than that of the MDV genome in productively infected cells. The repeat regions of the genome are particularly active transcriptionally during latency and these RNAs are, to some extent, also present in cytolytically infected cells.

3.1.2 HVT

Transcription of the HVT genome during latent infections has been studied by in situ hybridization (HOLLAND et al. 1996). Latent infections in these studies are defined as infections that are positive for in situ hybridization with probes from the repeat regions of the genome but negative for in situ hybridization with a glycoprotein B (gB)-specific probe, gB transcription being indicative of productive infection. Latent infections have been reported for lymphoid, nerve, and feather tissues of HVT-infected chickens (HOLLAND et al. 1998). Moreover, the patterns of latent gene expression were found to differ among spleen, thymus, and nerves, suggesting that even in latency, there is tissue specificity to gene expression patterns (HOLLAND and SILVA 1999).

3.2 Analysis of cDNAs from Lymphoblastoid Cells and Tumors

Analysis of cDNAs derived from the *Bam*HI-H region of the genome led to the identification of the pp38/pp24 gene (CHEN et al. 1992). See Sect. 4.2 for a more extensive discussion of pp38/pp24.

Two cDNAs derived from the *Bam*HI-I2 region of the MDV genome present in MKT-1 cells have been analyzed in detail (PENG et al. 1995). One of these cDNAs extends into the adjacent *Bam*HI-Q2 and -L fragments, represents an

abundantly expressed transcript in lymphoblastoid cells, and corresponds to the *meq* gene. The other cDNA is also related to the *meq* transcript but contains only the DNA-binding domain sequence spliced to sequences present in *Bam*HI-L. Both transcripts are present in both latently infected lymphoblastoid cells and lytically infected cells. A more detailed discussion of Meq is given in Sect. 4.1 and also in another chapter of this volume.

Analysis of cDNAs prepared from polyA+ RNA purified from MDCC-CU41 lymphoblastoid cells led to the identification of two cDNA clones that correspond to transcripts derived from the *Bam*HI-Q2 and -L regions (Ohashi et al. 1994b). These transcripts are 2.5, 0.8, and 0.6kb in size. The 0.6-kb transcriptional unit contains an ORF, termed ORF L1, that could encode a 107-amino-acid protein. ORF L1 corresponds to the *meq* gene and is related to the cDNAs described by Peng et al. (1995) (see above). Analysis of two MDV strain CVI988-based mutants in which ORF L1 has been disrupted indicated that ORF L1 is not required for virus replication in cell culture or in chickens, nor is it required for the establishment or reactivation from latency in chickens (Schat et al. 1998).

This same MDCC-CU41 cDNA library yielded three groups of MDV-specific clones that map to the IRS located within *Bam*HI-A. It is not entirely clear that these cDNAs represent latent transcripts since the amounts of the corresponding transcripts present in lymphoblastoid cells are much less than in lytically infected cells. One of the clones (A41) contained a small open reading frame (ORF). Antibody prepared against a bacterial fusion protein consisting of glutathione-*S*-transferase and the A41 ORF binds to a cytoplasmic antigen in lytically infected cells, but the antigen is not detectable in lymphoblastoid cells (Ohashi et al. 1994a).

3.3 Latency-Associated Transcripts and the RB1BLAT*lac* Mutant

We (Cantello et al. 1994, 1997), and others (Li et al. 1994, 1998; McKie et al. 1995), identified and characterized a group of latency-related RNAs all of which map antisense to the MDV ICP4 gene. Figure 1 shows a summary of our current understanding of the structures of the latency-associated transcripts (LATs) of MDV. The LATs reported so far include two small, spliced RNAs (0.9 and 0.75kb), named MSRs (Marek's disease virus small RNAs) (Cantello et al. 1994, 1997). The difference between the MSRs is unknown. The MSRs have also been referred to as SARs (Ross et al. 1996) and S RNA (Li et al. 1998). The splice junctions of the MSRs during latent and late productive infection were determined by sequencing RNA-PCR products generated with primers that flank the 3′ splice region (Cantello et al. 1997). The MSR contains four introns, the largest of which is 4852nt long and overlaps the ICP4 putative translational start site. In addition, a large 10-kb RNA first discovered by Northern analysis (Cantello et al. 1994) and also referred to as L RNA (Li et al. 1998) has been characterized. Other latency-related RNAs include three spliced, 3′ coterminal RNAs discovered by analyzing cDNAs (2.2, 1.8, and 0.5kb in size) that were grouped into classes based on three different splicing patterns (McKie et al. 1995). An additional 2.7-kb cDNA having

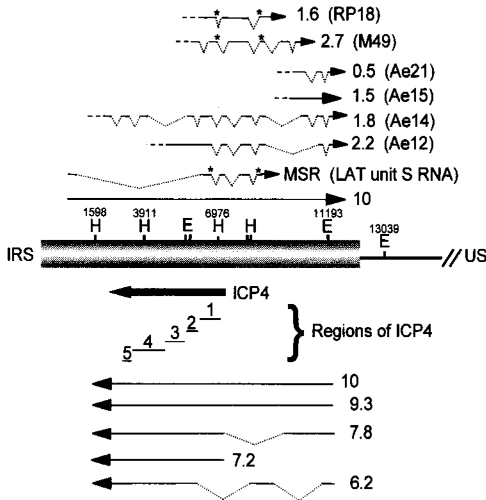

Fig. 1. Diagram of the known latency associated transcripts (*LATs*) and ICP4 transcripts of serotype 1 MDV. The *center portion* of the figure shows a portion of the MDV IRS and the US region. The positions of *Eco*RI and *Hind*III recognition sites are indicated and, in some cases, numbered using the IRS sequence published by McKie et al. (1995). The regions of the predicted ICP4 coding region are diagrammed (Anderson et al. 1992). The *upper portion* of the figure shows the LATs of MDV and their splicing patterns. *Asterisks* denote common splice sites. The *dashes* to the left of spliced LATs indicate that the precise 5′ ends of these species are unknown since they were identified by characterization of cDNAs. The sizes of the LATs are indicated to the *right* of the figure and alternative names for these RNAs used by Li et al. (1998) are indicated in parentheses. The 2.2-, 1.8-, and 0.5-kb cDNAs were identified by McKie et al. (1995), the 1.6- and 2.7-kb cDNAs were identified by Li et al. (1994 and 1998, respectively), the 10-kb RNA was identified by Cantello et al. (1994) and Li et al. (1994), and the MSR was identified by Cantello et al. (1994). The MSR is the same as SAR reported by Li et al. (Ross et al. 1996, 1997). In the *lower portion* of the figure, transcripts corresponding to the ICP4 coding region and its upstream region (which is in frame with the coding region) and their known splice sites are diagrammed (Morgan et al. 1996)

a different splicing pattern defines yet another LAT derived from this region of the genome (Li et al. 1994), as does a 1.6-kb cDNA (Li et al. 1998). Each of the small RNAs has a unique overall splicing pattern, although some splice donor and acceptor sites are shared. The MSRs are nonpolyadenylated, but the 10-kb RNA contains polyA (Cantello et al. 1997). The other small RNAs are presumed to be polyadenylated, since they were discovered by the analysis of oligo-dT-primed cDNAs (Li et al. 1994; McKie et al. 1995).

Based on RNase protection studies and Northern hybridizations, the 5′ end of the MSR has been placed at position 949 [numbering is based on the published sequence of the MDV IRS (McKie et al. 1995)] approximately 5kb upstream from the main body of the transcript. The nearest TATA box (TATAA) is located 26nt upstream at position 919–923, and another TATA box (TTATAT) is located 49nt upstream at positions 895–900. There is a potential αTIF-binding site 73nt upstream at position 866–876. αTIF is a component of the HSV-1 tegument that recognizes the core consensus sequence TAATGARAT in the promoter regions of immediate-early genes (Kristie and Roizman 1987), and a potential αTIF recog-

nition site lies within the promoter regions of HSV-1 LATs (BATCHELOR and O'HARE 1990; LAGUNOFF and ROIZMAN 1995; ZWAAGSTRA et al. 1990, 1991). It is possible that the other small LATs initiate at this same promoter since they were defined by cDNAs that might not have been full-length.

Although the precise 5′ end of the MDV 10-kb LAT is not known, based on Northern hybridizations and RNase protection data, it appears that the end is identical to or lies very near to the 5′ end of the MSR (CANTELLO et al. 1997; LI et al. 1998). The 10-kb RNA lies entirely within the repeats flanking the US region of the genome. Furthermore, the large LAT overlaps almost perfectly a large 10-kb ICP4 transcript (MORGAN et al. 1996; LI et al. 1998). It should be noted that remarkably similar results regarding the nature of this transcript have been obtained by two groups working independently with different cell lines and strains (CANTELLO et al. 1997; LI et al. 1998).

MDV LATs have been detected in at least three lymphoblastoid cell lines, namely MSB1 cells (CANTELLO et al. 1994; LI et al. 1994; MCKIE et al. 1995), MKT-1 cells (MCKIE et al. 1995), and RPL1 cells (LI et al. 1994), and in MDV-induced lymphoma tissue (CANTELLO et al. 1997; LI et al. 1994). Although these latency-related RNAs are abundant in MDV-induced lymphoblastoid cells, they are also detectable in productively infected CEF (LI et al. 1994; MCKIE et al. 1995), particularly late during infection (CANTELLO et al. 1997). At least for the MSRs and the 10-kb RNA, iododeoxyuridine treatment of MSB1 cells, which results in MDV reactivation, causes a decrease in the steady-state levels of the latency-related RNAs but an increase in the steady-state level of ICP4 RNA (CANTELLO et al. 1994). The pattern of expression of these LATs suggests that they are inversely related to MDV productive infection (CANTELLO et al. 1994; LI et al. 1994). Synthesis of the large RNA (and possibly all of the LATs) is sensitive to cycloheximide treatment and therefore requires protein synthesis (LI et al. 1998).

We have constructed a mutant, designated RB1BLAT*lac*, in which we disrupted the 5′ end of the MSR at the LAT promoter region. The transfer vector used to make the LAT mutant consisted of pMD206 (CANTELLO et al. 1997) in which the *Msc*I site at position 1055 was disrupted by addition of a *lacZ* cassette. The *Msc*I site lies just downstream from the TATA boxes, and disruption of this site should knock out transcripts that initiate at this particular promoter. The mutant was constructed in the genetic background of the oncogenic RB1B strain of MDV using procedures that we have used previously to construct RB1B-based mutants.

Southern hybridization indicated that the mutant had the expected genomic structure and did not contain parent virus (Fig. 2). Total DNAs from CEF infected with the parent virus (RB1Bp19), a derivative of the parent virus that was passed alongside the mutant during its construction (RB1Bp28), or three of the ten putative LAT mutants (RB1BLAT*lac*1-10) were digested with *Spe*I and *Nco*I, and Southern blots were prepared using standard methods. The MDV-specific probe detected a 2.2-kb fragment in DNA from the parent viruses. This fragment was not present in DNA from CEF infected with the putative RB1BLAT*lac* mutants and instead the MDV-specific probe detected two fragments of 5.5kb and 0.6kb, as expected. The *lacZ*-specific probe detected nothing in DNA from CEF infected

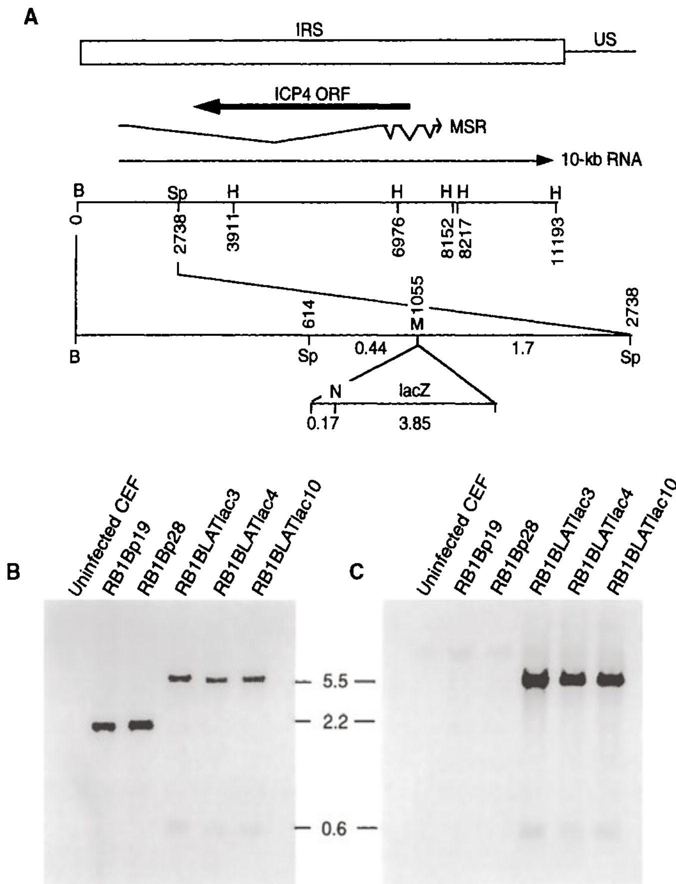

Fig. 2A–C. Southern hybridization analysis of the RB1BLAT*lac* mutant and the corresponding parent MDV strain. **A** Diagram of the relevant region of the MDV genome. Nucleotide positions correspond to the published IRS sequence (McKie et al. 1995). The positions of the ICP4 ORF, the MSR, and the 10-kb LAT are indicated. *B*, *Bam*HI; *H*, *Hind*III, *M*, *Msc*I; *N*, *Nco*I; *Sp*, *Spe*I. **B** The MDV-specific probe was a 1-kb *SpeI*/*Hind*III fragment purified from pMD206 (Cantello et al. 1997), which spans the *Msc*I insertion site. **C** The *lacZ*-specific probe was a 4.0-kb *Bam*HI fragment purified from p*lacZ* (Sondermeijer et al. 1993). For **B** and **C**, each lane contains 10μg of digested DNA and sizes are indicated in kb pairs. The MDV-specific probe detected a 2.2-kb fragment in DNA from the parent viruses. This fragment was not present in DNA from CEF infected with the putative RB1BLAT*lac* mutants and instead two fragments of 5.5kb and 0.6kb were present. The *lacZ*-specific probe detected nothing in DNA from CEF infected with the parent viruses but specifically hybridized to the 5.5-kb and 0.6-kb fragments in DNA from CEF infected with the putative RB1BLAT*lac* mutants

with the parent viruses but specifically hybridized to the 5.5-kb and 0.6-kb fragments in DNA from CEF infected with the putative RB1BLAT*lac* mutants. Thus, the Southern hybridization pattern indicated that insertion of the *lacZ* mutagenesis cassette occurred as expected and that the mutant virus stocks did not contain parent virus. In addition, the mutant stocks all appeared to be diploid; i.e., the *lacZ* cassette was present in both the IRS and TRS copies of the LAT promoter. We used PCR to confirm diploid insertion of the *lacZ* cassette and the absence of parental virus in our stock (data not shown). Finally, standard PCR reactions

(Silva 1992; Zhu et al. 1992) were used to verify that the RB1BLAT*lac* mutant stocks had not sustained expansion of the 132-bp repeat region but had a pattern of repeats that was indistinguishable from that of the parent viruses. The parent viruses and all of the mutants had 1–3 copies of the 132-bp repeat (data not shown).

Northern hybridizations indicated that the defect in the RB1BLAT*lac* mutant had some effect on, but did not totally ablate, LAT transcription (data not shown). It is likely that the observed transcription stems from numerous promoter-like elements downstream from the cassette insertion site. Indeed, a TATA-less HSV-1 LAT promoter, designated LAP2, is located between LAP1 (the major LAT promoter) and the 5′ end of the 2.0-kb LAT (Goins et al. 1994). LAP2 shows similarity to promoters of housekeeping genes. Secondary promoters for the MDV LATs remain to be described.

The RB1BLAT*lac* mutant and its parent virus have been tested in chickens and the results are shown in Table 1. One-day-old specific-pathogen-free single comb white leghorn chickens (SPAFAS, Norwich, Conn., USA) were wingbanded, randomly assigned to experimental groups, housed in isolation units, and maintained under negative pressure. At 1 day of age, chickens were inoculated intra-abdominally with the RB1Bp19 original parent virus, the equal passage level RB1B virus (RB1Bp28), the RB1BLAT*lac* mutant, mock-infected cells, or nothing at all. Titers were done on the actual inocula used in the experiment and are shown in Table 1. At 6, 12, and 18 days post-inoculation, spleen cells, thymocytes, and peripheral blood leukocytes (PBL) were isolated from three chickens per group for virus reisolations as described previously (Parcells et al. 1994a,b, 1995). Two weeks after the initiation of the experiment, 1-day-old chickens were obtained from the same breeder flock as the original chickens and placed in the isolation units with the 14-day-old inoculates. These chickens were exposed to MDV only by contact with the previously inoculated chickens, which at this time should have been shedding MDV. Two weeks after placement of the contact-exposed chickens (28 days after the initiation of the experiment), virus reisolations from spleen,

Table 1. Incidence of tumors in chickens exposed to RB1BLAT*lac*

Virus[a]	Inoculates		Contacts	
	No. of chickens[b]	No. of tumors (%)	No. of chickens[b]	No. of tumors (%)
None	4	0 (0)	4	0 (0)
RB1Bp19				
232 PFU	15	9 (60)	8	6 (75)
RB1Bp28				
151 PFU	12	0 (0)	10	0 (0)
1560 PFU	14	4 (29)	10	3 (30)
RB1BLAT*lac*p28				
169 PFU	15	0 (0)	9	0 (0)
2010 PFU	15	0 (0)	11	0 (0)

[a] For inoculates, the dose in PFU/chicken is indicated. For contacts, the indicated dose is that inoculated into cage-mates. RB1Bp19 is the original parent strain, RB1Bp28 is a derivative of the parent strain that was passaged in cell culture alongside the mutant during its construction, and RB1BLAT*lac* is the mutant.

[b] Number of chickens with gross Marek's disease lesions.

thymus, and PBL were done from three contact-exposed birds and three inoculates from each group. Both inoculates and contact-exposed chickens were observed daily for mortality until 8 weeks after the initiation of the experiment. At the end of the 8-week period, all remaining chickens were euthanized. Chickens were examined for gross MDV lesions such as tumors in the liver, spleen, gonads, kidney, heart, and intestines.

In vivo results indicated that the RB1BLAT*lac* mutant failed to cause tumors in either inoculates or contact-exposed chickens (Table 1). We do not know whether the lack of oncogenicity is due to the specific mutation at the putative LAT promoter or whether there have been other changes in this virus that result in an attenuated phenotype. We saw reduced tumorigenicity of the equally passaged parent virus, but tumors were clearly evident in both inoculates and contacts exposed to this parent virus derivative. Firm conclusions on the effect of impairing LAT expression in vivo await the construction of rescued derivatives of this mutant or of additional mutants.

4 Viral Proteins Expressed During Latency

The viral proteins discussed in this section are expressed in lymphoblastoid cells and/or tumors. It has not been possible to examine their expression in non-transformed latently infected cells. Nevertheless, they represent a group of MDV gene products whose expression parallels latency in some way and they have been considered to be potentially important in oncogenesis. The literature on these proteins is, in some cases, fairly extensive. Therefore, they are only briefly reviewed here.

4.1 Meq

An extensive discussion of Meq appears elsewhere in this volume. The *meq* gene was first identified because it is expressed in MDV-transformed lymphoblastoid cells (Jones et al. 1992; Tillotson et al. 1988). The gene encodes a 339-amino-acid bZIP protein that resembles the Fos/Jun family of oncoproteins. In addition to lymphoblastoid cells (Liu et al. 1998), the *meq* gene is expressed in lymphomas (Ross et al. 1997) and in lytically infected cells (Jones et al. 1992).

The Meq protein has domains for DNA binding, dimerization, transactivation, and intracellular localization (Qian et al. 1995, 1996). The amino terminal bZIP region of the protein is comprised of a basic region and a leucine zipper. The basic region has been further divided into two subregions; namely, BR1, which provides an auxiliary signal for nuclear translocation, and BR2, which has both the primary nuclear localization signal and the nucleolar localization signal (Liu et al. 1997). The leucine zipper can promote dimerization with other Meq molecules as well as

association with c-Jun, p53, and probably other proteins (Brunovskis et al. 1996; Qian et al. 1996). The carboxyl-terminal region of Meq contains a proline-rich activation domain, which, when fused to the yeast Gal4 DNA-binding domain, transactivates the chloramphenicol acetyltransferase reporter. The last 33 amino acids of Meq are essential for transactivation and contain a consensus sequence for an RNA-binding motif.

A variant of Meq has been described, termed Meq-sp, which includes the DNA binding/dimerization domain of Meq but lacks the transactivation domain (Peng and Shirazi 1996a). This variant can bind DNA and complex with Meq or with Jun, but cannot transactivate. It has been proposed that Meq-sp regulates Meq activity by competing for heterodimer formation and DNA binding. The C-terminus of Meq-sp shares significant homology with the CXC chemokine IL-8 (Liu et al. 1999a). Further analysis facilitated the identification of vIL-8, an MDV gene that shares its second and third exons with Meq-sp, but which has a signal peptide encoded within its unique first exon (Liu et al. 1999).

The *meq* promoter lies on a 268-kb *Eco*RI-*Xmn*I fragment that contains two tail-to-tail copies of the consensus heat-shock regulatory element (Jones and Kung 1992) and an AP-1-like sequence (Qian et al. 1995). The *meq* promoter can be transactivated by Meq-cJun heterodimers but transactivation by Meq-Meq homodimers is inefficient (Qian et al. 1995). Two sequences have been described, MERE1 and MERE2, to which Meq can bind (Qing et al. 1996). MERE1 contains a TRE or CRE core flanked by specific sequences and has significant homology to the Maf oncoprotein recognition site (Katoaka et al. 1994; Kerppola and Curran 1994). MERE1 is located in the *meq* promoter region and, when bound by Meq and c-Jun heterodimers, transcription of *meq* can be activated (Liu et al. 1999). MERE2 contains an ACACACA core flanked by important but varying sequences. A MERE2 site lies in the putative MDV origin of replication suggesting a possible role for Meq in MDV replication.

To further complicate matters, two cDNAs which map to the right end of the *Bam*HI-I2 region and which are derived from mRNAs that are antisense to *meq* transcripts have been described. These cDNAs encode 135- and 195-amino-acid polypeptides. Antibodies raised against the 195-amino-acid polypeptide detected a 23-kDa nuclear protein in lymphoblastoid cells (Peng and Shirazi 1996b). The function of this protein or of these transcripts remains unknown.

Meq is probably the strongest candidate oncoprotein or co-oncoprotein known for MDV. Its expression is important for the maintenance of transformation of MDCC-MSB1 lymphoblastoid cells (Xie et al. 1996). Overexpression of Meq results in transformation of Rat-2 cells (Liu et al. 1998). In transformed Rat-2 cells, apoptosis is inhibited (Liu et al. 1997). This inhibition of apoptosis has been attributed, at least in part, to induction of *bcl* expression and suppression of *bax* expression. Meq requires a complementing oncoprotein such as vRas to transform primary cells such as fibroblasts, suggesting that Meq requires a cooperating oncoprotein(s) to realize its full oncogenic potential (Liu et al. 1998).

The intracellular location of Meq further suggests a role in transformation. Meq has been shown to localize in the coiled bodies of the nucleolus of infected

cells (Liu et al. 1997). In transformed Rat-2 cells, Meq can co-localize with cyclin-dependent kinase 2 (CDK2) in the coiled bodies and nucleolar periphery, suggesting that Meq may affect the subcellular localization of CDK2 in these cells (Liu et al. 1999). The interaction between Meq and CDK2 is particularly evident during the G_1/S boundary and early S phase of the cell cycle. In addition, CDK2 can phosphorylate Meq at serine 42 and phosphorylated Meq has decreased DNA-binding activity. Thus, it appears that Meq and CDK2 may modify each other.

Meq exhibits a number of activities common to the DNA tumor virus oncoproteins such as SV40 T antigen, adenovirus E1A and E1B, and human papillomavirus E6 and E7 (McCance 1998). These activities include sequestering p53 and simultaneously inhibiting apoptosis. These activities are important for DNA tumor viruses since these viruses depend on the cell for their propagation. They must poise cells for DNA replication and simultaneously short-circuit apoptotic pathways that become stimulated as a result of inappropriate cell cycle progression.

4.2 pp38/pp24

The pp38 gene was first identified by λgt11 cloning using a monoclonal antibody that detected a 38-kDa viral antigen expressed in lymphoblastoid cell lines and in tumor cells (Cui et al. 1990). The pp38 gene maps to the *Bam*HI-H fragment, spans the IRL/UL junction, and encodes a 290-amino-acid ORF (Cui et al. 1991; Chen et al. 1992). The promoter for the pp38 gene overlaps that of the major 1.8-kb family of transcripts (see Sect. 4.3) and the gene is transcribed into a 1.8-kb unspliced mRNA leftward from this promoter in the direction opposite that of transcription of the 1.8-kb family. Antisera from chickens with MD will immunoprecipitate pp38. Homologs to the pp38 gene have been found in the genomes of serotype 2 (Ono et al. 1994; Cui et al. 1992) and 3 (HVT) (Cui et al. 1992) strains. A direct role of pp38 in MDV transformation is complicated by the fact that the gene is also expressed by nononcogenic attenuated serotype 1 strains and by nononcogenic serotype 2 and 3 strains, and there is no evidence showing that pp38 is related to known oncoproteins. Nevertheless, an indirect role of pp38 in pathways leading to transformation is quite possible, and pp38 has been called an MDV tumor antigen (Chen et al. 1992).

A related ORF, which maps to the *Bam*HI-D region of the genome, has been described (Becker et al. 1994; Makimura et al. 1994; Zhu et al. 1994). This 465-bp ORF encodes a 155-amino-acid protein named pp24. Along with pp38, pp24 is a member of a group of MDV phosphorylated proteins that can be immunoprecipitated using monoclonal antibodies against this phosphorylated protein complex (Zhu et al. 1994). The amino-terminal 65 amino acids of pp24 are shared with the amino-terminus of pp38 since the coding regions for these segments of the proteins are derived from sequences lying within TRL and IRL. In particular, residues 45–50 of the proteins comprise a shared antigenic domain. A function for pp24 is unknown at this time.

4.3 1.8-kb Gene Family

Comparison of oncogenic strains and their attenuated derivatives, which lose the ability to spread horizontally and to cause tumors in chickens, indicated that attenuated strains have sustained expansions in the *Bam*HI-D and *Bam*HI-H fragments, both of which map to the IRL (FUKUCHI et al. 1985; SILVA and WITTER 1985). These expansions consist of the accumulation of tandem 132-bp direct repeats (MAOTANI et al. 1986). The region in the immediate vicinity of the 132-bp expansion is transcriptionally active. Bradley et al. (BRADLEY et al. 1989a,b) have reported that a major 1.8-kb family of transcripts produced by oncogenic strains is replaced by a group of truncated transcripts in attenuated strains. These transcripts are derived from a bidirectional promoter shared with the pp38 gene. The 1.8-kb family and pp38 genes are transcribed in opposite directions from this bidirectional promoter.

Several groups have reported analysis of cDNAs derived from this region. Two to three copies of the 132-bp repeats have been found in cDNAs from RPL1 and MSB1 cells (KOPACEK et al. 1993). Two copies have also been reported in cDNAs derived from MD5-infected CEFs (IWATA et al. 1992). Four cDNAs 1.69, 1.5, 1.9, and 2.2kbp in length corresponding to transcripts of the 1.8-kb family have been sequenced (PENG et al. 1992). Two of these (1.69kbp and 2.2kbp) are not spliced; whereas two (1.5kbp and 1.9kbp) are singly spliced species. The cDNAs contain a number of potential ORFs. Others have reported that four groups of transcripts are produced in the region and that they can initiate or terminate in both rightward and leftward directions and at multiple sites relative to the 132-bp repeat region (CHEN and VELICER 1991). HONG and COUSSENS reported two cDNAs, designated C1 and C2, derived from mRNAs spanning the *Bam*HI-H and *Bam*HI-I_2 regions (HONG and COUSSENS 1994). These cDNAs appear to be derived from a major 1.6-kb immediate-early transcript, and each of these cDNAs contains two small ORFs.

Evidence that this region is related to oncogenicity includes the following. First, as stated previously, the 1.8-family of transcripts is present only in oncogenic strains and is replaced by truncated transcripts in attenuated derivatives (BRADLEY et al. 1989b). Second, some of the ORFs associated with the 1.8-kb family have been reported to have some homology to cellular oncoproteins, particularly the mouse T-cell lymphoma oncoprotein and the Fes/Fps family of protein kinase-related oncoproteins (PENG et al. 1992). However, these homologies have not been noted by all investigators (HONG and COUSSENS 1994). Third, oligonucleotides complementary to the predicted splice donor site in the 1.8-kb family can inhibit proliferation of MDV-induced lymphoblastoid cells (KAWAMURA et al. 1991). Fourth, when introduced into primary CEF by transfection, two of the transcripts from the 1.8-kb family can reduce the serum dependence and prolong proliferation of these cells (PENG et al. 1993).

Although several potential ORFs have been observed in cDNAs corresponding to the 1.8-kb family, only two proteins have been reported to date. Antibodies raised against a fusion protein consisting of an ORF (ORF A) from the 1.7-kb

unspliced transcript fused to glutathione-*S*-transferase immunoprecipitated a 7-kDa protein, called BHa (PENG et al. 1994). BHa was detectable from lymphoblastoid cells or from lysates of CEF infected with oncogenic MDV strains but not nononcogenic strains (PENG et al. 1994). In another study, antisera were raised against glutathione-*S*-transferase fusion proteins containing two ORFs derived from C1 and C2 cDNAs (HONG and COUSSENS 1994). Both antisera detected a 14-kDa species, designated pp14, in Western blots. The 14-kDa species was detected in lysates of cells infected with oncogenic and attenuated serotype 1 MDV strains as well as in MSB1 lymphoblastoid cells. Furthermore, this protein is phosphorylated and localized to the cytoplasm (HONG et al. 1995). The functions of these proteins, the relationship between them, and the role of the region in general in MDV-induced transformation remain obscure.

The 1.8-kb gene family and the pp38/pp24 genes share a common bidirectional promoter element. Transient expression studies using this promoter indicate that it functions in infected CEF but that promoter activities vary according to the nature of the MDV strain being examined (SHIGEKANE et al. 1999). Furthermore, the short enhancer region shared by the two genes can be bound by a nuclear factor present in infected cells.

4.4 Others

Although expression of the MDV ICP4 gene has not been demonstrated during latency, this protein will be included in this discussion since it is so central to MDV gene expression in general. The MDV ICP4 homolog gene has been identified and completely sequenced (ANDERSON et al. 1992). The gene is 4245 nucleotides long, maps to the *Bam*HI-A fragment of the genome, and shows similarity to ICP4 homolog genes of alphaherpesviruses. The MDV ICP4 protein is predicted to have a structure similar to that of ICP4-like proteins of other herpesviruses. Several potential transcriptional regulatory sites are present in the MDV ICP4 gene sequence, one of which is a putative autoregulatory site (FABER and WILCOX 1986) located 525bp into the predicted coding region. Transcription of the MDV ICP4 gene is complex. At least five RNAs with sizes of 10, 9.3, 7.8, 7.2, and 6.2kb have been reported (MORGAN et al. 1996). At least two of these RNAs (7.8kb and 6.2kb) are spliced. The nature of the 10-kb RNA is not well understood but it overlaps almost exactly the large LAT transcript. The MDV ICP4 protein can transactivate the pp38 gene (PRATT et al. 1994).

A nuclear antigen (MDNA) present in MKT-1 and MSB-1 cells has been reported to bind to two sites on the MDV genome (WEN et al. 1988). One site was located within the US (present in both MKT-1 and MSB1 cells) and the other (present only in MSB-1 cells) was within the IRS. DNAse digestion indicated that binding occurred to 60-bp and 103-bp sequences, respectively, both of which spanned palindromes. The biochemical nature of this factor has not been pursued and probably warrants further investigation.

5 Latency and Immunocompetence

Immunocompetence is important for initiating and maintaining MDV latency (Buscaglia et al. 1988). Latency can be maintained in cultured lymphocytes (as determined by suppression of expression of virus internal antigen) by a factor present in conditioned medium (Buscaglia and Calnek 1988). Conditioned media from ConA-stimulated spleen cells or from mixed-lymphocyte reactions is active. Some of this activity has been attributed to interferon (Volpini et al. 1992, 1995).

6 Summary and Conclusions

MDV latency is defined as the persistence of the viral genome in the absence of production of infectious virus except during reactivation. A number of systems for studying MDV latency exist, and most involve the use of lymphoblastoid cells or tumors. It has been difficult to divorce latency and transformation. Understanding the relationship between these two states remains a major challenge for the MDV system.

Based on their patterns of expression, the MDV LATs are apt to be important in the balance between latent and lytic infections. The LATs are a complex group of transcripts. The profile of gene expression that characterizes latency differs among all herpesviruses, and MDV is no exception. MDV LATs bear little resemblance to LATs of other alphaherpesviruses or to the LATs of other lymphotropic herpesviruses. LAT splicing patterns are complex and the relationships among various spliced species or between these species and the large 10-kb transcript are unknown. In addition, the existence of any protein gene products of significance is unknown at this time. More work is needed to further investigate the significance and function of these RNAs. Better technology to construct mutants in the MDV system is badly needed, since the analysis of mutants in the chicken is a powerful and unique advantage of the MDV system.

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