JOURNAL OF VIROLOGY, Mar. 1991, p. 1352–1363
0022-538X/91/031352-12$02.00/0

Vol. 65, No. 3

Two Spatially Distinct Genetic Elements Constitute a Bipartite DNA Replication Origin in the Minute Virus of Mice Genome

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Received 20 August 1990/Accepted 26 November 1990

Mutations were introduced into plasmid pMM984, a full-length infectious clone of the fibrotropic strain of minute virus of mice, to identify *cis*-acting genetic elements required for the excision and replication of the viral genome. The replicative capacity of these mutants was measured directly, using an in vivo transient DNA replication assay following transfection of plasmids into murine A9 cells and primate COS-7 cells. Experiments with subgenomic constructs indicated that both viral termini must be present on the same DNA molecule for replication to occur and that the viral nonstructural protein NS-1 must be provided in *trans*. The necessary sequences were located within 1,084 and 807 nucleotides of the 3′ and 5′ ends of the minute virus of mice genome, respectively. The inhibitory effect of deletions within the 206-bp 5′-terminal palindrome demonstrated that these sequences comprise a *cis*-acting genetic element that is absolutely essential for the excision and replication of viral DNA. The results further indicated a requirement for a stem-plus-arms T structure as well as for the formation of a simple hairpin. In addition, the removal of one copy of a tandemly arranged 65-bp repeat found 94 nucleotides inboard of the 5′-terminal palindrome inhibited viral DNA replication in *cis* by 10- and just greater than 100-fold in A9 and COS-7 cells, respectively. The latter results define a novel genetic element within the 65-bp repeated sequence, distinct from the terminal palindrome, that is capable of regulating minute virus of mice DNA replication in a species-specific manner.

Minute virus of mice (MVM) belongs to the family *Parvoviridae* whose members infect many animal species, including humans (10, 66, 70). Infectious virions contain linear single-stranded DNA genomes of about 5 kb with self-complementary sequences at their termini that fold into stable base-paired hairpins (4, 11, 17, 25, 27, 55, 56, 75). Most models of parvovirus DNA replication postulate that terminal palindromes constitute the viral DNA replication origin and that initiation of viral DNA synthesis is accomplished by site-specific nicking to generate DNA primers (4, 5, 17, 18, 70). In support of this model, the palindromic termini in replicative intermediates exist in covalently closed as well as extended configurations (21, 22) and two nucleotide sequence orientations have been demonstrated within the terminal inverted repeats of several parvovirus genomes (4, 8, 11, 17, 25). Consistent with these observations, hairpin termini of the type I defective interfering particles of MVM, H-1, and adeno-associated virus (AAV) are selectively retained upon serial undiluted passage (29) and mutations within the terminal palindromes of MVM (50), AAV (61, 64), LuIII (57), and bovine parvovirus (65) act in *cis* to inhibit viral DNA replication and viability. The effects of deletions and substitutions within a stem-plus-arms T structure in the AAV termini on viral DNA replication further indicate that secondary structure, as well as DNA sequence, is critical for maintaining the functional integrity of the genomic termini (12, 43).

All parvoviruses encode an analogous set of proteins known either as NS-1 or Rep that play a critical role in viral replicative-form (RF) DNA replication (36, 50, 57, 71, 72) and in the positive and negative regulation of viral gene expression (10). Infectious plasmid clones developed for several parvoviruses have been used to demonstrate that DNA replication defects caused by mutations in the NS-1 or Rep genes can be corrected by providing the wild-type NS-1 or Rep proteins in *trans* (36, 50, 57, 71) and that temperature-sensitive mutations in the NS-1 gene confer a temperature-sensitive phenotype for viral DNA replication (72). Apparently as a reflection of a direct role played by the Rep proteins in parvovirus DNA replication, the AAV Rep78 and -68 proteins bind specifically to the hairpin configuration of the AAV genomic termini (3, 40). The Rep68 protein also displays ATP-dependent site-specific endonuclease and DNA helicase activities (41, 67) that are required for hairpin resolution as predicted in parvovirus DNA replication models. Consistent with these enzymatic properties, the parvovirus NS-1 and Rep proteins have been shown to contain a highly conserved domain that displays homology to purine nucleotide binding proteins (1, 7, 15). NS-1 is also known to be covalently bound to the 5′ ends of single-stranded and duplex forms of viral DNA (22), likely via a tyrosine residue (8, 19), which provides further evidence that this viral protein is directly involved in the mechanism for resolving cross-linked hairpin termini and initiating viral DNA synthesis.

In this study, specific mutations were introduced into the 5′ noncoding region of pMM984 (51), the full-length infectious clone of MVM(p), to define the *cis*-acting elements required for MVM DNA replication. The effect of these mutations was assessed in an in vivo transient DNA replication assay. The results allow us to define at least two spatially distinct *cis*-acting genetic elements at the 5′ end of the MVM genome that are required for viral DNA replication. Contrary to what might have been expected based on

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previous studies, we find that one of these DNA replication elements maps to a 65-bp repeat that lies inboard of the 5′-terminal palindrome. The 65-bp repeat element displays a remarkable ability to modulate the efficiency of viral DNA replication in a species-specific manner. The possible significance of this previously undefined DNA replication element for the parvovirus DNA replication mechanism is discussed.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, New England BioLabs, Promega Biotec, Pharmacia, and Worthington and used according to the manufacturer's instructions. Radioactive nucleotides were from New England Nuclear or ICN. The Geneclean Kit used for purifying DNA fragments was purchased from BIO 101 and used according to instructions. The Dulbecco modified Eagle medium and fetal bovine serum were purchased from GIBCO. The pMM984 plasmid and the A9 ouab^{r}11 mouse L cells were a gift from P. Tattersall. The simian virus 40 (SV40)-transformed African green monkey kidney COS-7 cell line was purchased from the American Type Culture Collection.

Cells and strains. The A9 (45, 69) and COS-7 cells (32) were passaged by trypsinization and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 2.0 mM glutamine. The MVM recombinant plasmids were propagated in the *recBC sbcB recF Escherichia coli* JC8111 (13) except for pSVNS1-1, which was propagated in *E. coli* DH5α (34). The JC8111 strain permits the deletion-resistant propagation of plasmid clones containing the 5′-terminal palindrome of MVM (13). Plasmids propagated in JC8111 were grown in LB (48) containing 50 μg of ampicillin per ml and 20 mM $MgCl_2$. Frozen competent JC8111 cells were prepared and used in transformations as described by Hanahan (34) once cells reached an optical density at 550 nm of 0.6 to 0.7. The cells were made competent with freezing storage buffer (10 mM potassium acetate, 100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM $HaCoCl_3$, 10% glycerol, adjusted to pH 6.3 with 0.1 M HCl and filter sterilized). To transform the competent cells, DNA was added, the mixture was heat pulsed for 90 s at 42°C and then put on ice for 1 to 2 min. An aliquot of SOC (48) medium was added, and the cells were incubated at 37°C for 1 h in a bacterial shaker (225 rpm). Transformed cells were plated on LB plates containing 100 μg of carbenicillin per ml and 20 mM $MgCl_2$.

Construction of pMM984 mutants. Plasmids were constructed by using standard methods (48). To construct plasmid pRS5, pMM984 DNA was cleaved with *Acc*I and the ends were filled in by using the Klenow fragment of *E. coli* DNA polymerase I. Reaction mixtures contained 6.0 mM Tris HCl (pH 7.5), 6.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 50 mM NaCl, and a 1.0 mM concentration of each deoxynucleoside triphosphate per microgram of DNA with 1.0 U of *E. coli* DNA polymerase I Klenow fragment per μg of DNA. Incubation was for 15 min at 22°C. Following digestion with *Bam*HI, the 972-bp, 5′-terminal *Acc*I-blunted *Bam*HI fragment was purified and ligated to the 2.4-kbp *Nru*I-*Bam*HI pML-2 vector fragment, using T4 DNA ligase. To construct pRS3, the 3′-terminal, 1.1-kbp *Eco*RI-*Bam*HI fragment from pMM984 was isolated and ligated to the 2.6-kbp *Eco*RI-*Bam*HI fragment of pML-2. The pRS53 construct was made by ligating the 972-bp, 5′-terminal fragment described above with the 3.1-kbp fragment of *Bam*HI-*Nru*I-digested pRS3. Plasmid pMMdl21-84 was constructed by self-ligating the ends of the 5.7-kbp *Xba*I-*Eco*RI fragment of pMM984 following the Klenow fill-in reaction described previously.

Construct pSVNS1-1 was made by adding *Bam*HI linkers as described before (48) to the blunt-ended pMM984 *Hga*I-*Aha*II fragment (nucleotides [nt] 240 to 2290) followed by ligation to *Bam*HI-linearized pSVL vector (Pharmacia). Transformed *E. coli* DH5α colonies were screened to select a clone with the insert in the proper orientation. The pMM984-derived ins84A has an 18-bp insertion resulting from the addition of *Kpn*I linkers to the blunted *Xba*I site (nt 4342). This modification near the carboxyl-terminal portion of the capsid protein-coding sequences does not change the original reading frame. The ins20B construct has a 14-bp insertion made by the addition of *Mlu*I linkers to the blunted *Eae*I site (nt 1029). Therefore, ins20B contains a frameshift mutation expected to lead to the synthesis of a truncated form of NS-1 consisting of the N-terminal 258 amino acids of the wild-type protein followed by nine missense amino acid residues. Details of the construction and subsequent characterization of the ins plasmids and pSVNS1-1 are described elsewhere (unpublished data). The pCA3.0dl mutants were derived from construct pCA3.0, a clone made by inserting a *Bam*HI-*Sma*I adapter to the viral 5′ end following partial digestion of pMM984 DNA with *Bam*HI. Mutant pCA3.0dl12 was made by isolating the small 5′-terminal MVM *Xba*I-*Xma*I fragment and subjecting it to *Sau*3AI digestion. The two larger fragments were isolated and then ligated together and subjected to *Xba*I-*Xma*I digestion to ensure isolation of a unit-length terminal fragment of ~740 bp missing the *Sau*3AI fragment (nt 4741 to 4806). Finally, this fragment was ligated to the large *Xba*I-*Xma*I pCA3.0 fragment. To construct pCA3.0dl19 and pCA3.0dl16, *Sma*I-linearized pCA3.0 was digested with ExoIII to generate a series of 5′-terminal deletions. The ExoIII-treated DNA was then restricted with *Sal*I, and the nested large fragments were isolated and subsequently mixed with the small *Sal*I-*Sma*I fragment from pCA3.0 to ligate the MVM 5′-terminal deleted sequences back to the original vector.

The pTD2 and pTD3 plasmids were constructed independently, using a different approach. Briefly, a pSP64 recombinant plasmid containing the 5′-terminal *Acc*I-*Bam*HI fragment (nt 4177 to 5149) of pMM984 was digested with *Sac*I-*Bam*HI followed by ExoIII digestion, producing unidirectional deletions from the 5′ protruding *Bam*HI end. The DNA was subjected to mung bean nuclease digestion followed by the Klenow fill-in reaction to ensure complete conversion of staggered DNA ends to blunt ends. Commercially phosphorylated *Bam*HI 8mer (Pharmacia) was added to the ends, and the 5′-terminal *Xba*I-*Bam*HI fragments were isolated following *Xba*I digestion. This DNA was then ligated to purified 8.0-kbp *Xba*I-partial *Bam*HI-digested pMM984 fragment missing the 0.8-kbp 5′-terminal MVM *Xba*I-*Bam*HI fragment.

The ΔK deletion was constructed from pCA3.0. The small (~0.8 kbp) 5′-terminal *Xba*I-*Xma*I fragment was purified, digested with *Msp*I, ligated, and redigested with *Xba*I-*Xma*I. This ~0.8-kbp fragment, which migrated slightly faster than the original fragment due to the removal of the 9-bp *Msp*I fragment, was purified and religated with the large *Xba*I-*Xma*I fragment from pCA3.0, generating the ΔK plasmid. The ΔB and Bi forms of pMM984, with internal deletions within the 5′ hairpin, were isolated by screening colonies of pMM984-transformed *E. coli* DH1 or JC8111 cells, respectively, for the particular deletions. The MVM insert from pMM984ΔB was then transferred from its original pBR322

background and DH1 host to the pAT153 vector (73) and *E. coli* JC8111. Also, the viral genomes of the original pCA3.0dl mutants and the ΔK construct were excised from pBR322 and inserted into pAT153. These DNAs were subsequently used in the transfections. In all cases, the MVM recombinant plasmids used in these experiments have bacterial vector backgrounds missing the putative pBR322 "poison" sequences known to inhibit SV40 DNA replication in COS cells (47) and bovine papillomavirus DNA replication in mouse C127 cells (63).

Plasmid DNA preparation. Plasmid DNA used in the transfections was purified from an NaOH-sodium dodecyl sulfate (SDS) cleared bacterial lysate by two cycles of isopycnic CsCl-ethidium bromide equilibrium density gradient centrifugation (48).

Transient transfection assay. Transfection studies of pMM984-derived mutants were done in at least two separate experiments, normally done with duplicate samples. The results of the pRS3 and pRS5 transfection series represent a single experiment done in duplicate. In all cases, similar results were obtained, with experimental variation accounting for twofold differences or less as judged by direct comparison to wild-type pMM984 replication levels. A9 or COS cells were plated at 10^6 cells per 100-mm dish the day before transfection. The growth medium (pH 7.2 to 7.4) was replaced with 5 ml of fresh growth medium 2 to 4 h before the addition of DNA. The amount of plasmid DNA added to each plate was 10 μg. The total amount of DNA per dish was 20 μg, standardized with sonicated salmon sperm DNA when applicable. Cotransfections were done with 10 μg of each plasmid. Calcium-phosphate DNA precipitates were prepared by the method of Graham et al. (33), by adding 2× $CaCl_2$ solution (DNA in 250 mM $CaCl_2$) dropwise to 2× HeBS (1.64% [wt/vol] NaCl, 1.19% [wt/vol] HEPES [*N*-2-hydroxyethypiperazine-*N'*-2-ethanesulfonic acid]-NaOH and 0.04% [wt/vol] Na_2HPO_4, pH 7.10) and modified by agitating the solution with a mechanical pipettor. The precipitate was allowed to form for 20 to 30 min at room temperature, and 1 ml was added per dish. Cells were incubated for 4 h at 37°C and then rinsed with serum-free medium and incubated in 20% (vol/vol) glycerol in 1× HeBS (pH 7.1) for 1 min at room temperature. The cells were rinsed twice with medium, 10 ml of growth medium was added, and incubation was continued for 40 to 42 h. The cells were then collected in phosphate-buffered saline by scraping with a rubber policeman and pelleted in an IEC PR6000 centrifuge with a no. 269 rotor at 2,000 rpm for 10 min at 4°C. Viral DNA was extracted by a modified version of the Hirt procedure (38). Briefly, cell pellets were resuspended with 100 μl of Hirt wash buffer (150 mM NaCl, 20 mM Tris HCl [pH 7.5], 10 mM EDTA) and transferred to Eppendorf tubes. Four volumes of prewarmed Hirt lysis buffer (1.25 M NaCl, 0.75% SDS, 20 mM Tris HCl [pH 7.5], 10 mM EDTA) containing 100 μg of proteinase K per ml were added, and the tubes were incubated at 37°C for approximately 4 h. Following an overnight incubation on ice, the cell lysates were cleared by centrifugation at 4°C for 2 to 2.5 h at 13,000 rpm in a Biofuge B microcentrifuge. The DNA was precipitated in 70% ethanol and yeast tRNA, which served as carrier. The precipitate was pelleted, dissolved in TE (10 mM Tris HCl [pH 7.5], 0.1 mM EDTA) containing 50 μg of RNase A per ml, and incubated for 1 h at 37°C. Sodium chloride was added to a final concentration of 0.2 M, and the DNA was ethanol precipitated as above following extraction with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. Subsequent to centrifugation of the ethanol precipitate, DNA was resuspended in TE. To monitor the efficiency of DNA recovered from individual samples, equal aliquots of α-^{32}P-labeled pMM984 1.1-kbp *Eco*RI-*Bam*HI fragment (nt 0 to 1084) was added to the samples at the beginning of the extraction procedure. Radiolabeled nucleotides were incorporated into DNA by the fill-in reaction described earlier except one deoxynucleoside triphosphate was replaced with 80 μCi (3,000 Ci/mmol) of the corresponding α-^{32}P-labeled deoxynucleoside triphosphate. The sample volume is adjusted to 100 μl with TE following the reaction and the DNA is purified from unincorporated deoxynucleoside triphosphates by passing the mixture through a G-50 fine-spun column (48) equilibrated in TE.

Analysis of transfected DNA. The amount of radioactivity in the DNA samples was determined by liquid scintillation counting. Based on these values, normalized aliquots of Hirt procedure-extracted DNA were analyzed by *Dpn*I restriction endonuclease digestion alone or in combination with other restriction endonucleases when indicated. DNA was electrophoresed in 0.8% agarose gels at 40 V for 15 to 18 h in TBE (100 mM Tris-borate [pH 8.3], 2 mM EDTA) containing 1 μg of ethidium bromide per ml. The DNA was transferred by the method of Southern (68) to magnagraph membrane (Micron Separations Inc.) according to the manufacturer's instructions. Prehybridization solution (1 ml/10-cm^2 membrane) consisted of 50% formamide, 5× Denhardt solution (1.0 g of polyvinylpyrrolidone, 1.0 g of Ficoll, and 0.5 g of SDS per liter), 5× SSC (0.75 M NaCl, 75 mM sodium citrate [pH 7.0]), 0.1% (wt/vol) SDS, 0.1% (wt/vol) bovine serum albumin, 20 mM sodium phosphate (pH 7.0), and 200 μg each of sheared denatured salmon sperm DNA and yeast tRNA per ml. Hybridization was done in the aforementioned solution after adding the radiolabeled probe. Prehybridizations were performed for 2 to 4 h at 50°C and hybridizations were done for 18 to 22 h at the same temperature. Unless otherwise indicated, the blots were analyzed with a radiolabeled RNA probe complementary to the viral strand of the MVM genome synthesized with SP6 RNA polymerase and a pSP64 recombinant plasmid (pSP64-5) containing the nondeleted form of the 5′-terminal *Acc*I-*Bam*HI fragment of pMM984. Probe pSP64-3 has the 3′-terminal *Bam*HI-*Eco*RI fragment of pMM984 cloned in pSP64. The probes are synthesized in a reaction mixture that contains 0.5 to 1.0 μg of *Bam*HI or *Eco*RI linearized pSP64-5 or pSP64-3 plasmid template DNA, respectively, 1× transcription buffer (40 mM Tris HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), a 2.5 mM concentration of each of ATP, GTP, and UTP, 12 μM CTP, 10 mM dithiothreitol, 50 μCi of [α-^{32}P]CTP (752 Ci/mmol), 1 U of Rnasin RNase inhibitor, and 5 to 10 U of SP6 RNA polymerase in a total volume of 20 μl. After a 1-h incubation at 37°C, 0.5 μl of a 2.5-mg/ml DNase I solution is added and the incubation is continued for 15 min. Unincorporated ribonucleotides are removed from the RNA as described previously. Hybridized membranes were washed (1 to 2 ml/cm^2 of membrane) twice with 5× SSPE (0.75 M NaCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$, 5.0 mM Na_2 EDTA, pH 7.4)–0.5% SDS for 15 min at room temperature, twice with 1X SSPE–0.5% SDS for 15 min at 37°C, and twice with 0.1× SSPE–1.0% SDS at 65°C for 15 min. The filters were air dried and autoradiographed with Kodak XAR-5 film and an intensifying screen at −70°C.

Sequencing of pMM984 mutants. The deletions in the ΔB and Bi forms of pMM984 and the ΔK construct were sequenced by a modified version (9) of the Maxam and Gilbert method (49). As well, the 5′ palindrome of pCA3.0dl12 was sequenced by the chemical method and found to be intact. The DNA sequence of the 65-nt deletion

mutation in pCA3.0dl12 was determined essentially by the Sanger dideoxy-chain termination procedure (62) with minor modifications following the subcloning of the blunt-ended pCA3.0dl12 *Ava*II fragment (nt 4297 to 4872) into the MP13mp18 *Sma*I site and generation of recombinant single-stranded DNA. The deletions in pTD2 and pTD3 were determined by using a modified double-stranded plasmid sequencing procedure derived from established protocols (16, 62). In particular, a 10-μl reaction containing 2 μg (0.34 pmol) of pTD2 or pTD3 DNA and 2 μl of a 2 M NaOH–2 mM EDTA solution was incubated at room temperature for 5 min to denature the DNA. Following neutralization with 3 μl of 3 M sodium acetate (pH 5.2) and ethanol precipitation, the alkali-denatured DNA was annealed to 60 μg (9 pmol) of pBR322 *Bam*HI site clockwise primer (New England Bio-Labs) for 15 min at 42°C. Eight units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) was used to perform the typical DNA polymerization and subsequent chase reactions, both done at 42°C for 30 min.

RESULTS

Experimental design. We used an in vivo transient DNA replication assay (54) in which methylated plasmids were generated in *E. coli* and transfected into mouse A9 cells or COS-7 cells. The extent of viral DNA replication was determined by monitoring the production of *Dpn*I-resistant viral DNA molecules in a Southern blot analysis, using MVM-specific hybridization probes. Unless indicated otherwise, the extent of viral DNA replication was such that a 100-fold dilution of the signal obtained for monomer replicative-form (mRF) DNA in the standard assay could be detected. This level of DNA replication, representing 1% of the pMM984 replication level, was thus established as the lower limit of detection for the assay system that was used in the experiments described below.

Requirement for terminal MVM sequences. Previous studies of naturally occurring deletion mutants of MVM indicated that the *cis*-acting elements required for viral DNA replication are located within approximately 200 nt of the genomic termini. The deletion mutants were defective for DNA replication but could be propagated if the wild-type NS-1 protein was provided in *trans* by coinfection with wild-type virus (29). To study this directly, we constructed plasmids that contained DNA sequences from terminal regions of the MVM genome, but did not contain an intact NS-1 gene. The construction of these plasmids is depicted in Fig. 1. While none of these plasmids replicated when transfected into A9 cells individually, as expected due to the absence of an intact NS-1 gene, pRS53 and pMMdl21-84 did replicate in A9 cells when cotransfected with pMM984 (Fig. 2A). Replication of wild-type MVM DNA occurred normally, as judged by the appearance of relatively high levels of the 5.0-kbp monomer and 10.0-kbp dimer RF DNA species and lower amounts of an 8.0-kbp partially replicated dimer species and progeny single-stranded viral DNA. These forms of viral DNA are identical to those seen in extracts of A9 cells infected with MVM (28). pRS53 replicated as a linear 4.1-kbp DNA species at 10% of wild-type levels (Fig. 2A, lane 8). This relatively low level of DNA replication is not due to an inherent limitation of the complementation assay since pMMdl21-84 replicated at near wild-type levels under the same conditions (Fig. 2A, lane 10). The *Dpn*I-resistant, 1.9-kbp DNA species derived from pMMdl21-84 was resolved into two distinct DNA species (evident upon a lighter exposure of the autoradiogram) which can be attributed to the generation of extended and hairpin configurations of the genome termini that arise as a consequence of viral DNA replication (21, 22). The failure of pRS53 to replicate with the same efficiency as pMM984 could be due to the presence of plasmid sequences in the linear pRS53 molecule that somehow reduce replication efficiency, despite the absence of plasmid "poison" sequences, or it might reflect a reduced efficiency of conversion of the input circular plasmid DNA to a linear RF DNA due to the juxtaposition of the 3′ and 5′ palindromes in an unusual head-to-tail configuration. Nevertheless, since pRS53 replicated as a linear DNA species, the circular input pRS53 plasmid with juxtaposed MVM DNA termini appears to have been cleaved and replicated by a mechanism similar to that used by pMM984, albeit at a lower efficiency. Taken together, the results obtained with pRS53 and pMMdl21-84 show that the NS-1 defect in the pMMdl21-84 and pRS53 mutants could be complemented in *trans* in A9 cells by cotransfection with pMM984 and that the *cis*-acting sequences required for viral DNA replication are present within 1,084 nt at the 3′ end and 807 nt at the 5′ end of the MVM genome.

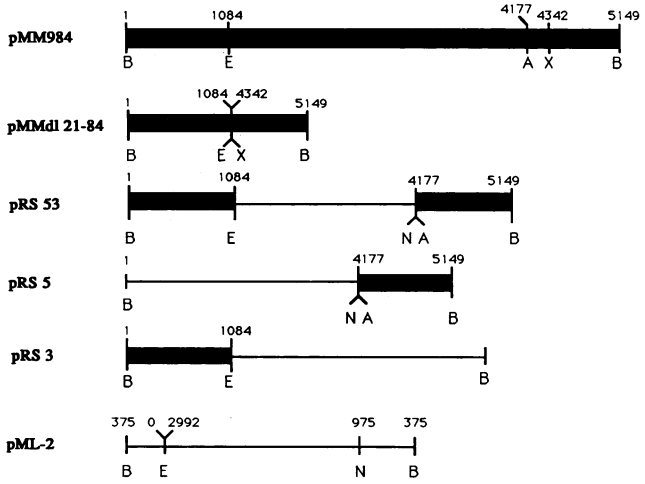

FIG. 1. Diagram of subgenomic plasmids containing terminal portions of the MVM genome. The construction of the plasmids depicted is described in Materials and Methods. Solid blocks represent sequences derived from pMM984 and thin lines represent plasmid sequences. pMM984 and pMMdl21-84 are represented as *Bam*HI restriction fragments excised from the pAT153 vector sequences (not shown). All other plasmids contain pML-2 vector sequences. Numbers above the solid boxes and thin lines represent the restriction endonuclease cleavage sites in pMM984 according to the published DNA sequence of the MVM(p) genome (6) and that of pBR322. A, *Acc*I; B, *Bam*HI; E, *Eco*RI; N, *Nru*I; X, *Xba*I.

Plasmids with single 3′ or 5′ genomic termini are Rep$^-$. To determine whether the 3′ and 5′ termini of the MVM genome must be present on the same DNA molecule in order to support viral DNA replication, we examined the ability of plasmids containing either the 3′ or the 5′ terminus to replicate in A9 cells. pRS5, which has the 5′-terminal 972 nt of the MVM genome, failed to replicate when transfected into A9 cells by itself (Fig. 2A, lane 5). This result was expected since this plasmid does not contain any of the viral sequences that encode the MVM NS-1 gene, which is known to be essential for viral DNA replication. When pRS5 was cotransfected with pMM984, under conditions that permit the replication of cotransfected NS-1 mutants (see below), a DNA species that appeared to correspond to an mRF DNA

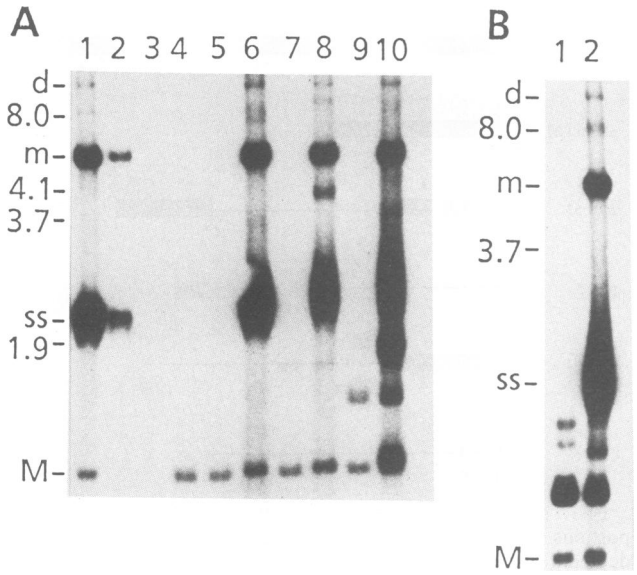

FIG. 2. Transient in vivo DNA replication in A9 cells of subgenomic plasmids containing MVM termini. Plasmids were transfected into A9 cells, and DNA samples were analyzed in Southern blots as described in Materials and Methods. The positions of dimer RF DNA (d), partially replicated dimer RF DNA (8.0 kbp), mRF DNA (m), progeny single-stranded DNA (ss), and ^{32}P-labeled DNA that was added to the samples as an internal normalization standard (M) are indicated. The sizes of linear forms of plasmids pRS5 (3.5 kbp), pRS3 (3.7 kbp), and pRS53 (4.1 kbp) and of the MVM sequences excised from pMMdl21-84 (1.9 kbp) are also indicated. Transfections involving pRS3 and pRS5 were done in duplicate and produced comparable results. (A) Lanes: 1 to 3, pMM984 at 1×, 0.1×, and 0.01× dilutions, respectively; 4, mock; 5, pRS5; 6, pRS5 and pMM984; 7, pRS53; 8, pRS53 and pMM984; 9, pMMdl21–84; 10, pMMdl21-84 and pMM984. (B) The Southern blot was hybridized to the pSP64-3 probe specific for the 3′-terminal sequences of the MVM genome (see Materials and Methods). Lanes: 1, pRS3; 2, pRS3, and pMM984.

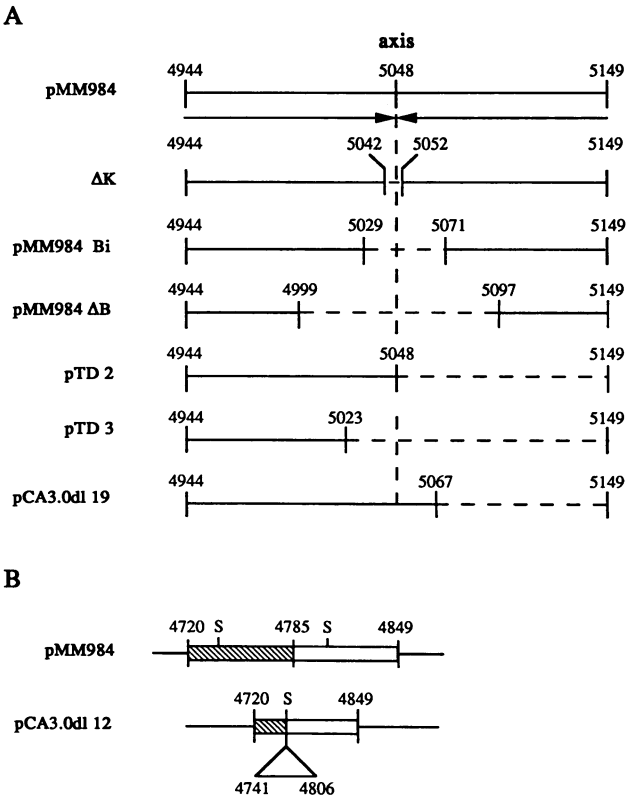

FIG. 3. Diagram of plasmid constructs derived from pMM984 that contain deletions in the 5′-terminal region of the MVM genome. The construction of the plasmids is described in Materials and Methods. (A) Diagram depicts deletion mutations within the 5′-terminal palindrome of the MVM genome. Arrows indicate the inverted repeat, and the axis of symmetry is depicted by the vertical dashed line at nt 5048. The horizontal dashed lines represent the sequences that are deleted in the mutants. The endpoints of the deletions were assigned on the basis of DNA sequence analysis as described in Materials and Methods. (B) Schematic representation of the deletion of pCA3.0dl12. Hatched and open boxes represent the two tandemly arranged 65-bp repeats in the MVM(p) genome as cloned in pMM984. pCA3.0dl12 has one copy of the repeat depicted by the hatched-open box resulting from the removal of a *Sau*3AI fragment (S) from nt 4741 to 4806. The deletion in pCA3.0dl12 was verified directly by DNA sequence analysis (see Materials and Methods).

of pRS5 (3.5 kbp) was produced at a relatively low level (1%) compared with the wild-type MVM genome (Fig. 2A, lane 6). However, additional analysis revealed that this DNA species is a *Dpn*I digestion product of unreplicated viral DNA and is not due to the replication of pRS5 DNA (data not shown). Failure of pRS5 to replicate in the presence of pMM984 indicates that the 5′-terminal 972 bp of MVM DNA is not sufficient to support the accumulation of replicated viral DNA molecules, despite the presence of NS-1.

pRS3, which contains the 3′-terminal 1,084 nt of MVM DNA, also failed to replicate (<5% of pMM984) when transfected into murine A9 cells either by itself or together with pMM984 (Fig. 2B). These results when taken together with the results of the experiments with pRS53 and pMMdl21-84, demonstrate that the 3′ and 5′ termini of the MVM genome must be present on the same DNA molecule for the viral DNA to replicate in A9 cells.

Effect of mutations in the 5′-terminal palindrome. Several mutations were introduced at the 5′ terminus of pMM984 either by in vitro manipulation or as the result of spontaneous deletions arising during propagation of pMM984 DNA in *E. coli* (13). Deletion mutants were obtained that were missing 9 (ΔK), 41 (Bi), or 97 (ΔB) bp that encompassed the axis of symmetry in the 206-bp, 5′-terminal palindrome (Fig. 3A). To study the effects of these mutations, each mutant was transfected into either A9 or COS-7 cells. As judged by the absence of *Dpn*I-resistant RF DNA in extracts of transfected cells, these mutants failed to excise and replicate their DNA in each of these cell lines (Figs. 4A and 5A). A similar result was obtained with pTD2, a mutant that possessed a 5′-terminal deletion of 101 bp, and with pTD3, which contains a terminal deletion of 126 bp (Fig. 3A and 4B). Replication of pCA3.01dl19, which is missing 82 bp from the 5′ end of the MVM genome, was detectable at a relatively low level (1% of pMM984) (Fig. 4A, lane 6).

In vivo complementation. To characterize the phenotype of the terminal deletion mutants in more detail, cotransfection experiments were done to determine whether the terminal deletion mutations acted in *cis*. Wild-type NS-1 was provided in *trans* from plasmid pSVNS1-1 in which the MVM NS-1 gene is under the control of the SV40 late promoter. The complementation assay using pSVNS1-1 is described in detail elsewhere (Skiadopoulos et al., unpublished data).

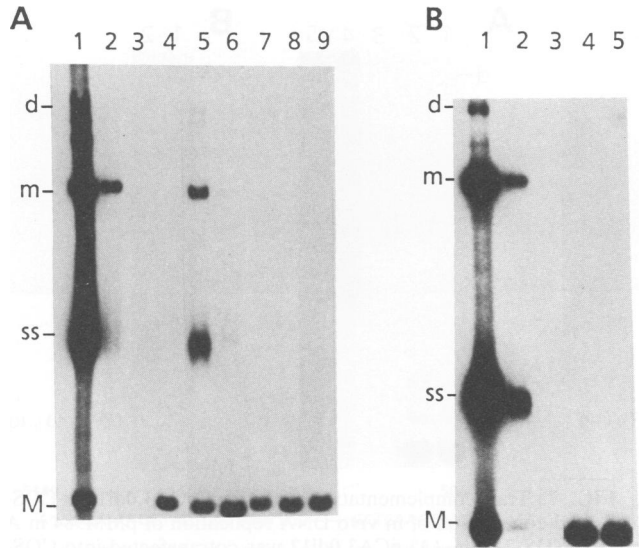

FIG. 4. Transient in vivo DNA replication analysis of 5′-terminal deletion mutants in A9 cells. DNA was analyzed in Southern blots after transfection of A9 cells with mutants containing deletions in the 5′-terminal region of the MVM genome. All transfections were done in duplicate and produced comparable results. (A) Lanes: 1 to 3, pMM984 at 1×, 0.1×, and 0.01× dilutions, respectively; 4, mock; 5, pCA3.0dl12; 6, pCA3.0dl19; 7, pMM984Bi; 8, pMM984ΔB; 9, ΔK. (B) Lanes: 1 to 3, pMM984 at 1×, 0.1×, and 0.01× dilutions, respectively; 4, pTD2; 5, pTD3. See legend to Fig. 2 for abbreviations.

When pSVNS1-1 was cotransfected into COS-7 cells with a replication-defective NS-1 frameshift mutant of pMM984 (ins20B), the ins20B genome replicated as judged by the appearance of a 4.1-kbp *Mlu*I fragment that is specific for ins20B (Fig. 5B, lane 5). However, pSVNS1-1 failed to promote the excision and replication of the pCA3.0dl19, ΔK, Bi, and ΔB deletion mutants in COS-7 cells, demonstrating directly that these deletion mutations in the 5′-terminal palindrome act in *cis* to block rescue and viral DNA replication (Fig. 5A, lanes 2, 4, 6, and 8).

Similar results were obtained in A9 cells. The ΔK mutant was cotransfected with ins84A, a capsid mutant that replicates its duplex DNA at approximately 30% of wild-type levels but cannot accumulate single-stranded progeny DNA (Fig. 6; unpublished data). Replication of ins84A could be distinguished from that of the cotransfected ΔK mutant by cleavage at its unique *Kpn*I site which produces characteristic 4.3- and 0.8-kbp fragments (Fig. 6, lane 2). Although a 5.0-kbp DNA species that could represent replication of the ΔK DNA was observed at approximately 1% of pMM984, additional experiments established that this DNA species arose by recombination between the parental mutant genomes (data not shown) and therefore does not represent replication of the ΔK mutant. The progeny single-stranded DNA observed in this experiment can also be accounted for by this mechanism. Failure of ins84A to complement the replication-defective phenotype of the ΔK mutant is not due to a defect in the NS-1 gene of ins84A or to a requirement for a wild-type capsid gene since ins84A is capable of complementing replication-defective NS-1 mutants to approximately 20% of the replication level observed for pMM984 (data not shown). We therefore conclude that the ΔK mutation acts in *cis* to prevent excision and replication of MVM DNA in A9 cells.

Importance of the 65-bp repeat sequence. The MVM(p) genome has two tandemly arranged 65-bp repeats spanning nt 4720 to 4849, inboard of the 5′-terminal palindrome. To investigate the significance of these sequences for viral DNA replication, plasmid pCA3.0dl12 was constructed in which

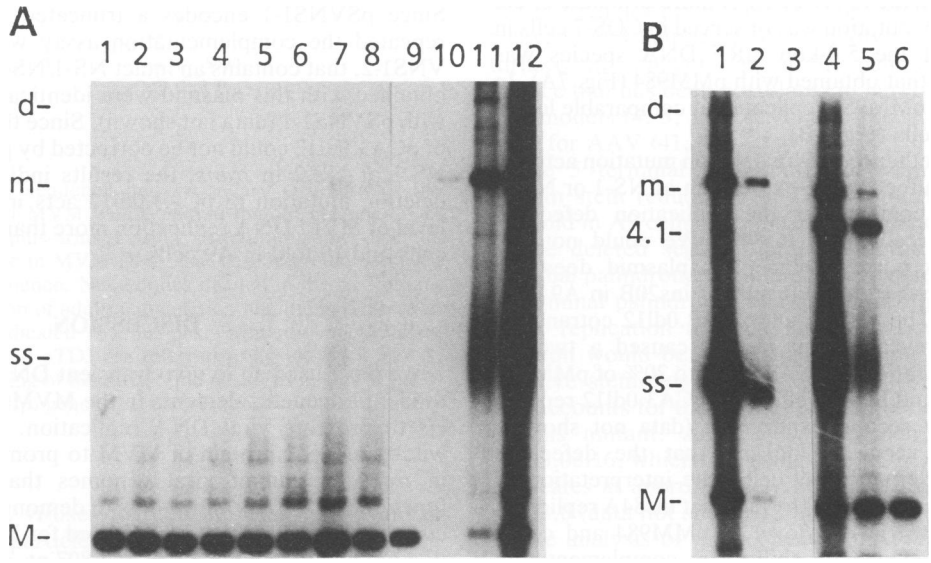

FIG. 5. *Trans* complementation analysis of mutant pMM984 constructs by pSVNS1-1 in COS-7 cells. Mutants with deletions in the 5′-terminal palindrome were transfected into COS-7 cells either alone or in a cotransfection with pSVNS1-1 as described in Materials and Methods. The DNA samples were analyzed in Southern blots. (A) Duplicate transfections of the terminal deletion mutants by themselves or with pSVNS1-1 gave similar results. Lanes: 1, ΔK; 2, ΔK and pSVNS1-1; 3, pMM984Bi; 4, pMM984Bi and pSVNS1-1; 5, pMM984ΔB; 6, pMM984ΔB and pSVNS1-1; 7, pCA3.0dl19; 8, pCA3.0dl19 and pSVNS1-1; 9, mock; 10 to 12, pMM984 at 0.01×, 0.1×, and 1× dilutions, respectively. (B) The frameshift NS-1 mutant ins20B was cotransfected with pSVNS1-1 or pMM984, and the transfected DNA was cleaved with *Mlu*I and *Dpn*I and analyzed in a Southern blot. The replicated ins20B genome is designated as the 4.1-kbp DNA species. Lanes: 1 to 3, pMM984, 1×, 0.1×, and 0.01× dilutions, respectively; 4, pMM984 and ins20B; 5, pSVNS1-1 and ins20B; 6, ins20B. See legend to Fig. 2 for abbreviations.

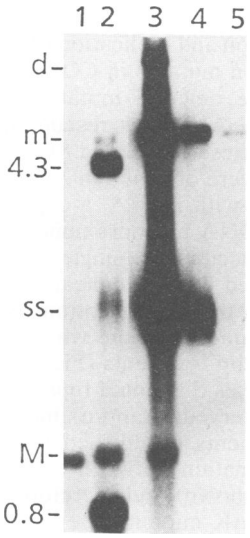

FIG. 6. Transcomplementation analysis of the ΔK mutant in A9 cells. The ΔK construct was cotransfected with ins84A, and the transfected DNA was cleaved with *Kpn*I and analyzed in a Southern blot. The experiment was done in duplicate with comparable results. The 4.3- and 0.8-kbp DNA species indicate replication of the ins84A genome (see text). Lanes: 1, ΔK; 2, ΔK and ins84A; 3 to 5, pMM984, 1×, 0.1×, and 0.01× dilutions, respectively. See legend to Fig. 2 for abbreviations.

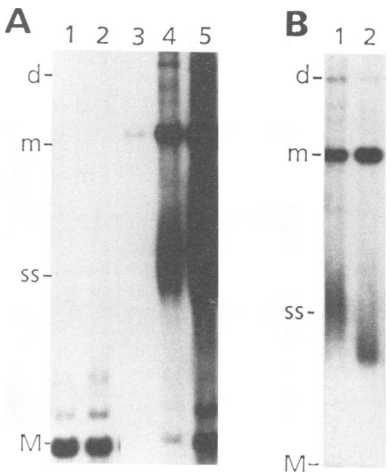

FIG. 7. Transcomplementation analysis of pCA3.0dl12 in COS-7 cells and comparison of in vivo DNA replication of pMM984 in A9 versus COS-7 cells. (A) pCA3.0dl12 was cotransfected into COS-7 cells with pSVNS1-1, and the transfected DNA was analyzed in a Southern blot. Transfections involving pCA3.0dl12 were done in triplicate with comparable results. Lanes: 1, pCA3.0dl12; 2, pCA3.0dl12 and pSVNS1-1; 3 to 5, pMM984, 0.01×, 0.1×, and 1× dilutions, respectively. (B) In this experiment, pMM984 transfections were done in duplicate with comparable results. Lanes: 1, pMM984 at 0.1× dilution in COS-7 cells; 2, pMM984 at 0.1× dilution in A9 cells. See legend to Fig. 2 for abbreviations.

one of the 65-bp repeats was deleted (Fig. 3B). When pCA3.0dl12 was transfected into A9 cells, viral DNA was excised and replicated at approximately 10% of pMM984 levels, as judged by the appearance of a linear 5.0-kbp RF DNA species (Fig. 4A, lane 5). Thus, the 65-nt deletion mutation has a moderate inhibitory effect on the ability of MVM DNA to replicate in A9 cells. A more dramatic effect of the 65-nt deletion mutation was observed in COS-7 cells in which the level of the 5.0-kbp mRF DNA species was reduced to <1% of that obtained with pMM984 (Fig. 7A). In contrast, wild-type pMM984 replicated at comparable levels in A9 and COS-7 cells (Fig. 7B).

To determine whether the 65-bp deletion mutation acted in *trans*, possibly by affecting the expression of NS-1 or NS-2, we attempted to complement the replication defect of pCA3.0dl12 in A9 cells with ins84A. We could not use pSVNS1-1 for this purpose since this plasmid does not complement the NS-1 frameshift mutant ins20B in A9 cells (data not shown). The ins84A and pCA3.0dl12 cotransfection experiments revealed that ins84A caused a twofold increase in the replication of pCA3.0dl12 to 20% of pMM984 in one experiment and had no effect on pCA3.0dl12 replication efficiency in a second experiment (data not shown). While this would seem to indicate that the defect in pCA3.0dl12 is *cis* acting in A9 cells, the interpretation of these results is complicated by the fact that ins84A replicates at approximately 30% of the level of pMM984 and could therefore be limited in its ability to complement the pCA3.0dl12 defect beyond this level. Experimental variation could also account for a twofold difference. To clarify this point, we performed a complementation analysis in COS-7 cells, in which the replication of pCA3.0dl12 was restricted to slightly <1% of pMM984 levels. We expected that this low background of replication in COS-7 cells relative to the 10% level seen in A9 cells would permit a more definitive interpretation to be placed on the degree to which complementation occurs in COS-7 cells. pSVNS1-1 consistently failed to enhance replication of pCA3.0dl12 in COS-7 cells beyond a background level of just below 1% (Fig. 7A, lane 2), although this plasmid reproducibly complemented the replication of the NS-1 frameshift mutant ins20B in *trans* to approximately 30% of pMM984 levels (Fig. 5B, lane 5). Since pSVNS1-1 encodes a truncated from of NS-2, we repeated the complementation assay with a plasmid, pSVNS1-2, that contains an intact NS-1/NS-2 gene. The results obtained with this plasmid were identical to those obtained with pSVNS1-1 (data not shown). Since the Rep^- phenotype of pCA3.0dl12 could not be corrected by providing wild-type NS-1 or NS-2 in *trans*, the results indicate that the 65-nt deletion mutation in pCA3.0dl12 acts in *cis* to reduce the level of MVM DNA replication more than 100-fold in COS-7 cells and 10-fold in A9 cells.

DISCUSSION

We have used an in vivo transient DNA replication assay to identify genetic elements in the MVM genome that act in *cis* to promote viral DNA replication. The ability of the wild-type NS-1 protein of MVM to promote the replication in *trans* of mutant viral genomes that do not produce functional NS-1 has allowed us to demonstrate that all of the *cis*-acting genetic elements required for MVM DNA replication are located within 1,084 and 807 nt, respectively, of the 3′ and 5′ termini of the MVM genome. Furthermore, both genomic termini must be present on the same DNA molecule for replication to occur. These conclusions are consistent with the mapping of the MVM, H-1, and AAV DNA replication origins to the genome termini based on an analysis of defective viral genomes (29) and with the demonstration that deletion mutations within the palindromic termini of bovine parvovirus, LuIII, MVM, and AAV severely interfere with

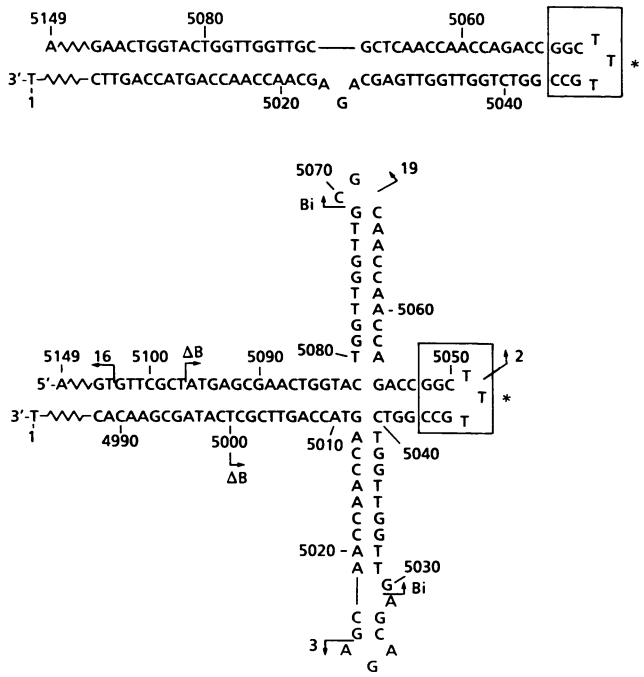

FIG. 8. Nucleotide sequence and secondary structure of the 5′-terminal hairpins of MVM wild-type and mutant genomes. Depicted are the stem-plus-arms hairpin structural isomers of the 5′-terminal palindrome in MVM DNA that can be predicted based on the nucleotide sequence. Nucleotides deleted in the ΔK mutant are boxed. The location of additional deletion mutations referred to in the text are indicated by arrows. Mutants pCA3.0dl16, pCA3.0dl19, pTD2, and pTD3 are referred to as 16, 19, 2, and 3, respectively. Numbering of the nucleotide positions begins at the 3′ terminus of the MVM(p) genome and corresponds to that given by Astell et al. (6).

the ability of these viruses to replicate their DNA and to produce infectious particles (50, 57, 61, 64, 65).

Two distinct genetic elements near the 5′ end of the MVM genome that are required for the efficient excision and replication of viral DNA following transfection of the infectious plasmid pMM984 into A9 or COS-7 cells were defined in this study. These elements map to a region within the 206-bp 5′-terminal palindrome and to a 65-bp tandemly repeated sequence located 94 nt inboard of the start of the palindromic sequence. Our results are in general agreement with previous studies in which the 97-bp ΔB mutation was found to block the production of infectious virus (51). We have extended these results by demonstrating an equally dramatic effect of 5′-terminal deletions of between 82 and 126 bp and of internal palindrome deletions of 9 and 47 bp. The Rep$^-$ phenotype of the palindrome mutants can most likely be explained in terms of the requirement for a putative stem-plus-arms T structure that may form due to the presence of an internal palindromic sequence (Fig. 8) (4). In the case of the ΔB (97 bp) and Bi (47 bp) deletions, such a stem-plus-arms T structure cannot form because of the removal of this internal palindromic sequence, although neither of these mutations would be expected to interfere with the formation of a terminal hairpin (4, 13). The phenotype of the ΔK mutant, which has a 9-bp deletion centered around the hairpin axis of symmetry, might also be explained on this basis if this mutation reduces the stability of the postulated stem-plus-arms T structure due to the loss of several GC base pairs (Fig. 8). Consistent with this interpretation, removal of an 11-base symmetrical sequence that is part of an internal palindrome in one of the cross arms of the T structure of the AAV terminal palindrome inhibited AAV DNA replication, and substitution of symmetrical sequences unrelated to the original sequence corrected the defect and allowed the propagation of the mutant sequences (12, 43). The secondary structure of the AAV inverted terminal repeat, rather than a specific sequence, was thus shown to be critical for its functional integrity. In the case of AAV, the T structure of the inverted terminal repeat is specifically recognized by nuclear proteins in extracts of infected cells (3, 40) and the AAV Rep78 and -68 proteins bind specifically to the hairpin configuration of the AAV termini (40). DNase I protection and methylation interference assays indicate that the AAV Rep proteins interact with most of the sequences that make up the palindrome (3, 40). By analogy with AAV, the palindrome mutations described here may exert their effect by preventing the binding of the MVM NS-1 protein to the hairpin terminus and thus interfere with subsequent cleavage and hairpin transfer involved in resolution of the turnaround configuration of the palindromic terminal sequence that has been postulated in parvovirus DNA replication models (4, 5, 17, 18, 70) and recently demonstrated in vitro for AAV (41, 67).

The 5′-terminal pCA3.0dl19 deletion of 82 bp of the hairpin stem reduced viral DNA replication just less than 100-fold in A9 cells. Presumably, sequences complementary to the deleted segment present in the intact half of the terminal palindrome facilitated the regeneration of the entire 5′-terminal palindrome by replication, thus permitting viral DNA replication to occur at a relatively low level. This deletion would be expected to prevent the formation of a putative stem-plus-arms T structure (Fig. 8), and perhaps this accounts for the large reduction in replication efficiency of this mutant. Another smaller hairpin stem deletant, pCA3.0dl16, which is missing only 46 nt of the hairpin stem, replicates at approximately one-third the level of wild-type pCA3.0 (data not shown). Furthermore, restriction endonuclease analysis of the replicated mRF DNA indicated that the 5′ hairpin was indistinguishable in size from that of wild-type DNA,thus supporting the notion of a repair mechanism. Moreover, the 46-nt deletion would affect neither the putative T-structure configuration nor the ability of the hairpin to prime DNA synthesis, nor would repair using the complementary template be affected. In fact, this result is consistent with AAV studies that used mutants with similar deletions (61, 64). The more extensive terminal deletions of 101 and 126 bp could not be repaired utilizing this mechanism, and therefore the Rep$^-$ phenotype of these mutants is likely due to the failure to form a terminal hairpin necessary to provide a primer for the initiation of DNA synthesis. Comparable results have been obtained for similar terminal deletions in the genomes of LuIII (57) and bovine parvovirus (65). Taken together with the results obtained here with the internal palindrome deletions, it appears that a self-complementary sequence that can fold back into a stable hairpin as well as an internal palindromic sequence involved in the formation of a putative T structure are both necessary to maintain the functional integrity of the 5′-terminal palindrome of the MVM genome. Thus, while the genetic studies with AAV and MVM point to the existence of a stem-plus-arms T structure with an important role in the function of the parvovirus DNA replication origin, it is important to note that physical evidence for such a structure has yet to be obtained.

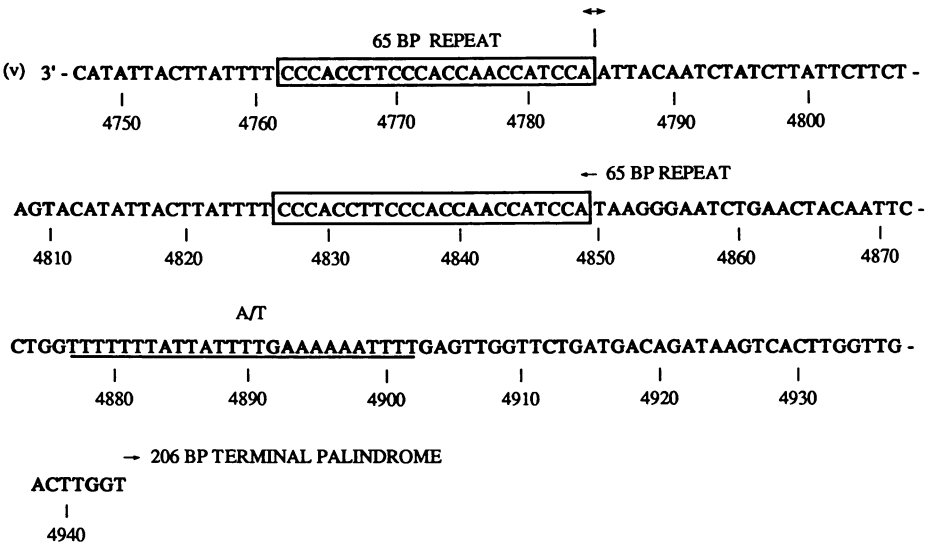

FIG. 9. Nucleotide sequence in the vicinity of the 65-bp deletion mutation in pCA3.0dl12. Depicted is the tandem 65-bp repeat in relation to the start of the 5'-terminal palindrome and to a neighbouring A+T-rich region (underlined). The CC(C/A) repeats that could act as high-affinity binding sites for DNA primase are boxed (see text). The boxed regions also contain potential transcriptional enhancer elements (6). Two 11-nt sequence elements each containing an 8-base match to the autonomously replicating core consensus-like sequence (see text) are located immediately to the 3' side each of the boxed regions. Nucleotides are numbered according to Astell et al. (6).

The results obtained with the 65-bp deletion mutant pCA3.0dl12 in A9 cells are in agreement with previous studies of the immunosuppressive variant MVM(i) in which a reciprocal host range determinant of MVM(i) and MVM(p) was mapped to a region within the capsid gene and to a region at the p38 promoter (2, 31). Like the pCA3.0dl12 mutant described here, MVM(i) has a single copy of the 65-bp DNA segment (6, 60). However, unlike MVM(i), which fails to grow in A9 cells (31), pCA3.0dl12 replicates in A9 cells, albeit at a reduced efficiency compared with wild-type MVM(p) DNA. The occurrence of only one copy of the 65-bp repeat sequence in its genome therefore cannot account for the failure of MVM(i) to replicate in A9 cells, in agreement with the conclusion that was reached in the aforementioned definitive mapping studies of the host range determinant in MVM(i). Previous studies of MVM(i) also showed that chimeric viral genomes of MVM(p) that contained MVM(i) sequences from map units 84 to 100, and therefore included only one copy of the 65-bp repeat, replicated approximately 10 times less efficiently in A9 cells than wild-type MVM(p) as judged by plaque formation (31). This observation is in complete agreement with the 10-fold reduction in DNA replication efficiency of the pCA3.0dl12 deletion mutant that occurred in A9 cells in the present study. It would be of considerable interest to determine the effect on viral DNA replication of a deletion in the MVMp genome that encompassed both copies of the 65-nt element as compared with a 65-nt deletant of MVM(i).

The results obtained in this study also clearly demonstrate that the 65-bp deletion mutation in pCA3.0dl12 affects MVM DNA replication in *cis*. Although MVM mRNAs are polyadenylated at nt 4885, just downstream of the 65-bp repeat (20), failure to polyadenylate viral mRNA cannot explain the *cis*-acting nature of the deletion mutation in pCA3.0dl12. We therefore conclude that at least a portion of the 65-bp tandemly repeated sequence that lies between 94 and 159 nt inboard of the start of the 5' palindromic region serves as the MVM DNA replication origin.

The *cis*-acting nature of the 65-bp deletion mutation suggests that this deletion affects the binding of an as yet unidentified factor(s) to the 65-bp repeat element. Since wild-type MVM DNA, transfected as pMM984, is excised and replicated at comparable levels in A9 and COS-7 cells, both cell lines appear to be capable of providing all necessary host cell permissive factors for MVM DNA replication and of producing adequate quantities of functional NS-1. The >10-fold difference in replication efficiency of pCA3.0dl12 seen between A9 and COS-7 cells is therefore directly related to the removal of one copy of the 65-bp repeat and suggests that a host cell factor rather than NS-1 binds to the 65-bp repeat element. The differential effect of the 65-bp deletion mutation on viral DNA replication in COS-7 and A9 cells could therefore be most readily explained if the murine host cell factor(s) has a higher affinity for the 65-bp repeat than the analogous factor(s) from monkey cells. The question as to whether the 65-bp repeat encompasses a minimal core DNA replication origin where viral DNA synthesis is initiated or whether these sequences represent auxiliary elements that stimulate the initiation of DNA replication, as occurs in papovaviruses (26, 44, 52, 53), remains to be resolved. One possibility is that multiple CC(C/A) repeats within the 65-bp repeat element (Fig. 9) serve as high-affinity binding sites for DNA primase, as discussed in detail elsewhere (23, 30). One problem with this interpretation of our results is that a role for DNA primase is not generally included in current models of parvovirus DNA replication in which DNA synthesis is postulated to initiate exclusively by using DNA primers. However, a role for DNA primase is compatible with the parvovirus DNA replication model of Rhode in which initiation of viral DNA replication occurs internally by RNA priming within or near the 5'-terminal palindrome of the viral genome and is followed by hairpin-primed DNA synthesis at a later stage in the replication mechanism (59). That the DNA polymerase α-primase complex is modified in MVM-infected cells supports the view that DNA primase is involved in MVM DNA

replication (39). Furthermore, the ability of plasmids with partial deletions of the AAV inverted terminal repeat to replicate as circular molecules (12) suggests that there may also be an internal DNA replication origin within the inverted terminal repeat in the AAV genome. It is worthwhile to note that a region analogous to the MVM 65-bp repeat is reiterated up to nine times in defective interfering viruses of H-1 parvovirus (29) and that such tandem reiterations of the replication origin have also been described for defective evolutionary variants of papovaviruses (42, 46). It is also of interest that a 26-bp, A+T-rich segment is present 28 nucleotides downstream of the 65-bp repeat in the MVM genome (Fig. 9). In the SV40 DNA replication origin, such an A+T tract directs DNA bending and coordinately influences DNA replication most likely by facilitating DNA strand separation (24, 37). Furthermore, the sequence 5'-TTTTATTCATT-3' present in the 65-bp repeat element (Fig. 9) contains an 8-base match to the 11-base core consensus sequence 5'-(A/T)TTTAT(A/G)TTT(A/T)-3' found in the yeast autonomously replicating sequence elements (14, 74). While all of these facts are consistent with the existence of an internal DNA replication origin in the MVM genome, proof for such a mechanism awaits the isolation and characterization of Okazaki fragments that are initiated in the 65-bp repeat region of the MVM genome. It should also be noted that, although an SV40 transcriptional enhancer core motif has been identified in the MVM 65-bp repeat element (6), attempts to demonstrate transcriptional enhancer activity in an analogous region of the H-1 parvovirus genome, which has a tandem repeat of 55 bp, have not been successful (58). Nevertheless, a dual role for the MVM 65-bp element in transcriptional activation and DNA replication would make this element comparable in function to enhancer elements in papovavirus genomes which are known to affect both gene expression and viral DNA replication (35, 52).

ACKNOWLEDGMENTS

These studies were supported by grants from the MRC to E. A. Faust and C. R. Astell and from the BCHCRF to C. R. Astell. Ralph Salvino is the recipient of an Ontario graduate studentship. Patrick Tam is the recipient of an MRC studentship, and Rosemary O. Shade was a postdoctoral fellow of the BCHCRF and Killam Foundation.

We thank Lora Harris for technical support.

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