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Identification and Characterization of a Protein Covalently Bound to DNA of Minute Virus of Mice

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We identified a protein which is covalently linked to a fraction of the DNA synthesized in cells infected with minute virus of mice. This protein is specifically bound to the 5′ terminus of the extended terminal conformers of the minute virus of mice replicative-form DNA species and of a variable fraction of single-stranded viral DNA. The chemical stability of the protein-DNA linkage is characteristic of a phosphodiester bond between a tyrosine residue in the protein and the 5′ end of the DNA. The terminal protein (TP) bound on all DNA forms has a relative molecular weight of 60,000; it is also seen free in extracts from infected cells. Immunologic comparison of the TP with the other known viral proteins suggests that the TP is not related to the capsid proteins or NS-1.

The genome of the autonomous parvovirus minute virus of mice (MVM) is a linear, single-stranded (ss) DNA molecule 5,081 nucleotides in length. Extensive studies on the replication mechanism of this virus show that the sequence and structure of the virion DNA (vDNA) termini play major roles at each stage of the replication process (1–3): the synthesis of double-stranded (ds) replicative-form (RF) DNA from the infecting parental vDNA, the synthesis of concatemer RF molecules, and the synthesis of progeny vDNA. From sequence and structural analyses of the vDNA, both the 3′- and 5′-terminal sequences are nonidentical palindromes, and the genomic termini spontaneously form hairpin duplexes in solution (1–4, 7, 13). In the RF DNA population, these sequences exist in two alternative structural conformations. They can form intrastrand base pairs with its complementary sequence within the palindrome, forming hairpin duplexes—the "turnaround" (t) conformation. Alternatively, these sequences on the viral (minus) strand can form interstrand base pairs with the sequences present on the complementary (plus) strand, forming a fully base-paired DNA duplex—the "extended" (e) conformation. In addition, the 3′-terminal palindromic sequence is unique, while the 5′ palindrome exists in two sequence orientations. Finally these palindromic sequences are found internally bridging concatemeric RF DNA molecules in a head-to-head orientation.

Structural characterization of the DNA termini on both MVM vDNA and RF DNA indicated that the termini were modified. We show here that a protein is covalently bound to the 5′ end of both viral and complementary strands. The protein is associated preferentially with only one of the two terminal conformations present in the RF population. Furthermore, the terminal protein (TP) is serologically distinct from all known viral structural and nonstructural proteins. These data suggest that the protein is not virally encoded. We propose that this covalently bound protein is important for the replication and maturation of the genomic termini.

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MATERIALS AND METHODS

Preparation of viral DNA-protein complexes. MVM RF DNAs were isolated from Ehrlich ascites or A9 cells after infection at high multiplicity of infection with MVM(p), the prototype strain of MVM. Two liters of cell suspension culture (3×10^5 cells per ml) was infected with MVM(p) at a multiplicity of infection of 1 or 10 PFU per cell (57). [^{3}H]thymidine (2 Ci/ml; New England Nuclear Corp.) was added between 6 and 8 h postinfection, and the cells were harvested 30 h postinfection. Two methods were used to isolate the complex. In both protocols, cells were lysed in the presence of 1 mM phenylmethylsulfonyl fluoride. Viral DNA was initially isolated by hydroxylapatite chromatography (50) or by a modified Hirt extraction procedure (43). The protein-DNA complex was subsequently purified from the protein-free viral DNA by phenyl-Sepharose chromatography. Because the TP in these preparations showed various degrees of proteolytic degradation (see Results), an alternative purification method was developed (5, 14, 26, 44). Briefly, MVM-infected A9 cells (harvested 24 to 30 h postinfection) were disrupted with a Hirt lysis procedure without any proteases and with protease inhibitors added. The Hirt supernatant was then brought to 2% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol and heated to 80°C for 5 min. The DNA-protein complex was purified by exclusion chromatography on Sepharose 4B-CL in the presence of 0.3% SDS, and most of the SDS was removed by dialysis against TE (10 mM Tris, 1 mM EDTA [pH 7.5]). The complex was concentrated by ethanol precipitation and resolubilized in TE. The adenovirus DNA-protein complex was isolated from purified virions by the method of Stillman and Bellett (53).

Phenyl-Sepharose 4B hydrophobic chromatography. Phenyl-Sepharose (Pharmacia, Inc.; ligand concentration, 40 mol/ml of resin) was cycled through distilled water (1 volume), ethanol (2 volumes), *n*-butanol (2 volumes), ethanol (2 volumes), and distilled water (1 volume) before equilibration with the starting column buffer (0.5 M $NaPO_4$ [pH 7.0]). Generally, for approximately 300 μg of MVM RF DNA

containing 100 μg of protein-DNA complex, a 5-ml phenyl-Sepharose column was used. The DNA sample in 0.5 M $NaPO_4$ (pH 7.0) was loaded onto the column, and the column was washed with 3 volumes of 0.5 M $NaPO_4$ (pH 7.0). The bound protein-DNA complex was eluted in TE containing 2% Sarkosyl (CIBA-GEIGY Corp.) and extensively dialyzed into TE.

Filter-binding assay. The amount of protein-MVM DNA complex was assayed by binding to nitrocellulose filters (Millipore Corp.; 0.65 μm pore size). For each assay, a sample of the [^{3}H]DNA sample was spotted directly onto a dry filter to measure the total amount of DNA in the sample. The rest of the sample was applied to a filter presoaked in filter buffer (FB; 40 mM Tris [pH 7.8], 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μg of bovine serum albumin per ml, 0.2 M NaCl), which had been washed with 2 ml of FB prior to addition of the sample; after sample filtration, the filter was washed with an additional 2 ml of FB. The filters were dried and counted in a toluene-base liquid scintillation cocktail. The filtration rate for all assays was between 2 ml/20 s and 2 ml/40 s, conditions which gave optimal discrimination between free ss and ds DNAs and protein-DNA complexes. Although binding of protein-DNA complexes is insensitive to low concentrations of NaCl (0.5 M), urea (1 M), and guanidine (4 M), the assay is sensitive to the presence of detergent (e.g., 0.1% SDS).

DNA gel electrophoresis. DNA fragments were separated by electrophoresis in agarose gels in the presence (11, 35) and absence (48) of SDS. Polyacrylamide gels (4%; 39:1 acrylamide/bisacrylamide ratio) were run in 40 mM Tris acetate–20 mM sodium acetate–2 mM EDTA [pH 8.0] and stained for 10 min with ethidium bromide (10 μg/ml) after running. ^{3}H-labeled DNA was detected by staining with ethidium bromide or by fluorography (12). ^{32}P-labeled DNA was detected by autoradiography.

Protein gels. Linear-gradient (8 to 16%) and constant-percentage (10%) polyacrylamide gels, were run as described by Laemmli (29) with a microslab apparatus as described by Matsudaira and Burgess (31). The gels were stained with Coomassie brilliant blue R-250 or silver (36). ^{125}I-labeled protein bands were detected by autoradiography.

Immunologic characterization. Antiserum to MVM capsid proteins was a gift of Peter Tattersall. Isolated MVM DNA-protein complexes were repurified on SDS-agarose gels, and the monomer RF DNA band was cut out and electroeluted in SDS prior to immunization. A guinea pig was injected subcutaneously with the complex (10 μg) in SDS and was boosted twice (10 μg) before bleeding. The serum of the immunized animal contained antibodies specific to the TP as assayed by protein A-Sepharose chromatography.

Protein A-Sepharose chromatography was based on a procedure developed by Gerace and Blobel (22) for immunological characterization of the highly insoluble nuclear lamina. Samples contained 1 μg of TP-DNA complex in 500 μl of a 0.05 M triethanolamine hydrochloride (pH 7.4) buffer containing 0.5% SDS, 0.10 M NaCl, and 2 mM EDTA. The samples were then heated at 80°C for 5 min and cooled to room temperature. The following solutions were added sequentially with vortex mixing between additions: 20% Triton X-100, 50 μl; 0.5 M iodoacetamide, 5 μl; appropriate antiserum, 25 μl. Antibody-containing samples were incubated for 2 h at 37°C, added to 25 μl of protein A-Sepharose (Pharmacia), and incubated with end-over-end agitation for 1 h at 37°C. The samples were then applied to pipette tips plugged with glass wool, and the retained beads were washed with the following: (i) 1 ml of 0.05 M triethanolamine hydrochloride buffer (pH 7.4) containing 2% Triton X-100, 0.5% SDS, 0.1 M NaCl, and 2 mM EDTA; (ii) 1 ml of 0.1 M triethanolamine (pH 7.4). To release the immunoprecipitated DNA-protein complex, the beads were incubated (30 min at 37°C) in 100 μl of solution containing 6% SDS, 15% sucrose, 0.1 M triethanolamine hydrochloride (pH 7.4), and 2 mM EDTA. Samples (25 μl) were loaded on agarose gels (1%) either directly or after 1 h of incubation at 37°C with pronase (1 mg/ml). After electrophoresis, the DNA was transferred to nitrocellulose filters and probed with ^{32}P-labeled, cloned MVM DNA (pMM984) as previously described (34).

For Western blot analysis, cells were synchronized, infected, and harvested (59). Subcellular fractions were prepared from synchronized, infected cells (5, 41). Samples of each cell fraction (1.5×10^5 cell equivalents) were electrophoresed as described above and transferred electrophoretically to nitrocellulose. The blots were probed with antiserum and developed with ^{125}I-labeled protein A (8, 60).

^{125}I labeling of DNA-protein complex. Bolton-Hunter reagent (6) was synthesized with 5 mCi of Na^{125}I (NEX-033L) and incubated with DNA-protein complex in 0.1 M sodium borate buffer (pH 8.5) (1.5 μg of DNA in 50 μl) on ice for 15 min. The reaction was stopped by addition of 0.5 M glycine, and the unincorporated label was removed by extensive dialysis against TE.

Alternatively, the MVM DNA-protein complex was labeled with ^{125}I via Iodogen (Pierce Chemical Co.) (45) and separated from unincorporated label by exclusion chromatography on SDS-Sepharose 4B-CL as described above.

Enzyme conditions. (i) Exonuclease III digestion. Radiochemical quantities of the DNA-protein complex were digested by exonuclease III (20 U; Biolabs) in 100-μl reaction mixtures containing 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 50 μg of calf thymus DNA per ml. Duplicate reactions were done at room temperature (22°C) to minimize digestion from nicked sites within the DNA. Samples (15 μl) were removed at specific times from the reaction mixture and precipitated onto Whatman GF/C filters with a solution of 10% trichloroacetic acid–10% saturated tribasic sodium phosphate–10% saturated sodium pyrophosphate (10% trichloroacetic acid-PO_4) and counted in a toluene-base scintillation cocktail.

(ii) λ Exonuclease digestion. [^{3}H]thymidine-labeled DNA was digested with λ exonuclease (6 U; a gift from Charles Radding) in 100-μl reaction mixtures containing 50 mM glycine-KOH (pH 9.5), 2 mM $MgCl_2$, 0.1 mM EDTA, and 45 μg of calf thymus DNA per ml. Duplicate digestions were done at 37°C. Samples (15 μl) were removed, acid precipitated onto GF/C filters, and counted in a toluene-base scintillation cocktail.

(iii) Restriction enzyme digestions. MVM RF DNA, with or without associated protein, was digested with *Eco*RI or *Mbo*I in 6 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–6 mM 2-mercaptoethanol–0.1 M NaCl. *Pst*I and *Hin*fI digestions were in 6 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–6 mM 2-mercaptoethanol–50 mM NaCl. Protease-treated samples were digested with 25 μg of proteinase K (E. Merck AG) or 200 μg of pronase (Calbiochem Behring) after restriction enzyme digestion. The pronase had been autodigested at 37°C for 6 h prior to use. If the DNA was analyzed by agarose gel electrophoresis immediately after enzymatic digestion, the reactions were stopped by precipitation with ethanol in the presence of 0.3 M sodium acetate–10 mM magnesium acetate–10 μg of carrier yeast tRNA. If the DNA was to be rerun on a phenyl-Sepharose

column, the reactions were stopped with EDTA and an equal volume of 1 M sodium phosphate buffer (pH 6.8) was added.

RESULTS

Identification of a protein moiety attached to MVM RF molecules. The presence of a protein associated with a fraction of the total MVM RF DNA population was suggested when MVM RF was isolated without prior protease digestion and examined by agarose gel electrophoresis after ethidium bromide staining (Fig. 1). Although a strong band corresponding to the monomer species and faint bands corresponding to ss vDNA and dimer MVM RF DNA species were observed, there was additional DNA fluorescent staining at the origin (lane 1). When the DNA was treated with pronase prior to gel analysis, the fluorescent intensity of the vDNA, monomer, and dimer RF forms increased, while fluorescent staining at the origin of the gel and above the monomer was reduced (lane 2). The prominent higher-molecular-mass band above the labeled dimer RF band was probably contaminating cellular DNA (Fig. 1A). This band was present in RF preparations made from mock-infected cells and failed to hybridize to MVM DNA on Southern blots (data not shown). Cellular DNA ran in a discrete band, because in a 1.4% agarose gel, high-molecular-mass DNAs migrated in the nonlinear region of the gel.

Digestion of DNA samples with *Eco*RI prior to electrophoresis clearly showed that staining at the origin was due to MVM RF DNA molecules complexed with protein. *Eco*RI cleaves MVM DNA at two sites, yielding three fragments: A (spanning 20.9 to 69.9% genome map units [20.9/69.9]), B (69.9/100), and C (0/20.9). The C fragment contains the viral 3′-terminal palindromic sequences, and the B fragment contains the 5′-terminal palindromic sequences. (The 3′- and 5′-terminal sequences of RF DNA refer, respectively, to the sequences present at the 3′ and 5′ regions of the viral strand.) Because the termini are structurally heterogeneous, *Eco*RI digestion of protease-digested RF generates doublets in the terminal fragments owing to the terminal sequences existing in the e and t conformations (1). The e conformer migrates as the slower DNA band of the doublet, and the t conformer migrates as the faster. When MVM RF, isolated without protease treatment, was digested with *Eco*RI and subsequently with pronase, the expected pattern of fragments A, Be, Bt, Ce, and Ct was observed (Fig. 1B, lane 3). However, this same DNA sample, when digested only with *Eco*RI, yielded only three of the expected bands (A, Bt, and Ct) (Fig. 1B, lane 1), and fluorescent staining was observed again at the origin. This staining at the origin disappeared as the DNA sample was extracted with phenol after digestion with *Eco*RI (Fig. 1B, lane 2) or digested with protease (Fig. 1B, lane 3), indicating that the DNA remaining at the origin was complexed with protein. In this RF preparation, the protein was clearly associated with the *Eco*RI B fragment in the e conformation, Be. The relative proportions of the two conformations for each of the termini varied from RF preparation to preparation. For this specific RF preparation, virtually all of the 3′-terminal sequences were in a t conformation (fragment Ct). The small amount of Ce present also ran into the gel only after pronase treatment. Qualitatively similar results were obtained with DNA purified by the standard Hirt procedure with pronase or by hydroxylapatite chromatography with and without prior pronase treatment. Thus, the protein-DNA complex was present in the RF DNA population independent of the method used to isolate RF DNA.

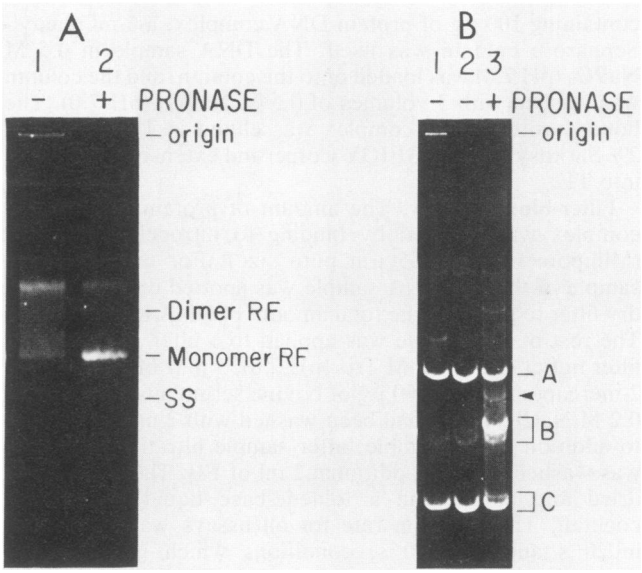

FIG. 1. Identification of a protein attached to MVM DNA molecules. DNA from MVM-infected mouse A9 cells was isolated in the absence of proteases and electrophoresed on 1.4% agarose gels. (A) DNA-protein complex isolated by Hirt lysis procedures without proteases. Lanes: 1, untreated complex; 2, complex digested with pronase prior to electrophoresis. (B) *Eco*RI digestion of RF DNA complex isolated without protease treatment. Lanes: 1, complex digested with *Eco*RI; 2, complex digested with *Eco*RI and then extracted with phenol; 3, DNA digested with *Eco*RI and then proteinase K. The arrowhead denotes the position of the dimer bridge fragment, i.e., the internal, fused, end-to-end *Eco*RI C fragments from dimer MVM RF.

The retention of DNA at the origin most likely was due to protein aggregation. In the presence of SDS, the protein-DNA complex entered the gel, suggesting that this aggregation was dissociated. This behavior was evident for the MVM DNA-TP complex which was labeled with ^{125}I-Iodogen (Fig. 2). Comparison of the stained gel (Fig. 2A) and autoradiogram (Fig. 2B) showed that the MVM DNA forms (dimer RF, monomer RF, and ss vDNA) were associated with ^{125}I label, which was removed by pronase digestion (Fig. 2, lanes 3 and 4). Note that pronase digestion removed the slight broadening and retardation of the DNA bands. When the DNA-protein complex was digested with *Eco*RI (Fig. 2A and B, lanes 5 and 6), the A, dimer bridge, Bt, and Ct fragments were protein free by two criteria. There was no ^{125}I label comigrating with the stained DNA bands (Fig. 2A and B, lane 5), and the intensity and position of the DNA bands were unchanged by protease treatment (Fig. 2A and B, lane 6). Again, the TP was present on both the Be and Ce fragment conformers. In the stained gel, both the Be and Ce conformers were absent in the lane which was not pronase treated (Fig. 2A, lane 5). However, in each case, very diffuse DNA bands were seen, which ran slower than the Be or Ce bands generated by *Eco*RI digestion of RF isolated with proteases. In the autoradiogram, there was ^{125}I label clearly comigrating with viral DNA, but none was evident at the position of the Be or Ce fragment (Fig. 2B, lane 5). However, there was ^{125}I label seen in diffuse bands at the same position as the diffuse DNA bands in the stained gel. These bands were excised and electroeluted in SDS. When these samples were digested with pronase and rerun on agarose gels, the eluted DNA bands comigrated with Be or Ce conformers (data not shown). In addition, when the *Eco*RI-

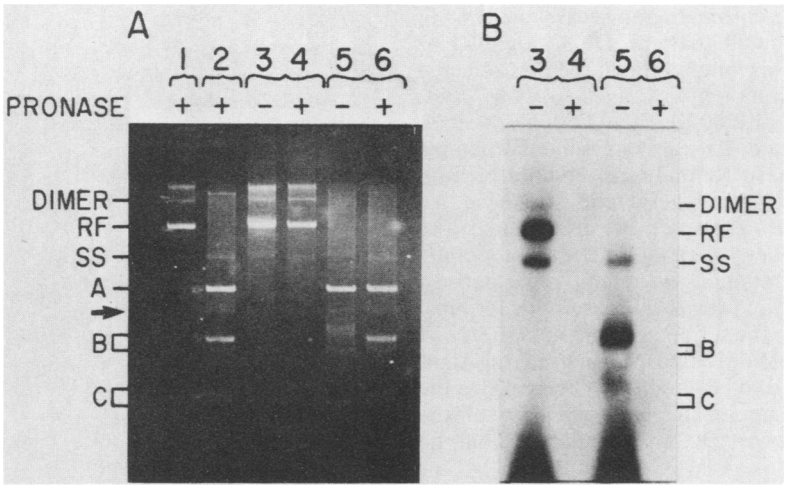

FIG. 2. SDS-agarose electrophoresis of the MVM DNA-protein complex. The complex was labeled with ^{125}I and electrophoresed on 1.4% agarose in the presence of SDS. (A) The gel, stained with ethidium bromide. (B) An autoradiogram of the ^{125}I-labeled proteins of the gel in A. Lanes: 1, RF DNA isolated from infected cells by the standard Hirt lysis procedure with proteases; 2, *Eco*RI digestion of RF DNA that was isolated with protease digestion; 3 and 4, isolated DNA-protein complex; 5 and 6, DNA-protein complex digested with *Eco*RI. Samples in lanes 4 and 6 were treated with pronase prior to electrophoresis. The dimer bridge fragment is noted with an arrow.

digested protein-DNA complex was treated with pronase (Fig. 2A and B, lane 6) the ^{125}I label disappeared, and both Be and Ce comigrated with the appropriate markers. In all preparations of the TP-RF DNA complex, the terminal *Eco*RI fragments in the e conformation were always associated with protein, and the internal fragments and t terminal fragments were protein free.

Protein attached to vDNA molecules. The ss vDNA isolated from virions had a variable fraction that was complexed with protein when assayed on agarose gels. This variability was consistent with the variable labeling efficiencies of vDNA with T4 polynucleotide kinase and [γ-^{32}P]ATP (Table 1). The amount of protein-DNA complex present in various [^{3}H]thymidine-labeled vDNA preparations was assayed with nitrocellulose filters. In parallel, these vDNA samples were 5′ end labeled with T4 polynucleotide kinase. All the preparations of vDNA were intact, genome-length molecules, as judged by their sedimentation in alkaline sucrose gradients and by their migration in agarose gels. Because all vDNA preparations bound to filters above background levels, they appeared to contain a subpopulation of DNA which was attached to protein; the percentage of [^{3}H]DNA binding to the filters varied inversely with the T4 labeling efficiencies observed. Thus, the 5′ termini of isolated vDNA preparations were blocked to various degrees. The labeling efficiency of T4 polynucleotide kinase did not improve when either the vDNA or the RF DNA was digested with either pronase or proteinase K, indicating that the protein was not completely removed by protease digestion and that the protein may be covalently bound to the 5′ terminus of the DNA.

TABLE 1. Percentage of [^{3}H]thymidine-labeled vDNA which is protein associated

DNA sample	% DNA retained on filters		% Labeling efficiency of T4 polynucleotide kinase
	Without proteinase K treatment	With proteinase K treatment	
φX174 ds	0.19	ND[a]	ND
φX174 ss	0.26	ND	ND
Adenovirus vDNA	100	0.4	ND
MVM vDNA prepn 1	72.6	1.4	1
MVM vDNA prepn 2	50.3	1.2	16
MVM vDNA prepn 3	28.8	1.5	51
MVM vDNA prepn 4	11.8	0.8	66

[a] ND, Not determined.

Isolation of protein-DNA complex. By gel analysis, a variable portion of the total isolated DNA was complexed with protein. In addition, the relative proportions of monomer and dimer RF DNAs that were complexed with protein varied from preparation to preparation. Thus, a method was sought to separate the complex from protein-free DNA for further characterization. Because the protein-DNA complex appeared to be highly hydrophobic in character, its ability to bind to several different hydrophobic resins was tested. Phenyl-Sepharose yielded the best and most reproducible separation of the two RF DNA subpopulations. Protein-free DNA failed to bind to the resin in 0.5 M PO_4 buffer (Fig. 3, lanes 3 and 4). Under these conditions, the MVM protein-DNA complex became resin bound but was eluted with greater than 95% recovery with 2% Sarkosyl (Fig. 3, lanes 5 and 6). Similar results were obtained with adenovirus DNA-protein complex (data not shown).

The adenovirus DNA-protein complex was selectively retained on columns of benzolated, naphthalated DEAE-cellulose and quantitatively eluted with 8 M urea–1% SDS (53). However, the MVM DNA-protein complex could not be eluted from benzolated, naphthalated DEAE-cellulose without treating the resin with pronase. The MVM DNA-protein complex also bound to octyl-Sepharose (Pharmacia); however, the on rate of the complex was extremely slow; the load had to be cycled 10 to 15 times for quantitative binding of the DNA-protein complex to the resin. In contrast, adenovirus DNA-protein complex bound more rapidly to octyl-Sepharose (data not shown).

Localization of the protein on the monomer DNA molecule. To further localize the sizes of protein binding, we used phenyl-Sepharose chromatography to isolate restriction endonuclease-cleaved fragments which are attached to protein. [^{3}H]thymidine-labeled protein-MVM RF complex was iso-

lated by phenyl-Sepharose chromatography. Gel analysis of the eluted RF DNA indicated that the DNA migrated as monomer RF (Fig. 3). This phenyl-Sepharose-preselected MVM RF DNA-protein complex was digested with *Mbo*I, *Pst*I, or *Hin*fI and rerun on 200-μl phenyl-Sepharose columns. The flowthrough and 2% Sarkosyl-eluted fractions were treated with proteinase K, dialyzed, ethanol precipitated, and electrophoresed in polyacrylamide gels (Fig. 4). Two *Mbo*I-, *Hin*fI-, and *Pst*I-generated fragments were specifically retained by phenyl-Sepharose chromatography (lanes labeled Ph). The retained fragments were derived from both genomic termini. As was the case with terminal *Eco*RI fragments B and C, the terminal fragments of *Mbo*I-, *Hin*fI-, and *Pst*I-digested MVM RF DNA migrated as doublets. The protein appeared to be attached specifically to the e conformer of each fragment. Because the size of the 3′-terminal *Hin*fI fragment was 230 base pairs and that of the 5′-terminal *Mbo*I fragment was 350 base pairs, the site of protein attachment to the DNA must be within 230 and 250 base pairs of the 3′ and 5′ molecular termini of MVM RF DNA, respectively.

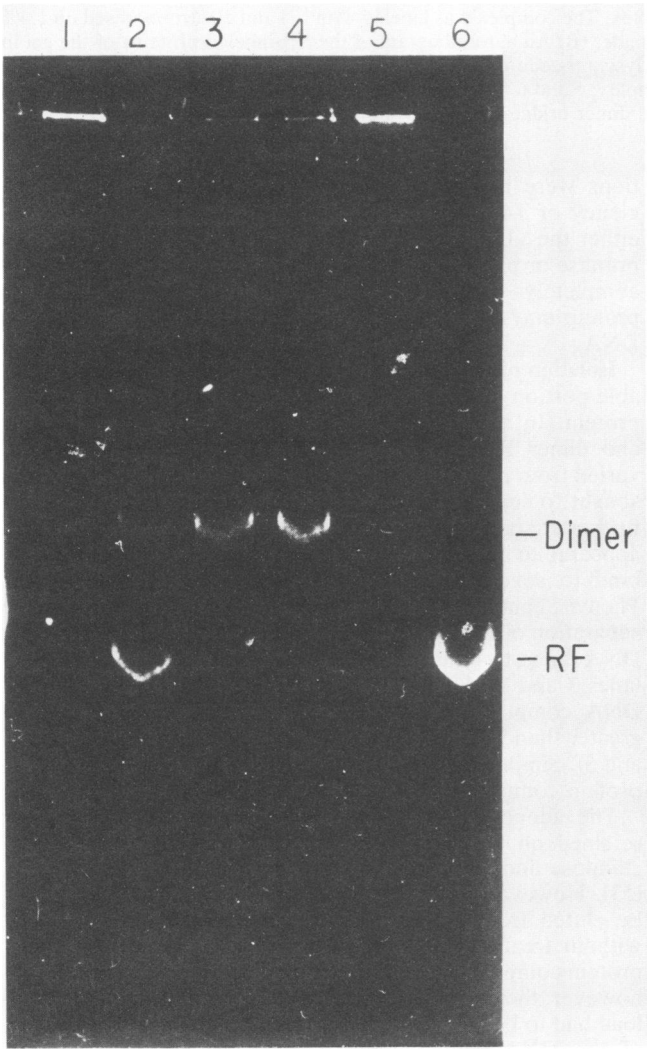

FIG. 3. Chromatography of the MVM RF on phenyl-Sepharose columns. The different fractions were electrophoresed on a 1.4% agarose gel, and the gel was stained with ethidium bromide. Lane 2, 4, and 6 samples were digested with pronase; lane 1, 3, and 5 samples were undigested. Lanes: 1 and 2, MVM RF which was loaded onto the phenyl-Sepharose column; 3 and 4, the flowthrough fraction from the column; 5 and 6, DNA which was eluted off the column with 2% Sarkosyl.

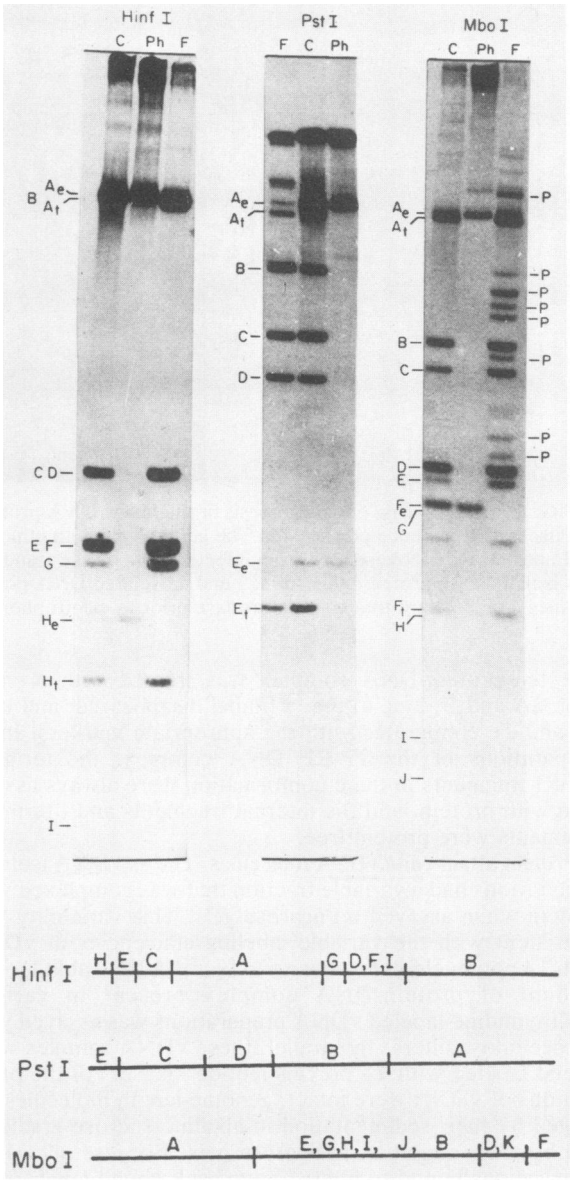

FIG. 4. Association of TP with specific regions of MVM DNA. MVM DNA-TP complex was digested with *Hin*fI, *Mbo*I, and *Pst*I and chromatographed on phenyl-Sepharose columns. Bound fragments were eluted with 2% Sarkosyl. The flowthrough (F) and eluted (Ph) fractions were digested with proteinase K and run on a 4% polyacrylamide gel. A sample of MVM monomer RF was also digested to completion (C) and run to indicate the positions of all fragments. The fluorograph of the gel is shown. Bands labeled P are partials due to incomplete digestion of the DNA with *Mbo*I.

Although the protein was found at both termini of the RF molecules, the protein could be attached to either the 3′ or 5′ end of each strand. Thus, the sensitivity of the protein-DNA complex to the two exonucleases was tested. It was assumed that, if the protein was bound to the 5′ end of each RF strand, the DNA would be sensitive to exonuclease III (a 3′→5′ ds-specific endonuclease) and resistant to λ exonucle-

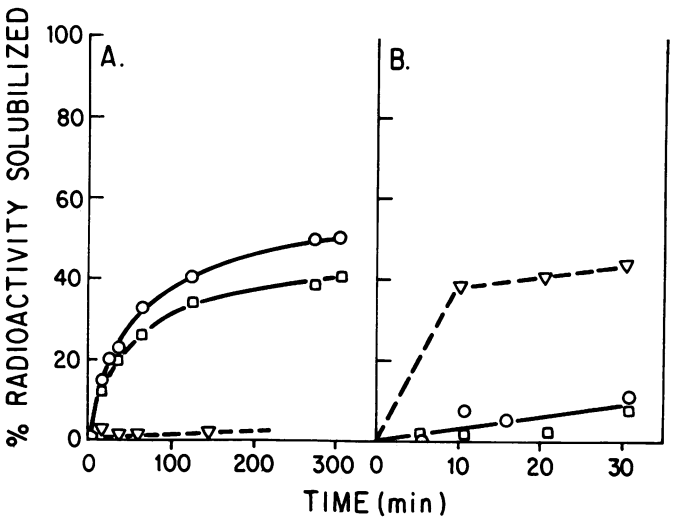

FIG. 5. Exonuclease digestion of MVM DNA-TP complex. (A) Digestion with exonuclease III, a 3'→5' exonuclease. (B) Digestion with λ exonuclease, a 5'→3' exonuclease. Symbols: ○, MVM DNA; □, adenovirus DNA; ▽, simian virus 40 DNA. Simian virus 40 DNA is form II in A and is linearized with *Bam*HI in B.

ase (a 5'→3' ds-specific exonuclease). This is the pattern of nuclease sensitivity seen with the adenovirus DNA-protein complex where the protein is known to be linked to each 5' terminus. If the protein were bound to the 3' end of both RF strands, then the sensitivities of the protein-DNA complex to the two exonucleases would be reversed. The MVM DNA-protein complex, like the adenovirus DNA-protein complex, was completely sensitive to exonuclease III (Fig. 5A), as the maximum digestion expected (50%) was achieved. The linear simian virus 40 DNA generated by *Bam*HI cleavage of simian virus 40 form I and II molecules also was completely sensitive to λ exonuclease, as expected. In contrast, the protein-DNA complexes of MVM and adenovirus were both highly resistant to λ exonuclease digestion (Fig. 5B). The 10% sensitivity to λ exonuclease of the adenovirus and MVM DNAs was probably due to a low level of contaminating nuclease in the λ exonuclease preparation used. Thus, the protein appeared to be covalently attached to the 5' terminus of each RF strand. This was entirely consistent with the 5'-terminal ^{32}P-labeling studies of vDNA with polynucleotide kinase.

Stability of the linkage between the protein and DNA. The stability of the protein-DNA complex was studied by filter-binding assay. Protein-DNA complexes labeled with [^{3}H]thymidine were treated under a variety of conditions which would normally dissociate noncovalent DNA-protein complexes. The presence of protein-DNA complexes was measured with a filter-binding assay, and the chemical stability of the complex was quantitated by the ratio of [^{3}H]DNA retained on the filter relative to that loaded (Table 2). The assay was dependent on binding of the protein to nitrocellulose filters, and neither φX174 ss vDNA nor ds RF form II bound to the filters. The adenovirus DNA-protein complex was completely retained; after proteinase K treatment, the percentage of ^{3}H-labeled adenovirus DNA retained on the filter dropped to background levels. Under these conditions, the MVM DNA-protein complex was completely retained on nitrocellulose, and binding was markedly reduced by the proteinase K treatment. However, because treatment of the MVM DNA-protein complex with proteinase K did not completely abolish binding, it was possible that the MVM-specific protein was more resistant to proteolysis or that a residual oligopeptide remained after proteinase K digestion which was bound to the filters less efficiently than was the intact protein. Because the efficiency of protein-DNA complex binding to nitrocellulose filters varied with the different treatment conditions, samples of adenovirus protein-DNA complex (as well as ss and ds φX174 DNAs) were filtered under each assay condition as internal controls. In addition, a time course for the stability of the MVM DNA-protein complex under each experimental condition was determined.

The protein-DNA linkage was stable in 1 M NaCl, 8 M urea, 4 M guanidine hydrochloride, 1% SDS, and 2% Sarkosyl (data not shown). In addition, the protein was not removed by phenol or phenol-chloroform extraction. When the MVM DNA-protein complex was heated at 98°C for 5 min and then chilled on ice for 10 min or treated with 0.3 M base or acid, only 50% of the label was retained (the reasons are outlined in the footnote to Table 2). However, because the amount of MVM DNA retained on the filters did not change over a period of 2 h (acid treatment) or 7 h (base treatment), it was concluded that the MVM DNA-protein complex is stable under the basic and acidic conditions tested. In contrast, the adenovirus DNA-protein complex is quite labile when treated with 0.3 M NaOH, exhibiting a half-life of less than 5 min. Adenovirus DNA is attached through a phosphoserine linkage to the 55,000-molecular-weight protein (17, 42); the observed base lability and acid stability are characteristic of this type of linkage. The stability of the MVM DNA-protein complex under condi-

TABLE 2. Stability of the MVM DNA-protein complex measured by specific retention of DNA-protein complexes on nitrocellulose filters

Treatment	Time (min)	% [^{3}H]thymidine-labeled DNA samples bound to nitrocellulose filters			
		φX174 (ss)	φX174 (ds)	Adenovirus DNA-protein complex	MVM DNA-protein complex[a]
None		1.26	0.4	100	100
Proteinase K (5 μg)	30	1.0	0.36	0.37	6.6
98°C (5 min) → 0°C (10 min)		1.1	0.5	97.3	50
1 M NaCl	60				100
	120	0.895	0.46	100	100
0.3 M NaOH–0.7 M NaCl (pH 14)	0			98	53.2
	5			19.4	
	120				62
	360	2.05	2.1	3.1	51.8
0.3 M CH_3COOH–0.7 M NaCl (pH 3.5)	0				48.3
	60			95.8	52.5
	120	1.1	0.5	98.8	50.2

[a] The MVM RF DNA used in these studies contained protein at the 5'-terminal *Eco*RI fragment (Be) and virtually no 3'-terminal *Eco*RI fragment (Ce) (Fig. 1). Because all RF molecules in this preparation did not have covalently continuous viral and complementary strands (data not shown), only 50% of the ^{3}H label (that corresponding to the DNA linked to the 5' e fragment) would be expected to bind to the filters after denaturation.

tions that normally disrupt noncovalent protein-DNA interactions indicated that the protein was covalently linked to the MVM RF DNA. The base and acid stability of the complex suggested that the protein may be linked through the a phosphotyrosine residue to the DNA.

Molecular mass of the TP. To obtain an estimate of the protein's molecular mass, we purified the DNA-protein complex by hydroxylapatite and phenyl-Sepharose chromatography and labeled it with Bolton-Hunter reagent. Alternatively, the complex was purified by protease-free Hirt lysis and exclusion chromatography and then labeled with Iodogen. The ^{125}I-labeled complexes were digested with DNase, and DNase-resistant samples were electrophoresed on SDS-acrylamide gels. In both cases, a single, major polypeptide species was seen by autoradiography of the gel. Using hydroxylapatite/phenyl-Sepharose purification, the TP had a molecular mass of 60 kilodaltons (kDa) when measured against protein standards (Fig. 6, lane 3). However, this purification procedure sometimes showed additional bands at lower molecular masses that were heterogeneous and of various intensities, suggesting that some degradation had occurred. The second purification method

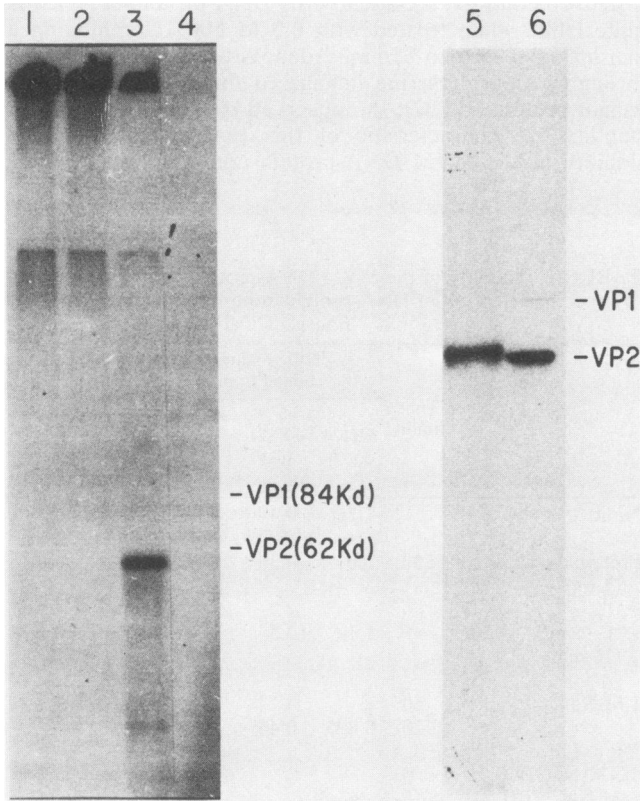

FIG. 6. Autoradiographic identification of MVM TP. SDS-polyacrylamide gel analysis of MVM DNA-protein complex. Lanes 1 through 4, TP-DNA complex isolated by hydroxylapatite and phenyl-Sepharose chromatography and labeled with ^{125}I-Bolton-Hunter reagent; lanes 5 and 6, complex isolated by SDS exclusion chromatography and ^{125}I labeled with Iodogen. Lanes: 1, labeled complex; 2, TP-DNA complex after phenol extraction; 3 and 5, TP-DNA complex treated with DNase; 4, TP-DNA complex digested with pronase; 6, ^{125}I-labeled MVM capsids. For lanes 1 through 4 the positions of VP1 and VP2 capsid proteins, which were coelectrophoresed and detected by staining with Coomassie blue, are indicated, along with their molecular masses.

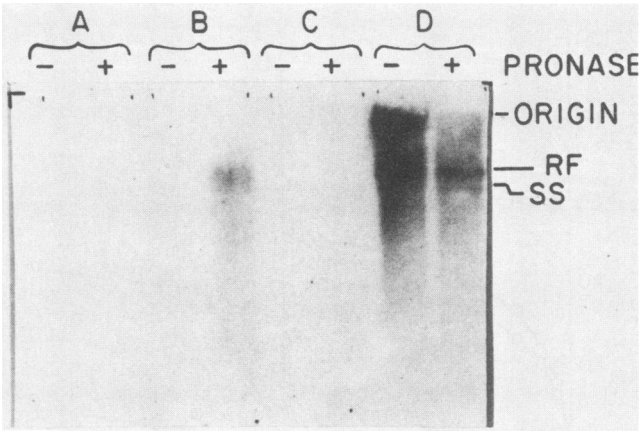

FIG. 7. Antigenicity of the MVM DNA-protein complex. Purified complex was reacted with normal serum (A), anti-TP serum (B), or anti-capsid protein serum (C). Immunocomplexes were immobilized on protein A-Sepharose, eluted with SDS, and electrophoresed on agarose gels with (+) or without (−) prior digestion with pronase. The DNA was transferred to nitrocellulose and probed with labeled MVM DNA. Lane D is the DNA-protein complex run directly on the gel.

also yielded a single protein band in gels. However, the measured molecular masses varied slightly from preparation to preparation between 62 and 65 kDa (Fig. 6, lane 5). This apparent difference in molecular mass is probably due to incomplete DNase digestion because of residual SDS present in the sample. To resolve this inconsistency, bands corresponding to the Be and Ce RF fragments and vDNA from the SDS-agarose gel (Fig. 2) were excised; the DNA was electroeluted in SDS and digested with DNase, and samples were run on SDS-acrylamide gels. In each case, a single polypeptide of approximately 60 kDa was seen (data not shown). This molecular mass was confirmed by the immunological data described below.

The change in buoyant density of the DNA-protein complex in CsCl is consistent with the molecular mass measurements. When compared with the DNA complex after protease digestion, the TP-DNA complex banded at $\rho = 1.695$, a position slightly less dense than the position of the RF after protease digestion ($\rho = 1.710$) (data not shown). Assuming that free protein forms a band at a CsCl density of 1.290 and that the molecular mass of the MVM RF DNA is 3×10^6 daltons, to obtain a shift of approximately 0.02 density units, a protein of approximately 110 to 130 kDa or two protein molecules of approximately 55 to 65 kDa molecular mass would have to be bound per RF molecule.

Immunological characterization of the TP. Because the molecular mass of the TP was very similar to that of the 62-kDa VP2 capsid protein, it was possible that the TP was actually one of the capsid proteins or a modified derivative thereof. Thus, to determine the origin of the TP, we raised an antiserum against the MVM DNA-TP complex. This antiserum was reacted with purified MVM DNA-TP complex, and the immunocomplexes were immobilized on a protein A-Sepharose column. The column was eluted with 2% SDS, a sample was treated with pronase, and the samples were run on an agarose gel. The DNA was transferred to nitrocellulose and probed with radiolabeled, cloned MVM DNA. MVM RF was specifically immobilized on the protein A-Sepharose column by anti-TP antiserum; the DNA was protein associated (Fig. 7, lane B). Parallel samples were run with normal serum (Fig. 7, lane A) and antiserum to MVM

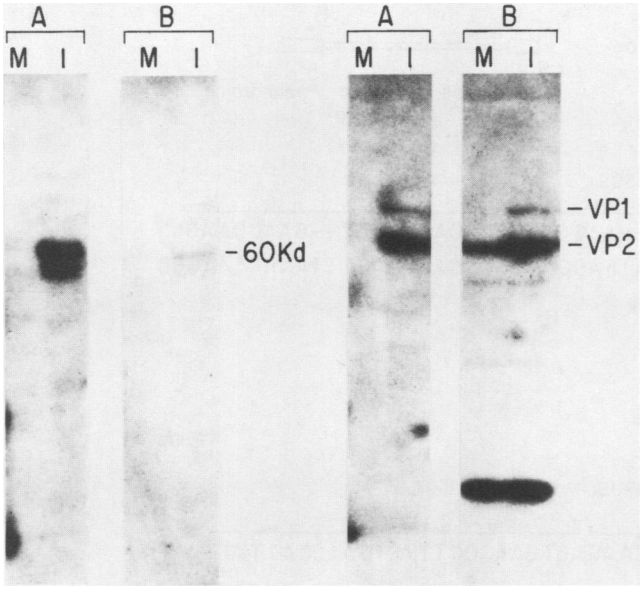

FIG. 8. Western blot analysis of intracellular MVM proteins. Mouse A9 cells were synchronized, infected with MVM, and harvested 24 h postinfection. The cytoplasmic (A) and nuclear (B) extracts were electrophoresed on 10% SDS-polyacrylamide gels, transferred electrophoretically to nitrocellulose, and probed with antibodies to MVM TP or MVM capsid proteins. The antibody complexes were detected with ^{125}I-labeled protein A. M, Mock-infected cells; I, MVM-infected cells.

capsids (lane C). The TP-DNA complex was not retained on the protein A-Sepharose column with either the normal or the anticapsid serum.

The antiserum to TP was also used in Western blot analysis of MVM-infected A9 cell extracts (Fig. 8). MVM- or mock-infected cells were harvested at 24 h postinfection, and cytoplasmic and nuclear extracts were made. The proteins in these extracts were separated on SDS-acrylamide gels, transferred to nitrocellulose, and probed with antiserum to MVM capsids or MVM TP. As expected, the antiserum to MVM capsids detected both VP1 (84 kDa) and VP2 (62 kDa) in the cytoplasmic and nuclear extracts from infected cells. However, the antiserum to TP detected only a major band at 60 kDa (and minor bands of lower molecular mass that probably represent degradation products). The VP2 amino acid sequences are completely represented within VP1 (58); thus, any polyclonal antiserum that reacted with VP2 should recognize VP1. Because the anti-TP serum failed to identify any protein larger than 60 kDa, the 60-kDa protein recognized by anti-TP serum was not VP2. In addition, the anti-TP serum recognized virtually no proteins in the nuclear extract. Thus, the free, uncomplexed TP is apparently antigenically unrelated to the capsid polypeptides and is distributed differently within the cell. Analysis of the TP and VP2 by one-dimensional partial protease cleavage maps is consistent with the immunological and cell fractionation data (data not shown).

The anti-TP antiserum did not recognize in cell lysates any protein larger than the 60-kDa protein, suggesting that it is unlikely that a large precursor to the TP exists. Thus, the molecular mass of the MVM TP is apparently 60 kDa, whether isolated from the termini of RF DNA or ss vDNA or found free in the cell. In this respect, the MVM TP differs from the adenovirus TP, which is synthesized as a larger precursor (80 kDa) that is subsequently cleaved to a 55-kDa TP during adenovirus maturation (10).

DISCUSSION

The data clearly show that a protein is covalently linked to a select population of MVM monomer RF DNA molecules. In addition, a variable fraction of the MVM vDNA and dimer RF DNA is found associated with protein. The protein is attached to the 5′ terminus of both viral and complementary strands. The stability of the protein-DNA complex to denaturing agents indicates that the protein is covalently bound to the DNA. The base and acid stability of the protein-DNA linkage shows that the linkage is not through a serine, threonine, or amide residue and suggests that the bond may be a phosphodiester bond between the 5′-terminal deoxynucleotide and a tyrosine residue in the protein. The protein has an estimated molecular mass of 60 kDa. Immunological analysis of the TP with other known viral proteins indicates that the TP is antigenically unrelated to the capsid proteins or to the 84K nonstructural protein NS-1; its genetic origin is unknown.

On monomer RF, the terminal *Eco*RI fragments in the e conformation were always protein associated. The internal fragments and t terminal fragments were protein free. The fraction of monomer RF that was protein bound varied among preparations, but this fraction reflected variability of the relative populations of RF molecules with t or e ends. Similarly, dimer RF DNA had a variable fraction that was associated with the TP. The exact localization of the TP on dimer RF was not determined, although the internal fragment containing the 3′-terminal sequences (dimer bridge) was protein free (Fig. 1B and 2A). Unfortunately, in these preparations, other MVM DNA forms, such as tetramer RF and the 8.0-kilobase replicative intermediate (20), were not present in sufficient concentration to analyze. Because the characterization of any TP associated with these RF DNAs is crucial in understanding the role of the TP in MVM DNA replication, similar studies are necessary to characterize the TP on dimer RF, tetramer RF, and the 8.0-kilobase intermediate.

Viral TP systems. The characteristics of the MVM DNA-protein complex are very similar to those reported for the protein covalently linked to the 5′ terminal of the related parvovirus H-1 (43). Although the H-1 protein-DNA complex was not characterized further, the molecular mass of the H-1 protein moiety was estimated to be 60 to 80 kDa from the decrease in buoyant density of the DNA due to the bound protein. The protein H-1 RF DNA complex also was extremely base stable, suggesting that the protein is linked to the DNA via a tyrosine residue. Of interest is the observation that, based on its buoyant density, H-1 vDNA contains no protein attached to it. Since filter-binding analysis of several MVM vDNA preparations showed different fractions of the protein complex being present, and this fraction inversely correlated with the 5′-end-labeling efficiencies of these different vDNA preparations, the protein may be removed from DNA fated to be packaged. The removal of the MVM protein (possibly by a required host function) may vary in efficiency from one infection to another or from one cell type to another; thus, the absence of H-1 protein from vDNA may reflect host cell differences.

Proteins also have been found to be covalently linked to genomes of several other viruses or bacterial phages. These include the gene A protein of φX174 (19, 27, 30), the gene 3 protein of φ29 (28, 46), the RNAs of picornaviruses (21, 25, 37, 47) and a number of plant viruses (16, 24, 32, 52), and the

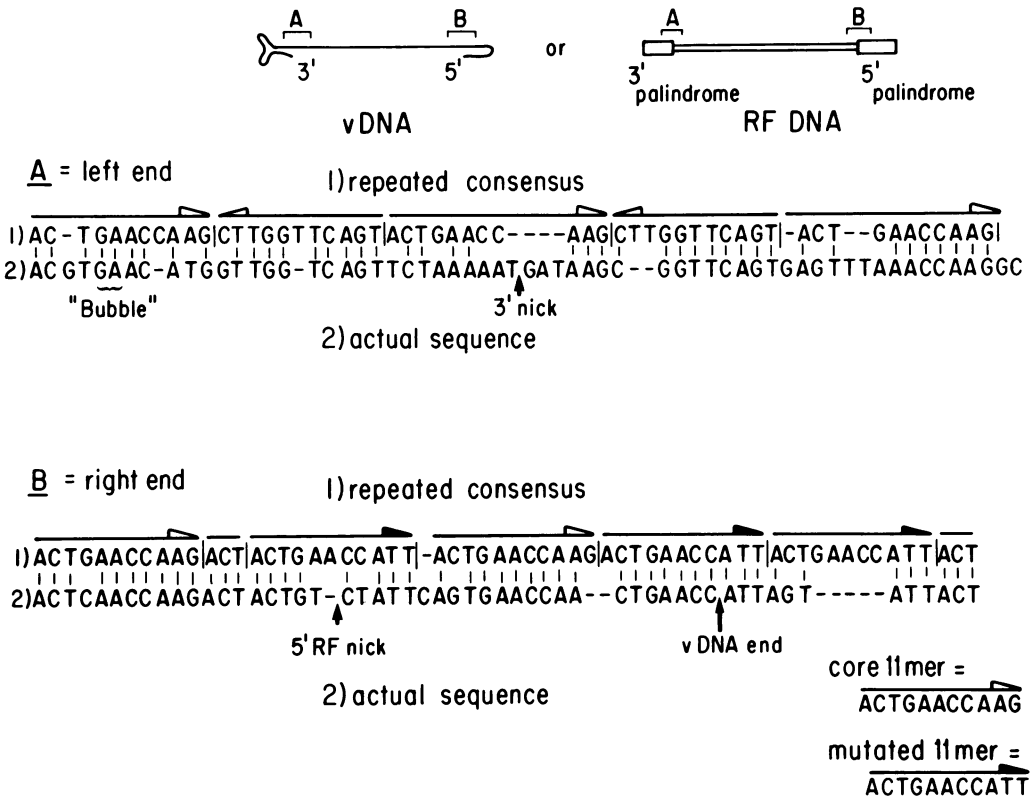

FIG. 9. Repetitive sequence elements near site-specific nicks in MVM DNA. The sequences around the ends of the palindromes on both the 3′ or left end (A) and the 5′ or right end (B) of MVM DNA can be constructed from imperfect repetitions of the 11-mer ACTGAACCAAG. The repeated consensus is compared with the actual sequence observed around each nick.

DNAs of adenoviruses (14, 42, 53) and hepatitis B virus (23, 35). The analogy of MVM RF DNA with adenovirus DNA is particularly noteworthy, since both are linear ds DNA molecules with proteins covalently bound at their 5′ termini; their replication origins are at or near the termini, and the terminal DNA fragments of both DNAs are found enriched in the nuclear matrix fraction (Bodnar, unpublished data).

Role of TP in MVM replication. TP is found attached to the e conformation of both the 3′ and 5′ termini of MVM DNA. However, the DNA palindromic sequences at the MVM termini are different (4). Therefore, if the interaction of the TP with the terminal palindrome is site specific, there must be common sequence elements near these sites of TP interaction. Analysis of the palindromic sequences shows common sequence motifs present around both termini which might be recognized by the TP prior to covalent binding. There are several imperfect copies of the consensus ACTGAACCAAG, an 11-mer (Fig. 9). These copies are present at each terminal sequence. At the MVM 3′ end, this consensus appears five times in head-to-head configuration. At the 5′ end, there are five copies of the consensus ACTGAACCAAG in head-to-tail configuration, with the last two positions either AG or TT. Thus, there are common sequence elements near both MVM termini that may be potential sites for TP recognition, even though the palindromes themselves have different sequences. In addition, processing and maturation of both termini is believed to involve nicking at specific sites (Fig. 9). For each terminal palindrome, the nick is at the same approximate position in the consensus sequence and on the same strand. The clustering of potential TP-binding sites around nick sites is consistent with a previously described model suggesting that site-specific nicking by a TP is required for processing and maturation of the MVM termini and for initiating MVM DNA replication from the RF molecules (1, 3).

Although the specific function of the proteins bound to the DNA or RNA may vary from system to system, most covalently linked proteins appear to play an essential role in initiating the replication of viral genomes by providing a primer for the polymerase (reviewed in reference 62). Current data suggest that such proteins play a part in initiating replication by either of two mechanisms: (i) site-specific nicking which generates a free 3′ end or (ii) presenting of the first nucleotide (which is already covalently bound to the protein) to provide a free 3′ hydroxyl group. In both cases, nucleotides are added to the free 3′ hydroxyl end by DNA polymerase. Site-specific nicking has been implicated in initiation of DNA replication of ϕX174 (18, 19, 30) and hepatitis B virus (35). Priming by a nucleotide-protein complex is believed to be involved in initiation of adenovirus (9, 42, 55) and ϕ29 (33, 38, 40, 49, 61) replication. Whereas the role of the MVM TP is unknown, we suggest that the MVM TP role in MVM replication may be very similar to those previously described for TPs in the other systems.

Origin of the MVM-TP complex. For covalent protein-DNA complexes, in both procaryotes and eucaryotes, the protein has been shown to be virus encoded in every case for which data are available (25, 27, 33, 40, 54). Assuming that the TP is MVM encoded, attempts have been made to identify the gene within the viral genome. In all cases, these approaches have been unsuccessful. From analyses of the complete genome sequence (5,081 nucleotides in length),

only two regions have been identified which have large open reading frames (3, 4). During a productive lytic infection, three major mRNAs are produced. These mRNAs have been mapped, and they encompass these two reading frames (39). Two of the RNAs encode the capsid proteins, and the third mRNA produces the 84-kDa nonstructural protein NS-1 (15). Thus, if the TP is also produced from these mRNAs, it should share protein sequences in common with either the capsid proteins or NS-1. The data presented here indicate that anti-TP antibodies do not cross-react with any identified MVM viral proteins (VP1, VP2, or NS-1) on Western blots. In addition, anti-capsid protein antibodies do not recognize the TP-DNA complex. The lack of cross-reactivity between the TP and VP1 or VP2 is consistent with the observation that one-dimensional partial peptide maps of the MVM TP differ from those of the capsid proteins (unpublished data). Finally, the lack of cross-reactivity between the TP and NS-1 is supported by studies with anti-NS-1 serum. In vitro translation experiments of MVM mRNAs followed by immunoprecipitation with anti-NS-1 antibodies have failed to find a 60-kDa protein antigenically related to NS-1, although the 84-kDa protein is synthesized and present in the immunoprecipitate (S. Cotmore and P. Tattersall, personal communication). Thus, it is unlikely that the TP is encoded by either of the large reading frames. It is formally possible that the TP might be encoded by smaller alternate reading frames by an identified but unmapped minor MVM transcript. However, to synthesize a 60-kDa protein, an extremely complex splicing pattern would be required to generate this RNA, and this RNA would have to be translated with extremely high efficiency to synthesize the amounts of protein seen within the infected cell. Thus, the data suggest that the TP is not MVM encoded. Proof of this hypothesis will require identification of the TP in uninfected cells. Although on Western blots the TP was not observed in lysates from mock-infected cells, the TP may still be a host-encoded protein. MVM DNA replication requires host-specific factors that are present only during the S phase (51, 56, 63). If the TP is this host factor, few of the mock-infected cells are in S phase, and detection of the TP would be very difficult. Alternatively, the TP may be a host protein induced upon MVM infection.

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