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MOLECULAR CHARCTERIZATION OF A DEFECTIVE MAREK'S DISEASE VIRUS: IDENTIFICATION OF A FUNCTIONAL MDV REPLICATION ORIGIN AND A GENE ENCODING A POTENTIAL NUCLEAR DNA BINDING PROTEIN

By

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ABSTRACT

MOLECULAR CHARACTERIZATION OF A DEFECTIVE MAREK'S DISEASE VIRUS: IDENTIFICATION OF A FUNCTIONAL MDV REPLICATION ORIGIN AND A GENE ENCODING A POTENTIAL NUCLEAR DNA BINDING PROTEIN

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Characterization of molecular events involved in Marek's disease virus (MDV) DNA replication is essential to understand the entire scope of the MDV replication cycle. Defective MDV genomes (MDV replicons) contain limited viral sequences important for MDV DNA replication. Therefore, they can replicate as separate entities from that of standard virus DNA, creating a useful model system to study MDV DNA replication. Based on DNA sequencing and transient plasmid amplification assays, a functional serotype 2 MDV replication origin was identified within the MDV replicon DNA. The serotype 2 replication origin is approximately 90-bp long, arranged in an imperfect palindrome centered around an AT-rich region. Sequence analysis of the 90-bp region revealed a sequence and structure strikingly similar to origins of alpha-herpesvirus replication. In addition, the 90-bp MDV origin sequence contains a 9-bp sequence (CGTTCGCAC) sharing 82% sequence similarity to an 11-bp motif recognized by the herpes simplex virus (HSV) origin binding protein (OBP). Mobility shift and

competition assays were performed to investigate viral or cellular proteins interacting with the potential OBP recognition site of the MDV replication origin. A virus specific protein-DNA complex, complex B, was identified using synthetic 22-mer oligonucleotide probe containing the potential OBP binding site of MDV. The 5'-region of MDV replicon DNA revealed a significant sequence identity to the HSV *a* sequence, responsible for cleavage/packaging process. Several potential open reading frames (ORF) were identified within the MDV replicon DNA. ORF-A codes for a putative 204 amino acid protein that shares 21% and 36% amino acid sequence identity to nuclear DNA binding proteins such as a nuclear antigen (EBNA-1) of Epstein-Barr virus (a gamma-herpesvirus) and galline, a chicken sperm histone protein, respectively. The MDV replicon, therefore, appears to contain characteristics of both gamma- and alpha-herpesviruses at the molecular level.

As alyways, to love of my life, Steve and Lindsay.

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Table of contents

Chapter I

Literature review

1 Biology of Marek's disease virus (MDV)

In 1907, a Hungarian scientist, Joseph Marek first described the clinical signs of Marek's disease (MD) (Marek, 1907). MD is a readily transmissible, lymphoproliferative disease of chickens, often accompanied by leg paralysis and lymphoid tumors (Payne and Biggs, 1967). During the early 1960's, it was discovered that the etiologic agent of MD is a highly cell-associated avian herpesvirus, termed Marek's disease virus (MDV) (Churchill and Biggs, 1967; Nazerian and Burmester, 1968).

The structure of the MDV capsid is that of a typical herpesvirus containing 162 capsomeres with icosahedral symmetry (Nazerian and Burmester, 1968). Nucleocapsids containing intact viral DNA, approximately 85-100 nm in diameter, are present in MDV infected cells. Only a few enveloped particles, approximately 130-170 nm in diameter, are observed budding from the infected cell inner nuclear membranes (Nazerian and Burmester, 1968; Hamdy et al, 1974). The only source of a large number of cytoplasmic enveloped particles (270-400 nm) is the infected feather follicle epithelium.

MDV is classified into 3 serotypes based on agar gel precipitation and indirect fluorescent antibody assays (Bulow and Biggs, 1975; Bulow and Biggs, 1975b). Serotype 1 MDV includes all oncogenic viruses and their attenuated derivatives.

With regards to oncogenic potential, different pathotypes exist within serotype 1 MDV. For example, "classical forms" of MDV which include GA and JM strains can cause high incidences of MD in genetically susceptible, unvaccinated chickens. RB-1B and Md/11 are classified as very virulent strains of MDV (vvMDV) and are responsible for causing outbreaks of MD in vaccinated chickens (Witter, 1985). Only genetically resistant lines of chickens can be protected from vvMDV by current vaccine programs.

Non-oncogenic, naturally occurring chicken herpesviruses are represented in the serotype 2 group. Though tumors have never been observed in susceptible chickens infected with serotype 2 strains, certain strains of serotype 2 MDV, such as SB-1, can cause a low level of cytolytic infection in lymphoid organs resulting in immunosuppression in infected birds (Schat and Calneck, 1978). Serotype 3 MDV is the non-oncogenic herpesvirus of turkeys (HVT) which is antigenically related to MDV. HVT does not occur naturally in chickens, but is now prevalent in commercial flocks due to its widespread use as a vaccine against MDV-induced lymphomas. Attenuated serotype 1 strains and bivalent vaccines containing HVT and serotype 2 strains are also being used as vaccines to protect birds against MD. MDV is of interest not only because of its immense economic importance to the poultry industry, but also because of its role as a model system to study virus-induced malignancy.

MDV can be readily cultured from viable lymphocytes and MDV tumor cells. Cell-free MDV can be recovered only from the feather follicle epithelium of infected birds. Upon initial isolation, MDV can be propagated in chicken kidney

cells (CKC), duck embryo fibroblast (DEF) cells, chicken embryo fibroblast (CEF) cells or other primary avian cell cultures (Purchase, 1974). In tissue culture, MDV establishes strictly cell-associated infections resulting in poor virus recovery from the culture media.

Most studies on the biological characterization of MDV have been done with serotype 1 and serotype 3. More attention was given to serotype 2 strains when Bacon et al. (1989) reported that serotype 2 MDV-based vaccines caused the enhancement of lymphoid leukosis in certain strains of chickens co-infected with exogenous avian leukosis virus (ALV). Further reports suggest that serotype 2 MDV may encode a trans-activation protein(s) which stimulates the retrovirus long terminal repeat promoter region (Tieber et al., 1990), leading to an increase in ALV gene expression and virus production (Pulaski et al., 1992). Further biological characterization of all three serotypes is needed to understand the nature and relationships among all MDV serotypes at the molecular and antigenic levels.

2. Pathologenesis of MDV.

The pathogenesis of MDV has been well defined in experimental settings using mostly "classical forms" of MDV and genetically susceptible maternal antibody-free chicks (Payne, 1985). MDV is readily transmitted horizontally by direct contact with infected birds and through the air (Sevoian et al, 1963). MDV infection usually occurs via the respiratory tract, followed by a cytolytic infection in lymphoid organs at 3-6 days post-inoculation. Mononuclear infiltration of

peripheral nerves, gonad, iris, various visceral organs, muscle, and skin can be observed at approximately 2 weeks post infection. Clinical signs and gross lesions generally appear after about 3-4 weeks.

The most common clinical signs of MD are leg paralysis primarily due to lymphocyte infiltration and nerve demeylination of spinal cords. Clinical signs may vary, however, among infected birds depending upon the location of infected nerves. For example, the head will be paralyzed if the nerves controlling neck muscles are affected. Vagal nerve involvement can lead to paralysis of the crop. In addition, a certain strain of MDV can cause depigmentation of the iris, resulting in blindness. Infected visceral organs, particularly the gonads, liver, lungs, heart, muscle, and skin, may have diffuse or grayish-white lymphoid tumors (Payne and Biggs, 1967). In addition, severe atrophy of the thymus and bursa of Fabricius often occurs in infected chickens. However, several factors can affect the outcome of MDV pathogenesis such as the age of host, strain and species of host, and strain of viruses (Payne, 1988).

There are three types of MDV infections *in vivo*; lytic infection, latent infection and transformation. The lytic infection can be sub-divided into fully-productive and semi-productive infections. Fully-productive infections are characterized by the production of cell-free enveloped virus particles which occurs only in the infected chicken feather follicle epithelium. Semi-productive infections result in the production of predominantly nonenveloped nucleocapsids in the nuclear membrane of B lymphocytes from infected spleen, bursa, thymus, and peripheral blood (Calneck et al., 1970; Schat et al., 1978). The establishment of latent

infections by MDV is usually detected at the end of the early cytolytic infection and results from development of host cell immune responses. Latent infections are primarily restricted to T-lymphocytes and are characterized by the presence of viral genomes without viral antigen expression or virus particle production. T-lymphocytes are also the target for oncogenic transformation by MDV (Calneck et al., 1970; Nazerian, 1973). As with most of the other DNA tumor viruses, the oncogenic potential of MDV infections may be due to either the presence of viral oncogenes, or the induction of host cell immune response.

3. Molecular biology of MDV

A. MDV genome structure.

The MDV genome consists of a linear double stranded DNA molecule of approximately 165-180 kilo-basepairs (kbp) with nicks and gaps as in other herpesviruses (Lee et al., 1971; Hirai et al., 1979; Cebrian et al., 1982; Wilson et al., 1991). The density of MDV DNA in CsCl gradients is 1.705 g/cm3, close to that of chicken cell DNA, presenting difficulties isolating pure virus DNA.

Originally, MDV was classified as a gamma-herpesvirus, based primarily on its lymphotrophic nature, similar to Epstein-Barr virus (EBV). However, recently MDV has been re-classified as an alpha-herpesvirus based on MDV genomic structure and gene collinearity with those of other alpha-herpesviruses (Buckmaster et al., 1988; Roizman, 1990; Roizman, 1992; Brunovski and Velicer, 1992; Velicer and Brunovski, 1992). In addition, random nucleotide sequencing of MDV genomes strongly indicate that MDV has a greater sequence similarity

to the alpha-herpesviruses than to gamma-herpesviruses (Buckmaster et al., 1988).

The genome structure of MDV belongs to the *Herpesvirdae* group E genome family along with HSV and Varicella-Zoster virus (VZV) (Cebrian et al., 1982). The group E genome structure is composed of covalently linked long (L) and short (S) regions, each consisting of unique sequences (U_L or U_S) flanked by inverted repeats (Wadsworth et al., 1975) (Fig. 1A). The inverted repeat regions flanking the L component are designated ***ab*** and ***b'a'***, whereas the inverted repeats of the S component are termed ***a'c'*** and ***ca***. The standard group E virus genome can thus be represented by ***ab***-U_L-***b'a'c'***-U_S-***ca***. The ***a*** sequence in HSV genomes, approximately 250-500-bp long, is variable among different virus isolates (Mocarski et al., 1985). A single unit of the HSV ***a*** sequence is composed of two copies of a 20-bp terminal direct repeat (DR1), a unique 64-bp sequence (U_b), 19-22 tandem repeats of a 12-bp sequence (DR2), 3 repeats of a 37-bp sequence (DR4), and a unique 58-bp sequence (U_c) (Fig. 1B). Therefore, the HSV ***a*** sequence can be represented by DR1-U_b-$DR2_{(19\text{-}22)}$-$DR4_3$-U_c-DR1. Numerous reports suggest that the ***a*** sequence present in the L-S junction and both termini of HSV genomes contains two *cis*-acting recognition signals commanding inversion of L-S components relative to each other, as well as for proper cleavage/packaging of unit length viral DNA. A more extensive literature review on the functional property of the ***a*** sequence will be presented in the *DNA replication* section (p. 11).

In addition to the standard herpesvirus group E genome structure, serotype 1 MDV contains several sets of direct repeats consisting of more than 100-bp

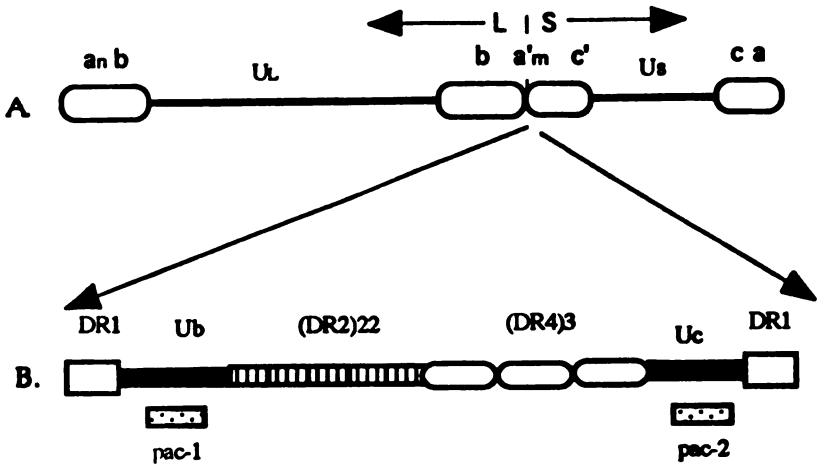

Fig. 1 (A) The group E herpesvirus genome structure, (B) A single copy of the HSV *a* sequence

(designated DR1 to DR5) scattered throughout the genome. Direct repeat elements of MDV DNA are reminiscent of direct repeats present in EBV DNA (Hirai, 1988).

Based on gene colinearity between MDV and HSV, DR5 sequences (located in both termini of the MDV serotype 1 genome) are of interest in regards to a potential *a*-like sequence. Southern blot analysis depict DR5 regions as heterogenous EcoRI bands hybridizing to appropriate probes, suggesting the existence of terminal heterogeneity (Hirai et al., 1988). However, DR5 sequences are not present within the L-S junction of the MDV genome, indicating that DR5 sequences may play different functional roles than the HSV *a* sequence. In support of this notion, Kishi et al. (1991) reported the identification of an *a*-like sequence (269-bp) located within the L-S junction and both termini of the serotype 1 MDV genome. DNA sequencing analysis of the 1.6-kbp BamHI to HindIII

subfragment of the serotype 1 MDV BamHI-A genomic clone revealed 215 nucleotides containing two copies of a 12-bp repeat (DR1), 17 tandem repeats of a 6-bp sequence (DR2), two copies of a 13-bp sequence (DR4) and two unique sequences within the region flanking the 12-bp repeats (Ub and Uc, respectively) (Fig. 2). Therefore, the putative *a*-like region of serotype 1 MDV genome contains a structural similarity to the HSV *a* sequence. The *a*-like region of serotype 1 MDV, however, did not contain any nucleotide sequence similarity to the HSV *a* sequences. On the contrary, DR2 sequences (17 tandem repeats of GGGTTA) of serotype 1 MDV were found in human herpesvirus type 6 DNA (Kishi et al.,1988).

A potential *a*-like sequence in serotype 3 MDV was reported by Reilly and Silva (1993). Based on southern hybridizations and exonuclease digestions of the termini of HVT genomes, a 1.2-kbp fragment which is located in both termini and the L-S junction region of HVT genome was identified. The 1.2-kbp fragment was reported to share some structural similarity to the HSV *a* sequence. The number of copies of this 1.2-kbp region were variable among different HVT isolates as in HSV. Additionally, different HVT isolates exhibited intra-strain variations in the number of copies of 1.2-kbp *a*-like sequence within L component termini and L-S junctions. The S termini contained only a single copy of the 1.2-kbp fragment. Interestingly, the 1.2-kbp *a*-like region in HVT is similar in size to the 950-bp *a* sequence of cytomegalovirus, a beta-herpesvirus (Tamashiro et al., 1984). Nucleotide sequences of the 1.2-kbp region, however, are not yet available for direct comparison with *a*-like sequences reported for other herpesviruses.

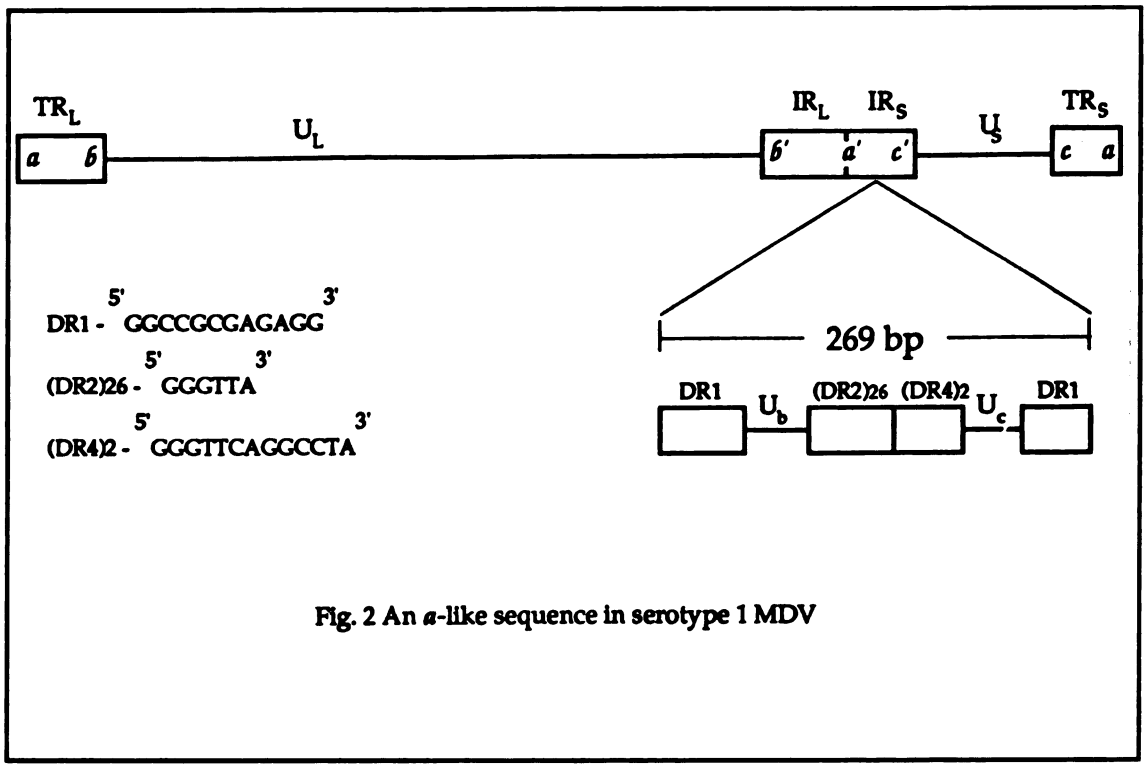

Fig. 2 An *a*-like sequence in serotype 1 MDV

B. Physical maps of MDV

The construction of physical maps of serotype 1 and HVT DNA were accomplished by Fukuchi et al.(1985) and Igarashi et al. (1987), respectively (Fig. 3A and 3B). More recently, Ono et al. (1992) reported the construction of restriction endonuclease (RE) maps for serotype 2 DNA, strain HPR16 (Fig. 3C). The availability of RE maps and genomic clones of MDV DNA have greatly facilitated our understanding of the molecular nature of MDV.

Although all three serotypes of MDV are related antigenically, their genomic RE patterns are very different, and these differences can be used to evaluate new MDV isolates (Silva and Barnett, 1991). There have been conflicting results regarding the overall degree of homology between serotype 1 MDV and HVT; 5% to 70% homology was reported based on reassociation kinetics and low stringency

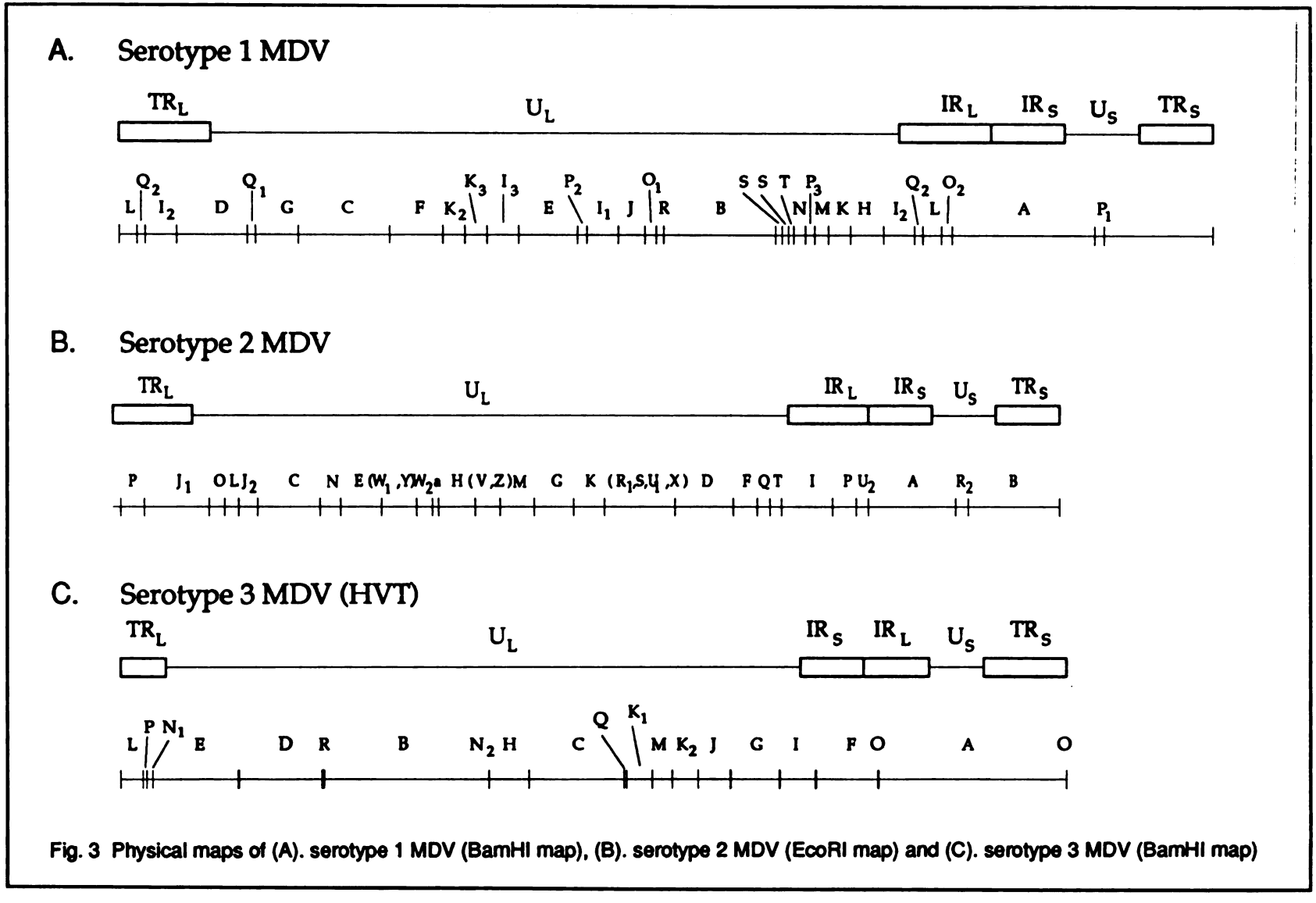

Fig. 3 Physical maps of (A). serotype 1 MDV (BamHI map), (B). serotype 2 MDV (EcoRI map) and (C). serotype 3 MDV (BamHI map)

southern blot analysis, respectively (Kaschka-Dierich et al., 1979; Gibbs et al., 1984). A recent report by Ono et al. (1992) suggested that serotype 2 MDV is similar to serotypes 1 and 3 MDV, with regards to genome structure and gene colinearity. Determination of complete nucleotide sequences for all three MDV serotypes would enhance our understanding of MDV biology.

C. DNA replication.

Much of the interest in animal virus genome replication comes from a desire to understand the events that occur during replication of eucaryotic chromosomes. Viruses offer many advantages as model DNA replication systems. The relatively simple genome structure of most viruses allows easy manipulation at the molecular level. Studies of Simian virus 40 (SV40) and adenovirus DNA replication, which relies mostly on host-cell replication machinery, have led to the discovery of many eucaryotic proteins which are necessary for viral replication processes. In contrast to SV40 and adenovirus, herpesvirus genomes are more complex and encode many of the proteins required for replication of their genomes. Thus herpesviruses represent a model system in which to study interactions of virus-encoded and cellular proteins involved in DNA synthesis (Olivo and Challberg, 1989). Genetic and molecular dissection of herpesvirus replication events, will therefore increase our understanding of eucaryotic genome replication.

Study of MDV DNA replication is important for several reasons. First, MDV exhibits characteristics of both alpha-and gamma-herpesviruses; the MDV genome

structure and gene arrangements are similar to HSV and VZV (alpha-herpesviruses), whereas the biological properties of MDV are similar to EBV (a gamma-herpesvirus) (Buckmaster et al., 1988; Roizman, 1982; Roizman, 1992; Brunovskis and Velicer, 1992). Based on ethidium bromide-CsCl equilibrium centrifugation, several investigators have reported that MDV genomes exist as closed circular DNA in lymphoblastoid cell lines established from MDV tumors (Tanaka et al., 1978; Hirai et al., 1981). The closed circular structure of MDV DNA in established MDV cell lines resembles that of EBV genomic structure in latently infected cells. However, based on Gardella gel electrophoresis and *in situ* hybridization techniques, Delecluse and Hammerschmidt (1993) recently reported that the majority of MDV genomes are integrated into the host cell chromosomes in various MDV lymphoblastoid cell lines. Only a minor population of MDV genomes were detected as linear or covalently closed circular DNA. As in the MDV system, the integration of EBV DNA was observed in a number of Burkitt's lymphoma cell lines, together with circular forms of EBV DNA (Anvret et al., 1984; Adams et al., 1987). Study of MDV DNA replication, therefore, will enhance our understanding of MDV lineage in the herpesvirus family.

Secondly, MDV still causes a great economic loss in domestic poultry industries. Although successful vaccines exist, the mechanisms of anti-tumor immunity are virtually unknown. An emergence of very virulent MDV strains adds further complications in controlling MD. Additionally, conditions appropriate for the establishment of latency and transformation by MDV infection are not clear at present. It is likely that multiple steps are involved in MDV

transformation rather than a single viral oncogene product. Restriction of viral DNA replication has been implicated in DNA virus transformation. Integration of viral genomes into host cell chromosomes can lead to activation or repression of certain cellular proteins, leading an imbalance of host gene expression and, thereby contributing to the transformation process. The mode of MDV DNA replication during lytic and latent infections, and its association with host cell replication, are poorly understood. One can ask a series of questions regarding MDV DNA replication. 1). Does MDV contain origins of DNA replication? 2). Does MDV contain signals specifying the cleavage-packaging process? 3). Are there viral-specific proteins necessary for MDV DNA replication? 4). Are there host factors involved in MDV DNA replication? 5). How does MDV DNA replicate and maintain its genome in lytic and transformed/latent infection stages?

The current literature review will be focused on comparisons of DNA replication from all three herpesvirus classes (alpha, beta, and gamma-herpesviruses). Reviewing studies and results on replication of other herpesviruses will help us gain some insights regarding future experimental designs in MDV DNA replication research.

i). Alpha-herpesvirus DNA replication.

HSV is an alpha-herpesvirus, and is the most extensively characterized of all herpesviruses. The importance of defective herpesvirus genomes in study of viral replication is accentuated in the HSV system. Existence of three separate HSV

origins of DNA replication was first inferred from studies of two different types of defective herpesviruses (amplicons) (Frenkel, 1981). A more extensive literature review on the description of HSV amplicons will be addressed in a later section entitled,"*defective herpesviruses*" (see p.32). All three HSV origin sequences have been cloned from the standard virus genome and analyzed in some detail.

Origin function analyses are usually performed by transient plasmid amplification assays. Standard plasmid amplification assay is performed by transfecting plasmids containing origin sequences into HSV infected cells, or co-transfecting cells with origin containing plasmids and standard virus DNA (Mocarski and Roizman, 1982; Stow, 1982; Stow and McMonagle, 1983). Subsequent studies revealed the identification of two copies of lytic origins within the inverted repeats flanking the S component (ori_S). A third origin, ori_L was localized to the center of the L component (ori_L). A core ori_L is located in a 425-bp segment containing a perfect 144-bp AT-rich palindrome (Weller et al., 1985). Ori_S and ori_L sequences share extensive nucleotide sequence similarity, except for sequences extending to rightward from the AT-rich palindrome (Murchie and McGeoch, 1982; Weller et al., 1985). To determine functional significance of the existence of three separate HSV origins, several mutant viruses were created. Mutant viruses lacking ori_L, or with one copy of ori_S replicated normally *in vitro* (Longnecker and Roizman, 1986; Polvino-Bodnar et al., 1987). Attempts to construct mutant viruses lacking both copies of ori_S have not been successful, implying that HSV DNA replication requires at least one copy of ori_S or, alternatively, at least two origin sequences (ori_S and ori_L, or two copies of ori_S).

Functional significance of three separate origins in HSV DNA replication is not clear.

Deletion analysis of ori_S sequences revealed a core origin of 75-bp region which included both arms of a 56-bp palindrome centered on alternating AT residues, and an additional 30-bp leftward of the AT-rich palindrome (Stow and McMonagle, 1983; Lockshon and Galloway, 1988). Substitution of the AT-rich region with GC-rich sequences impaired ori_S replication activity (Lockshon and Galloway, 1988). A large insertion of AT dinucleotide within the AT-rich motif resulted in an oscillating effect on the replication activity; the insertion of 3 copies of an AT dinucleotide caused a dramatic decrease in DNA synthesis, the insertion of 5 or 6 copies of the AT dinucleotide returned replication efficiency to the normal level. Insertion of 8 AT dinucleotides decreased replication efficiency. These results suggest that spacing between the two palindromic arms in ori_S is critical for DNA replication.

Deb and Doelberg (1988) performed systematic base substitutions in a triple tandem arrangement across the AT- rich region, and found that changing the first 3-bp of the left end of the AT-rich sequences caused a dramatic loss of replication function. This suggests that the AT-rich sequences at the center of the palindrome are essential and have an unknown functional role in replication which is not clear at present.

Two origin-binding protein (OBP) recognition sites (designated sites I and II) overlap the ends of each arm of the ori_S palindrome (Fig. 4) (Elias and Lehman, 1988; Elias et al., 1990). The HSV OBP is one of seven viral proteins that are

required for HSV DNA replication: a helicase and primase complex (UL5, UL8, UL52), the OBP (UL9), the major single-stranded DNA-binding protein (ICP8), DNA polymerase, and a polymerase accessory protein (UL42) (McGeoch et al., 1988; Wu et al., 1988).

Based on site-directed mutagenesis and methylation interference, the OBP recognition site was localized to a sequence YGYTCGCACT; where Y represents C or T (Koff and Tegtmeyer, 1988; Deb and Deb, 1989; Elias et al., 1990). The deletion of OBP-binding sites I and II completely eliminates both the OBP-binding

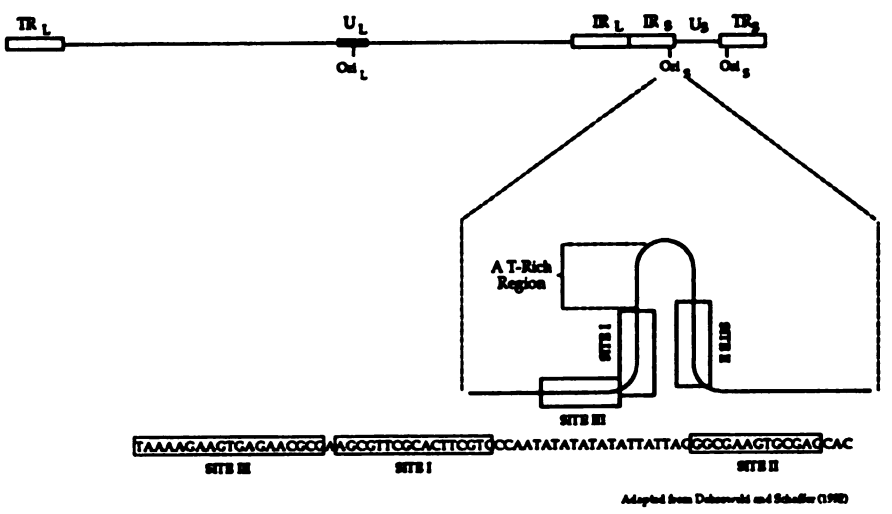

Fig. 4 HSV origin Binding protein (UL9) recognition sites

activity and HSV DNA replication in transient plasmid replication assays (Deb and Deb, 1989; Weir and Stow, 1990). Therefore, the interaction of OBP with ori_S is critical to optimal HSV DNA replication. As mentioned earlier, an oscillating

activity was observed with plasmids containing different copy numbers of AT dinucleotide within the AT-rich region (Lockshon and Galloway, 1988). This phenomenon may reflect a requirement for OBP, bound on each arm of the palindrome, to be located on the same side of the DNA helix (Challberg and Kelly, 1989).

An 11-bp motif containing the OBP-binding domain of site I is highly conserved in ori_S and ori_L of HSV-1 and HSV-2, as well as in VZV, equine herpesvirus (EHV), and MDV origins of DNA replication (Stow and Davison, 1986; Baumann et al., 1989; Camp et al., 1991).

A third presumptive OBP-binding site (III) within ori_S has been identified and shown to contain sequence similarity to OBP-binding sites I and II, but no sequence-specific OBP binding has been reported at site III (Elias et al., 1990; Weir and Stow, 1990) (Fig. 4). Weir and Stow (1990) reported that the deletion of site III within ori_S of HSV-1 resulted in only a moderate decrease in replication efficiency. On the contrary, the deletion of part of a sequence corresponding to site III in HSV-2 resulted in a dramatic decrease in DNA replication (Lockshon and Galloway, 1986). A more recent report by Martin et al. (1991) indicated that all three OBP-binding sites are required for optimal DNA replication, suggesting the functional importance of all three OBP binding sites in HSV replication.

The lack of OBP binding to site III in *in-vitro* assays, and the decrease in DNA replication by mutagenesis within site III, suggest that other factors may interact with site III. These "accessory factors" may change the overall origin structure, thereby making site III more accessible to OBP *in-vivo*. Sequence-specific binding

of OBP to sites I and II within HSV origins, the loss of replication activity in origins with mutagenized OBP binding sites, and the helicase and ATPase activities contained within purified OBP, suggest that the OBP may represent an initiator protein for HSV DNA replication.

Like other DNA viruses such as SV40, adenovirus, and bovine papillomavirus, HSV origin sequences are flanked by an auxiliary component containing transcriptional regulatory regions. Ori_L is located between the divergent transcriptional start sites of the genes encoding the major DNA-binding protein ICP8 and DNA polymerase (Weller et al., 1985; Polvino-Bodnar et al., 1987). Ori_S is positioned between the genes encoding immediate-early proteins ICP4 and ICP22/47 (Preston and Tannahill, 1984; Preston et al., 1984; Preston et al., 1988). Many transcription factor binding sites are located within the promoter regions of ICP4 and ICP22/27, such as a VP16 transactivator recognition site (TAATGARAT motif), Sp1 binding sites, and a nuclear factor III binding site. Wong and Schaffer (1991) reported that mutations within these transcription factor binding sites resulted in a significant reduction in ori_S replication activity. In addition, Wong and Schaffer postulated that transcription factors which bind to, and regulate immediate-early genes may promote a secondary effect on ori_S activity, increasing accessibility of initiation protein complexes to ori_S. Alternatively, these transcription factors may interact directly with DNA replication proteins. Hubenthal-Voss and Roizman (1988) have identified two transcripts which transverse ori_S sequences in both HSV-1 and HSV-2, and postulate that these transcripts may have functions in DNA replication by either

inducing alterations in the superhelical density of the template or serving as primers for DNA replication.

The current understanding of the mode of HSV DNA replication is primarily based upon pulse-labeling HSV DNA and fractionation of resulting viral DNA molecules of different buoyant densities. HSV DNA replicative intermediates from buoyant density fractionation have been extensively studied by electron microscopy (Jacob and Roizman, 1977; Jacob et al., 1979). There are at least five different types of HSV DNA structures: 1) double-stranded circular molecules, 2) unit length linear molecules containing internal gaps and single-stranded termini, 3) lariat structures containing circular-linear molecules, 4) linear molecules with "eye" and "D" loops, and finally 5) large "tangled masses" of DNA. With the exception of lariat molecules, the same types of replicative intermediates were observed in pseudorabies virus (an alpha-herpesvirus) infected cells (Ben-Porat et al., 1976; Jean et al., 1977).

On the basis of these observations, it has been proposed that double-stranded linear HSV DNA circularizes upon entry into host cells. As mentioned earlier, the group E herpesvirus genome structure can be written as $ab\text{-}U_L\text{-}b'a'a'c'\text{-}U_S\text{-}ca$. Because the *a* sequence is repeated at both ends of the HSV genome, it is likely that the linear HSV DNA can fold back upon itself to form circular molecules by ligating ends through the *a* sequences. The resulting circular molecules are then serve as templates to generate linear concatemers consisting of tandemly repeated unit size viral genomes via a rolling circle mechanism (Jacob and Roizman, 1977). Additional evidence to support concatemeric formation by a rolling circle

mechanism was reported by Jacob and co-workers (1979). Restriction enzyme analyses of linear replicative intermediates revealed a decrease in the molar concentration of termini fragments and an increase in the molar concentration of the L-S Junction fragments suggesting that linear replicative intermediates exist as head-to-tail concatemers. Furthermore, HSV was reported to induce an amplification of integrated simian virus 40 (SV40) DNA by generating large, tandemly reiterated SV40 DNA, which may have arisen by a rolling circle mechanism (Matz, 1987). In a natural setting, SV40 replicates in a theta mode of DNA replication, generating only circular monomers. This result suggests that HSV contains signals directing the formation of concatemers, possible via rolling circle mechanism.

Further evidence for rolling circle HSV DNA replication was reported by Rabkin and Hanlon (1990) who performed an artificial in vitro DNA synthesis using preformed replication forks consisting of a nicked, double-stranded, circular DNA molecule with a 5′ single-strand tail as templates with partially purified HSV infected cell protein extracts. Analysis of replicated DNA structures from those templates revealed linear concatemers with HSV infected protein extracts, but not with mock infected protein extract, suggesting that DNA synthesis occurred via a rolling circle mechanism. The suggestion that HSV DNA replication occurs via a rolling circle mechanism thus is very plausible and supported by numerous studies (Jacob and Roizman, 1977;Jacob et al., 1979; Matz, 1987; Rabkin and Hanlon, 1990). However, further studies are required to explain the existence and functions of other types of replicative intermediates in HSV

infected cell nuclei. For example, it is difficult to explain the formation of "tangled masses of DNA" by a rolling circle mechanism alone.

Difficulties associated with the study of herpesvirus DNA replication are due primarily to the large genome size of the herpesviruses and, to the isomerization of herpesvirus genomes, in which the L and S components invert relative to each other. As a consequence, viral DNA extracted from virions or from infected cells consists of four equimolar isomers that differ in the orientation of the L-S components relative to each other (Hayward et al., 1975).

It has been suggested that inversion of HSV genomes is mediated by homologous recombination via the *a* sequence, present at both termini and the L-S junctions of HSV genomes (Smiley et al., 1981). Further experiments revealed that the *a* sequence contains *cis*-acting elements through which a site-specific recombination occurs (Mocarski and Roizman, 1981; Chou and Roizman, 1985). Chou and Roizman (1985) reported that DR4 elements of the *a* sequence are important for site-specific recombination, whereas Smiley et al. (1990) concluded that both ends of the *a* sequence are required for the inversion process (Fig. 1B, p7). To the contrary, some investigators have suggested that the *a* sequence inversion is mediated by a general, rather than a site-specific recombination process (Pogue-Geile et al., 1985; Longnecker and Roizman, 1986; Weber et al., 1988). However, numerous reports suggest that the *a* sequence may be considerably more recombinogenic than other DNA fragments of equivalent length and therefore, mediates high-frequency recombination events. Furthermore, Poffenberger and Roizman (1985) constructed a mutant virus lacking

most of the internal inverted repeats including the *a* sequence. This mutant (inverted repeat minus) virus was frozen in one arrangement suggesting an inability of the mutant virus L and S components to invert.

A different approach was undertaken to quantitate HSV inversion frequency by Dutch et al. (1992). To briefly explain, plasmid constructs containing the E. *coli* lacZ gene flanked by directly repeating *a* sequences, with or without ori_s, were measured for its frequency of lacZ gene deletion. Deletion of the lacZ gene was determined by using white and blue color selection of the resulting bacterial colonies. In this system, lacZ gene deletion most likely occurred by homologous recombination between two intramolecular *a* sequences. Dutch et al (1992) concluded that the recombination frequency was higher with plasmids containing an origin of HSV replication and two copies of the *a* sequences, compared to plasmids containing only one copy of the *a* sequence with or without the origin sequences. In addition, the processes of replication and recombination occur in parallel.

An additional role of the HSV *a* sequence is in the virus maturation process. The *a* sequence contains signals specifying a site-specific cleavage/packaging process (Spaete and Frenkel, 1982; Stow and McMonagle, 1983). As mentioned previously, HSV generates replicative intermediates consisting of head-to-tail concatemers. It has been proposed that concatemeric molecules are cleaved at the *a* sequence thus ensuring that only unit length viral DNA is packaged into nucleocapsids (Spaete and Frenkel, 1982). Comparative analysis of the *a* sequences of other herpesviruses revealed two highly conserved sequence

elements, termed *pac1* and *pac2* (Davison, 1984; Matsuo et al., 1984; Albrecht et al., 1985; Bankier et al., 1985; Tamashiro and Spector, 1986; Marks and Spector, 1988). *pac1* and *pac2* sequences are located in the Ub and Uc regions of the HSV *a* sequence, respectively (Fig. 1B, p. 7). Deiss et al. (1986) concluded that *pac1* and *pac2* sequences play important roles in the site-specific cleavage/packaging process in HSV propagation.

ii). Beta-herpesvirus DNA replication.

Cytomegalovirus (CMV) is a beta-herpesvirus, and contains the largest genome (240-kbp) among herpesviruses. Human cytomegalovirus (HCMV) can cause serious clinical consequences in acquired immunodeficiency syndrome or immunocompromised patients (Alford and Britt, 1985). Like other herpesviruses, CMV exhibits both lytic and latent infection cycles. Although much less is known about the molecular aspects of CMV DNA replication in comparison to alpha-herpesviruses, there is some evidence to indicate that linear molecules of CMV DNA undergo circularization, via terminal regions, upon initial infection of host cells (Marks and Spector, 1988).

At the present, only a single copy of the lytic origin of CMV DNA replication (ori*Lyt*) has been identified and analyzed in some detail. Based on a novel approach utilizing the ganciclovir-induced chain termination method, Hamzeh et al. (1990) located a region encoding a potential lytic origin of CMV DNA replication near the center of the CMV U_L component (**EcoRI**-V fragment). Subcloning and deletion analyses of the potential CMV lytic replication origin

revealed that the functional ori*Lyt* was located on a 2.4-kbp fragment (Anders and Punturieri, 1991). In contrast to alpha-herpesvirus lytic origins of replication, the HCMV ori*Lyt* is intriguing for its large size and complexity. Several novel repeated sequence elements and known transcription factor binding sites such as ATF/CREB, MLTF/USF, and Sp1 within the HCMV ori*Lyt* were identified (Anders et al., 1992). It is noteworthy that the overall base composition of the HCMV ori*Lyt* is approximately 62% G+C with an asymmetric nucleotide distribution; the leftward boundary of ori*Lyt* contains an AT-rich segment (up to 70%) and the rightward boundary contains extremely G+C rich sequences.

Several lines of evidence suggest that the HCMV ori*Lyt* is functionally analogous to lytic origins of alpha-herpesviruses. Moreover, the functionality of the HCMV ori*Lyt* appears to be similar to the ori_L of HSV, based on several observations; 1). HCMV ori*Lyt* is localized near the center of the U_L (approximately 1-kbp upstream of UL57 which encodes a single-stranded DNA binding protein) as in the HSV ori_L, 2). Plasmids containing the 2.4-kbp HCMV ori*Lyt* can induce the generation of high-molecular-weight tandem oligomers, 3). HCMV ori*Lyt* requires the virally encoded DNA polymerase (Hamzeh et al., 1990; Anders and Punturieri, 1991; Anders et al., 1992).

The lytic origin of simian cytomegalovirus (SCMV) has also been located and analyzed (Anders and Punturieri, 1991). As with the HCMV ori*Lyt*, the structure of the SCMV ori*Lyt* appears to be complex. A core functional unit of the SCMV ori*Lyt* was localized to a 1.3-kbp fragment containing four distinct domains; a 9-bp repeated sequence, an AT-rich region, an 11-bp direct repeat sequence, and a

47-bp direct repeat sequence.

The overall CMV DNA replication process is unclear. Hamzeh et al. (1990) proposed that CMV DNA replication occurs bidirectionally, and not by a rolling circle mechanism. However, based on transient plasmid amplification assays, HCMV ori*Lyt* containing plasmids can induce the amplification of tandem oligomers as in HSV lytic origins of replication. This result suggests that HCMV DNA replication occurs via a rolling circle mechanism as in HSV (Anders et al., 1991).

iii). Gamma-herpesvirus DNA replication.

EBV is a human herpesvirus belonging to the gamma-herpesvirus subfamily. EBV infects and immortalizes human B lymphocytes *in vitro*, and is associated with mononucleosis, nasopharyngeal carcinoma, and Burkitt's lymphoma (zur Hausen, 1981). The structure of EBV genome consists of five unique sequence domains which are divided by four classes of internal repeat elements (IRs) and a variable number of directly repeated 500-bp sequences (TR) located at both ends of the EBV genome (Dambaugh et al., 1980) (Fig. 5). Thus, overall structure of the EBV genome can be represented as TR-UI-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR.

B-cells immortalized by EBV express relatively few EBV genes and are, therefore said to be latently infected with EBV. There are two distinct *cis*-acting elements required for EBV DNA replication during lytic and latent phases of the EBV life cycle (Yates et al., 1984; Hammerschmidt and Sugden, 1988). In latently infected cells, EBV genomes exist as multiple copies of plasmids containing the

plasmid origin of replication (oriP) which is derived from the U1 region of EBV genomes (Fig. 5) (Yates et al., 1984). OriP is approximately 1,700-bp in length, and contains two *cis*-acting components required for activity; approximately 20 imperfect copies of a 30-bp sequence and a 65-bp region of dyad symmetry located 1-kbp away from the family of 30-bp sequences (Reisman et al., 1985). Deletion analysis within both *cis*-acting components of oriP, results in loss of oriP activity. The distance and orientation of two *cis*-acting elements did not alter the oriP function, suggesting that neither a precise distance nor a particular orientation of the two components relative to one another, is essential for oriP activity (Reisman et al., 1985; Chittenden et al., 1989). The overall structure of oriP is very complex and distinct from that of alpha-herpesvirus lytic origins of replication. Most alpha-herpesviruses generate head-to-tail concatemers during their replication, whereas the products of oriP replication are circular plasmid molecules (Yates and Camiolo, 1988). In addition, replication of plasmids containing oriP is synchronized with host cell proliferation, replicating only once during S phase of the cell cycle (Adams, 1987).

In EBV latently infected B lymphocytes, four distinct EBV proteins are expressed; EBV nuclear antigens 1 (EBNA1), 2 (EBNA2), 3(EBNA3) and a plasmid membrane protein (LMP) (van Santen et al., 1981; Hennessy et al., 1984; Hennessy et al., 1985; Hennessy and Kieff, 1985; Sample et al., 1986). The oriP domain and its flanking sequences are of great interest, primarily due to the presence of 5′ regions for all three EBV nuclear antigen RNAs. All three nuclear antigens are, presumably, derived from a single primary transcript which is modified by

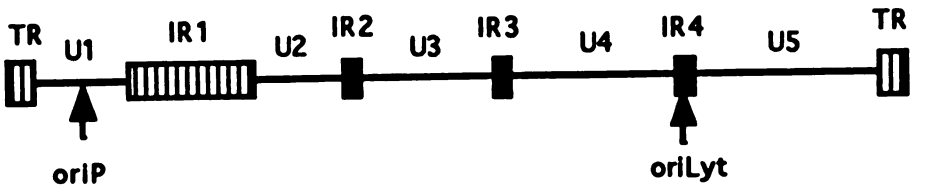

Fig. 5 Epstein-Barr virus genome structure. Terminal repeats (TR), internal repeats (IR1, IR2, IR3, and IR4), unique sequences (U1, U2, U3, U4, and U5)), origin of plasmid (oriP) and lytic (ori*Lyt*) replication are indicated.

alternate splicing. EBNA-1 has the ability to transactivate oriP by binding to the family of 30-bp repeats within the enhancer region of oriP, allowing maintenance and replication of EBV episomes (Reisman, et al., 1985).

Treatment of EBV latently infected cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can reactivate EBV and subsequently induce the lytic phase of the EBV cycle (zur Hausen et al., 1981). During lytic infection, EBV utilizes a separate replication origin, distinct from oriP (ori*Lyt*). Hammerschmidt and Sugden (1988) first described the ori*Lyt*, which is present twice in genomes of most EBV isolates and serves as a lytic origin of EBV DNA replication. One copy of ori*Lyt* is centrally located within the EBV genome, approximately 40-kbp away from oriP (Fig. 5). The second copy of oriLyt is located at the right end of the EBV genome. As in HSV ori_S, EBV isolates containing only one copy of ori*Lyt* can replicate as efficiently as EBV isolates containing both copies of ori*Lyt*. Several observations suggest that EBV Ori*Lyt* is structurally and functionally different from EBV oriP,

but functionally analogous to HSV lytic origins of replication; 1). Replication products via ori*Lyt* are composed of concatemeric EBV DNA molecules, similar to the products of HSV ori_S and ori_L, 2). Plasmids containing ori*Lyt* induce a 100-1000 fold amplification of the input plasmid molecules in cells that support the lytic life cycle of EBV, whereas templates containing oriP replicate only once during S phase of the cell cycle, and 3). Ori*Lyt* requires viral DNA polymerase as do HSV lytic origins of replication, whereas oriP containing plasmids require only EBNA-1 for replication.

The structure of ori*Lyt* is more complex than HSV ori_S and ori_L consisting of arrays of direct and inverted repeat sequences. Auxiliary sequences containing potential transcription factor binding sites have been localized near ori*Lyt* sequences. These binding sites accentuate ori*Lyt* replication activity. Therefore, as for HSV DNA replication, a combination of cellular and virally encoded transcription factors may play important roles in the lytic cycle of EBV DNA replication.

iV). Marek's disease virus DNA Replication.

Knowledge of the *cis*-acting sequences required for viral DNA replication is essential to understand viral replication mechanisms. Utilizing a defective MDV genome, a functional origin of DNA replication in serotype 2 MDV was identified (Camp et al., 1991). The serotype 2 MDV replication origin is located in inverted repeats flanking the L fragment, indicating the existence of at least two copies of origin sequences within serotype 2 MDV genomes. The replication origin of

serotype 2 MDV was localized to a 90-bp region arranged in an imperfect palindrome containing 30-bp of alternating AT sequences. The structure and sequence of the serotype 2 replication origin is strikingly similar to that of alpha-herpesvirus replication origins. In addition, the 90-bp sequence contains a 9-bp motif highly conserved among origins of alpha-herpesviruses. The 9-bp sequence is a subset of an 11-bp motif recognized by the HSV origin binding protein (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988). Although MDV shares biological properties similar to those of gamma-herpesviruses, no significant nucleotide sequence similarity was found between the serotype 2 replication origin and origins of beta- or gamma-herpesviruses.

Based on nucleotide sequence analysis, a putative replication origin of serotype 1 MDV was reported (Bradley et al., 1989). The putative serotype 1 MDV origin sequence is located in inverted repeats flanking the L component of the serotype 1 MDV genome, and contains several transcription factor binding sites (Sp1 and Oct-1 binding sites) within flanking regions of the presumed origin of replication (Bradley et al., 1989; Morgan et al., 1991). As in HSV, CMV, and EBV, these auxiliary components may play important roles in MDV DNA replication. Morgan and co-workers (1991) observed a decrease in MDV plaque formation when cells were co-transfected with standard viral DNA and a plasmid containing the auxiliary component of the putative serotype 1 origin sequence. Constructs containing deletions within those transcription factor binding sites did not result in plaque inhibition, indicating that there may be competitions for transcription factors between the standard virus and plasmids containing the

putative origin sequences.

Transcription factor binding sites located at close proximity to the putative serotype 1 MDV replication origin are most likely utilized to express a family of MDV genes; a gene encoding pp38 (Silva and Lee, 1984; Cui et al., 1991; Chen and Velicer, 1992) and a family of transcripts thought to play roles in MDV transformation (Bradley et al., 1989; Chen and Velicer, 1991). A gene encoding pp38, a MDV phosphoprotein, is located within the junction of long unique and long internal repeat regions of the serotype 1 MDV genome. The 5'-promoter region of the pp38 gene is located upstream of the putative serotype 1 MDV origin sequence, and transcribes right to left with respect to the putative origin sequence. Expression of pp38 is insensitive to phosphonoacetic acid treatment (inhibits viral DNA synthesis), suggesting that pp38 may belong to one of the immediate-early or early proteins (Chen and Velicer, 1992). Based on immunoprecipitation analyses, pp38 appears to be serotype 1 specific (Silva and Lee, 1984; Ikuta et al., 1985). It has been proposed that pp38 plays a role(s) in MDV tumorigenicity and latency due to its high expression in MDV lymphoblastoid cell lines (Cui et al., 1991; Chen and Velicer, 1992).

Within the downstream region of the putative serotype 1 MDV replication origin, a family of transcripts potentially responsible for MDV tumorigenicity was identified (Bradley et al., 1989; Chen and Velicer, 1991). Expression of these transcripts is very complex followed by extensive post-transcriptional modifications. It is noteworthy that these transcripts are directly associated with a heterogeneous region (het) which is presumed responsible for MDV

transformation. The het region consists of 132-bp repeats and undergoes expansion (0.6 to 5.4-kbps) in different serotype 1 MDV isolates upon serial *in vitro* passages (Hirai et al., 1981; Hirai, 1988; Fukuchi et al., 1985; Silva and Witter, 1985). In addition to those transcripts, a potential immediate early gene was localized in the downstream region of the putative serotype 1 replication origin. Construction of a cDNA library from serotype 1 MDV infected cell RNA revealed a 1.3-kbp cDNA clone transcribing in the left to right direction away from the putative origin sequence (Hong and Coussens, personal communication). It is intriguing that multiple transcripts that are implicated in MDV transformation are located near the putative serotype 1 MDV replication origin. It is of interest, therefore, to study the affects of those transcripts and their promoter/enhancer regions in MDV DNA replication.

Despite electron microscopic studies on MDV DNA structures were reported (Nazerian and Burmester, 1968; Cebrian et al., 1982), the mode(s) and kinetics of MDV DNA replication have not been determined. Based on the comparison of genome structures between MDV and HSV, it is likely that MDV genomes undergo isomerization and formation of concatemers during the MDV lytic infection stage, but this has not been proven. Reilly and Silva (1993) observed two different sizes of TR_L fragments for serotype 3 MDV genomes, suggesting that MDV genomes, may, in fact, isomerize similar to HSV genomes. Further studies are required to understand the molecular events of MDV DNA replication and genome arrangement.

4. Defective herpesviruses

Defective virus particles represent any virus genomes that are lacking the complete genetic information of non-defective standard virus genomes. As a consequence, such particles are not infectious and they need a "helper virus" to provide functions necessary for replication and propagation. Defective viruses are present in many animal, bacterial, and plant virus stocks that have gone through *in vitro* undiluted serial passaging (Huang, 1973). The first description of defective herpesvirus particles was reported by Bronson et al. (1973). Since then, characterization of defective variants from other herpesviruses, such as pseudorabies virus (PrV), equine herpesvirus (EHV), cytomegalovirus (CMV), Epstein-barr (EBV) virus, herpesvirus of saimiri (HVS) and Marek's disease virus (MDV) has been reported (Fig. 6) (Frenkel, 1981; Carter and Silva, 1990). Because defective HSV variants have been the most extensively studied, the current review will primarily focus on defective HSV particles, and when appropriate, studies reported on other defective herpesviruses will be included.

There are at least three important aspects to the study of defective virus genomes. First, they represent deleted versions of their parental virus genomes and yet retain *cis*-acting replication recognition sites (an origin of replication and a cleavage-packaging signal). Defective virus genomes thus represent simpler model systems in which to study *cis*-acting elements essential for virus propagation, modes of viral DNA replication, and viral or cellular factors required for viral DNA replication. Secondly, because of their ability to replicate in the

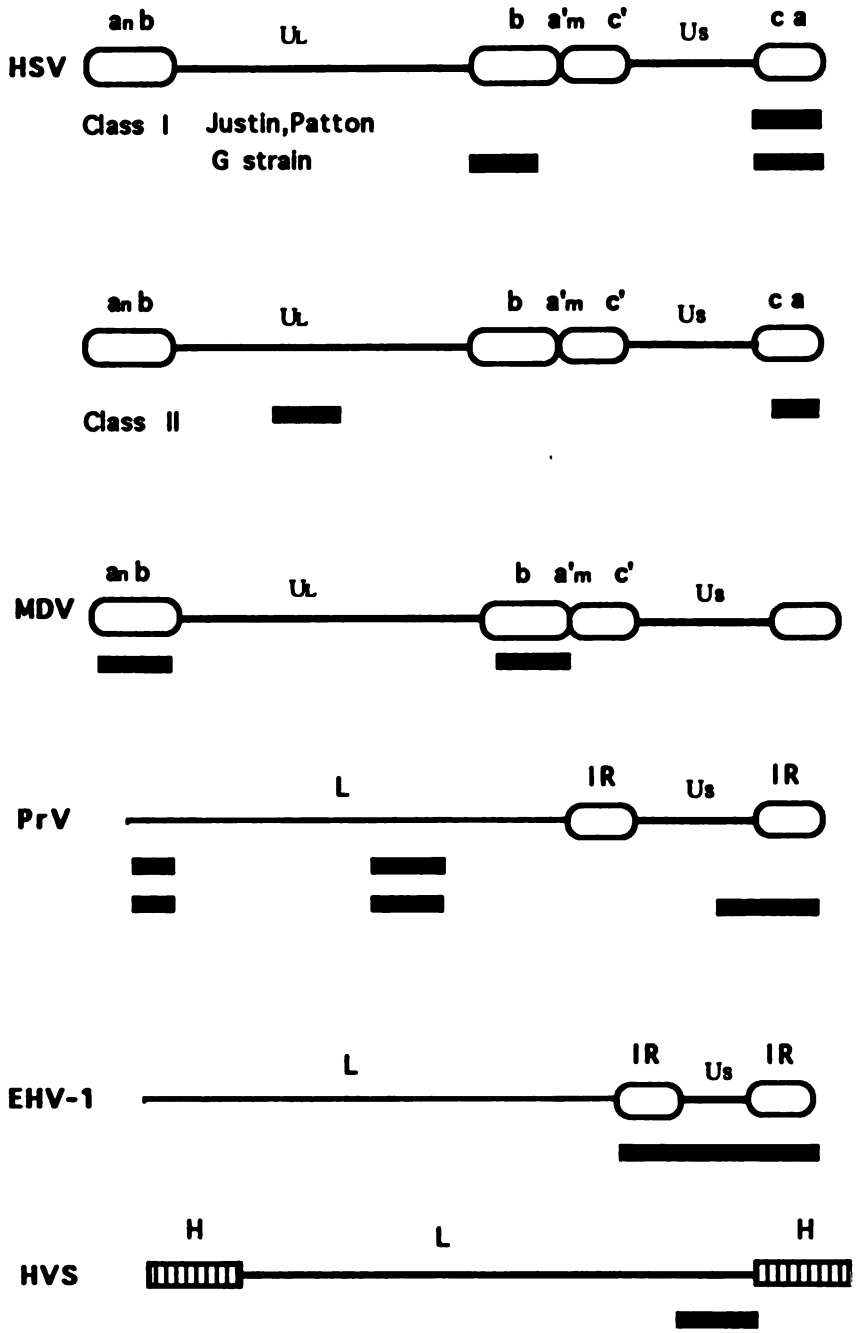

Fig. 6 Black boxes represent selected viral sequences retained in defective herpesviruses; class I and class II defective herpes simplex virus (HSV), defective Marek's disease virus (MDV), defective pseudorabies virus (PrV), defective equine herpesvirus type 1 (EHV-1) and defective herpesvirus saimiri (HVS).

presence of *trans*-functions (enzymes and factors) provided by helper viruses, defective virus DNA can be utilized as a shuttle vector to deliver and express foreign gene products in eucaryotic cells. Finally, overall virus gene expression and virus-host cell interactions are altered in cells infected with virus stocks containing a large percentage of defective particles. Therefore, defective particles have implications in the overall outcome of virus pathogenesis and the virus evolutionary process.

A. Generation of defective particles.

Propagation of plaque purified virus in tissue culture cells at high multiplicity of infection (M O.I), often results in accumulation of defective viral variants. It is noteworthy, however, that defective virus particles were frequently observed in cells infected with herpes virus saimiri (HVS), a lymphotropic gamma-herpesvirus, at low input multiplicities (Fleckenstein et al., 1975). In addition, cells transfected with standard HVS DNA resulted in generation of defective virus particles only after a few replication cycles. This result suggests that other factors are involved in generation of defective particles other than high M.O.I. Defective HVS DNA, which contains reiterated repetitive sequences, have been found in autopsy materials from tumors induced by HVS and in lymphoblastoid cell lines derived from lymphomatous HVS tumor organs (Fleckenstein et al, 1975). Thus, results with HVS are raising questions in regard to a possible role(s) defective particles play in overall viral pathogenesis and virus-host cell interactions.

In contrast to most other herpesviruses, MDV is highly cell-associated and,

therefore, it is impossible to establish high M.O.I in tissue culture cells. One defective variant, however, was identified in CEF cells infected with 281MI/1 passage 94, a serotype 2 MDV strain (Silva et al., 1988; Carter and Silva, 1990). As in HSV amplicons, defective MDV genomes (MDV replicon) exist as multiple reiterated head-to-tail concatemers (Fig. 7). However, MDV replicon differs from HSV amplicons in at least two aspects; 1). MDV replicon DNA is derived from inverted repeats flanking the L component, whereas the analogous class I HSV amplicons originate from repeats flanking the S component, 2). CEF cells co-transfected with a plasmid construct containing a single repeat unit of MDV defective genome and standard virus DNA resulted in the generation of a recombinant replication competent virus (Silva et al., 1988; Silva and Witter, 1991), whereas such a phenomenon has never been observed with HSV amplicons, and 3). Defective MDV particles were observed in cell culture passages of greater than 90, whereas defective HSV particles are often observed in cell culture passages 10-15. As in HVS, the occurrence of defective MDV genomes is not likely due to high M.O.I, but rather may be due to repeated multiple passages of the virus stocks and mechanisms that we do not understand at the present.

Most defective virus DNA contains selected viral sequences (*cis*-acting replication elements) derived from parental virus genomes. A model suggesting that, during high M.O.I., inter-molecular recombinational events generate defective variants containing limited DNA sequences of viral origins. Intra-molecular recombination events are favored over other types of genomic rearrangements (Kwong and Frenkel, 1984). Conservation of *cis*-acting replication

elements retained in most defective virus DNAs suggest that preferential selective treatment of those DNA sequences during undiluted serial passages.

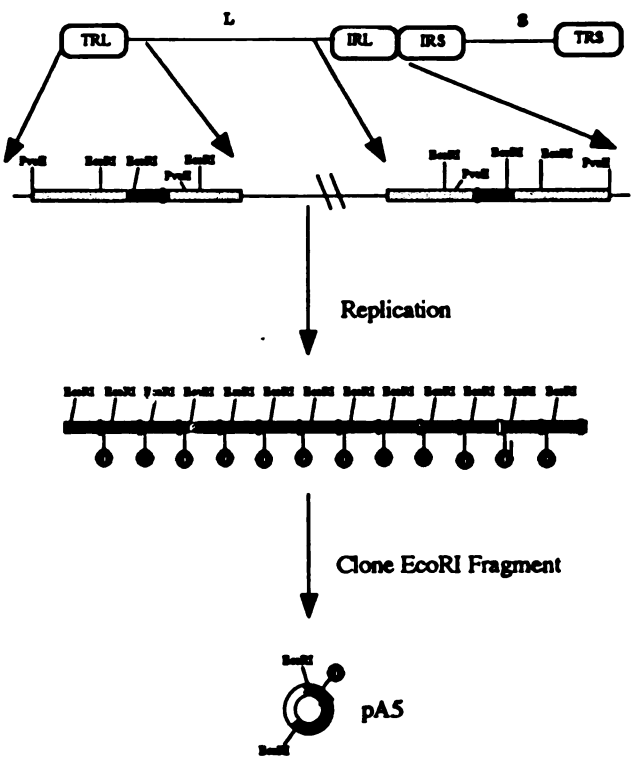

FIG. 7 Generation of MDV replicon (pA5).

B. Structure of defective genomes

In general, most defective virus DNA contains multiple reiterations of selected viral sequences, derived from parental virus genomes in head-to-tail

arrangements. The overall size of packaged defective virus DNA is indistinguishable from that of standard virus DNA, with the exception of human CMV defective virus particles, which are smaller than parental virus DNA (Stinski et al., 1979).

There are two predominant species of defective variants in HSV type 1 and 2 strains. Class I defective HSV genomes contain viral sequences derived from the right end of the S component of parental virus DNA in head-to-tail tandem arrays (Bronson et al., 1973; Frenkel et al., 1976; Murray et al., 1975). Because of the S component of HSV genomes contains relatively high G+C content, class I defective HSV DNA display a higher buoyant density than that of parental virus DNA. Class II defective HSV genomes contain multiple reiterated sequences predominantly from within the central unique portion of the L segment, covalently linked to DNA sequences from the end of the S segment (Cuifo and Hayward, 1981; Stegman et al., 1978; Frenkel et al., 1981). Unlike class I series, class II defective HSV variants display buoyant density that is indistinguishable from that of parental virus DNA (Frenkel, 1981). Both classes of HSV defective DNA contain relatively homogeneous populations of DNA molecules consisting of head-to-tail concatemers. Different size repeat units are observed among different defective viral isolates. Repeat size differences are primarily due to the size of contiguous sequences flanking the S portion or the central L segment. In certain cases, deletions or deletion/substitutions were found in both types of defective HSV DNA (Frenkel, 1981).

Pseudorabies virus (PrV) is an alpha-herpesvirus that infects porcine. In vitro,

rabbit kidney cells infected with PrV at high M.O.I have generated two different types of defective PrV particles (Pr1 and Pr2) (Ben-Porat and Kaplan, 1976). Pr1 and Pr2 defective particles differ from each other in terms of their overall DNA composition. Yet Pr1 and Pr2 share some common structural characteristics (Ben-Porate and Kaplan, 1976; Rixon et al., 1980; Rixon and Ben-Porate, 1979). Pr1 contains DNA sequences derived from two noncontigous regions of PrV genomes (middle and end of the L segment), consisting of highly reiterated DNA sequences that have a lower buoyant density than standard viral DNA (Rixon et al., 1980). In contrast, Pr2 contain DNA sequences with only a few reiterated sequences and have a buoyant density indistinguishable from that of standard virus DNA (Ben-Porate and Kaplan, 1976; Rixon and Ben-Porate, 1979). However, the molecular weights of both Pr1 and Pr2 are very similar to each other, and only slightly smaller than that of standard virus genomes.

C. Replication of defective particles.

Murray et al. (1975) reported that serial passages of undiluted HSV types 1 and 2 resulted in a "cyclic pattern" of infectious standard and defective virus production. Moreover, cells infected with virus stocks containing a mixture of standard and defective virus particles resulted in a loss of viral infectivity with a concomitant increased level of defective virus DNA synthesis. It is noteworthy, that interference with standard virus replication by defective particles was absent in cells infected with standard virus prior to challenge with defective viruses, suggesting that the level of interference may have occurred during an early event

of the viral replication process.

Time course infection studies with virus stocks containing a mixture of standard and defective virus particles were reported by Vlazny and Frenkel (1981). They proposed existence of a "temporal and enzymatic" relationship between defective and standard viruses in DNA synthesis. Replication of defective virus DNA occurs most actively during the late phase of infection, and is inhibited by the treatment of infected cells with phosphonoacetic acid (an inhibitor of HSV DNA polymerase), indicating that both defective and standard virus DNA require the HSV DNA polymerase activity. In addition, they observed an inverse relationship between the rate of DNA synthesis of defective and standard viruses.

In contrast to the kinetics of defective HSV DNA synthesis, similar time course infection studies on defective PrV synthesis indicate that defective PrV genomes replicate preferentially at an early stage of infection (Wu et al, 1986). Different kinetics observed in defective HSV and PrV DNA synthesis are not well understood. A possible answer may be due to enrichment of different types of origin sequences that have different functions in DNA replication.

Becker et al. (1978) first proposed a model for defective virus DNA replication. Based on electron microscopy studies, Backer et al. observed several "replicative intermediates" in cell fractions containing only defective virus DNA; circular molecules varying in size with contour lengths (10, 5, 2.5 and less than 2.5um), linear DNA molecules with the length of intact virion DNA (52um), and circular-linear DNA molecules, with circular components of either 2.5um or 5.0um, and

linear components varying in length from 1-50um. The observation of different types of "replicative intermediates", and especially the presence of circular-linear molecules suggest that the S component of standard virus DNA (common to all defective HSV particles) is excised out, and fragmented into smaller circular molecules during undiluted serial passages. These small circular molecules serve as templates to generate linear concatemers via a rolling circle mechanism (Becker et al., 1978).

Additional evidence supporting the rolling circle model of defective HSV DNA replication was reported by Vlazny and Frenkel (1981). Co-transection of rabbit skin cells with a monomeric repeat unit of defective virus DNA resulted in re-generation of progeny defective virus genomes that were identical to the structure of the original defective virus genome (Vlazny and Frenkel, 1981). These results indicate that the single monomeric repeat unit can serve as a template to generate full length head-to-tail concatemers via a rolling circle mechanism.

D. *Cis*-acting recognition sites within defective virus genomes.

Successful regeneration of defective progeny DNA from a single repeat unit parent, provided evidence for two important *cis*-acting replication elements within the HSV genomes (Vlazny and Frenkel, 1981; Stow and McMonagle, 1983; Spaete and Frenkel, 1985). Stow and McMonagle (1983) first reported the identification of two *cis*-acting functions necessary for propagation of HSV class I amplicons (a recombinant vector containing a single repeat unit). In addition, Spaete and

Frenkel (1985) have mapped ori_L and a cleavage/packaging signal site within the amplicon pP2-102 containing a 3.9-kb repeat unit of HSV-1 class II defective genomes. HSV class I defective genomes contain a replication origin derived from repeat regions flanking S segments of the parental virus genome (ori_S). Whereas, HSV class II defective genomes contain a replication origin derived from a central portion of the L segment (ori_L).

Other defective herpesvirus particles were utilized to identify origins of DNA replication, such as Equine herpesvirus I (EHV-1). EHV-1 is an alpha-herpesvirus that infects horses, and causes spontaneous abortions in infected pregnant mares (O'Callaghan et al., 1978). Defective EHV-1 particles have been obtained from serial undiluted passages of standard virus genomes. Defective EHV-1 genomes were utilized to map and determine nucleotide sequences of an EHV-1 origin of replication (Baumann et al., 1989). Thus, defective particles represent tools to investigate *cis*-acting replication functions necessary for the replication of standard and defective viruses.

In addition to a functional origin of replication, both classes of HSV amplicons retained sequences derived from S component termini, responsible for the cleavage/packaging process (*a* sequence). Detailed analysis of maturation and cleavage of concatemeric defective genomes based on transfection-propagation assays was reported by Deiss and Frenkel (1986). To summarize, functional HSV amplicons and their deletion mutants were transfected into cells along with helper virus DNA. Transfection-derived virus stocks were further propagated through several serial passages and analyzed for the presence of regenerated progeny

defective genomes. Only the HSV amplicons containing a functional origin of replication and a cleavage/packaging signal site were expected to be recovered in transection-derived virus stocks after serial propagation. In summary, the results of Deiss and Frenkel led to the following conclusions; 1). The cleavage/packaging signal site resides within the *a* sequence of defective virus genomes, 2). The cleavage process for viral DNA concatemers is coupled to the packaging process, 3). The cleavage/packaging event involves amplification of the *a* sequence.

E. Role (s) of defective particles in pathogenesis and virus-host cell interactions.

Accumulation of defective virus particles is often accompanied by a decrease in standard virus production with reduction in virus titers. Since the first description of this phenomenon by Von Magnus (1951), decrease in standard virus titers have been recognized in the growth of almost all viruses in the presence of defective particles. Based on available literature, it appears that the interference of standard virus growth by defective particles is either due to the ability of defective particles to compete with standard virus at the level of viral DNA replication or transcription; defective genomes are enriched for origins of DNA replication due to their concatemeric genomic arrangements, and often contain several transcription factor binding sites. Alternatively, interference may occur at the cleavage/packaging process (Frenkel, 1981; Rixon and Ben-porate, 1980).

In HSV systems, the study of standard and defective genome replication revealed a quantitative correlation between the capacity of various passages containing different concentrations of defective particles to interfere with standard virus replication (Schroder et al., 1975/76; Frenkel et al., 1975; Vlazny and Frenkel, 1981). In all cases, the degree of interference was dependent upon the initial ratios of defective to standard virus particles present within a given virus stock. However, other undetected small alterations within standard virus genomes that may have arisen during undiluted serial passages were not taken into an account. Seemingly small alterations in standard virus genome structure may have a profound effect on the overall outcome of infectious standard virus production.

Analyses of viral transcripts and infected cell polypeptides (ICPs) in cells infected with HSV virus stocks containing a mixture of standard and defective viruses revealed the overproduction of ICP4 (Class I defective HSV series) and ICP8 (Class II defective HSV series) (Murray et al., 1975; Frenkel et al., 1975). Overproduction of certain ICPs was dependent upon the gene templates located in each repeat unit of a given defective virus genome. In addition, Frenkel and co-workers (1981) observed a linear relationship between the degree of ICP overproduction and the number of reiterated sequences present in defective HSV genomes. In contrast to overproduction of ICP4 and ICP8, most early (E) and late (L) gene products were underproduced in cells infected with a mixture of defective and standard HSV particles (Frenkel et al., 1981).

Development of defective particles from cells infected with Pseudorabies virus (PrV) at high M.O.I occurs rather slowly when compared to defective HSV or

defective EHV-1 particles (Ben-Porate et al., 1974). In addition, cyclical increases and decreases in infectious virus titers observed upon infection of cells with virus stocks containing defective HSV or EHV-1 particles, were not observed with defective PrV particles (Ben-Porate and Kaplan, 1976). In the PrV systems, overproduction of some transcripts, for which the defective PrV DNA is enriched, was observed in cells co-infected with standard and defective PrV viruses (Rixon and Ben-Porate, 1980). However, the protein profile of the resulting co-infection was indistinguishable from that observed in cells infected with standard PrV alone. This indicates an apparent lack of translation of the RNA species that were overproduced by defective PrV particles. Overproduction of IE gene products such as HSV ICP4 was not observed and therefore, could not account for the ability of defective Pr viruses to interfere with standard virus production. It is noteworthy, that mechanisms controlling the kinetics of gene expression (IE, E and L) located within defective PrV genomes were identical to mechanisms controlling standard PrV gene expression. Wu and co-workers (1986) reported that interference of standard PrV by defective PrV particles occurs at an early stage of infection and is accompanied by transient replicative advantages for defective PrV, due to enrichment of origin sequences. Moreover, interference occurs at the level of cleavage and encapsidation of standard virus. Precise mechanisms regarding interference of standard virus growth by defective virus particles, however, are yet to be clarified.

F. Defective herpesviruses as eucaryotic expression vectors

In general, proteins expressed in mammalian cells require post-translational modifications that are unique to animal cells. Consequently, the development of eucaryotic expression vectors that can deliver a high production of authentic, biologically active protein products is of great interest.

Utilization of defective HSV particles as eucaryotic expression vectors stems from several considerations; 1). defective HSV particles contain an origin of DNA replication and a cleavage/packaging signal site that are essential for virus propagation (Frenkel, 1981; Spaete and Frenkel, 1985), 2). a single monomeric repeat unit of defective HSV genomes can regenerate full-length defective genomes consisting of head-to-tail reiterations, offering the potential for amplification of foreign DNA ((Vlazny and Frenkel, 1981; Vlazny et al., 1982), 3). defective HSV genomes appear to be relatively stable upon serial passage *in vitro* (Kwong and Frenkel, 1984; Spaete and Frenkel, 1982).

Defective HSV particles (HSV amplicons) have been utilized as an efficient cloning-amplifying vector for expression of a chimeric chicken ovalbumin gene in rabbit skin cells (Kwong and Frenkel, 1985). Briefly, a chimeric amplicon was constructed by cloning a modified chicken ovalbumin gene, under control of the HSV-1 ICP4 promoter sequences, followed by co-transfection of cells with the chimeric amplicon and helper virus DNA. Analysis of polypeptides synthesized in cells infected with the resultant virus stocks containing regenerated defective virus genomes revealed an abundant chicken ovalbumin polypeptide of the correct size.

Because of the ability of HSV-1 to infect and establish latency in neurons, HSV

amplicons have attracted much attention in the development of systems to deliver foreign genes in post-mitotic neuronal cells. Infection of neurons with a chimeric HSV amplicon vector containing the E. *coli* lacZ gene driven by the HSV-1 immediate early 4/5 promoter in the presence of a helper virus resulted in a high expression of stable beta-galactosidase activity (Geller and Breakefield, 1988).

As mentioned earlier, the replication of the EBV genome in latently infected human B-cells requires only oriP and EBNA-1 nuclear antigen. Several reports indicate that plasmids containing oriP and EBNA-1 can stably replicate as episomes in various uninfected mammalian cells without helper viruses (Yates et al., 1985; Lupton and Levine, 1985). Thus, EBV derived replicons have been utilized as cloning vehicles for the stable episomal replication of cDNA expression libraries in human lymphoblastoid cells (Margolskee et al., 1988).

Jalanko et al. (1988) conducted extensive comparative studies on mammalian cell expression vectors, one containing EBV replicon sequences fused to the SV40 early promoter directing the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene (EBV-SV40-CAT) and the other with the same construct without the EBV replicon sequences (SV40-CAT) in various mammalian cell lines. Three important observations were reported; 1). Overall, cells transfected with the EBV-SV40-CAT construct have clearly higher CAT expression levels than the SV40-CAT plasmid, 2). The level of CAT expression was highly variable among tested cell lines, 3). Cells expressing a low level of CAT activity with the EBV-SV40-CAT plasmid contained mostly oligomeric forms of the transfected DNA, possibly resulting from the integration of vector DNA into host-

cell chromosomes.

Defective MDV genomes (MDV replicon) represent excellent tools to deliver foreign gene products in avian species. The MDV replicon has advantages over other defective herpesviruses in several respects. First, the use of natural host will greatly facilitate study of gene dosages in *in vivo* settings. Secondly, a naturally occurring non-oncogenic virus can be used as a helper virus to provide *trans*-acting functions required for the replication of MDV replicons. Further characterization of MDV replicon, therefore, will increase our understanding of molecular events involved in MDV DNA replication. Additionally, basic knowledge concerning the molecular nature of the MDV replicon can be applied to construction of delivery systems for expression of foreign genes *in vitro* as well as *in vivo.*

The present dissertation is primarily focused on four objectives: 1). To locate MDV replicon sequences within the parental virus genome, 2). To locate and define a functional origin of MDV DNA replication, 3). To examine and compare MDV replication origin with other herpesvirus origins, 4). To study viral or cellular origin binding proteins.

Chapter II

Identification and characterization of a Marek's disease virus origin of replication.

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ABSTRACT

Previously, we isolated a replicon from a defective Marek's disease virus (MDV) which was shown to be analogous to defective herpes simplex viruses (amplicons). Most defective viruses contain cis-acting elements required for DNA synthesis and virus propagation such as an origin of DNA replication and a packaging/cleavage signal site. In this report, the MDV replicon was utilized to locate an origin of MDV DNA replication. A comparison of MDV replicon sequences to other herpesvirus replication origin sequences revealed a-90 bp sequence containing 72% identity to the lytic origin (ori_L) of Herpes simplex virus type 1 (HSV-1). This 90 bp sequence displayed no similarity to beta-herpesvirus or gamma-herpesvirus replication origins. The 90-bp sequence is arranged as an imperfect palindrome centered around an A-T rich region. This sequence also contains a 9-bp motif (5'CGTTCGCAC3') highly conserved in alpha-herpesvirus replication origins.

To test functionality of the 90-bp putative MDV replication origin, DpnI replication assays were conducted with subclones generated from the 4 kbp MDV replicon. Results of these experiments revealed a 700-bp MDV replicon subfragment containing the 90-bp putative MDV replication origin sequence, capable of replicating in chicken embryo fibroblast (CEF) cells co-transfected with standard MDV DNA. In conclusion, we have identified a functional origin of DNA replication in MDV. Similarity of MDV origin sequences to those of alpha-herpesviruses supports the current contention that MDV is more closely related to alpha-herpesvirses than gamma-herpesviruses.

INTRODUCTION

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus. In chickens, MDV is the etiologic agent of Marek's disease (MD), a malignant T-cell lymphoma (Calneck and Witter, 1991). There are three MDV serotypes: Serotype 1 includes oncogenic MD viruses and their attenuated derivatives, serotype 2 includes all naturally occurring non-oncogenic chicken herpesviruses and serotype 3 is the antigenically related non-oncogenic turkey herpesviruses (HVT). MDV pathology has been extensively characterized (Payne, 1989). Molecular analysis of MDV, however, has lagged behind that of other herpesviruses, primarily due to technical difficulties presented by the tightly cell-associated nature of MDV infection.

The MDV genome, a double-stranded linear DNA molecule of approximately 160-180 Kbp, consists of a unique long (U_L) and a unique short (U_s) segment flanked by inverted repeats (TR_L, IR_L, IR_s, TR_s). MDV has been classified as a gamma-herpesvirus based on its lymphotropism (Roizman, 1982). However, the overall genomic structure and colinearity of many MDV genes to those of alpha-herpesviruses such as herpes simplex virus (HSV) and Varicella-zoster virus (VZV), suggest that MDV should be re-classified (Buckmaster, 1988).

Previously, we reported the isolation and characterization of a defective serotype 2 MDV (Carter and Silva, 1990). The defective MDV genome exists as a high molecular weight head-to-tail concatemer consisting of 4 kbp viral monomeric repeats. The defective serotype 2 MDV virus (replicon) is analogous to HSV amplicons (Frenkel et al., 1976).

HSV amplicons contain multiple head-to-tail reiterations of monomeric repeat units derived from either end of the U_s fragment or from two noncontiguous regions within the U_s and U_L fragments of HSV genomes (Frenkel, 1980). HSV amplicons replicate and propagate in the presence of a helper virus (Frenkel, 1980; Spaete and Frenkel, 1985; Weller et al., 1985). Molecular analysis of HSV amplicons revealed three lytic origins (ori_L and two copies of ori_s) of HSV DNA replication and a packaging/cleavage signal site (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1985).

In this study, MDV replicon DNA was utilized to locate an origin of MDV DNA replication. We have identified a 90-bp replicon sequence that shares 72% identity to the HSV-1 ori_s and ori_L. No significant similarity has been found with replication origins from either beta- or gamma-herpesviruses (Gahn et al., 1989; Hamzeh et al., 1990). The 90-bp sequence is arranged in an imperfect palindrome with alternating A-T-rich sequences. Within its 90-bp sequence is a 9-bp motif (CGTTCGCAC) that is highly conserved in alpha-herpesvirus origins of replication and is known to be recognized by the HSV-1 origin binding protein (Elias and Lehman1988; Koff and Tegtmeyer, 1988). Based on functional replication assays, we have identified a 700-bp subfragment of the replicon that contains the 90-bp sequence. This 700-bp subfragment was capable of replicating in CEF cells in the presence of helper virus.

MATERIALS AND METHODS

Cells and virus stock

Strain 281MI/1, a serotype 2 MDV field isolate (Witter, 1983), was plaque purified from cell-free virus following the fifth cell-culture passage. Preparation and maintenance of primary or secondary chick embryo fibroblast (CEF) cultures have been previously described (Silva and Lee, 1984). Secondary CEF were infected with cell-associated 281MI/1 passage 15 virus stocks.

Isolation of high-molecular weight viral DNA

High-molecular weight viral DNA was isolated in association with cellular DNA from CEF infected with cell-associated 281MI/1 virus stocks. Upon evidence of extensive cytopathic effect (CPE), cells were lysed in 150 mM NaCl, 100 mM EDTA, 1% sodium dodecyl sulfate and 100 µg/ml proteinase K for 4-24 hr at 37°C. DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 vol/vol/vol), once with chloroform-isoamyl alcohol (24:1 vol/vol), and precipitated with 2.5 volumes of 100% ethanol. The DNA was resuspended in TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and stored at 4°C.

DNA sequence analysis

Isolation and cloning of pA5 was described previously (Carter and Silva,

1990). pA5I was constructed by cloning the 4 kbp replicon into pUC18 in the inverse orientation with respect to pA5. Unidirectional deletion mutants were created from pA5 and pA5I with exonuclease III (Boehringer Mannheim Corp., Indianapolis, IN.) and S1 nuclease (Boehringer Mannheim Corp., Indianapolis, IN.), followed by re-circularization of deleted clones using T4 DNA ligase (Henikoff, 1987). DNA sequencing was performed using double stranded plasmid templates and the dideoxy chain termination method (Sanger et al., 1977). In all sequencing reactions, the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH.) was used as recommended by the manufacturer. The MDV replicon sequences were used to search for homologous sequences from GENBANK data base using the MacVector (International Biotechnologies, Inc., New Haven, CT.) computer program.

Generation of subclones from pA5

pNOTA5 was derived from pA5. In essence, a 2.5 kbp HpaI and ClaI fragment was replaced with NotI linkers (a gift from A. Finkelstein). p281MI-1 was constructed by cloning a 2 kbp BamHI fragment from the 281MI/1 genome into pUC18 at the BamHI site. pCK300 contains a 300 bp ClaI to KpnI subfragment of pA5 inserted between the ClaI and KpnI sites in pUC18. A 700 bp PstI to KpnI subfragment of pA5 was isolated and cloned into pUC18 using the PstI and KpnI sites, to create pA700.

DpnI resistance assay

DpnI resistance assay

4 kbp EcoRI replicon fragment was isolated from pA5 and re-ligated. Subclones of pA5 as well as the circular form of the 4 kbp replicon were tested for replication activity by conducting DpnI resistance assays (Stow and McMonagle, 1983). Secondary CEF were co-transfected with 500 nanograms (ng) of plasmid DNA and 20 µg of 281MI/1 DNA by calcium phosphate co-precipitation (Morgan et al., 1990), with the following modifications: Dulbecco minimal essential medium (DMEM) containing 5% calf serum, 5% fetal bovine serum and 25% tryptose phosphate buffer was used to seed 5 x 10^6 secondary CEF cells in 60 mm tissue culture plates. Four hours after adding the calcium phosphate-DNA co-precipitates, cells were treated with 15% glycerol in serum free DMEM for 3 min. Cells were rinsed carefully three times with serum free DMEM, and fresh DMEM was added. 24 hours following co-transfection, cells were maintained with F10-199 media containing 4% calf serum. When extensive CPE (5-8 days post co-transfection) was evident, total high-molecular weight cellular DNA was isolated as described before.

Approximately 6 µg of total cellular DNA was digested with EcoRI and DpnI (International Biotechnologies, Inc., New Haven, CT.) using conditions recommended by the manufacturer. Digested DNA fragments were resolved on 0.8% agarose gels and transferred to Zeta Probe membranes (Bio-Rad, Inc., Richmond, CA.) as recommended by the supplier. Probes were prepared with [alpha-^{32}P]dCTP using a random primed labeling kit (Bethesda Research Laboratories, Gaithersberg, MD.) as specified by the manufacturer. Nylon

membranes were subsequently hybridized with appropriate ^{32}p-labeled probes (Budowle and Baechetel, 1990).

GENBANK ACCESSION NUMBER

The nucleotide sequences reported in this paper have been submitted to the GenBank database. The accession number is M64132.

RESULTS

DNA sequence analysis

We attempted to locate a potential origin of MDV DNA replication by initiating DNA sequencing of the 4 kbp replicon. As previously reported (Carter and Silva, 1990), the MDV replicon was derived by cloning a 4 kbp monomeric repeat unit from a defective MDV genome into the EcoRI site of pUC18. The restriction endonuclease (RE) map of the resulting clone, designated pA5, is shown in Fig. 1.

DNA sequences of the MDV replicon were compared with published sequences of herpesvirus replication origins. A Pst1 to BamHI fragment of pA5 contained a 90-bp sequence homologous to consensus replication origins of HSV and VZV replications (Fig. 2A). The 90- bp sequence was arranged in a A-T rich imperfect palindrome and contained a 9 bp sequence (CGTTCGCAC) that is highly conserved in alpha-herpesvirus replication origins. Overall, the 90-bp sequence shares 72% identity to core regions of the HSV-1 ori_s and ori_L and also contained 66% identity to origins of VZV and Equine herpes virus type 1 (EHV-1) replication (Fig. 2B). Neither origins of Epstein-Barr virus (EBV) (oriP and oriLyt) nor the origin of cytomegalovirus (CMV) replication contained specific sequences similar to the putative origin of serotype 2 MDV replication (Anders et al., 1991; Hammerschmidt and Sugden, 1988; Hamzeh et al., 1990).

Functional replication analysis of the 4 kbp replicon and pA5

DpnI resistance assays were performed to test functionality of the putative origin of MDV replication identified by DNA sequencing analysis. DpnI cleaves only methylated GATC sequences, therefore DNA propagated from dam+ strains of E. coli is methylated and susceptible to DpnI digestion. DNA replicated in eucaryotic cells is not methylated at GATC sequences and therefore is resistant to DpnI cleavage.

CEF cells were co-transfected with 281MI/1 DNA and either pA5 or a circular form of the 4 kbp replicon contained within pA5. When extensive CPE were evident on transfected monolayers, total cellular DNA was isolated and digested with EcoRI and DpnI. The resulting fragments were resolved by agarose gel electrophoresis and transferred to nylon membranes. Membranes were hybridized with a ^{32}P-labeled 4 kbp replicon probe. EcoRI and DpnI cleavage of 281MI/1 infected CEF DNA produced 5.5, 5.2, and 2.8 kbp fragments which hybridized to the 4 kbp replicon probe (Fig. 3, lane 2). The 4 kbp replicon probe, however, did not hybridize to uninfected CEF DNA (Fig. 3, lane 1). Co-transfection of CEF cells with circular EcoRI 4 kbp DNA and 281MI/1 DNA, resulted in a DpnI resistant EcoRI fragment of 4 kbp (Fig. 3, lanes 3), in addition to the three EcoRI fragments from 281MI/1 helper virus genome. DNA isolated from CEF cells co-transfected with intact pA5 and 281MI/1 DNA also yielded a 4 kbp EcoRI fragment resistant to DpnI cleavage (Fig. 3 lane 4) in addition to the three EcoR1 fragments. Methylated input pA5 DNA mixed with 281MI/1 infected CEF DNA was cleaved by DpnI, indicating that DpnI resistance is not a

result of inhibition of DpnI activity (Fig. 3 lane 5).

Functional replication analysis of pA5 subclones

Based on DNA sequence analysis, a series of subclones from pA5 were constructed to further localize MDV sequences important for replication of pA5 (Fig. 4). Following co-transfection of CEF cells with each of the pA5 subclones and 281MI/1 DNA, total cellular DNA was isolated and subjected to DpnI replication analysis as described in Materials and Methods. Replication of pA5 would be indicated by the presence of unit-length DpnI resistant plasmid DNA following digestion of transfected cell DNA with EcoRI and DpnI (EcoRI introduces a single cut within pA5 subclones) by hybridization to a ^{32}P-labeled pA5 probe. As expected, intact pA5 was able to replicate in the presence of helper virus DNA (Fig. 5, lane 5) generating Dpn1 resistant fragments of 4 kbp and 2.7 kbp of viral and plasmid portions respectively. However, pA5 alone did not show replication activity (Fig. 5, lane 3). Among tested subclones, only pA700, which contains the putative MDV origin of replication identified by DNA sequence analysis, replicated as indicated by a DpnI resistant fragment of 3.4 kbp (2686 bp pUC18 plus 700 bp PstI to BamHI MDV DNA) (Fig. 5, lane 9). CEF cells co-transfected with 281MI/1 DNA and either pNOTA5, p281MI-1 or pCK300 (Fig. 4) representing the remainder of MDV replicon DNA did not contain any DpnI resistant plasmid sequence, suggesting they do not contain an origin of replication (Fig. 5, lanes 6, 7 and 8). Methylated input pA5, used as a control, was susceptible to DpnI cleavage (Fig. 5, lane 1).

DISCUSSION

Localization and characterization of MDV replication origins will allow construction of efficient MDV replicons for use as shuttle vectors. For example, HSV amplicons have been utilized as cloning vectors to deliver and express foreign genes in vitro as well as in vivo (Geller and Breakefield, 1988; Kwong and Frenkel, 1984; Sawtell and Thompson, 1990). In addition, isolation of an MDV replication origin will help to identify MDV or cellular proteins required for efficient replication of MDV genomes in infected cells.

DNA sequencing and functional replication assays of the MDV replicon suggest that we have identified a functional origin of DNA replication in MDV. The 4 kbp circular form of the replicon is able to replicate in CEF cells in the presence of 281MI/1 helper virus DNA. pA5, containing the 4 kbp replicon inserted into the EcoRI site of pUC18, is also replicated as an episome in CEF cells co-transfected with 281MI/1 helper virus DNA. Therefore, sequences spanning the EcoRI site within the MDV replicon are not essential for amplification of replicon DNA. However, failure of pA5 to replicate in the absence of helper virus DNA, suggests that functions provided in trans by helper virus are essential for pA5 replication.

Results of DpnI resistance assays indicated that the functional origin of MDV replication contained within pA5 is located in a 700-bp PstI to KpnI fragment of MDV replicon DNA. However, the exact localization of the MDV replication origin in the 281MI/1 viral genome is unknown, due to the lack of complete maps or genomic clones of serotype 2 MDV. Studies from our laboratory indicate there

are two copies of replicon sequences within the 281MI/1 viral genome (Silva et al., 1988). This suggests there may be at least two replication origins in MDV. A complete RE map of the serotype 2 MDV genome will allow precise localization of the serotype 2 MDV replication origin within the viral genome.

HSV contains two copies of ori_s within TR_s and IR_s localized by utilizing HSV amplicons (Frenkel et al., 1976; Frenkel, 1980). By analogy with HSV, the MDV replicon sequence is most likely to be present in repeats flanking the U_s segment of the MDV genome; assuming that the structure of serotype 2 MDV DNA is similar to serotype 1 and 3 MDV DNA. However, based on DNA sequence identity to lytic origins of alpha-herpesvirus replication, Bradly et al. (1989) and Morgan et al. (1990) have located a putative serotype 1 MDV origin of replication in repeats flanking the U_L region. MDV serotype 2 functional replication origin sequences, identified in this paper, share 82% identity to putative serotype 1 MDV replication origin sequences.

The serotype 2 MDV replication origin displays an imperfect palindrome containing 30 bp of alternating A-T residues, whereas the ori_s and ori_L of HSV contain a nearly perfect palindrome with an 18 bp A-T rich region (Deb and Doelberg, 1988; Stow and McMonagle, 1983). Lockshon et al. (1988) reported that the center of palindrome containing the 18 bp A-T rich sequence is essential for HSV-2 replication. However, expansion of the 18 bp A-T rich region to 52 bp by introducing alternating A-T sequences did not abolish HSV-2 replication function.

Interestingly, MDV replication origin also contains a 9-bp sequence, CGTTCGCAC, shown to be recognized by the HSV-1 origin-binding protein, the

product of UL9 gene expression (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988). This 9-bp sequence is a subset of an 11-bp motif (CGTTCGCACTT), highly conserved among HSV and VZV replication origins. The lytic origin of EHV-1 replication also contains this 9-bp sequence, suggesting that conservation of this region must be essential in replication functions among alpha-herpesviruses. Recently, Bruckner el al. (1991) reported that the HSV-1 origin-binding protein contains helicase activity, possibly used for unwinding DNA duplex at the origin. Others have recently reported that proper interaction of origin-binding protein with ori_s containing the 11 bp motif, is essential for DNA replication (Hernandez et al., 1991).

Although MDV is classified as a gamma-herpesvirus, analysis of the MDV replicon sequences has revealed significant sequence identity to the consensus replication origins of HSV, VZV and EHV-1 and no identity to EBV replication origins. Our data supports recent findings by others (Brunovskis and Velicer, 1992; Buckmaster et al., 1988) that MDV is more closely related to the alpha-herpesvirus, than the gamma-herpesvirus.

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Figure 1. RE map of pA5.

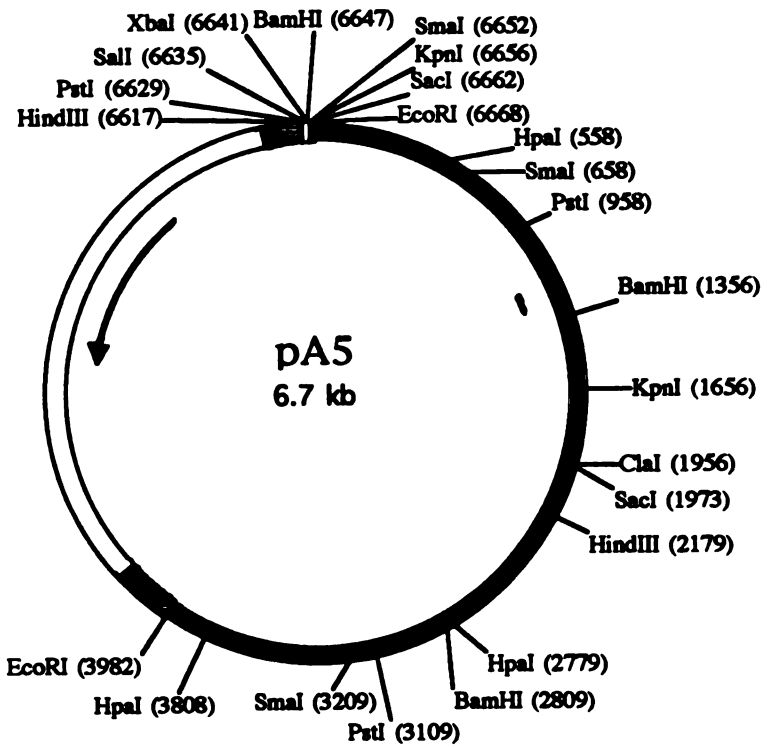


Figure 2. Identification of a putative origin of MDV serotype 2 replication by DNA sequence analysis of the MDV replicon.

DNA sequencing was performed by the dideoxy chain-termination method. A. Location and DNA sequence of the 90 bp putative origin sequence of MDV serotype 2 replication. Arrow indicates the 9 bp sequence shown to be recognized by the HSV-1 origin binding protein (9). B. Alignment of the alpha-herpesvirus lytic origins of replication with MDV serotype 2 replication origin. Boxed gray areas indicates identical sequences among other origins of herpesvirus replication.

A. MDV ori

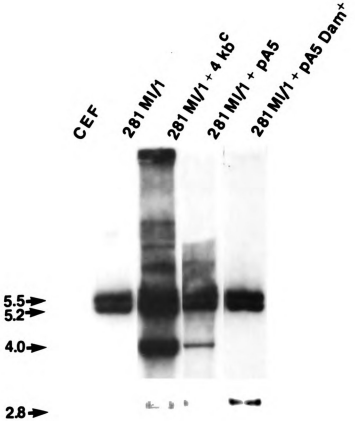
```
                    10         20         30         40         50
ACGCGTCAGC GTTCGCACCG CGAACCAATA TAAGATTATA TATATAATAT

                    60         70         80         90
ATTATTGGCG CAAGGTGCGA ACGCCGTCCG CGCAATCGGG
```


B.

```
                    10         20         30         40         50         60         70         80
MDV ori     ACGCGTCAGC GTTCGCACCG CGAACCAATA TAAGATTATA TATATAATAT ATTATTGGCG CAAGGTGCGA ACGCCCGTCC GCGCAATCGG
HSV-1 oris ACGCG-.AGC GTTCGCAC.. CG..CCAATA T---A-TATA TATAT----T ATTA-.GGCG -AA-GTGCGA AC...CG.C. GC.
HSV-1 oriL ACGCG-.AGC GTTCGCAC.. .G-.CC.A-A TAA---TATA TATAT----T ATTA--GG-. CAA.GTGCGA ACG--C.TC. G
VZV ori     -----.CA.C GTTCGCAC.. .......ATA T-A.A-TATA TATAT-ATAT A-TAT.G.-G -AA.G.G.GA ..----GT
EHV-1 oris--.G.G.-..C GTTCGCACC. ...ACCA--A TAA-.T-.-. T-.ATAAT-. ATTATTG... CAA.GTGCGA AC.
```

Figure 3. Replication of the 4 kbp replicon and pA5.

The 4 kbp replicon and pA5 were subjected to DpnI resistance assay to test their ability to replicate in CEF cells. CEF cells were co-transfected with either the circular form of 4 kbp replicon DNA (lane 3) or pA5 (lane 4) and 281MI/1 DNA. 7 days post co-transfection, total cellular DNA was digested with EcoRI and DpnI, resolved through a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P-labeled 4 kbp replicon DNA and visualized by autoradiography. Lanes: (1) Mock-infected CEF DNA; (2) DNA from CEF transfected with 281MI/1 DNA; (3) DNA from CEF co-transfected with 281MI/1 DNA and the circular form of the 4 kbp replicon DNA; (4) DNA from CEF co-transfected with 281MI/1 DNA and pA5; (5) 281MI/1 DNA was mixed with methylated input pA5.


CEF
281 MI/1
281 MI/1 + 4 kb^c
281 MI/1 + pA5
281 MI/1 + pA5 Dam+
5.5
5.2
4.0
2.8


1
2
3
4
5

Figure 4. Generation of pA5 subclones.

All clones were inserted into pUC18. The location of the putative MDV origin of replication is indicated as an open circle (○). E = EcoRI, Hp1 = HpaI, Sm = SmaI, P = PstI, BH = BamHI, K = KpnI, Cl = ClaI and H3 = HindIII.

pA5 subclones

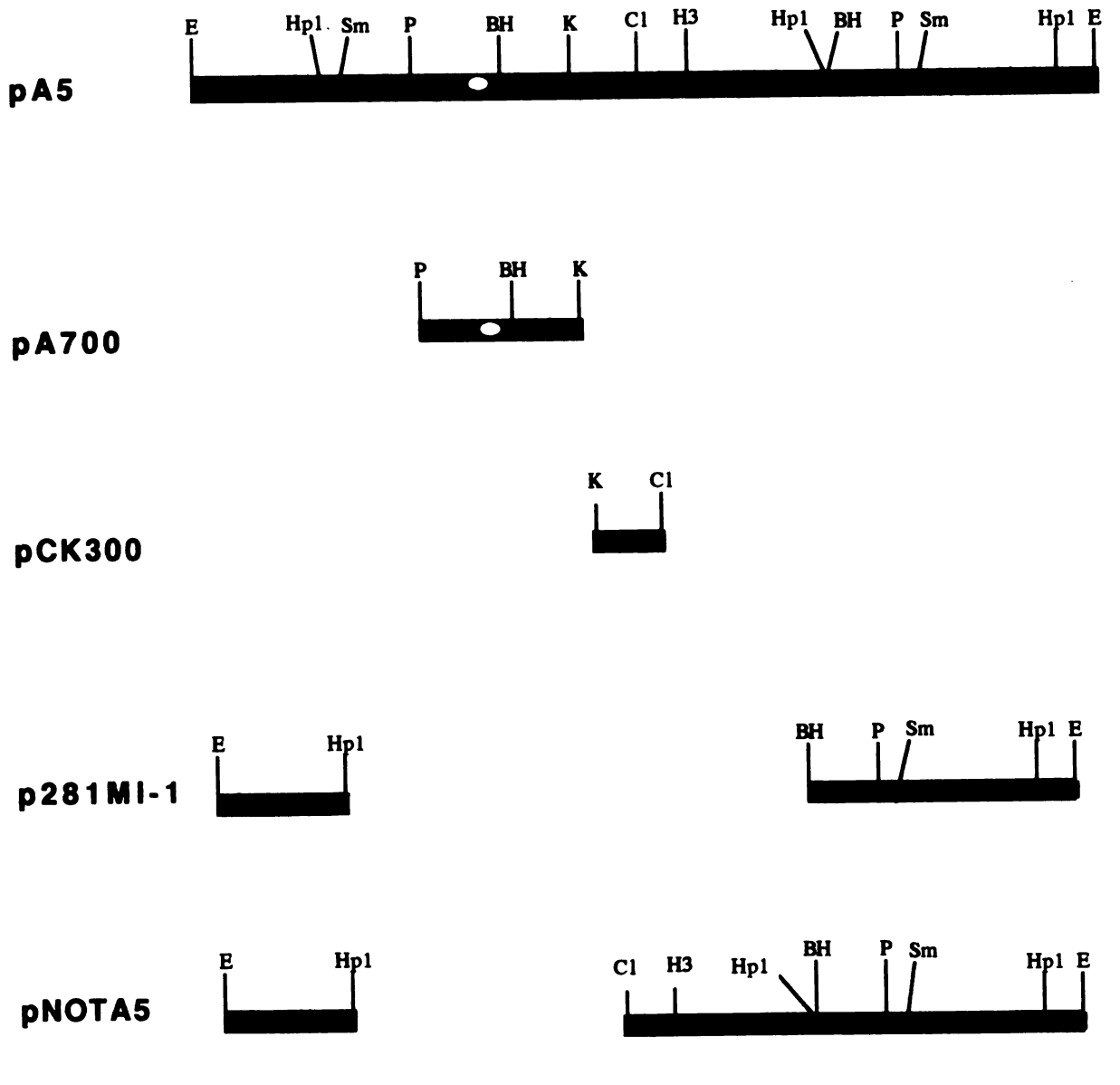
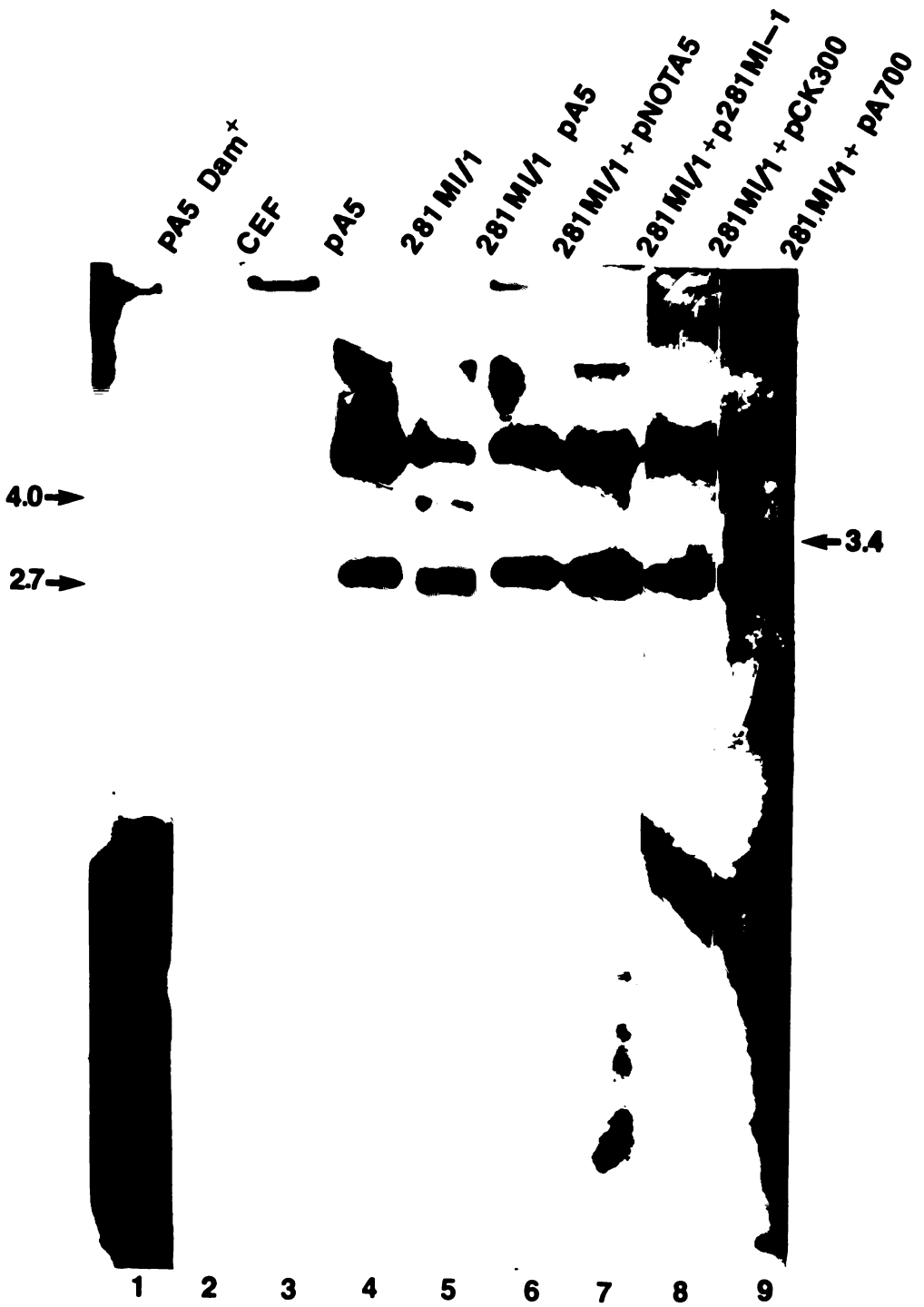


Figure 5. Replication analysis of pA5 subclones.

Each of the pA5 subclones were tested for replication activity based on DpnI resistance assay as described in Fig. 3. Each lane contains total transfected cellular DNA digested with EcoRI and DpnI. ^{32}P-labeled pA5 was used to detect plasmid DNA as well as 281MI/1 DNA. Lanes: (1) pA5 Dam+; (2) Mock-infected CEF DNA; (3) DNA from CEF cells transfected with pA5; (4) DNA from CEF cells transfected with 281MI/1 DNA; (5) DNA from CEF cells co-transfected with 281MI/1 DNA and pA5; (6) DNA from CEF cells co-transfected with 281MI/1 DNA and pNOTA5; (7) DNA from CEF cells co-transfected with 281MI/1 DNA and p281MI-1; (8) DNA from CEF cells co-transfected with 281MI/1 DNA and pCK00; (9) DNA from CEF cells co-transfected with 281MI/1 DNA and pA700.


PA5 Dam+
CEF
pA5
281MI/1
281MV1 pA5
281MI/1 + pNOTA5
281MI/1 + p281MI–1
281MI/1 + pCK300
281MI/1 + PA700
4.0
2.7
3.4
1
2
3
4
5
6
7
8
9

Chapter III

Marek's disease virus DNA contains a gene encoding a potential nuclear DNA binding protein and a HSV a-like sequence.

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(Submitted to Virology)

ABSTRACT

Four RNA transcripts from chicken embryo fibroblast cells infected with Marek's disease virus (MDV) strain 281MI/1 hybridized to the 4-kbp MDV replicon DNA. In an attempt to identify open reading frames encoding the four transcripts, we determined the nucleotide sequences of 4-kbp replicon DNA (represents a single monomeric repeat unit of defective MDV genome). Computer analysis indicates that the 4-kbp MDV replicon DNA contains two intact open reading frames (ORFs) with common promoter regulatory elements. ORF-A codes for a putative 204 amino acid protein that shares 21% and 36% amino acid sequence identity to nuclear DNA binding proteins such as the EBNA-1 of Epstein-Barr virus and galline, a chicken sperm histone protein, respectively. ORF-B encodes for a potential 350 amino acid protein, which did not show any significant amino acid sequence similarity to known protein sequences wthin Swiss-Protein data base. ORF-B may, therefore, encode a MDV specific protein. The 5'-region of MDV replicon DNA revealed seven reiterated copies of an 11-bp motif sharing 8 out of 11 nucleotide sequence identity to DR2 elements of the herpes simplex virus strain USA-8 a sequence.

INTRODUCTION

Marek's disease, a lymphoproliferative disorder of chickens, is caused by Marek's disease virus (MDV), a highly contagious oncogenic avian herpesvirus. MDV has been classified as a gamma-herpesvirus based on its lymphotropism (Roizman, 1984). However, the overall genomic structure of MDV and colinearity of MDV genes to those of alpha-herpesviruses such as herpes simplex virus (HSV) have led to re-classification of MDV as an alpha-herpesvirus (Roizman et al., 1992). The MDV genome, a double-stranded linear DNA molecule of approximately 160 to 180 kilobase pairs (kbp), consists of unique long (U_L) and unique short (U_S) segments, each flanked by inverted repeats (TR_L, IR_L, IR_S, TR_S). Based upon agar-gel precipitation and virus neutralization, MDV is divided into three groups (Bulow and Biggs, 1975; Bulow and Biggs, 1975b). Serotype 1 MDV includes oncogenic strains and their attenuated forms. Serotype 2 MDV consists of naturally occurring non-oncogenic chicken herpesviruses. Serotype 3 MDV is an antigenically related non-oncogenic turkey herpesviruses (HVT).

A defective MDV variant, analogous to HSV amplicons, was isolated from a high-passaged (HP) stock of 281MI/1, a serotype 2 MDV strain (Carter and Silva, 1990). MDV defective genomes exist as high-molecular-weight head-to-tail concatemers consisting of 4-kbp viral monomeric repeat units derived from repeat regions of 281MI/1 virus genomes (Carter and Silva, 1990; Silva et al, 1988). Most defective herpesviruses contain two important cis-acting elements necessary for DNA synthesis and virus propagation; an origin of DNA replication and a

packaging/cleavage signal site (Frenkel, 1980; Frenkel and Roizman, 1976; Spaete and Frenkel, 1985; Vlazny and Frenkel, 1981). Because of genetic complexity of parental virus genomes, defective viruses represent simpler model systems to study the process of viral DNA replication. Furthermore, defective genomes can be engineered as shuttle vectors to introduce and express foreign gene products in eucaryotic cells. Defective genomes replicate as episomes, and properly package defective DNA into virions in the presence of helper virus. (Kwong and Frenkel, 1985; Geller and Breakfield, 1988; Margolskee et al., 1988; Jalanko et al., 1988; Silva et al., 1988; Jalanko et al., 1989).

Utilizing defective MDV genomes (MDV replicon), we have identified a functional origin of MDV DNA replication, based on DNA sequencing analysis and DpnI resistance replication assays (Camp et al., 1991). Structure and nucleotide sequence of serotype 2 MDV DNA replication origin are strikingly similar to those of alphaherpesvirus origins (Camp et al., 1991; Baumann et al., 1989; Deb and Doelberg, 1988; Lockshon and Galloway, 1988; Stow and McMonagle, 1983). We now extend our earlier findings by reporting the analysis of the entire 4-kbp MDV replicon DNA. Northern blot analysis of infected cell RNA revealed four poly(A)+ RNA species (0.9, 1.4, 1.8 and 4.5-kbp) encoded within or near MDV replicon sequences. MDV replicon DNA contains two intact potential open reading frames (ORF-A and ORF-B). ORF-B may encode a 350 amino acid protein unique to MDV. Computer-aided translation of ORF-A revealed a potential protein product of 204 amino acids sharing amino acid sequence similarity to nuclear DNA binding proteins, such as the EBNA-1 of EBV

and galline, a chicken sperm histone.

The 5'-end of the MDV replicon DNA contains 90% G+C content, and shares significant nucleotide sequence identity to DR2 elements of the HSV a sequence. The HSV a sequence is thought to play important roles in generating four equimolar isomers by inverting L-S components relative to each other, and in cleavage/packaging processes of replicative intermediate forms of HSV DNA into preformed virions. The linear arrangement of MDV replicon DNA within the parental virus genome and strong nucleotide similarity to DR2 elements of the HSV a sequence suggest that MDV replicon DNA contains a portion of an a-like sequence.

MATERIALS AND METHODS

Cells and virus stocks

Strain 281MI/1, a serotype 2 MDV field isolate has been described previously (Witter, 1983). Preparation and maintenance of primary or secondary chicken embryo fibroblast (CEF) cells was essentially as described by Silva and Lee (1984). Secondary CEF cells were infected with cell-associated 281MI/1 passage 25 (LP) or passage 100 (HP). Virus stocks were determined to be free of reticuloendotheliosis and avian leukosis viruses by either complement fixation or ELISA (Smith et al., 1979).

Northern blot analysis

Using a guanidinium-phenol:chloroform isolation method (Chomczynski and Sacchi, 1987), total RNA was isolated from uninfected CEF cells and CEF cells infected with LP 281MI/1, HP 281MI/1 virus or 52 virus, a recombinant 281MI/1 virus containing two copies of pUC18 sequences (Silva and Witter, 1992). Total RNA pellets were resuspended in 500 ul of diethylpyrocarbonate treated water and subjected to poly(A)$^+$ RNA isolation using the PolyATack mRNA kit (Promega, Madison, WI) as recommended by the manufacturer. Approximately 0.5 ug of poly(A)$^+$ RNA was resolved on 1.2% formaldehyde gels and transferred to Hybond^{TM}-Nylon membranes (Amersham corp.,Arlington Heights, IL) using conditions recommended by the manufacturer. MDV replicon DNA was labeled with [α-^{32}P]dCTP using a random primed labeling kit (Bethesda Research

Laboratories, Gaithersberg, MD.) as specified by the manufacturer. Northern blots were pre-hybridized in 5 x SSPE, 5 x Denhardt's solution, 0.5% (w/v) SDS and 100 ug/ml of sonicated salmon sperm DNA in a 65°C shaking water bath for 1 hour. Denatured, labeled probe was added and incubated at 65°C overnight. High stringency washing conditions were employed by washing filters twice in 2x SSPE, 0.1% (w/v) SDS at room temperature, and twice in 1 x SSPE, 0.1% (w/v) SDS at 65 C for 15 min. Filters were removed and subjected to autoradiography at -70° C.

Nucleotide sequencing

Isolation and cloning of pA5 and pA5I were described previously (Carter and Silva, 1990; Camp et al., 1991). Unidirectional deletion mutants were constructed from pA5 and pA5I double digested with XbaI and SphI using exonuclease III (Boehringer Mannheim Corp., Indianapolis, IN.) and S1 nuclease (Boehringer Mannheim Corp.), followed by re-circularization of deleted clones using T4 DNA ligase (Henikoff, 1987). DNA sequencing was performed using double-stranded plasmid templates and the dideoxy chain-termination method (Sanger et al., 1977). In all sequencing reactions, [^{35}S]-dATP (NEN, Boston, MA) and the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH.) were used as recommended by the manufacturer. Both strands of MDV replicon DNA were sequenced completely. M13 forward and reverse sequencing primers were used to determine nucleotide sequences of p281MI-1, a genomic subclone containing the left flanking region of MDV replicon DNA within the parental virus genome.

Construction of p281MI-1 subclone has been described previously (Camp et al., 1991).

Computer analysis

Nucleotide sequences of deletion mutants were entered and compiled into contiguous segments using SigMan (DNASTAR, Inc., Madison, WI). MDV replicon sequences were compared with sequences from GENBANK data base using MacVector 3.5 (International Biotechnologies, Inc., New Haven, CT). Amino acid sequences of potential ORFs were compared against entries in the Swiss-Protein data base. Final alignment of amino acid sequences was performed using the GAP program of the University of Wisconsin Genetics Computer Group (Devereus et al., 1984)

RESULTS

Detection of transcripts from within or near the MDV replicon sequences

The generation and amplification of a defective MDV genome is depicted in Fig. 1 adapted from Carter and Silva (1990). pA5 is a recombinant vector containing a single copy of the 4-kbp monomeric repeat unit derived from a defective MDV genome. The 4-kbp replicon DNA was used as a probe to detect transcripts encoded within or near replicon sequences of 281 MI/1 genomes. CEF cells infected with LP 281MI/1 virus stock contained four transcripts of 0.9, 1.4, 1.8 and 4.5-kbp which specifically hybridized to MDV replicon DNA (Fig. 2, lane 2). CEF cells infected with HP 281MI/1 virus stock, from which the MDV replicon DNA was initially isolated, did not contain 4.5 and 1.8-kbp transcripts (Fig. 2, lane 3).

We have previously described isolation of a replication competent recombinant virus (52-virus) that contains two copies of pUC18 sequences integrated within the MDV replicon DNA at the same location as pUC18 sequences appear in pA5 (Silva et al., 1988; Silva and Witter, 1992). To examine changes in transcription due to integration of pUC18 sequences, transcripts from CEF cells infected with 52-virus were also examined. These transcripts were indistinguishable from those observed in CEF cells infected with LP 281MI/1. Thus, the insertion of the pUC18 sequences did not appear to disrrupt any local genes.

Native structure and nucleotide sequence of MDV replicon DNA

To determine the nucleotide sequence of the MDV replicon DNA, overlapping unidirectional deletion mutants were constructed from pA5 and pA5I. DNA sequence analysis (Fig. 3) indicated that the MDV replicon DNA is 3926-bp long and has an overall 65% G+C content. To determine the true linear arrangement of MDV replicon DNA within the parental LP 281MI/1 virus genome, a 507-bp segment of p281-1 subclone (Fig. 4B) containing the left flanking region of MDV replicon DNA were aligned with the MDV replicon sequences. Based on this alignment, MDV replicon DNA begins with a guanosine (G), 15 bp upstream of a HpaI site (Fig. 4B). Information on native linear arrangement was critical to allow accurate assessment of potential ORFs by linearizing MDV replicon sequences as they would arranged in the parental virus genome (Fig. 3).

Analysis of potential open reading frames in MDV replicon DNA

Computer-aided translation of MDV replicon sequences in all six reading frames revealed various potential ORFs (Fig. 5). Nucleotide postions of potential methionine initiation codon, TATA box, CAAT like sequences and polyadenylation signal sequences (AATAAA homologies) of ORF-A are indicated in the legend to Figure 3. Computer alignment of translation products from ORF-B did not show any significant amino acid sequence similarity to known protein sequences, suggesting that ORF-B may encode a MDV specific protein. ORF-A, transcribed from left to right, away from the origin of DNA replication, could potentially encode 204 amino acids (Fig. 5). Comparison of ORF-A with entries

in the Swiss-protein data base revealed that ORF-A could encode for a protein that has amino acid sequence similarities to nuclear DNA binding proteins; 1) amino acid residues 70 to 132 of ORF-A displayed 36% identity and 56% overall amino acid sequence similarity to galline, a sperm histone of chicken (Fig. 6A), and 2) the entire 204 amino acid sequence of ORF-A contained approximately 22% identity and 41% similarity to the 3'-exon of the nuclear antigen EBNA-1 of Epstein-Barr virus (EBV) (Fig. 6B). ORF-A contains a high concentration of arginine, a feature shared by the sperm histone of chicken, which is composed primarily of basic amino acid residues. A hydrophilicity plot (window size of 7) indicated that ORF-A may encode a strongly hydrophilic protein, and did not contain leader signal sequences which are normally found in virus glycoproteins (data not shown). To identify a transcript(s) associated with ORF-A, pCK300 subclone (Camp et al., 1991) was used as a probe. The same filter from Fig. 2 was stripped and re-probed with pCK300. Only the 0.9-kbp transcript hybridized to labeled pCk300 DNA, suggesting that MDV DNA comprising ORF-A encodes a specific mRNA (Fig. 7).

HSV-1 a-like sequence at the 5'-end of the MDV replicon sequences

Overall G+C content of MDV replicon sequences was 65%. However, the 5'-end (1 to 500-bp) of MDV replicon sequences were 90% G+C (Fig. 8A). Further analysis revealed that this region is related to the HSV-1 a sequence. Comparison of MDV replicon sequences with the HSV-1 a sequence displayed overall 53%

nucleotide sequence identity in a 400 bp overlap (Fig. 8B). In particular, seven copies of an 11-bp repeat motif (CGGTCGTCGCC) within MDV replicon sequences were strikingly similar to internal short direct repeats (DR2) (CGCTCCTCCCC) of the HSV-1 strain USA-8 a sequence. Three cytosine residues were converted to guanine in the DR2 sequence of serotype 2 MDV in comparison to the DR2 element of HSV-1. In addition to the 11-bp motif, two copies of direct repeat element of a 25-bp sequence was present at the 5′ end of the MDV replicon DNA (Fig. 3). Although an a-like sequence in serotype 1 MDV was recently reported by Kishi et al (1991), we did not detect any sequence similarity between the putative a-like sequence in serotype 1 MDV and MDV replicon sequences.

DISCUSSION

Based on the recently published RE map of serotype 2 MDV (Ono et al., 1992) and our own partial EcoRI map of the 281MI/1 genome, generated from a cosmid library of 281MI/1 DNA (unpublished results), MDV replicon DNA originates from inverted repeat regions (TR_L and IR_L) flanking the U_L segment. Four poly(A)$^+$ RNA transcripts (0.9, 1.4, 1.8 and 4.5-kbp) were detected in CEF cells infected with LP 281MI/1 using the 4-kbp replicon DNA as probe. However, the 1.8 and 4.5-kbp transcripts were absent in HP 281MI/1 virus infected cells. Alterations in RE patterns of cells infected with 281MI/1 upon serial in vitro passage have been extensively studied (Silva and Barnett, 1991). Except for the hypermolar 4-kbp fragment representing the defective genome in HP 281MI/1, no changes in EcoRI fragments specifically hybridizing with 4-kbp MDV replicon DNA (5.5, 5.2, 2.8-kbp), have been detected (Carter and Silva, 1990; Silva and Barnett, 1991). Serial passage in vitro, however, induced alterations in overall RE patterns of 281MI/1 genome (Silva and Barnett, 1991). In addition, visible changes in plaque morphology of HP 281MI/1 in comparison to LP 281MI/1 were observed (data not shown). Altered expression of the 1.8 and 4.5-kbp transcripts observed in HP 281MI/1 infected cell RNA may be the result of point mutations within MDV replicon sequences. It is possible that the 1.8 and 4.5-kbp transcripts are encoded partially within MDV replicon DNA and mutations which may affect expression of these transcripts may have occurred upstream or downstream of MDV replicon DNA during serial in vitro passage. Alternatively,

MDV gene products required for expression of 1.8 and 4.5-kbp transcripts may be altered or absent in HP 281MI/1. For example, expression of MDV A antigen (gp57-65) is greatly reduced in attenuated MDV strain of Md11 relative to LP Mdll. Loss of gp57-65 expression at the transcription level occurs without any gross alterations in gp57-65 gene structure (Wilson et al., 1992).

Insertion of two copies of pUC18 at an EcoRI site of MDV replicon DNA (recombinant 52-virus) did not alter the expression of transcripts associated with the 4-kbp replicon DNA. The EcoRI/pUC18 integration sites also occur outside of ORF-A and ORF-B. Therefore these positions may represent non-essential sites for virus gene expression and replication in vitro. Repeated serial in vitro passages of 52-virus in cell cultures did not cause any deletions within 52-virus DNA, suggesting pUC18 integration sites are stable (Silva, personal communication). However, in vivo passage of 52-virus resulted, in some cases, in deletion of pUC18 sequences, suggesting that this site is not entirely stable in vivo (Silva and Witter, 1992).

Based on DNA sequence analysis, we have located two intact potential ORFs containing conventional promoter elements. Because of a striking nucleotide sequence identity of serotype 2 MDV origin of DNA replication to those of alpha-herpesvirus origins of DNA replication (Camp et al., 1991) and biological similarities between defective HSV and defective MDV, we expected that ORFs flanking the MDV origin of replication would encode for MDV homologues of HSV ICP4, ICP22, or ICP47. However, ORF-A, which is located approximately 20-bp upstream of the serotype 2 MDV origin of DNA replication, revealed

overall 41% amino acid sequence similarity to the 3' EBNA-1 exon (641 amino acids) of EBV (Baer et al., 1984); Sample et al., 1990). EBNA-1 is a nuclear antigen expressed in human B lymphocytes latently infected with EBV. EBNA-1 functions in trans by binding to "oriP", the EBV episome replication origin, permitting episome maintenance and enhancing transcription (Yates et at., 1984; Reisman et al., 1985; Rawlins et al., 1985). The size of EBNA-1 varies considerably, from 68 to 85 kilodaltons among different EBV isolates (Hennessay and Kieff, 1983) and is expressed from a multiply spliced mRNA. It is possible that ORF-A encodes an EBNA-1 like MDV protein with a smaller molecular weight than EBV EBNA-1. Alternatively, ORF-A may represent one exon of a spliced gene. Based on immunofluorescence microscopy and subnuclear fractionations, EBNA-1 was localized in association with metaphase chromosomes (Petti et al., 1990). This association may ensure proteins neccessary for episome maintenance are immediately available to daughter cells.

A MDV nuclear DNA binding protein (MDNA), which may be responsible for the maintenance of MDV genomes in latently infected lymphoblastoid cell lines, was reported by Wen et al. (1988). MDNA is a 28-kilodalton (Kd) nuclear antigen from MSB-1, a T-lymphoblastoid cell line established from MDV tumors. Because of MDNA is only expressed in latently infected cell lines and not in cells undergoing productive infection with MDV, it has been proposed that MDNA may share some biological properties with EBV EBNA-1. Although the size of MDNA is consistant with the predicted size of ORF-A (25 Kd), the functional relationship of MDNA to ORF-A is, at present, unclear.

An 11-bp sequence, tandemly repeated seven times within the 5'-end of MDV replicon DNA shares 8 out of 11 nucleotide sequence identity to DR2 elements present in the a sequence of HSV-1 strain, USA-8. An unusual property of HSV genomes is isomerization due to inversion of L-S components (Hayward et al., 1975). The HSV a sequence, located at the termini and L-S junction of HSV genomes, contains a cis-acting site(s) for inversion of L-S components, and a signal responsible for the packaging/cleavage process during virus replication (Vlazny et al., 1982; Mocarski and Roizman, 1981; Dutch et al., 1992). Size of HSV a sequences (250 to 500-bp) is strain specific with size differences mainly due to the number of DR2 elements (Davison and Wilkie, 1981; Varmuza and Smiley, 1984; Umene, 1991).

A potential a-like sequence (256-bp), structurally similar to the a sequence of HSV-1, has been localized in the L-S junction of serotype 1 MDV (Kishi et al., 1991). Serotype 1 MDV contains five and ten copies of the a-like sequence within both termini and the L-S junction, respectively. Serotype 1 MDV a-like sequences, however, do not contain any sequence similarity to the a sequence of HSV-1. Potential DR2 elements of serotype 1 MDV contain a 6-bp sequence (GGGTTA) tandemly repeated 17 times, where as the DR2 sequence of HSV-1 contains 19 to 22 copies of a 12-bp sequence (CGCTCCTCCCCC). On the contrary, the 6-bp repeat sequences (GGGTTA) in serotype 1 MDV were also found in human herpesvirus-6 DNA (Kishi et al., 1988). Our data indicates that the potential DR2 element of serotype 2 MDV is more closely related to the DR2 element of HSV-1 than to serotype 1 MDV.

Defective HSV genomes (amplicons), contain a functional origin of HSV DNA replication and a packaging/cleavage signal recognition site (a sequence) (Deiss and Frenkel, 1986). It is noteworthy that HSV amplicon (justin) contains two copies of an 11-bp sequence (CGCTCCTCCCC) identical to DR2 elements of strain USA-8 of HSV-1. Based on location of MDV replicon sequences within the MDV genome, similarity of molecular structures between MDV replicon DNA and HSV amplicons, and the fact that MDV replicons are packaged into defective virus particles, it is likely that MDV replicon DNA contains an a-like sequence.

Recently, Reilly and Silva (1992) have located a 1.2-kbp region of HVT that is similar in structural arrangement to the HSV a sequence. The number of 1.2-kbp fragment copies, directly repeated at both termini and in the HVT L-S junction region, was variable among different HVT isolates. DNA sequences of the HVT a-like region, however, is not yet available. However, size and nucleotide sequence of potential a-like elements in the three MDV serotypes vary considerably.

Nucleotide sequences between 650-750 of MDV replicon DNA encode a functional replication origin of serotype 2 MDV (Camp et al., 1990). This region also shares a significant sequence similarity to a putative origin of serotype 1 MDV DNA replication within the BamHI-H fragment (Bradly et al., 1988; Morgan, et al., 1991). The putative serotype 1 origin of replication contains bi-directional promoter regulatory elements presumably used to express pp38 (Cui et al., 1991), a serotype 1 specific phosphoprotein and an uncharacterized immediate-early transcript (Hong and Coussens; personal communication). However, putative

regulatory elements (Sp1 binding sites and an Oct-1 binding site) (Cui et al., 1991; Morgan et al., 1991) present within the serotype 1 bi-directional promoter/origin region are not present in serotype 2 MDV replicon sequences. These differences accentuate previous contention that MDV serotype 1 and 2 have diverged more than serotype 1 and 3 MDVs (Silva and Lee, 1984).

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Fig. 1. Generation and amplification of a defective MDV genome (Carter and Silva, 1990).

(A). Schematic diagram of regions of MDV genome consists of unique long (U_L) and unique short (U_S) segments flanked by inverted repeats (TR_L, IR_L, IR_S, TR_S). (B). Partial RE map of IR_L and TR_L regions of serotype 2 MDV genome. Black boxes represent MDV replicon DNA. Grey shaded areas represent 281MI/1 virus DNA flanking MDV replicon sequences. Vertical white bars indicate serotype 2 MDV origin of DNA replication. (C). Schematic diagram of generation of defective MDV genomes in head-to-tail concatemeric forms during serial in vitro passages of 281MI/1 virus stock. Junction regions joining each monomeric 4-kbp replicon DNA are depicted as J. (D). A single 4-kbp EcoRI monomer was isolated and cloned into pUC18, to generate pA5.

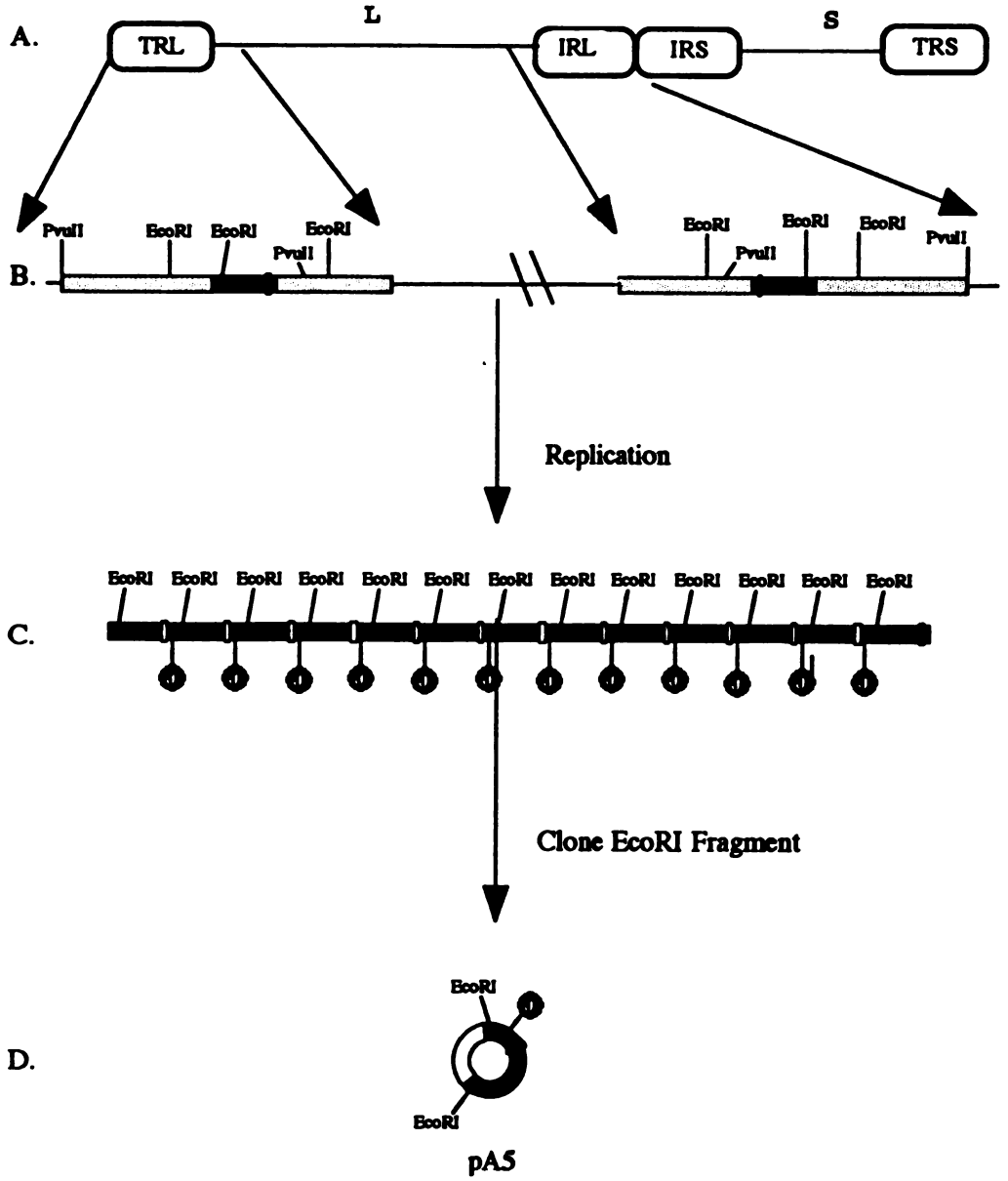
A.
L
S
TRL
IRL
IRS
TRS
B.
PvuII
EcoRI
EcoRI
EcoRI
PvuII
EcoRI
EcoRI
EcoRI
PvuII
PvuII
Replication
C.
EcoRI
Clone EcoRI Fragment
D.
EcoRI
EcoRI
pA5

Fig. 2. Detection of transcripts using MDV replicon DNA as a probe.

Poly(A)$^+$ RNA was isolated from uninfected CEF cells (lane 1), CEF cells infected with LP 281MI/1 (lane 2), HP 281MI/1 (lane 3) , or 52-virus (lane 4). 0.5 ug of each mRNA was resolved in a 1.2% formaldehyde/agarose gel, transferred to Hybond-Nylon membrane, and hybridized with the 4-kbp MDV replicon DNA derived from EcoRI digested pA5.

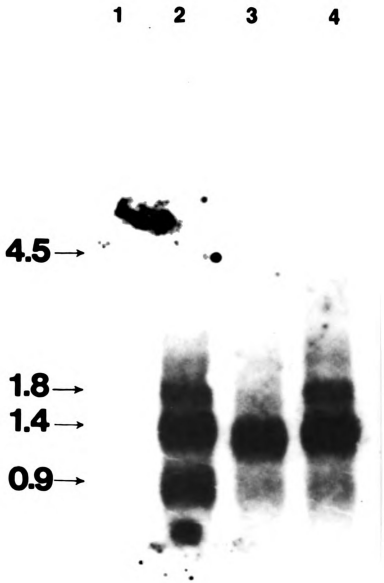
1
2
3
4
4.5
1.8
1.4
0.9

Fig. 3. Complete nucleotide sequence of monomeric MDV replicon DNA. Both strands of MDV replicon DNA were sequenced using the Sanger dideoxy chain termination method (1976) from overlapping pA5 uni-directional deletion mutants as described in materials and methods. Nucleotide sequence of MDV replicon DNA is shown in the reverse complement orientation of pA5. Translations of two intact ORFs are located under the specific coding nucleotide sequences. Potential methionine initation codon, TATA box, CAAT like sequences and polyadenylation signal sequences (AATAAA) are indicated by circles. Underlines represent a functional serotype 2 replication origin. Arrors represent 25-bp direct repeat elements, and seven copies of 11-bp repeat motif are indicated by rectangles.

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   1 GCCCG CGCCC GGGTC GTCGC CCGCG CCCGC GCCCG CGCCC GGGTC GTCGC CGGCG TCGTC

  61 GCCCG GTCGT CGCCC GGTCG TCGCC CGGTC GTCGC CCGGT CGTCG CCGGG TCGTC GCCCG

 121 GTCGT CGCCC GGTCC GCGCC CGGTC CGCCT CCGGC GTCGT TCTCT CCGGC GTCCG GGTCG

 181 CCCCC TCCGT CCCCG GCTCG CTCTC GCGCT CCGCA TCCCT CTCGG CGTCC GGAGG GGCGC

 241 GCGGA TCAGC GGTCG GGTCG CGATC GCGTC CGTCG GATCG GCGCC TTTTC CCCCG CCGCA

 301 TCGTT CCGCC GGGCC GGTCG GACGG GAGAA GAAGG GGGAG GGGGG GAAGG AGGAG AGGGG

 361 GGAGG GAGGG TAGCC GGCCG GCCTG CAGTT CGGGA AGAGC GGGGG AGGCG CCGTC CGAGG

 421 CGCCG GTGAG GAGGT TGTGG GGGCG GGCGG ATGTG TGGGG GAAGG GAAGG GGGAG ACGGC

 481 CGAAA CCTAC GCGTT CGCCG CGCTC CGATC CGGGA TCGCT CCCGA CGGGC TCTCG TTCGG

 541 CGATC GCTTA TCCTC TGCCG CCACC TTGCG TCCGT TCGCG GGAAG CCGGA CCGGC GCTCT

 601 AAGCG GAGAT CCGGC GCCCT CCGCT TCTTA TGACC GGGCC GGTCG TGAGG GCGTA ACGAT
                                ORIGIN
 661 CACGT GATGC AATGC AAACG AGCGG GGCAG ACGCG TCAGC GTTCG CACCG CGAAC CAATA

 721 TAAGA TTATA TATAT AATAT ATTAT TGGCG CAAGG TGCGA ACGCC GTCCG GGCCA ATCGG

 781 GAAGC GGGAT CCTAT GCCAC GTGTT CGTGT CCGGC CGCGG CCCGC GCCGG GGCTA GAAAC
                     M   P   R   V   R   V   R   P   R   P   A   P   G   L   E   T
 841 GCCGC CCCCC TCCCA CGGGG GCGAT TCGGG GACCT CCGGC CTACA AATAC GCGAG CGGAG
       P   P   P   S   H   G   G   D   S   G   T   S   G   L   Q   I   R   E   R   R
 901 GTCCG GCGGG GACCG TCGTT CCGCT GGCCG GCCCG CCGTC CGAAA GCGCG GGACC GCGGT
       S   G   G   D   R   R   S   A   G   R   P   A   V   R   K   R   G   T   A   V
 961 AATAA AGCGC CCGCC GTCGC GGATC GGATT TTCTG GTCGT TCTTT TACCG CCGGG CGAAC
       I   K   R   P   P   S   R   I   G   F   S   G   R   S   F   T   A   G   R   T
1021 CGCGC GGCGA ACGAA CCCGT CCCGT TGGGA TCGCA GGCGG CCGGG AAGCG ATCGC GCGCC
       A   R   R   T   N   P   S   R   W   D   R   R   R   P   G   S   D   R   A   P
1081 GTCCC GAGAA CGTCG TCTAC GGCTC GCGTT CGCGG GGGTC GCCGA CGGGT GGAAG GGGGA
       S   R   E   R   R   L   R   L   A   F   A   G   V   A   D   G   W   K   G   D
1141 TGGGT ACCGA GGGCA TCGAA CTGGC CGAGC TCGGC ATCTC CGCCT TCGGG CCGCG ACCCT
       G   Y   R   G   H   R   T   G   R   A   R   H   L   R   L   R   A   A   T   L
1201 CGCGG CGTCC GGAAC CGTCG GACGA GAACG AGGGA GGACG CCCCC TCGTT CTCGT CCGCG
       A   A   S   G   T   V   G   R   E   R   G   R   T   P   P   R   S   R   P   R
1261 GATCG TCCCG ATCCC TCGCA CGAGA ACGAG ATCCG ACCCC GCGTC GGCGA GCGCC GAACG
       I   V   P   I   P   R   T   R   T   R   S   D   P   A   S   A   S   A   E   R
1321 CCGCT CGCGG AACAC GGTCG CGGGT CGCCC CCCGA CTTTC CCCCC GGCCC GCACC CGCGG
       R   S   R   N   T   V   A   G   R   P   P   T   F   P   P   A   R   T   R   G
1381 CGAGG TCGGA ACCTC GGGAA TCGAT TGAAA ACGGG CCGGT CGAGA TAGGA CCGCT CTCTC
       E   V   G   T   S   G   I   D
1441 GTTCG GAAGC GGAGC AATAA AGCCG TTCGG CGTGA GCTCG GGATG AGTCT CGGAC GCGCG

1501 TCGGT CCGTT TCTGC CCTTG CGGCG CTCTA CGAAA GAACC CGATG AGGCC GCGTT CTGTT

1561 CGGGA GCGGT CCCGA GCTGT AGGGA TCGGC TCGGT AGTAA CTCGT CTCGG GTTTC GATCG

1621 AGTTC TAGCG AACCC GAAAG GGGGG GGTAC GAGGA AGCTT GGCCT GCCGC AAAAA GACTC

1681 CGAGG AGAAT AGCTT ATCCG CGATA CGCCG CCCCC TCAAC CGTTC AGCAT TCACT TCGGC

1741 GTCAG TTCCG AAGTG ACTTT GTCTG GCCGG CTCGG CGCGA GCCGG CCTCG GAGAG AACGC
```

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1801 GTACA TAGCG ATCGT GGGCT GGGCA ACGAG ATAGG AGTAG AGGGA TGGGG AAAAA GTGAA

1861 GTCCC GCGCT GGCTC TGGAC GAGGC GGAAC GAAAT GGGGG AGGGG ACGGC CTCTC GAACG

1921 AGAGC GGGGG TGGGG CATGG TCGGT GGAGG GGTGT GGGAA ACGCC GCGAC GCCGC GGCTC

1981 GTCCA TGTGG TAATG CGGGG AATAG AGTTC CCTGA CGGGG AGGAG TTGTC AGCTC ACCGG

2041 TCCCG TGATC AACGC ATATG CCACA TATAT GCAAC CCCGC GACCC TCTCC TCACG AACAG
                                    M   Q   P   R   D   P   L   L   T   N   R
2101 GGTAG AGGGT CTCGC TTTGT TGCAC ACACA AGCAC ACACT CACTT TTGCG GCGAC AATGA
       V   E   G   L   A   L   L   H   T   Q   A   H   T   H   F   C   G   D   N
2161 AAATC GTGAG CCCAG ACCAA TTGCC CCTAT ATGTG TTAAC GGCCT CCGAC GGAAC AACGG
     E   N   R   E   P   R   P   I   A   P   Y   V   L   T   A   S   D   G   T   T
2221 AGTTC AGTAT TCGAG ATTGG TCGCT ATGGG ATCGG GATCC GGAAT CCCGT GCGGG TACGC
     E   F   S   I   R   D   W   S   L   W   D   R   D   P   E   S   R   A   G   T
2281 ACCGC GGGCT CCGTC AAACG GAACG CTCTC TTTTT GGAGC GATGT CGTTT GGGTC CGGGC
     H   R   G   L   R   Q   T   E   R   S   L   F   G   A   M   S   F   G   S   G
2341 CCGAG TAGGG TTCGG CCGGC TATTT CCCTC CCCCC CCCCC CCCGA AGCCG TGCGG ACCGA
     R   V   G   F   G   R   L   F   P   S   P   P   P   P   E   A   V   R   T   E
2401 GGTTG AGTGG GAAGG TCTCG CCTGC CGGAT CGGGG GGGGG AGGGG AAAAG ACGTT TGCGT
     V   E   W   E   G   L   A   C   R   I   G   G   G   R   G   K   D   V   C   V
2461 TATCC GGAAT CGAGC CGGAC CGGTT TACGT TTCAG AGTTC GAACA CGCTC TCACG AGCTG
     I   R   N   R   A   G   P   V   Y   V   S   E   F   E   H   A   L   T   S   C
2521 CAGCC ACCGG CTCAA GCCCT CAGAC CGCGT CCGCG CCCCG GGCCA GCCTA GCGTC GCAAA
     S   H   R   L   K   P   S   D   R   V   R   A   P   G   Q   P   S   V   A   N
2581 CGTCT GGAAG AGTCT GTGGA GTTTG GTTTC GAGGG CCGTG CGGGG AAACG CAGAG GGCT
     V   W   K   S   L   W   S   L   V   S   R   A   V   R   G   N   A   E   G   S
2641 CTACG GACGA CCGGA GAGGG AGACG CCGAA ACTGC CGTGT AAACG AATGC TTAGC GGACG
     Y   G   R   P   E   R   E   T   P   K   L   P   C   K   R   M   L   S   G   R
2701 CGCGC CCGGG ACGCT CGTTT CTTGC GAAAC GGGGT GGACT GCTCG CCCGC TCTTA GCCCT
     A   P   G   T   L   V   S   C   E   T   G   W   T   A   R   P   L   L   A   L
2761 CGCGT ACAGT GCGTC GATCG TTAAG GTGTA CGTGC GTGTA CGAGA CCGCG TGCGA GCGGT
     A   Y   S   A   S   I   V   K   V   Y   V   R   V   R   D   R   V   R   A   V
2821 CTCGT CGTAG TGCGT ATGAA CGTGT TTATT AAAAA GTCTT TCTCG CTTCG GTCCC GTTTC
     S   S
2881 GGTGT CTGGC GATGG CGCGG GCGGG GGAGT CCGTC GCGTA AGTCG GGGAA CGTAC TTTGT

2941 CCTCT CCTCT CCCCG AGGCG GATGT GCGAG GGGGG GGGGC GACGG GCGAC CGGGA ATGCG

3001 TGCGA TTAGT GTTGT GGAGA ACGTA CCGCG CCGAG GCCCG GTGCG CGCGA AGGGT TAGTT

3061 GGGGG AGGGG CACGC GCATC ATAAG TCGCT CGTGG ATCGT CCAGG TGTTT GTGCG GGGGA

3121 TACAG TTTCG CGACG GGGAG GAGCC GTTAG CTCAC CGGCC CCGCG GGAGA CGCAG ATGCG

3181 ATATA AACAG CCCCG CGGTC CACCT CCTTG TTCGC GACCT GCTAC TTAGC CTCGC TTCGT

3241 TCCCA CGTAT AAGCA CCACT GACTT ACGCG GCGAC CGTGG AAGTC GCGAG TTCCG ACCAG

3301 TGTCG CGTAT AAGTG TTAAC GGCCC CCGTC GGAAC CGAAT TCCAC ATTCG AGATG AAGTG

3361 CTACG GAATC AGGAA TCCCG TCCCG GCCGC GCGCA CACCC ACACA CACAC CCCCA CACAC

3421 CCCCA CACAC ACCGC GGCTA CAGAG CGGCT GTCGT AGTAC GTATA GGCGC GACGC GAACA

3481 CTTGC ACGGA CTCTC GTCGT AGTAC GTTCA AATCG GTTTA TTAAA GCCCT CATGC TCAGA
```

3541 CTCCG TGTCG ATGGC GGGGG GGGGG GGGGG GGGCG GTGTG AGTGA CACGA ACTTA CTTTC

3601 CTGCG ACGCT CCCGA GAGGA GGTGT GCACC GGGTC GTTTT CTCCG AGACT GAAAA GAGAA

3661 AGAAA ACGTC CGCCG TTCGC CCGGC CAAGC GCCCG GACGC ACCGG TGTCC GTACG GCCTT

3721 CGGAC GCTTA GAGAG CGCGG GCGTT TGCGG CTGCG GCGTT TAGGC GCGCA GCGGC GCGTT

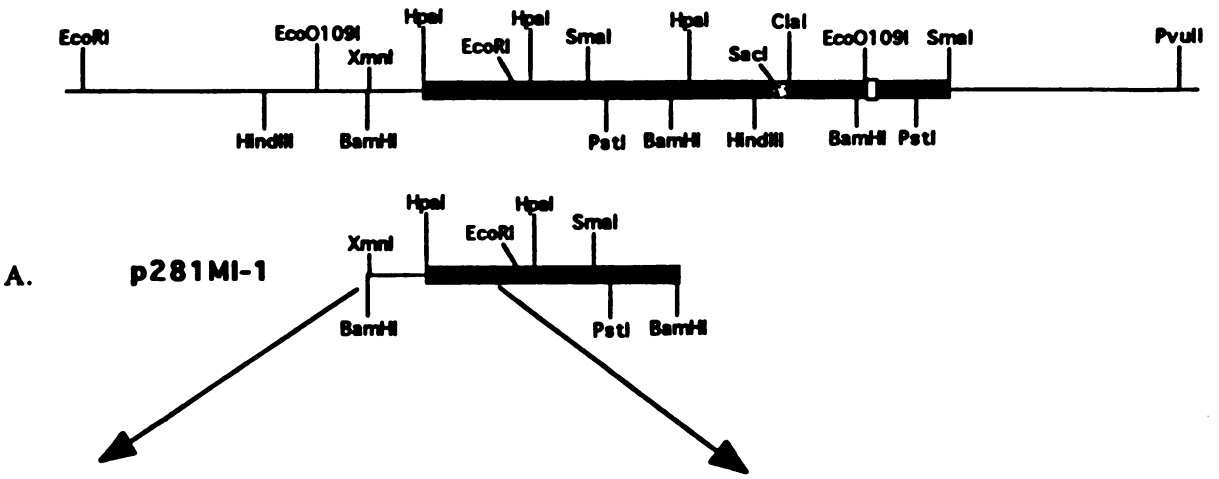
3781 TGCAA AAAAA AAAGA ATGTA CGGAG AGACC CGCCC GCCAG CCTCT CCGGC CCGGA GCGGA

3841 AACGC GCGGT TATCT CTACG CCCCC GACCC GCACA TCCGA ATGCG CGGTC GGTCG CATCG

3901 CGAGG TTAAC TCCCT GCCCG CGCCC

Fig. 4. Linear arrangement of MDV replicon DNA within the parental virus genome.

(A). 281MI/1 sub-genomic map containing MDV replicon DNA (black bar). The white rectangle represents serotype 2 MDV replication origin. p281MI-1 is a genomic subclone containing a 2-kbp BamHI fragment which hybridizes to the 4-kbp replicon DNA (see materials and methods). (B). 507-bp nucleotide sequence of p281MI-1 was compared to the 3926-bp MDV replicon sequence. Sequences from p281MI-1 identical to the MDV replicon sequences are underlined.



B.

TTCGGATGGC AACCGTGCGG CGGGAAGGCT CCGAGTCCGA CCGGAAACGA

AAAAGGCGGC ACGGGGCGCT CAACGCACAG GGGATAGGAA GAATTTCCCT

CGGCCCGCGG ATTCGGATCG CGCTCRRRRR CCTTTTCTCC GCCGACTCGC

CGACCGCCCG GAACGCCGGT ACTTACRCGC TGGAGAAGCC CCGTATTACT

CTTCTTCCCG GATTCGGCCC CGGCGCTTCT GCGTCTGCGC GGCGGACATA

CCAGTTGCGC TTCGTCGACG TATTCAATCT AAACAAGGAA GTCGGCCTGC

GACTTCCGCG ATCGCGATCG CGAACCGGTA AACAACGCCA GGGGGAGCGC

Hpa I

GGAGGGCGCG GGCAGGGAGT TAACCTCGCG ATCCGACCGC GCATTCGGAT

GTGCGGGTCG GGGCGTACAC ATAACGCGGC GTTTCCGTCC GGGCCGGAGA

GGTCCACGGG CGGGTCTCTC CGTACATTCT TTTTTTTTAG CAAACGCGCC

GCTGCTG

Fig. 5. Potential ORFs encoded within MDV replicon DNA.

Various of ORFs containing greater that 85 amino acids were located in all three reading frames (1, 2, and 3) of both replicon strands using the universal start and stop codons (McVector, IBI). ORFs in the plus strand are depicted as black boxes, whereas ORFs in the minus strand are indicated as white boxes.

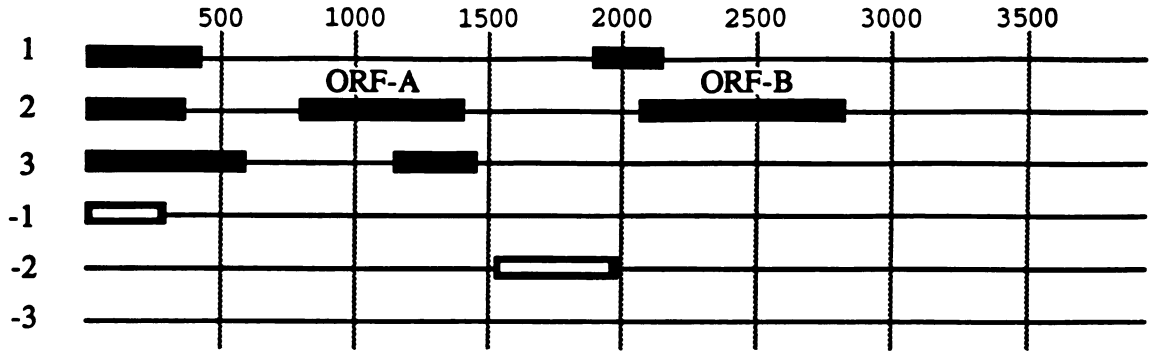

500
1000
1500
2000
2500
3000
3500
1
2
3
-1
-2
-3
ORF-A
ORF-B

Fig. 6. Amino acid alignments of ORF-A to nuclear binding proteins.

(A). Amino acids 70 to 132 of ORF-A shares 36% amino acid identity to the galline sperm histone of chicken, and (B). the entire ORF-A shares 22% amino acid identity to the 3′ exon of EBNA-1 protein. Vertical lines represent identical amino acids between two aligned sequences while colons and dots represent similar amino acid sequences calculated according to the BESTFIT program.

A.

```
Galline    2 RYRRSRTRSRSPRSRRRRRRSGRRRSPRRRRR....YGSARRSRRSVGGR
             .:  :|| .|. .||: |||.|. |.|.| ||    :::.  :::: | |
ORF-A     70 SFTAGRTARRTNPSRWDRRRPGSDRAPSRERRLRLAFAGVADGWKGDGYR

Galline   48 RRRYGSRRRRRRR
              :| |. |: | |
ORF-A    120 GHRTGRARHLRLR
```

B.

```
EBNA1     365 SRERARGRGRGRGEKRPRSPSSQSSSSG..SPPRRPPPGRRPFFHPVGEA
              .| |.|.|. .  |..|.|.:::|:.||   ..||...:||.
ORF-A       2 PRVRVRPRPAPGLETPPPSHGGDSGTSGLQIRERRSGGDRRS........
                .         .         .         .         .
EBNA1     413 DYFEYHQEGGPDGEPDVPPGAIEQGPADDPGEGPSTGPRGQGDGGRRKKG
                     :|..: .. ..:.|...|.  . .|.| .:   | .:||...
ORF-A      44 ........AGRPAVRKRGTAVIKRPPSRIGFSGRSFTA...GRTARRTNP
                .         .         .         .         .
EBNA1     463 GWFGKHRGQGGSNPK........FENIAEGLRALLARSHVERTTDEGTWV
              :::::::|..:..|.         |..:|:|:::   |:| .  . .  :
ORF-A      83 SRWDRRRPGSDRAPSRERRLRLAFAGVADGWKGDGYRGHRTGRARHLRLR
                .         .         .         .         .
EBNA1     505 AGVFVYGGSKTSLYNLRRGTALAIPQCRLTPLSRLPFGMAPGPGPQPGPL
              |:.:. :|. ..     || . : ...|:.|:.| .   .|:.:... .
ORF-A     133 AATLAASGTVGR....ERGRTPPRSRPRIVPIPRTRTRSDPASASAERRS
                .         .
EBNA1     555 RESIVCYFMVFLQTHIFAEVLKDAIK
              |:.:..   .| ..:. :|| ..:|.
ORF-A     179 RNTVAGRPPTFPPARTRGEVGTSGID
```

Fig. 7. Detection of transcripts using pCK300 as a probe.

The northern blot filter from Fig. 2 was stripped and re-probed with pCK300 DNA which is located at nucleotide positions from 1143 to1400.

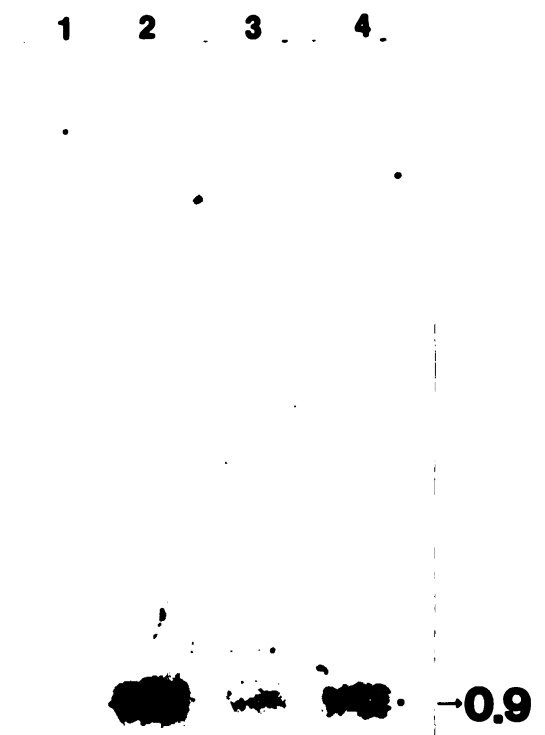
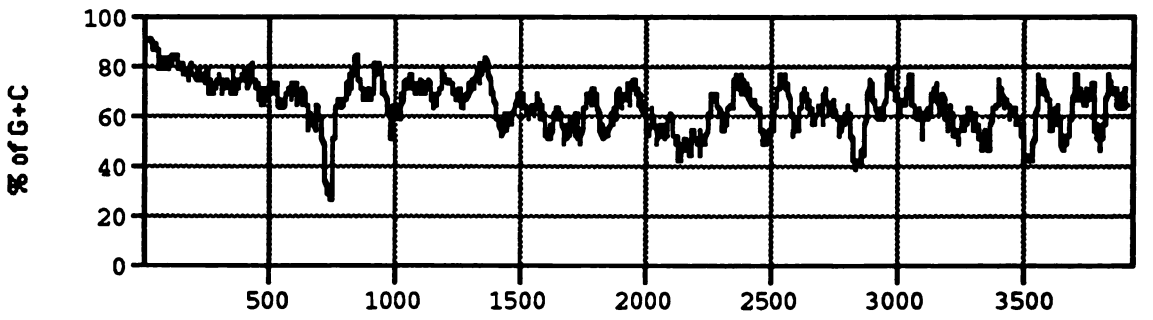

1
2
3
4
0.9

Fig. 8. (A). Percent G+C chart of MDV replicon DNA. (B). Nucleotide sequence alignment of the 5′-end of MDV replicon DNA containing the 90% G+C content with the HSV-1 a sequence. Dots represent mismatched nucleotide sequences and horizontal bars indicate gaps. Alignment of DR2-like elements in MDV replicon DNA is underlined.

A



```
                  10          20          30          40          50          60
                   *           *           *           *           *           *
MDV"a"SEQ   GCCCG CGCCC GGGTC GTCGC CCGCG CCCGC GCCCG CGCCC GGGTC GTCGC CCGCG TCGTC

HSV-1 a           370         380         390         400         410
[  500 ]    .CCC. C-... GGG-. GT... .C-.G ...G. GCCCG ..... GGG.. .-.G. CCG-. TC.TC>

                  70          80          90          100         110         120
                   *           *           *           *           *           *
MDV"a"SEQ   GCCCG GTCGT CGCCC GGTCG TCGCC CGGTC GTCGC CCGGT CGTCG CCCGG TCGTC GCCCG

HSV-1 a     420         430         440         450         460         470
[  500 ]    .CCCG .TC.T C.CCC G.TC. TC.CC CG.TC .TC.C CCG.T C.TC. CCCG. TC.TC .CCCG>

                  130         140         150         160         170         180
                   *           *           *           *           *           *
MDV"a"SEQ   GTCGT CGCCC GGTCC GCGCC CGGTC CGCCT CCGGC GTCGT TCTCT CCGGC GTCCG GGTCG
HSV-1 a     480         490         500             510         520         530
[  500 ]    .TC.T C.CCC G.TCC .-.CC C.GTC .--.- ..GG. G..G. T-.CT CC..C GTC.G .G...>

                  190         200         210         220         230         240
                   *           *           *           *           *           *
MDV"a"SEQ   CCCCC TCCGT CCCCG GCTCG CTCTC GCGCT CCGCA TCCCT CTCGG CGTCC GGAGG GGCGC
HSV-1 a           540         550         560         570         580         590
[  500 ]    ....C TCC.. CCCCG .CT.. ...T. G.GCT CC.C. .CC.T CT..G .GT.. GG... ..C.C>
```

```
                250         260         270         280         290         300
                 *           *           *           *           *           *
MDV"a"SEQ   GCGGA TCAGC GGTCG GGTCG CGATC GCGTC CGTCG GATCG GCGCC TTTTC CCCCG CCGCA

HSV-1 a           600         610         620             630         640
[  500 ]    .CG.. T..-. GGT.G GG... C...C .CGTC .G-.G G.-.G -.G.C T..TC CCCCG C..C.>

                  310         320         330         340         350         360
                   *           *           *           *           *           *
MDV"a"SEQ   TCGTT CCGCC GGGCC GGTCG GACGG GAGAA GAAGG GGGAG GGGGG GAAGG AGGAG AGGGG
.                                                                   |
HSV-1 a     650         660         670         680         690 |       700
[  500 ]    .C-.. C-.CC G..CC ...-. G.C.G ..-.. -..G. G.G.G .G..G ...GG .G..G ...G.>

                  370         380         390         400[1]      410         420
                   *           *           *           *           *           *
MDV"a"SEQ   GGAGG GAGGG TAGCC GGCCG GCCTG CAGTT CGGGA AGAGC GGGGG AGGCG CCGTC CGAGG

HSV-1 a         710         720         730         740         750
[  500 ]    ..... ..G.- ..GCC G.CC. GCC.G C.G.. .G... .G..C G.... AGGC>
```

53% overall nucleotide sequence identity

Chapter IV

Cellular and viral protein interactions with Marek's disease virus replication origin

Heidi S. Camp, Robert F. Silva and Paul M. Coussens

ABSTRACT

Previous reports indicate that a serotype 2 Marek's disease virus (MDV) replication origin contains a region highly homologous to the herpes simplex virus (HSV) type 1 origin-binding protein (OBP) recognition site I (Camp et al., 1991). Origin-specific cellular and viral proteins have been investigated using a synthetic 22 base-pair (bp) oligonucleotide containing a potential OBP recognition site of serotype 2 MDV. Three protein-DNA complexes were identified in uninfected chicken embryo fibroblast (CEF) cell extracts. Incubation of the labeled 22-bp MDV ori oligonucleotide with MDV-infected cell extracts revealed two virus specific protein-DNA complexes (complexes A and B). Competition analysis indicates that only the complex B is sequence-specific. In addition, we have detected a protein-DNA complex (complex K) between the 22-bp MDV DNA and HSV-1 infected cell extracts suggesting the interaction of HSV-1 specific protein(s) with MDV origin sequences.

INTRODUCTION

As with most herpesviruses, genes in MDV appear to be sequentially regulated in a cascade fashion. Genes of MDV are divided into immediate-early (IE), early (E) and late genes (L) (Maray et al., 1988). In herpesvirus systems, IE genes are the first set to be expressed upon initial infection, and transcription of these genes does not require de novo protein synthesis. The expression of E genes requires functional IE proteins, whereas the expression of L genes requires both the functional IE proteins and viral DNA synthesis.

In a previous report, Camp et al. (1991) provided evidence that the structure and properties of serotype 2 MDV DNA replication origin are strikingly similar to HSV lytic origins of replication (ori_S and ori_L). Within the serotype 2 MDV replication origin, a 9-bp motif, sharing 9 out of 11 nucleotide sequence identity to the 11-bp consensus sequence which is recognized by the herpes simplex virus type 1 origin binding protein (OBP) (Elias and Lehman, 1988), was identified (Camp et al., 1991). OBP is the product of UL9, an E gene (McGeoch et al., 1988; Wu et al., 1988). Based on the following observations, it has been proposed that OBP is an initiator of HSV DNA replication: 1). In DNase I footprinting and methylation interference assays, OBP recognizes an overlapping small binding domain of 8-bp (5'-GTTCGCAC-3'/ 3'-CAAGCGTG-5') located within flanking regions of ori_S palindrome (Koff and Tegtmeyer, 1988), 2). Site-specific deletion analysis of HSV OBP-binding site I revealed a consensus sequence (CGTTCGCACTT) which is required for specificity and binding affinity to OBP, 3). Mutations within the OBP recognition sites inhibit HSV DNA replication in

vitro, 4). OBP contains DNA-dependent nucleoside 5'-triphosphatase and ATP-dependent helicase activities, and 5). OBP exists primarily as a homodimer in solution and that these dimers specifically interact with lytic origins of HSV DNA replication (Hernandes, et al., 1991; Debrowski and Schaffer, 1991; Koff et al., 1991; Bruckner et al., 1991).

As with many other DNA viruses, it is likely that MDV encodes proteins which specifically interact with replication origin(s) during DNA synthesis. In this report, a 22-mer synthetic oligonucleotide containing a potential OBP binding site present within the serotype 2 MDV replication origin was used as a probe to detect origin-specific viral or cellular proteins using mobility-shift assays. Incubation of MDV-infected whole cell extracts with the 22-mer MDV ori oligonucleotide resulted in formation of two virus-specific protein-DNA complexes (complex A and B), while incubation with uninfected cell extracts resulted in formation of three protein-DNA complexes (complex M, M', and M"). Based on competition analysis, only the protein-DNA complex B from the MDV infected cell extracts was formed by in a sequence-specific manner. In addition, whole cell extracts isolated from vero cells or HSV (KOS strain) infected vero cells contained proteins capable of sequence-specific binding to the labeled 22-mer MDV ori oligonucleotide. Preliminary data suggests that MDV may encode a protein product analogous to HSV-1 OBP during MDV DNA replication.

MATERIALS AND METHODS

Cells and virus stocks

Strain 281MI/1, a serotype 2 MDV field isolate (Witter, 1983), was plaque purified from cell-free virus following the fifth cell-culture passage. Preparation and maintenance of primary or secondary chick embryo fibroblast (CEF) cultures have been previously described (Silva and Lee, 1984). Secondary CEF were infected with 281MI/1 passage 15 virus stocks.

African green monkey kidney (vero) cells were grown and maintained as described (Weller et al., 1983). Stocks of the HSV-1 wild-type virus, strain KOS, were grown and assayed as described (Debrowski and Schaffer, 1991).

Preparation of whole cell extracts.

Whole cell extracts were prepared from uninfected or infected CEF cells with a serotype 2 MDV strain, 281MI/1. Cell extracts prepared from MDV infected CEF cells were isolated from different time points post-inoculation (PI). In addition, whole cell extracts were isolated from vero cells or HSV-1 strain KOS infected vero cells. Isolation of whole cell extracts and mobility-shift DNA binding assays were performed essentially as described (Dabrowski and Schaffer, 1991). Final cell suspensions were aliquoted and stored at -70°, until use.

Preparation and labeling of oligonucleotide and DNA probes.

A 22-mer synthetic oligonucleotide (CA**GCGTTCGCACC**GCGAACCAA) containing an HSV OBP binding site I homologe (**bold** letters) from the serotype 2 MDV replication origin, was synthesized at the Michigan State University Macromolecular Structure, Synthesis, and Sequence facility. For mobility-shift assays, double-stranded 22-mer MDV ori oligonucleotide was annealed by heating to 65° C for 5 min. followed by slow cooling to room temperature. Radiolabeled oligonucleotide was prepared by incubating approximately 100 ng of the annealed 22-mer oligonucleotide with [γ -^{32}P]ATP (7000 Ci/mmol, Dupont New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Boehringer-Mannheim). Unincorporated labeled nucleotides were separated from probe DNA by spin-column chromatography (Maniatis et al. 1982) through Sephadex G-50 (Pharmacia-LKB, Piscataway, NJ) followed by ethanol precipitation. DNA pellets were resuspended in TE [10 mM Tris-hydrochloride, pH8.0 and 1 mM ethylenediaminete-tracetate (EDTA)] at 1 ng/ul and stored at -20° C.

A plasmid construct, p130.HC, containing the entire 90-bp core serotype 2 MDV origin sequence was constructed from the parental construct, pA5 (Carter and Silva, 1990). First, pPB400 was created by cloning a 400-bp PstI to BamHI subfragment of pA5 and inserted between the PstI and BamHI sites in pUC18. A 130-bp DpnI fragment containing the origin sequences was then isolated from pPB400 and cloned into the SmaI site of pUC18 using a blunt-end ligation procedure (Maniatis et al., 1989). For mobility-shift assays, the ends of the 130-bp fragment were dephosphoryated by treating DNA with 1 unit of alkaline

phosphatase (Boehringer Mannheim Corp., Indianapolis, IN.) for 30 min. at 37° C , followed by extraction of DNA with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 vol/vol/vo/), once with chloroform-isoamyl alcohol (24:1 vol/vol), and precipitation with 2.5 volumes of 100% ethanol. Recovered DNA was resuspended in TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and stored at 4° C. DNA probe was end-labeled by T4 DNA polymerase as described above.

Mobility-shift assays

DNA binding assays were performed as described by Debrowski and Schaffer (1991), except the N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) was omitted. Briefly, 1-2 ng of labeled probes were incubated with approximately 10 ug of uninfected or infected cell extracts in the presence of 2 ug of poly (dI-dC) (Pharmacia, Bern, Switzerland) in DNA binding buffer (10% glycerol, 50 mM N-2-hydroxyethyl peperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol, 100 mM Nacl) in a final volume of 20 ul. Competition experiments were performed by combining labeled probe with 100-fold excess of cold specific or non-specific competitor DNA prior to initiation of binding reactions. Non-specific competitor DNA contains a 20-mer synthetic oligonucleotide derived from HSV IE promoter gene sequences. All reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) on 5% nondenaturing gels buffered with Tris-borate-EDTA buffer (TBE) (Maniatis et al., 1990; Garner and Revzin, 1981). Bands of unbound and protein-bound DNA complexes were visualized by autoradiography.

RESULTS

Detection of protein-DNA complexes with 22-mer MDV ori oligonucleotide and whole cell extracts isolated from uninfected or MDV infected CEF cells.

Conservation of a potential HSV OBP binding site within the origin of serotype 2 MDV replication prompted us to initiate experiments to detect cellular or viral proteins that may interact directly with the MDV replication origin. Initially, the p130.HC construct containing the entire 90-bp MDV origin sequence was used as a probe in mobility-shift gel assays. Two protein-DNA complexes, M and M′ were detected with mock infected whole cell extracts (Fig. 1, lane 2). Incubation of the labeled 130-bp DNA with infected cell extracts, isolated 12 hrs post infection (PI) resulted in formation of a distinct protein-DNA complex (complex A) (Fig. 1, lane 4), which was absent in mock infected or 5 hrs PI MDV cell extracts (Fig. 1, lane 3). Protein-DNA complexes from 12, 24, and 48 hrs PI MDV cell extracts were essentially identical (Fig. 1, lanes 4-6).

Conservation of a potential OBP binding site I within the MDV core origin of replication suggested that MDV origin binding proteins may interact with this sequence. To analyze this possibility, a 22-mer oligonucleotide containing the potential MDV OBP binding site was constructed (Fig. 2). Mobility-shift assays were performed with the labeled 22-mer oligonucleotide as described in materials and methods. Three protein-DNA complexes were detected in reactions containing mock infected cell extracts (M, M′ and M″) (Fig. 3, lane 2). Incubation

of labeled 22-mer oligonucleotide with MDV infected cell extracts, isolated at different time points PI, revealed two viral specific protein-DNA complexes (A and B), which were absent in mock infected cell extracts (Fig. 3, lanes 3-6).

To test the specificity of protein-DNA complexes observed with MDV infected cell extracts, competition mobility-shift assays were performed. As expected, protein-DNA complexes, M, A and B were observed with 12 hrs PI cell extracts (Fig. 4, lane 1). Unlabeled 22-mer oligonucleotide competed effectively for binding of all three protein-DNA complexes to the labeled 22-mer MDV oligonucleotide (Fig. 4, lane 2). Unlabeled non-specific competitor, however, also competed for binding of complexes M and A. No competition was observed for complex B formation. These results suggest that only protein-DNA complex B was formed in a sequence-specific manner.

Interaction of MDV origin sequences with KOS infected cell proteins.

A potential HSV OBP-binding site (**CGTTCGCA**CCG) within MDV replication origin shares 9 out of 11 nucleotide sequence identity (**BOLD**) to consensus sequences recognized by HSV-1 OBP. To determine if there are any interactions between the potential OBP-binding site found in MDV DNA and KOS infected cell proteins, the radiolabeled 22-mer MDV DNA was incubated with whole cell extracts isolated from vero cells or KOS infected vero cells. Two protein-DNA complexes were identified (V′ and V") with either mock-infected or KOS-infected vero cell extracts (Fig. 5, lane 2). One protein-DNA complex, K, was observed specifically in KOS- infected cell extracts (Fig. 5, lane 3).

To determine if the formation of protein-DNA complex K was due to the interaction of the OBP in HSV-1 infected cell extracts, different concentrations of antiserum against a carboxy terminal peptide of HSV-1 OBP (a gift from M. Challberg) were added to DNA binding reactions for antibody supershift assays. Although the intensity of the protein-DNA complex K was less with the increasing concentrations of OBP antiserum, the expected supershift banding patterns did not occur (data not shown). In addition, immunoprecipitation of MDV-infected cell proteins with the OBP antiserum did not detect OBP-like protein in MDV (data not shown). Further experiments are necessary to analyze the interaction between the HSV OBP and a putative OBP-binding site from MDV replication origin.

DISCUSSION

The formation of complexes between viral or cellular proteins with origins of DNA replication has been reported and characterized in a number of virus systems (Challberg and Kelly, 1989). T antigens of Simian virus 40 (SV40) and polyomavirus, the terminal protein of adenovirus, and the OBP of HSV-1 interact directly with correspponding origins of virus DNA replications (Borowiec et al., 1990; Gaudray et al., 1981; Debrowski and Schaffer, 1991).

As in other herpesviruses, MDV likely encodes many DNA metabolic proteins, necessary for DNA replication. The expression of MDV late genes is prevented by phosphonoacetic acid (PAA) which is known to specifically inhibit herpesvirus DNA polymerase activity (Lee et al., 1976). These results suggest a functional similarity between MDV polymerase and those of other herpesviruses. Identification of a conserved OBP recognition site within a functional MDV replication origin, similar to the HSV-1 OBP binding sequences, strongly suggests existence of an OBP-like protein that may interact directly with MDV DNA replication origins.

Initially, Elias and Lehman (1988) identified and purified the HSV-1 OBP which interacts directly with Ori_S. Since then, it has been proposed that OBP is one of the required components for origin-dependent initiation of HSV-1 DNA replication. Interestingly, the 11-bp motif which was shown to interact physically with the HSV-1 OBP is highly conserved among origins of alphaherpesvirus replication, such as VZV, EHV-1, and MDV (Stow and Davison, 1986; Baumann et al., 1989; Camp et al., 1991). The 22-mer MDV oligonucleotide containing

a potential OBP recognition site is derived from the left end of the serotype 2 MDV origin sequence palindrome. At least one virus-specific protein-DNA complex (complex B) is formed via a sequence-specific binding with labeled 22-mer MDV oligonucleotide, suggesting the existence of a HSV OBP-like protein in MDV infected cells. However, failure of HSV-1 OBP antiserum to interact with protein-DNA complexes in MDV infected cell extracts suggests that MDV may encode an OBP that has different antigenic properties than HSV-1 OBP. Alternatively, the specificity of HSV-1 OBP antiserum, which is directed against the carboxy termini of the OBP, is not sufficient to bind to MDV OBP-like protein-DNA complexes.

Based on the work of Debrowski and Schaffer (1991), conservation of the 9-bp OBP binding sequence in MDV should be sufficient for interaction with the HSV-1 OBP. In support of this hypothesis, a virus-specific protein-DNA complex, K, was observed in reactions containing HSV-1 strain KOS-infected cell extracts and the 22-mer MDV oligonucleotide. However, further investigation is required to determine if protein-DNA complex K is related to the HSV-1 OBP.

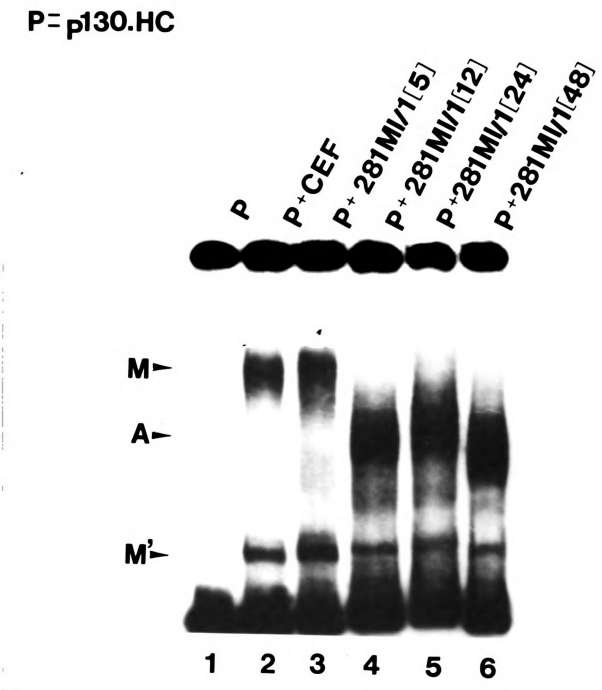


Figure 1. Mobility-shift assay using 1 ng og end-labeled 130-bp fragment probe and 10 ug of CEF whole cell extracts (lane 2), or 10ug of MDV-infected whole cell extracts from 5, 12, 24 and 48 hrs PI (lanes 3-6). Control reaction (lane 1) contained labeled probe only without cell extracts.

Figure 2. Serotype 2 MDV origin sequences. 22-mer oligonucleotide containing a potential HSV OBP binding site is indicated.

MDV Ori

ACGCGTCAGC GTTCGCACCG CGAACCAATA TAAGATTATA TATATAATAT

22-mer Oligo

ATTATTGGCG CAAGGTGCGA ACGCCCGTCG GGCCAATCG

Figure 3. Mobility-shift assay using end-labeled 22-mer ds MDV oligonucleotide probe (1 ng) and 10 ug of CEF whole cell extracts (lane 2), or 10 ug of MDV-infected whole cell extracts from 5, 12, 24 and 48 hrs PI (lanes 3-6). Control reaction (lane 1) contained labeled 22-mer DNA only without cell extracts.

P = 22-merOri oligo

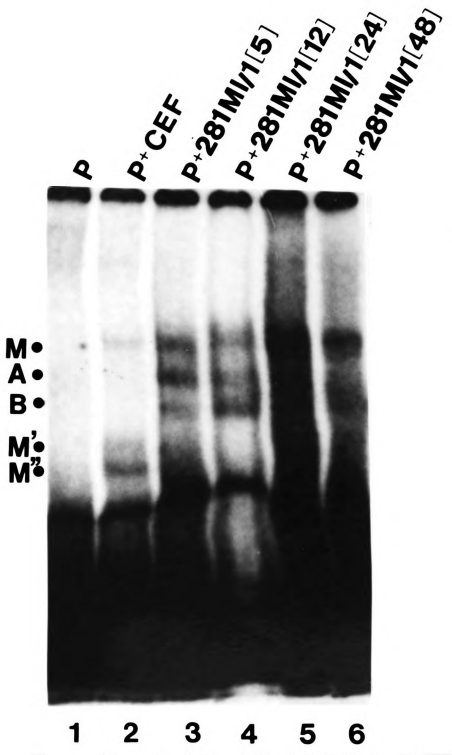
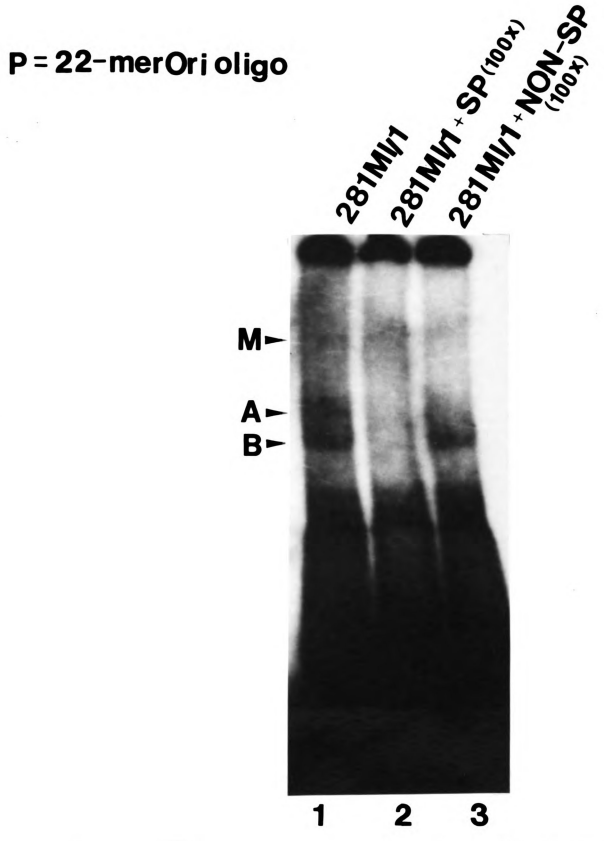


Figure 4. Competition mobility-shift assay using end-labeled 22-mer ds MDV oligonucleotide probe (1 ng/lane). Probe DNA was incubated with 100 fold excess of unlabeled specific (lane 2) or non-specific (lane 3) double-stranded oligonucleotides at 37° C for 30 min in the presence MDV-infected protein extracts. Control reaction (lane 1) contained labeled probe with MDV-infected protein extracts without any competitor DNA.


P = 22-merOri oligo
281Mv1
281Mv1 + SP(100x)
281Mv1 + NON-SP (100x)
M
A
B
1
2
3

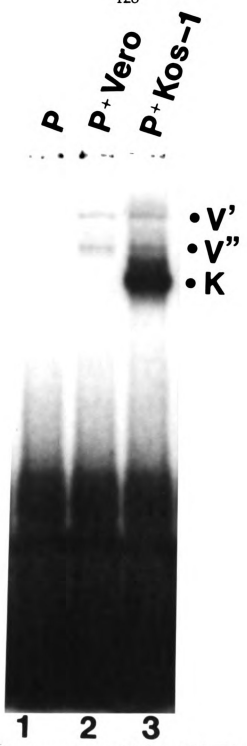


Figure 5. Mobility-shift assay using end-labeled 22-mer ds MDV oligonucleotide (1 ng) with 5 ug of uninfected vero cell extracts (lane 2), or with 5 ug of KOS infected vero cell extracts (lane 3). Lane 1 contained labeled probe only as a control.

Chapter V

Summary and Conclusions

Defective MDV genomes represent a useful system to study MDV DNA replication and for construction of a shuttle vector system for delivery of foreign gene products into eucaryotic cells. Previous studies indicated defective MDV genomes exist as high-molecular-weight viral DNA consisting of head-to-tail concatemers (Silva et al., 1988; Carter and Silva, 1990). A single monomeric repeat unit of a defective MDV genome was isolated and cloned into a bacterial plasmid, designated pA5 (Carter and Silva, 1990).

By analogy with defective HSV particles (HSV amplicons), a single monomeric repeat unit of defective MDV genomes should contain at least a subset of the same *cis*-functions (replication origin and cleavage/packaging signal site) operating in the replication and packaging of standard virus. In search of *cis*-acting elements necessary for standard and defective MDV DNA replication, a 4-kbp MDV replicon DNA was completely sequenced and analyzed. Comparisons of MDV replicon DNA sequences with other viral replication origin sequences revealed a potential serotype 2 MDV replication origin within a 90-bp fragment. Sequences within this fragment are arranged in an imperfect palindrome, centered around 30-bp of alternating A-T residues. Primary structure of this MDV replication origin is very similar to alpha-herpesvirus replication origins; the 90-bp sequence shares 72% nucleotide sequence identity to core regions of HSV-1 ori_S and ori_L, and 66% nucleotide sequence identity to replication origins of VZV and EHV-1. Additionally, replication origin sequences of serotype 2 MDV were

similar (75% nucleotide sequence similarity) to a 76-bp region in the inverted repeats flanking the unique long region of the serotype 1 MDV genome (Bradley et al., 1988). Although MDV is a lymphotropic herpesvirus like EBV, neither origins of EBV (oriP and ori*Lyt*) contained significant sequence similarity to the potential origin of serotype 2 MDV replication.

To test functionality of the putative serotype 2 MDV replication origin identified by DNA sequence analysis, DpnI resistance assays were performed. DpnI recognizes and cleaves only methylated GATC sequences. Progeny DNA molecules from dam+ strains of E. *coli* are methylated at GATC sequences and thus susceptible to DpnI digestion. In contrast, progeny DNA molecules replicated in eucaryotic cells are not methylated at GATC sequences and therefore are resistant to DpnI cleavage. Several subclones (pNOTA5, p281MI-1, pCK300 and pA700) of pA5 were constructed and tested for their replication activity via DpnI resistance assays. Among tested subclones, only pA700, which contains the 90-bp putative MDV replication origin replicated in the presence of a helper virus. Therefore, the MDV replicon DNA contains a functional origin of MDV DNA replication.

In addition to the functional replication origin sequence, MDV replicon DNA revealed various potential open reading frames (ORFs). Two ORFs (ORF-A and ORF-B) containing common promoter elements were further analyzed. ORF-B encodes a potential MDV-specific 350 amino acid protein product; computer alignment of the translation products of ORF-B did not show any significant amino acid similarity to known protein sequences. Due to significant nucleotide

sequence similarity between the serotype 2 MDV replication origin and HSV lytic replication origins (ori_S and ori_L), and biological similarity between defective MDV and defective HSV led us to expect that the ORFs flanking the MDV origin of replication may encode for MDV homologues of HSV ICP4, ICP22, or ICP47. On the contrary, ORF-A, which is located approximately 20-bp upstream of the serotype 2 MDV replication origin, revealed a potential 204 amino acid protein product sharing overall 21% amino acid sequence identity to the 3' exon of EBNA-1 encoded by Epstein-Barr virus. EBNA-1 is one of three nuclear antigens expressed only in cells latently infected with EBV. Although functional relationships between EBNA-1 and the potential protein product of ORF-A are unknown, several lines of observation indicate that ORF-A could code for a potential nuclear DNA binding protein. First, MDV and EBV are both lymphotropic oncogenic herpesviruses, and therefore, can infect and establish latency in lymphocytes. A population of MDV and EBV genomes exists as circular forms in latently infected lymphocytes, or integrate into host cell chromosomes. EBNA-1 functions in *trans* by binding to "oriP", allowing maintenance of the circular EBV genome structure and enhancing transcription of other nuclear antigens. Secondly, a 28-kilodalton (Kd) MDV nuclear antigen (MDNA) was isolated from lymphoblastoid cell line established from MDV tumors (Wen et al., 1988). The MDNA was not detected in lytically infected with MDV cells, suggesting that MDNA may share some biological properties with EBV EBNA-1. The functional relationship of MDNA to ORF-A is unclear at present, however, it is likely that MDV encodes a protein product(s) required for

maintenance of MDV genomes as circular forms. Thirdly, although defective MDV and defective HSV genomes share some biological similarity, they differ in several respects. Based on recently reported restriction endonuclease maps of a serotype 2 MDV genome (Ono et al., 1992), MDV replicon DNA originates from repeats flanking the U_L component, whereas HSV class I amplicons originate from the terminal repeat region of the S component. Therefore, gene templates located within defective MDV and HSV genomes are most likely different.

Overall %G+C content of MDV replicon DNA was 65%, significantly greater than the proposed G+C content of standard virus genome (45%). The 5′ end region of MDV replicon DNA is composed of approximately 90% G+C. Further analyses revealed that the 5′ end region of MDV replicon contained considerable nucleotide sequence identity to the HSV ***a*** sequence, which is located in the L-S junction and both termini of the HSV genome (overall 53% sequence identity). The HSV ***a*** sequence plays important roles in inversion of L-S components relative to each other, and in packaging/cleavage process of HSV replicative intermediates into nucleocapsids. Although further investigation is required to test the functionality of a potential ***a***-like region of MDV replicon DNA, it is likely that MDV replicon DNA contains a *cis*-acting element that controls the cleavage/packaging process of head-to-tail concatemers of defective MDV DNA. The highly cell-associated nature of MDV infection, however, will cause difficulties in performance of functional propagation assays, in which the production of cell-free virus is essential. Additionally, MDV infection often results in cell-to-cell fusion, presenting a different route of MDV infection other than via

infectious enveloped particles.

Most herpesviruses encode proteins necessary for viral DNA replication. Within the serotype 2 MDV replication origin, two copies of a 9-bp sequence present in the flanking regions of AT-rich sequences share a significant nucleotide sequence identity to the HSV origin binding protein (OBP) recognition site I. A synthetic 22-bp oligonucleotide containing a single copy of the 9-bp sequence was constructed (ori 22), and used as a probe to detect virus-specific proteins interacting with the serotype 2 MDV replication origin. Mobility shift and competition assays were performed with labeled ori 22 and whole cell extracts prepared from uninfected or MDV infected cells. A virus-specific protein (protein-DNA complex B) interacting specifically with ori 22 probe DNA was identified. To test if protein-DNA complex B was related to the HSV OBP, antibody super-shift assays and immunoprecipitations were performed using anti-serum directed against the carboxyl terminus of HSV OBP. Super-shifted bands representing the protein-DNA complex B were not observed even with a high concentration of the HSV OBP anti-serum. Additionally, HSV OBP antiserum failed to precipitate a protein from infected cells. Several factors are implicated in these results. First, anti-serum directed against HSV OBP may not recognize any epitope in an MDV OBP homologue. The HSV OBP recognizes two additional sites (site II and III) within lytic origins of HSV DNA replication. OBP recognition site II is similar in sequence to site I of HSV, whereas the potential OBP binding site I of MDV is repeated twice in the MDV replication origin. The OBP binding site III has not been observed in MDV. Therefore, MDV may

encode a protein product(s) similar in function but different structurally when compared to the HSV OBP. Further studies are necessary to investigate MDV-specific protein(s) that may function as a initiator during MDV DNA replication.

Optimization of MDV whole-cell or nuclear extract preparations is pertinent to continue MDV OBP research. MDV-infected cell extracts degrade rapidly when compared to HSV-infected cell extracts. Addition of proteinase inhibitors in extract preparations, thus will be helpful to isolate, and store intact MDV-infected cell extracts.

Additionally, future experiments should include the identification of MDV UL9 gene homologue based on low stringency hybridizations using HSV UL9 gene as a probe, which will facilitate understanding of MDV DNA repliation process. Based on gene colinearity of MDV genes and HSV and VSV, it is likely that MDV UL9 gene homologue is located in the unique long region of MDV genomes.

An intriguing aspect of this report is that MDV contains properties of both alpha- and gammaherpesviruses at the molecular level. Although MDV has been re-classified as an alpha-herpesvirus based on MDV gene colinearity and sequence homology to alpha-herpesviruses, the results obtained from this study suggest that further investigation is required to clearfy the lineage of MDV within the herpesvirus family.

Study of MDV DNA replication is far behind those of other herpesviruses. In essence, MDV DNA replication research has just begun. Results obtained from this research project can be utilized for future experimentation and research

direction. A systematic deletion/substitution analyses across the serotype 2 MDV replication origin sequences will required to delimit the core MDV origin sequences necessary for its replication activity. Additionally, mutational analyses within the potential OBP recognition site I of MDV and its affect on MDV DNA replication are of interest. Comparisons of alphaherpesvirus replication origins indicate that they contain variable numbers of alternating A-T residues in the center of palindromic origin regions. A putative serotype 1 MDV replication origin contains 30-bp alternating A-T sequences, whereas HSV lytic origins of replication contain 18-bp A-T sequences. Functions implicated for this common AT-rich sequence are not clear. However, it is likely that the AT-rich region may play an important role in formation of secondary structure that is essential for the initiation of DNA replication. Deletion analyses of the AT-rich region of serotype 2 MDV replication origin may help us to understand the role of AT-rich regions play during herpesvirus replication.

Based on nucleotide sequencing analysis, the MDV replicon DNA contains at least two potential open reading frames. ORF-A is especially interesting because of its potential amino acid sequence similarity to EBNA-1 of EBV. Generation of a fusion protein product and corresponding anti-sera from ORF-A will be helpful in examining a functional relationship between EBNA-1 and MDV ORF-A.

Identification of a potential *a*-like sequence in MDV suggests that the MDV replicon DNA is packaged into nucleocapsids as in standard virus genome, suggesting that defective MDV genomes can be propagated continuously in the presence of a helper virus. Because of a highly cell-associated MDV infection, it

is difficult to perform standard functional virus propagation assays. Additionally, the densities of MDV DNA and host cell DNA are very similar and thus, isolation of pure viral DNA from total infected cell DNA is difficult. Nucleotide sequencing analysis of MDV replicon DNA indicated that the overall % G+C content of MDV replicon DNA is 65%, which is greater than the % G+C content of standard virus DNA (45%). This result offers a possibility to isolate pure defective MDV DNA, separated from host and standard virus DNA by standard density centrifugation methods. Isolation and analysis of intact defective MDV genomes are essential to understanding the native structure of defective MDV genome, and for manipulation of MDV replicon DNA as a shuttle vector to deliver foreign genes *in vitro* and *in vivo*.

Only 281MI/1 strain has been used as a helper virus to propagate the MDV replicon DNA. Identification of other MDV strains which may function as helper viruses will be of great interest in light of recombinant virus technology. For instance, serotype 3 MDV (HVT) is the most widely used vaccine against MD. Also, bivalent vaccines containing SB-1 have been reported to offer a greater protective immunity against MD. Thus, it would be useful to introduce recombinant MDV replicon DNA, containing genes of interest, in chickens inoculated with HVT vaccine or bivalent vaccine strains.

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