CIS-ACTING SEQUENCES FOUND WITHIN THE MVM GENOME REQUIRED FOR DNA REPLICATION

by

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B.Sc. (Hon), University of British Columbia, 1988

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry and Molecular Biology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1994

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Date Oct.5, 1994

Abstract

Minute virus of mice (MVM) belongs to the Parvovirus genera of the Parvoviridae family of eukaryotic viruses. Its genome consists of approximately 5 kb of primarily single stranded negative sense DNA. Since the primary sequences at the genomic termini are palindromic, duplex hairpin structures are found at the termini. It is clear that parvoviral terminal hairpins are important for viral DNA replication.

In order to determine the sequence requirements for MVM DNA replication, an *in vivo* DNA replication assay was developed. In this assay, two plasmids were required. First, the plasmid vector, pPTLR, was constructed such that it encoded a minigenome of MVM containing 411 nt and 807 nt of the left and right termini, respectively. This minigenome was designed to contain only the terminal sequences since the analysis of DNAs found within defective interfering (DI) particles suggested that the *cis*-acting sequences required for viral DNA synthesis are found at or near the termini (Faust and Ward, 1979). When pPTLR was co-transfected into mouse LA9 cells or COS-7 cells with a second plasmid which expresses MVM NS-1, the major viral non-structural protein, the viral minigenome was rescued from the plasmid sequences and replicated in the host cell. The replicated DNA exhibited heterogeneous termini suggesting that both terminal hairpins were functional during viral DNA replication.

Deletion analysis of the pPTLR minigenome suggested that in addition to previous studies which partially defined the requirements of the right end palindrome, two additional regions of the MVM genome are important for viral

DNA replication. First, analysis of the left terminal hairpin suggested that an element(s) between MVM nucleotide position 11 and 25 (or 31) is important.. Although the nature of the left terminal hairpin allows minor deletion mutants to regenerate wild type terminal hairpins, it is hypothesized that the bubble sequence which, results from nucleotide positions 25-26 being mispaired with nucleotide positions 91-89 in the duplex hairpin, is altered during DNA replication. The potential effect of this change in the DNA template on viral DNA replication is discussed. Second, deletion analysis of the region internal of the right hairpin suggested that two adjacent elements, A (nucleotide positions 4489-4636) and B (nucleotide positions 4636-4695) are important for minigenome DNA replication. Two Rsa I restriction fragments which partially span elements A and B, Rsa A (nucleotide positions 4431-4579) and Rsa B (nucleotide positions 4579-4662), were used to probe nuclear extracts for sequence specific DNA binding proteins in electrophoretic mobility shift assays. A number of DNA-protein interactions were discovered. The binding site of one relatively abundant cellular factor, MVM DNA replication factor B5 or MRF B5, was determined. MRF B5 protected two regions of the Rsa B probe, site I (~nucleotide positions 4589-4610) and site II (~nucleotide positions 4616-4646), in DNase I footprinting assays. Although the function of MRF B5 in viral DNA replication is unknown, it is speculated that it might activate DNA replication at the right terminus.

Analysis of the replication of identical end minigenome mutants showed that viral DNA can also be replicated using either two left termini (LL) or two right termini (RR). Replication of the RR minigenome was observed to be substantially greater that of the LL minigenome. However, it was demonstrated that the Xba I (nucleotide position 4342)/Sau3a (nucleotide position 4741) fragment, which contained elements A and B, could stimulate DNA replication of LL type

minigenomes. These data confirm the finding that sequences at both termini encode origins of DNA replication.

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Abbreviations

AAV	adeno-associated virus
ACS	ARS consensus sequence
Ad2	adenovirus type 2
ARS	autonomously replicating sequence
ATP	adenosine 5'-triphosphate
BPV-1	bovine papillomavirus type 1
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CMV	cytomegalovirus
cpm	counts per minute
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DEAE	diethylaminaoethyl
dGTP	deoxyguanosine 5'-triphosphate
DI	defective interfering particles
dLL	dimer LL
dLR	dimer LR
dRF	dimer replicative form
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dRR	dimer RR
DS	dyad of symmetry element

DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
DUE	DNA unwinding elements
E.coli	*Escherichia coli*
EBNA-1	Epstein-Barr nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N', tetraacetic acid
EP	early palindrome
EtBr	ethidium bromide
exo III	exonuclease III
FR	family of repeats
HEPES	N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid
HSV-1	herpes simplex virus type 1
ILE	internal left end
IP	input plasmid
IRE	internal right end
kb	kilo base(s)
kbp	kilo base pair(s)
kDa	kilo dalton(s)
min	minute(s)
mLL	monomer LL
mLR	monomer LR
MRF	MVM(p) replication factor
mRF	monomer replicative form
mRR	monomer RR

MVM(i)	minute virus of mice, immunosupressive strain
MVM(p)	minute virus of mice, prototypical strain
NS	non-structural protein
nt	nucleotide(s)
OD	optical density
PBS	phosphate buffered saline
PCV	packed cell volume(s)
PMS	plasmid maintenance sequence
pol	polymerase
RF	replication factor
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RV	rat virus-like
SDS	sodium dodecylsulphate
SSC	standard saline citrate
SSPE	standard saline phosphate EDTA
SV40	simian virus 40
T-ag	SV40 large tumour antigen
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
Tris	tris(hydroxymethylamino)methane
tRNA	transfer RNA
U	unit(s)
UV	ultraviolet
V	volts

VP	viral protein

Acknowledgements

First, I wish to thank Dr. Caroline Astell for the opportunity to work in her laboratory. I also thank Dr. Michael Smith and Dr. Frank Tufaro for being on my supervisory commitee. The research presented in this thesis would not have been possible without the constant encouragement of Amy, Jan, and the other current members and ex-members of this lab. Special thanks go to Wesley and Brendan of the Sadowski lab for technical advice and late night talks. Last but not least, I wish to thank my parents for putting up with all the nonsense throughout the years.

I Introduction

Studies on the initiation and regulation of DNA replication in mammalian cells have been generally unyielding because of a lack of useful genetic tools and approaches. However, the study of small defined genomes of some mammalian DNA viruses has allow detailed genetic and biochemical dissection of essential cellular processes such as DNA replication and gene expression, albeit in the context of viral function. Well studied viruses such as simian virus 40 (SV40) or adenovirus have served as relatively simple model systems for understanding such cellular processes. Initially, the parvovirus minute virus of mice (MVM) was thought to be another simple model system to study mechanisms of DNA replication. Although the mechanism of MVM DNA replication is still not fully understood, what is currently known shows that MVM requires complex reactions at or near the sequences encoding the hairpin termini. Understanding the molecular mechanisms of MVM DNA replication may contribute to a fuller understanding of parvovirus replication as well as mammalian DNA replication. A clear account of DNA replication and its regulation is essential to understanding the growth and development of organisms. In addition, this understanding may also contribute to the comprehension of disease states which arise from aberrant growth and replication of cells.

1.1. Review of parvoviruses

1.1.1. General Characteristics

Parvoviruses form a large family of physically similar viruses which infect both invertebrate (insects) and vertebrate (mammals) animal species (Berns, 1990; Tijssen, 1990). A typical parvovirus virion is composed of 4.6 to 6.0 kb of single stranded DNA encapsidated in an icosahedral coat 20-25 nm in diameter. The genomic DNAs of all parvoviruses analyzed to date have terminal palindromes which are able to form hairpin structures (Astell, 1990). The hairpin sequences of several parvoviruses (MVM, AAV) are essential for viral replication.

1.1.2. Classification of parvoviruses

Historically, parvoviruses have been divided into three genera: Parvovirus, Densovirus and Dependovirus (Tijssen, 1990) (Fig. 1). This classification system is based on two criteria: requirement of a helper virus for viral replication and the host species which is infected. Autonomous parvoviruses are vertebrate viruses which are able to replicate in the absence of a helper virus (Cotmore and Tattersall, 1987). Originally, it was thought that autonomous parvoviruses have non-identical terminal hairpins and package only minus sense DNA. It has since been shown that some autonomous parvovirus contain identical terminal palindrome and package both plus and minus sense DNA. Dependoviruses, (also called the adeno-associated viruses or AAV) (Berns and Bohenzky, 1986), are vertebrate viruses which were originally thought to

Parvoviridae Family

A. Genus Parvovirus

Kilham rat virus (KRV)
Minute virus of mice (MVM)
LuIII
H-1
Feline parvovirus (FPV)
Canine parvovirus (CPV)

RV-like

B-19
Simian parvovirus (SPV)
Bovine parvovirus (BPV)
Aleution disease virus (ADV)

B. Genus Dependovirus

Adeno-associated virus (AAV)

C. Genus Densoviruses

Galleria DNV
Bombyx DNV

Fig. 1. Parvoviridae Family.

The family Parvoviridae comprises of three genera. Within the Parvovirus genus, MVM is classified in the RV-like subgroup whose members have similar capsid structure characteristics. The Dependovirus genus consists of adeno-associated viruses (AAVs) which is a group of serotypically distinct viruses. Most molecular studies of AAVs have been carried out on the human AAV-2 serotype. The Densovirus genus includes a number of viruses which infect insects.

require a helper virus for productive viral replication. It has since been shown that AAV is able to replicate in cell lines in the absence of a helper virus. AAV genomes typically contain either plus and minus sense DNA with identical terminal palindromes. The Densoviruses are parvoviruses which infect insect species (Tijssen *et al.*, 1990). Although the molecular characterization of Densovirus genomes has lagged behind that of the Parvovirus and Dependovirus genera, the DNA sequences of several Densoviruses have now been published. Interestingly, this genus contain viruses which use both strands to code protein sequences. In contrast, all vertebrate parvoviruses contain one coding strand. The Densovirus genomes have been shown to contain plus and minus sense DNAwith identical terminal palindromes and (Bando *et al.*, 1990).

1.2. Molecular Biology of MVM

1.2.1. Historical Aspects

MVM was first isolated as a contaminant virus in mouse adenovirus stocks (Crawford, 1966). The contaminant virus was separable from adenovirus and polyomavirus by CsCl density equilibrium centrifugation. Material from a relatively dense band on CsCl gradients (1.43g/ml) was found to contain hemagglutinating activity. Furthermore, mouse embryo cultures infected with the newly discovered virus also produced hemagglutinating activity. MVM was eventually plaque purified and distributed to other laboratories interested in the molecular biology of MVM (Tattersall, 1972). Thus, this original plaque purified strain was designated the prototypical strain of MVM, MVM(p). An immunosupressive strain of MVM designated MVM(i) has also been isolated and sequenced (Sahli *et al.*, 1985; Astell *et al.*, 1986).

1.2.2. Genomic Organization of MVM(p)

MVM(p) has been cloned into plasmid vectors and the complete nucleotide sequence determined in part from cloned fragments and viral DNA (Astell *et al.*, 1979; Astell *et al.*, 1983; Merchlinsky *et al.*, 1983). By convention, the nucleotide sequence of MVM(p) is numbered beginning at the first nucleotide of the 5' terminal of the positive sense strand or complementary strand DNA. The 3' terminal hairpin and 5' terminus hairpin of the viral DNA are also designated as the left and right hairpin, respectively, and are used interchangeably in this thesis.

1.2.3. Viral Transcripts

All vertebrate parvoviruses studied to date appear to have their entire protein coding sequences found on one DNA strand, the plus strand. MVM(p) contains two overlapping transcription units driven by promoters at map unit 4 (P4) and 38 (P38) (Fig.2)(Cotmore and Tattersall, 1987, Pintel *et al.*, 1983). Three major size classes of spliced and polyadenylated cytoplasmic transcripts designated R1 (4.8 kb), R2 (3.3 kb) and R3 (3.0 kb) have been identified (Pintel *et al.*, 1983). All three classes of transcripts are polyadenylated at a single site found near the right terminal palindrome (Clemens and Pintel, 1987). In addition, sequence analysis of cDNAs demonstrated that one of three possible small introns, involving two potential splice donor and splice acceptor sites between map units 46 and 48, is removed in all mature transcripts (Jongeneel *et al.*, 1986;

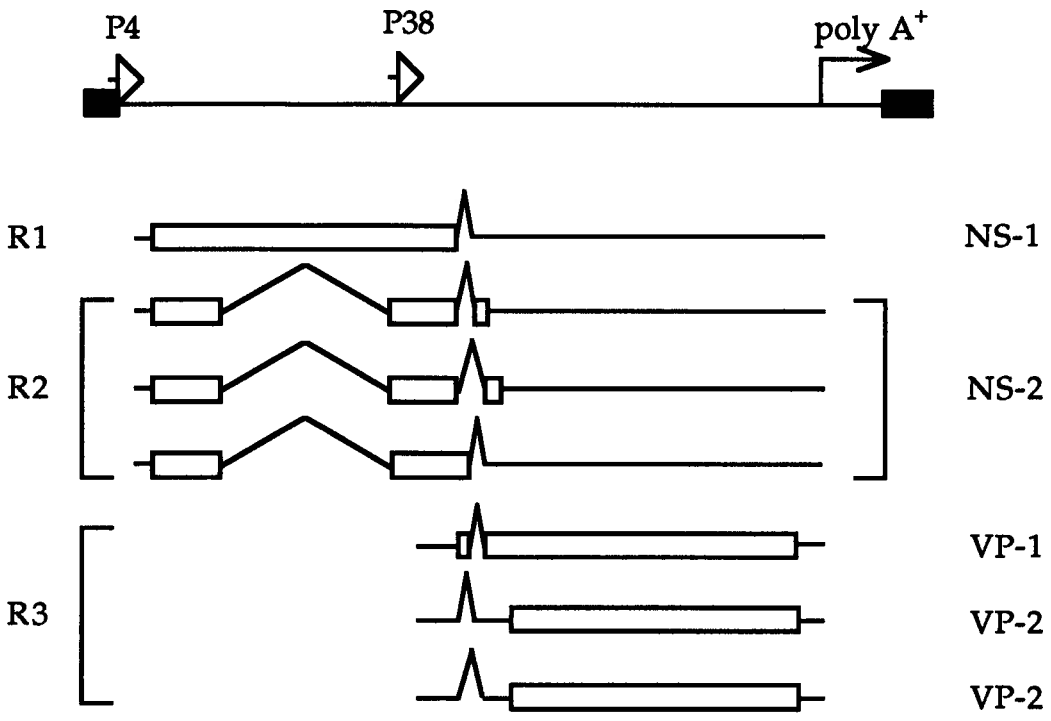


Fig. 2. MVM(p) Transcription Map.

The top schematic shows the MVM(p) genome containing the left (3') and right (5') hairpins sequences (black boxes), the transcriptional promoters located at map unit 4 and 38 (P4 and P38) and the polyadenylation signal (poly A^+) located at nucleotide position 4885. The transcript map below shows the three classes of transcripts R1, R2 and R3. Open boxes and thin horizontal lines represent the protein coding portion and the non-coding portion of the transcript. All transcripts have one of three possible introns removed due to the utilization of two splice donor sites (nt 2280 and 2316) and two acceptor sites (nt 2399 and 2377). In addition, mature R2 transcripts also have a large intron removed between nt 514 and 1990. The protein synthesized from each transcript is indicated on the right.

Morgan and Ward, 1986). Synthesis of transcripts coding for NS-1 (R1) and NS-2 (R2) is directed by P4 and starts approximately at nucleotide 201 (Ben-Asher and Aloni, 1984). In addition to the removal of the small intron, R2 transcripts also have a large intron removed between map units 10 (nt 514) and 40 (nt 1990) (Jongeneel *et al.*, 1986). Thus the N-terminal 84 amino acid residues of NS-1 and NS-2 are identical (Fig. 2). Three possible C-terminal tails of NS-2 are generated by the multiple splicing events removing the small intron. These splicing events do not affect the NS-1 protein since the termination ochre codon (TAA) at nucleotide 2277 occurs before the two potential splice donor sites at nt 2280 and 2316. Synthesis of the R3 transcripts is directed by P38 and starts at approximately nucleotide 2005 (Ben-Asher and Aloni, 1984). Again the multiple splicing events between map units 46 and 48 result in R3 transcripts which code for two possible translation products, VP-1 and VP-2. The two R3 transcripts utilizing the splice donor at nt 2280 code for the smaller VP-2 protein. R3 transcripts utilize the downstream splice donor at nt 2316 code for the larger VP-1 protein. This splicing pattern allows the translational start codon at nt 2286 to be used for the initiation of the VP-1 protein. Utilization of alternative splicing is thought to promote the correct molar amounts of VP-1 and VP-2 (approximately one to five) observed in infected cells and virions (Tattersall *et al.*, 1976; Cotmore, 1990).

1.2.4. MVM(p) Polypeptides

In vitro translation of transcripts isolated from MVM(p) infected cells shows four major viral encoded primary translation products are synthesized (Cotmore *et al.*, 1983; Cotmore and Tattersall, 1986b). These products were detected using antisera raised against bacterial fusion proteins containing portions of the NS-1 and NS-2 ORFs or sera from animals infected with different vertebrate parvoviruses. A

third viral capsid protein, VP-3, thought to be a proteolytic cleavage product of VP-2, probably arises during virus particle maturation (Tattersall *et al.*, 1976).

Sequence analysis of the large ORF in the R1 transcript predicts that the large non-structural protein of MVM(p), NS-1, is 672 amino acids in length with a molecular weight of approximately 77 kDa (Astell *et al.*, 1983, Cotmore *et al.*, 1983). It has been shown that NS-1 is a nuclear phosphoprotein which migrates as an 83 kDa polypeptide in SDS-polyacrylamide gels (Cotmore *et al.*, 1983; Cotmore and Tattersall, 1986a). The NS-1 proteins from parvoviruses from the RV-like group show a high degree of antigenic conservation since sera from animals infected with any of the RV viruses recognize MVM(p) NS-1 (Cotmore and Tattersall, 1987). Further comparison shows that a 405 nucleotide sequence (nt 1428-1833) found within the MVM(p) NS-1 ORF, encodes a 135 amino acid sequence which shares a significant degree of sequence homology with the other parvoviral large non-structural proteins as well as SV40 and polyomavirus large T-antigens and the E1 protein of papillomaviruses (Astell *et al.*, 1987). Within this conserved 135 amino acid domain, a conserved purine nucleotide binding motif was identified suggesting that NS-1 requires the binding and subsequent hydrolysis of ATP for its function. It has subsequently been shown that purified recombinant NS-1 from the baculovirus expression system contains ATP binding, ATPase and ATP-dependent DNA helicase activities (Wilson *et al.*, 1991). Point mutations within the purine nucleotide binding domain abrogated the ability of mutant NS-1 to replicate MVM(p) minigenomes *in vivo*, and severely reduced the ATP-dependent DNA helicase activity in purified mutant protein preparations (Jindal *et al.*, 1994). In another study, mutation of the lys 405 residue within the purine nucleotide binding domain also eliminated the ability of mutant NS-1 (in nuclear extracts) to replicate cloned MVM(p) palindromic junction fragments *in vitro* (Nuesch *et al.*, 1992).

Interestingly, all mutant proteins were localized in the nucleus and were able to transactivate the p38 promoter (except for mutations at lys 405) (Nuesch *et al.*, 1992; Jindal *et al.*, 1994).

In addition to the replication function(s) of NS-1, it has also been demonstrated that NS-1 from MVM(p) (Doerig *et al.*, 1988) as well as H-1 (Rhode, 1985; Rhode, 1987; Rhode and Richard, 1987) are able to transactivate the P38 promoter. Also, the NS-1 of B-19 (Doerig *et al.*, 1990) and Rep gene products from AAV (Labow *et al.*, 1986; Trempe and Carter, 1988) are able to regulate their own promoters. Although genetic analysis of MVM NS-1 has shown that it can transactivate P38, there is no evidence that the protein is able to bind to DNA *in vitro*. At this point it is not known if NS-1 requires a cognate DNA binding protein(s) to localize itself near the promoter for transcriptional activation. However, the activation domain of NS-1 has been mapped to the C-terminal 129 residues by assaying the ability of various Gal4-NS-1 fusion proteins to transactivate a Gal4-CAT construct (Harris and Astell, 1994).

Relatively little is known about the activities and functions of the smaller NS-2 proteins (25 kDa). It has been shown that NS-2 is required for efficient DNA replication and virus production in certain cell types (Naeger *et al.*, 1990; Naeger *et al.*, 1993). In addition, NS-2 is required for viral replication and pathogenesis in infected mice since mice infected with a mutant MVM(i) deficient in NS-2 synthesis caused an asymptomatic infection whereas wild type MVM(i) causes a lethal infection (Brownstein *et al.*, 1992). NS-2 has been shown to be phosphorylated and localized to both the cytoplasmic and nuclear compartment of infected cells (Cotmore and Tattersall, 1990). It appears that heavily phosphorylated forms of NS-2 are found primarily in the cytoplasm whereas non-phosphorylated forms of

NS-2 were shown to be found in the nucleus as well as the cytoplasm (Cotmore and Tattersall, 1990). Indirect immunofluorescence experiments and pulse chase experiments show that NS-2 molecules appear to be rapidly degraded compared to NS-1. Since all three forms of the NS-2 protein appear to share the same degradation, phosphorylation and localization patterns (Cotmore and Tattersall, 1990), the significance of the three alternative C-terminal tails of NS-2 remains to be determined.

Differential splicing of the R3 class of transcripts results in two transcripts (Fig. 2) encoding VP-2 (64 kDa) and one transcript encoding VP-1 (83 kDa) (Labieniec-Pintel and Pintel, 1986). Although the entire protein sequence of VP-2 is found within the sequence of VP-1 (Tattersall *et al.*, 1977), the larger VP-1 protein contains an additional N-terminal region which contains a significant number of basic residues. It is thought that this basic N-terminal domain may interact with the ss genomic DNA inside the capsid (Tsao *et al.*, 1991). A third viral coat protein, VP-3 (62 kDa), is derived by a proteolytic cleavage of a short N-terminal peptide of VP-2 (Tattersall *et al.*, 1976). Although the nature of the proteolytic cleavage has not been characterized, it has been shown that purified empty capsids contain only VP-1 and VP-2, but preparations of full infectious virus contains at least a small portion of VP-3 (Tattersall *et al.*, 1976; Santaren *et al.*, 1993). Pulse chase experiments also show that the proportion of VP-3 in infected cells increases during the infection progression. These data suggest the VP-2 to VP-3 conversion takes place during virion maturation or internalization (Tattersall *et al.*, 1976; Santaren *et al.*, 1993). The significance of this conversion is not known since full virions appear to be infectious irrespective of the VP-2/VP-3 ratios. In addition, all viral structural proteins have been shown to be phosphorylated (Santaren *et al.*, 1993). It is thought

that phosphorylation of the capsid proteins may contribute to the morphogenesis of the virion.

1.2.5. Structure of MVM Terminal Hairpins.

The genomic DNA of MVM contains primarily minus sense single stranded DNA (Crawford *et al.*, 1969; Bourguignon *et al.*, 1976) with imperfect palindromic sequences which may form stable hairpin duplexes at the left and right termini (Bourguignon *et al.*, 1976). Imperfections in the palindromic sequence result in mispaired nucleotides in the hairpin structure (Fig. 3). Unlike AAV, which contain inverted terminal repeats (Lusby *et al.*, 1980), MVM and other RV-like viruses contain a unique primary sequence at each terminus (Astell, 1990). The terminal hairpins provide parvoviruses with a novel method of circumventing the problem that all linear DNA molecules have in replicating their 5' ends. This problem results from the fact that all DNA polymerases require a primer (3′ OH) for DNA synthesis. Cavalier-Smith proposed the hairpin transfer mechanism as a method by which linear DNA molecules containing terminal palindromes can replicate their 5' ends (Cavalier-Smith, 1974). Imperfections in the palindromic sequences which undergo hairpin transfer result in two sequence orientations termed the flip and flop forms (for example, compare Fig. 3C and 3D).

Sequence determination of the left hand hairpin of MVM(p) shows that it is 115 nucleotides in length and assumes a stem plus arms or Y shaped configuration (Fig. 3A) (Astell *et al.*, 1979). Mismatches between nucleotide

A

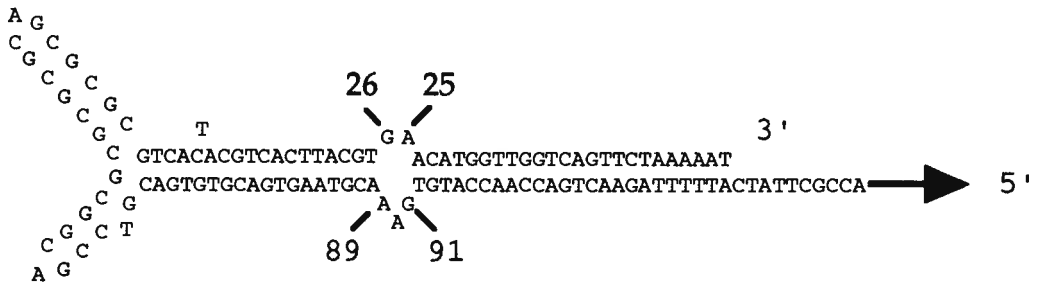


Fig. 3. Structure of the MVM(p) termini.

The nucleotide sequence of the viral 3' terminus is shown in the hairpin configuration (A). Only 104 out of 115 nt of this left hand (3') terminus are base paired in the hairpin configuration. The bubble structure found within the stem results through a mismatch between nucleotide position 25-26 to 89-91. The nucleotide sequence of the right hand (5') viral termini is shown in various configurations. B shows the perfect base pairing between nucleotide positions 4944-4993 to 5149-5100. This portion of the palindrome is in common to the other structures shown in C,D and E. The nucleotide sequence of the remaining DNA (position 4994-5099) in the flip (C) and flop (D) forms is shown in straight hairpins configuration. The flip and flop forms are inverted complement of each other. E shows the nucleotide sequence of the flip form of the hairpin in the stem plus arms structure. Both flip and flop forms may assume a stem plus arms structure. The nucleotide positions of the imperfections in the hairpin structures are indicated.

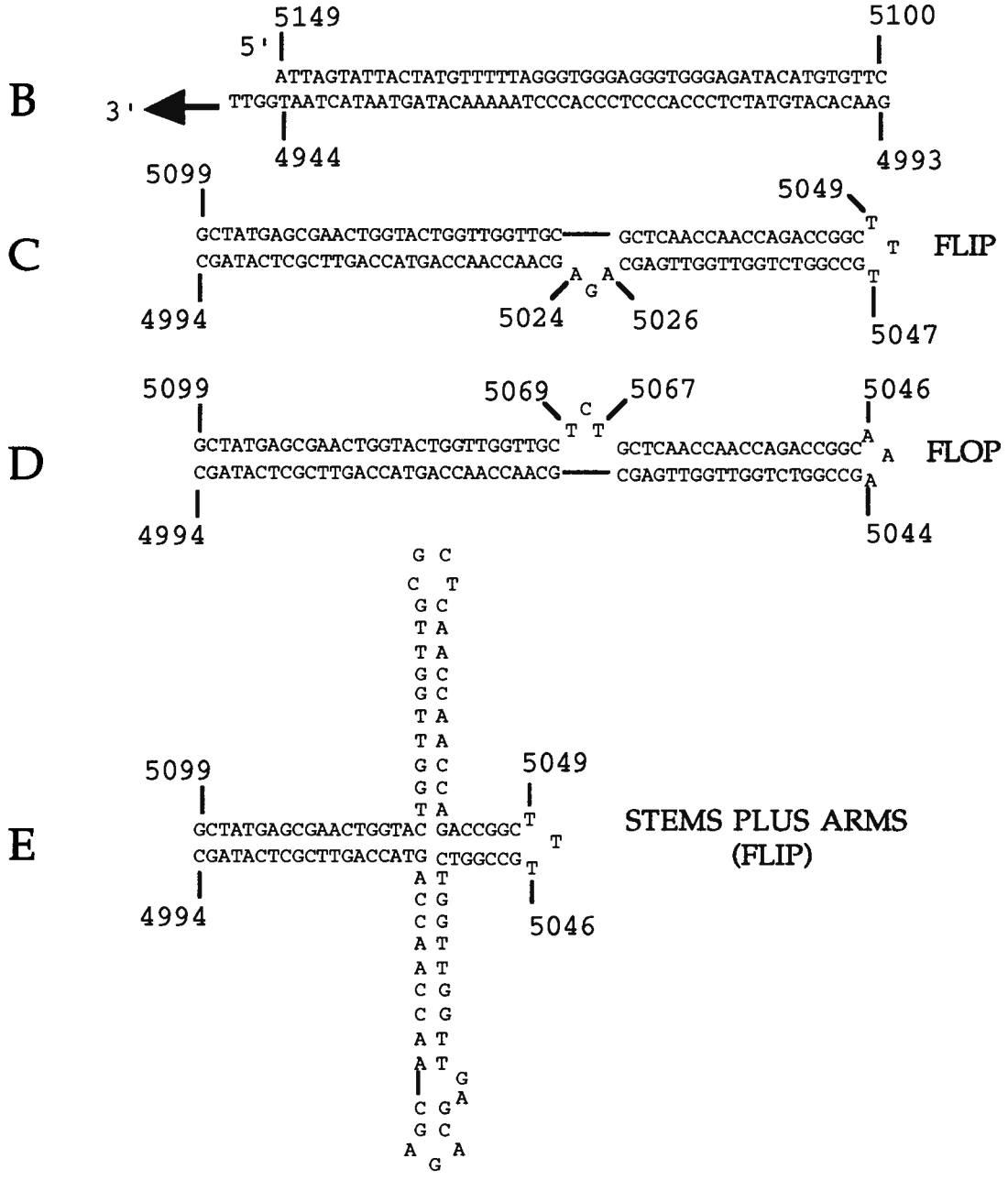

B
5149
5100
5'
ATTAGTATTACTATGTTTTTAGGGTGGGAGGGTGGGAGATACATGTGTTC
3'
TTGGTAATCATAATGATACAAAAATCCCACCCTCCCACCCTCTATGTACACAAG
4944
4993
C
5099
5049
GCTATGAGCGAACTGGTACTGGTTGGTTGC
GCTCAACCAACCAGACCGGC
FLIP
CGATACTCGCTTGACCATGACCAACCAACG
CGAGTTGGTTGGTCTGGCCG
4994
5024
5026
5047
D
5099
5069
5067
5046
GCTATGAGCGAACTGGTACTGGTTGGTTGC
GCTCAACCAACCAGACCGGC
FLOP
CGATACTCGCTTGACCATGACCAACCAACG
CGAGTTGGTTGGTCTGGCCG
4994
5044
E
5099
5049
GCTATGAGCGAACTGGTAC GACCGGC
STEMS PLUS ARMS
(FLIP)
CGATACTCGCTTGACCATG CTGGCCG
4994
5046

Fig. 3B-E

positions 25-26 and 89-91 in the stem predicts that a bubble structure is formed in the stem of the hairpin. Although the bubble structure and sequence is a conserved feature among some members of the RV-like viruses (H-1, KRV, ADV, LuIII) (Astell *et al.*, 1979), its significance if any is has not been determined. It has been predicted that the bubble may play a role in the asymmetrical resolution of dimer RF molecules (Astell *et al.*, 1983). Only one sequence orientation (flip) was found at this terminus in both the genomic DNA and intracellular replicative DNA forms (Astell *et al.*, 1983; Astell *et al.*, 1985). The right hand (5') hairpin was determined to be 206 nucleotides in length and exists in both the flip and flop sequence orientation (Fig.3C and D) (Astell *et al.*, 1983; Astell *et al.*, 1983). In addition to the extended hairpin conformation, the flip and flop forms of the right hand (5') palindrome can potentially form a stem plus arms structure (Fig.3E). Deletion mutants which eliminate the potential stem plus arms structure but not the formation of an extended hairpin were unable to replicate (Salvino *et al.*, 1991). Genetic analysis of AAV hairpins also suggest that the stem plus arms conformation of the terminal palindrome is important for DNA replication (Lefebrve *et al.*, 1984). In fact, all parvovirus sequences determined to date have terminal palindromes which can potentially form a stem plus arms configuration (Astell, 1990). Although the significance of this conserved structural feature is not fully understood, these data suggest that the stem plus arms structure is important for parvovirus DNA replication. Since the primary sequence of the terminal palindromes of unrelated parvovirus genomes are different, this suggests that the topology rather than the sequence of a stem plus arms palindrome may be important in the hairpin function.

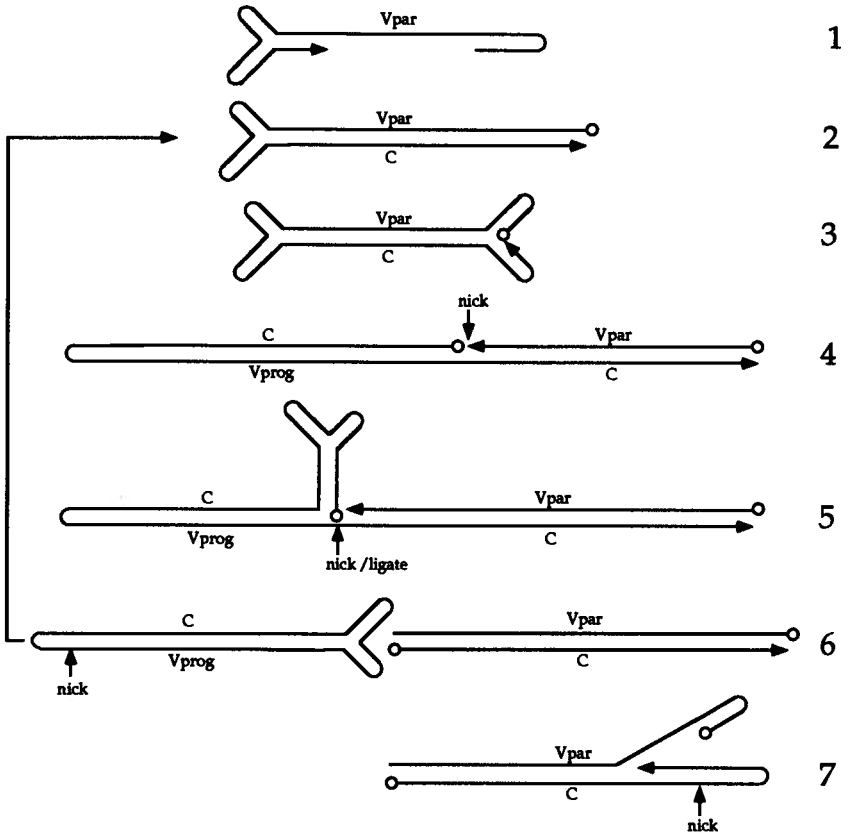


Fig. 4. Modified Rolling Hairpin Model of MVM DNA Replication.

The schematic diagram shows the Modified Rolling Hairpin Model of MVM DNA replication (*Astell et al.*, 1985). Vpar and Vprog denote the parental and progeny viral strands. The complementary strand (C) represents the plus strand. The arrows indicate the 3' hydroxyl groups which are used to prime DNA synthesis. The open circles represent NS-1 which is known to be covalently attached to the 5' ends of viral DNAs. Details of this model are discussed in the text.

1.2.6. Model of MVM DNA Replication

A modification of the Rolling Hairpin Model for MVM DNA replication (Tattersall and Ward, 1976), which took into account the observed DNA sequences found at the right and left end, has been proposed (Fig. 4) (Astell *et al.*, 1983; Astell *et al.*, 1985). In this model, the left hand (3') hairpin is used to prime DNA synthesis using the incoming single stranded DNA as the template (Fig. 4, Step 2). The resulting monomer replicative form (mRF) is then further amplified using the right hand (5') hairpin to prime DNA synthesis to produce the dimer replicative form (Fig. 4, step 2-4). This replicative intermediate contains essentially two genome equivalents arranged in a head to head arrangement with the left (3') terminal sequence joining the two genomes. The DNA joining the two monomer genomes is termed the 3' junction or 3' dimer bridge fragments. Since there is only one sequence orientation, flip, found at the genomic 3' hairpin and in intracellular replicative intermediates, the 3' junction fragment must be resolved in an asymmetric manner to generate only the flip sequence orientation at the 3' terminus (Fig.4, steps 4-6). The Modified Rolling Hairpin Model predicts that a site specific nickase recognize a specific site on the Vparental strand (Fig. 4, step 4). The 3' hydroxyl group provided by the nicking event primes DNA synthesis across the 3' junction fragment to a second nick introduced at the corresponding Vprogeny strand (Fig. 4, step 5). A ligation event which crosslinks the Vparental 3' hairpin with the Vprogeny strand producing a molecule which is covalently closed at both termini. The second mRF molecule generated contains extended hairpin forms at both termini. The two resulting mRF molecules are used as templates for further DNA amplification or for packaging genomic DNA. Synthesis of genomic single stranded DNA is primed at the 5' hairpin sequence displacing the genomic single

stranded DNA which is then packaged (Fig. 4, step 7). Multiple rounds of hairpin transfer at the 5' palindrome sequences followed by strand displacement generates ss genomic DNA (Fig. 4, step 7) containing equal proportions of flip and flop sequence configurations at the 5' terminus. In contrast, the specific manner in which the 3' junction fragments are resolved retains the unique flip sequence orientation at the 3' terminal. Site specific nicks introduced at the right hand palindrome sequence must be at least 18 nt inboard of the viral DNA sequence since intracellular DNA replicative forms are an additional 18 nt longer than the genomic viral DNA (Astell *et al.*, 1985). The finding that NS-1 is found covalently linked to the ss genomic DNA, outside MVM particles, with approximately 24 nt found outside of the particles (Cotmore and Tattersall, 1989b) confirms this hypothesis. It is thought the NS-1 and the external 5' nucleotides are not required for infection since removal of the genome linked NS-1 and the external ss DNA does not affect infectivity. Presumably, removal of the exposed ss DNA (and NS-1) *in vivo* accounts for the "extra" 18 nt found in intracellular replicative intermediates that is not found in ss genomic DNA.

Evidence which supports the model has been recently reported. Since NS-1 is found covalently bound to the 5' ends of intracellular replicative forms as well as the genomic single stranded DNA, it implies that NS-1 acts as the site specific nickase (Cotmore and Tattersall, 1988; Cotmore and Tattersall, 1989). Further evidence to support an asymmetrical resolution mechanism of the 3' junction fragment was obtained when recombinant forms of NS-1 were shown to specifically resolve the junction fragments *in vivo* and *in vitro* . First, it was shown that cloned 3' and 5' junction fragments of MVM(p) NS-1 were resolved only when plasmids encoding the junction fragments were transfected into mouse cells and co-infected with MVM(p) (Cotmore and Tattersall, 1992). Further experiments showed that

extracts containing recombinant NS-1 from the vaccinia or baculovirus expression systems were able to resolve cloned 3' junction fragments into the predicted covalently closed and extended hairpin structures in an *in vitro* replication system (Cotmore *et al.*, 1993; Liu *et al.*, 1993). In addition, the 5' junction fragment were also resolved into extended ends with NS-1 covalently attached to the 5' ends (Cotmore *et al.*, 1992; Cotmore and Tattersall, 1992). Although NS-1 has been shown to possess the activities (ATPase, helicase and nickase) thought to be required to direct the resolution of dimer replicative form (dRF) molecules, the precise details of sequence specific nicking and role of cellular proteins in the resolution reaction have not been determined fully.

1.2.7. AAV DNA Replication

Replication of genomes containing inverted terminal repeats, such as AAV, is thought to follow a similar, but not identical scheme to that of MVM (Fig.5). Since AAV genomic DNA contains single strands from both polarity (plus and minus) (Mayor *et al.*, 1969) and both flip and flop sequence orientation are found at the left hand (3') and right hand (5') terminus (Lusby *et al.*, 1980), it suggests that both termini utilize the hairpin transfer mechanism to replicate the genomic termini. Sequence inversion was further demonstrated when a plasmid containing the AAV genome with flip sequences at both ends generated AAV genomes containing approximately equal proportions of flip and flop at both ends (Samulski *et al.*, 1982). In order to demonstrate that AAV terminal hairpins undergo hairpin transfer, an *in vitro* replication system using extracts from

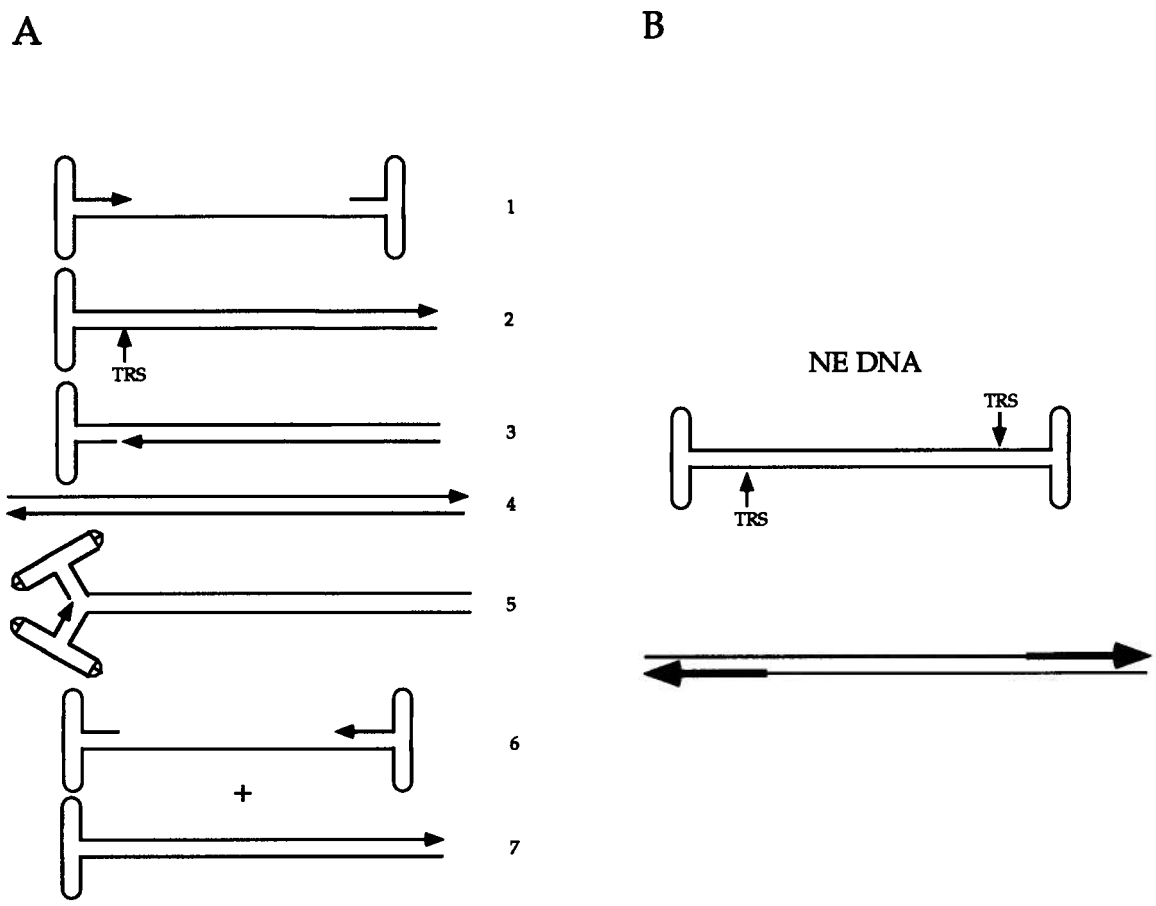


Fig. 5 Model of AAV DNA Replication

(A) AAV DNA replication uses the terminal hairpin primers (arrows) to initiate synthesis of the mRF (2). The covalently closed hairpin is then resolved in a series of reactions initiated by a site specific nick at the terminal resolution site (TRS). The newly created 3' OH then serves as a primer for synthesis of the palindrome. The fully extended terminus of the resulting intermediate can then be denatured to form terminal hairpin structures to facilitate DNA synthesis and strand displacement to generate genomic ss DNA and a duplex mRF. (B) The terminal resolution reaction shows synthetic no end (NE) DNA is resolved

AAV/adenovirus infected HeLa cell extracts were shown to resolve covalently closed AAV hairpin "no end" (NE) DNA (Fig.5A step 2-4 and 5B) (Snyder *et al.*, 1990). In addition, it was also shown that purified AAV Rep68, one of the AAV replication proteins, contain ATP-dependent site specific nickase, DNA helicase and terminal hairpin binding activities *in vitro*. (Im and Muzyczka, 1990; Snyder *et al.*, 1990). Like MVM(p) NS-1, Rep68 is found covalently attached to the 5' end of nicked hairpin DNA (Im and Muzyczka, 1990). Although it has not been demonstrated, it's likely that Rep proteins can also resolve AAV junction fragments.

1.3. Origins of DNA replication

The initiation of DNA replication has been hypothesized to depend on two determinants: a *cis*-acting element termed the origin or the replicator and a *trans*-acting factor termed the origin activator or initiator (Stillman, 1989). Identification of either determinant in mammalian cells has been hampered by a lack of useful genetic techniques. Yet origins of DNA replication and their initiator proteins have been identified in mammalian DNA tumor viruses such as SV40, bovine papillomavirus (BPV-1), herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV) and in the budding yeast, *Saccharomyces cerevisiae* (Kelly *et al.*, 1988; Challberg and Kelly, 1989; Marahrens and Stillman, 1992). For DNA tumor viruses such as SV40, adenovirus and BPV-1, the development of *in vitro* replication systems using cellular extracts or fractionated cellular extracts has allowed detailed biochemical studies of initiation and regulation of viral DNA replication (Kelly *et al.*, 1988; Stillman, 1989). Although in many cases, the origin and initiator protein have been identified, the mechanism of initiation have not been fully described. The most complete description of the initiation of DNA replication comes from the studies of SV40.

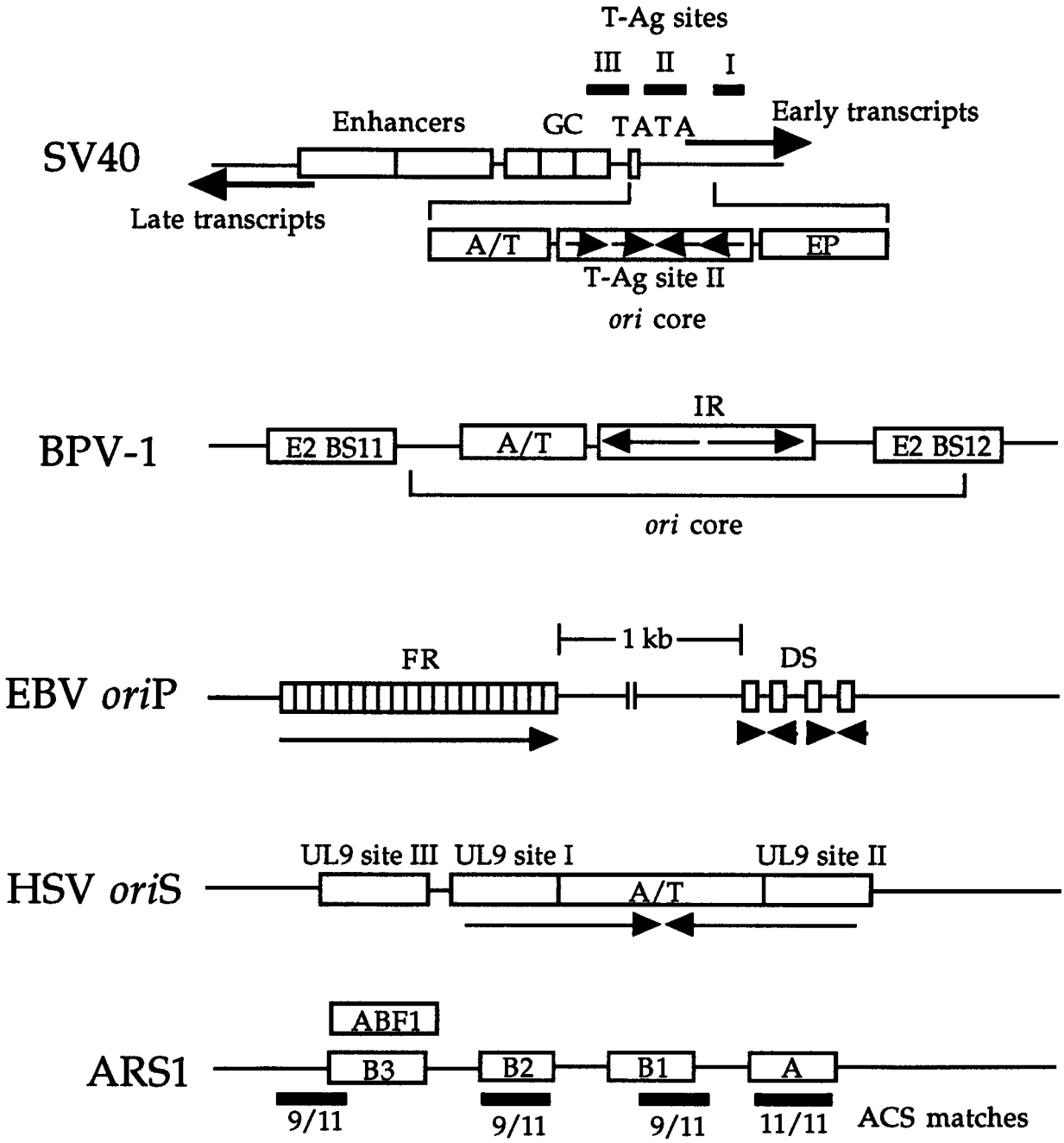
1.3.1. SV40 Origin of DNA replication

The interactions between SV40 and its host cell have been amenable to dissection by scientists partly because of its apparent simplicity. The viral chromosome is 5243 bp of duplex circular DNA containing one origin of DNA replication and two divergent transcriptional units coding for six proteins (Fanning and Knippers, 1992). The SV40 origin of DNA replication is embedded in the complex non-coding region of the SV40 genome located between the early and late transcriptional units (Fig. 6A)(Deb *et al.*, 1986; Dean *et al.*, 1987; Deb *et al.*, 1987). The arrangement of the transcriptional control and DNA replication elements in a central control region allows the virus to efficiently coordinate viral gene expression and replication of the viral genome in the host cell. The minimal sequence which is sufficient and necessary for the initiation of DNA replication *in vitro* is contained within a 64 bp region termed the *ori* core (Fig. 6A) (Deb *et al.*, 1986). Although the transcriptional control elements such as the enhancers and Sp1 binding sites are not required for DNA replication *in vitro*, maximal DNA replication *in vivo* requires the presence of these auxiliary replication elements (DeLucia *et al.*, 1986; Hertz and Mertz, 1986; DePamphillis, 1988). Only one viral encoded protein is required for the initiation of DNA replication at the *ori* core, the SV40 large T-antigen (T-Ag). This multifunctional protein plays several key roles in the viral life cycle: the coordinate control between transcription and DNA replication, the initiation of DNA replication at the *ori* core and the transformation of infected cells (Fanning and Knippers, 1992). This discussion will only encompass functions of T-Ag with respect to its role as the initiator protein at the *ori* core.

Fig. 6A Organization of Several Eukaryotic Origins of DNA Replication.

Eukaryotic origins of replication from DNA tumor viruses (SV40, BPV-1, EBV, HSV-1) and yeast (ARS1) contain multiple *cis*-acting elements. Each box represent functional elements found in the respective origin of replication and are discussed in detail in the text. In the case of SV40, both the control region and the core *ori* (exploded view) is shown. The core *ori* contains three elements, T-ag site II, which is composed of four binding sites (arrows) for T-ag arranged in a perfect 27 bp palindrome, an A/T rich element and an early palindrome (EP). Transcriptional control elements like the 72 bp enhancers, GC boxes and TATA box and the T-ag binding sites I, II and III (thick lines) are shown. The BPV-1 origin of replication is composed of an 18 bp inverted repeat and an A/T rich element. Flanking the core elements are two E2 binding sites (E2 BS11 and E2 BS12). The Epstein-Barr virus *ori*P contains two elements, the family of repeats (FR) element and dyad symmetry (DS) element. Each element is made up of degenerate copies of a 30 bp sequence (small box), the EBNA-1 binding site, arranged in either 30 tandem copies (FR) or in two pairs of inverted repeats (DS). The DS and FR elements are separated by approximately 1 kbp of DNA. The HSV-1 *ori*S contains three binding sites for UL9. Site I and II are inverted with respect to each other and are separated by an A/T rich element. A third UL9 binding site is found flanking UL9 site I. Yeast ARS1 contains four elements. The A element contains a perfect 11/11 match with the ars consensus sequence (ACS) The B1 and B2 sites have a 9/11 match to the ACS. The B3 site corresponds to the binding site of the yeast transcription factor ABF1.

A


T-Ag sites
III II I
Enhancers
GC
TATA
Early transcripts
SV40
Late transcripts
A/T
EP
T-Ag site II
ori core
IR
BPV-1
E2 BS11
A/T
E2 BS12
ori core
1 kb
FR
DS
EBV oriP
UL9 site III
UL9 site I
UL9 site II
HSV oriS
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9/11
9/11
11/11
ACS matches

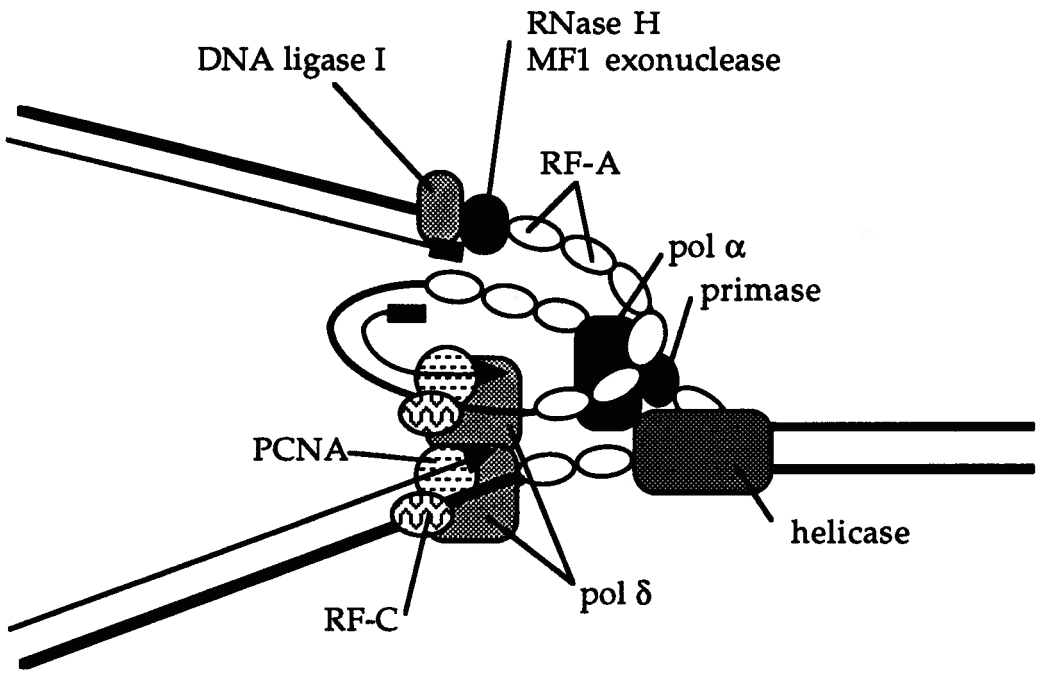

Fig. 6B Model of DNA replication at a replication fork by a multiprotein complex.

In vitro replication of SV40 DNA using highly purified proteins suggest that leading and lagging strand synthesis requires a polymerase switching mechanism (Waga and Stillman, 1994). Thick and thin black lines represent the parental and newly synthesized DNA strands, respectively. Pol α/primase complex is required to synthesize DNA primers for both leading and lagging (as shown) strand synthesis. Dual pol δ/RF-C/PCNA complexes elongate DNA as the helicase (SV40 T-ag) unwinds the DNA at the head of the fork. RF-A binds and stabilizes the ss DNA. After RNA primers (black box) are removed by MPF exonuclease and RNase H, lagging strand synthesis is completed when the 3' end of the current Okazaki fragment reaches the 5' end of the previous fragment and is ligated with DNA ligase I. This figure is adapted from that of Waga and Stillman (1994).

Although three T-ag binding sites have been identified, only site II, found in the 64 bp *ori* core, is required for initiation of DNA replication (Deb *et al.*, 1986). The *ori* core also includes a 17 bp A/T rich element and 15 bp palindrome termed the early palindrome (EP). Both of these elements are essential for the initiation of DNA replication. T-Ag binding site II contains four copies of the pentanucleotide sequence, GAGGC, arranged in two pairs in opposite orientation forming a 27 bp palindromic sequence. Each pentanucleotide is capable of binding one molecule of T-Ag (Tjian, 1978). Although a tetrameric T-Ag complex is able to form on site II (Mastrangelo *et al.*, 1985), it can not initiate DNA replication. However, when T-Ag is incubated with DNA containing the *ori* core in the presence of ATP at 37° C, T-Ag complexes with the *ori* core give footprints covering the entire *ori* core (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988a). Further studies using sedimentation centrifugation and scanning transmission electron microscope techniques revealed that T-Ag assembles into two hexamers forming a two lobed structure which covers the entire *ori* core on both strands of DNA (Mastrangelo *et al.*, 1989). The assembly of the two lobed structure requires ATP but not ATP hydrolysis since the complex structure is able to form in the presence of non-hydrolyzable analogs of ATP (Borowiec and Hurwitz, 1988a). This suggests that ATP acts as an allosteric molecule which induces a shift in T-Ag conformation to allow specific protein-protein interaction at the *ori* core.

The formation of the T-Ag two lobed structure on the *ori* core induces structural changes in both the early palindrome and A/T rich element (Borowiec and Hurwitz, 1988b; Borowiec *et al.*, 1990). Although the structural alteration in the SV40 *ori* core do not require the hydrolysis of ATP, complete strand separation requires the helicase activity of T-Ag and ATP hydrolysis (Wiekowski *et al.*, 1988).

The activated *ori* then recruits a three subunit protein, replication factor A (RF-A), which acts as a single stranded DNA binding protein (Melendy and Stillman, 1993) (see Fig. 6B). This protein presumably prevents the reannealing of the denatured DNA and allows further unwinding of the duplex DNA at the origin. The T-Ag/RF-A/*ori* complex then associates with a DNA polymerase α (pol α) /primase complex. A RNA primer is synthesized by primase and extended by pol α (Murakami *et al.*, 1992). The cellular replication factor C (RF-C) protein then recognizes and binds to the 3' end of the newly synthesized DNA primer (Tsurimoto and Stillman, 1991b; Tsurimoto and Stillman, 1991a). RF-C then allows DNA polymerase δ (pol δ) and proliferating cell nuclear antigen (PCNA), a processivity factor for pol δ (Prelich *et al.*, 1987; Prelich and Stillman, 1988), to be loaded into the replication fork. The processive RF-C/PCNA/pol δ complex then elongates the nascent DNA primer to synthesize the continuous leading strand of the replication fork (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991b).

The lagging strand synthesis is also primed by Okazaki fragments synthesized from the pol α/primase complex. The lagging strand is thought to loop around in such a manner that the RNA primed Okazaki fragment is able to be elongated by a second RF-C/PCNA/pol δ complex formed at the replication fork (Waga and Stillman, 1994). The RNA primer of the Okazaki fragments is removed by a 5' to 3' MPF exonuclease and RNase H (Ishimi *et al.*, 1988; Turchi and Bambara, 1993; Waga and Stillman, 1994). The Okazaki fragments are then ligated to complete synthesis of the lagging strand. The dual RF-C/PCNA/pol δ complex at the replication fork allows leading and lagging strand synthesis to take place in concert. This polymerase switching mechanism on both the leading and lagging strand synthesis is supported by experiments using the *in vitro* replication of either SV40 DNA or synthetic template DNA with highly purified proteins (Tsurimoto *et*

al., 1990; Tsurimoto and Stillman, 1991b; Waga and Stillman, 1994). Since the complete replication of naked SV40 DNA can be reconstituted *in vitro* with purified T-Ag and highly purified cellular proteins mentioned above plus purified topoisomerase I and II, activation of the core *ori* by other *trans*-acting factors such as transcription factors can now be explored biochemically in the SV40 system.

1.3.2. Bovine Papillomavirus Origin of Replication

Papillomaviruses are small double stranded DNA tumor viruses which are able to be maintained extrachromasomally in terminally differentiated cells (Kelly *et al.*, 1988; Challberg and Kelly, 1989). BPV is presented as an attractive model for molecular studies on DNA replication for many of the same reasons as SV40. The BPV-1 genome contains 7945 bp of covalently closed circular DNA. The BPV-1 origins of DNA replication or plasmid maintenance sequences (PMS) were defined genetically by testing plasmids containing cloned restriction fragments of the BPV-1 genome for their ability to be maintained extrachromasomally in BPV-1 transformed mouse cells (Lusky and Botchan, 1984; Lusky and Botchan, 1986). Although two segments of the BPV-1 genome were able to maintain plasmids (PMS-1 and PMS-2) , two-dimensional gel mapping techniques (Yang and Botchan, 1990) localized the origin of replication to a small region of the upstream regulatory region (URR) near PMS-1. The *ori* core of BPV-1 has been shown to contains two elements: an 18 bp inverted repeat and an 8 bp A/T rich element (Fig. 6A). An *in vitro* replication system for BPV-1 showed that two proteins, E1 (68 kDa) and E2 (48 kDa), were required to efficiently initiate DNA replication when supplemented with soluble cell extracts from uninfected cells (Yang *et al.*, 1991). E1 has been identified as the initiator protein for BPV-1 DNA replication (Lusky and Botchan, 1986) since E1, alone, is capable of supporting DNA replication *in vitro* (Yang *et al.*,

1991). E2 was first characterized as a transcriptional activator, but it is now known that E2 is also required for DNA replication *in vivo*. Although E1 alone is capable of supporting low levels of DNA replication *in vitro*, it is only able to do so at relatively high E1 concentrations. Upon addition of E2, the *in vitro* replication of BPV-1 DNA is greatly stimulated. Thus DNA replication of BPV-1 is absolutely dependent on the presence of E2 when E1 is present at low concentrations. Since it was shown that E1 is capable of forming a complex with E2, it was thought that the activation of DNA replication by E2 is a result of the formation of the E1-E2 complex (Mohr *et al.*, 1990). The E1-E2 complex was shown to protect a region covering the 18 bp inverted repeat found in the *ori* core (Yang *et al.*, 1991). Curiously, the two E2 binding sties flanking the *ori* core are not required for DNA replication *in vitro*. It is thought that E2 functions by targeting the E1 initiator protein to the *ori* core through the E1-E2 complex. Once the E1 protein is localized and stabilized to the *ori* core via the E1-E2 complex, it is believed that the E1 protein acts as an ATP-dependent helicase to unwind DNA at the *ori* (Yang *et al.*, 1991) to initiate DNA replication.

1.3.3. Herpes Simplex Virus 1 (HSV-1) and Epstein-Barr Virus (EBV) Origins of DNA Replication

HSV-1 (153 kbp) and EBV (172 kbp) are large linear double stranded DNA viruses which belong to the herpesvirus family of viruses. Although the EBV genome is linear, EBV is maintained as supercoiled plasmid in the nucleus of EBV immortalized cells (Middleton *et al.*, 1991). The EBV origin of DNA replication, *ori*P, was first defined by testing recombinant plasmids containing segments of the EBV genome to replicate in EBV transformed or EBV nuclear antigen 1 (EBNA-1) expressing cell lines (Yates *et al.*, 1984; Yates *et al.*, 1985). Deletion analysis of *ori*P

shows that it is composed of two functional elements, both of which contain multiple copies of a degenerate 30 bp sequence (Fig. 6A) (Reisman *et al.*, 1985). One element contains 20 copies of the 30 bp sequence arranged as direct repeats and is termed the FR element (for family of repeats). The second element contains a region of dyad symmetry, termed the DS element, where four copies of the repeat sequence are arranged as a pair of inverted repeats. The DS and FR elements are separated by approximately 1 kbp of DNA. The exact spacing the these elements appears not to be critical since the intervening DNA can be lengthened or shortened without impairing *ori*P activity (Reisman *et al.*, 1985). EBNA-1 (80 kDa) is the viral *trans*-acting initiator protein which binds to both the FR and DS elements (Rawlins *et al.*, 1985). Although both elements are required for *ori*P activity, the initial unwinding and DNA synthesis is thought to occur at the DS element (Gahn and Schildkraut, 1989). Initiation is thought to include a DNA looping mechanism which links the DS and FR elements. Biochemical (Middleton and Sugden, 1992) and electron microscopy studies (Frappier and O'Donnell, 1991) show that EBNA-1 complexes binding to each of the DS and FR elements can interact with each other through protein-protein interaction, looping out the intervening DNA. This may explain why spacing between the two elements is not critical for *ori*P function. How EBNA-1 promotes the initiation of DNA replication from the looping structure is not known. EBNA-1 does not appear to have a helicase or an ATP binding activity that is seen in the other replicator proteins discussed. Thus, in addition to the structural effects induced by EBNA-1, it may be possible that EBNA-1 is able to recruit cellular replication factors to the activated *ori*P promote formation of initiation complexes.

HSV-1 is known to contain three functional origins of replication. *Ori*L is found in the middle of the long unique region of the HSV-1 genome whereas *ori*S is

found in the inverted repeat sequences found flanking the unique short region of the HSV-1 genome (Challberg and Kelly, 1989). Most molecular studies have used *ori*S since plasmids containing *ori*L appear to be highly unstable in bacteria. Unlike the large EBV *ori*P, the HSV-1 origin of replication, *ori*S, is relatively compact. OriS consists of a 45 bp palindrome (Deb and Doelberg, 1988; Lockshon and Galloway, 1988) containing two high affinity binding sites (site I and site II) for the HSV-1 origin binding protein, UL9 (82 kDa) (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988; Olivo *et al.*, 1988). The two high affinity UL9 binding sites are inverted with respect to each other and are separated with an A/T rich element. In addition, a third, low affinity UL9 binding site (site III) flanking site I appears to be important for DNA replication. The UL9 protein is thought to bind to each site as a dimer (Koff and Tegtmeyer, 1988) since UL9 ia a dimer in solution (Fierer and Challberg, 1992). After both high affinity sites are filled, protein-protein interactions between the two UL9 dimers occurs, looping out the intervening A/T rich DNA (Koff *et al.*, 1991; Rabkin and Hanlon, 1991). Chemical probing experiments suggest that looping out of DNA distorts the A/T rich element (Koff *et al.*, 1991), which then may promote the formation of replication complexes. It has been recently shown that purified UL9 protein contains an ATP-dependent helicase activity (Fierer and Challberg, 1992; Boehmer *et al.*, 1993; Dobson and Lehman, 1993) which is stimulated by the viral encoded ss DNA binding protein, ICP8. This finding suggests that UL9 (complexed with ICP8 (Boehmer and Lehman, 1993)) is the helicase which unwinds the DNA at *ori*S. The mechanism of assembly of replication complexes on the activated *ori*S has not been reported yet.

1.3.4. The yeast chromosomal ARS1 origin of replication

The origins of replication described in this review have been limited to a set of mammalian DNA viruses thus far. Identifying yeast chromosomal origins of replication was relatively simple since short chromosomal sequences can be cloned into plasmid vectors and tested for their ability to replicate in yeast along with the host chromosome in plasmid stability assays (Fangman and Brewer, 1991). Sequences which are able to maintain plasmids in yeast were termed autonomously replicating sequences or ARS. An ARS consensus sequence (ACS), (T/A)TTTA(T/C)(A/G)TTT(T/A) (Van Houten and Newlon, 1990), was defined by comparing several ARS sequences from yeast chromosome III. Recently, the chromosomal ARS1 origin was shown to contain four functional elements (Marahrens and Stillman, 1992) (Fig 6A). The A element contains an 11/11 match to the ACS. The B1 and B2 elements are adjacent to the A element and contain a 9/11 match to the ACS. The B3 element is located most distal to the A element and corresponds to the binding site for the yeast transcription factor ABF1. It was shown that the B3 element could be replaced with the binding sites for either transcription factors RAP1 or Gal4 without loss of ARS activity. Only the A element is essential for ARS activity since plasmids containing linker insertions into of each of the B1, B2 or B3 elements, individually, are maintained in yeast strains (Marahrens and Stillman, 1992). A multiprotein complex, origin recognition complex (ORC), was purified from yeast and shown to bind to the A element of ARS1 and other A type elements in other chromosomal ARS sequences, in DNase I footprinting assays (Bell and Stillman, 1992). Although the binding of ORC to the A element is dependent on the presence of ATP, it is not clear if the hydrolysis of ATP is required since purified ORC preparations do not appear to have ATPase activity. SDS-PAGE analysis of purified ORC shows that ORC contains possibly six

polypeptides (120, 72, 62, 56, and 50 kDa). Although the ORC is considered the initiator in yeast, its role in the initiation of chromosomal DNA replication has not been elucidated.

1.4. The present study

The objective of this study was to identify elements within the MVM(p) genome which are necessary for viral DNA replication. Although it was clear from previous studies that the viral terminal palindromes were essential for DNA replication, sequences inboard of the terminal palindromes had not been investigated. Since the MVM(p) genome has been cloned into a plasmid vector, pMM984 and this has been shown to be infectious, the first objective was to develop an *in vivo* DNA replication system based on the original infectious clone. After the DNA replication assay was developed, mutant plasmid constructs of the viral genome ("minigenomes") were generated to determine sequences which affect viral DNA replication. Deletion mutants made in the left hand (3') palindrome, internal right end (IRE) and internal left end (ILE) regions were tested. Mutant genomes containing identical termini were also constructed and tested. It was determined that sequences found internal of the right palindrome were important for DNA replication of MVM minigenomes. This sequence was used as a probe to detect DNA binding factors in nuclear extracts. The factors which bind to elements which affect the DNA replication of the mutant virus may be potential replication factors.

II. Materials and Methods

2.1. Materials

Chemicals were purchased from BDH, Fisher Scientific, or Sigma Chemical Co. unless otherwise specified. Polyacrylamide and agarose gel electrophoresis supplies were obtained from GIBCO/Bethesda Research Laboratories (BRL) or Bio-Rad Laboratories.

Tissue culture media and supplies were purchased from either GIBCO/Bethesda Research Laboratories or Sigma Chemical Co. Bactotryptone, yeast extract and Bactoagar were supplied by Difco Laboratories. Ampicillin (Penbritin) was purchased from Ayerst Laboratories.

All restriction and DNA modification enzymes were purchased from either GIBCO/BRL, Promega or New England Biolabs unless otherwise specified. DNA sequencing kits using the Sequenase enzyme were supplied by United States Biochemical Company (USB).

2.2. Bacteria

The recombination deficient strains of *E.coli*, JC8111 (Boissy and Astell, 1985) and Sure (Stratagene), were used to maintain and propagate plasmids which contain the right hand (5') terminal palindrome sequence of MVM(p) since it has been shown that specific deletions in the right hand terminal sequences tend to be generated and propagated in standard strains. The DH5α strain was used for all other routine cloning. Bacteria were routinely grown in YT medium (8 g tryptone,

5 g yeast extract and 5g NaCl per liter) supplemented with either 100 μg/ml ampicillin or 10 μg/ml tetracycline (when necessary) for the selection of plasmids.

2.3. Mammalian Cell Lines

2.3.1. COS-7 Cells

COS-7 cells (obtained from T. Maniatis, Harvard University) were derived by transforming the simian kidney cell line, CV-1, with an origin-defective SV40 genome which is integrated into the host cell genome (Gluzman, 1981). In this cell line, SV40 large T-Antigen is expressed and is thus able to maintain plasmids which contain the SV40 origin of replication. COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 mM HEPES-NaOH, pH=7.4 and 10% fetal bovine serum at 37° C in 5% CO_2. Cells were passaged approximately once every three days by trypsinization and dilution (1:10).

2.3.2. LA9 Cells

Murine LA9 cells (Littlefield, 1964) used in this study are a ouabain resistant isolate of the $HGPRT^-$ mouse fibroblast cell line A9 and were obtained from P. Tattersall (Yale University). These cells have been shown to be permissive to infection by MVM(p) and are routinely used for the molecular analysis of MVM(p) (Tattersall, 1972). LA9 cells were grown in DMEM supplemented with 10 mM HEPES-NaOH, pH=7.4 and 5% fetal bovine serum and passaged as described above.

2.4. Basic Cloning Techniques

Many of the basic cloning procedures and the growth of bacteria were described previously (Sambrook *et al.*, 1989).

2.4.1. Isolation of Plasmid DNA

Large scale isolation of plasmid DNA was performed using the alkaline lysis method followed by either density equilibrium centrifugation in CsCl-ethidium bromide (EtBr) gradients or the polyethylene glycol precipitation method. Final DNA concentrations were determined by A_{260} readings. Small scale isolation of DNA was performed by the alkaline lysis method followed by a phenol/chloroform extraction and ethanol precipitation.

2.4.2. Isolation of DNA Fragments From Agarose Gels

Restriction fragments were routinely isolated from agarose gels after electrophoresis in Tris-Acetate-EDTA (0.04 M Tris-Acetate, 1 mM EDTA pH=8.0) (TAE) buffer. DNA was stained with 0.2 μg EtBr/ml added to the gel and running buffer and visualized by exposure to UV light. DNA from gel slices was isolated by electroelution followed by ethanol precipitation or by adsorption to glass beads using the GeneClean DNA purification reagents (BIO 101 Inc.). Purified DNA was redissolved in 5-10 μl TE (10mM Tris-HCl, pH=8.0, 1mM EDTA).

2.4.3. Cloning of Restriction Fragments into Plasmid Vectors

Small restriction fragments were routinely cloned into plasmid vectors. Typically, vector and insert fragments were ligated in a 10 µl reaction containing 50 mM Tris-HCl, pH=7.6, 10 mM $MgCl_2$, 1 mM ATP, 1mM dithiotheritol (DTT) and 1 U T4 DNA ligase. The ligation reaction was allowed to proceed for 2 hours at room temperature before transformation of competent *E.coli* with the ligation products. When blunt-ended restriction fragments were cloned, the vector DNA fragment was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). The reaction was terminated by adding SDS to a final concentration of 1% and incubating at 75°C for 15 min. Vector DNA was then extracted once with phenol/chloroform and precipitated with 3 volumes of ethanol. Dephosphorylation of 5' ends of DNA inhibits self-ligation and increases the efficiency of cloning blunt-ended molecules Blunt ended ligation reactions were incubated at room temperature for 4 hours or at 16° C overnight before transformation.

2.4.4. Preparation and Transformation of Competent Cells

Competent Sure cells and DH5α were prepared using the $CaCl_2$ method. Briefly, 100 µl of a fresh overnight culture was inoculated into 35 ml of YT medium and allowed to grow in a 37° C waterbath, shaking vigorously. Cells were harvestedwhen cultures reached OD_{590}= 0.2 by centrifugation (10 min X 4000 rpm). The cells were resuspended in 15 ml ice cold 50 mM $CaCl_2$ by gentle aggitation with a vortex mixer. After incubation on ice for 30 min with occasional agitation, the cells were` recentrifuged and resuspended in 3.0 ml ice cold 50 mM $CaCl_2$ containing 15% glycerol. Two hundred microliter aliquots were flash-frozen in an

ethanol/dry ice bath before being stored at -80° C. JC8111 cells were made competent by the Hanahan method (Hanahan, 1983) since this strain can not be made competent using the standard $CaCl_2$ method. In this method, 100 ml cultures were grown in SOB medium to a OD_{590}=0.7 before being harvested by centrifugation. The cells were resuspended in 30 ml ice cold frozen storage buffer (FSB) and incubated on ice for 30 min. After recentrifugation, the cells were resuspended in 8.0 ml of ice cold FSB. After the addition of 240 µl of DMSO, cells were flash-frozen in 200 µl aliquots as described above.

In standard transformations, 200 µl of frozen competent cells were allowed to thaw on ice before being added to ice cold plastic culture tubes containing 2-5 µl of a ligation reaction. The transformation mixture was incubated on ice for a further 30 min. Cells were heat shocked at 42° C for 90 seconds and 0.8 ml YT was added. Transformed cells were incubated in a 37° C water bath, gently shaking, for 45-60 min. Usually 20 and 200 µl of each transformation mixture were spread on YT-agar plates containing the appropriate antibiotics.

2.5. DNA Sequencing

Plasmid DNA to be sequenced by the dideoxy chain termination DNA method (Sanger *et al.*, 1977) was isolated using the standard mini-prep method. Usually 2 µg of each plasmid was boiled with 2-5 pmol of the appropriate primer for 2 min in 0.2 M NaOH in a final volume of 20 µl and neutralized with 3 µl 3 M sodium acetate (pH=5.2). The DNA was then precipitated with ethanol, centrifuged, washed and dessicated. The resulting DNA was then redissolved in 10 µl of 1X sequencing buffer (40 mM Tris-HCl, pH=7.5, 20 mM $MgCl_2$ and 50 mM NaCl) and incubated at 37° C for 15 min before proceeding with the labelling and

termination reactions using the DNA sequencing mixes and Sequenase enzyme supplied by USB. Labelled DNA fragments in the termination reactions were then separated by electrophoresis through a 6 or 8% polyacryamide gel containing 8 M urea and Tris-Borate-EDTA (TBE) buffer (89 mM Tris-Borate, pH=8.3, 2 mM EDTA). After electrophoresis, gels were dried and subjected to autoradiography.

2.6. Plasmid Construction

The original infectious clone of MVM(p), pMM984 (obtained from D.C. Ward, Yale University), contains the double stranded sequence of MVM(p) cloned into the BamHI site of pBR322 (Merchlinsky *et al.*, 1983). This clone was shown to produce infectious virions upon transfection into cells permissive for MVM(p). Both pMM984 and the variant pCA4.0, which contains a SmaI site adjacent to the left end BamHI site of MVM(p), were used as starting points for most plasmid constructions.

2.6.1. Construction of pCMVNS-1

The NS-1 expression vector, pCMVNS-1, was constructed in two steps. First, the 2.3 kbp Hga I fragment of pMM984 was blunt ended using T4 DNA polymerase and ligated to phosphorylated BamHI linkers. After digestion with BamHI, this insert was then cloned into the BamHI site of pUC 19 (Wilson *et al.*, 1991). The BamHI fragment was transferred to the BamHI site of the pCMV-5 SV40 origin based mammalian expression vector (Andersson *et al.*, 1989). The expression of NS1 is directed by the strong immediate early CMV promoter.

2.6.2. Construction of pPTLR

Since most of the internal coding portion of the MVM(p) genome is not required in *cis* for the replication of the viral DNA, I sought to make a minigenomic construction which contained only the terminal sequences. The right terminal 0.8 kbp Xba I (4342)/Bam HI fragment of pMM984 was cloned into the Xba I/Bam HI sites of pUC 19 and designated pUCRH. The 0.7kb Hind III/Pst I fragment containing the 411 bp of the left terminus was subsequently cloned into pUCRH. This plasmid, designated pPTLR, contains both the right and left terminal palindrome sequences as well as the flanking internal sequences. The P4 promoter and transcriptional start site remains intact at the left end. The single polyadenylation site and 65 bp tandem repeat were also left intact in the right terminal fragment.

2.6.3. Construction of Left Palindrome Deletions

In order to analyze the sequence requirements of the left palindrome, the left end containing the 0.4 kbp Sma I/Alu I (408) fragment of pCA4.0 was cloned into the Sma I site of pUC 19 and designated pCA408. Plasmid pCA408F, containing the Sma I/Alu I fragment oriented such that the 3' palindrome sequence is proximal to the EcoRI end of the multiple cloning site, was digested with Sst I and Sma I and used to generate exonuclease III deletions (Henikoff, 1984). After sequence analysis, clones containing deletions from MVM(p) nucleotide one to nucleotide 12, 26, 31, 57, and 78 bp were selected. The 0.9 kbp Xba I (4342)/Sph I fragment of pMM984 which contains the 5' terminal palindrome was then cloned into the Xba I/Sph I site of the deletion clones resulting in a set of minigenomes containing deletions in the left terminal palindrome.

2.6.4. Construction of Internal Left End (ILE) and Right End (IRE) Deletions

Deletion of the internal portions of the minigenome encoded within pPTLR were made in both the internal left end (ILE) and internal right end (IRE) regions. Unidirectional IRE deletions were generated using XbaI/PstI digested pPTLR as a template for exonuclease III digestion (Henikoff, 1984). ILE deletions were generated by digesting pPTLR with HincII/NcoI and HincII/StyI. The DNA fragments were blunt ended using T4 DNA polymerase and ligated. Plasmids from recombinant clones were first sized on agarose gels. Clones containing approximately the desired size deletions were then sequenced to determine the exact nucleotide junctions.

In order to use the EcoRV (381) site within the MVM(p) minigenomes for subsequent analysis of the IRE deletions, the EcoRV site in the vector sequences was removed by replacing the EcoRV(381)/HindIII left end containing fragment with the EcoRV(381)/HindIII fragment from pCA408F. The subsequent series of clones is essentially identical to the parental clones except it contains 408 bp (Alu I(408)) of the left terminus instead of 411 bp. with respect to the minigenomic sequences. The SV40 origin of DNA replication was cloned into the resulting minigenomes by ligating a blunt ended 0.3 kbp SmaI/PstI fragment of pSV(-), which contains the Bgl I/PvuII fragment of the SV40 genome (SV40 nucleotides 5235-270), into the unique EcoRV site, resulting in the SVR series of minigenomes. In order to use the Xba I(4342)/Sau3a(4741) fragment (SX fragment), the SX fragment from pUCRH was subcloned into the BamHI/XbaI site of pUC 19 and designated pUCXbaSau. Then the blunt ended SmaI/PstI fragment pUCXbaSau which contains the SX fragment

was ligated into the unique EcoRV (381) site of the modified minigenomes resulting in the SXR series of minigenomes..

2.6.5. Construction of Minigenomes Containing Two Left Termini or Two Right Termini

The 4.5kb NheI (pBR322 sequence)/XbaI(4342) fragment of pMM984 was cloned into the XbaI site of pCA408F to create a plasmid, pPTLL, encoding a 4.7 kbp MVM(p) minigenome containing two left hairpins. A smaller version of the LL minigenome was constructed by deleting the internal XhoI(2070)/XbaI(4342) restriction fragment from pPTLL, resulting in pPTLLX. In order to introduce restriction fragments into the LLX minigenome, the pPTLLX was digested with EcoRV and Bgl II linkers were ligated onto the EcoRV blunt ends. After digestion with Bgl II, the vector containing the identical left terminal fragments (pPTLLX-Bgl) was purified and used in ligation reactions. In order to insert the SX fragment into pPTLLX-Bgl, the SmaI/HincII fragment of pUCXbaSau, which contains the SX fragment, was purified before the ligation of Bgl II linkers. After digestion with Bgl II, the fragment was purified and ligated into the pPTLLX-Bgl vector giving rise to the LLSX series of minigenomes. In addition to the LLXS clones, the 350 bp BamHI/BglI fragment of the FUS3 gene of yeast was also inserted into pPTLLX-Bgl vector giving rise to the LLFUS3 series of minigenomes.

The right end containing 0.9 kbp EcoRI/HindIII fragment of pUCRH was ligated to the large EcoRI/Hind III(3996) 5.1 kbp fragment of pMM984. The resulting plasmid, designated pPTRR, encodes an MVM(p) minigenome containing two right termini oriented as inverted terminal repeats with the MVM(p) XbaI(4342)/HindIII(3996) fragment separating the two right termini. Construction

of minigenomes containing inverted terminal right or left end repeats without spacer DNA was unsuccessful.

2.7. Transfection of DNA into Mammalian Cells

DNA was introduced into COS-7 and LA9 cells by the DEAE-dextran transfection method (Lopata *et al.*, 1984). Cells were usually passaged at dilution of 1:5 the night before the transfection. The next morning, the cells were washed twice with DMEM before the transfection mixture was added to the cells. The transfection mixture was made by adding 5-10 μg of the appropriate plasmid DNAs to 0.3 ml DEAE-Dextran solution(Mr=20,000) (2 mg/ml). The mixture was made up to a final volume of 3.0 ml with DMEM. After the addition of the transfection mixture, the cells were incubated at 37° C in 5% CO_2 for 8 hours. The cells were shocked with PBS (0.20 g KCl, 0.24 g KH_2PO_4, 8.0 g NaCl, 1.4 g NA_2PO_4) containing 10% DMSO for 2 min (LA9 cells) or 4-5 min (COS-7 cells). This procedure has been shown to increase transfection efficiency in a variety of cell lines. The transfected cells were allowed to grow in complete medium for 3 days before harvesting for further analysis.

2.8. Isolation of Low Molecular Weight DNA From Mammalian Cells

Low molecular weight DNA was isolated by a modified Hirt extraction procedure (Hirt, 1967). Transfected cells were washed twice with PBS then lysed with 1.0 ml of Hirt lysis buffer (100 mM NaCl, 1% SDS, 10 mM EDTA, pH=8.0) added to the culture dish. After incubation at room temperature for 15-20 min, the lysate was transferred to a fresh 1.5 ml microfuge tube. NaCl was added to a final concentration of 1.1 M and the lysate was mixed by inversion 10-15 times. After the

extract was incubated on ice overnight, the fluffy white precipitate containing mainly genomic DNA was pelleted by centrifugation for 30 min at 4°C in a microfuge. The supernatant was transferred to a tube and digested with proteinase K (BRL) at a final concentration of 500 μg/ml at 37° C for 2 hours. After one phenol/chloroform extraction, the nucleic acids were precipitated with one volume of isopropanol.

2.9. Use of Restriction Endonuclease, Exonuclease III and S1 nuclease to Characterize Replicated DNA

In order to characterize the DNA in Hirt extracts, samples were digested with various restriction and other nucleases. DpnI and MboI digestions were performed in a buffer supplied by the manufacturer. Duplicate exonuclease III (exoIII) digestions of Hirt DNA samples were performed in a 100 μl volume containing 66 mM Tris-HCl pH=8.0, 77 mM NaCl, 5 mM $MgCl_2$, 10 mM DTT and 660 U exoIII for 1 hour at 37°C. The reactions were terminated by the addition of 100 μl of ice cold 2X S1 nuclease buffer (60 mM potassium acetate pH=4.6, 500 mM NaCl, 2 mM $ZnCl_2$, 10% glycerol). Ten units of S1 nuclease were added to one of the duplicate sets of exoIII reactions and incubated at 37°C for 30 min. All samples were extracted once with phenol/chloroform and the DNA was precipitated with ethanol before agarose gel electrophoresis and Southern blot analysis (Southern, 1975).

2.10. Southern Blotting and Hybridization

DNA was electrophoresed through a 1.0% agarose gel containing TAE buffer and 0.2 µg/ml EtBr at 10-20 volts for 16-20 hours. The DNA was then transferred to a nylon membrane (GeneScreenPlus, Dupont) using the LKB 2016 VacuGene vacuum blotting system. The gel was first soaked in 0.25 M HCl for 10-15 min (until the xylene cyanol dye turned yellow). The gel was then laid on top of the nylon membrane supported by a porous screen. A vacuum of 40-50 cm of H_2O was applied. DNA in the gel was then denatured by adding a denaturation solution (1.5 M NaCl, 0.5 M NaOH) on top of the gel for approximately 15-20 min. After the denaturation solution was removed, the gel was neutralized by adding a neutralization solution (1.5 M Tris-HCl, pH=5.0, 1.5 M NaCl) on top of the gel for another 15-20 min. The transfer was then allowed to proceed in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH=7.0) for one hour. The nylon membrane was then washed in 5X SSC for 5 min to remove any agarose left on the membrane and allowed to dry.

Nylon membranes were prehybridized in 10 ml of the hybridization solution (5X SSPE, 1.0% SDS, 0.1 % Tween 20, 50% formamide and 100 µg/ml denatured sheared salmon sperm DNA) before the addition of the denatured ^{32}P labeled DNA probe. Hybridization was carried out in a water bath at 43-44°C for at least 16 hours. Membranes were then washed twice in 1XSSPE, 0.1% SDS at room temperature for 30 min each, and twice in 0.1X SSPE, 0.1% SDS at 65°C for 30 min each. The membranes were wrapped in Saran Wrap and subjected to autoradiography.

2.11. Preparation of ^{32}P labeled DNA Probes

^{32}P labeled DNA probes were prepared by the random hexamer priming method (Feinberg and Vogelstein, 1983). Usually, the template DNA was denatured in boiling water for 5 min in a total volume of 8.0 µl containing 50-100 ng of a purified restriction fragment, 1.6 µl of 100 A_{260} units/ml of random hexamers. The reaction was cooled on ice for 5 min before the addition of 5 µl 1 M HEPES-NaOH, 5 µl 5X dNTP (100 mM of each dCTP, dGTP, dTTP, 50 mM β–mercaptoethanol and 25 mM $MgCl_2$), 5 µl 3000Ci/mmol [$\alpha^{32}P$]dATP, 1 µl 10 mg/ml acetylated BSA and 6U DNA plymerase I (Klenow fragment). The labeling reaction was allowed to proceed at room temperature for 4 hours. The labeled DNA was then precipitated with ethanol and washed 5-6 times with 1.0 ml 70% ethanol to remove the majority of the unincorporated label. The labeled probe was dissolved in 100 µl of dH_2O and denatured in boiling water for 5 min before being used in Southern hybridization experiments. Probes contained approximately 10^8 cpm/ug DNA as determined by TCA preciptiation.

2.12. Western Blot Analysis

In order to determine the levels of NS-1 in transfected LA9 cells and COS-7 cells, lysates of transfected cells were probed with the NS-1 specific monoclonal antibody, CE10 (Yeung *et al.*, 1990), using the Enhanced Chemiluminescence detection (ECL) kit supplied by Amersham. Transfected cells were washed twice with 10 ml PBS before lysis in 200 µl sample buffer (62.8 mM Tris-HCl, pH=6.8, 2% SDS, 5% β–mercaptoethanol, 10% glycerol and 0.05% bromophenol blue). The viscous lysate was transferred to a microfuge tube and boiled for 10 min. Aliquots (2-10 µl) were loaded onto a 4%/12% discontinuous polyacrylamide mini-gel (Mini

Protean II, Bio-Rad) and electrophoresed for one hour at 100 V (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose membranes by electroblotting in transfer buffer (25mM Tris, 192 mM glycine pH=8.3 and 20 % methanol). The membranes were incubated with undiluted hybridoma culture supernatant containing the CE10 primary antibody for one hour. The membranes were washed and incubated with the secondary goat anti-mouse antibody conjugated to horseradish peroxidase (1:3000 dilution) for one hour. Immunoreactive proteins were detected using a chemiluminescence reaction during exposure to Kodak X-AR film as described by the protocol from the supplier.

2.13. Preparation of Nuclear Extracts

Nuclear extracts were prepared from isolated nuclei from MVM(p) infected or uninfected LA9 cells (Dignam *et al.*, 1983). Large scale growth of LA9 cells was done in Bellco spinner culture flasks. Ten to 20 subconfluent plates of LA9 cells were trypsinized, pelleted by centrifugation and resuspended in Joklik's Modified Eagles Medium (S-MEM) supplemented with 5% fetal bovine serum, 10 mM HEPES-NaOH, pH=7.4. The culture was then introduced into a 250 ml spinner culture flask and gassed with 5% CO_2 balanced air. Cells were allowed to grow in suspension at 37° C with stirring at a 3.5 setting on a Bellco 4-Spin magnetic stirrer. Cultures were maintained continuously at a cell density of 1.0×10^6 to 6×10^6 cells per ml. Approximately 2×10^8 LA9 cells were infected at a multiplicity of infection (moi) of 10 in a final volume of 50 ml containing DMEM supplemented with 1% fetal bovine serum and 10 mM HEPES-NaOH, pH=7.4 for one hour. The cells were then diluted to a final volume of 1000 ml of complete medium in a 2 liter spinner flask and gassed with 5% CO_2 balanced air. Infected cells were allowed to grow for 16 hours before harvesting.

Nuclear extracts were prepared using the protocol from Dignam *et al.* (1983). All steps were performed at 4° C. Infected or uninfected LA9 cells were harvested by centrifugation. The cells were washed once in 5 PCV (packed cell volume) of ice cold PBS and once in 5 PCV of buffer A (10 mM HEPES-KOH, pH=7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml each antipain, leupeptin and pepstatin). The resulting cells were resuspended in 2 PCV of buffer A and homogenized in a 25 ml Dounce Homogenizer using 20 strokes of the loose fitting "B" pestle (Kontes). The rupture of the cells was monitored visually by phase contrast microscopy. The nuclei were pelleted in a 15 ml COREX tube (2000 x g, 10 min). The supernatant was removed and the pellet was recentrifuged (25,000 x g, 20 min) to remove the residual cytoplasmic material. The nuclei were resuspended in 2 ml buffer C (20 mM HEPES-KOH, pH=7.9, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.5mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 1 µg/ml each of antipain, leupeptin and pepstatin, and 20% glycerol) and homogenized again using 10 strokes of the B pestle in a Dounce homogenizer. The ruptured nuclei were stirred for 30 min and centrifuged (25,000 x g, 30 min). The supernatant was then dialyzed against one liter of buffer D (20 mM HEPES-KOH, pH=7.9, 0.1 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5mM DTT, 0.5 mM PMSF and 25% glycerol) for 8 hours. Particulate matter in the dialysate was removed by centrifugation (25,000 x g, 30 min). The supernatant, designated nuclear extract, was stored at -70° C in 100-200 µl aliquots.

2.14. Fractionation of Nuclear Extracts

Typically, 5-10 ml of uninfected LA9 nuclear extract was loaded on a 10 ml DEAE-Sephacel (Pharmacia) column equilibrated with buffer D containing 1 μg of each/ml antipain, leupeptin and pepstatin. The column was washed with 50 ml of buffer D followed by 50 ml buffer D containing 0.6 M KCl. Fractions were immediately assayed for binding activities and relative protein concentration (A_{280}). Fractions containing DNA binding activity were pooled and loaded on a 10 ml heparin-agarose (Sigma) column equilibrated with buffer D. The heparin-agarose column was then washed with 60 ml of buffer D containing 0.1 M KCl, 0.3 M KCl and 0.6 M KCl. Peak protein fractions were assayed for DNA binding activity, dialyzed against one liter of buffer D for 6-8 hours and stored at -70° C.

2.15. Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays (EMSA) were performed in a final volume of 20 μl containing 10 mM Tris-HCl (pH=7.5), 50 mM NaCl, 1.0 mM DTT, 250 ng/μl double stranded poly dI-dC (Pharmacia) and 2.5-5.0 μg of nuclear extracts. The proteins were preincubated for ten min at room temperature followed by addition of 10,000 cpm (0.2-1.0 ng) of the indicated ^{32}P end labeled probe. After a 30 minute binding reaction, DNA-protein complexes were loaded onto a native 4% polyacrylamide gel (Hoeffer SE600) containing 0.5 X TBE and 1% glycerol. Gels were pre-electrophoresed for 2-3 hours at 200V in 0.5 X TBE/1% glycerol prior to sample loading. Samples were electrophoresed at 150 V for 2.0-2.5 hours before the gel was dried on Whatman 3MM paper and subjected to autoradiography.

2.16. DNase I Footprinting

In DNase I footprinting experiments, proteins from column fractions were used in binding reactions as described above except the final incubation volume was 50 µl and contained 10 mM $MgCl_2$ and 5 mM $CaCl_2$. The DNA in the binding reaction was tpartially digested with DNase I by the addition of 2 µl of 1/25th dilution of the enzyme (Promega) in 150 mM NaCl, 1 mM $CaCl_2$ and 50% glycerol. The DNase I digestion was stopped after 60 seconds with 50 µl DNase I stop buffer (8 M ammonium acetate, 40 mM EGTA and 100 µg/ml yeast tRNA). Samples were immediately extracted once with phenol/chloroform and precipitated with ethanol. The precipitated DNA was dissolved in 2 µl of formamide dye buffer and boiled for 2 min before being electrophoresed through denaturing 10% polyacrylamide/8 M urea sequencing gels. Samples were electrophoresed at 32 W constant power until the bromophenol blue dye reached approximately two-thirds the length of the gel. Gels were dried on Whatman 3MM paper and subjected to autoradiography.

2.17. Preparation of Probes for DNA-Protein Interaction Studies

2.17.1 RsaA and RsaB Restriction Fragments

The RsaA and RsaB probes were prepared by cloning the respective Rsa I fragments (nt 4431-4579 and 4579-4662) into the Sma I site of pUC19 and releasing then with BamHI and EcoRI digestion. Digestion products were loaded onto a 5% polyacrylamide gel containing 1 X TBE and electrophoresed 16-20 hours at 100V. After the gel was stained by soaking in 1 X TBE containing 0.5 µg/ml EtBr for 10-15 min, the desired EcoRI/BamHI fragments were isolated by electroelution.

The EcoRI/BamHI fragments containing Rsa A and Rsa B were end labeled using DNA polymerase I (Klenow fragment). Approximately 300-500 ng of each probe was labeled in a 20 µl reaction volume containing 20 mM Tris HCl, pH=8.0, 7 mM $MgCl_2$, 40 µCi [α^{32}P]dATP (3000 Ci/mmol) and 5 U DNA polymerase I (Klenow fragment). The reaction was incubated for 15 min at room temperature before the probe was precipitated with ethanol. The radioactive probe was pelleted for 30 min in a microfuge and washed 5-6 times with 70 % ethanol to remove the unincorporated label. The pellet was dried briefly and dissolved in 20 µl TE. The specific activity was determined by precipitating a 1µl sample with 5% trichloroacetic acid. The precipitable material was collected by suction filtration through glass fibre filters (Whatman) and counted. The probe was then diluted with TE just before use to 10,000 cpm/µl.

2.17.2 Synthetic Oligonucleotides

Three pairs of synthetic oligonucleotides, B oligo site I (5'AGCTTTCATATATTATTAAGACTAATAAAGATACAA3' and 5'AGCTTTGTATCTTTATTAGTCTTAATAATATATGAA3'), B oligo site II (5'AATTCATAGAAATATAATATTACATATAGATTTAAGAAATAG3' and 5'AATTCTATTTCTTAAATCTATATGTAATATTATATTTCTATG3') and FREBP oligo A (5'GATCCGGGAGCTGCATCCGGAGTAGG3' and 5'GATCCTACTCCGGATGCAGCTCCCGG3') were synthesized (ABI 391 DNA synthesizer) and annealed before being used in competition EMSA experiments.

III. Results

3.1 Replication of a Defective LR Minigenome in COS-7 and LA9 Cells

In order to study the sequence requirements for MVM DNA replication, a transient DNA replication assay was developed. In this assay system, a plasmid encoding a defective genome or minigenome of MVM(p) is co-transfected with a vector expressing the viral encoded enzymes into cells permissive for MVM(p) DNA replication. Thus, the ability of the minigenome to replicate is dependent on having the essential sequences present on the plasmid containing the minigenome. Previous studies have shown that plasmids containing a single copy of either the left or right palindrome were incapable of replicating in permissive cells (Merchlinsky *et al.*, 1983; Salvino *et al.*, 1991). The minigenome encoded by the pPTLR plasmid contains 411 nt of the left terminus fused to 807 nt of the right terminus (Fig. 7). Thus the majority of the viral coding sequences have been deleted. The hypothetical sizes of the monomer and dimer replicative forms, termed mLR and dLR, respectively, of the minigenome is expected to be approximately 1.2 and 2.3 kbp respectively.

In order to determine if the minigenome encoded by pPTLR replicates in COS-7 or LA9 cells, pPTLR linearized at the unique EcoRI site was co-transfected with either pMM984 or pCMVNS-1 into the two cell lines (Fig. 8). Low molecular weight DNA was isolated from transfected cells and analyzed by Southern blot analysis. When pPTLR was transfected in the absence of vectors expressing viral proteins, no replicative intermediates were seen at the expected molecular weight. When pPTLR was co-transfected with either pMM984 or pCMVNS-1,

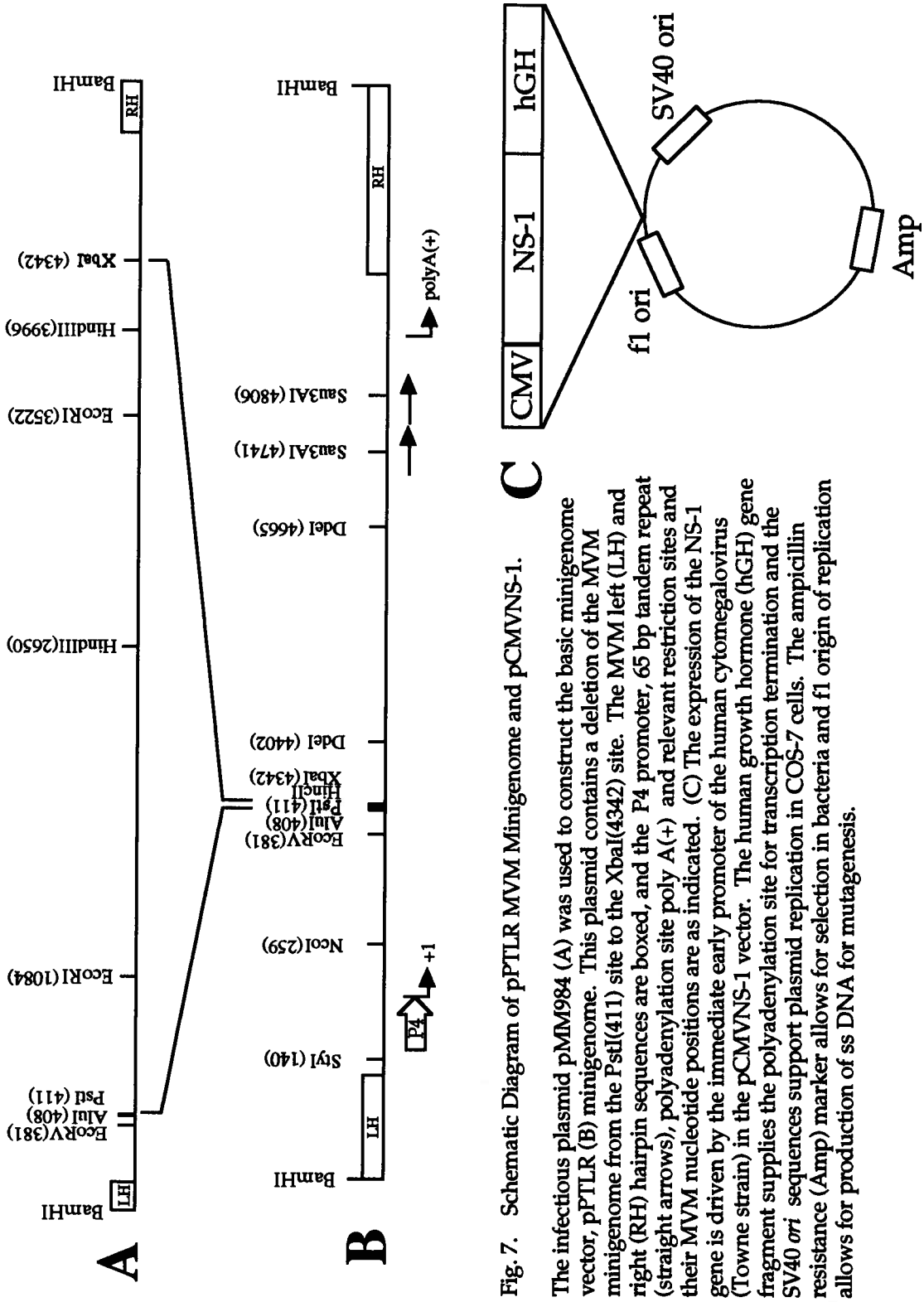

Fig. 7. Schematic Diagram of pPTLR MVM Minigenome and pCMVNS-1.

The infectious plasmid pMM984 (A) was used to construct the basic minigenome vector, pPTLR (B) minigenome. This plasmid contains a deletion of the MVM minigenome from the PstI(411) site to the XbaI(4342) site. The MVM left (LH) and right (RH) hairpin sequences are boxed, and the P4 promoter, 65 bp tandem repeat (straight arrows), polyadenylation site poly A(+) and relevant restriction sites and their MVM nucleotide positions are as indicated. (C) The expression of the NS-1 gene is driven by the immediate early promoter of the human cytomegalovirus (Towne strain) in the pCMVNS-1 vector. The human growth hormone (hGH) gene fragment supplies the polyadenylation site for transcription termination and the SV40 *ori* sequences support plasmid replication in COS-7 cells. The ampicillin resistance (Amp) marker allows for selection in bacteria and f1 origin of replication allows for production of ss DNA for mutagenesis.

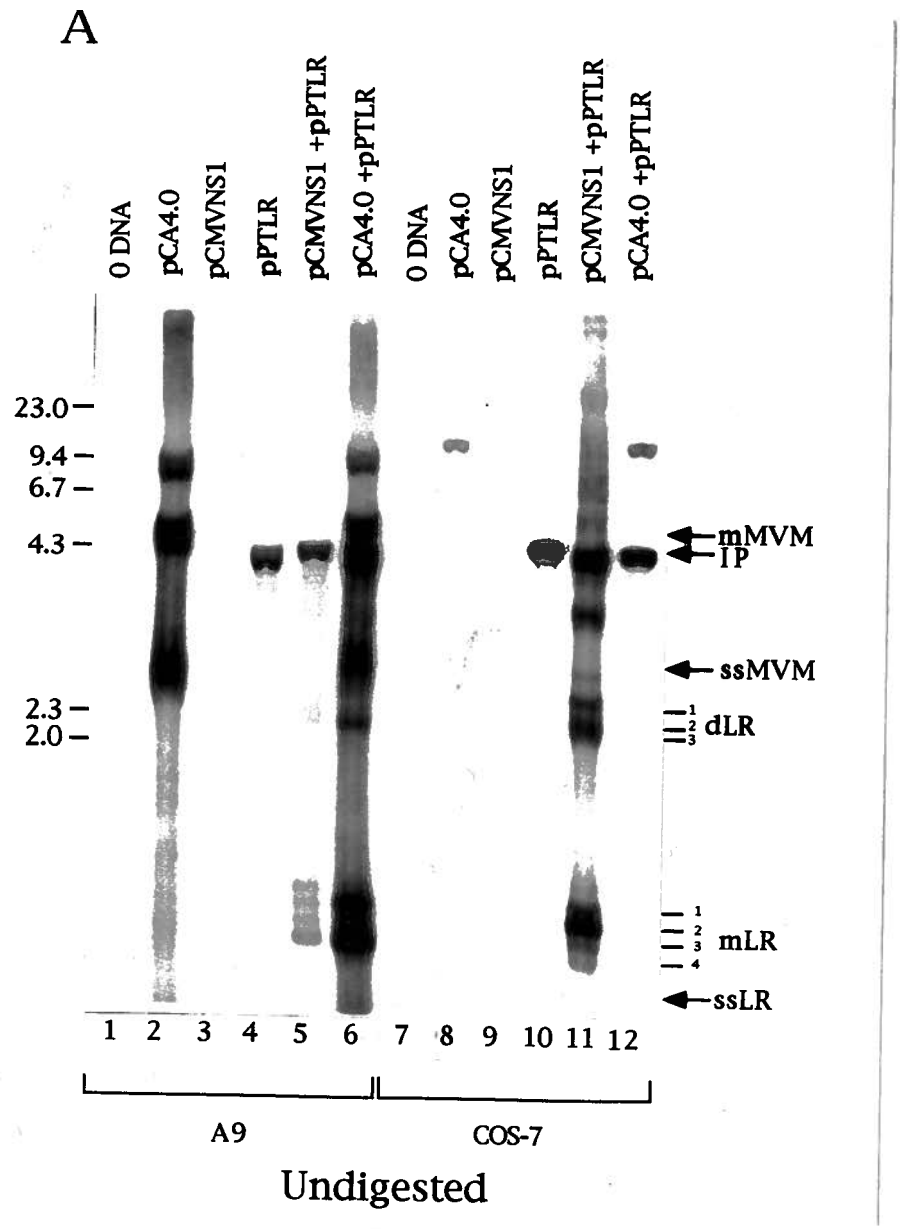

Fig. 8. Replication of the pPTLR Minigenome.

Both pCA4.0 and pPTLR were linearized with SmaI (pCA4.0) or EcoRI (pPTLR) before transfection. Five micrograms of each of the indicated plasmids were transfected into either mouse LA9 or COS-7 cells as outlined in the Materials and Methods. Southern blots of undigested (A), Dpn I (B), and Mbo I (C) digested Hirt DNA samples isolated from the indicated transfections experiments were performed. The monomer RF (m), dimer RF (d) and single stranded DNA (ss) are as indicated for both the minigenomic LR and wild type MVM(p) constructs. The MboI resistant input minigenomic plasmids (IP) are also indicated. At least four discrete monomer LR (mLR) and 3 discrete dimer (dLR) species are seen at the 1.1 kbp and 2.2 kbp regions, respectively.

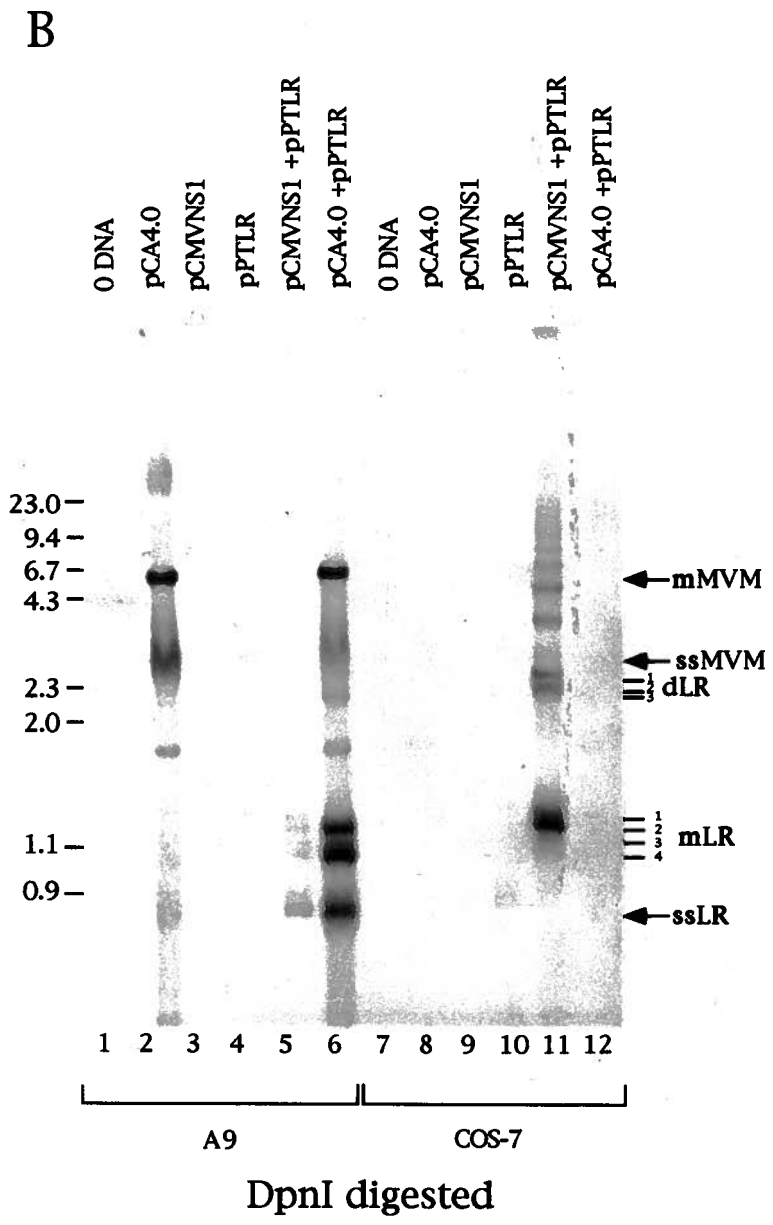
B
0 DNA
pCA4.0
pCMVNS1
pPTLR
pCMVNS1 +pPTLR
pCA4.0 +pPTLR
0 DNA
pCA4.0
pCMVNS1
pPTLR
pCMVNS1 +pPTLR
pCA4.0 +pPTLR
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
mMVM
ssMVM
dLR
mLR
ssLR
1 2 3 4 5 6 7 8 9 10 11 12
A9
COS-7
DpnI digested

Fig. 8B

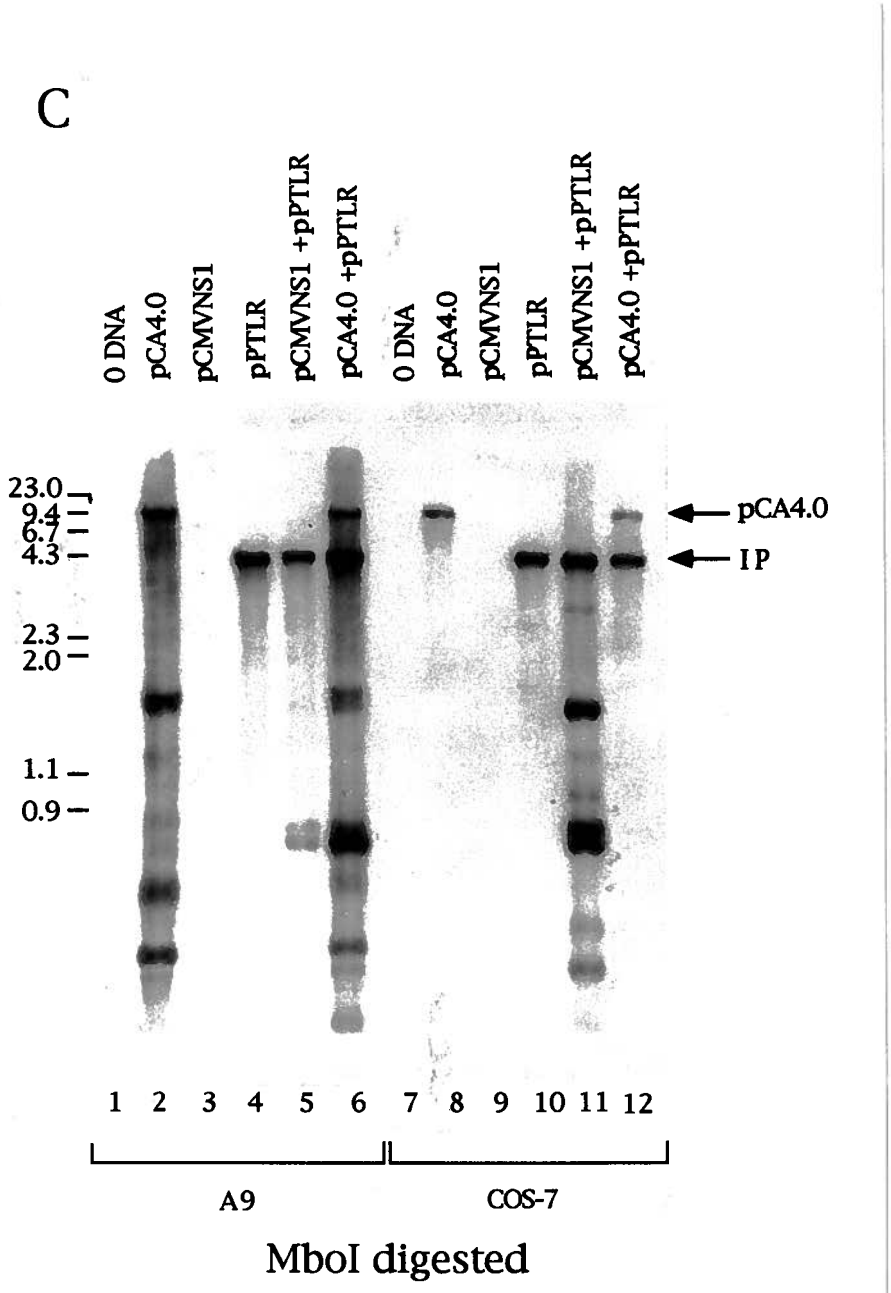
C
0 DNA
pCA4.0
pCMVNS1
pPTLR
pCMVNS1 +pPTLR
pCA4.0 +pPTLR
0 DNA
pCA4.0
pCMVNS1
pPTLR
pCMVNS1 +pPTLR
pCA4.0 +pPTLR
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
pCA4.0
IP
1 2 3 4 5 6 7 8 9 10 11 12
A9
COS-7
MboI digested

Fig. 8C

multiple discrete bands appear at approximately 1.1 (mLR) and 2.2 kbp (dLR) (Fig. 8A, lanes 5, 6, 11). Since these bands migrated approximately at the expected minigenomic mLR and dLR molecular weights and only appear in the presence of vectors expressing *trans*-acting viral factors, the viral minigenome encoded in pPTLR must be excised from the plasmid sequences and replicated in the host cells.

In order to show that these putative replicative intermediates have been replicated in eukaryotic cells, DNA samples were subject to digestion with Dpn I (Fig. 8B) and Mbo I (Fig. 8C) prior to Southern blot analysis. DNAs replicated in eukaryotic cells may be distinguish from plasmid DNA from bacterial sources by digestion with either Dpn I or Mbo I. Although these restriction enzymes are isoschizomers, recognizing and cutting at the GATC sequence, Dpn I only cleaves at the recognition site when the N-6 position of A residues is methylated on both strands. Since DNAs replicated in eukaryotic cells do not contain this methylation pattern and DNA replicated in the *E.coli* strains used to propagate plasmids in this study do contain these methylated A residues, DNA replicated in mammalian cells are resistant to Dpn I digestion whereas plasmid DNA is susceptible to Dpn I cleavage. In contrast, Mbo I does not cleave methylated plasmid DNA but does cut unmethylated DNA replicated in mammalian cells. When only pPTLR plasmid DNA was transfected into COS-7 or LA9 cells, the input plasmid DNA (IP) was shown to be both Dpn I susceptible (Fig. 8B, lane 4 and 10) and Mbo I (Fig. 8B, lane 4 and 10) resistant, confirming that the indicated DNA came from bacterial sources. The minigenomic replicative intermediates, mLR and dLR were shown to be Dpn I resistant (Fig. 8B, lane 5, 6 and 11) as well as Mbo I susceptible (Fig. 8B, lane 5, 6 and 11) confirming that DNAs contained within both bands have been replicated in the mammalian host cells. Wild type MVM(p) mRF (mMVM) also exhibited the same

digestion properties as mLR and dLR (Fig. 8B and C, lanes 2 and 6). In addition, it was also observed that higher molecular weight Dpn I resistant bands were present at approximately 1.2 kbp intervals in COS-7 cells (Fig. 8A and B, lane 11). It is likely that these molecules represent higher order replicative intermediates such as trimers, tetramers, etc. Previous analysis of intracellular concatemer RFs purified from MVM(p) infected cells consists of dimers, tetramers and octamers (Ward and Dadachanji, 1978). These are probably synthesized by consecutive primer extensions from the 3' OH hairpin termini. Although these intermediates were observed with the LR minigenome, other odd numbered intermediates such as trimers and pentamers were not found in MVM(p) infected cells. It may be possible that these odd numbered intermediates were generated when base concatemers such as tetramers and octamers are resolved into secondary concatemer products which are always smaller but are multiples of the unit minigenome length. For example, it may be possible that an octamer may be resolved into two other intermediates such as a trimer and a pentamer.

Although the replication of the minigenome in LA9 cells using either pMM984 or pCMVNS-1 and in COS-7 cells using pCMVNS-1 is observed readily, replication of the minigenome in COS-7 cells using pMM984 (Fig. 8A and B, lane 12) was relatively low since longer exposures were required to detect the presence of mLR and dLR (data not shown). Western blot analysis of NS-1 using a NS-1 specific monoclonal antibody, CE10 (Yeung *et al.*, 1991), showed that the expression of NS-1 in pMM984 transfected COS-7 cells was very low compared with pCMVNS-1 transfected COS-7 cells (Fig. 9, lane 5 vs. 6). It is not surprising that the level of expression of NS-1 in LA9 cells is greater when the infectious

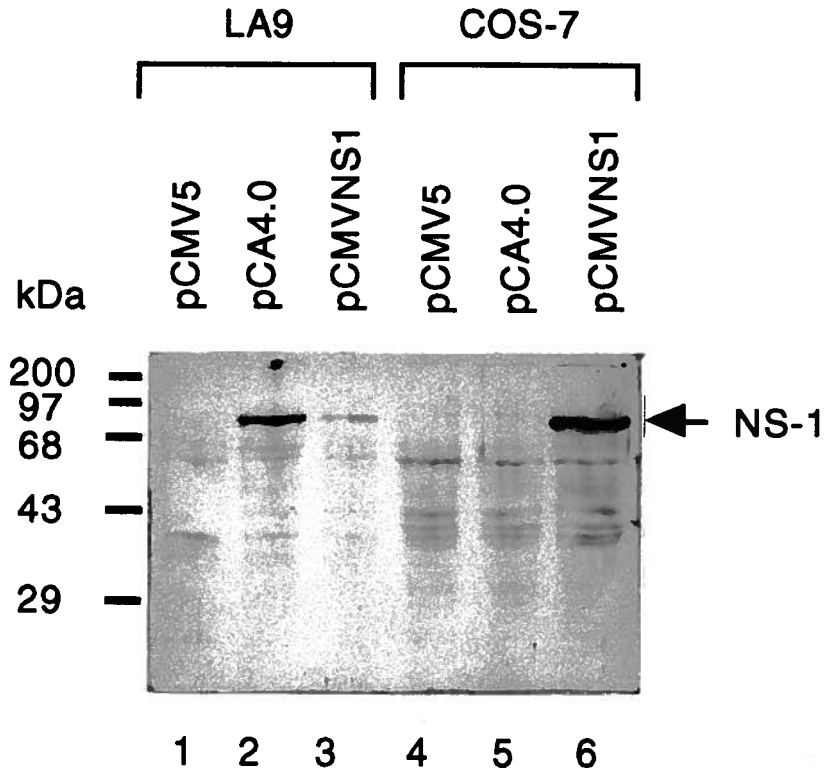

Fig. 9. Expression of NS-1 in LA9 and COS-7 Cells.

Protein lysates from pCMV5 (lanes 1 and 4), pCA4.0 (lanes 2 and 5) and pMM984 lanes 3 and 6) transfected LA9 and COS cells were probed for NS-1 using the western blot analysis. NS-1 specific CE10 monoclonal antibody was used as the primary antibody to probe proteins electroblotted onto a nitrocellulose membrane. The band corresponding to NS-1 as indicated and is only found in cells transfected with either pCMVNS-1 or pMM984.

clone, pCA4.0, was used rather that the SV40 based pCMVNS-1 (Fig. 9, lane 2 vs. 3) since the infectious clone is able to replicate in these cells. When the result of the western blot is compared to that of the replication assays, it appears that the level of viral DNA replication is more or less proportional to the level of NS-1 expression. Since replication of the LR minigenome was more extensive in LA9 cells with pCA4.0 and in COS-7 cells with pCMVNS-1 than the reciprocal experiments, subsequent co-transfection experiments were carried out using pCA4.0 in LA9 cells and pCMVNS-1 in COS-7 cells.

3.2. Analysis of the Termini of the mLR Replicative Intermedates

It was observed that at least 4 bands were present at the mLR position (designated mLR1, mLR2, mLR3 and mLR4) and at least 3 bands in the dLR position (designated dLR1, dLR2, and dLR3) (Fig. 8A and B, lanes 5, 6 and 11). Since previous analysis of intracellular viral DNA showed that the termini existed in both the covalently closed hairpin form and open extended form (Astell *et al.*, 1985; Cotmore *et al.*, 1989), it was suspected that the multiple banding patterns at the mLR and dLR position reflected this heterogeneity at viral termini of minigenomic replicative intermediates found in co-transfected cells. In order to investigate this possibility, DNA samples from Hirt lysates were digested with either exo III, an enzyme which digests double stranded DNA progressively from either a blunt end or 3' recessed end to single stranded DNA, or with exo III followed by S1 nuclease, an enzyme which digests single stranded DNA (Fig. 10). After exhaustive digestion with exo III, all mLRs were degraded to ssLR except for mLR4 in each set of Hirt samples (Fig. 10, lanes 6, 8 and 11). This experiment shows that mLR4 is devoid of 3' OH ends suggesting that this intermediate

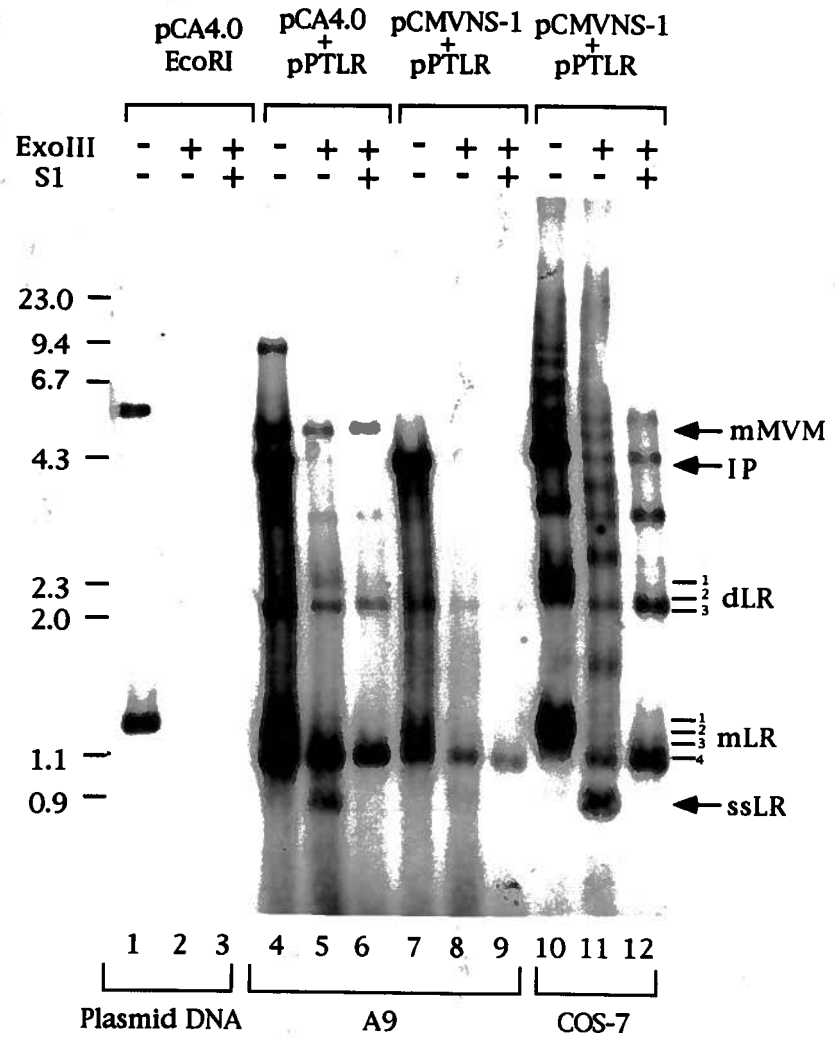

Fig. 10. Exonuclease III/ S1 Nuclease Digestion Patterns of the Minigenomic LR Replicative Intermediates.

Hirt DNA samples of the indicated transfection experiments or EcoRI linearized pCA4.0 plasmid were analyzed by nuclease digestions. Either undigested (lanes 1, 4, 7 and 10) , exoIII digested (lanes 2, 5, 8 and 11) or exoIII and S1 digested (lanes 3, 6, 9 and 12) DNA samples were analyzed by Southern blotting as described in the Materials and Methods.

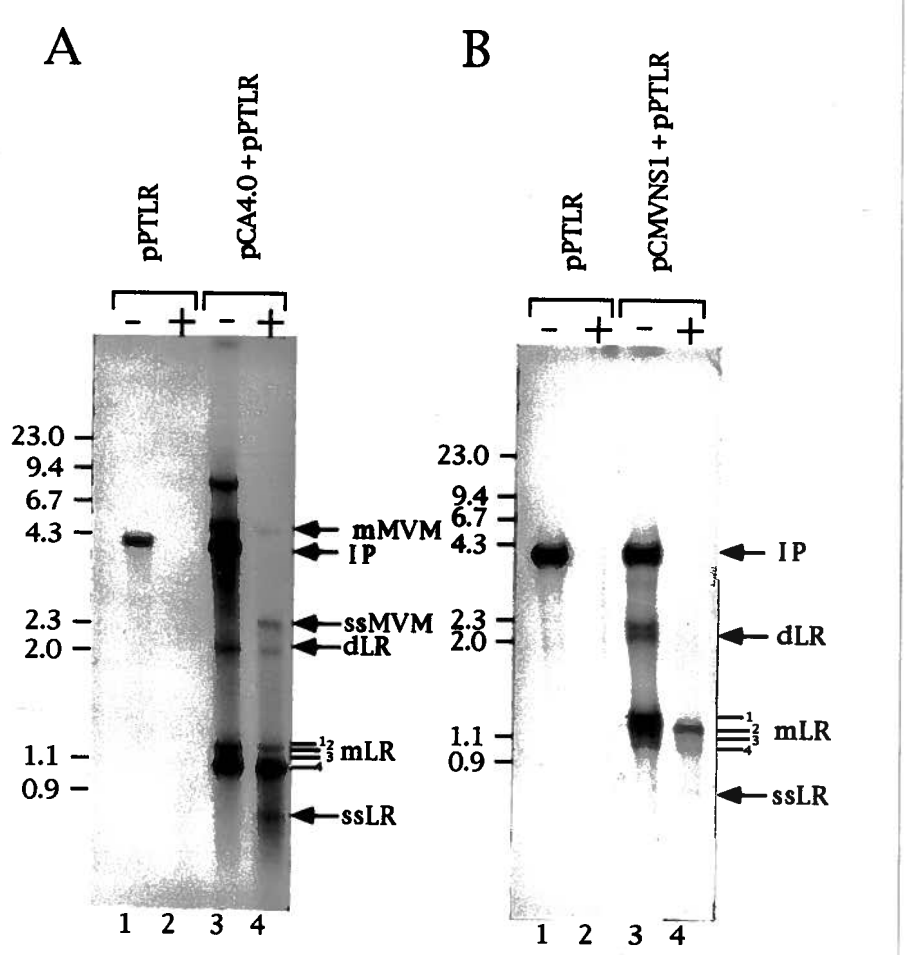

Fig. 11. Heat Denaturation/Quick Chill Properties of Minigenomic LR Replicative Intermediates

Hirt DNA samples from A9 transfected cells (A) or COS-7 transfected cells (B) were thermally denatured by incubation in boiling water for 5 minutes followed by a rapidly chilling in ice water for 5 minutes (lanes 2 and 4). These DNAs along with untreated samples (lanes 1 and 3) were analyzed by gel electrophoresis and Southern blotting. (C) indicates the proposed structures for the various mLR species. The rabbit ear hairpin structure represents the left hairpin, and the straight hairpin structure represents the right hairpin. The arrow tip represents the 3'OH end of the DNA molecules, a substrate for exo III digestions.

C

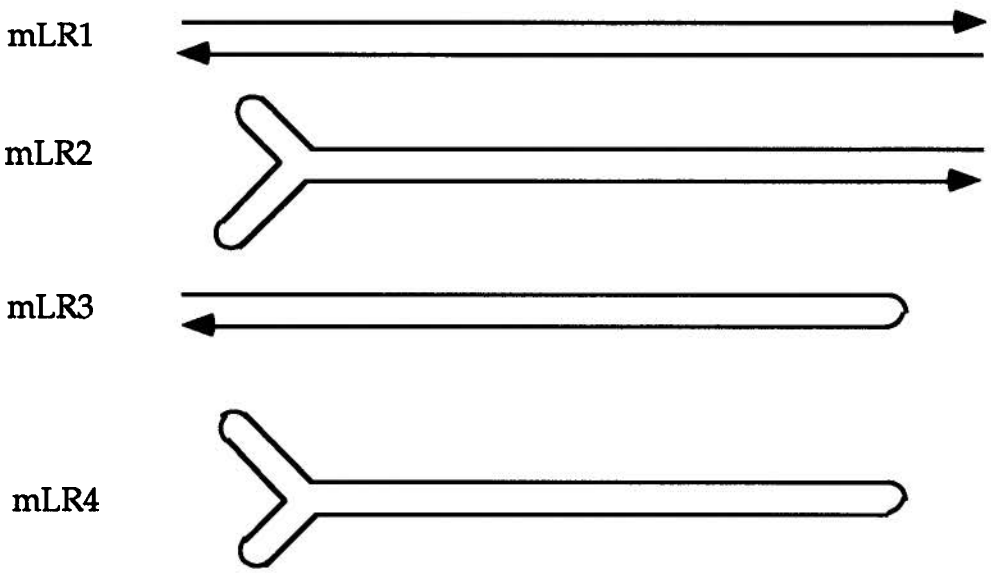

Fig. 11C

contains covalently closed hairpins at both termini whereas the other intermediates must contain at least one open 3' OH end. It was also observed that a portion of mMVM in transfected LA9 cells (Fig.9, lanes 4) and other higher molecular weight minigenomic intermediates in transfected COS-7 cells (Fig. 9, lanes 10-12) were resistant to exo III. The single stranded ssLR was confirmed to be mostly single stranded DNA since it was removed by S1 nuclease. In order to further characterize these intermediates, Hirt DNA samples were boiled and rapidly cooled on ice water to test for the presence of closed hairpin molecules (Fig. 11). Under these conditions, mLR1 molecules were denatured to ssLR material whereas mLR2, mLR3 and mLR4 were resistant to this treatment. Results from samples from transfected LA9 (Fig. 11A) and COS-7 cells (Fig. 11B) were the same. This analysis shows that mLR2, mLR3 and mLR4 molecules can snap back to their double stranded form via the hairpin sequences. These data also confirm that mLR4 contains covalently closed hairpins at both termini. Furthermore, these data in conjunction with that in Fig. 10 suggest that mLR2 and mLR3 contain one covalently closed hairpin at one terminus (boil/chill resistant) and an open extended terminal at the opposite terminus (exo III susceptible). Since the size of the right terminal hairpin (206 nt) is larger than the left terminal hairpin (115nts), it is likely that mLR2 contains the closed left hairpin and open extended right terminal and mLR3 contains the reverse configuration (Fig. 11C). Since it was also observed that a portion of replicative intermediates were exo III resistant at the dLR level (in both A9 and COS-7 cells) and higher multimer levels (in COS-7 cells), it suggests that there may also exist processed hairpin forms at each concatemer level, supporting the notion that base concatemers are processed to secondary concatemer forms.

3.3. Replication of Left Hand (3') Terminal Deletion Mutants.

All parvovirus sequences determined thus far contain terminal palindromic sequences which can potentially fold into a stem plus arms structure (Astell, 1990). Deletion analysis of the right hand (5') terminal palindrome of MVM(p) suggests that the potential to form the stem plus arms structure is required for viral DNA replication (Salvino *et al.*, 1991). Furthermore, analysis of AAV terminal repeats also suggests that the structure of the stem plus arms is required for viral DNA replication (Lefebrve *et al.*, 1984). In order to determine the structural requirements of the 3' terminal palindrome, a nested set of unidirectional deletions originating at the extreme 3' terminal palindrome was constructed (Fig. 12). Deletion clones with deletions of 11, 25, 30, 56 and 77 nt, designated dl12, dl26 dl31, dl57 and dl 78, respectively, of the viral DNA sequences were chosen . Removal of the first 11 nt removes only a small portion of one strand of the stem of the hairpin. Deletion of 25 or 30 nt is predicted to remove a greater portion of the stem structure and alter or remove the bubble structure found in the middle of the stem. Further deletion of 56 and 77 nt removes completely one strand of the stem and bubble feature, but the former deletion only removes one arm while the latter deletion removes both arms.

These deletions were then assembled into minigenome constructs similar to pPTLR (except that the original left terminal fragment is shorter by 3 bp, see Materials and Methods) and transfected into LA9 and COS-7 cells . When the deletion mutants were co-transfected into LA9 cells with pMM984, all minigenomes were able to replicate to approximately comparable levels (Fig.12B). It was surprising to see that deletion mutants in which either

A

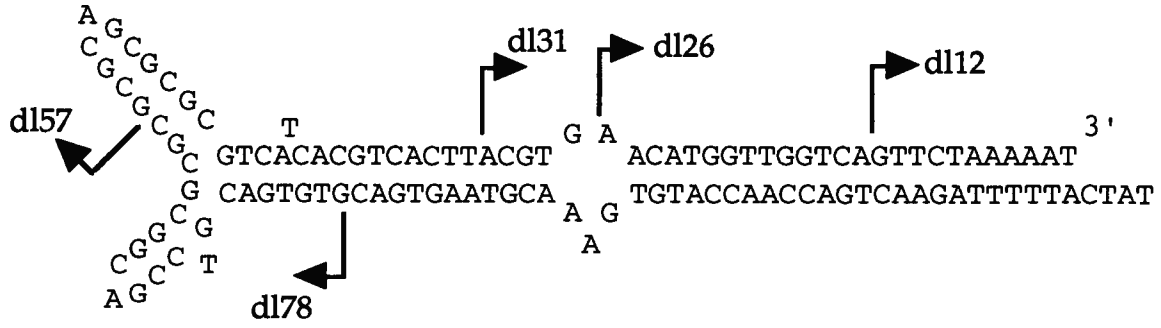

Fig. 12. Replication of Left Hand (3') Terminal Deletion Mutants.

The schematic diagram of the 3' hairpin sequence is shown (A) with the deletion endpoints indicated by the arrows. Minigenomic constructs containing the deletions in (A) were transfected into LA9 (B) and COS-7 cells (C, D and E). The mRF and dRF of wild type MVM(p) (mMVM and dMVM) and the mutant minigenomes (mLR and dLR) are as indicated. IP represent input plasmid DNA. (A) and (B) show undigested Hirt DNA samples from transfected LA9 and COS-7 cells. In addition, Dpn I (D) and Mbo I digested (E) Hirt DNA samples from transfected COS-7 cells are shown. In the case of the Mbo I digest, the Southern blot was probed with a ^{32}P radiolabeled 0.4 kbp BamHI/EcoRV fragment containing the 3' terminal sequences. The bands indicated on this figure identify the 3' bridge dimer fragment (3' br) and the covalently closed and open extended 3' hairpin. Although the pPTLR and pPTLRdl12 minigenomes produce both types of 3' termini, pPTLRdl26 and pPTLRdl31 only generate covalently closed 3' hairpins.

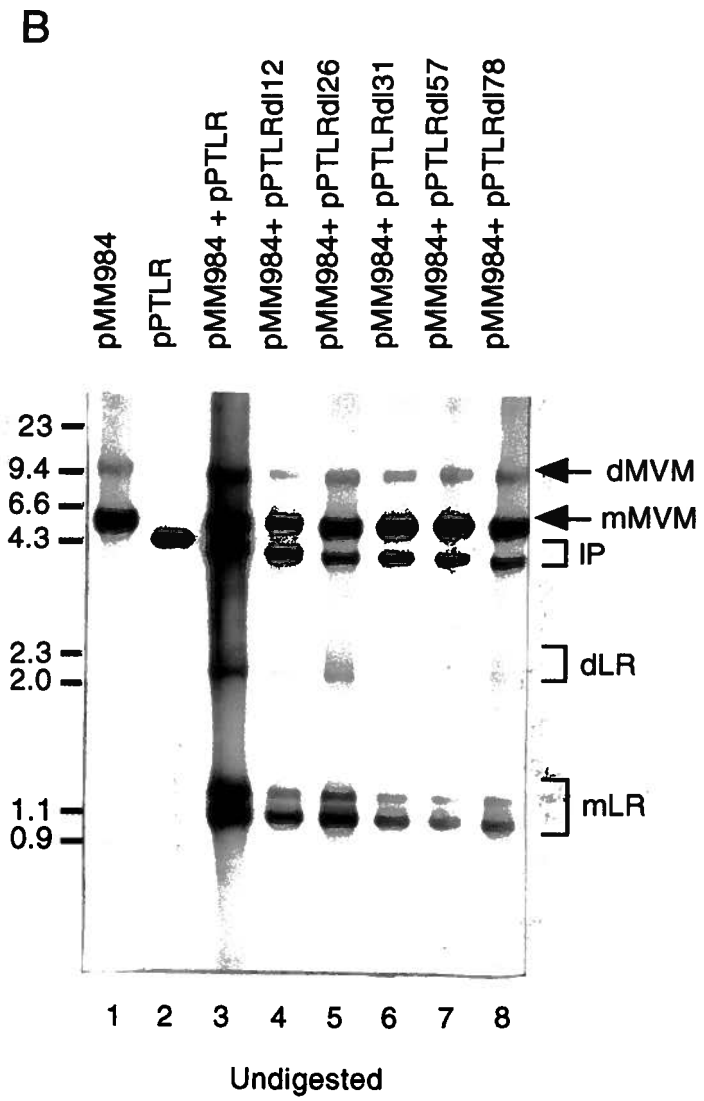
B
pMM984
pPTLR
pMM984 + pPTLR
pMM984+ pPTLRdl12
pMM984+ pPTLRdl26
pMM984+ pPTLRdl31
pMM984+ pPTLRdl57
pMM984+ pPTLRdl78
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
dMVM
mMVM
IP
dLR
mLR
1 2 3 4 5 6 7 8
Undigested

Fig. 12B

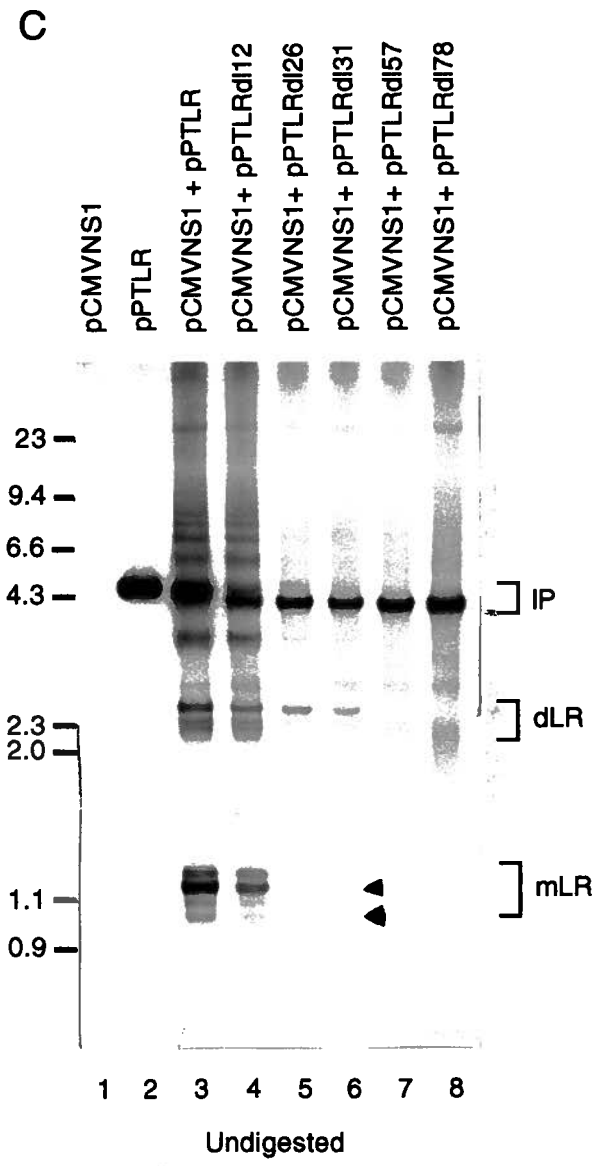
C
pCMVNS1
pPTLR
pCMVNS1 + pPTLR
pCMVNS1+ pPTLRdl12
pCMVNS1+ pPTLRdl26
pCMVNS1+ pPTLRdl31
pCMVNS1+ pPTLRdl57
pCMVNS1+ pPTLRdl78
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
IP
dLR
mLR
1 2 3 4 5 6 7 8
Undigested

Fig. 12C

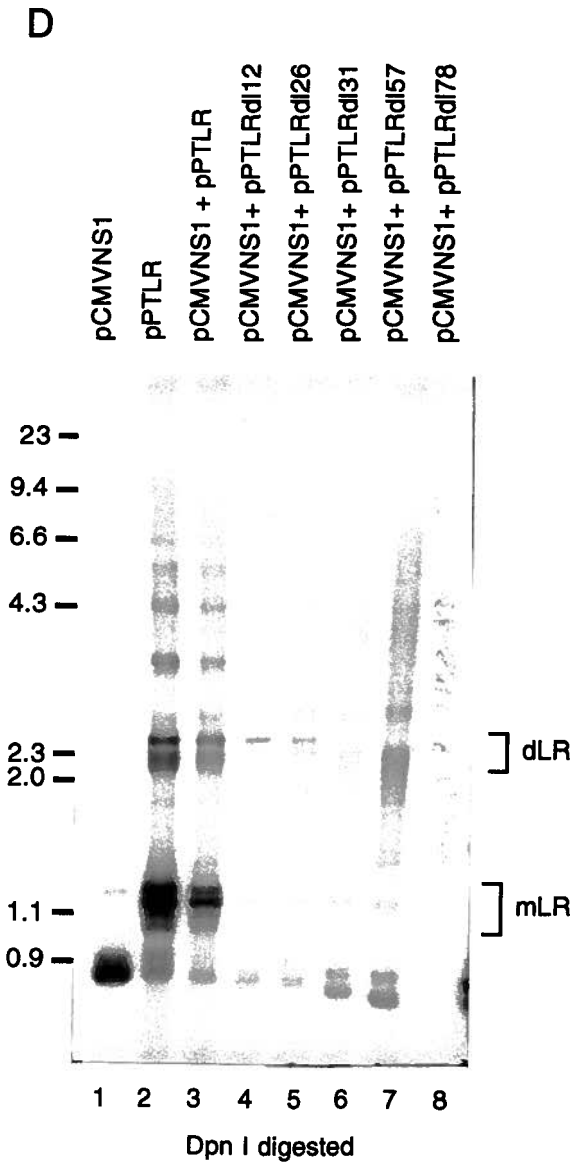
D
pCMVNS1
pPTLR
pCMVNS1 + pPTLR
pCMVNS1+ pPTLRdl12
pCMVNS1+ pPTLRdl26
pCMVNS1+ pPTLRdl31
pCMVNS1+ pPTLRdl57
pCMVNS1+ pPTLRdl78
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
dLR
mLR
1 2 3 4 5 6 7 8
Dpn I digested

Fig. 12D

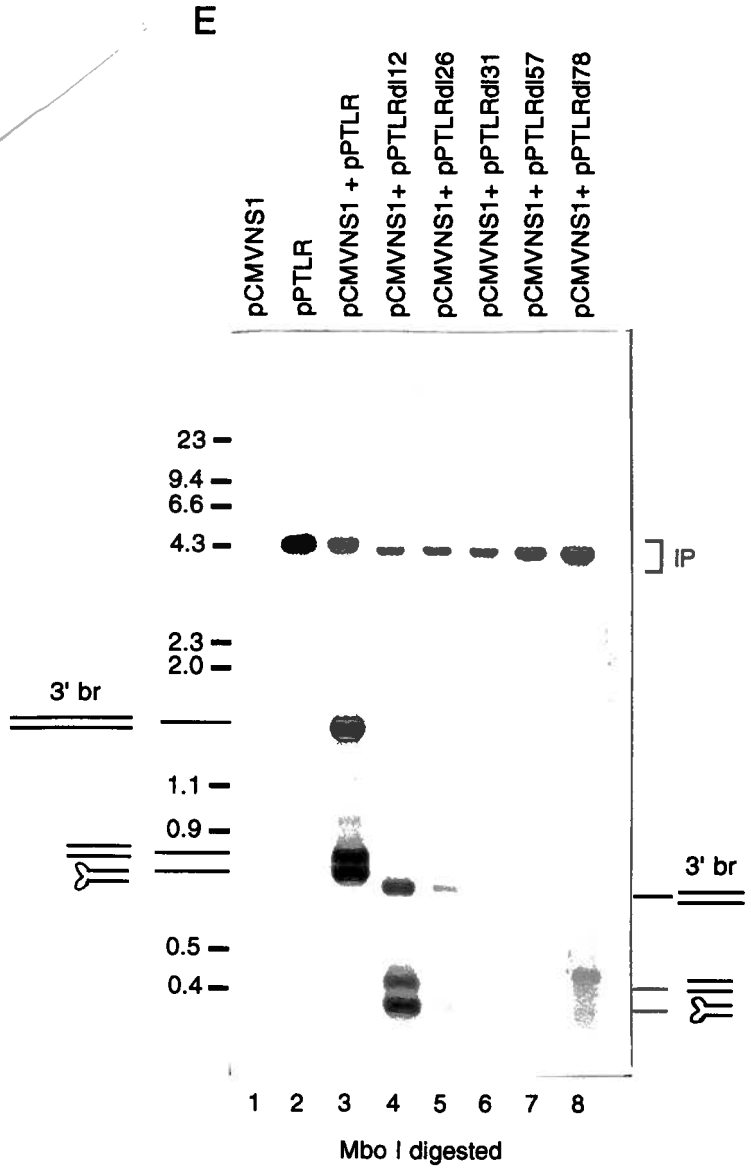
E
pCMVNS1
pPTLR
pCMVNS1 + pPTLR
pCMVNS1+ pPTLRdl12
pCMVNS1+ pPTLRdl26
pCMVNS1+ pPTLRdl31
pCMVNS1+ pPTLRdl57
pCMVNS1+ pPTLRdl78
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
0.5
0.4
IP
3' br
3' br
1 2 3 4 5 6 7 8
Mbo I digested

Fig. 12E

one arm (pPTLRdl57) or two arms (pPTLRdl78) were removed from the stem plus arms structure were able to replicate as effectively as pPTLRdl12 which only removes a small portion at the base of the stem (Fig. 12B). In addition, it appears that the mLR intermediates of all the mutant 3' palindromes are of approximately the same size. This result suggests that all mutants were able to repair their hairpin sequences to the wild type sizes. Although it is easy to envision that the pPTLRdl12 mutant is able to repair its hairpin sequences by using the existing hairpin to prime DNA synthesis to fill in the rest of the missing sequences, this mechanism can not be applied to the pPTLRdl78 mutant since the possibility of hairpin formation is highly unlikely. It is more probable that the repair of pPTLRdl78 mutant may involve a recombination mechanism which uses wild type MVM(p) sequences derived from pMM984 as a template for repair.

In order to determine the replication levels of the 3' palindrome deletion mutants in the absence of the wild type viral sequence, the minigenomic constructs were co-transfected into COS-7 cells with pCMVNS-1 (Fig. 12C, D, E). Replication of pPTLRdl12 replicates to levels similar to pPTLR (Fig.12C, lane 3 vs. 4). In contrast, pPTLRdl26 and pPTLRdl31 replicated to a very low level (1-5%) (Fig.12C and D, lanes 3 vs 5 and 6). Interestingly, there is a considerable level of the dLR generated by these mutants despite exhibiting relatively low levels of mLR. It also appears that only mLR2 and mLR4 (arrowheads, Fig. 12C, lanes 5 and 6) are the only detectable monomers species generated by these two mutants. Both pPTLRdl57 and pPTLRdl78 also generated very low levels of mLR and no detectable dLR (Fig.12C and D, lanes 7 and 8). Thus, in the absence of wild type viral hairpin sequences, only pPTLRdl12 was able to replicate to levels comparable with pPTLR. It is probable that the pPTLRdl12 mutant is able to repair its 3' hairpin sequences probably by primer extension of the remaining hairpin structure. It was

interesting that both pPTLRdl26 and pPTLRdl31 could not replicate as efficiently as pPTLR or pPTLRdl12 even though the formation of a useable hairpin at the 3′ end would be predicted to form. Both pPTLRdl26 and pPTLRdl31 mutations also disrupt the bubble structure located at nt 25 and 26. If the hairpin is repaired by primer extension, the mismatches which form the bubble in the wild type sequence will be altered such that duplex DNA will be generated instead. Since these mutants are able to generate dLR, but not all species of mLR, it may be possible that these mutants exert their effect during the resolution of dLR. This hypothesis is consistent with data from a Southern blot of Mbo I digests of Hirt DNA probed the left (3′) terminal sequences (Fig 12E). Clearly pPTLR gives rise to three prominent bands corresponding to the 3' dimer bridge (3' br, 1.4kbp), open left hairpin fragment (0.81kb) and the covalently closed left hairpin (0.75 kbp) fragment (Fig 12E, lane 3). Similarly, pPTLRdl12 generated the same banding pattern but the fragments migrate at lower molecular weights since an extra Mbo I site was introduced during the cloning procedures (Fig 12E, lane 4). These data suggest that both pPTLR and pPTLRd12 clones generate both the open extended (0.41 kbp) and covalently closed hairpin forms (0.35 kbp) at the left terminus. In contrast, pPTLRdl26 and pPTLRdl31 produced a 3' dimer bridge fragment (3' br, 0.7 kbp) and a band which co-migrates with the covalently closed hairpin. Interestingly, the band corresponding to the extended left hairpin is not present, suggesting that mLRs with extended hairpin conformation are either not present or present at undetectable levels. It may be possible that replicative intermediates containing an open extended left end might not be generated because the resolution of dLR type molecules may be affected by the sequence changes during the repair of the 3' palindrome of these mutants. Further deletion of one (pPTLRdl57) or both (pPTLRdl78) arms of the left hairpin clearly inhibits viral DNA replication even further. Although no dimer replicative intermediates were observed, a low level of

a single species of mLR exists. It may be possible that these intermediates are excised from the plasmid sequences and are allowed to proceed with synthesis of a monomer species by an unknown mechanism possibly using the intact right hand hairpin sequences to prime DNA synthesis. The structures of these intermediates were not examined.

3.4. Analysis of Internal Right End (IRE) Deletions

Although it is clear that the terminal palindrome sequences are essential for MVM(p) DNA replication, it is not clear whether sequences internal to the right or left palindromes is required. Some evidence has been reported that the 65 bp tandem repeat near the right terminus was important for DNA replication (Salvino *et al.*, 1991). It is curious to note that the wild type immunosuppressive variant of MVM(p), MVM(i), has only one copy of the 65 bp element (Sahli *et al.*, 1985; Astell *et al.*, 1986). It was shown that MVM(i), which normally infects T lymphocytes, could acquire fibrotropism by changing codons 317 and 321 of the MVM(i) VP-2 gene to the MVM(p) equivalent codons (Ball-Goodrich and Tattersall, 1992). This fibrotropic mutant virus and wild type MVM(p) were able to replicate their DNA equally well in LA9 fibroblasts (Ball-Goodrich and Tattersall, 1992). Although this result suggests that the 65 bp repeat may not be essential for MVM DNA replication its role has not been elucidated. In order to perform a more comprehensive deletion analysis in this region, a nested set of unidirectional exo III deletions was created in the LR minigenome using the unique Xba I site as the origin of deletion (Fig.13B). In A9 cells it was shown that a LR minigenome containing a deletion of nt 411-4436

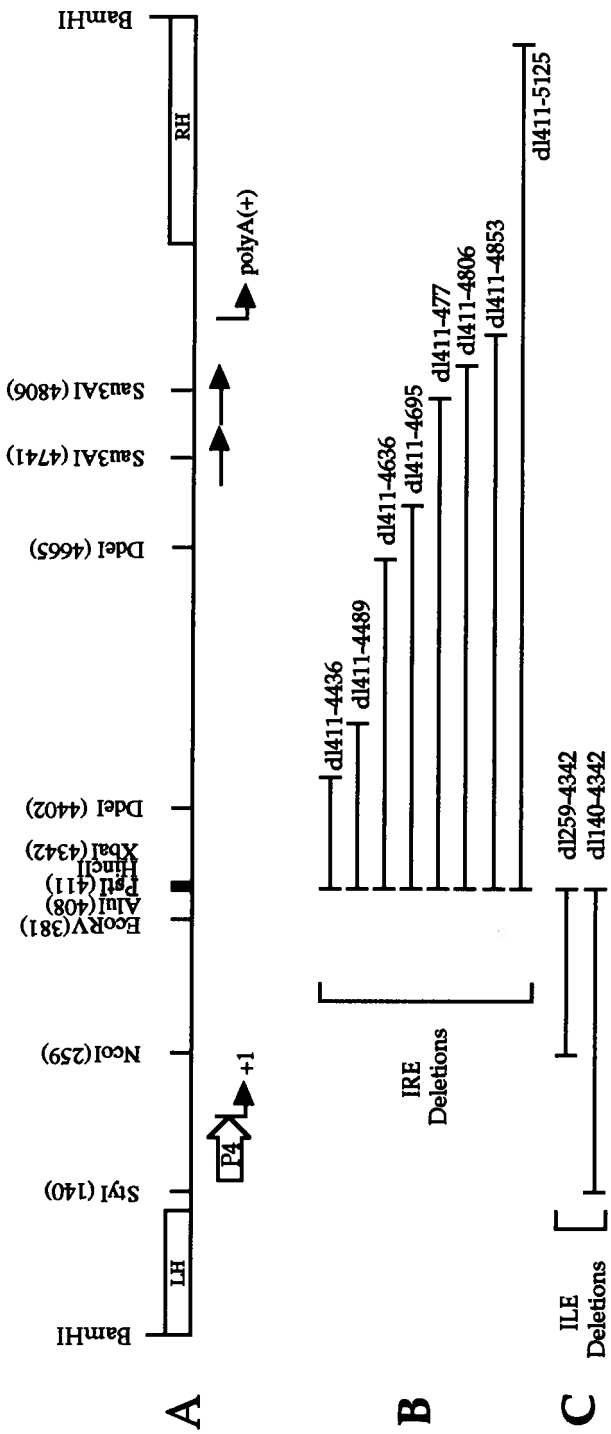

Fig. 13. Schematic Diagram of Internal Right End (IRE) and Left end (ILE) Deletion Mutants.

(A) represents the parental pPTLR construct used to generate the IRE (B) and ILE deletion (C) mutants. The size of each deletion (dl) is indicated by the size of the horizontal bar and the corresponding nucleotide positions. IRE deletions were generated using the XbaI(4342) site as the origin of unidirectional exo III deletions. ILE deletions were obtained by fusing the Hinc II site, adjacent to the PstI(411) site, with either the NcoI (259) or StyI(140) site of the MVM genome.

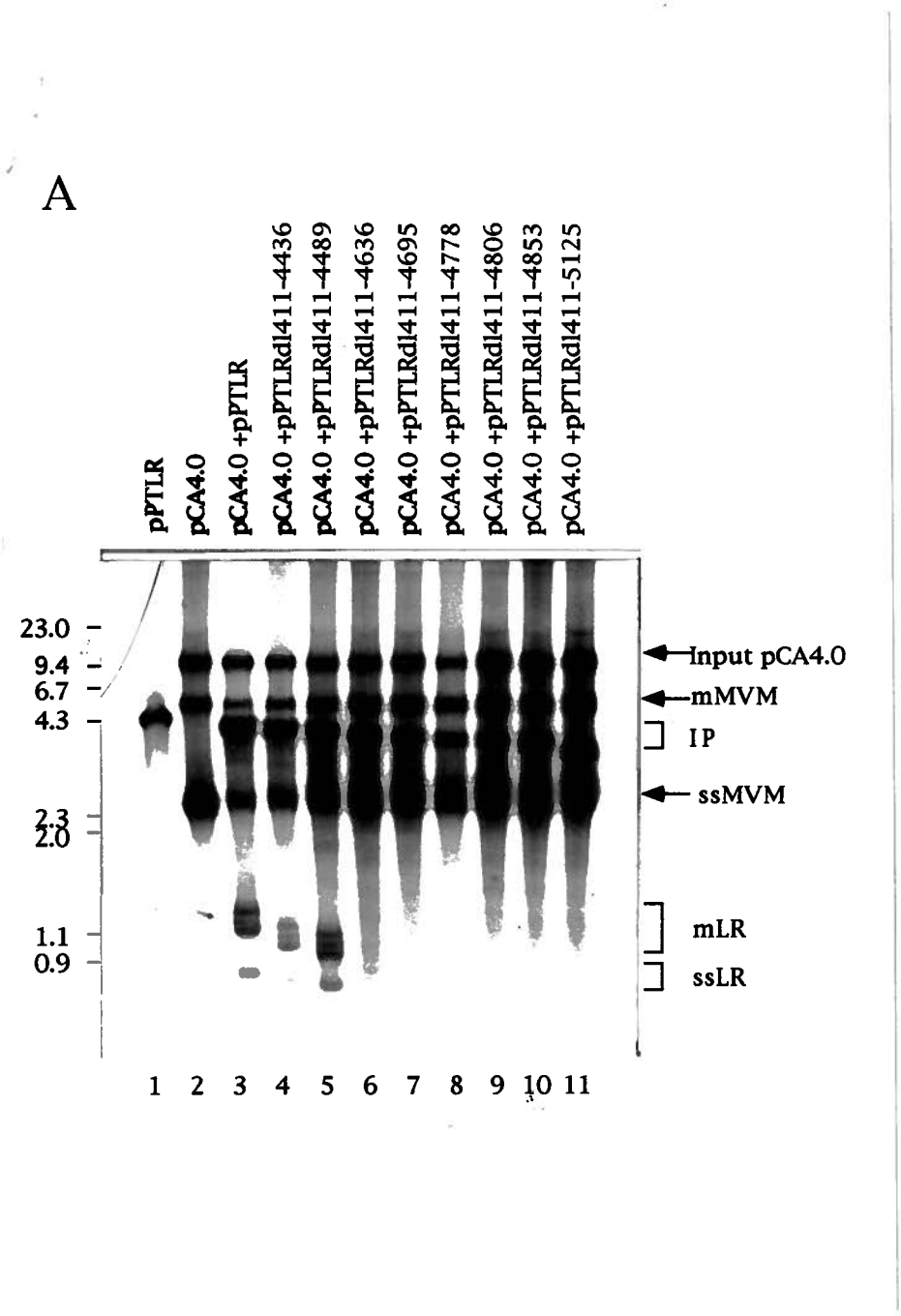

Fig. 14 Replication of Internal Right End (IRE) Deletion Mutants.

The IRE deletion mutants (see Fig.13 for description) were linearized with EcoRI before transfection into either A9 cells (A) or COS-7 cells (B). Hirt DNA samples were analyzed by gel electrophoresis and Southern blotting. The immobilized DNA was probed with a left terminal BamHI/EcoRV (381) ^{32}P radiolabeled restriction fragment. Replication was monitored by the appearance of mLRs.

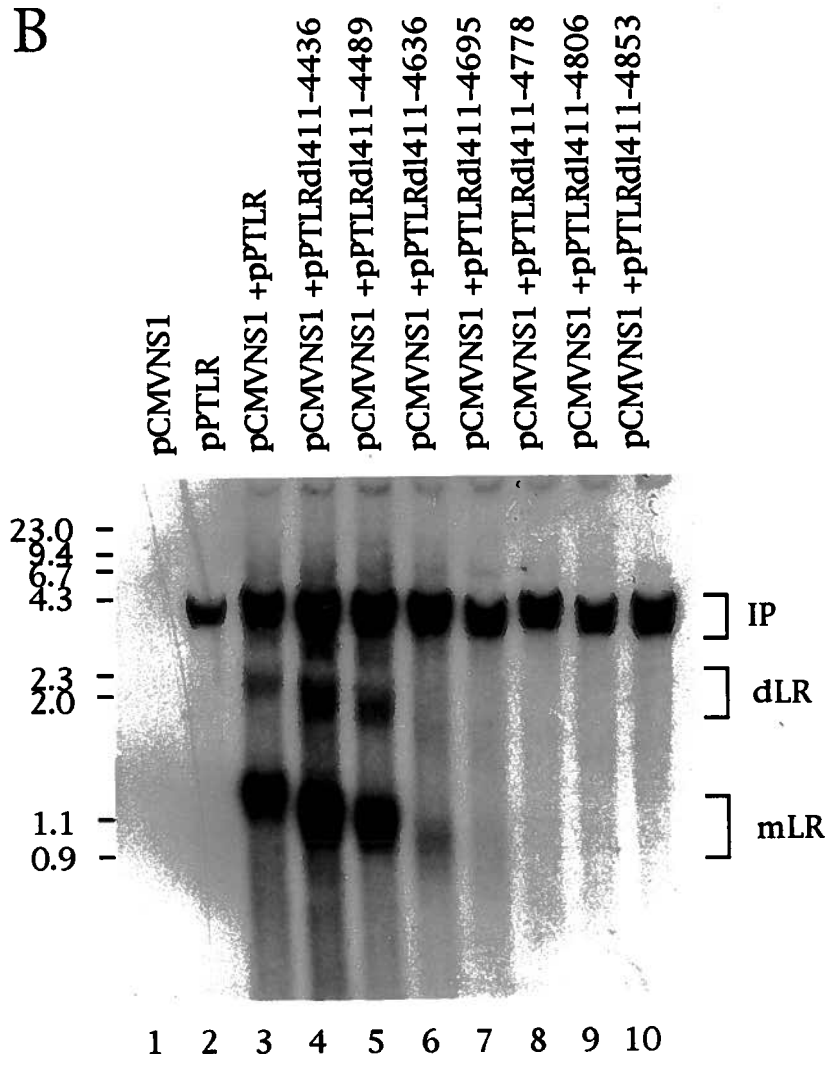
B
pCMVNS1
pPTLR
pCMVNS1 +pPTLR
pCMVNS1 +pPTLRdl411-4436
pCMVNS1 +pPTLRdl411-4489
pCMVNS1 +pPTLRdl411-4636
pCMVNS1 +pPTLRdl411-4695
pCMVNS1 +pPTLRdl411-4778
pCMVNS1 +pPTLRdl411-4806
pCMVNS1 +pPTLRdl411-4853
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
IP
dLR
mLR
1 2 3 4 5 6 7 8 9 10

Fig.14B

and nt 411-4489 (Fig. 14A, lane 4 and 5) did not affect replication compared to the LR control (lane 3), but when the deletion was extended to nt 4636 (Fig. 14A, lane 6), replication was abolished. Similarly, in COS-7 cells (Fig.14B), LR minigenomes containing deletions of nt 411-4436 and nt 411-4489 (Fig. 14B, lane 4 and 5) replicated as well as the pPTLR control (lane 3). Unlike in LA9 cells, the LR minigenome deletion of nt 411-4636 was able to replicate, but at a much lower level than that of the pPTLR minigenome in COS-7 cells (Fig.14A and B, lane 6). Extension of the deletion to nt 4695 (Fig.14 B, lane 7) totally abolished replication of the LR minigenome. Although ability of dl411-4636 to replicate in COS-7 cells but not A9 cells may reflect that the COS-7 cell system is a more sensitive replication assay system than the A9 cell system, the basis for this difference is unknown. In addition, the data from this analysis suggested to us that there may be two specific DNA elements found between nt 4489-4436 (element A) and 4636-4695 (element B) which are required for efficient MVM DNA replication.

In order to confirm this hypothesis, I attempted to rescue the replication phenotype of dl411-4636 and dl411-4695 by inserting the MVM(p) Xba I (4342)/Sau3a (4741) fragment (SX fragment) into the unique EcoRV (381) site, a site common to all the IRE deletion minigenomes (Fig. 15). When the MVM(p) specific fragment was inserted into the dl411-4636 and dl411-4695 mutants, replication was restored to control LR levels in both A9 (Fig. 16A, lanes 4-6) and COS-7 cells (Fig. 16B, lanes 4-6). It was also observed that the ability of the MVM(p) specific fragment to rescue the dl411-4636 mutant was orientation independent since dl411-4636SXL and dl411-4636SXR replicated to similar levels in A9 and COS-7 cells (Fig. 16A and B, lanes 4 vs 5). In addition, I also tried to

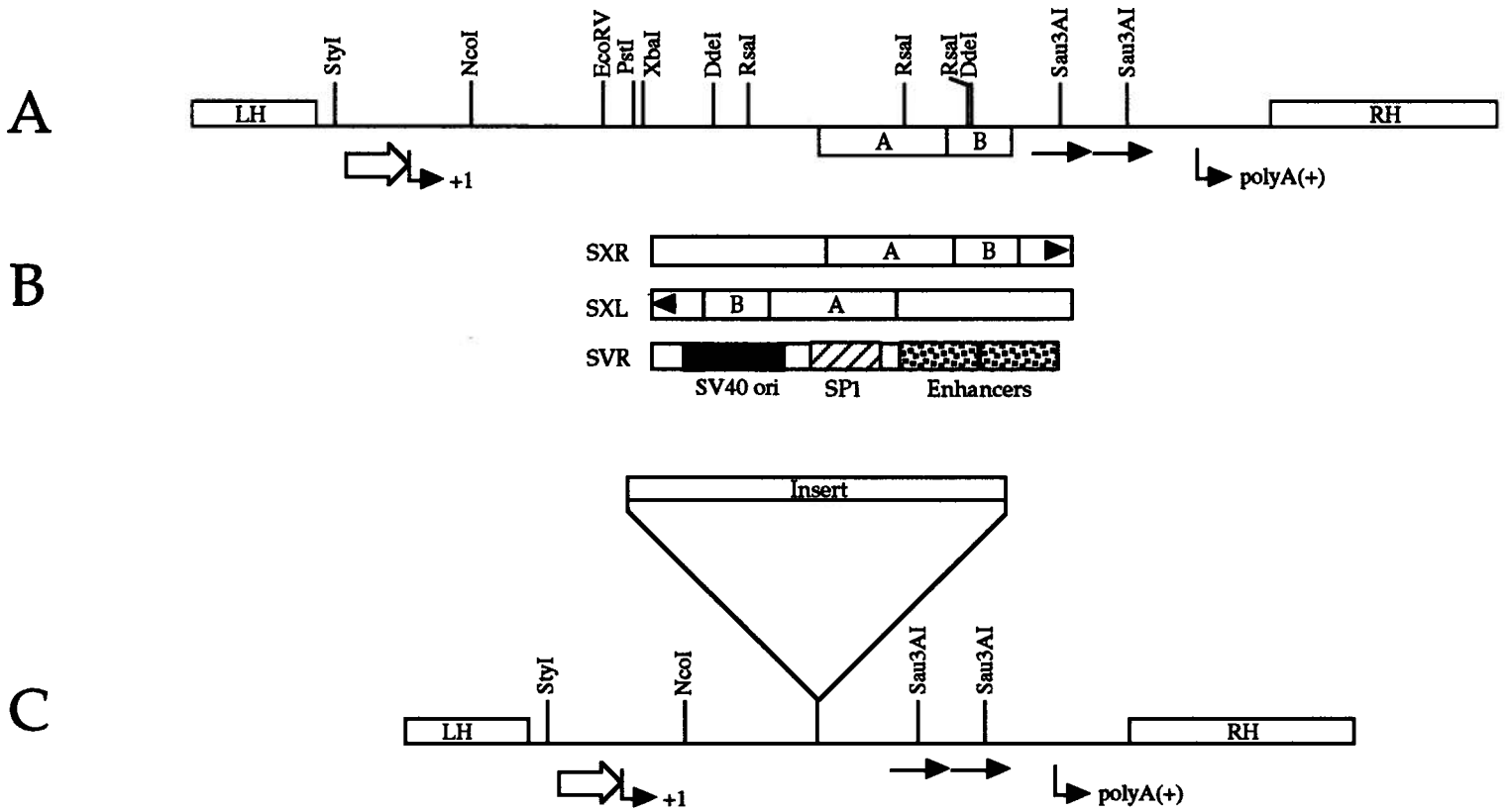

Fig. 15. Schematic Diagram of DNA Fragments Used to Rescue Replication of IRE Deletion Mutants.

(A) Represents the pPTLR construct. (B) Two restriction fragments were used to rescue the IRE deletion mutants. The MVM insert is the XbaI(4342) /Sau3a(4741) fragment which contains both elements A (nt 4489-4636) and B (nt 4636-4695). SXR and SXL insertions indicate the relative orientation of the XbaI(4342) /Sau3a(4741) restriction fragment. The SV40 insert (SVR) was the BglI(5235)/PvuII(270) fragment of the SV40 genome. This fragment contains the entire SV40 control region including the transcriptional enhancers found in the 72 bp tandem repeat (stippled boxes), 6 SP1 binding sites (cross hatched box) and minimal origin of SV40 DNA replication (black box) . (C) A single EcoRV (381) site in the IRE deletion mutants was used to clone either the SV40 (SVR mutants) or MVM (SXR or SXL mutants) restriction fragments.

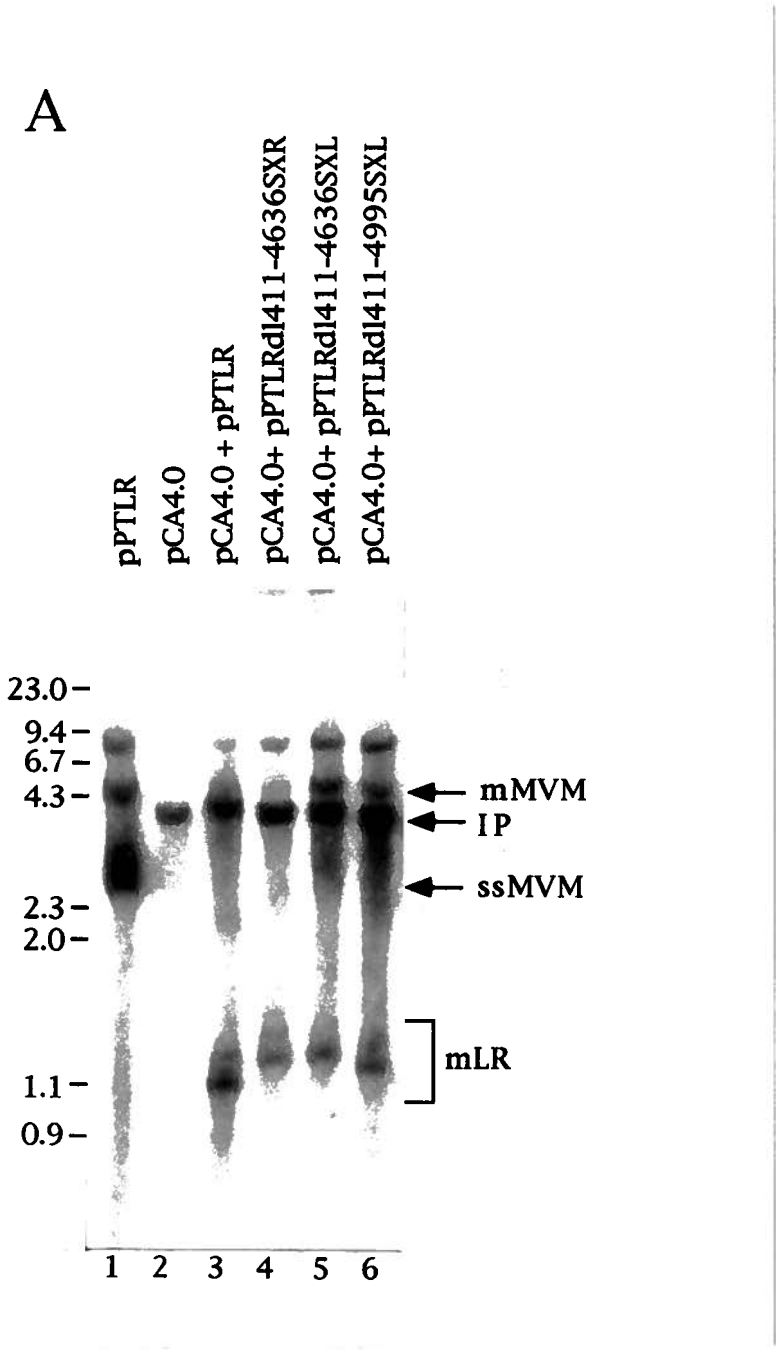

Fig. 16. Replication of IRE SXL and SXR Rescue Mutants.

The MVM(p) specific XbaI(4342)/Sau3a(4741) SX fragment was cloned into IRE deletion mutants dl411-4636 and dl411-4695 in either orientation (Fig.15). The resulting SXL and SXR mutants were linearized with EcoRI before transfection into A9 cells (A) or COS-7 cells (B). Hirt DNA samples were analyzed by Southern blotting and probed as in Fig.14.

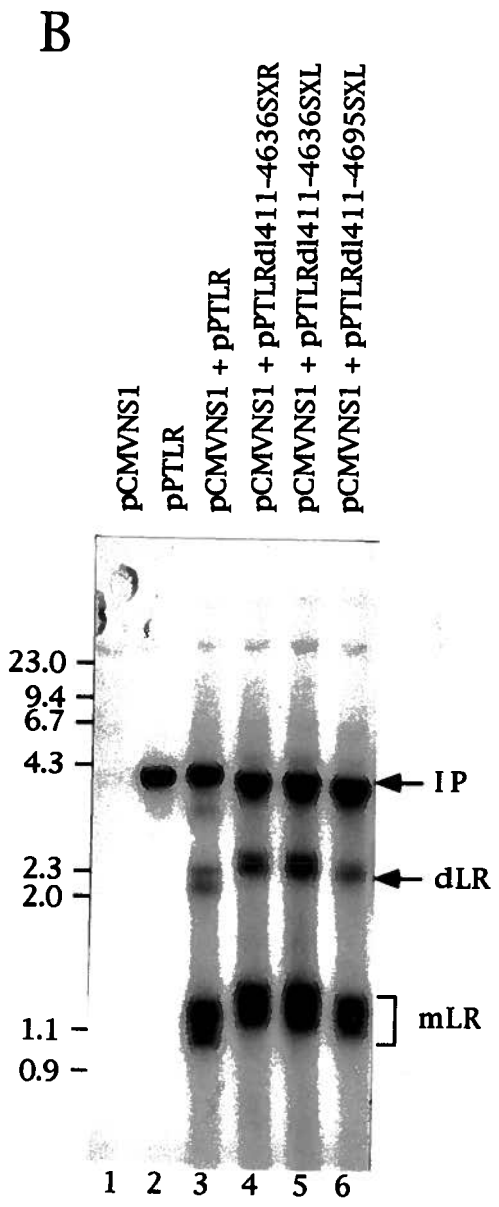
B
pCMVNS1
pPTLR
pCMVNS1 + pPTLR
pCMVNS1 + pPTLRdl411-4636SXR
pCMVNS1 + pPTLRdl411-4636SXL
pCMVNS1 + pPTLRdl411-4695SXL
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
IP
dLR
mLR
1 2 3 4 5 6

Fig. 16B

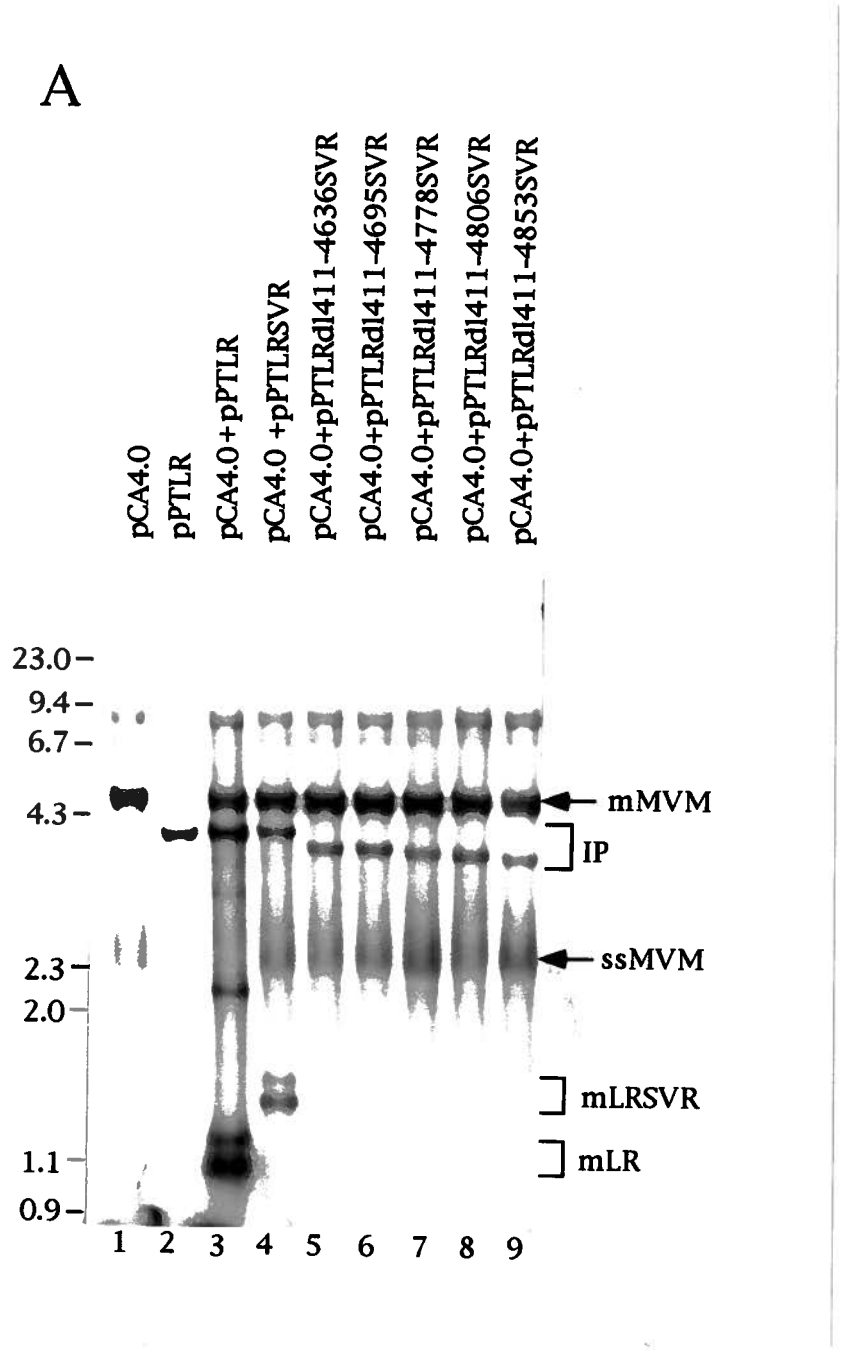

Fig. 17. DNA Replication of IRE SVR Rescue Mutants.

The SV40 control region was cloned into replication defective IRE deletion mutants as well as the parental pPTLR vector (Fig.15). The resulting SVR mutants were linearized with EcoRI before transfection into either A9 cells (A) or COS-7 cells (B). Hirt DNA samples were analyzed by Southern blotting and probed as in Fig.14.

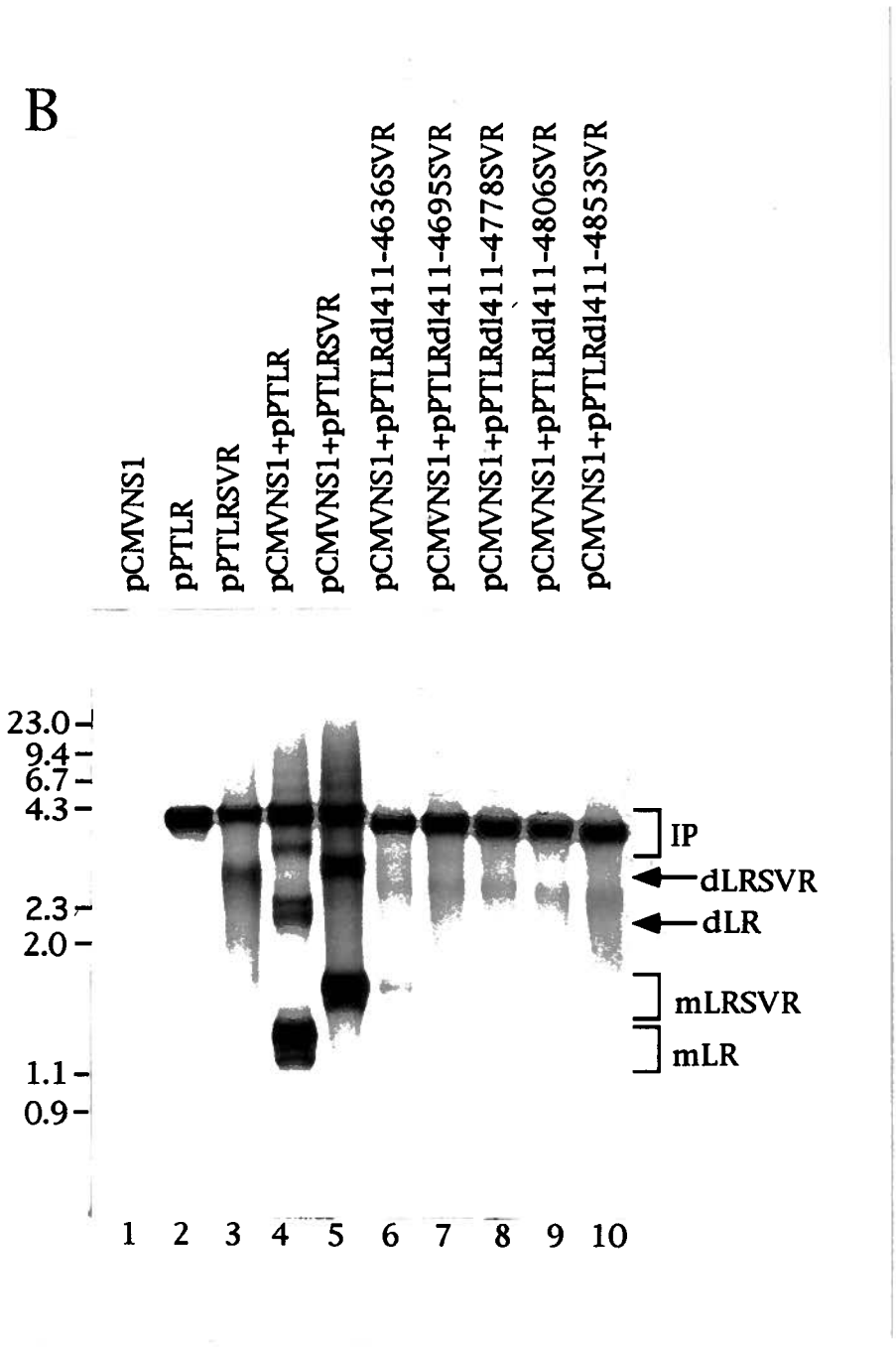
B
pCMVNS1
pPTLR
pPTLRSVR
pCMVNS1+pPTLR
pCMVNS1+pPTLRSVR
pCMVNS1+pPTLRdl411-4636SVR
pCMVNS1+pPTLRdl411-4695SVR
pCMVNS1+pPTLRdl411-4778SVR
pCMVNS1+pPTLRdl411-4806SVR
pCMVNS1+pPTLRdl411-4853SVR
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
IP
dLRSVR
dLR
mLRSVR
mLR
1 2 3 4 5 6 7 8 9 10

Fig. 17B

rescue replication of the defective minigenomes by introducing the well characterized SV40 control region (Fig. 15). In Fig. 17, the results show that the SV40 control region was not able to rescue the replication defective IRE deletion mutants in either A9 cells (Fig. 17A, lanes 5-9) or COS-7 cells (Fig. 17B, lanes 7-10) since no replication intermediates were detected at the expected sizes. It was also noted that the dl411-4636 SVR mutation replicates at a low level in COS-7 cells (Fig. 17B, lane 6) but not A9 (Fig. 17A, lane 7) cells. The parental minigenome, dl411-4636, also exhibited the same phenotypes (Fig.14B, lane 6). It was concluded that the insertion of the SV40 fragment is a neutral mutation since its insertion into the LR control or dl411-4636 did not affect the replication in COS-7 cells. Insertion of the SVR fragment into pPTLR appeared to have a small negative influence on the replication of the resulting minigenome, pPTLRSVR, when the results are normalized with respect to the input plasmid (IP) DNA band (Fig.17A, lane 3 vs. lane 4). It was predicted that the SV40 origin of replication would have a positive effect if any on the replication of SVR mutants in COS-7 cells since they express the SV40 T-Ag which initiates SV40 DNA replication. However, this was not apparent in Fig. 17B and may be due to the fact that the input DNA was linearized prior to transfection. The last two experiments, taken together, suggested that the replication defective phenotype of the two deletion mutants, dl411-4636 and dl411-4695, was not due to the fact I have reduced the size of the minigenome, but due to the deletion of specific MVM(p) DNA elements, A and B, which lie between nt 4489-4636 and 4636-4695, respectively.

In order to further define the *cis*-acting sequence in this region, two small Rsa I fragments, designated Rsa A (nt 4489-4579) and Rsa B (nt 4579-4665), found

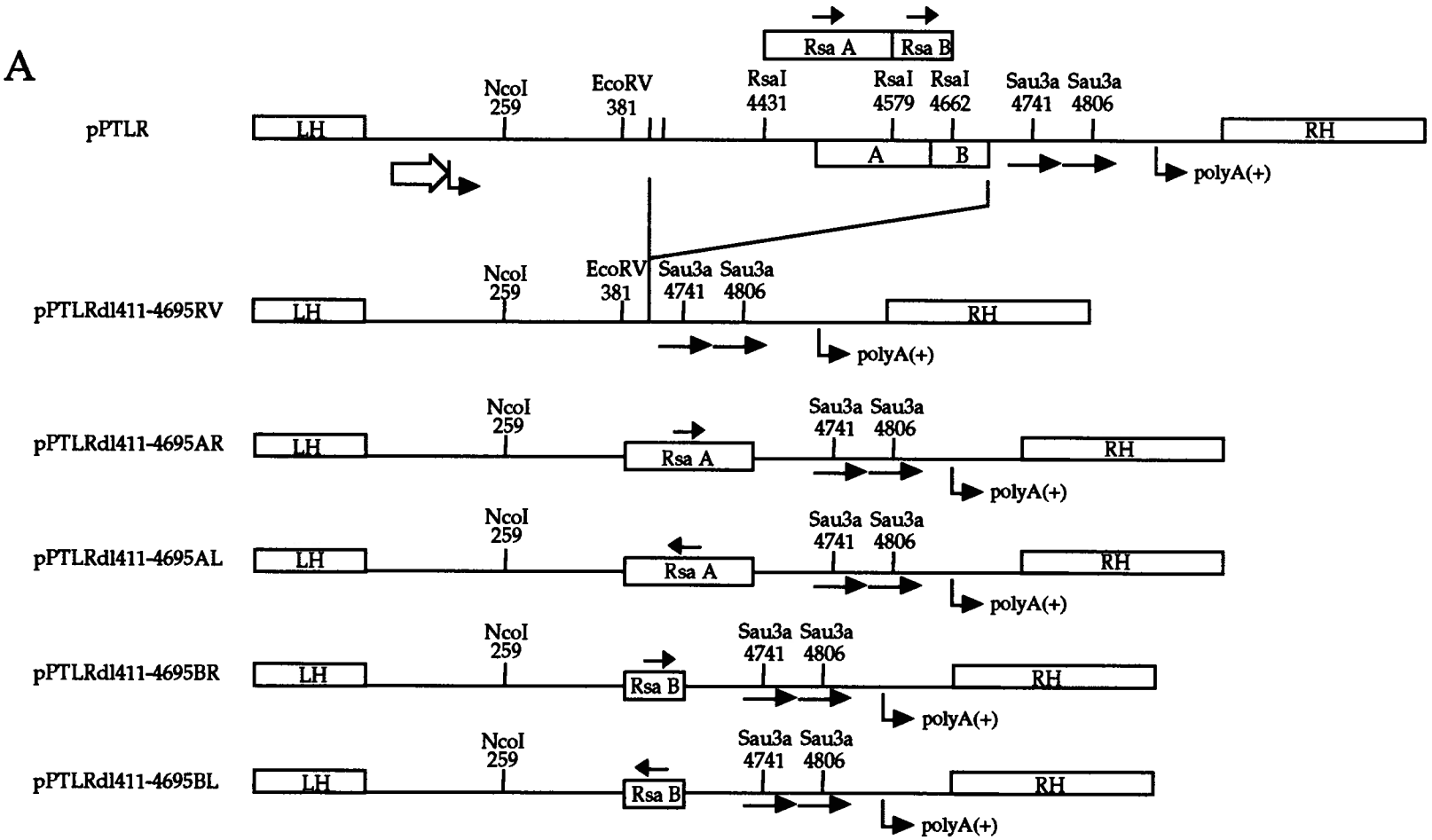

Fig. 18. Rsa A and Rsa B Activates DNA Replication of dl411-4695 IRE.

(A) Schematic diagram of dl411-4695 minigenomic constructs which contain the Rsa A (nt 4431-4636) and Rsa B (nt 4636-4695) inserts cloned into the unique EcoRV(381) site is shown. Elements A (nt 4489-4636) and B (nt 4636-4695) are boxed and are shown beneath the pPTLR construct .The cloning strategy is as outlined in Fig. 15. The Rsa A and Rsa B fragments (boxed) were cloned in either R or L orientation as indicated by the arrow above the box. All minigenomic constructs were linearized with EcoRI prior to transfection. Southern blots of Hirt DNA samples isolated from transfected COS-7 cells (B) undigested and (C) digested with Dpn I are shown. The Dpn I resistant monomer (mLR) replicative forms are as indicated.

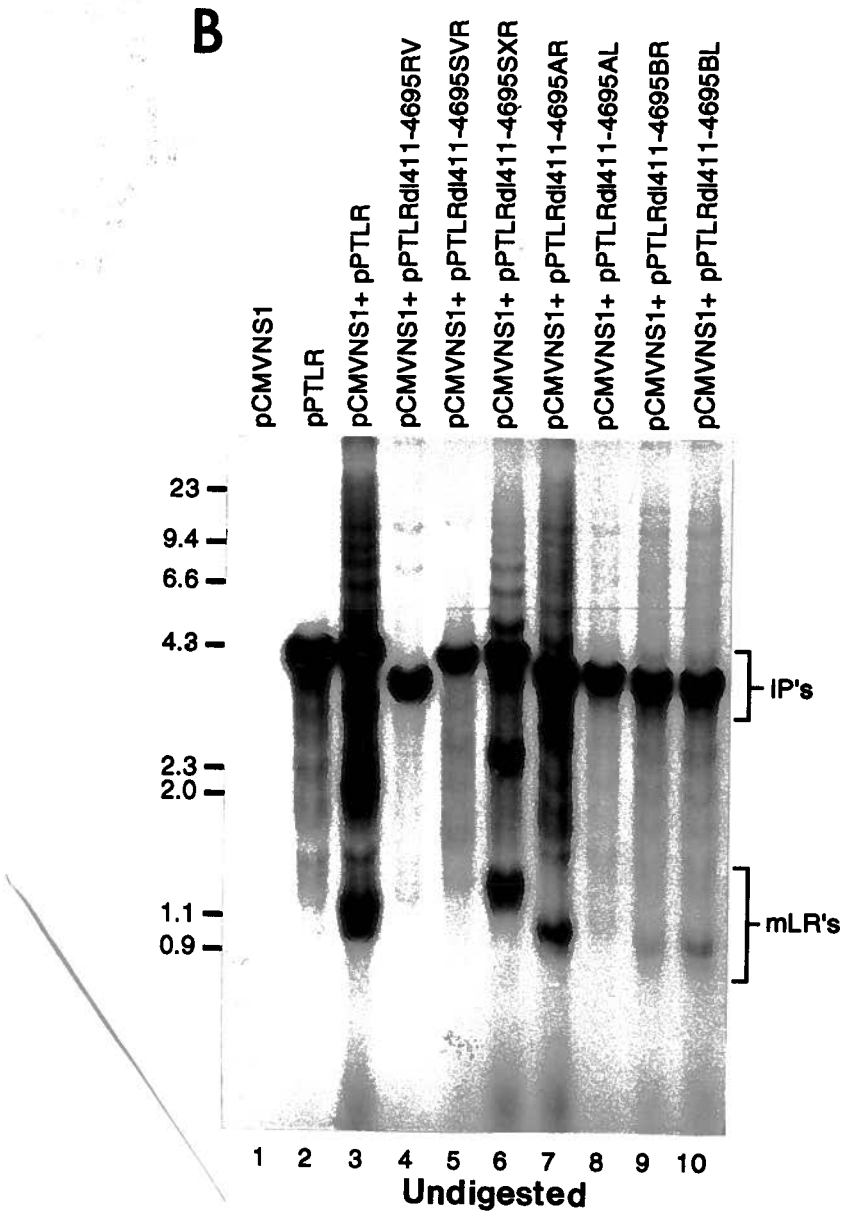
B
pCMVNS1
pPTLR
pCMVNS1+ pPTLR
pCMVNS1+ pPTLRdl411-4695RV
pCMVNS1+ pPTLRdl411-4695SVR
pCMVNS1+ pPTLRdl411-4695SXR
pCMVNS1+ pPTLRdl411-4695AR
pCMVNS1+ pPTLRdl411-4695AL
pCMVNS1+ pPTLRdl411-4695BR
pCMVNS1+ pPTLRdl411-4695BL
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
IP's
mLR's
1 2 3 4 5 6 7 8 9 10
Undigested

Fig. 18B

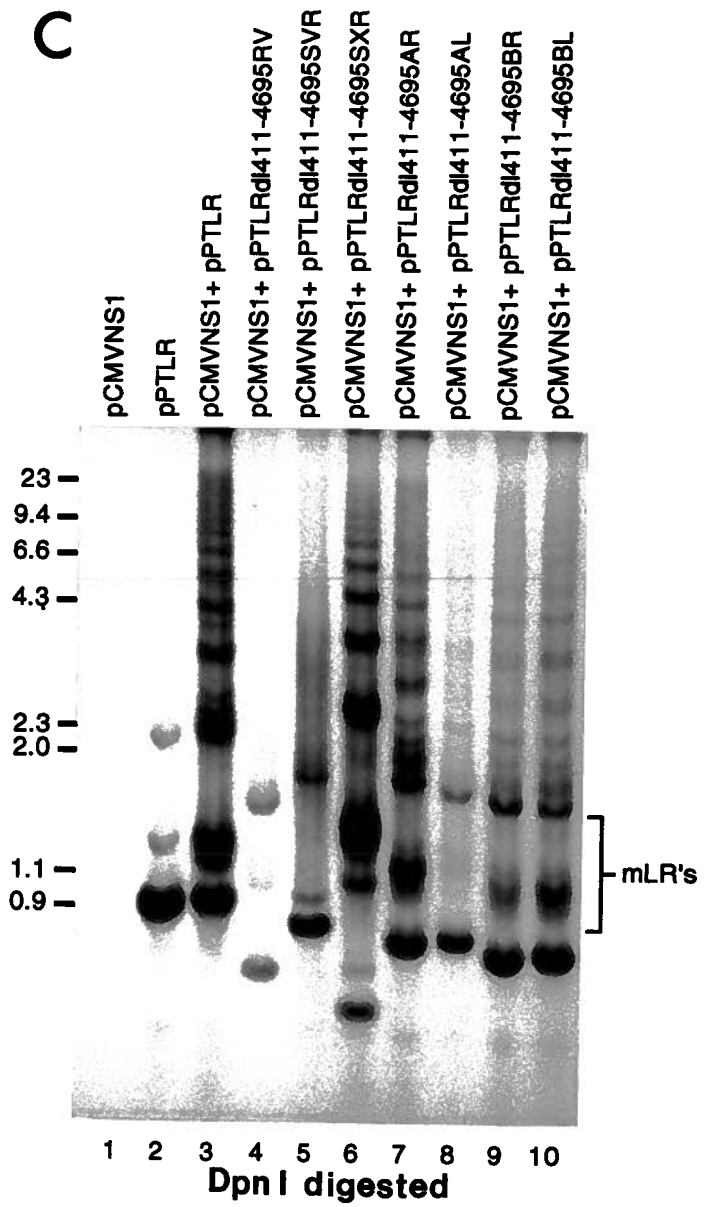
C
pCMVNS1
pPTLR
pCMVNS1+ pPTLR
pCMVNS1+ pPTLRdl411-4695RV
pCMVNS1+ pPTLRdl411-4695SVR
pCMVNS1+ pPTLRdl411-4695SXR
pCMVNS1+ pPTLRdl411-4695AR
pCMVNS1+ pPTLRdl411-4695AL
pCMVNS1+ pPTLRdl411-4695BR
pCMVNS1+ pPTLRdl411-4695BL
23
9.4
6.6
4.3
2.3
2.0
1.1
0.9
mLR's
1 2 3 4 5 6 7 8 9 10
Dpn I digested

Fig. 18C

within the complementing SX fragment were cloned into the unique EcoRV (381) site of the pPTLRdl411-4695(RV) minigenome (Fig.18A). Fragments which are able to activate DNA replication are predicted to either partially or fully rescue the replication phenotype. As expected, when the XbaI(4342)/Sau3a(4741) (SX) fragment was inserted, the replication phenotype was fully restored (Fig. 18B and C, lane 6). When the SV40 origin of replication was inserted, the minigenome remained replication deficient (Fig. 18B and C, lane 5). Again, this confirmed that the elements contained within the SX fragment were specific for MVM(p) DNA replication. The presence of the Rsa A fragment (nt 4431-4579) appeared to activate replication to approximately 60% of the control LR levels in the "R" orientation (Fig. 18B and C, lane 7), but only 7% in the opposite "L" orientation (Fig. 18B and C, lane 8). Furthermore, the presence of the adjacent Rsa B fragment (nt 4579-4662) also stimulated replication to approximately 20-30% of LR levels in either orientation. These results suggested the elements within the two Rsa I fragments are specifically required for efficient DNA replication of MVM(p) minigenomes. Since the larger SX fragment was able to fully rescue replication levels to LR levels and neither of the two Rsa I fragments were able to do so, it appears that the effects from the two Rsa I fragments may be additive.

3.5. Replication of Internal Left End (ILE) Deletions

Since *cis*-acting sequences were found distantly inboard of the right hairpin, two left end internal deletions were constructed to determine if other DNA elements found distantly inboard of the left hairpin are required for efficient DNA replication (Fig. 13). The dl259-4342 construct deleted the entire

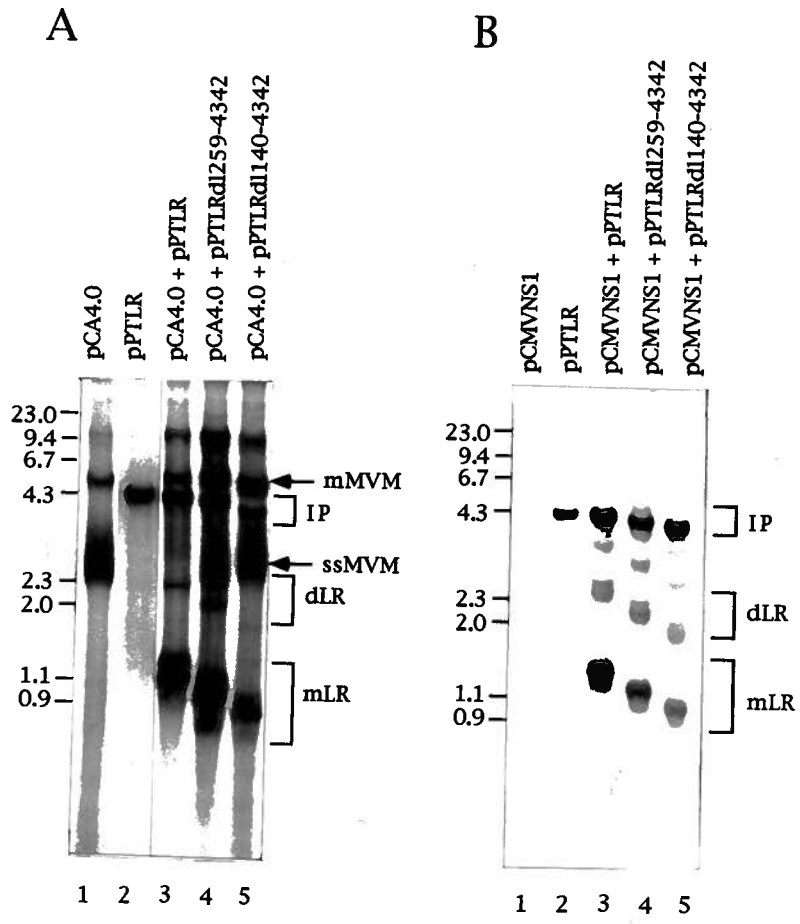

Fig. 19. Replication of Internal Left End (ILE) Deletion Mutants.

Two ILE deletion mutants dl259-4342 and dl140-4342 were constructed by removing the Nco I (259)/Hinc II or Sty I (140)/Hinc II restriction fragments from pPTLR (see Fig. 13C). The resulting plasmid constructs were linearized with EcoRI and transfected into LA9 (A) and COS-7 cells (B). Hirt DNA samples were analyzed by Southern blot analysis using a ^{32}P labeled probe generated with the right terminal Xba I(4342)/BamHI fragment.

NS-1 and NS-2 ORFs while the dl140-4342 construct further deleted the promoter elements including a functional Sp1 binding site found within P4 (Ahn *et al.*, 1989; Pitluk and Ward, 1991). The results show that the dl259-4342 mutation was replication competent when compared with the LR control minigenome in both A9 and COS-7 cells (Fig.19A and B, lane 3 vs 4). However, the dl140-4342 mutant replicated to at most 60-70% of the control levels (Fig.18A and B, lane 3 vs 5). These data suggested that deletion of the transcriptional control elements found in P4 may reduce the efficiency of minigenome replication, but the signals for processing of the viral hairpins were still present since the multiple banding patterns at the mLR level remains unchanged. Interestingly, deletion of Sp1 binding sites within the SV40 control region also reduces but does not abolish DNA replication *in vivo* (Hertz and Mertz, 1986). It is possible that Sp1 may provide the same function in both cases. This interpretation does not exclude the possibility that there may be as yet unidentified transcriptional control or replication elements found in the hairpin sequences.

3.6. Replication of Identical End Genomes

Although MVM(p) belongs to the autonomously replicating parvoviruses which contain two non-identical terminal hairpin sequences and package primarily minus strand viral genomes, other parvoviruses such as AAV and B-19 contain inverted terminal repeats (which result in identical hairpin termini) and package both plus and minus strands in approximately equal proportions. Can MVM(p) also engage in a mode of DNA replication that is similar to that of AAV? To this end, two plasmids (pPTRR and pPTLL) were constructed to encode two minigenomes which contains either two right termini or two left termini (Fig.20). The RR minigenome is approximately 1.9kbp and consists of two copies of the right

terminal 807 bp fragment oriented as inverted terminal repeats separated by a 0.3 kbp Hind III(3996)/Xba I(4342) restriction fragment. The LL minigenome is approximately 4.7 kbp and contains two copies of the left terminal 408 bp fragment oriented as terminal inverted repeats separated by a 3.9 kbp Alu I (408)/Xba I(4342) fragment. When these plasmids were transfected into COS-7 cells in the absence of pCMVNS-1, no additional bands corresponding to the expected replication intermediates were seen. However, when these constructs were co-transfected with pCMVNS-1, replication intermediates migrating at the predicted sizes corresponding to the monomer and dimer RF's of the LL (mLL and dLL) and RR (mRR and dRR) minigenomes appeared (Fig. 20A, lanes 5, 6 and 7). These replicative intermediates were shown to be both Dpn I resistant (Fig. 20B, lanes 5, 6 and 7) and Mbo I sensitive (Fig. 20C, lanes 5, 6 and 7), confirming that these intermediates were replicated in COS-7 cells. It was not surprising that replication of the LL and RR minigenomes was dependent on NS-1 since it has been shown that NS-1 is required for MVM DNA replication. This result also confirms that NS-1 plays a role in the replication of both the right and left termini. These results agree with other experiments showing that NS-1 is able to resolve concatemer junction fragments containing the 3' and 5' terminal DNA sequences (Cotmore *et al.*, 1992; Cotmore and Tattersall, 1992; Cotmore *et al.*, 1993). These results also confirm that each terminus contains a latent origin of DNA replication. Although both the LL and RR minigenomes replicated in COS-7 cells, it was observed that only the RR minigenome replicated at similar if not higher levels than the LR minigenome. In contrast, the LL minigenome consistently replicated at much lower levels (<5%) than that of the LR and RR minigenomes. The reason for this difference is unknown, but it is possible that the replication origin at the right terminus may

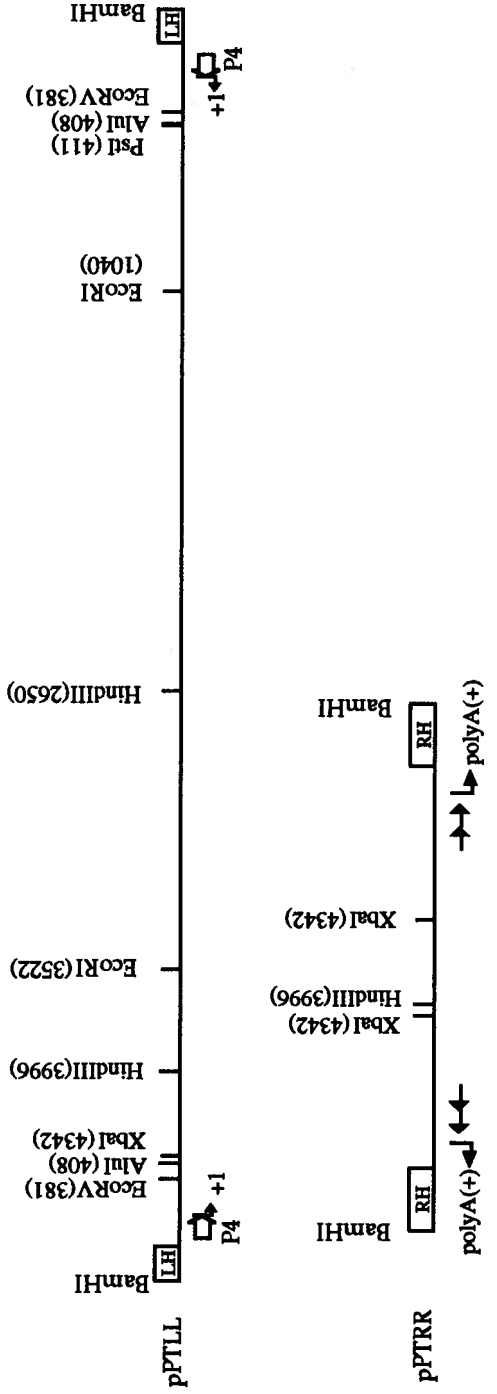

Fig. 20. Replication of identical end genomes.

Identical end minigenomes containing two left termini (pPTLL) or two right termini (pPTRR) are shown above. The plasmids were linearized with either with Sma I (pPTLL) or EcoRI (pPTRR) before co-transfection with pCMVNS-1 into COS-7 cells. Undigested (A), Dpn I (B) or Mbo I digested (C) Hirt samples were analyzed by Southern blot analysis. The input plasmid (IP), mRF (m) and dRF (d) of the variant RR and RR minigenomes are indicated. Under the gel conditions used, three mRR species were observed. Only one mLL intermediate was observed. All putative replicative intermediates were Dpn I resistant and Mbo I sensitive.

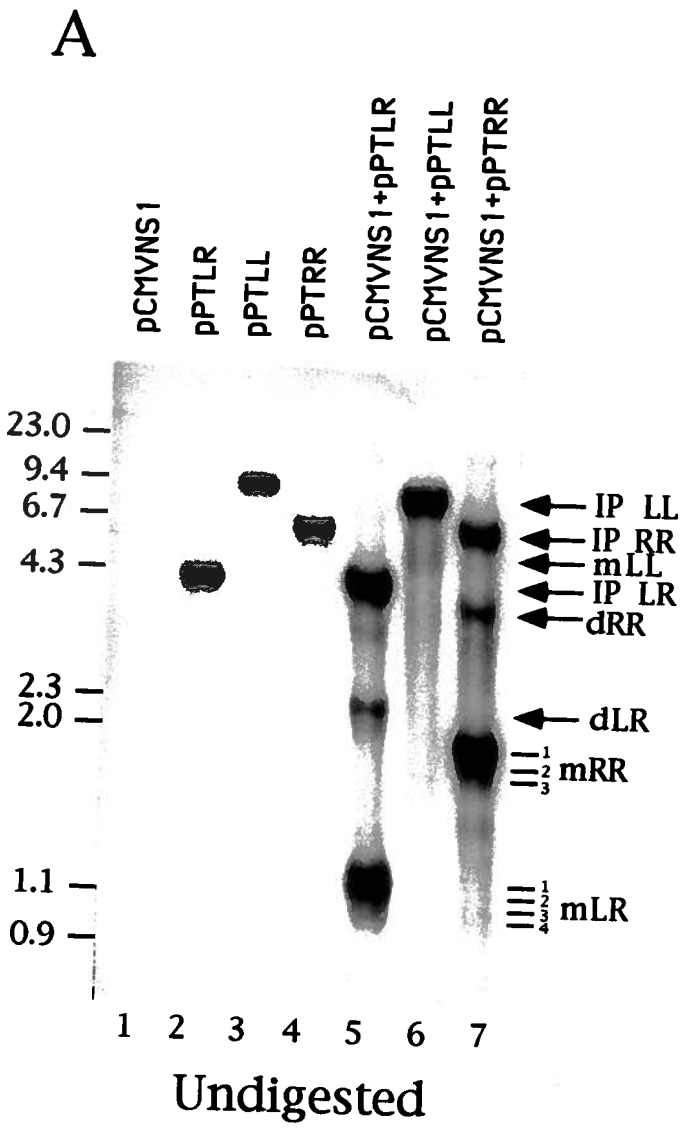
A
pCMVNS1
pPTLR
pPTLL
pPTRR
pCMVNS1+pPTLR
pCMVNS1+pPTLL
pCMVNS1+pPTRR
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
IP LL
IP RR
mLL
IP LR
dRR
dLR
mRR
mLR
1 2 3 4 5 6 7
Undigested

Fig. 20A

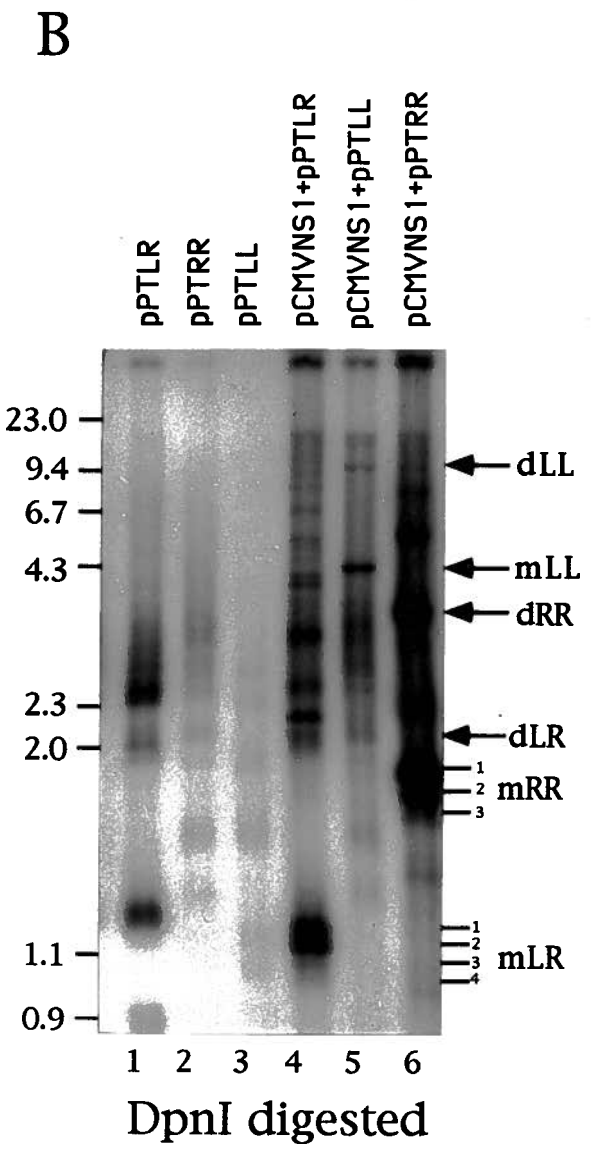
B
pPTLR
pPTRR
pPTLL
pCMVNS1+pPTLR
pCMVNS1+pPTLL
pCMVNS1+pPTRR
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
dLL
mLL
dRR
dLR
mRR
mLR
1 2 3 4 5 6
DpnI digested

Fig. 20B

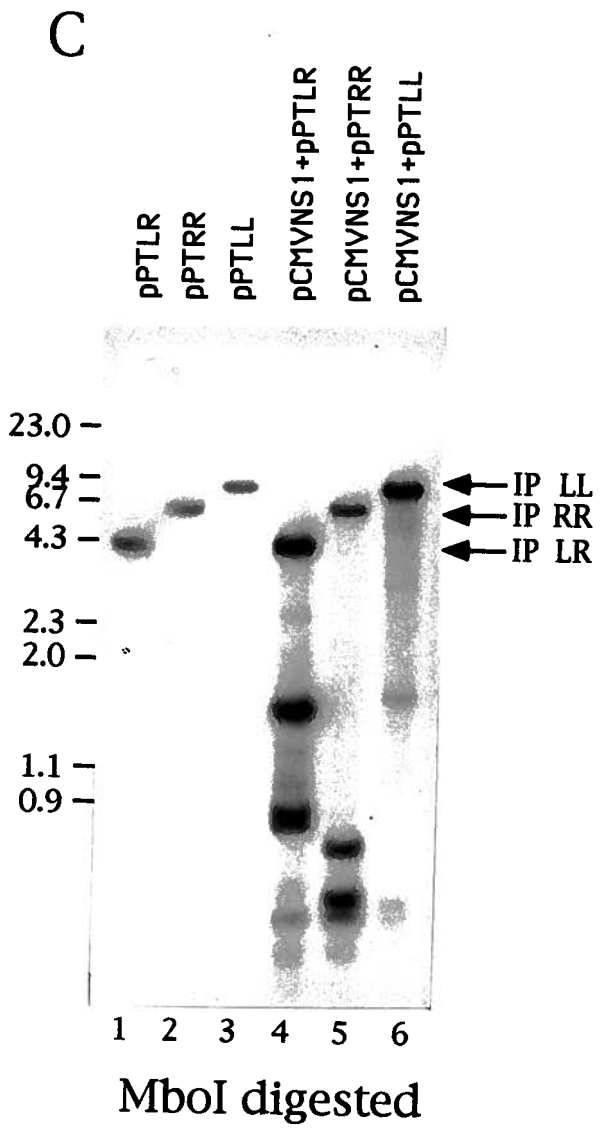
C
pPTLR
pPTRR
pPTLL
pCMVNS1+pPTLR
pCMVNS1+pPTRR
pCMVNS1+pPTLL
23.0
9.4
6.7
4.3
2.3
2.0
1.1
0.9
IP LL
IP RR
IP LR
1 2 3 4 5 6
MboI digested

Fig. 20C

potentially be more active than that of the left terminus. Although the left terminal sequences provide resolution sites in 3' dimer bridge fragments for dimer resolution, it might be predicted that the right terminal sequences contain a more active origin since this terminus serves as the origin of DNA replication for the synthesis of higher concatemeric forms as well as single stranded genomic DNA.

Since it was observed that mRR is composed of at least three bands, mRR1, mRR2 and mRR3, it was suspected that the terminal ends of the RR minigenomes contained various processed hairpin forms. When Hirt DNA samples from the pPTRR co-transfection were digested with exo III or with exo III followed by S1 nuclease, a similar digestion pattern was seen as with mLR (Fig. 21A). Again, only the smallest mRR, mRR3, was resistant to exoIII (i.e. absence of 3'OH end) whereas mRR1 and mRR2 were digested to smaller exoIII digestion products, e1 and ssRR (Fig. 21A, lane 6). These data suggest that mRR3 contains a closed hairpin structure at both termini. When these samples were subsequently digested with S1 nuclease, two resistant bands migrating between e1 and ssRR were observed, es1 and es2 (Fig. 21A, lane 7). Since ssRR was degraded by S1 nuclease, ssRR must be mostly single stranded DNA. In contrast, e1 represents a product which contains both single stranded and double stranded structures since e1 appears to be partially resistant to the S1 nuclease treatment, generating the smaller double stranded es1 and es2 products. I propose that e1 might represent an exoIII resistant panhandle structure that is present in the degradation pathway of mRR1 and/or mRR2. To confirm this, Hirt DNAs were boiled and rapidly cooled before gel electrophoresis (Fig. 21A, lane 8). As suspected, both mRR2 and 3 were resistant to this treatment

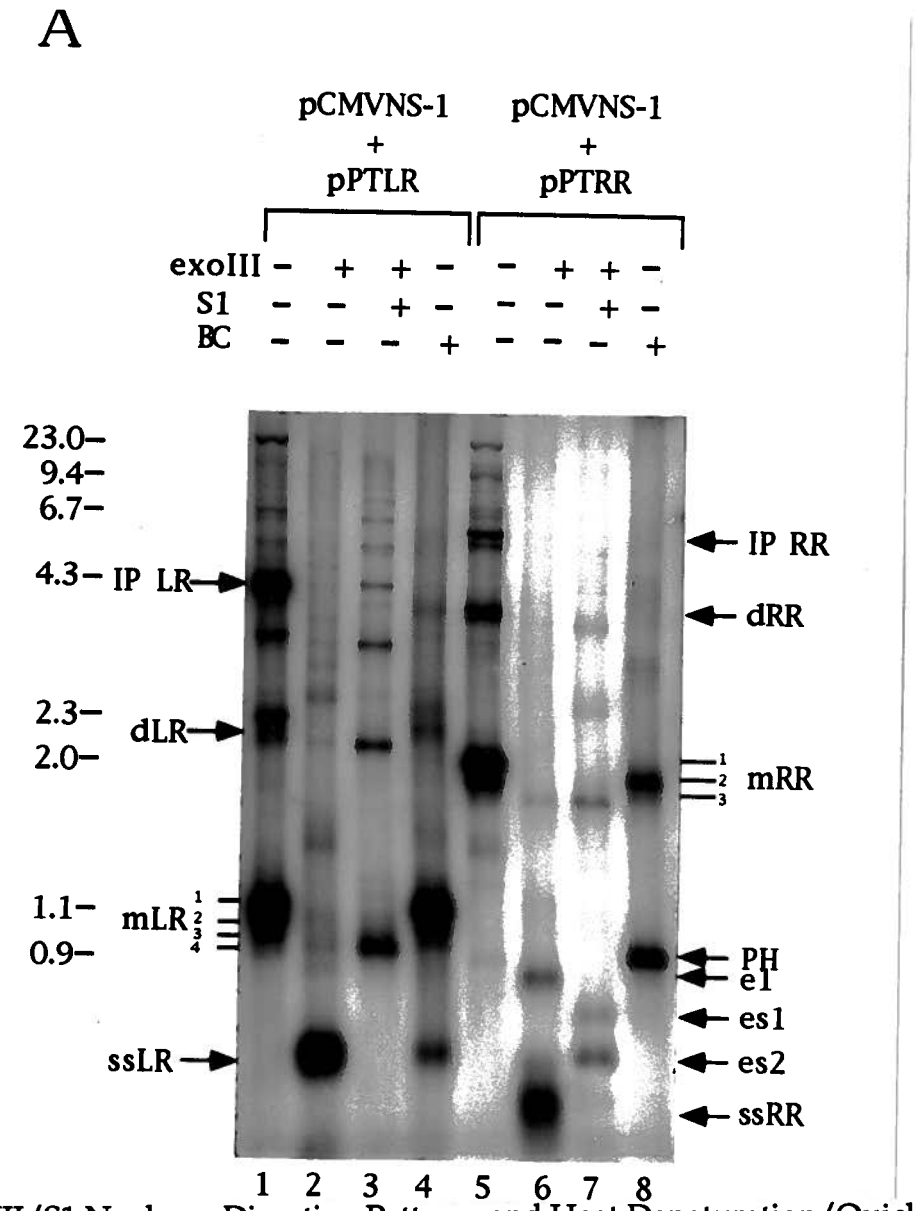

Fig. 21. Exo III/S1 Nuclease Digestion Patterns and Heat Denaturation/Quick Chill Properties of the Minigenomic RR Replicative Intermediates.

(A) Hirt DNA samples containing either minigenomic LR or RR replicative intermediates were either digested with exoIII (lanes 2 and 6), exoIII followed by S1 nuclease (lanes 3 and 7), or boiled and rapidly chilled (lanes 4 and 8) before analysis by gel electrophoresis and Southern blotting. The exoIII degradation products, e1 and ssRR (lane 6), and exoIII/S1 degradation products, es1 and es2 (lane 7), are indicated. The band indicated by PH (lane 8) is predicted to be a panhandle structure. (B) The proposed structure of mRR1, mRR2, mRR3 and PH is shown based on data shown in (A). The arrowheads indicate (3' OH) ends which are susceptible to exoIII digestion.

B

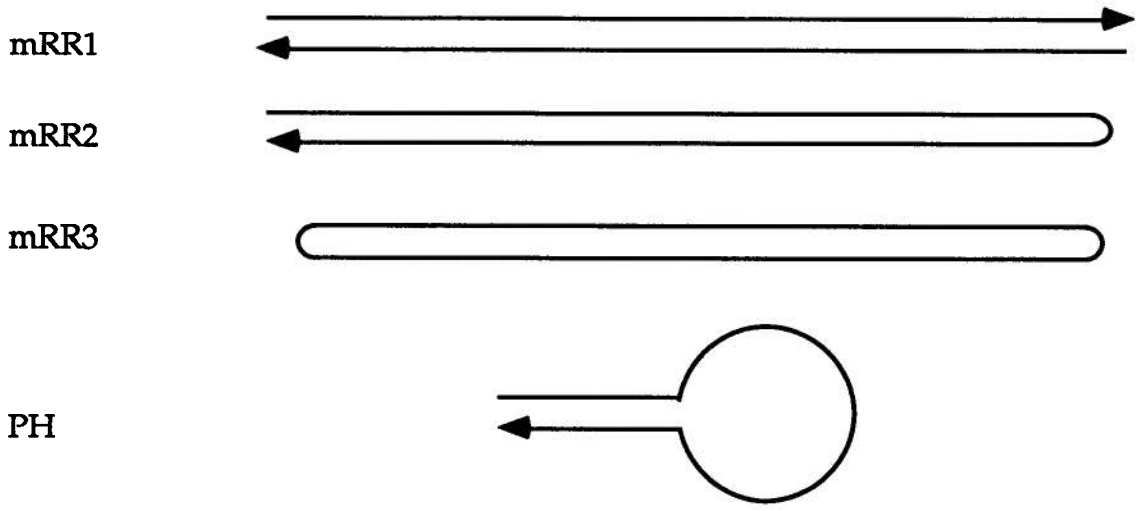

Fig. 21B

showing that these intermediates contain at least one closed right hairpin end but the heat denatured/quick chilled mRR1 material co-migrated with a similar band in the undigested sample and migrated slightly slower with the exoIII degradation product e1. These data suggested that mRR1 contained two open extended right termini. When mRR1 is heat denatured and rapidly cooled, a panhandle (PH) structure is predicted to form due to the presence of the inverted 5' terminal sequence repeat in the duplex RR sequences. It may be possible that e1 is slightly smaller than PH because of a limited exo III degradation of the terminal sequences ends during the exo III reaction. In Fig. 21B, the proposed structures of the mRRs based on the exoIII/S1 degradation patterns, heat denaturation properties and electrophoretic mobility are indicated. The presence of multiple mLLs were not observed probably because the LL minigenome is relatively large and thus various mLLs may not be resolved during electrophoresis.

When the same transfection experiments were performed in A9 cells, either very low levels of replication (RR) or no replication (LL) were observed (data not shown). Although it is not know why this is the case, it may be possible that in the COS-7 transfections, our helper plasmid is not an infectious clone, hence there is no competing replicating genome. When A9 cells are used, the infectious wild type helper genome likely competes with the LL and RR minigenomes for replication factors. If this is the case, it suggests that the normal MVM(p) genome configuration (LR) may be a more efficient replication template than the aberrant LL and RR minigenomes. There is other evidence for this line of reasoning. Studies of MVM(p) defective interfering (DI) particles (Faust and Ward, 1979) showed that there are two types of DI particles, Type I particles contain the LR configuration whereas Type II DI particles maintain the RR configuration. Upon further

undiluted serial passages, the population of the Type II particles is reduced relative to the Type I particles suggesting that either the Type I genomes are preferentially packaged or Type I genomes have a competitive advantage during the replication of the DNA. The data suggest that the latter explanation is more likely since the RR minigenome clearly replicates as efficiently as the LR minigenome only in the absence of a competing LR genome.

3.7. Activation of LL minigenome DNA replication

The analysis of IRE deletion mutants suggested that there may be elements found within the SX fragment which are required for efficient replication of LR minigenomes. Since the SX fragment was able to rescue the replication of the dl411-4695 minigenome, it is possible that this fragment may also activate DNA replication at the left terminus. To test this hypothesis, a series of MVM(p) minigenomes was constructed containing two left hand (3') termini with either the activating SX fragment or an unrelated fragment from the yeast FUS3 gene (Fig. 22). First, the smaller LL type minigenome, LLX (2.4 kbp), was constructed by deleting the internal Xho I(2070)/Xba I(4342) fragment from the large LL minigenome encoded by pPTLL. The LLX minigenome replicates poorly but consistently better than the larger LL minigenome (Fig. 22C, lanes 5 vs. 6). A minigenome with one copy of the FUS3 restriction fragment replacing the internal 1.7 kbp EcoRV fragment of pPTLLX (LLFUS3.1) replicated at a very low level when compared to the LR control (Fig. 22C, lane 11 vs 4). When four copies of the FUS3 fragment were inserted (pPTLLFUS3.4), no replication intermediates were detected (Fig. 22C, lane 12).

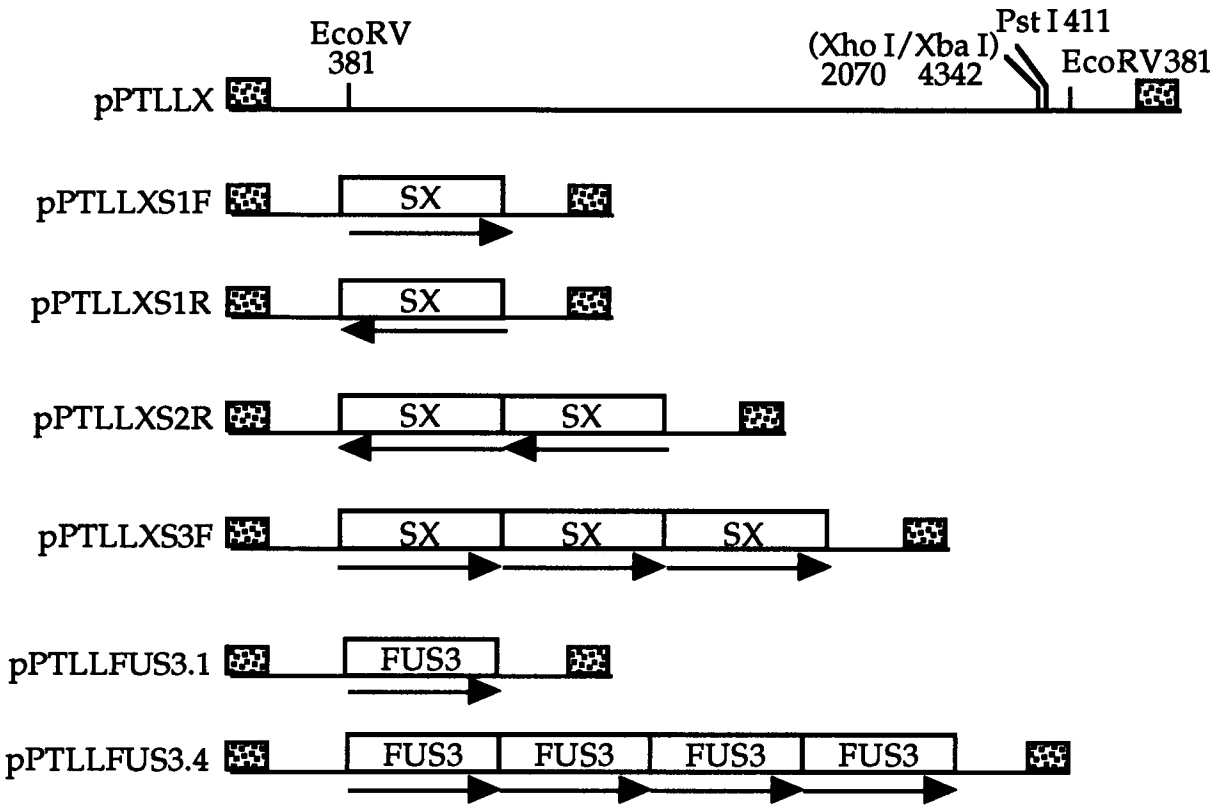

Fig. 22. Activation of Replication of MVM(p) LL Minigenomes.

Plasmid clones encoding LL minigenomes are described in (A). The stippled boxes at the end of each line represents the left hairpin sequences. A deletion of the Xho I (2070) to Xba I (4342) restriction fragments from pPTLL (Fig. 20A) resulted in the pPTLLX clone. One (pPTLLXS1F or pPTLLXS1R), two (pPTLLXS2R) or three (pPTLLXS3F) copies of the SX replaced the 1.7 kbp internal EcoRV fragment of the pPTLLX vector. In addition, one (pPTLLFUS3.1) or four (pPTLLFUS3.4) copies of the FUS3 gene were also used to replace the internal EcoRV fragment of pPTLLX. The arrows below each SX or FUS3 fragment (box) indicate the relative direction of each insert. Southern blots of low MW DNAs isolated from transfected COS-7 cells (B) undigested or (C) Dpn I digested were performed as described in the Materials and Methods. The monomer and dimer replicative forms of the LR (mLR and dLR) and RR genomes (mRR and dRR) are as indicated. The predicted monomer (triangle) and dimer (diamond) replicative forms of the various LL minigenomes is indicated to the left of each lane. Only two monomer forms are observed for minigenomes containing the LL type configuration.

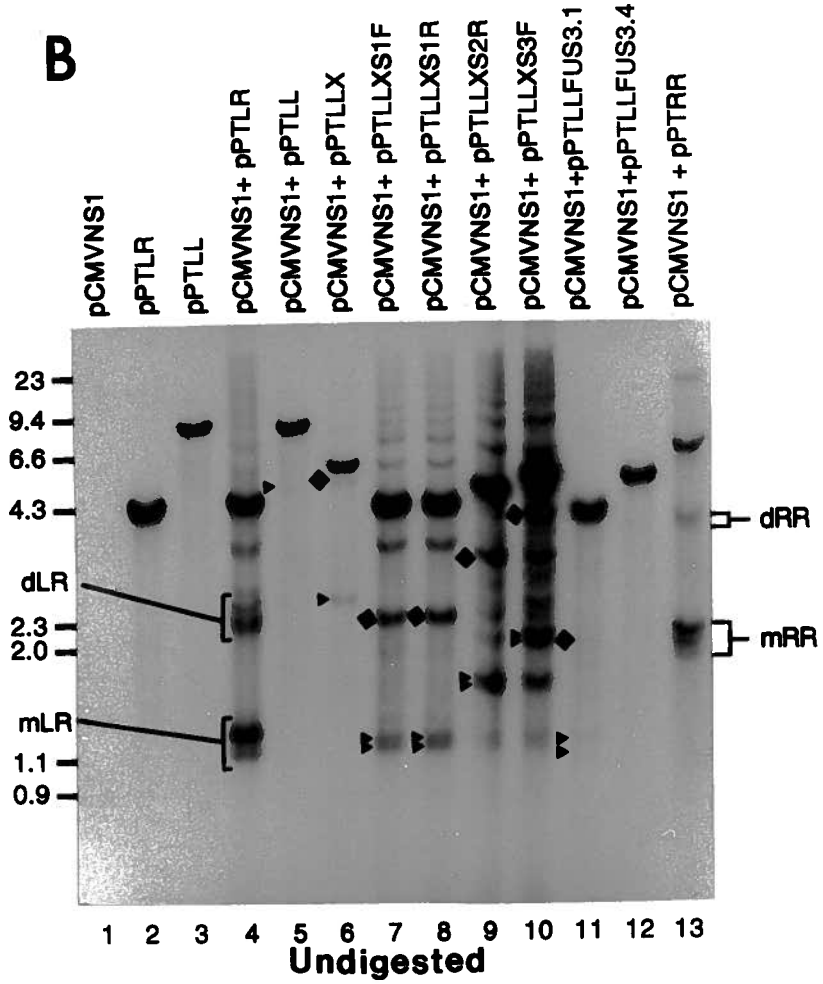
B
pCMVNS1
pPTLR
pPTLL
pCMVNS1+ pPTLR
pCMVNS1+ pPTLL
pCMVNS1+ pPTLLX
pCMVNS1+ pPTLLXS1F
pCMVNS1+ pPTLLXS1R
pCMVNS1+ pPTLLXS2R
pCMVNS1+ pPTLLXS3F
pCMVNS1+pPTLLFUS3.1
pCMVNS1+pPTLLFUS3.4
pCMVNS1 + pPTRR
23
9.4
6.6
4.3
dLR
2.3
2.0
mLR
1.1
0.9
dRR
mRR
1 2 3 4 5 6 7 8 9 10 11 12 13
Undigested

Fig.22B

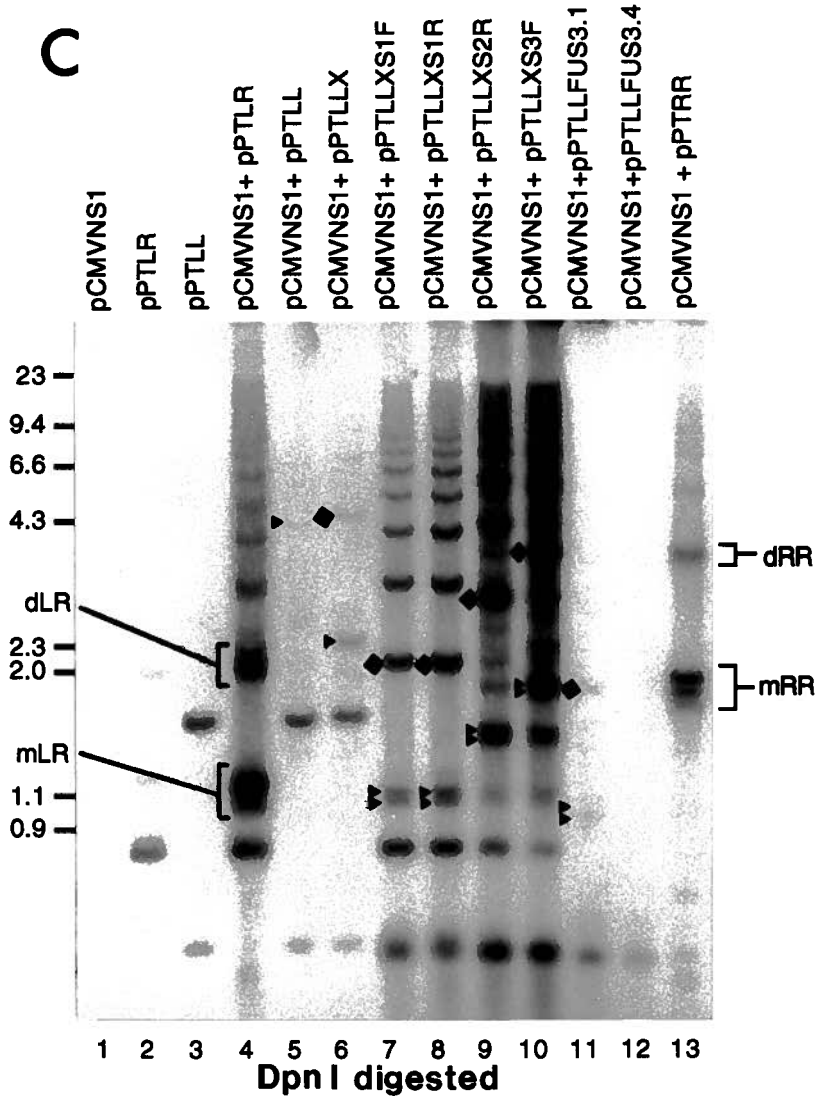
C
pCMVNS1
pPTLR
pPTLL
pCMVNS1+ pPTLR
pCMVNS1+ pPTLL
pCMVNS1+ pPTLLX
pCMVNS1+ pPTLLXS1F
pCMVNS1+ pPTLLXS1R
pCMVNS1+ pPTLLXS2R
pCMVNS1+ pPTLLXS3F
pCMVNS1+pPTLLFUS3.1
pCMVNS1+pPTLLFUS3.4
pCMVNS1 + pPTRR
23
9.4
6.6
4.3
dLR
2.3
2.0
mLR
1.1
0.9
dRR
mRR
1 2 3 4 5 6 7 8 9 10 11 12 13
Dpn I digested

Fig. 22C

In contrast, when the internal 1.7 kbp EcoRV fragment of pPTLLX was replaced by the SX fragment, the predicted Dpn I resistant monomers and dimers were easily detectable (Fig. 22C, lanes 7 and 8). A single copy of the SX fragment activated DNA replication approximately 30-60 times over that of the LLX minigenome as judged by the Dpn I resistant material in the Hirt DNA samples (Fig. 22C, compare lane 6 with 7 or 8). It was noted that the LLXS1F and LLXS1R minigenomes replicate equally well since they are identical minigenomes even though the SX fragment is oriented in opposite directions with respect to the plasmid sequences. Thus no comment can be made on the orientation dependence of the SX fragment on the activation of LL type minigenomes. Although activation of the LL minigenomes by the SX fragment is unequivocal, some unexpected replication intermediates were observed. When two (LLXS2R) or three (LLXS3F) copies of the SX fragment were used (Fig. 22C, lanes 9 and 10), monomer forms corresponding to the LLXS1F or LLXS1R were present. These data suggested that LL minigenomes containing multiple copies of the SX fragment may be able to excise copies of the SX fragment by either recombination or slipped mispairing during replication. An intramolecular slipped mispairing mechanism has been implicated in the generation of deletion variants of parvovirus genomes (Faust and Hogan, 1990)

I also observed only two monomer RF forms of the LLXS1F and LLXS1R constructs (Fig. 22B and C). In order to determine the nature of the termini of monomer replicative forms of the LLXS1F minigenome, Hirt DNA samples were digested with exonuclease III (Fig. 23). Only mLLXS-2 was resistant to exonuclease III digest suggesting that both termini are covalently closed. To determine the status of mLLXS-1, Hirt DNA samples were denatured in boiling water and quickly cooled in ice water before gel electrophoresis. It was observed that both

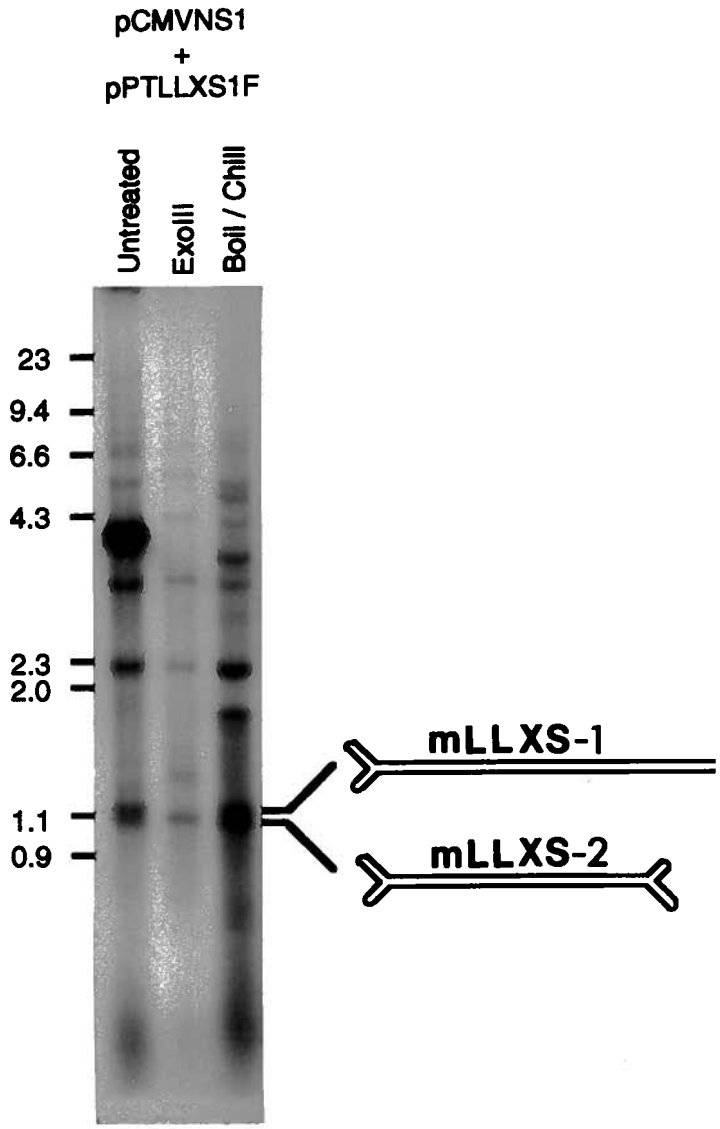

Fig 23 Exo III Digestion Patterns and Heat Denaturation/Quick Chill Properties of Minigenomic LL Replicative Intermediates.

Hirt DNA samples containing the indicated transfected DNA were either undigested (lane 1), exonuclease III digested (lane 2) or boiled and rapidly cooled (lane 3) before gel electrophoresis and Southern blot analysis. The mLLXS-2 indicates a structure which contains covalently closed hairpin ends at both termini whereas mLLXS-1 contains one covalently closed hairpin end and one open duplex terminus.

mLLXS-1 and 2 are both able to snap back suggesting that both molecules have at least one covalently closed hairpin terminus. Taken together, mLLXS-2 must contain one open extended duplex palindrome terminus and one covalently closed hairpin terminus (Fig. 23 cartoon). The absence of detectable molecules containing two open duplex termini is consistent with what is known of MVM DNA replication. First, the MVM left hairpin cannot undergo hairpin transfer like AAV (Fig. 5) to produce extended duplex palindrome sequence at either end since all ends are required to remain in the flip sequence orientation. Second, the proposed asymmetric resolution mechanism of the Modified Rolling Hairpin Model of MVM DNA replication (Fig. 4) predicts that one covalently closed left (3′) hairpin is produced in each dimer resolution reaction (Astell *et al.*, 1985).

It is also interesting to note that minigenomes containing two right termini generate three monomer forms (Fig. 21) while minigenomes containing two left termini generates two monomer forms (Fig. 22). The difference appears to be that the RR minigenome is capable of generating a monomer replicative form with extended duplex palindromes at each end. The extended right palindrome may be generated either by a hairpin transfer reaction or by a resolution of the 5' bridge dimer fragment. The latter reaction has been demonstrated *in vivo* and *in vitro.* (Cotmore and Tattersall, 1992; Cotmore *et al.*, 1992). A closed hairpin at the right terminus is thought to arise through hairpin formation followed by DNA replication using the 3'OH as the primer. Clearly the presence of a mRR3 which contains two covalently closed hairpin termini (Fig. 21B) cannot be explained using the model of MVM DNA replication in Fig. 4. It may be possible that mRR3 arises through recombination of two mRR2 type molecules. Alternatively, a ligation event at the right terminus of mRR2 type molecules may also generate the mRR3. This

has been previously postulated to explain the existence of mLR4 type molecules, which also have covalently closed right and left termini (Cotmore *et al.*, 1989). In any case, if mRR3 is a legitimate replicative intermediate, the covalently closed ends must be resolved in a manner similar to the resolution of AAV covalently closed hairpins. Curiously, the generation of mLLXS-2 type molecules (see Fig 23) presents a problem to the replication of LL type molecules. It is thought that covalently closed left hairpins cannot be resolved by a hairpin transfer type mechanism since the flip sequence orientation must be maintained at the left terminus. If both ends are covalently closed, then this molecule may not participate in further amplification of the LL minigenome since both ends are "locked" into the closed hairpin form. Thus amplification of LL type minigenomes may be dependent on the amplification of mLLXS-1 type molecules.

3.8. Identification of Binding Activities in Nuclear Extracts of A9 and MVM(p) Infected A9 Cells

Since it was determined that the SX fragment, as well as the RsaA and RsaB fragments are able to specifically rescue DNA replication of the dl411-4695 mutant MVM(p) minigenome, it is plausible that elements A (nt 4489-4636) and B (nt 4636-4695), as defined by deletion analysis, may constitute elements of a viral origin of DNA replication. Previous studies have shown that sequence specific DNA binding proteins such as transcription factors are able to activate DNA replication in a variety of viral systems as well as yeast. In order to determine if proteins bind to elements A and B, nuclear extracts were prepared from uninfected and MVM(p) infected LA9 cells and probed with end labeled fragments which contain Rsa A or Rsa B in electrophoretic mobility shift assays (EMSA) (Fried and Crothers, 1981). In this assay, ^{32}P labeled restriction fragments were mixed with proteins from a

nuclear extract. DNA-protein complexes were separated from the unbound ^{32}P labeled probe by electrophoresis through a non-denaturing 4% polyacrylamide gel . At least six DNA-protein complexes were seen when Rsa A was used as a probe (Fig. 24A, lane 2). In order to determine specificity of these complexes, identical binding reactions were carried out in the presence of a 200 fold excess by weight of either unlabeled Rsa A, RsaB or 70 bp RsaI fragment of unrelated sequence from pUC19 designated Rsa70. The slowest migrating complex was likely to be a non-specific DNA-protein complex (NS) since it is completely removed upon addition of any of the three unlabeled fragments. In contrast, five other DNA-protein complexes designated MVM(p) DNA Replication Factors (MRF) A2, A3, A4, A5 and A6 demonstrated specific competition. The majority of MRF A2 was competed off with excess of unlabeled Rsa A and Rsa B probe whereas MRF A3, A4 and A5 were completely removed only in the presence of unlabeled Rsa A. Addition of Rsa B or Rsa 70 did reduce the intensity of MRF A3, A4 and A5, but failed to completely remove these complexes. These data suggest that these complexes are likely to be specific for the Rsa A probe, but the protein involved may also have some non-specific interactions with Rsa B and Rsa 70. MRF A2 appears to be specific for Rsa B and Rsa A but not Rsa 70. Since identical results were seen when nuclear extracts from MVM(p) infected LA9 cells were used, it suggests that factors binding to elements within the Rsa A probe are cellular in origin. When ^{32}P labeled Rsa B was used in EMSA experiments, four complexes were observed and were designated MRF B2, B3, B4 and B5 (Fig. 24B). Again the majority of MRF B2 was competed off in the presence of an excess of unlabeled Rsa A or Rsa B but not Rsa 70. This observation suggests the protein factor involved in MRFA2 and MRF B2 may be the same as both bind specifically to

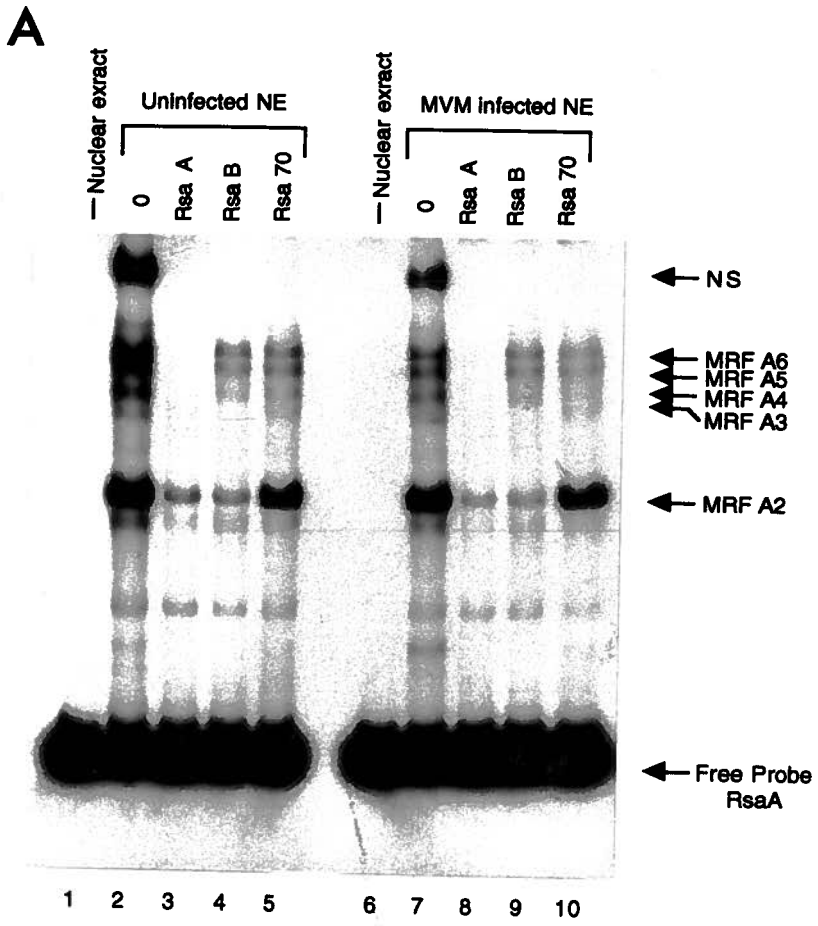

Fig. 24. Cellular Proteins in Nuclear Extracts Bind to Rsa A and Rsa B.

Radiolabeled Rsa A (A) and Rsa B (B) were incubated in the presence of 5 μg of either uninfected A9 nuclear extracts (lanes 2-5) or MVM(p) infected LA9 nuclear extracts (lanes 7-10). Addition of a 200 fold excess of either unlabeled Rsa A fragment (lanes 3 and 8), Rsa B fragment (lanes 4 and 9) or the non-specific Rsa 70 fragment (lanes 5 and 10) in competition binding reactions determined specificity of binding. Lanes 2 and 7 contained no unlabeled restriction DNA fragment competitor. Lanes 1 and 6 contained no protein added to the binding reaction. Specific protein-DNA complexes were designated MVM(p) replication factors (MRF's). Non-specific (NS) complexes were as indicated.

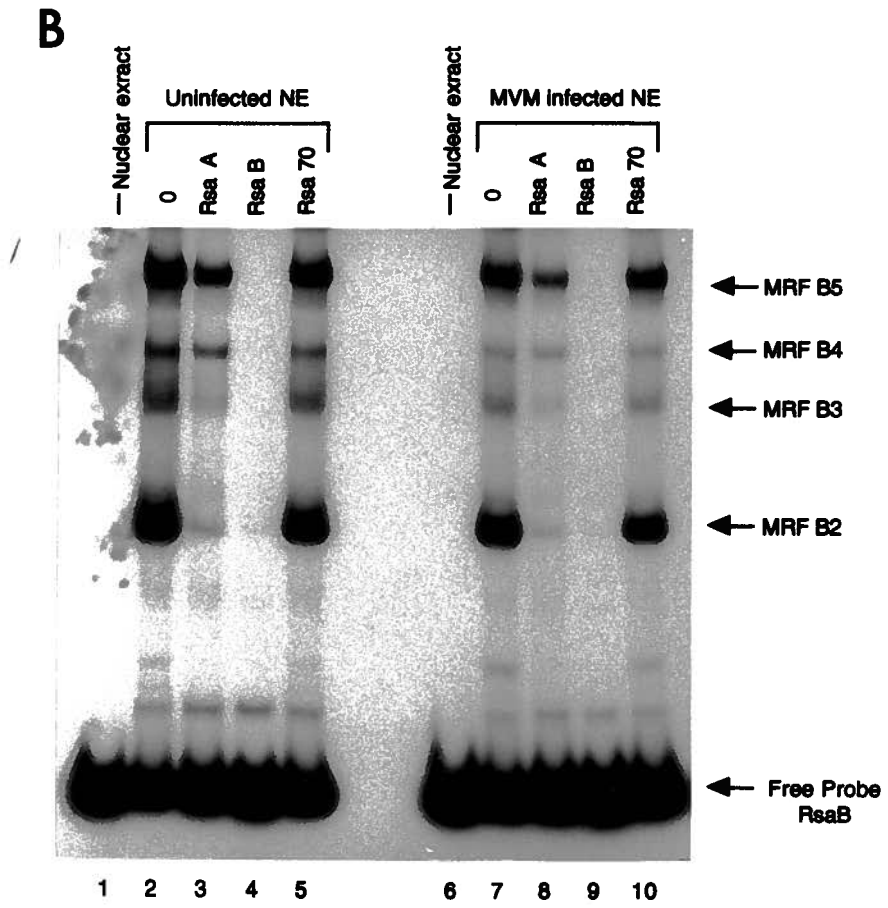
B
— Nuclear exract
Uninfected NE
0
Rsa A
Rsa B
Rsa 70
— Nuclear exract
MVM infected NE
0
Rsa A
Rsa B
Rsa 70
MRF B5
MRF B4
MRF B3
MRF B2
Free Probe
RsaB
1 2 3 4 5 6 7 8 9 10

Fig. 24B

RsaA and Rsa B. MRF B3, B4 and B5 appear to be specific for Rsa B since addition of unlabeled Rsa B completely removed the radiolabeled complexes. Addition of unlabeled Rsa A reduced the intensity of MRF B3, B4 and B5 but not to the same extent as Rsa B. Again these observations suggest that these proteins may have a non-specific interaction with Rsa A. Identical results were seen when nuclear extracts from MVM(p) infected LA9 cells were used, suggesting protein factors binding to elements within Rsa B are cellular in origin.

3.9. Fractionation of Nuclear Extracts

In order to study further the binding sites of the proteins binding to the RsaB probe, it was necessary to fractionate nuclear extracts since DNase I footprinting using crude nuclear extracts yielded ambiguous results (data not shown). DEAE-Sephacel and heparin-agarose (HA) chromatography were used to fractionate nuclear extracts (Fig. 25). The majority of the MRF binding activities appeared in the flow through fractions of a DEAE-Sephacel column (0.1 M KCl D wash buffer) (Fig.25A, lane 3). The 0.1 M KCl D fractions were immediately loaded onto a heparin-agarose column and developed with a step gradient containing 0.1 M KCl, 0.3 M KCl and 0.6 M KCl wash buffers. EMSA using radiolabeled Rsa B showed that the majority of MRF B2, B3 and B5 were found in the 0.3 M KCl HA fraction whereas the majority of MRF B4 is found in the 0.1 M KCl HA fractions. Small amounts of MRF B3 and B5 were found in the 0.1 M KCl HA flow through whereas a small amount of MRF B2 eluted in the 0.6 M KCl buffer. MRF A2 had the same elution profile when radiolabeled Rsa A was used in EMSA (data not shown). This result agrees with the suggestion that MRF B2 is similar to MRF A2. Although, the majority of MRF B4 is found in the 0.1 M KCl HA flow through, a minor amount of MRF B4 is found in the 0.3 M KCl HA fractions. The finding that each of MRF B2,

B3, B4 and B5 binding activity eluted in two different KCl concentrations suggests either that the binding factor found in each salt fraction represents a distinct protein factor which has identical mobilities in EMSA or that the protein factors may be differentially modified (phosphorylated ?) resulting in their elution by different salt concentrations.

3.10. Determination of MRF B5 Binding Site by DNase I Footprinting

Samples from peak protein fractions of the 0.1 M KCl, 0.3M KCl and 0.6 M KCl eluates of the heparin-agarose column were used in DNase I footprint assays (Fig.25 B and C). In this assay, DNA-protein complexes are formed as in EMSA analysis. The DNA-protein complexes were then digested briefly with DNase I before electrophoresis on sequencing gels. Sites which are normally susceptible to DNase I cleavage are protected from digestion when a protein binds to that region of the labeled probe. Aliquots from the 0.1 and 0.6 M KCl HA fractions did not exhibit a DNase I footprint pattern, but two regions of DNase I protection were detected on both the plus and minus strands of the Rsa B probe when samples from the 0.3 M KCl HA fraction were used. When the plus strand was end labeled (Fig. 25B) nt 4589-4610 (site I) and 4616-4646 (site II) were protected from DNase I digestion. When the minus strand was labeled (Fig. 25C), nt 4590-4609 (site I) and 4618-4631 (site II) were protected. (See Figure 25D for a summary of the binding sites). I also noted that single nucleotides outside of the major binding regions were also either protected from DNase I digestion or hypersensitive to DNase I action (Fig. 25C). This suggests that the structure of the DNA template is somewhat different in the presence of the factors binding to the labeled Rsa B fragment.

Fig. 25 Determination of MRF B5 Binding Site.

(A) A9 nuclear extracts were fractionated using DEAE-Sephacel chromatography followed by heparin-agarose chromatography as outlined in the Materials and Methods. All specific binding activities flowed (60 ml/hour) through the initial DEAE-Sephacel column in the 0.1 M KCl fraction (0.1 M KCl D) The 0.1 M KCl D fraction was immediately loaded onto a 10 ml heparin agarose column and washed with 60 ml of 0.1 M, 0.3 M and 0.6 M KCl buffers. Three milliliter fractions were collected (20 ml/hour)and assayed in a standard EMSA using ^{32}P radiolabeled Rsa B as outlined in the Materials and Methods. (B) and (C) show MRF B5 bound multiple regions of Rsa B in DNase I footprinting experiments. Five, 7.5 or 10 microliters of the peak protein fractions (as measured by A_{280} readings) of the 0.1 M KCl , 0.3 M KCl and 0.6 M KCl eluates were incubated with Rsa B, radiolabeled on either the plus strand (B) or minus strand (C) , before a limited digestion with DNase I and denaturing gel electrophoresis. Maxam and Gilbert ladders of Rsa B were used to determine the binding site of MRF B5. Two sites of protection, designated site I and site II, are indicated by the boxes. Nucleotides which are protected from DNase I digestion at the edge of site II on the positive strand are indicated by arrows. Residues which are hypersensitive to DNase I are indicated by asterisks. (D) Regions of protection are shown graphically on both strands as indicated by the black bars and arrows. The numbers above the DNA sequence represent the MVM(p) nucleotide positions.

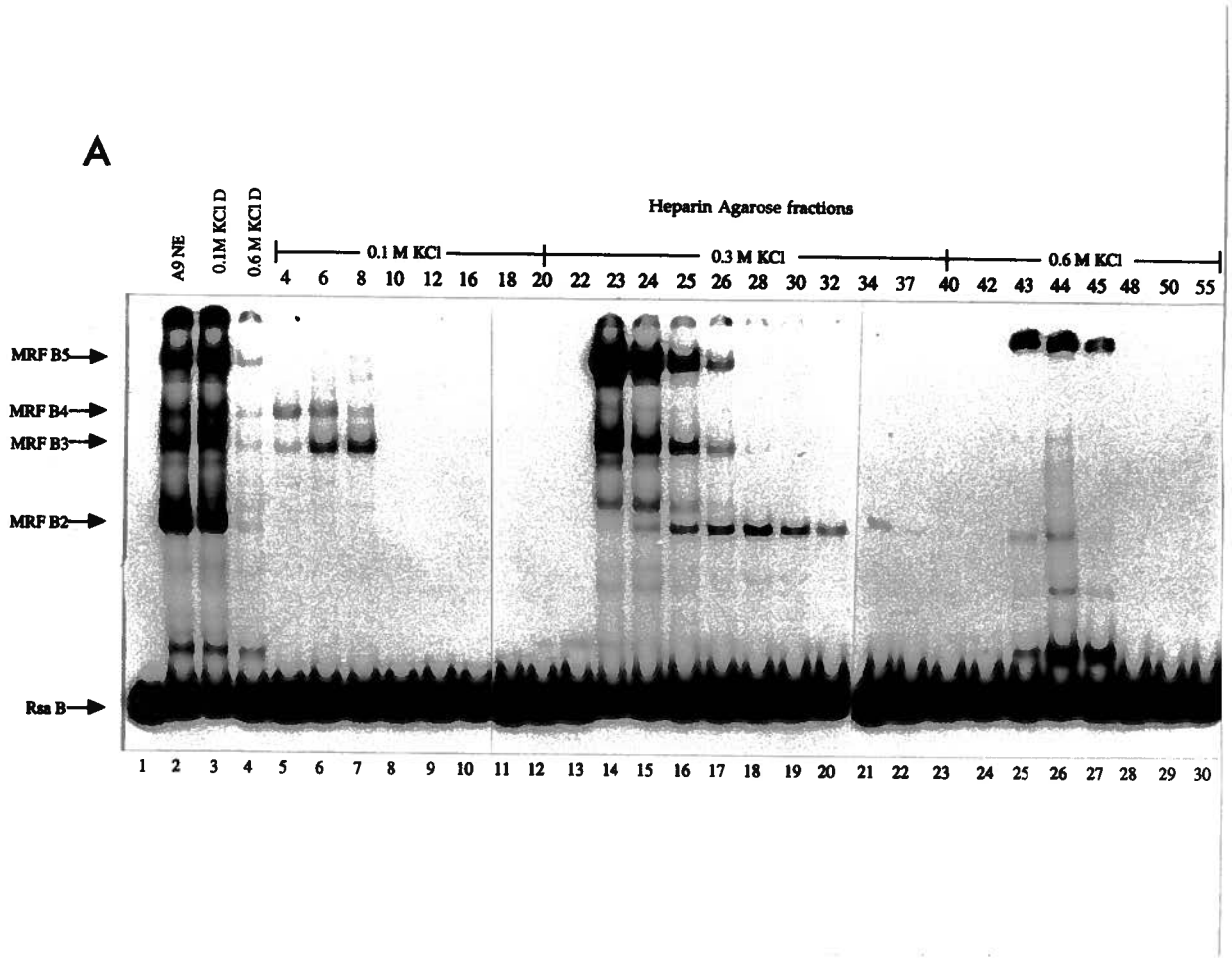
A
A9 NE
0.1M KCl D
0.6 M KCl D
Heparin Agarose fractions
0.1 M KCl
0.3 M KCl
0.6 M KCl
4 6 8 10 12 16 18 20 22 23 24 25 26 28 30 32 34 37 40 42 43 44 45 48 50 55
MRF B5
MRF B4
MRF B3
MRF B2
Rsa B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig.25 A

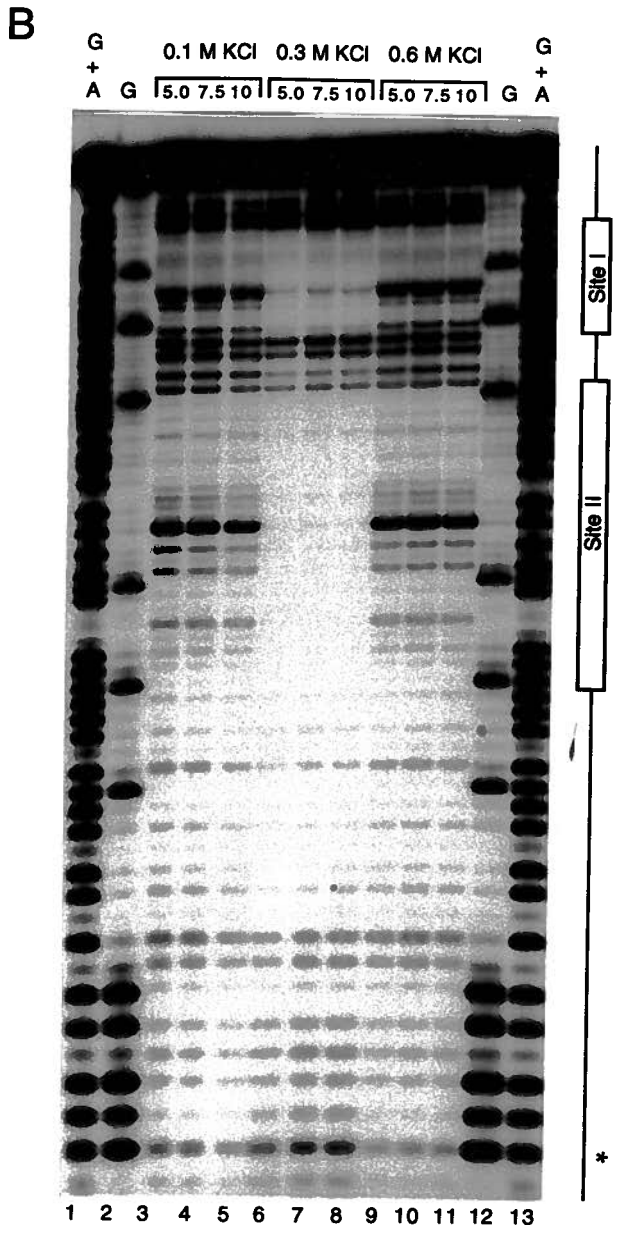
B
G + A
G
0.1 M KCl
0.3 M KCl
0.6 M KCl
5.0 7.5 10
5.0 7.5 10
5.0 7.5 10
G
G + A
Site I
Site II
*
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig.25 B

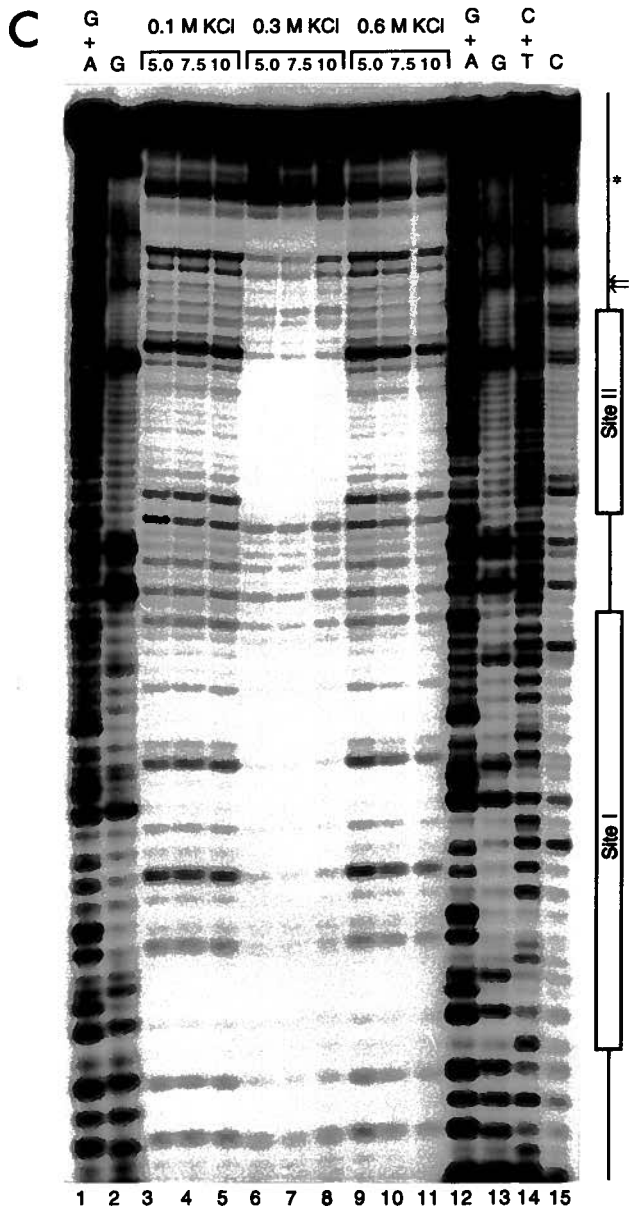
C
G + A
G
0.1 M KCl
5.0 7.5 10
0.3 M KCl
5.0 7.5 10
0.6 M KCl
5.0 7.5 10
G + A
G
C + T
C
*
Site II
Site I
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 25 C

D

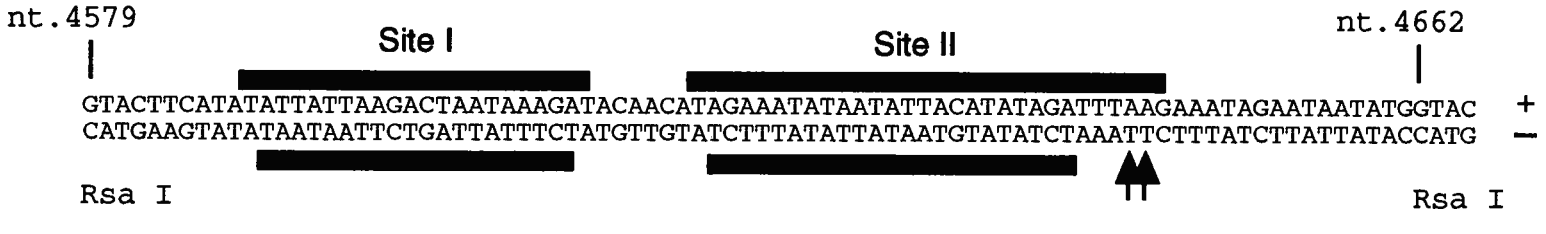
nt.4579
Site I
Site II
nt.4662
GTACTTCATATATTATTAAGACTAATAAAGATACAACATAGAAATATAATATTACATATAGATTTAAGAAATAGAATAATATGGTAC
CATGAAGTATATAATAATTCTGATTATTTCTATGTTGTATCTTTATATTATAATGTATATCTAAATTCTTTATCTTATTATACCATG
+
-
Rsa I
Rsa I

Fig. 25D

When the 0.3 M KCl HA fraction containing MRF B5 was titrated against Rsa B in EMSA (Fig. 26), MRF B5 appeared to be the only factor present in sufficient quantities to saturate the labeled probe. These data suggest that the two footprints generated from using the 0.3 M KCl HA fraction came from MRF B5. It may be possible that MRF B5 is a protein complex containing two DNA binding factors or a single polypeptide which contains two DNA binding domains. In an attempt to determine the composition of MRF B5, synthetic oligonucleotide pairs corresponding to site I (Bsite I) and site II (Bsite II) were used in a competition EMSA experiment (Fig. 27). When an excess of Bsite I oligo was introduced, only MRF B4 was specifically competed (Fig. 6, lane 3 and 4). When an excess of Bsite II oligo was added, only MRF B3 was competed (Fig. 6, lane 6 and 7). A non-specific oligonucleotide corresponding the *fps* responsive binding protein (FREBP) binding site (Bell and Sadowski, 1992), FREBP Oligo A, did not reduce the binding of any of the MRF's to Rsa B. These data suggest that the MRF B5 footprinting pattern may be generated by at least two binding activities, possibly MRF B3 and MRF B4, each of which binds to the Rsa B probe. If this hypothesis is correct, it is not known whether MRF B5 exists as a heterodimeric complex in solution or whether MRF B5 requires dimerization of MRF B3 and B4 on the DNA template in a cooperative manner. The former explanation is more likely since Bsite I or Bsite II oligos do not titrate MRF B5 binding to Rsa B at these oligo concentrations. At much higher concentrations of either unlabeled Bsite I or Bsite II oligos, MRF B5 binding activity to the radiolabeled probe is abolished (data not shown). Competition EMSA using both Bsite I and Bsite II oligos in the same tube was not performed. More detailed studies will be required in order to determine the exact nature of factor MRF B5.

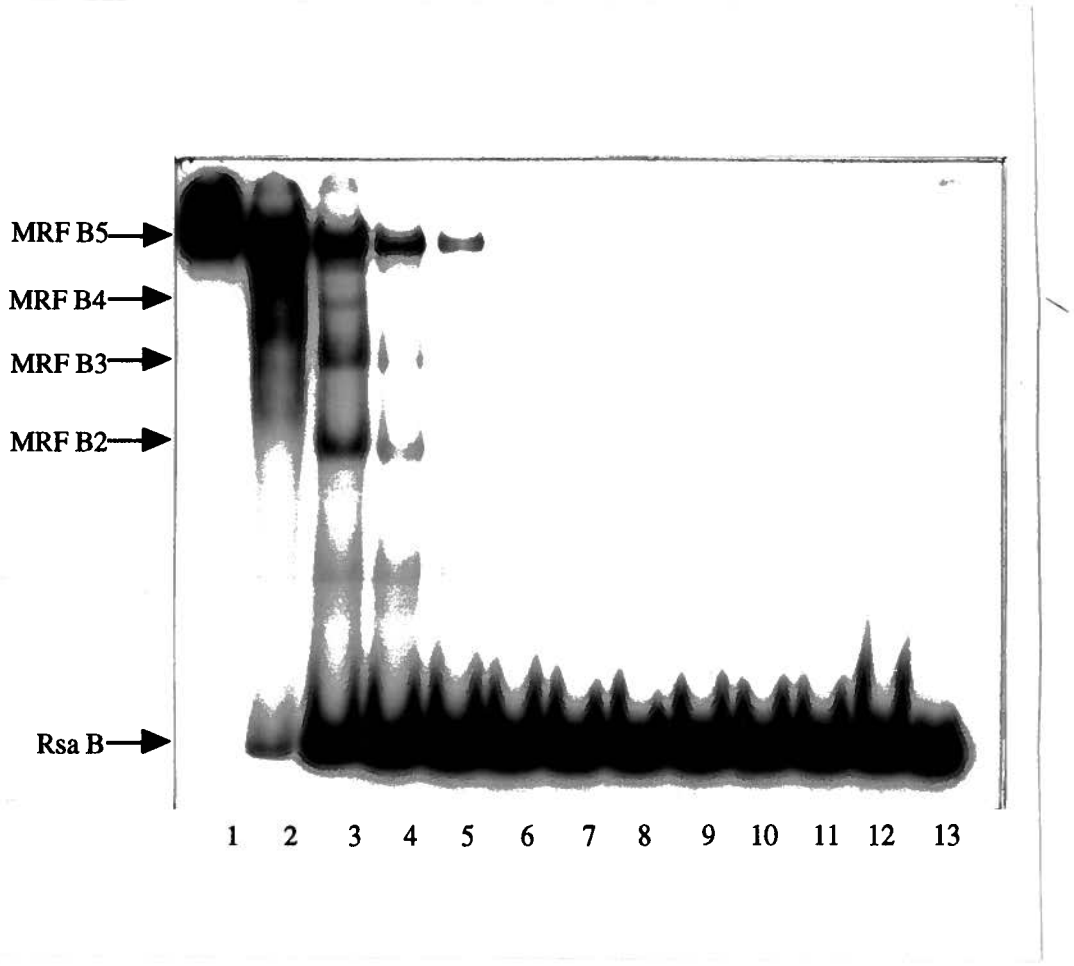

Fig. 26 MRF B5 can saturate Rsa B probe in EMSA.

Five microliters (11 μg protein) of the 0.3 M KCl HA extracts containing MRF B5 were introduced into a standard EMSA binding reaction (lane 1) and serially diluted 1:2 eleven times (lanes 2-12) before the addition of 10,000 cpm of radiolabeled Rsa B. Lane 13 contains no extract. The positions of free Rsa B, MRF B2, B3, B4 and B5 are indicated. Only MRF B5 was able to saturate the radiolabeled Rsa B probe.

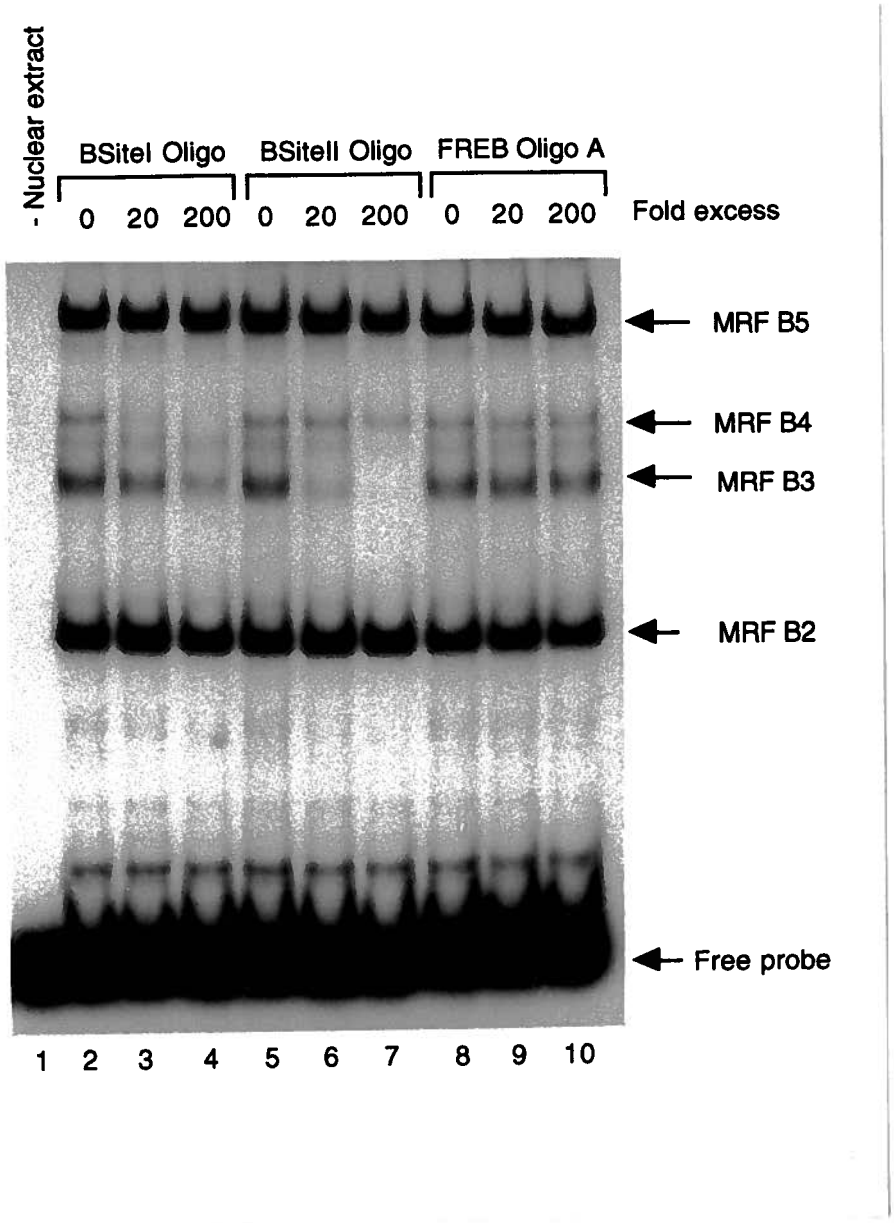

Fig. 27 Competition EMSA Suggests MRF B3 and MRF B4 are Components of MRF B5.

Radiolabeled Rsa B was incubated with 5 μg of uninfected nuclear extract in the absence (lanes 2, 5, 8) and presence of 20 and 200 fold molar excess of Bsite I Oligo (lanes 3 and 4), Bsite II Oligo (lanes 6 and 7) or FREBP Oligo A (lanes 9 and 10) in a standard EMSA experiment. The Bsite I and Bsite II oligos span the sequences defined by the DNase I footprints (site I and site II) of MRF B5 (Fig.5). FREBP Oligo A is a non-specific oligo which contains the binding site for the *fps*-responsive element binding protein. Lane 1 contains no nuclear extract.

IV. Discussion

4.1. Replication of MVM(p) Minigenomes

The data presented in this study show that a MVM(p) minigenome encoded by pPTLR was able to replicate in COS-7 or LA9 cells when NS-1 is provided in *trans* (Fig. 8). The pPTLR encoded minigenome contains 411 nt of the left terminus and 807 nt of the right terminus. When these regions are separated onto different plasmids, the clones are rendered replication defective. Although the pPTLR plasmid in this report was linearized close to the right terminus with EcoRI, minigenome replication was equally efficient when plasmids were linearized close to the left end palindrome (data not shown). The minigenome sequences must be excised or "rescued" from the plasmid sequences to generate a product which is suitable for MVM DNA replication. Although the mechanism of excision is currently not known, the minigenome sequences are rescued efficiently when the input plasmid is linearized before transfection. Replication of minigenomes rescued from covalently closed circular input plasmids was not examined.

Although the presence of covalently closed and open extended hairpin forms at either end of the pPTLR minigenome monomer pool (Fig. 10 and Fig. 11) implies that both terminal palindromes are actively used in viral DNA replication in COS-7 and LA9 cells, it was observed that there are differences in the replication of pPTLR in the two cell lines used in this study. First, the relative amounts of each double stranded monomer replicative form (mLR) is different in each cell line. In LA9 cells, mLR2 and mLR4 (Fig. 8A, lane 5) are the most abundant species, whereas mLR1 and mLR2 (Fig. 8B, lane 11) are the predominant species in COS-7 cells. Second, the amount of higher MW RFs in COS-7 cells is much greater that in LA9 cells (Fig. 8B,

lane 6 vs. 11). Third, the IRE deletion mutants dl411-4636 or dl411-4636SVR were able to replicate in COS-7 cells to a low efficiency, but not in LA9 cells. These differences suggest that the reaction kinetics for minigenome replication is different in each cell line. For example, if the resolution of dLR into mLRs is relatively less efficient, then the synthesis of higher MW RFs might be favoured. Resolution of higher MW RFs such as tetramers and octamers will affect the ratios of mLR species. This explanation is consistent with what is seen in COS-7 cells. If this hypothesis is correct, it is not unreasonable to suggest that the cellular factors involved in minigenome DNA replication in COS-7 and LA9 cells, and possibly NS-1, may replicate minigenome DNA at slightly different efficiencies.

4.2. Analysis of the Left Hand (3') Terminal Deletions Mutants Suggests That Bubble Sequences are Important

The original objective of the deletion analysis of the left (3') palindrome sequence was to determine if the stem plus arms structure of the left hairpin was required for DNA replication. It was surprising to see that mutants which cannot form a stem plus arms hairpin structure (pPTLRdl57 and 78) were able to replicate to substantial levels in transfected LA9 cells (Fig. 12B, lanes 7 and 8). A self-repair mechanism cannot be proposed for pPTLRdl78 since a stable duplex hairpin formation is not possible. A self-repair mechanism is possible for pPTLRdl12, 26, 31 and 57 since these mutants can potentially form a usable hairpin to prime DNA synthesis. But if this mechanism is used, only the pPTLRdl12 minigenome can regenerate the wild type MVM sequences at the left terminus. Thus in LA9 cells, regeneration of the left terminus of pPTLRdl78 must occur by an alternative mechanism such as recombination which uses wild type viral sequence.

In the COS-7 cell system, no recombination with wild type MVM(p) is possible since the NS-1 expression vector does not contain the hairpin sequences. Hence, in contrast with LA9 cells, sequences in the stem region found between nt 12 and 26, are important for DNA replication in COS-7 cells (Fig. 12C). Since deletion of 30 nt (pPTLRdl31) of the left terminus had the same effect as deletion of the 25 nt (pPTLRdl26), it is possible that both mutations resulted in a similar defect in the viral DNA template. In Fig. 28, a self-repair mechanism of the left (3') hairpin is proposed for pPTLRdl12 and 31. Nucleotide 25 and 26 are mismatched with nt 89-91 to form a bubble structure within the stem of the hairpin. The proposed mechanism predicts that only pPTLRdl12 is able to regenerate the wild type left hairpin sequences. This is possible because 3' terminal deletions up to the bubble can be repaired back to wild type MVM sequences by using the existing hairpin to prime DNA synthesis (Fig. 12A, dl12). The experimental result in Fig.12C is consistent with this theory since pPTLRdl12 replicates like pPTLR in COS-7 cells. Larger 3' deletions which extend past nt 24 (the bubble), but not past nt 45 (base of the short arm), if self-repaired, are predicted to alter the bubble sequences (Fig. 28A, dl31). For example, if pPTLRdl31 is self-repaired, the GAA sequence (nt 91-89) is used as template for DNA synthesis. Thus the imperfection of the palindrome sequence at nt 25-26 in the wild type left hairpin is changed in self-repaired pPTLRdl31. The consequence of this mechanism is two fold. First, the sequence of the repaired left hairpin will contain TTC base paired with AAG instead of GA

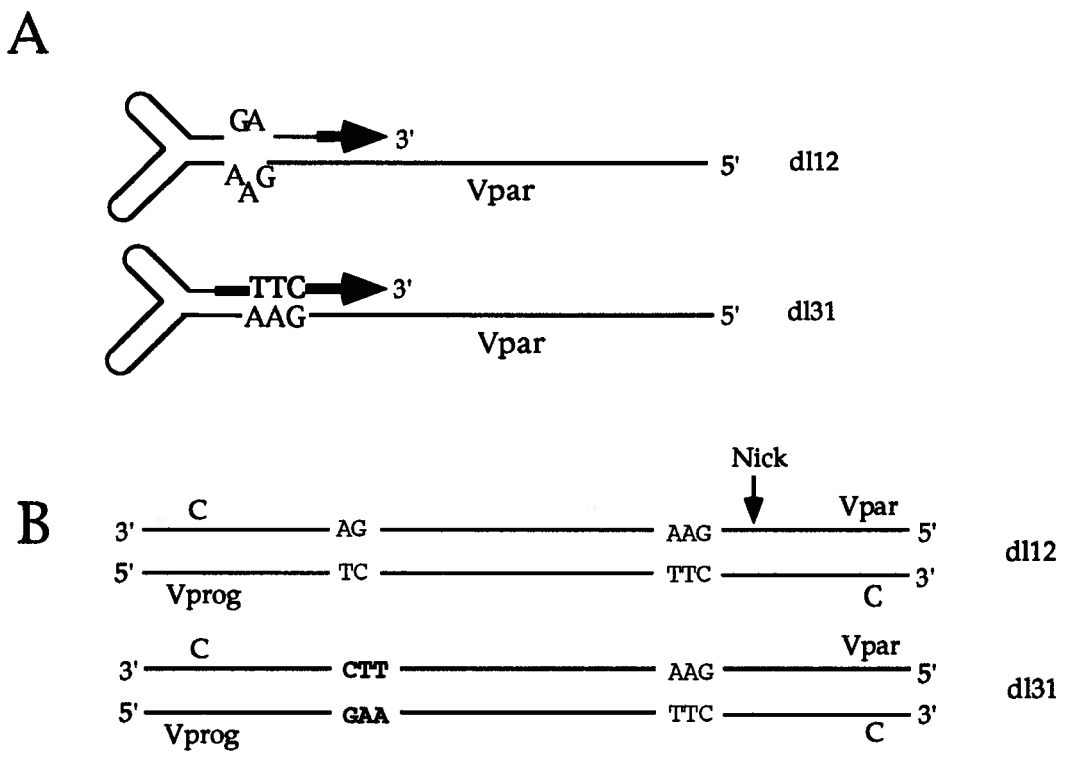

Fig. 28 Possible repair mechanism of 3' hairpin deletions.

(A) The hypothetical left hand (3') hairpin of two left hand hairpin deletion mutants, pPTLRdl12 and pPTLRdl31 is schematically drawn (thin line). Both mutants still can potentially utilize the existing hairpin primer for synthesis of mRF (thick line with arrow), facillitating the repair of the 3' terminus. In the case pPTLRdl12, the 3' terminal sequences are predicted to be exactly like wild type MVM. This is not the case for pPTLRdl31 since DNA synthesis through the bubble sequence abolishes the bubble structure. The predicted nucleotide changes are denoted in bold type. (B) The predicted sequence of the 3' dimer bridge of the two deletion mutants are shown. The Modified Rolling Hairpin Model of MVM DNA replication (Fig. 4) predicts the initial nick required for the resolution of the wild type 3' dimer bridge is located near the GAA sequence. This nick is shown in the 3' bridge dimer of dl12. The nucleotide sequence of the bridge dimer generated from pPTLRdl31 is predicted to also change such that the asymmetrical part of the dimer bridge fragment due to the bubble sequences becomes symmetrical. The implications of these sequence changes are discussed in the text. The bubble sequence is one of several asymmetrical sequences found in the 3' bridge fragment.

mismatched to GAA. This minor sequence alteration is predicted to replace the wild type bubble with duplex DNA, but the stem plus arms hairpin is predicted to form. Second, since bubble sequences within the wild type 3' palindrome result in asymmetry elements in the left (3') dimer bridge fragments, self-repaired pPTLRdl31 is predicted to have symmetrical DNA sequences in the same positions. For example, wild type left (3') end palindromes or self-repaired pPTLRdl12 left (3') palindromes are predicted to be $\overset{GA}{CT}$ on one side of the dimer bridge fragement, and $\overset{GAA}{CTT}$ on the other side of the dimer bridge fragment (see Fig. 28B, dl12). Self-repaired pPTLRdl31 is predicted to have identical but inverted $\overset{GAA}{CTT}$ sequences on both sides of the 3' bridge dimer fragment (Fig. 28B, dl31).

How might the alteration of the bubble sequences affect the function of the hairpin if the stem plus arms structure is not changed? It may be possible that deletion of the first 25 nt impaired the ability of transfected cells to rescue the minigenome sequence of pPTLRdl26 to a form suitable for DNA replication. It may also be possible that the change from the wild type bubble structure to the mutant duplex DNA in the stem may impair hairpin function. Several observations seem to be inconsistent with the notion that the mutant left (3') hairpin cannot funtion to prime DNA synthesis. First, analysis of Mbo I digests of Hirt DNAs (Fig. 12D) shows that mLRs generated from pPTLRdl25 and 31 contained covalently closed left hairpins. Second, the presence of significant levels of dLRs generated from these mutants show that both hairpins must function to some extent (Fig. 12B). The inablity of pPTLRdl26 and 31 to generate mLRs containing open extended hairpins at the left terminus is intriguing because this configuration of the left palindrome is generated through resolution of dLR molecules, not by hairpin transfer. These

observations suggest that pPTLRdl26 and 31 minigenomes are able to generate mLR and dLR molecules, but may not be able to resolve dLR molecules. According to the Modified Rolling Hairpin Model, the asymmetric resolution of the left (3') dimer bridge is initiated by a site specific nick (Fig. 4, step 4). It was also hypothesized that asymmetric elements within the left (3') dimer bridge fragment serve as the basis for the specificity of the first nick (Astell *et al.*, 1985; Cotmore and Tattersall, 1987). The self-repaired genome of pPTLRdl31 is predicted to generate a left (3') dimer bridge fragment that is different from that of pPTLRdl12 or pPTLR (Fig. 28B). However, the Modified Rolling Hairpin Model predicts that a nick occurs on the $\overset{GAA}{CTT}$ side of the dimer bridge (Astell *et al.*, 1985), hence molecules shown in Fig. 28B (dl31) would be expected to be nicked on both sides of the dimer bridge. The fact that the change in the dimer bridge fragment apparently inhibits resolution suggests that the precise details of the Modified Rolling Hairpin Model require further examination. This observation is, however, in agreement with the data of Cotmore and Tattersall, (1994), who have suggested nicking occurs on the $\overset{GA}{CT}$ arm of the dimer bridge, no longer present in the predicted dimer bridge of pPTLRdl31 (Fig. 28B).

4.3. Replication of Identical End Genomes

Previous data suggests that in MVM(p), and probably in other related parvoviruses, there are two biochemically distinct reactions occurring at the left and right terminal sequences during replication the viral DNA (Cotmore *et al.*, 1992; Cotmore *et al.*, 1993). In contrast, viruses that contain identical hairpin ends such as AAV likely use a single reaction at both ends (Im and Muzyczka, 1990; Snyder *et al.*, 1990). Is MVM able to replicate its DNA using a single reaction mechanism like

AAV ? The results in Fig. 20 show that a RR minigenome replicates efficiently in COS-7 cells whereas the LL minigenome replicates at a greatly reduced level in COS-7 cells (<5%). Since both types of genomes are able to replicate, both termini contain sequences required for excision of the viral sequences from the plasmid DNA and initiation of DNA replication. The data presented here indicate that sequences at the right termini may potentially contain stronger initiation signals than those at the left terminus (Fig. 20 and 22). The right terminal sequences are believed to initiate DNA replication at a double stranded template during three steps in the replication of MVM DNA: (1) conversion of the mRF to the dRF and other higher order intermediates, (2) right hairpin transfer reaction in the generation of genomic s.s. minus sense DNA and (3) resolution of the 5' junction fragments. Although the latter reaction has been reproduced *in vitro* (Cotmore *et al.*, 1993), the former two reactions have not been demonstrated *in vitro.*

The left hand terminal sequences are thought to be responsible for priming DNA replication for the conversion of the parental ss genomic DNA to the double stranded mRF DNA and the resolution of the 3' dimer bridge fragments, but only the latter reaction initiates DNA replication from a double stranded DNA template. Why is it that the replication of the LL minigenome is far less efficient than the RR minigenome? It has been shown previously that a plasmid containing the left bridge dimer fragment is resolved *in vivo* into a linear plasmid containing two conformationally different ends, one end an extended open end with NS-1 covalently attached to the 5'end while the other end is a covalently closed hairpin end (Cotmore and Tattersall, 1992). This resolution mechanism of the left bridge dimer was previously proposed to account for the fact that only one sequence orientation (flip) is found at the left end in both genomic DNA and intracellular replicative intermediates (Astell *et al.*, 1983; Astell *et al.*, 1985). As a consequence,

the left hairpin does not undergo a traditional hairpin transfer mechanism. A replicating LL genome would then accumulate dead end intermediates (i.e. mLL containing covalently closed hairpinned ends at both termini) which are generated through resolution of dLL or concatemer LL intermediates.

Since the right end of MVM is known to contain approximately equal proportions of flip and flop sequences (Astell *et al.*, 1985), a hairpin transfer mechanism may be involved in the resolution of the covalently closed hairpin forms. Such resolution reactions have been described *in vitro* for covalently closed AAV hairpins (Snyder *et al.*, 1990). A replicating RR genome would then generate intermediates similar to AAV. Our experiments indicate that both the extended and the closed hairpin forms are present in the mRR pool. The appearance of the covalently closed intermediate mRR3 was unexpected since resolution of the right bridge fragment produces only linear duplex ends. It is possible that mRR3 is generated by recombination events between genomes. An alternative explanation for the generation of mRR3 is a ligation event at the right end (Cotmore *et al.*, 1989). In any case, if mRR3 is a legitimate intermediate, the covalently closed ends are likely resolved in a manner similar to the resolution of AAV covalently closed hairpins.

4.4. Origins of Replication Contain Multiple *Cis*-Acting Elements

Studies of viral origins of replication such as SV40, BPV-1, EBV, HSV-1 or yeast ARS1 suggests that eukaryotic origins of DNA replication contain multiple functional *cis*- acting elements. Core *ori* elements include the replicator element (recognized by the initiator protein), DUE (DNA unwinding elements) and/or A/T rich elements. Replicator sequences such as the SV40 T-Ag site II, UL9 binding sites I and site II in HSV-1 *ori*S, or ACS in the yeast ARS1 are absolutely necessary for the origin activity. The initiator proteins have been either identified or implicated in each of the mentioned replicator sequence. Other *cis*- acting sequences which act as the initial site(s) for DNA unwinding (DUE) are also a feature in many origins. For example, the A/T rich elements HSV-1 *ori*S and the early palindrome (EP) of the SV40 core *ori* have been shown to be unusually sensitive to chemical probes if the initiator protein is bound to the *ori* demonstrating that the DNAs in these elements are denatured during the initial events of replication (Borowiec and Hurwitz, 1988b; Koff *et al.*, 1991). In SV40, the structure of the A/T rich element was also shown to be distorted but probably not denatured. Other elements which bind *trans* -acting factors such as transcription factors are also known to activate but not directly initiate DNA replication at the origin (DePamphillis, 1993). These elements constitute a third class of elements termed auxiliary replication elements. In the case of EBNA-1 of EBV, and perhaps the E2 protein of BPV-1, the transcription factors form the initiator complex or part of the initiator complex.

Auxiliary replication elements can activate or stimulate *ori* activity through several mechanisms. Transcription factors may stimulate the formation of replication complexes. The classic example of this type of activation comes from the

transcription factor nuclear factor (NF-1). It has been shown that NF-1 is able to stimulate initiation of replication of adenovirus type 2 (Ad2) at the *ori* located at the termini (Stillman, 1989). NF-1 binds immediately adjacent to the *ori* and facilitates the binding and stabilization of the Ad2 pre-terminal protein-dCMP/polymerase (Ad2 p-TP-CMP/pol) complex to the *ori* (Mui and Van der Vliet, 1992). NF-1 activation is important *in vitro* when concentrations of the Ad2 p-TP-CMP and Ad2 pol are subsaturating. Another example is the EBNA-1 protein of EBV. Although the initiation of replication is thought to start as the DS element (Gahn and Schildkraut, 1989), the wild type FR element, which also acts as a transcriptional enhancer (Reisman *et al.*, 1985; Reisman and Sugden, 1986), functions to facilitate the looping out of the intervening DNA through protein-protein interactions of the EBNA-1 complexes bound at the DS and FR element. Although the wild type FR element contains 20 copies of the EBNA-1 binding sites, as little as 8 binding sites is required for *ori* activity. It is thought that the looping out of the DNA facilitates the eventual formation of a replication complex at the DS element. Factors binding to auxiliary replication sequences may also target cellular replication factors to the *ori*. For example, it has been demonstrated that the yeast transcription factor, Gal4, is able to activate DNA replication when Gal4 binding sites are placed near the polyomavirus *ori*. Further studies demonstrate that a Gal4-VP16 chimeric protein is able to transactivate BPV-1 DNA replication only when Gal4 DNA binding sites are inserted near the *ori* (Li and Botchan, 1993). The finding that the activation domain of Gal4 and VP16 binds to RF-A (He *et al.*, 1993), the ss DNA binding protein, suggests that the RF-A replication factor could be localized to an origin via Gal4 DNA binding function and mediate unwinding of DNA at the *ori*. Transcription factors may also stimulate the *ori* core by derepressing the effects of nucleosome formation there by allowing easy access of the replication factors to the *ori*. An example of this type of "activation" was observed when a NF-1 site was

placed adjacent to the 64 bp *ori* core of SV40 (Cheng and Kelly, 1989). It was shown that a single NF-1 site was able to stimulate DNA replication *in vivo*, but no stimulation was observed when naked plasmid DNA was replicated *in vitro*. Assembly of nucleosomes onto this plasmid greatly repressed DNA replication *in vitro*, but the repression of DNA replication was relieved when the plasmid was incubated with NF-1 prior to nucleosome assembly.

4.5. Possible Roles for Cis-Acting Elements Found Inboard of the Right Palindrome

The genetic analysis of the DNA replication of MVM(p) minigenomes reported in this study suggest that two elements, A (nt 4489-4636) and B (4695) are important in MVM DNA replication (Fig. 14). Further experiments show that two Rsa I fragments, Rsa A (nt 4431-4579) and Rsa B (nt 4579-4662) were able to partially rescue the ability of pPTLRdl411-4695 minigenome to replicate in COS-7 cells (Fig. 18). Since sequence specific DNA binding proteins are important in the regulation and initiation of DNA replication in other characterized systems, I attempted to determine if such proteins were able to bind to the Rsa A and Rsa B fragments. Although a number of specific DNA/protein complexes were detected in EMSA experiments, the binding site of one factor, MRF B5, was determined. The two regions protected by MRF B5 from DNase I, are referred to as site I and site II. Although the composition of MRF B5 remains undetermined, competition EMSA experiments suggest that MRF B5 may contain MRF B3 and MRF B4. This suggestion does not exclude the possibility that other factors are involved in the formation of MRF B5. Clearly more experiments are required in

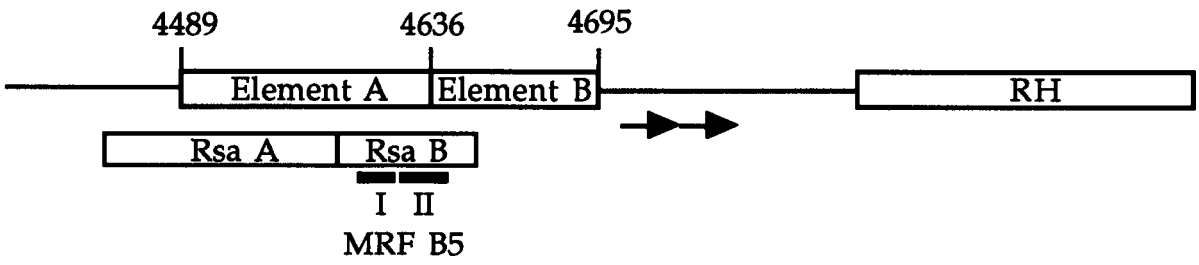

Fig. 28 Organization of Putatative Replication Elements at the Right Terminus of MVM.

Element A (nt 4489-4639), element B (nt 4636-4695) and the right (5') palindrome are boxed. The 65 bp tandem repeats are indicated by the arrows and are located downstream of element B. The position of the two MVM Rsa I fragments used in this study, Rsa A (nt 4431-4579) and Rsa B (nt 4579-4662), are also boxed and indicated below elements A and B. The positions of the MRF B5 binding site I (nt 4589-4610) and site II (nt 4616-4646) are indicated below the Rsa B fragment as thick lines.

order to determine the biochemical make up of MRF B5. If MRF B5 site I and site II are indeed elements important for MVM DNA replication, what type of DNA replication elements are they? Deletion analysis of the internal right end (IRE deletions) shows that pPTLRdl411-4636 in COS-7 cells are able to replicate at low levels. This implies that the *ori* core sequences are still present on this minigenome since core *ori* sequences are defined as absolutely essential for DNA replication. Thus core *ori* elements are not found in element A. The fact that no replication was seen in the pPTLRdl411-4695 minigenome suggests that at least one of the *ori* core elements may have been deleted. It is also possible that very low levels of replication occurs and was undetected in the data presented. Since both Rsa A and Rsa B were able to partially rescue the replication of the pPTLRdl411-4695 minigenome, it argues that *ori* core elements lies downstream of the Rsa I site at nt 4662. It cannot be definitively determined if any *ori* core elements reside within element B at this time. MRF B5 site I is found entirely within element A but MRF B5 site II (approximately nt 4610-4646) spans the element A and B junction, lying mostly within element A. It is very likely that MRF B5 site II is not functional in the pPTLRdl411-4636 minigenome since most of the binding site has been deleted. If MRF B5 site II is not functional in the pPTLRdl411-4636 minigenome then it is likely that MRF B5 binding site I and site II do not encode replicator elements.

What are the possible roles of MRF B5 in MVM DNA replication? Since these elements are found near the right (5') palindrome, it is likely that MRF B5 acts at the right end, but it also possible that MRF B5 also affects replication at the left palindrome sequence. Activation of genomes containing two left termini with the SX fragment (which contains the MRF B5 site I and II) is consistent with this possibility. According the current models of MVM DNA replication, several events are predicted to occur at the right terminus. First, synthesis of dimer replicative

forms requires the formation of a "rabbit ear" structure (Fig. 4, step 3) at the right terminus. In order to generate the rabbit ear structure, the double stranded DNA right terminus must be denatured and allowed to fold into "turnaround" hairpins. This would then provide a primer for DNA synthesis. A similar event must also take place when single stranded viral DNA is synthesized (Fig.4, step 7). It may be possible that MRF B5 (in addition to other factors binding to potential auxiliary replication elements) may facilitate these reactions. MRF B5 site I and site II appear to be relatively distant (~400 bp) to the right palindrome when compared to auxiliary elements in other origins of replication such as the Sp1 binding sites in the SV40 origin (Fig. 6A). However, an example when elements are distant to the origin of replication is found at *ori*P of EBV (Gahn and Schildkraut, 1989). The FR elements in the EBV *ori*P are approximately 1 kbp from the DS element, the site of initiation of DNA replication.

If parvoviral origins of replication resemble other origins of replication, then MVM should contain auxiliary replication elements, DUE, and at least one replicator element. In AAV, it is clear the terminal hairpin is the replicator and the Rep proteins are the initiators. For MVM, the replicator has not been determined yet, but it is likely to be near or at the terminal palindrome. Although MVM NS-1 is clearly the key replication enzyme for viral DNA replication, it has not been reported that NS-1 binds double stranded DNA in the traditional sense like SV40 T-ag or EBV EBNA-1. The basis for sequence specific initiation of MVM DNA replication is not currently known, but it is possible that cellular factors may contribute to this process. The data in this thesis suggest that there may be auxiliary elements found near the right palindrome of the MVM genome. Although any mechanism of activation suggested at this point is highly speculative, only further analysis of this region will help develop our understanding of the

biochemical events that occur at the right hand palindrome. Analysis of site specific mutants of the MRF B5 site I and II in the full length infectious clones may help elucidate the functional roles of the MRF B5 factor. Identification and characterization of the gene(s) coding for MRF B5 will likely contribute to our understanding of MVM DNA replication as well as DNA replication of the host cell. The data shown in this thesis also provide evidence that other proteins binds to the Rsa A and Rsa B fragments. The binding sites for these factors may also function as additional auxiliary replication elements.

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