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Multiple Cellular Factors Bind to *cis*-Regulatory Elements Found Inboard of the 5′ Palindrome of Minute Virus of Mice

PATRICK TAM AND CAROLINE R. ASTELL*

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Previous genetic analysis of the DNA replication of minute virus of mice (MVM) minigenomes suggested that specific elements, A (nucleotides [nt] 4489 to 4636) and B (nt 4636 to 4695), found inboard of the 5′ palindrome are required for efficient MVM DNA replication (P. Tam and C. R. Astell, Virology 193:812–824, 1993). In this report, we show that two MVM *Rsa*I restriction fragments (*Rsa*I A [nt 4431 to 4579] and *Rsa*I B [nt 4579 to 4662]) are able to activate DNA replication of an MVM minigenome containing deletions of both elements A and B. We also show that sequences inboard of the right palindrome are able to activate replication of minigenomes containing two left termini. In order to investigate the importance of the *Rsa*I fragments, we demonstrate the presence of a number of sequence-specific DNA-protein interactions by electrophoretic mobility shift assays. After partial fractionation of A9 nuclear extracts, DNase I footprinting analysis was used to determine the binding sites for MVM replication factor (MRF) B5. MRF B5 protects two distinct regions (sites I and II) of the *Rsa*I B probe from DNase I digestion. Competition electrophoretic mobility shift assays with synthetic oligonucleotides corresponding to sites I and II suggest that MRF B5 is composed of two factors, MRF B3 and MRF B4, which bind DNA independently in a sequence-specific manner. It may be possible that these replication factors are proteins which are able to transactivate MVM DNA replication and hence are accessory replication factors.

Minute virus of mice (MVM) is a nondefective parvovirus which contains 5,149 nucleotides (nt) of minus-sense DNA. The terminal palindrome sequences at either end are unique and are thought to fold into stable hairpin duplexes which serve as primers for DNA synthesis (6). Thus, the single-stranded genomic DNA is converted to a double-stranded monomer replicative intermediate during the initial steps of DNA replication. Since stable hairpins may form at both termini, further DNA synthesis generates dimer and higher concatamer replicative DNAs (i.e., tetramers) in which the monomer units are in a head-to-head and/or tail-to-tail arrangement (9, 24). Current models of MVM DNA replication suggest that site-specific nicks introduced in the bridge fragment create 3′-OH primers necessary for DNA synthesis and the subsequent resolution of the dimer replicative form (RF) (1, 2). The viral protein required for MVM DNA replication is NS-1, an 83-kDa nuclear phosphoprotein. MVM NS-1 has been shown to be covalently attached to the 5′ ends of intracellular replicative DNAs and single-stranded genomic DNA (7, 8). Furthermore, NS-1 has been shown to resolve the 3′-to-3′ bridge fragment in vivo (9) and in vitro with recombinant NS-1 from vaccinia virus (5) and baculovirus (19) expression systems. Purified recombinant NS-1 from insect cells contains an ATP binding motif and has ATPase and DNA helicase activities thought to be required for resolution of the 3′-to-3′ bridge fragment (25).

Although it is clear that the terminal palindromes are essential for MVM DNA replication, recent genetic evidence suggests that sequences found inboard of the hairpin sequences are required for efficient DNA synthesis. Deletion of sequences inboard of the 5′ palindrome were shown to impair or abolish replication of MVM minigenomes. We show in this report that MVM-specific sequences are able to activate a DNA replication-defective MVM minigenome as well as poorly replicating MVM LL minigenomes, i.e., containing two 3′ termini. Furthermore, we present data to suggest that host-cell sequence-specific DNA binding proteins may be responsible for this activation of DNA replication.

* Corresponding author. Phone: (604) 822-2142. Fax: (604) 822-5227.

MATERIALS AND METHODS

Cell lines and virus. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.3]) and 10% fetal bovine serum. A9 oub^{r}11 variant mouse L cells (18) were grown in suspension in Joklik's modified Eagle's medium supplemented with 10 mM HEPES (pH 7.3) and 5% fetal bovine serum.

Plasmid constructs. The construction of pPTLR, pPTLRdl 411-4695(RV), and pCMVNS1 has been described previously (23). Restriction fragments containing either the simian virus 40 (SV40) origin of replication or the MVM *Xba*I(4342)-*Sau*3a(4741) (SX) fragment were inserted into the unique *Eco*RV site of pPTLRdl411-4695(RV) to generate pPTLR SVR and pPTLRSXR, respectively (Fig. 1A). Two *Rsa*I restriction fragments (nt 4431 to 4579 and 4579 to 4662) which partially overlap the A (nt 4489 to 4636) and B (nt 4636 to 4695) elements were inserted into the unique *Eco*RV site of pPTLRdl411-4695(RV) in either rightward (AR and BR) or leftward (AL and BL) orientations.

Construction of pPTLL has been described previously (23). A deletion of the *Xba*I(4342)-*Xho*I(2070) fragment resulted in a smaller version of an LL minigenome, designated pPTLLX (Fig. 2A). In order to insert fragments into the pPTLLX vector, it was digested with *Eco*RV and a *Bgl*II linker was inserted (pPTLLX-*Bgl*II). The vector containing two identical left-terminal fragments was purified. In order to insert the SX fragment into the pPTLLX vector, the SX fragment was first

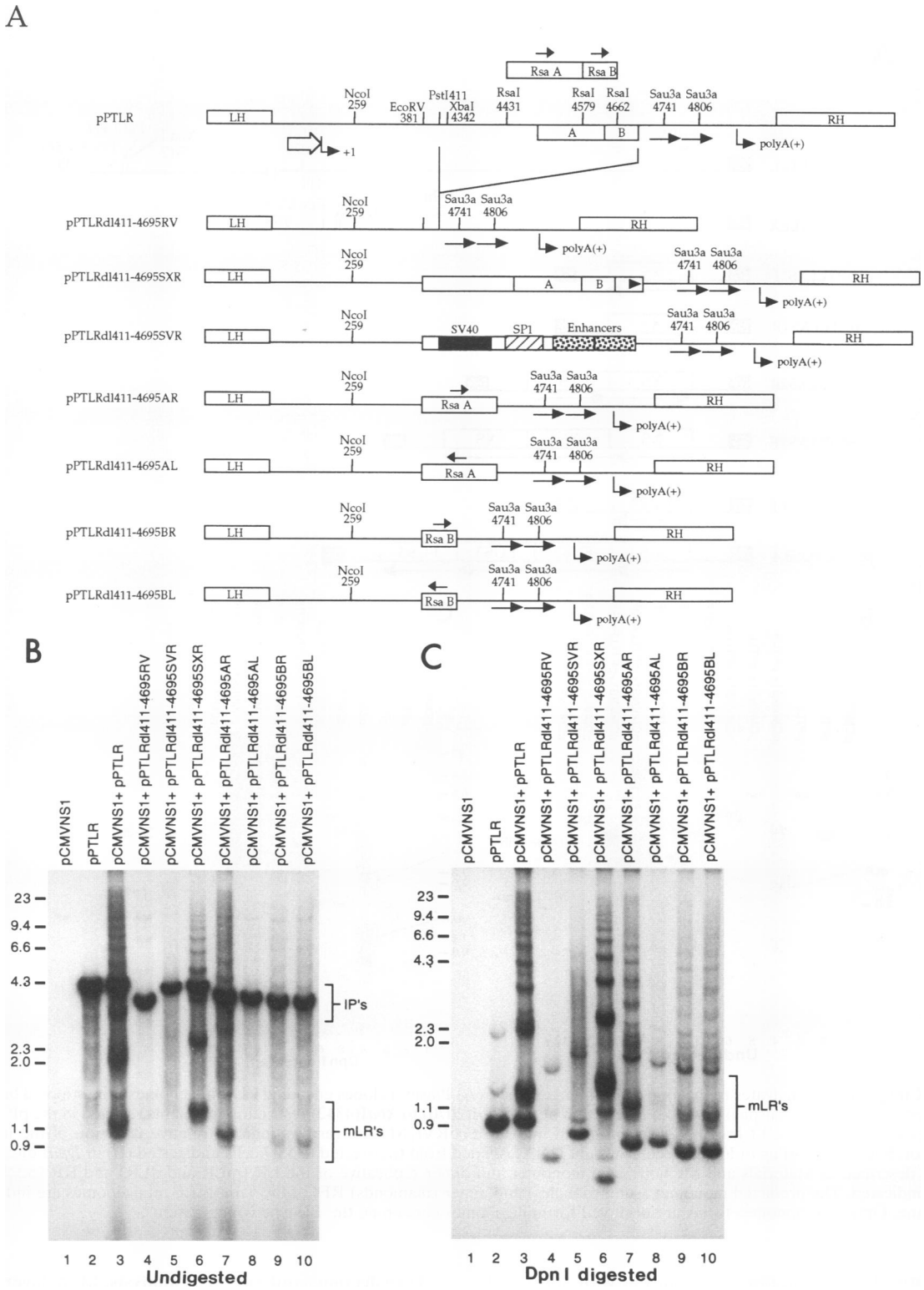

FIG. 1. *Rsa*I A and B fragments activate DNA replication. (A) Plasmid clones encoding MVM minigenome constructs. The plasmid pPTLR encodes a minigenome with the left 411 nt fused with 807 nt of the right terminus. Boxes with LH and RH indicate the left (3′) and right (5′) hairpin sequences. The P4 promoter (open arrow), 65-bp tandem repeats (solid arrows), and polyadenylation site are indicated. Elements A (nt 4489 to 4636) and B (nt 4636 to 4695) are boxed, as are the two *Rsa*I fragments, A (nt 4431 to 4579) and B (nt 4579 to 4662), used in this report. The plasmid pPTLRdl411-4695 (RV) contains a deletion between *Xba*I(4342) and *Xba*I(4695) of the pPTLR clone. Four fragments have been inserted into the unique *Eco*RV(381) site in this vector: the SX fragment; the entire SV40 origin of replication, including the enhancers (stippled boxes), GC boxes which code for the SP1 binding sites (hatched box), and the core SV40 origin of replication (solid box); the *Rsa*I A fragment; and the *Rsa*I B fragment. All minigenomic constructs were linearized with *Eco*RI prior to transfection. Southern blots of low-molecular-weight DNAs isolated from transfected COS-7 cells undigested (B) and digested (C) with *Dpn*I are shown. The *Dpn*I-resistant monomer (mLR) RFs are as indicated. IP's are input, unreplicated DNA.

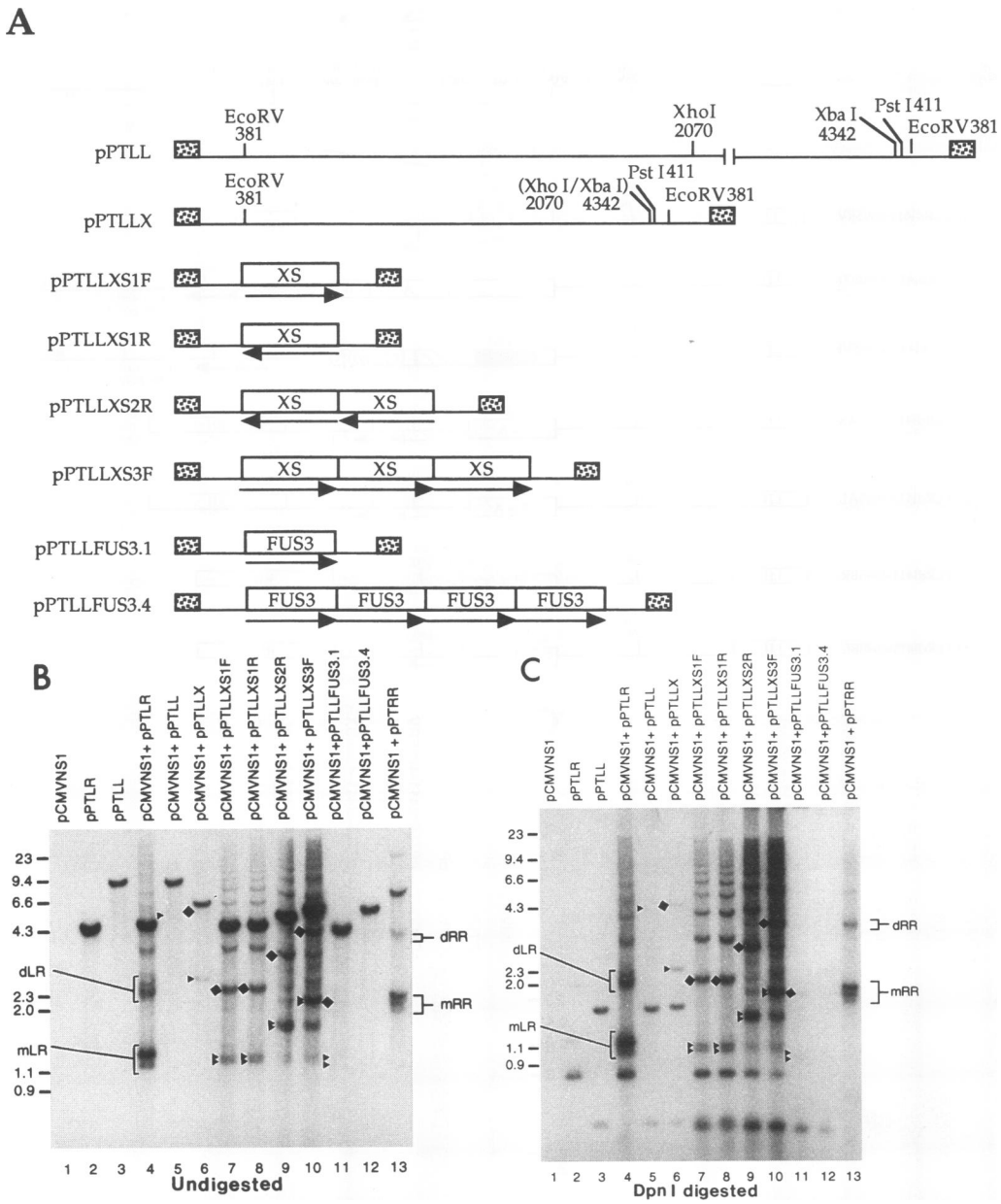

FIG. 2. SX fragment also activates replication of LL minigenomes. (A) Plasmid clones encoding LL minigenomes. The stippled box at the end of each line represents the left hairpin sequences. A deletion of the *Xho*I(2070) to *Xba*I(4342) restriction fragments resulted in the pPTLLX clone. Various copies of the SX or *FUS3* fragments were inserted into the *Eco*RV(381) site (arrows indicate relative direction of the insert) of the pPTLLX vector. Southern blotting of low-molecular-weight DNAs isolated from transfected COS-7 cells undigested (B) or *Dpn*I digested (C) was performed as described in Materials and Methods. The monomer and dimer replicative of the LR (mLR and dLR) and RR (mRR and dRR) genomes are indicated. The predicted monomer (solid triangles) and dimer (diamonds) RFs of the various LL minigenomes are indicated to the left of each lane. Only two monomer forms are observed for minigenomes containing the LL-type configuration.

subcloned into pUC19. The insert was cut out with *Sma*I and *Hinc*II, purified, ligated with *Bgl*II linkers, and digested with *Bgl*II. This fragment was purified and ligated to pPTLLX-*Bgl*II. Clones containing one (pPTLLXS1F and pPTLLXS1R), two (pPTLLXS2R), and three (pPTLLXS3F) inserts were obtained. (F and R refer to forward and reverse orientation of the insert.) In addition, we also cloned one and four copies, respectively, of the *Bam*HI and *Bgl*II fragments of the *FUS3* gene of *Saccharomyces cerevisiae* into the pPTLLX-*Bgl*II vector.

Transfections and Southern analysis. Monolayers of COS-7 cells were transfected by the DEAE-dextran method as described previously (23). Low-molecular-weight DNAs were isolated by the method of Hirt 72 h posttransfection (23). Cells were rinsed twice with phosphate-buffered saline before lysis with 100 mM NaCl–10 mM EDTA (pH 8.0)–1% sodium dodecyl sulfate for 15 min. The viscous lysate was transferred to a 1.5-ml microcentrifuge tube, and NaCl was added to a final concentration of 1.1 M. After incubation on ice overnight, samples were spun at 12,000 × *g* for 30 min. Supernatants

were digested with 0.5 mg of proteinase K per ml for 2 h before extraction with phenol-chloroform and isopropanol precipitation. Nucleic acids were rinsed, desiccated, and resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0). The Hirt DNAs were digested and analyzed by Southern hybridization as previously described (23).

Preparation and fractionation of nuclear extracts. Nuclear extracts of A9 cells were prepared by the method of Dignam et al. (13). For MVM-infected A9 cell nuclear extracts, 1 liter of cells was harvested by low-speed centrifugation and infected with MVM (multiplicity of infection of 10) in a volume of 50 ml of Joklik's modified Eagle's medium supplemented with 10 mM HEPES (pH 7.3) and 1% fetal bovine serum. After infection for 1 h at room temperature, the cells were recentrifuged and resuspended in complete media for 24 h before harvesting. Five milliliters of A9 nuclear extracts was loaded on a 10-ml DEAE-sephacel column (Pharmacia) equilibrated with buffer D (10 mM HEPES-NaOH (pH 7.9), 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing 1 μg (each) of antipain, leupeptin, and pepstatin per ml. The column was washed with 50 ml of buffer D followed by 50 ml of buffer D containing 0.6 M KCl. Protein fractions containing binding activities were pooled and immediately loaded on a 10-ml heparin-agarose column (Sigma) equilibrated with buffer D. This column was washed with buffer D containing 0.1, 0.3, and 0.6 M KCl. Fractions containing binding activities were dialyzed against 1 liter of buffer D and stored at −70°C.

Gel retardation assays and DNase I footprinting. Electrophoretic mobility shift assays (EMSAs) were performed in a final volume of 20 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 mM KCl, 1.0 mM dithiothreitol, 250 ng of double-stranded poly(dI-dC) per μl, and 2.5 to 5.0 μg of nuclear extracts. The proteins were preincubated for 10 min at room temperature, followed by addition of 10,000 cpm (0.2 to 1.0 ng) of the indicated end-labelled probe. After a 30-min binding reaction, DNA-protein complexes were separated from the free probe by gel electrophoresis on a native 4% polyacrylamide gel containing 0.5× Tris-borate-EDTA buffer and 1% glycerol. In DNase I footprinting experiments, proteins from column fractions were used in binding reaction mixtures as described above, except the final incubation volume was 50 μl and contained 10 mM $MgCl_2$ and 5 mM $CaCl_2$. The binding reaction mixture was then subjected to partial DNase I digestion by the addition of 2 μl of a 1/25th dilution of the enzyme (Promega) in 150 mM NaCl–1 mM $CaCl_2$–50% glycerol. The DNase I digestion was stopped after 60 s with 50 μl of DNase I stop buffer (8 M ammonium acetate, 40 mM EGTA, 100 μg of yeast tRNA per ml). Samples were extracted once with phenol-chloroform and precipitated with ethanol. Digestion products were electrophoresed on 10% polyacrylamide–8 M urea sequencing gels.

The *Rsa*I A and B probes were prepared by cloning the respective *Rsa*I fragments (nt 4431 to 4579 and 4579 to 4662) into the *Sma*I site of pUC19 and releasing them with *Bam*HI and *Eco*RI digestion. The *Bam*HI-*Eco*RI fragments were purified by gel electrophoresis before end labelling with [α-^{32}P]dATP and DNA polymerase I (Klenow fragment). Three pairs of synthetic oligonucleotides, B site I oligonucleotide (5'AGCTTTCATATATTATTAAGACTAATAAAGATACAA3' and 5'AGCTTTGTATCTTTATTAGTCTTAATAATATATGAA3'), B site II oligonucleotide (5'AATTCATAGAAATATAATATTACATATAGATTTAAGAAATAG3' and 5'AATTCTATTTCTTAAATCTATATGTAATATTATATTTCTATG3'), and FREB oligonucleotide A (a nonspecific oligonucleotide which contains the binding site for *fps* responsive element binding protein [3] [5'GATCCGGGAGCTGCATCCGGAGTAGG3' and 5'GATCCTACTCCGGATGCAGCTCCCGG3']), were synthesized (ABI 391 DNA synthesizer) and annealed before being used in competition EMSA experiments.

RESULTS

***cis*-regulatory sequences in the *Rsa*I A and B fragments activate MVM minigenome DNA replication.** Previous genetic analysis of the replication of MVM minigenomes in murine LA9 and COS-7 cells showed that *cis*-regulatory elements are present inboard of the 5'-terminal palindrome of MVM (23). The two replication elements required for efficient MVM minigenome replication, A (nt 4489 to 4636) and B (nt 4636 to 4695), were defined by deletion analysis of the parental MVM minigenome vector pPTLR (LR). MVM DNA replication intermediates were observed only when the LR vector was cotransfected with the NS-1-complementing expression vector pCMVNS1 (Fig. 1B and C, compare lane 2 with lane 3). The *Dpn*I-resistant replication products (Fig. 1C, lane 3) contained various monomer (mLR), dimer (dLR), and higher-order RFs, which are multiples of unit genomic length (i.e., trimers, tetramers, etc.). The heterogeneous compositions of the mLR bands were shown to be alternative extended duplex DNA or covalently closed hairpin forms of the palindromic sequence at either or both termini. These RFs are not seen when nt 4342 to 4695 are deleted from the LR minigenome (Fig. 1B and C, lane 4). In order to further define the *cis*-regulatory sequence in this region, we have inserted various fragments into the unique *Eco*RV (nt 381) site of the pPTLRdl411-4695(RV) minigenome. Fragments which are able to activate DNA replication should be able to rescue the replication phenotype. As expected, when the SX fragment was inserted, the replication phenotype was fully restored (Fig. 1B and C, lane 6). When the SV40 origin of replication was inserted, the minigenome remained replication deficient (Fig. 1B and C, lane 5). This suggested to us that the elements contained within the SX fragment are specific for MVM DNA replication. The presence of the *Rsa*I A fragment (nt 4431 to 4579) appeared to activate replication to approximately 60% of the control LR levels in the R orientation (Fig. 1B and C, lane 7) but activated replication to only 7% in the opposite, L, orientation (Fig. 1B and C, lane 8). Furthermore, the presence of the adjacent *Rsa*I B fragment (nt 4579 to 4662) also stimulated replication to approximately 20 to 30% of LR levels in either orientation. These results suggested to us that the two *Rsa*I fragments are specifically required for efficient DNA replication of MVM minigenomes. Since the larger SX fragment was able to fully rescue replication levels to LR levels and neither of the two *Rsa*I fragments was able to do so, it appears that the activation from the two *Rsa*I fragments may be additive. When genomes are excised completely from the plasmids before transfection, they replicate with the same efficiency as when the plasmids are linearized (23a). Hence, we conclude that sequences within the SX and *Rsa*I A and B fragments do not affect rescue (release of the minigenomes from the plasmid sequences). However, we do not know whether these sequences are involved in translocation of the minigenomes into the nucleus.

Activation of replication of MVM LL minigenomes. Although we have shown that the SX fragment is able to rescue replication of the defective minigenome *dl411-4695*, we wished to see whether this fragment will also activate DNA replication at the 3' terminus. To further test this hypothesis, we constructed a series of MVM minigenomes containing two 3' termini with either the activating SX fragment or a neutral

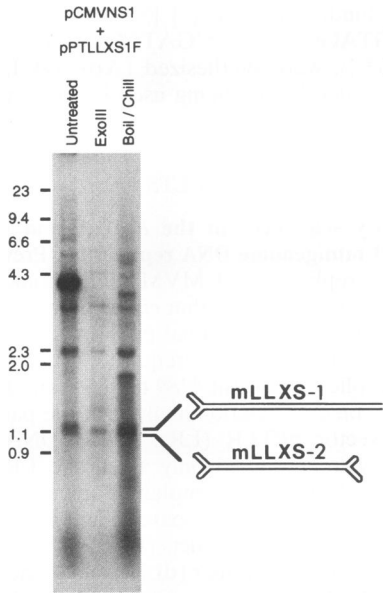

FIG. 3. Analysis of LL RFs. Hirt DNA samples containing the indicated transfected DNA were either undigested (untreated), exonuclease III digested (ExoIII), or boiled and rapidly cooled (Boil/Chill) before gel electrophoresis and Southern blot analysis. mLLXS-2 indicates a structure which contains covalently closed hairpin ends at both termini, whereas mLLXS-1 contains one covalently closed hairpin terminus and one open duplex terminus.

fragment from the yeast *FUS3* gene (Fig. 2A). We have shown previously that LL minigenomes replicate poorly compared with the LR genome or a minigenome containing two 5′ termini (RR) (Fig. 2C, compare lane 5 with lanes 4 and 13) (23). A smaller LL genome, the LLX minigenome, also replicates poorly, although it replicates consistently somewhat better than the LL genome (Fig. 2C, compare lanes 6 and 5). In the absence of the SX fragment, all LL minigenomes replicate poorly compared with LR minigenomes (Fig. 2C, compare lane 4 with lanes 5 and 6). When the SX fragment was introduced as a single copy or multiple copies in either orientation, replication was activated to high levels (approximately 30 to 60 times that of the LLX minigenome) (Fig. 2C, lanes 7 to 10). Insertion of the *FUS3* insert in one or four copies did not activate replication (Fig. 2C, lanes 11 and 12). Although activation of the LL minigenomes by the SX fragment is unequivocal, some unexpected replication intermediates were observed. When multiple copies of the SX fragment were used, monomer forms corresponding to the LLXS-1F or LLXS-1R were present. This suggested to us that minigenomes containing multiple copies of the SX fragment may be able to be removed 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 (14). We also observed only two monomer RFs of the LLXS-1F and LLXS-1R constructs (Fig. 2B and C). In order to elucidate the nature of the palindromic termini, we digested Hirt DNA samples with exonuclease III (Fig. 3). Only mLLXS-1 was resistant to exonuclease III digestion, suggesting that both termini are covalently closed. To determine the status of mLLXS-2, Hirt DNA samples were denatured in boiling water and quickly cooled in ice water before gel electrophoresis. We observed that both mLLXS-1 and mLLXS-2 are 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. 3). The absence of detectable molecules containing two open duplex termini may be the result of the unique method used by MVM to resolve the 3′ bridge fragment that joins the monomer RF. The asymmetric nature of the resolution of such fragments is thought to produce two termini—one that is an open extended form and one that is covalently closed (5), with both retaining their flip sequence orientation.

Identification of binding activities in nuclear extracts of A9 cells and MVM-infected A9 cells. We have determined that the *Rsa*I A and B fragments are able to activate DNA replication of MVM minigenomes. In order to provide biochemical evidence to support our genetic analysis, we wished to determine whether sequence-specific DNA binding proteins are responsible for this activation. Previous studies have shown that proteins such as transcription factors are able to activate DNA replication in a variety of viral as well as yeast systems. To this end, we have prepared nuclear extracts from uninfected and MVM-infected A9 cells and probed them with end-labelled fragments which contain the *Rsa*I A or B fragment in EMSAs (Fig. 4A and B, respectively). At least five sequence-specific DNA-protein complexes are seen when the *Rsa*I A fragment is used as a probe. These complexes were designated MVM DNA replication factors (MRFs) A2, A3, A4, A5, and A6 (Fig. 4A). Competition binding reactions suggest that MRF A2 appears to bind to both the *Rsa*I A and B fragments, whereas MRF A3, A4, A5, and A6, are specific for the *Rsa*I A fragment. When the radiolabelled *Rsa*I B fragment was used, at least four specific DNA-protein complexes, MRF B2, B3, B4, and B5, were observed (Fig. 4B). Competition binding reactions suggest that MRF B2 binds to both the *Rsa*I A and B fragments. These data suggest that the factors responsible for both MRF A2 and B2 are either similar or identical. The MRF B3, B4, and B5 complexes appear to be specific for the *Rsa*I B probe. Using both the *Rsa*I A and B probes, the pattern of DNA-protein complexes appears to be same in uninfected and MVM-infected nuclear extracts, suggesting that the factors binding to these elements are cellular in origin.

Determination of B5 factor binding site by DNase I footprinting. In order to determine the exact binding sites of the proteins binding to the *Rsa*I B probe, we found it necessary to fractionate nuclear extracts, since DNase I footprinting with crude nuclear extracts yielded ambiguous results. All binding activities are present in the flowthrough fraction of a DEAE-sephacel column (0.1 M KCl–wash buffer D) (Fig. 5A, lane 3). The 0.1 M KCl–wash buffer D fractions were immediately loaded onto a heparin-agarose column and developed with buffer D containing 0.1, 0.3, and 0.6 M KCl. MRF B3 and B4 are found in both the 0.1 and 0.3 M KCl wash buffers. MRF B5 activity was found only in the 0.3 M fraction, whereas B2 eluted at a slightly greater volume in the 0.3 and 0.6 M KCl wash buffers.

Samples from peak protein fractions of the 0.1, 0.3, and 0.6 M KCl eluates were used in DNase I footprint assays (Fig. 5B and C). We observed two regions of protection from DNase I cleavage on both the positive and minus strands when samples from the 0.3 M KCl fraction were used. These activities appear to be found exclusively in the 0.3 M KCl fraction, since samples from the other fractions did not produce any regions of protection. When the positive strand was labelled (Fig. 5B), nt 4589 to 4610 (site I) and 4616 to 4646 (site II) were protected from DNase I digestion. When the minus strand was labelled (Fig. 5C), nt 4590 to 4609 (site II) and 4618 to 4631 (site II)

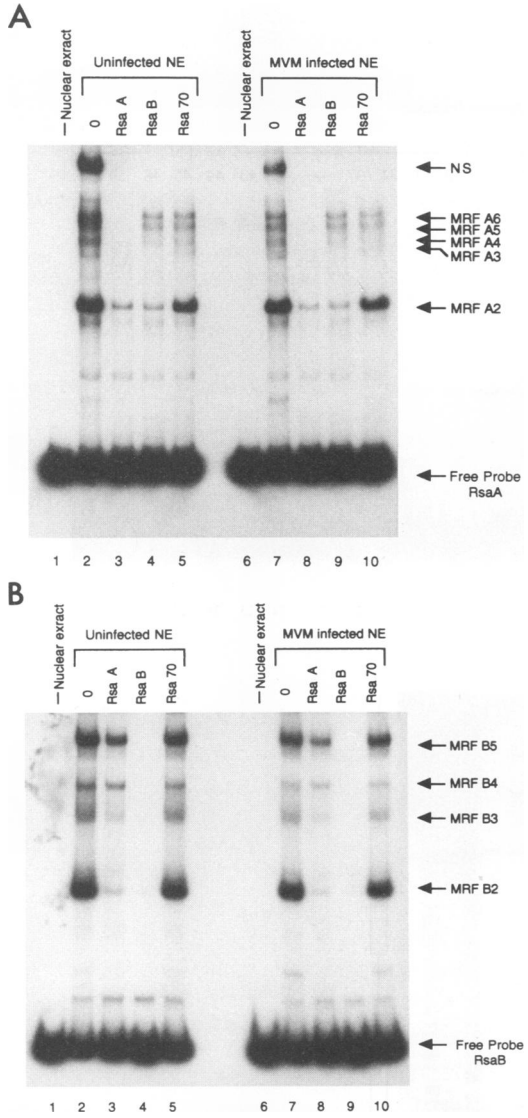

FIG. 4. Cellular proteins in nuclear extracts (NE) bind to the *Rsa*I A and B fragments. The radiolabelled *Rsa*I A (A) and B (B) fragments were incubated in the presence of 5 μg of either uninfected A9 nuclear extracts (lanes 2 to 5) or MVM-infected A9 nuclear extracts (lanes 7 to 10). Competition by addition of 200-fold excess of the unlabelled *Rsa*I A fragment (lane 3), B fragment (lane 4), and the nonspecific *Rsa*I 70 fragment (lane 5) was performed to determine the specificity of binding. Lanes 2 and 7 contained no unlabelled restriction DNA fragment competitor. Lanes 1 and 6 contained no protein added to the binding reaction mixture. Specific protein-DNA complexes were designated MRFs. Nonspecific (NS) complexes are indicated.

were protected. (See Fig. 5D for a summary of the binding sites.) We also note that single nucleotides outside of the major binding regions either were protected from DNase I digestion (Fig. 5C) or were hypersensitive to DNase I action. This suggests that the structure of the DNA template is somewhat different in the presence of the factors binding to the labelled *Rsa*I B fragment. Since only MRF B5 was present in quantity sufficient to produce the bipartite footprints as shown by titration EMSA experiments (data not shown), we wished to determine whether MRF B5 was composed of a single polypeptide with two DNA binding domains or whether MRF B5 consisted of at least two other factors binding to the *Rsa*I B fragment simultaneously. Synthetic oligonucleotides corresponding to sites I and II were used in a competition EMSA experiment (Fig. 6). When an excess of site I oligonucleotides was introduced, only MRF B4 was specifically inhibited (Fig. 6, lanes 3 and 4). When an excess of site II oligonucleotides was added, only MRF B3 was inhibited (Fig. 6, lanes 6 and 7). FREB oligonucleotide A (3) did not reduce the binding of any of the MRFs to the *Rsa*I B fragment. These data suggest that the MRF B5 activity may be composed of at least two other binding activities, MRF B3 and MRF B4, each of which binds to DNA. We do not know 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. We believe that the former explanation is more likely, since both site I and site II oligonucleotides do not titrate off MRF B5 at these oligonucleotide concentrations. At extreme concentrations of either unlabelled site I or site II oligonucleotides, MRF B5 binding activity to the radiolabelled probe is abolished (data not shown). It is possible that the extreme concentrations of site I and II oligonucleotides are required because they contain restriction endonuclease site tails to facilitate cloning; hence, they may be somewhat sterically hindered in their ability to bind simultaneously with MRF B5. More detailed studies will be required in order to determine the exact nature of factor MRF B5.

It is interesting that a search of the data bases for sequences corresponding with sites I and II (23b) found matches with other related parvoviruses (as expected) and the yeast *ARS* core consensus sequence (22).

DISCUSSION

Previous evidence shows that MVM contains at least two latent origins of replication, one at either end of the viral genome (9, 23). We have also shown that MVM minigenomes containing two left palindromes replicate poorly compared with a minigenome containing two right palindromes, suggesting that the right-terminal region may contain stronger replication signals relative to the left origin (23). The data in this report support this hypothesis since sequences inboard of the right palindrome are able to enhance MVM LL minigenome replication over the level of the LL minigenomes lacking these activating sequences. The nature of the replicating LL minigenome is distinguishable from minigenomes with the LR and RR configuration, since it appears that LL minigenomes must have at least one covalently closed hairpin at one end, whereas the LR and RR minigenome pool contains monomer species with extended duplex ends at both termini. These results are consistent with the observation that resolution of the 3′ bridge dimer specifically results in one end which is a covalently closed hairpin form and one end which is an extended duplex DNA (5, 19).

Studies of viral origins of replication such as those with bovine papilloma virus type 1 (20, 26), SV40 (10, 11, 16), or yeast *ARS1* (21) suggest that eukaryotic origins of DNA replication are multipartite in nature (12). For example, it has been shown that *ARS1* contains at least three elements, of which the *ars* consensus sequence is absolutely essential for plasmid replication. Since sequence-specific DNA binding proteins have been shown to contribute to the regulation of cellular processes such as transcription of RNA polymerase II genes and initiation of DNA replication, we sought to determine whether the activating sequences at the right terminus contained binding sites for proteins. DNAse I footprint analysis of the *Rsa*I B probe shows that MRF B5 protects two

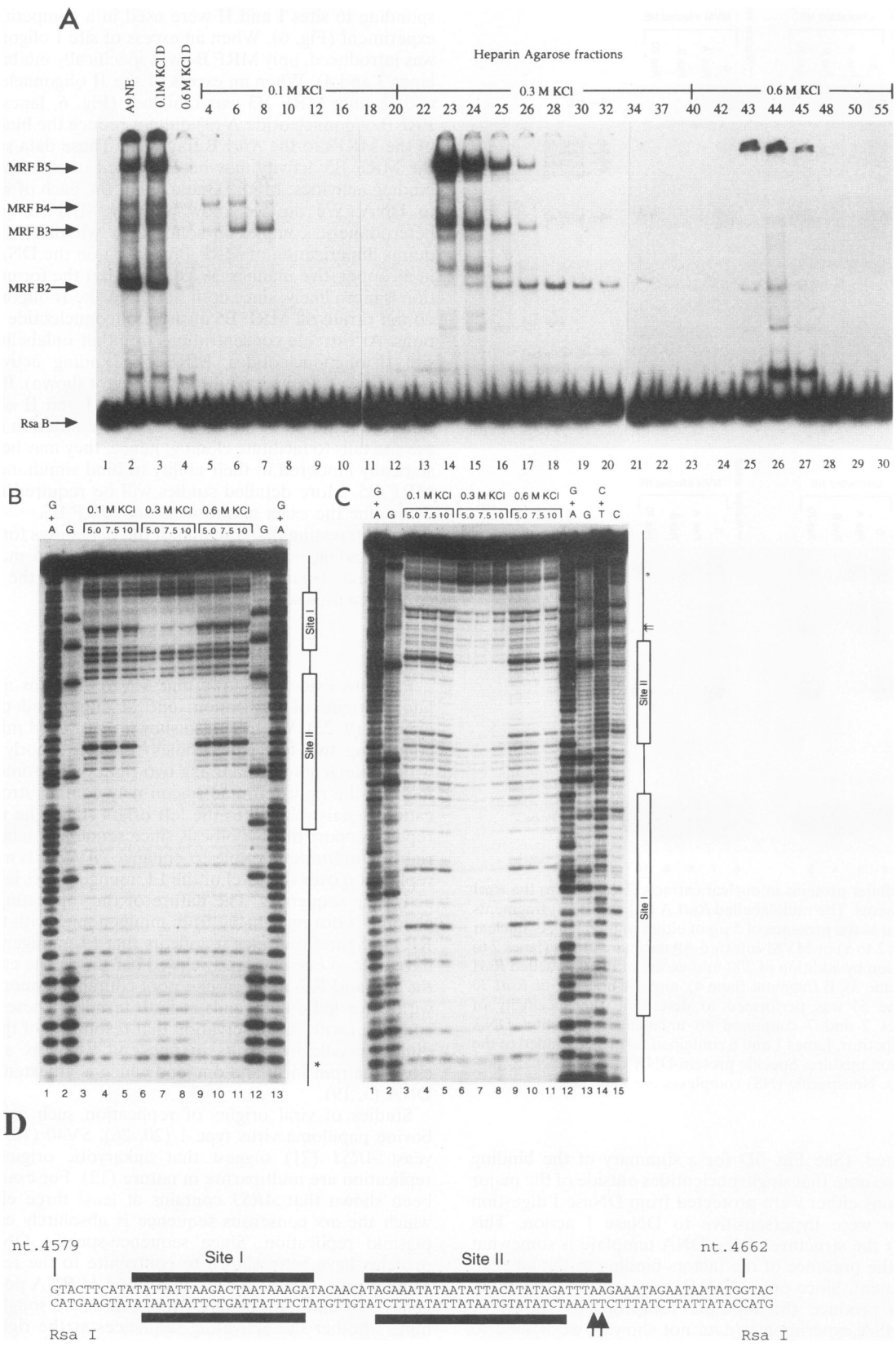
A
Heparin Agarose fractions
A9 NE
0.1M KCl D
0.6 M KCl D
0.1 M KCl
0.3 M KCl
0.6 M KCl
4 6 8 10 12 16 18 20 22 23 24 25 26 28 30 32 34 37 40 42 43 44 45 48 50 55
MRF B5
MRF B4
MRF B3
MRF B2
Rsa B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
B
G + A G
0.1 M KCl 0.3 M KCl 0.6 M KCl
5.0 7.5 10
G + A
Site I
Site II
1 2 3 4 5 6 7 8 9 10 11 12 13
C
G + A G
C + T C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
nt.4579
nt.4662
Site I
Site II
GTACTTCATATATTATTAAGACTAATAAAGATACAACATAGAAATATAATATTACATATAGATTTAAGAAATAGAATAATATGGTAC
CATGAAGTATATAATAATTCTGATTATTTCTATGTTGTATCTTTATATTATAATGTATATCTAAATTCTTTATCTTATTATACCATG
Rsa I
Rsa I

FIG. 5. Determination of MRF B5 binding site. (A) A9 nuclear extracts were fractionated by DEAE-sephacel chromatography followed by heparin-agarose chromatography as outlined in Materials and Methods. All specific binding activities flowed through the initial DEAE-sephacel column in the 0.1 M KCl fraction (0.1 M KCl–wash buffer D). The 0.1 M KCl–wash buffer D fraction was immediately loaded onto a 10-ml heparin-agarose column and washed with 60 ml of 0.1, 0.3, and 0.6 M KCl buffers. Three-milliliter fractions were collected and assayed with a standard EMSA, using the ^{32}P-radiolabelled *Rsa*I B fragment as outlined in Materials and Methods. (B and C) MRF B5 binds multiple regions of the *Rsa*I B fragment in DNase I footprinting experiments. Five, 7.5, or 10 μl of the peak protein fractions of the 0.1, 0.3, and 0.6 M KCl eluates was incubated with the *Rsa*I B fragment and radiolabelled on either the positive (B) or minus (C) strand before a limited digestion with DNase I and denaturing gel electrophoresis. Maxam and Gilbert ladders of the *Rsa*I B fragment were used to determine the binding site of MRF B5. Two sites of protection, designated sites I and 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 solid bars and arrows. The numbers above the DNA sequence represent the MVM nucleotide positions. Site II contains a 9 of 11 match to the yeast *ARS* core consensus sequence (23b).

regions within the probe. Furthermore, competition EMSA experiments suggest that the MRF B5 is composed of an MRF B3-B4 complex. The nature of the MRF B5 complex formation remains unknown, but MRF B3 and MRF B4 may dimerize in solution before binding to DNA or the individual factors may bind independently.

What are the possible roles of these factors during the MVM replication cycle? To account for the preferential packaging of genomic minus-strand DNA, current models of MVM DNA replication suggest that the right palindrome must be able to fold into a rabbit-eared hairpin structure before subsequent DNA synthesis, strand displacement, and packaging (2). A similar process must also take place when the MRF is converted to higher concatamer replicative intermediates. It seems likely that MRF B3, B4, and/or B5 binding activities are either required for or activate processing of the right terminus and hence may be referred to as replication accessory proteins. The recent finding that transcriptional activators such as VP16 are able to interact directly with the large subunit of RPA-I, a single-stranded DNA binding protein which is a component of the DNA replication complex, may be relevant (15, 17). Alternatively, other transcription factors are thought to enhance DNA replication by preventing the binding of nucleosomes to the replication origin (4). The MRFs described in this report may supply one or both of these functions. Although the mechanism of processing of the right terminus remains unknown at this point, further analysis of this region in the infectious clone may improve our understanding of events at the right terminus.

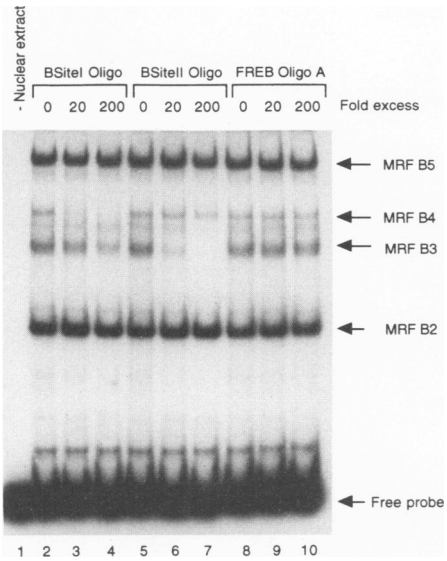

FIG. 6. Competition EMSA suggests MRF B5 is composed of MRF B3 and MRF B4. The radiolabelled *Rsa*I B fragment was incubated with 5 μg of uninfected nuclear extract in the absence (lanes 2, 5, and 8) and presence, respectively, of 20- and 200-fold molar excess of B site I oligonucleotide (lanes 3 and 4), B site II oligonucleotide (lanes 6 and 7), or FREB oligonucleotide A (lanes 9 and 10) in a standard EMSA experiment. The B site I and B site II oligonucleotides span the sequences defined by the DNase I footprints (sites I and II) of MRF B5 (Fig. 5). Lane 1 contains no nuclear extract.

We have genetic evidence that shows that elements within the *Rsa*I B restriction fragment are important for the replication of MVM minigenomes. Biochemical data suggest that sequence-specific DNA binding factors MRF B3, B4, and B5 specifically bind to *cis*-elements in the *Rsa*I B probe. The MRFs described here are likely to be cellular, since the binding activities are found in both uninfected and MVM-infected A9 nuclear extracts. These *cis*-regulatory elements may constitute a portion of the origin of DNA replication at the right terminus. Since packaging and DNA synthesis are thought to be concomitant events, these sequences may also be important for the packaging of genomic single-stranded DNA. The roles of these proteins in viral and cellular DNA replication may be elucidated when the genes for these proteins are cloned and characterized.

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