Cloning, Sequencing, and Functional Analysis of a Marek’s Disease Virus Origin of DNA Replication

HEIDI S. CAMP,1 PAUL M. COUSSENS,1 AND ROBERT F. SILVA2*

Molecular Virology Laboratory, Department of Animal Science, Michigan State University, East Lansing, Michigan 48824,1 and Avian Disease and Oncology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 3606 East Mount Hope Road, East Lansing, Michigan 488232

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Previously, we isolated a replicon from a defective Marek’s disease virus (MDV), analogous to defective herpes simplex viruses (amplicons). 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 with other herpesvirus replication origin sequences revealed a 90-bp sequence containing 72% identity to the lytic origin (oriL) of herpes simplex virus type 1. This 90-bp sequence displayed no similarity to betaherpesvirus or gammaherpesvirus 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 (S’CGTTCG CAC3’) highly conserved in alphaherpesvirus replication origins. To test functionality of the 90-bp putative MDV replication origin we conducted DpnI replication assays with subclones generated from the 4-kbp MDV replicon. A 700-bp MDV replicon subfragment containing the 90-bp putative MDV replication origin sequence is capable of replicating in chicken embryo fibroblast cells cotransfected with helper virus DNA. In conclusion, we identified a functional origin of DNA replication in MDV. Similarity of MDV origin sequences to those of alphaherpesviruses supports the current contention that MDV is more closely related to alphaherpesviruses than to gammaherpesviruses.

Marek’s disease virus (MDV) is a highly cell-associated avian herpesvirus. In chickens, MDV is the etiologic agent of Marek’s disease, a lymphoid-destructive T-cell lymphoma (7). There are three MDV serotypes. Serotype 1 includes oncogenic MDVs and their attenuated derivatives, serotype 2 includes the closely related, naturally occurring nononcogenic chicken herpesviruses, and serotype 3 is the antigenically related nononcogenic turkey herpesviruses. MDV pathology has been extensively characterized (7, 23). Molecular analysis of MDV, however, has lagged behind that of other herpesviruses, primarily because of technical difficulties presented by the tightly cell-associated nature of MDV infection.

The MDV genome, a double-stranded linear DNA molecule of approximately 160 to 180 kbp, consists of a unique long (U1) and a unique short (U2) segment flanked by inverted repeats (TRL, IRL, IRS, TRS). MDV has been classified as a gammaherpesvirus based on its lymphotropism (25). However, the overall genomic structure and colinearity of many MDV genes to those of alphaherpesviruses such as herpes simplex virus (HSV) and varicella-zoster virus suggest that MDV should be reclassified (5).

Previously, we reported the isolation and characterization of a defective serotype 2 MDV (8). The defective MDV replicon genome exists as a high-molecular-weight head-to-tail concatemer consisting of 4-kbp viral monomeric repeats and appears to be analogous to HSV amplicons (12). A 4-kbp repeat was cloned into the EcoRI site in pUC19 and was designated pA5 (8).

HSV amplicons contain multiple head-to-tail reiterations of monomeric repeat units derived from either end of the Us fragment or from two noncontiguous regions within the U1 and U2 fragments of the HSV genome (11). HSV amplicons replicate and propagate in the presence of a helper virus (11, 12, 19, 30, 34, 35). Molecular analysis of HSV amplicons revealed three lytic replication origins (oriL and two copies of oriS) and a packaging-cleavage signal site within the HSV genome (11, 12, 30, 31, 33-35).

DNA sequencing. DNA sequencing of the 4-kbp MDV replicon was initiated to identify a potential origin of replication in MDV. pA5I was constructed by cloning the 4-kbp replicon into pUC19 in the inverse orientation with respect to pA5. Unidirectional deletion mutants were created from pA5 and pA5I with exonuclease III and S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and then deleted clones were recircularized with T4 DNA ligase (16). DNA sequencing was performed with double-stranded plasmid templates and the dideoxy chain termination method (26). In all sequencing reactions, the Sequenase enzyme (United States Biochemical Corp., Cleveland, Ohio) was used as recommended by the manufacturer. MDV replicon sequences were compared with sequences in the GenBank data base with the MacVector (International Biotechnologies, Inc., New Haven, Conn.) computer program.

A 90-bp A+T-rich sequence, closely related to HSV and varicella-zoster virus replication origin sequences, was located between a PstI-to-BamHI site of pA5 (9, 20, 33) (Fig. 1A). Overall, the 90-bp sequence contains 72% sequence identity to core regions of the HSV-1 oriL and oriS, and 66% sequence identity to origins of varicella-zoster virus and equine herpesvirus 1 replication (2, 9, 20, 24, 32, 33) (Fig. 1B). Neither the replication origins of Epstein-Barr virus (oriP and oriLyt) nor those of cytomegalovirus contained sequences similar to the putative origin of serotype 2 MDV replication (1, 13, 14, 15, 37).

The 90-bp sequence is arranged in an imperfect palindrome containing 30 bp of alternating AT residues, whereas
the ori and ori of HSV contain a nearly perfect palindrome with an 18-bp A+T-rich region (9, 33). Lockshon and Galloway (20) reported that the palindrome center, containing the 18-bp A+T-rich sequence, is essential for HSV type 2 (HSV-2) replication. However, expansion of the 18-bp A+T-rich region to 52 bp by introducing alternating AT sequences did not abolish the HSV-2 replication function (20). Interestingly, the putative MDV replication origin also contained a 9-bp sequence (CGTTCGCAC) that is highly conserved in alphaherpesvirus replication origins (2, 24, 32, 33). This 9-bp sequence is a subset of an 11-bp motif (CGTTGCACTT) shown to be recognized by the HSV type 1 (HSV-1) origin-binding protein, the product of UL9 gene expression (10, 18). Recently, Bruckner et al. (4) 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, containing the 11-bp motif, is essential for DNA replication (17).

Plasmid replication. To determine whether the putative MDV origin, identified by DNA sequence analysis, could function as a replication origin, we constructed a series of subclones from pA5 (Fig. 2). pNOTA5 was derived from pA5 by replacing a 2.5-kbp HpaI and ClaI fragment of pA5 with NdiI linker (a gift from A. Finkelnstein). 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-KpnI subfragment of pA5 inserted between the ClaI and KpnI sites in pUC18. A 700-bp PstI-KpnI subfragment of pA5 was isolated and cloned into pUC18 by using the PstI and KpnI sites to create pA700. All pA5 subclones as well as pA5 were propagated in Escherichia coli DH5α competent cells, a Dam+ E. coli strain. Also, the 4-kbp EcoRI replication fragment was isolated from pA5 and religated.

We conducted DpnI resistance assays (33) on pA5 subclones as well as the circular form of the 4-kbp replicon. DpnI cleaves only methylated GATC sequences; therefore, DNA propagated from Dam+ strains of E. coli is methylated at the adenine residues within GATC and susceptible to DpnI digestion. DNA replicated in eukaryotic cells is not methylated at GATC sequences and is therefore resistant to DpnI cleavage.

High-molecular-weight DNA was isolated from chicken embryo fibroblast (CEF) cells infected with 281MI/1 passage 15, a serotype 2 MDV (36). Preparation and maintenance of primary or secondary CEF cultures have been previously described (28). Virus stocks were determined to be free of reticuloendotheliosis and avian leukosis viruses by either complement fixation or enzyme-linked immunosorbent assay (29). Upon evidence of extensive cytopathic effect, cells were lysed in 150 mM NaCl-100 mM EDTA-1% sodium dodecyl sulfate-100 μg of proteinase K per ml for 4 to 24 h at 37°C. DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and 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.

Secondary CEF cells were cotransfected with 500 ng of

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FIG. 1. Identification of a putative origin of MDV serotype 2 replication by DNA sequence analysis of the MDV replicon. DNA sequencing was performed by the dyelex chain termination method. (A) Nucleotide sequence of the 90-bp putative origin of MDV serotype 2 replication. Black bars indicate a 9-bp sequence shown to be recognized by the HSV-1 origin-binding protein. (B) Alignment of MDV serotype 2 replication origin with alphaherpesvirus lytic origins of replication. Black bars indicate highly conserved sequences among other origins of herpesvirus replication. Dots (·) represent mismatched sequences, and dashes (—) indicate gaps.

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FIG. 2. Generation of pA5 subclones. All subclones were inserted into pUC18. The location of the putative MDV origin of replication is indicated as an open circle (○). E. EcoRI; HpaI; HpaI; Sm; Smal; P; PstI; BH; BamHII; K; KpnI; Cl; ClaI; and H3, HindIII.
The 4-kbp replicon probe, however, did not hybridize to uninfected CEF DNA (Fig. 3, lane 1). Cotransfection 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, lane 3) in addition to the three EcoRI fragments from the 281MI/1 helper virus genome. DNA isolated from CEF cells cotransfected 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 EcoRI fragments. Methylated input pA5 DNA mixed with 281MI/1-infected CEF DNA was cleaved by DpnI, indicating that DpnI resistance was not a result of inhibition of DpnI activity (Fig. 3, lane 5).

Results of the DpnI resistance assay confirmed that the 4-kbp circular form of the MDV replicon was 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 pUC19, was also replicated as an episome in CEF cells cotransfected with 281MI/1 helper virus DNA. Therefore, we concluded that sequences spanning the EcoRI site within MDV replicon DNA are not required for amplification of replicon DNA. However, replication of the 4-kbp replicon was at least fivefold more efficient than that of pA5. Insertion of pUC19 sequences within the EcoRI site of replicon DNA may have reduced the ability of pA5 to replicate. The decrease in replication activity of pA5 may also be due to some unknown inhibitory sequences within pUC19.

Replication activity of pA5 subclones was indicated by the presence of unit-length DpnI-resistant EcoRI subclone DNA following digestion of cotransfected total cell DNA with EcoRI and DpnI (EcoRI introduces a single cut within the pA5 subclones). During cotransfection experiments, we observed MDV plaque reduction by certain pA5 subclones. Thus, 32P-labeled pA5 was used as a probe to detect both plasmid DNA as well as helper virus DNA. As expected, intact pA5 was able to replicate in the presence of helper virus DNA (Fig. 4, lane 5), generating DpnI-resistant EcoRI fragments of 4 and 2.7 kbp corresponding to virus- and plasmid-specific bands, respectively. However, pA5 alone did not show replication activity, suggesting that functions provided in trans by a helper virus are essential for replication (Fig. 4, lane 3). Methylated input pA5, used as a control, was susceptible to DpnI cleavage (Fig. 4, lane 1). 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 EcoRI fragment of 3.4 kbp (2,686-bp pUC18 plus 700-bp PstI-to-BamHI MDV DNA) (Fig. 4, lane 9). CEF cells cotransfected with 281MI/1 DNA and pNOTA5, p281MI-1, or pCK300 (Fig. 2) representing the remainder of MDV replicon DNA yielded a DpnI-resistant subclone. In all cases, a fraction of input plasmid DNA was susceptible to DpnI cleavage and migrated as low-molecular-weight material, evident after prolonged exposures of the autoradiogram (data not shown).

Results of DpnI resistance assays indicated that a functional origin of MDV replication is located in a 700-bp PstI-to-KpnI subfragment of the MDV replicon. The complete nucleotide sequence of the 700-bp fragment is shown in Fig. 5. Comparison of pA700 nucleotide sequences with the GenBank data base revealed no significant sequence homology to any known viral sequences. The exact location of the MDV replication origin in the 281MI/1 viral genome is unknown because of the lack of complete restriction endonuclease maps or genomic clones of serotype 2 MDV.
CEF cells transfected cellular resistance was pA5, pA5
Lanes: 1, pA5 Dam*; 2, mock-infected CEF DNA; 3, DNA from CEF cells transfected with 281MI/1 DNA and pA5; 4, DNA from CEF cells transfected with 281MI/1 DNA; 5, DNA from CEF cells cotransfected with 281MI/1 DNA and pA5; 6, DNA from CEF cells cotransfected with 281MI/1 DNA and pNOTA5; 7, DNA from CEF cells cotransfected with 281MI/1 DNA and p281MI-1; 8, DNA from CEF cells cotransfected with 281MI/1 DNA and pCK300; 9, DNA from CEF cells cotransfected with 281MI/1 DNA and pA700.

Studies from our laboratory indicate that there are two copies of the replicon sequences within the 281MI/1 viral genome (27). This suggests that there are at least two replication origins in MDV.

HSV contains two copies of ori, within TR and IR, and was localized by utilizing HSV amplicons (11, 12). By analogy with HSV, the MDV replicon sequence is most likely to be present in the repeats flanking the U segment of the MDV genome, assuming that the structure of serotype 2 MDV DNA is similar to that of serotype 1 and 3 MDV DNA. However, on the basis of DNA sequence identity to lytic origins of alphaherpesvirus replication, Bradley et al. (3) and Morgan et al. (21) have located a putative serotype 1 MDV origin of replication in repeats flanking the U region. MDV serotype 2 functional replication origin sequences, identified in this study, share 82% identity to the putative serotype 1 MDV replication origin sequences.

In conclusion, we identified a functional origin of MDV replication within a 700-bp subfragment of the MDV replicon. Systematic deletion analysis within the 700-bp fragment will be useful to determine the minimum sequences required for MDV DNA replication.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequences reported here is M74523.

FIG. 4. Replication analysis of pA5 subclones. Each of the pA5 subclones was tested for replication activity based on the DpnI resistance assay as described in Fig. 3. Each lane contains total transfected cellular DNA digested with EcoRI and DpnI. 32P-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 281MI/1 DNA and pA5; 4, DNA from CEF cells transfected with 281MI/1 DNA; 5, DNA from CEF cells cotransfected with 281MI/1 DNA and pA5; 6, DNA from CEF cells cotransfected with 281MI/1 DNA and pNOTA5; 7, DNA from CEF cells cotransfected with 281MI/1 DNA and p281MI-1; 8, DNA from CEF cells cotransfected with 281MI/1 DNA and pCK300; 9, DNA from CEF cells cotransfected with 281MI/1 DNA and pA700.

FIG. 5. Nucleotide sequences of pA700. pA700, containing a 700-bp subfragment of the MDV replicon DNA, was sequenced in both orientations by using dye-deoxy chain terminations (26). Underlines represent an MDV replication origin sequence that shares a significant sequence identity to alphaherpesvirus replication origins. Black bars indicate the 9-bp motif (CGTTCCGAC) highly conserved among alphaherpesvirus replication origins. Rectangles represent possible SpI-binding sites (GGCGGG), and circles (◆) depict possible CAAT binding regions.

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REFERENCES
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