JOURNAL OF VIROLOGY, Mar. 1988, p. 851–860
0022-538X/88/030851-10$02.00/0

Vol. 62, No. 3

The NS-1 Polypeptide of Minute Virus of Mice Is Covalently Attached to the 5′ Termini of Duplex Replicative-Form DNA and Progeny Single Strands

SUSAN F. COTMORE[1] AND PETER TATTERSALL[1,2]*

Departments of Laboratory Medicine[1] and Human Genetics,[2] Yale University School of Medicine, New Haven, Connecticut 06510

Received 29 September 1987/Accepted 20 November 1987

When A9 cells are infected with minute virus of mice, a small proportion of the virally coded NS-1 polypeptide becomes covalently attached to newly synthesized viral DNA. Antisera directed against NS-1 will specifically precipitate two forms of monomer duplex replicative-form DNA, multimeric duplex intermediates and progeny single strands, and restriction analysis of the duplex forms in these precipitates reveals that NS-1 is exclusively associated with extended-form conformers of the genomic termini. Pulse-labeled viral DNA, harvested at various times in a highly synchronized infection, can be almost quantitatively precipitated with any one of a series of antisera directed against different protein domains distributed throughout the NS-1 molecule but not with antibodies directed against other viral proteins. In each case the interaction with NS-1 can be shown to involve both termini of duplex DNA and single-strand forms, suggesting that in each case a full-length (83-kilodalton) copy of NS-1 is present. Precipitation of the replicating viral DNA with an antibody directed against a synthetic 16-amino-acid peptide containing the sequence at the extreme carboxy terminus of NS-1 can be quantitatively and specifically inhibited with the immunizing peptide in its unconjugated form, showing that the antibodies responsible for precipitating viral DNA are directed against the NS-1 sequence itself and not against a trace contaminant. Exonuclease digestion studies show that the association effectively blocks the 5′ ends of the DNA molecules. Very little (less than 0.1%) of the newly synthesized [^{35}S]methionine-labeled NS-1 made in highly synchronized cells during a 15-min pulse early in infection (6.25 to 6.5 h into the S phase) becomes associated with viral DNA immediately. However, pulse-chase experiments show that later in infection (10 to 13 h into the S phase), when viral DNA replication is reaching its peak, a few percent of the molecules in these preexisting pools of NS-1 do become covalently attached to the newly replicated DNA. Isolated viral DNA-protein complexes labeled with [^{35}S]methionine in this way can be obtained by fractionation of the immunoprecipitated complexes on Sepharose CL4B in sodium dodecyl sulfate. Digestion of the purified complexes with nuclease releases an 83-kilodalton molecule which exactly comigrates with authentic NS-1 in sodium dodecyl sulfate-polyacrylamide gels.

Minute virus of mice (MVM) has a linear, negative-sense, single-stranded DNA genome of approximately 5 kilobases (11). The coding strand of this virus contains two major blocks of open reading frame, of which one, the left-hand open reading frame, contains the entire sequence used to encode the 83-kilodalton (kDa) NS-1 polypeptide, which is the major nonstructural protein of the virus (10). Frameshift mutations introduced into this left-hand open reading frame are lethal and prevent the excision and/or the subsequent replication of the viral DNA sequences (M. J. Merchlinsky, Ph.D thesis, Yale University, 1984), but the exact role(s) of NS-1 in viral DNA replication remains uncertain. Most models of MVM DNA replication invoke a "rolling-hairpin" mechanism, in which self-priming at the genomic termini is coupled with a series of hairpin-transfer steps (1, 2, 30). All such models hypothesize the use of a number of DNA-modifying enzymes, including site-specific nickases, single-strand binding proteins, and ligases which have yet to be identified. The limited genetic complexity of these viruses means that they must rely heavily on purloining cellular mechanisms for their own preferential replication. However, the replicative intermediates of MVM are multimeric, and to excise unit-length viral genomes from these intermediates at least one site-specific nickase is required. For this function a specialized, virally coded, protein such as NS-1 might seem most appropriate.

There have been several reports of proteins covalently associated with the 5′ ends of replicative-form (RF) DNA from a number of different autonomous parvoviruses (2, 8, 15, 24, 32) and with the 5′ ends of a proportion of progeny single-strand DNA (8, 21). Because of their location, these proteins are obvious candidates for the site-specific nickases required to process the viral termini, although they might easily perform other functions as well. Previous studies, however, have suggested that in MVM these "terminal" proteins have apparent molecular weights of around 60,000 and are antigenically distinct from any of the known virally coded proteins (2, 8). We have recently prepared a series of antisera directed against individual protein domains in the nonstructural proteins of MVM (10); while analyzing in vivo forms of the nonstructural proteins with these antisera, we have come to the inescapable conclusion that NS-1 is covalently associated with newly replicated viral DNA. The experiments presented in this paper develop this observation, showing not only that full-length, 83-kDa, copies of the NS-1 polypeptide are attached to the 5′ ends of both the viral and complementary strands of RF DNA and to all newly synthesized progeny single-strands, but also that in our hands they are the only viral proteins which are covalently associated with intracellular MVM DNA.

* Corresponding author.

MATERIALS AND METHODS

Materials. Restriction endonuclease and other DNA-modifying enzymes were obtained from New England BioLabs, Inc., Beverly, Mass. (unless otherwise stated). Radiochemicals were from Amersham Corp., Arlington Heights, Ill.

Cells and viruses. The prototype strain of MVM was grown in the mouse L-cell derivative A9 ouab^{r}11 as previously described (29). For experimental purposes cells were synchronized by using a double-block schedule described elsewhere (11). Briefly, cells were first allowed to accumulate in the G_0 phase by starving them of isoleucine for 48 h and then released from the isoleucine block while simultaneously being exposed to the DNA polymerase alpha inhibitor aphidicolin. Under this regime cells leave G_0 somewhat asynchronously in the 20 h after application of the aphidocolin block and accumulate at the G_1-S boundary. MVM virions, added to the cells along with the aphidicolin, are able to penetrate the cells and accumulate in the nucleus in the 20 h before the polymerase inhibitor is washed out of the culture. All times of infection referred to here initiate at the point when the aphidicolin is removed from the cells and they become free to enter the S phase of the cell cycle.

Cell labeling and extraction procedures. Cells were labeled with $^{32}P_i$ at 0.05 mCi/ml in Dulbecco modified Eagle minimal medium containing 1/10 of the normal concentration of unlabeled phosphate and 5% dialyzed fetal calf serum at the time points and for the periods of time specified below. Labeling with [^{35}S]methionine was carried out at 0.16 or 0.5 mCi/ml (as specified below) in Dulbecco modified Eagle medium containing 0.3 mg of methionine (one 1/100 of the normal amount of this amino acid) per liter and 5% dialyzed fetal calf serum, for the time periods indicated below. Cells were washed into serum-free medium, harvested by scraping, and washed once in phosphate-buffered saline containing 0.01% bovine serum albumin. Typically, pellets of approximately 10^6 cells were stored frozen at −20°C until required and then thawed immediately into 0.45 ml of buffer A (10 mM EDTA, 1% Nonidet P-40 [Sigma Chemical Co., St. Louis, Mo.], 0.01 M Tris hydrochloride [pH 8], 0.15 M NaCl) containing 2% sodium dodecyl sulfate (SDS), vortexed, and incubated at 37°C for 10 min, 60°C for 20 min, and 70°C for a final 10 min with repeated vortexing. Extracts were then incubated with 5 μl of nonspecific rabbit serum at room temperature for 20 min, and the immunoglobulin was removed by tumbling with prewashed, Formalin-fixed *Staphylococcus aureus* (Boerhinger-Mannheim Biochemicals, Indianapolis, Ind.). After clearance, sample volumes were made up to 3.6 ml with buffer A to give a final SDS concentration of 0.25%, and each extract was used for 12 immunoprecipitations of 0.3 ml each. Extracts were incubated with 3 μl of specific antiserum overnight at 4°C, antigen-antibody complexes were adsorbed by tumbling for 20 min with 50 μl of a prewashed 10% suspension of Formalin-fixed *S. aureus* and collected by centrifugation, and the resulting pellets were washed three times by suspension and tumbling in cold buffer A. Finally pellets were washed once with either just 0.01 M Tris hydrochloride (pH 7.5) or this buffer supplemented with the divalent cations required for any subsequent enzyme digestion. Failure to wash immunoprecipitates sufficiently well can inhibit some DNA-modifying enzymes. The anti-NS-1 sera used in these experiments are relatively strong sera, such that 0.036 ml of specific serum (12 precipitates at 3 μl each) is able to scavenge at least 95% of the NS-1 expressed in 10^6 MVM-infected cells harvested 12 h after release from aphidicolin. However, at later time points extraction volumes and numbers of precipitates must be progressively increased to keep precipitates relatively quantitative.

Antisera. Most of the antisera used in these experiments have been described in detail elsewhere (10). Briefly, antiserum A is a rabbit serum directed against a gel-purified bacterial fusion protein which expresses 22 amino acids of the *cro* protein specified by phage lambda and the 84-amino-acid amino-terminal peptide which is shared by both NS-1 and NS-2. Antiserum B is directed against a similar *cro* fusion protein, but in this case the MVM sequence is derived from the middle of the NS-1 molecule (nucleotides 1110 through 1638), and the antiserum is thus totally specific for NS-1. Antiserum D is directed against a *cro* fusion protein in which the MVM sequence is derived from the carboxy-terminal half of NS-2 (nucleotides 2075 through 2291 expressed in reading frame 2) and is totally specific for NS-2. Antiserum C is a rabbit serum directed against a synthetic peptide of 16 amino acids derived from the extreme carboxy terminal of NS-1 and conjugated to hemocyanin via an additional cysteine residue added at the amino terminus of the peptide. The full sequence of this peptide is NH_2-Cys-Gly-Ala-Glu-Pro-Leu-Lys-Lys-Asp-Phe-Ser-Glu-Pro-Leu-Asn-Leu-Asp-COOH. Rabbit anti-MVM capsid serum was raised against gradient-purified MVM virions.

Enzyme digests. Immunoprecipitates were suspended in 25 μl of the appropriate buffer as directed by the supplier and digested for 30 min at 37°C as follows: bovine pancreatic DNase (Bethesda Research Laboratories, Bethesda, Md.), 0.1 mg/ml; proteinase K (Boehringer), 0.2 mg/ml in buffer containing 0.05% SDS; mung bean nuclease (PL Biochemicals, Inc., Milwaukee, Wis.), 17.5 U; micrococcal nuclease (Pharmacia Inc., Piscataway, N.J.), 0.1 mg/ml; *Eco*RI, 10 U; lambda exonuclease, 0.125 U; exonuclease III, 25 U. After *Eco*RI digestion, pellets were centrifuged, the supernatants were removed and stored, and the remaining pellets were washed by suspension in 0.2 ml of cold restriction buffer and repelleting.

Gel electrophoresis. Discontinuous SDS-acrylamide gels were prepared by the method of Laemmli (17), and autoradiographs were prepared as previously described (9). SDS-agarose gels were prepared as normal for DNA analysis (18), except that 0.2% SDS was included in both the gel and the running buffer, and samples were preincubated in normal sample buffer supplemented with 1% SDS and 0.05 M dithiothreitol (Sigma) and incubated at 60°C for 5 min before loading.

Sepharose CL4B chromatography. Sepharose CL4B (40 ml) was equilibrated in buffer B (0.01 M Tris hydrochloride [pH 8], 0.15 M LiCl, 0.0005 M EDTA, 0.3% SDS), and samples (0.4 ml) were loaded in buffer B containing 2% SDS.

RESULTS

Antisera specific for NS-1 precipitate viral DNA. The presence of nucleic acid covalently attached to NS-1 was first suggested when immunoprecipitates of this molecule were prepared from extensively denatured extracts of $^{32}P_i$-labeled cells and analyzed on Laemmli-type, SDS-polyacrylamide gels (Fig. 1). Whenever NS-1 (83 kDa) was present in the precipitate, the stacking gel and the top of the running gel contained large amounts of labeled material (lanes 2 and 5), which could be quantitatively removed by digesting the precipitate with nuclease before electrophoresis (lanes 3 and 6). In the experiment illustrated in Fig. 1, micrococcal nuclease was used, but essentially similar results were

obtained with bovine pancreatic DNase, indicating that the labeled material was DNA rather than RNA. Precipitates containing other viral proteins, for example, the various forms of NS-2 (25 kDa) seen in lanes 8 and 9, did not contain similar, nuclease-sensitive material.

Similar precipitates analyzed on SDS-containing agarose gels (Fig. 2) show that the nucleic acid coprecipitated with NS-1 is viral in origin (compare Fig. 2A, lanes 1 through 4 and 5, with lane 9), the higher-molecular-weight cellular constituents (c) being largely excluded from the pellets. After the extensive denaturation steps used to prepare cell extracts for precipitation (see above), sera directed against the viral capsid proteins or the other major nonstructural protein group, NS-2, totally failed to precipitate viral DNA (Fig. 2A, lanes 6 through 8). However, antisera directed against gel-purified, procaryotic fusion peptides expressing 22 amino acids of the phage lambda *cro* protein together with either 84 amino acids from the common amino-terminal peptide shared by NS-1 and NS-2 (lane 1, antiserum A) or 176 amino acids from an NS-1-specific sequence (lane 3, antiserum B) both efficiently precipitated two distinct forms of monomer RF DNA (m1 and m2 in Fig. 2), dimer RF DNA (d), and progeny single strands (s). Figure 2B shows the monomer RF region of lanes 1 through 5 at higher magnification to facilitate identification of the various molecular forms. When similar anti-NS-1 precipitates were electrophoresed on neutral or alkaline agarose gels in the absence of SDS, labeled nucleic acid failed to enter the gel unless the precipitates were predigested with proteinase K. Although we have no reason to believe that these antisera were in any way contaminated with antibodies directed against any eucaryotic or procaryotic proteins except the lambda *cro* sequence and, perhaps, lysozyme (10), it is theoretically possible that the protein to which viral DNA was attached was a minor contaminant in the immunogen rather than NS-1 itself. This interpretation is, however, rendered less probable by the precipitate shown in lane 5 of Fig. 2A and B and by those shown in Fig. 3, where the immunizing antigen used to evoke the anti-NS-1-specific antiserum, antiserum C, was a synthetic 16-amino-acid peptide expressing the sequence located at the extreme carboxy terminus of NS-1, [MVM(p) nucleotides numbers 2229 through 2276, expressed in frame 3 as designated in reference 10]. Despite its rather different origin, this antiserum effectively precipitated the same viral DNA species as other anti-NS-1 sera (compare lanes 1 and 2 in Fig. 3), and its ability to precipitate these forms could be titrated out by adding increasing amounts of the immunizing peptide to cell extracts before precipitation (Fig. 3, lanes 3 and 4). The addition of an unrelated peptide (lanes 6 and 7) failed to inhibit DNA precipitation with this antibody, and

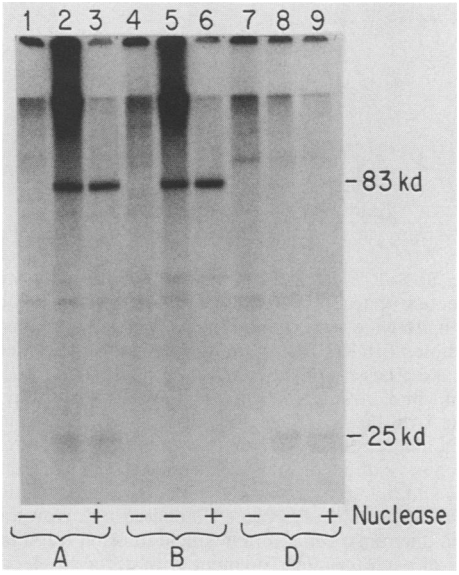

FIG. 1. Autoradiograph of an 11% SDS–polyacrylamide gel showing material immunoprecipitated from synchronized MVM-infected A9 cells (lanes 2, 3, 5, 6, 8, and 9) and from similar uninfected cells (lanes 1, 4, and 7) labeled with $^{32}P_i$ 10 to 12 h after release from aphidicolin, harvested immediately, and precipitated with antisera directed against the common amino-terminal region of NS-1 and NS-2 (serum A; lanes 1, 2, and 3), against NS-1 alone (serum B; lanes 4, 5, and 6), and against NS-2 alone (serum D; lanes 7, 8, and 9). Duplicate precipitates from infected cells were either treated (designated + in lanes 3, 6, and 9) or not treated (designated − in lanes 2, 5, and 8) with micrococcal nuclease before electrophoresis.

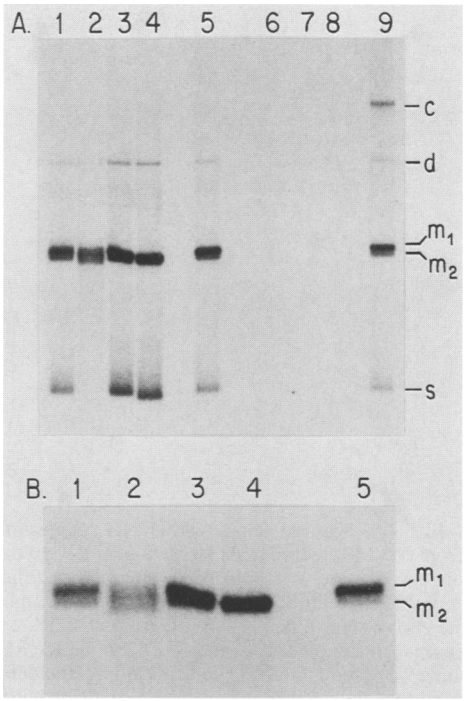

FIG. 2. (A) SDS–1% agarose gel showing DNA species immunoprecipitated from MVM-infected cells labeled with $^{32}P_i$ 10 to 12 h after release from aphidicolin, with antisera directed against the common amino-terminal region of NS-1 and NS-2 (serum A, lane 1), against a region in the middle of the NS-1 molecule (serum B, lane 3), against the 16-amino-acid carboxy-terminal peptide of NS-1 (serum C, lane 5), prebleed serum from the rabbit used to prepare serum C (lane 6), serum monospecific for NS-2 (serum D, lane 7), or a serum raised against purified MVM capsids (lane 8). The total cell extract from which these precipitates were prepared (lane 9) contains cellular DNA species (c) as well as several forms of viral DNA, including unit-length single-stranded progeny DNA (s), two forms of monomer duplex RF (m1 and m2), and dimer duplex RF (d). Pretreatment of immunoprecipitates with mung bean nuclease before electrophoresis (lane 2, serum B) destroys single-stranded progeny DNA (s) and shifts and obscures the banding of monomer RF species. Proteinase K digestion of precipitates before electrophoresis (lane 4, serum B) collapsed monomer RF forms into a single band with a relatively high mobility and substantially increased the mobility of single-stranded DNA. (B) The monomer RF region of lanes 1 through 5 from Fig. 2A is shown at increased magnification to show details of the banding pattern.

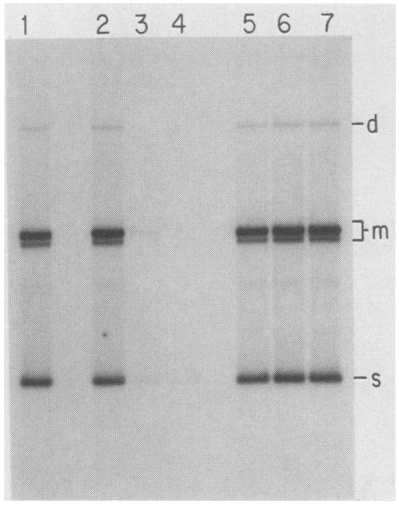

FIG. 3. SDS–1% agarose gel showing DNA species immunoprecipitated from MVM-infected cells labeled with $^{32}P_i$ 10 to 12 h after release from aphidicolin, with antisera directed against the middle of the NS-1 molecule (serum B, lane 1) or against the 16 amino acids at the extreme carboxy terminus of NS-1 (serum C, lanes 2 and 5). As in other figures, m denotes monomer RF, d is dimer RF, and s is single-stranded progeny DNA. The addition of the unconjugated NS-1 carboxy-terminal synthetic peptide (used as the hapten to raise serum C), to the cell extract at a final concentration of 1 μg/ml (lane 3) or 100 μg/ml (lane 4) before precipitation with serum C effectively inhibited precipitation of the viral DNA by competing for the antibody-binding sites. However, the addition of 1 μg/ml (lane 6) or 100 μg/ml (lane 7) of an unrelated synthetic peptide (corresponding to the carboxy terminus of the major form of NS-2) had no effect on the efficiency of RF precipitation with this serum.

nonimmune prebleed serum from these rabbits also failed to precipitate viral DNA (Fig. 2A, lane 6).

The single-stranded nature of the species designated as progeny DNA (marked "s" in all figures) was confirmed by digesting anti-NS-1 immunoprecipitates with single-strand-specific mung bean nuclease before electrophoresis (Fig. 2, lanes 2). This pretreatment left duplex RF DNAs relatively intact, although it shifted and obscured the distinct banding of monomer RF species (Fig. 2B, lane 2). Additional data (not shown) suggested that this mobility shift may be due to the partial removal of terminal protein from these forms by the nuclease, presumably because the protein disrupts base pairing of the terminal nucleotides and thus exposes short stretches of single-stranded DNA to the nuclease. The effects of protease treatment per se are seen in Fig. 2, lane 4, where treatment of an anti-NS-1 precipitate with proteinase K before electrophoresis increased the mobility of single-strand progeny DNA and collapsed the various forms of monomer RF into a single broad band with a higher electrophoretic mobility.

The data shown in Fig. 1, 2, and 3 were obtained with cells pulse-labeled with $^{32}P_i$ 10 to 12 h after the onset of S-phase in a highly synchronized cell system (11) and harvested immediately. This labeling period covered the peak of viral DNA amplification and progeny virus synthesis, but similar experiments with cells labeled between 5 and 7 h after the start of the S phase, i.e., near the start of the amplification stage of replication, gave essentially identical results with the various anti-viral protein antibodies, except that the extracts contained less single-stranded DNA.

NS-1 exclusively associated with extended conformers of the viral termini. The termini of duplex RFs of viral DNA exist in two conformers (Fig. 4): a covalently closed "turnaround" form, which has a single copy of the palindrome, and an "extended" form with two copies of the palindrome. Thus the restriction endonuclease *Eco*RI, which cuts each monomer twice, yielded five distinct fragments: a single fragment from the middle of the genome (M in Fig. 4) and two forms of each end (Re and Rt, Le and Lt). A sixth fragment, twice the size of fragment Lt, and designated db in Fig. 4 (lanes 4, 5, and 7), was derived from the head-to-head bridge of dimer RF DNA. When anti-NS-1 immunoprecipi-

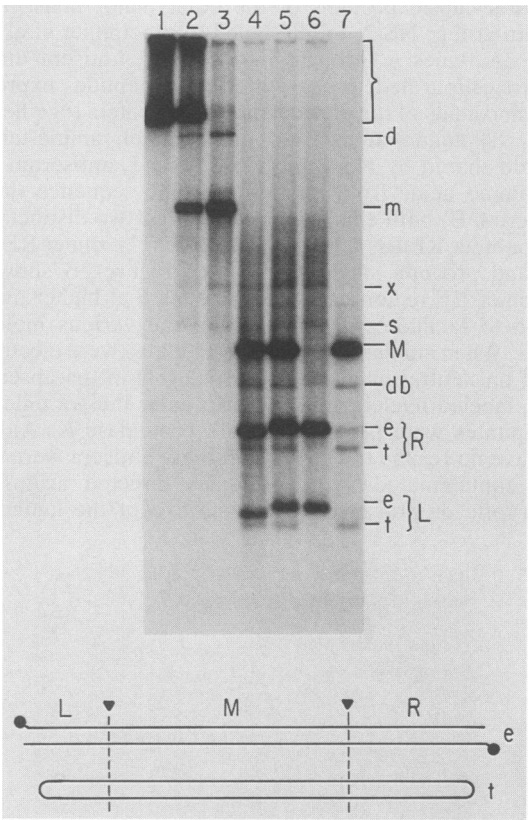

FIG. 4. SDS–1.4% agarose gel showing total cell extracts from MVM-infected (lane 2) and uninfected cells (lane 1), labeled with $^{32}P_i$ 5 to 7 h after the start of the S phase. Lane 3 contains monomer (m) and dimer (d) RF DNA, unit-length single-stranded progeny DNA (s), and a largely uncharacterized (but single-strand nuclease-sensitive) form of viral DNA (x) precipitated from a similar infected-cell extract with the NS-1 specific antiserum B. As depicted in the diagram, monomer duplex RF contains a mixed population of forms which can have extended (e) or turnaround (t) configurations at both the left (L) and right (R) ends of the genome. The restriction enzyme *Eco*RI cuts such molecules twice (indicated by arrows and dotted lines in the diagram), generating a single internal fragment (M) and two forms of each terminal fragment with different electrophoretic mobilities. Lane 5 shows material immunoprecipitated with serum B and then digested with *Eco*RI, and lane 4 shows that incubation of a similar digest with proteinase K before electrophoresis only influences the mobility of the extended form (Re and Le) terminal fragments. When *S. aureus*-bound immunoprecipitates were centrifuged after *Eco*RI digestion, the pellet contained DNA fragments which were directly attached to the antibody-bound NS-1, whereas the supernatant contained NS-1-free DNA. In these experiments the pellet contained the extended forms of both terminal fragments (Re and Le, lane 6), confirming that the DNA–NS-1 linkage occurs within these forms of each terminus, whereas turnaround forms and internal fragments are devoid of NS-1.

tates were digested with *Eco*RI before electrophoresis in SDS-agarose gels (lane 5), single-strand forms (s and x) remained intact, whereas duplex forms yielded the expected fragments (lane 5). When these restriction digests were then further digested with proteinase K before electrophoresis (lane 4), most fragments retained the same mobility, but the extended conformers from both the right and left halves of the genome showed an increased mobility, suggesting that these forms were previously complexed with protein. Restriction digests were carried out by directly suspending the *S. aureus* pellet carrying the immunoprecipitate in restriction buffer. Thus, after digestion, the bacterial pellet could be removed by centrifugation, taking with it any restriction fragments that were physically attached to NS-1 in the antigen-antibody complexes while leaving behind fragments that were not directly associated with NS-1. The pellet fraction from such a separation was massively enriched for the extended conformers of both duplex termini in addition to single-strand forms of DNA (Fig. 4, lane 6), whereas the supernatant (lane 7) contained all of the other forms.

Quantitative precipitation of viral DNA with antisera against the two termini of NS-1. To assess what proportion of the newly synthesized viral DNA was associated with NS-1, we performed repeated immunoprecipitations on the same sample of cell extract and analyzed the bound and unbound material on SDS-agarose gels (Fig. 5). Since it was possible that full-length copies of NS-1 might be associated with one of the termini of duplex DNA while cleaved forms of the protein were attached to the other terminus or to single-strand DNA, we performed these serial precipitation experiments with antisera against both termini of the NS-1 molecule and analyzed the resulting precipitates by *Eco*RI digestion and centrifugation as described above for Fig. 4.

Antiserum A, directed against the 84-amino-acid, amino-terminal peptide of NS-1, removed 90 to 95% of the viral DNA in the sample in a single round of precipitation (Fig. 5, lanes 3 through 5). The *Eco*RI pellet fraction from a similar precipitate (lane 2) showed that single-strand DNA and both the right and left terminal restriction fragments from duplex DNA were physically associated with NS-1 molecules which had relatively intact amino termini. Precipitation of the small amount of material remaining in the extract after a single round of precipitation removed most of the remaining viral DNA (Fig. 5, lanes 5 through 7), indicating that effectively all of the newly synthesized DNA in the cell is associated with NS-1 molecules carrying at least part of the 84-residue amino-terminal peptide. In contrast to these results, antiserum C, directed against the extreme carboxy-terminal 16 amino acids of NS-1, only removed 75 to 80% of the DNA in a single round of precipitation (Fig. 5, lanes 9 through 11). Moreover, repeated rounds of precipitation with this antibody failed to remove most of the remaining viral DNA (Fig. 5, lanes 11 through 13). Since both duplex and single-strand DNA remained in the unbound fraction from these precipitates (Fig. 5, lanes 11 and 13), this suggests that 20 to 25% of both of these species carry NS-1 molecules which have lost their extreme carboxy termini, whereas the remaining 75 to 80% of all DNA forms carry full-length (83-kDa) copies of NS-1. The unbound forms remaining after precipitation with antiserum C could be removed from the extract by reprecipitation with anti-NS-1 sera directed against other protein domains; this indicates that the association between protein and DNA is not mediated by the extreme carboxy terminus of NS-1. As seen in Fig. 5, lane 15, the *Eco*RI pellet derived from an initial immunoprecipitate prepared with antiserum C contained single-strand DNA and both termini of duplex

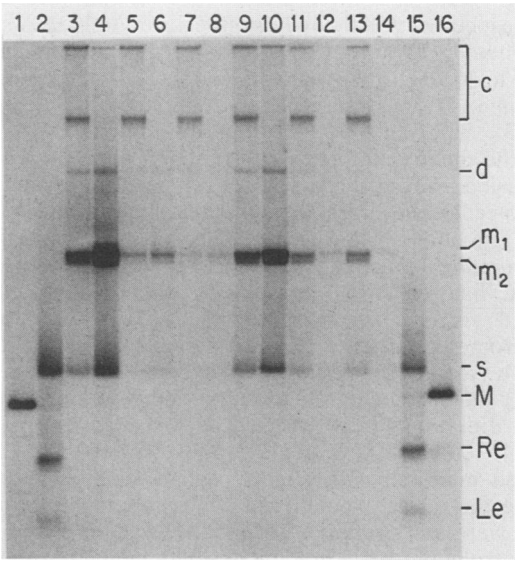

FIG. 5. SDS–1.2% agarose gel showing serial immunoprecipitates and unbound fractions from $^{32}P_i$-labeled, MVM-infected A9 cell extracts with antisera directed against the extreme amino-terminal 84 amino acids of NS-1 (serum A, lanes 1 through 8) and the extreme carboxy-terminal 16 amino acids of NS-1 (serum C, lanes 9 through 16). Lanes 3 and 9 contain equal cell equivalents of the initial cell extract before precipitation. Lanes 4 and 10 each contain the first precipitates, obtained with serum A and C, respectively, from five cell equivalents of extract. Lanes 5 and 11 each contain one cell equivalent of the unbound material remaining after the first precipitation. Lanes 6 and 12 each contain five cell equivalents of precipitate from the second set of precipitations, whereas lanes 7 and 13 each contain one cell equivalent from the unbound material from the second precipitation. Finally, lanes 8 and 14 each contain five cell equivalents of material precipitated in the third round of immunoprecipitation, with the same antibodies (A and C, respectively). Letter designations are as in Fig. 4. First-round precipitates equivalent to those shown in lanes 4 and 10 were digested with *Eco*RI and separated into pellet and supernatant fractions. Pellet fractions are shown in lanes 2 (serum A) and 15 (serum C), and supernatant fractions are shown in lanes 1 (serum A) and 16 (serum C). M denotes the uniques *Eco*RI fragment from the middle of the genome, Re is the extended-form of the terminal restriction fragment from the right end of the genome, and Le is the extended-form terminal fragment from the left end. Turnaround fragments from RF termini are not visible at this exposure.

DNA, confirming that NS-1 molecules containing this peptide are associated with all three types of termini. The material which remained unprecipitated by antiserum C was remarkably enriched for the lower form of monomer RF (m2 in Fig. 5; compare lanes 5 and 11). In a subsequent paper (Cotmore and Tattersall, manuscript in preparation), we show that the top form of monomer RF (m1 in Fig. 5) has extended forms of both right and left termini, whereas m2 has only one extended-form terminus, located predominantly at the right hand end of the molecule. Thus the substantial enrichment of form m2 in the unbound fraction from these precipitates probably reflects the fact that antiserum C has only a single chance of finding an NS-1 molecule with an intact carboxy terminal on this species, whereas it has two chances of finding such a molecule on the m1 species.

NS-1 blocks the 5′ termini of all newly synthesized viral DNA. Bacteriophage lambda exonuclease and *Escherichia coli* exonuclease III are double-stranded DNA-specific exonucleases which digest DNA in a 5′-to-3′ and 3′-to-5′ direc-

tion, respectively. All newly synthesized viral DNA species precipitated with anti-NS-1 serum were resistant to digestion in a 5′-to-3′ direction by lambda exonuclease (Fig. 6, lane 2), but duplex DNA forms were susceptible to exonuclease III (lane 3). Since this nuclease digests a single DNA strand from the duplex and ceases to function once the enzymes processing in from the two 3′ termini meet in the middle of the molecule, this digestion generates single strands of DNA which are one-half unit length. This fragment, marked b, is seen running as a collapsed rod in Fig. 6, lane 3, whereas a second limit-digestion product of unknown structure is marked a. Single-strand progeny DNA has a duplex 3′ palindrome of 116 nucleotides. Exonuclease III was able to remove part of this palindrome, leaving a molecule with a slightly increased electrophoretic mobility (lane 3). Proteinase K digestion of the exonuclease III digestion products (lane 4) left collapsed single-strand forms of DNA which then all migrated with slightly enhanced mobilities, confirming that after exonuclease III treatment the remaining forms were still protein associated. Digestion of the *Eco*RI pellets from anti-NS-1 precipitates with lambda exonuclease (lane 6) or exonuclease III (lane 7) confirmed that both ends of duplex DNA had blocked 5′ termini and free 3′ termini. *Eco*RI supernatant fractions from these precipitates (lane 8)

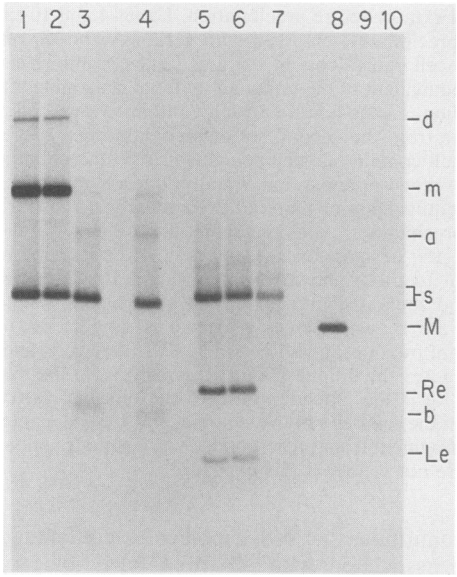

FIG. 6. SDS–1.4% agarose gel showing material immunoprecipitated from MVM-infected A9 cells labeled with $^{32}P_i$ 10 to 12 h after release from aphidicolin with antiserum B (NS-1 specific). Immunoprecipitates equivalent to that shown in lane 1, containing single-stranded viral DNA (s) and monomer (m) and dimer (d) duplex RF, were digested with the 5′-to-3′ exonuclease from bacteriophage lambda (lane 2) or the 3′-to-5′ *E. coli* exonuclease III (lane 3). Single-stranded DNA fragments half the length of monomer RF are labeled b, and an additional limit-digestion product generated by exonuclease III is labeled a. Lane 4 contains the forms generated when an exonuclease III digest similar to that shown in lane 3 was further digested with proteinase K before electrophoresis. The pellet fraction from an *Eco*RI digest of anti-NS-1 precipitated DNA is shown in lane 5, and similar pellets are shown in lanes 6 and 7 after digestion with lambda exonuclease and exonuclease III, respectively. The supernatant fraction from an *Eco*RI digest is shown in lane 8, and the results of digesting such fractions with lambda exonuclease or exonuclease III are shown in lanes 9 and 10, respectively. Letter designations are as in Fig. 6. Turnaround forms of the termini are not visible at this exposure.

were not associated with protein, and these fragments were totally susceptible to digestion with both lambda exonuclease (lane 9) and exonuclease III (lane 10).

Incorporation of [^{35}S]methionine into terminal protein. In the cell synchronization system used for these experiments, amplification of viral duplex DNA first becomes readily detectable between 4 and 5 h after the beginning of the S phase (11) and reaches peak levels by 10 h after release from aphidicolin. However, NS-1 synthesis can be detected within the first 2 h of the S phase and has reached maximal levels by 7 h (11). Given these two rather different time scales, we decided to carry out pulse-chase experiments, pulsing with [^{35}S]methionine at the time of maximal NS-1 synthesis (6.5 to 6.75 h into S) and then looking for incorporation of labeled protein into total cell DNA and immunoprecipitable DNA as a function of time. Under these conditions there was a substantial time lag between the incorporation of label into protein and the subsequent incorporation of that protein into viral DNA-protein complexes (Fig. 7A). Parallel anti-NS-1 immunoprecipitates from each of these time points were analyzed on SDS-acrylamide gels, and autoradiographs of this gel (data not shown) indicated that the total amount of labeled NS-1 remained fairly constant during the experimental period (6.5 to 13.25 h after the start of S), although there was perhaps a slight decline in the amount available at later time points. However, incorporation of this labeled protein into DNA-protein complexes was barely detectable until approximately 2 h after synthesis, and progressively more label then became associated with viral DNA as time progressed (up to 13.25 h after the start of S). Comparison of the labeling pattern seen for total cell DNA (Fig. 7A, lanes 2 through 6) and for immunoprecipitated DNA (lanes 7 through 11) confirmed that the appearance of label in material which could be precipitated with anti-NS-1 serum paralleled the incorporation of label into total DNA-protein complexes. In both cases the label first appeared associated with monomer RF DNA and only later became associated with single-stranded progeny DNA (Fig. 7A, lanes 6 and 11). Label also accumulated in dimer RF DNAs near the end of the experimental period (lanes 10 and 11), perhaps because the ability to nick the 3′ termini became a rate-limiting factor at this stage in the amplification of viral DNA. Figure 7B shows an ethidium stain of the gel in Fig. 7A taken before autoradiography. Comparison of these two figures shows that incorporation of labeled NS-1 into DNA-protein complexes simply follows the overall expansion of the viral DNA pool.

Isolated DNA-protein complexes release an 83-kDa polypeptide after digestion with nuclease. [^{35}S]methionine-labeled DNA-protein complexes were partially purified and concentrated by immunoprecipitation with anti-NS-1 serum. The antigen-antibody complexes were then released from the precipitating *S. aureus* with SDS buffer, and the resulting mixture was chromatographed on Sepharose CL4B in the presence of 0.3% SDS. Under these conditions all free protein (including NS-1) was included in the column, whereas the much larger DNA and DNA-protein complexes were excluded and appeared in the void volume. Figure 8 is an autoradiograph of an SDS-acrylamide gel showing selected fractions from such a column separation. In this particular experiment cells were labeled with [^{35}S]methionine 6 to 7 h after the beginning of the S phase and harvested 4 h later. Without nuclease treatment, all labeled material in the void volume remained at the top of the stacking and running gels (lane 1), whereas after DNase digestion (lane 2) a major band with an apparent molecular mass of 83 kDa

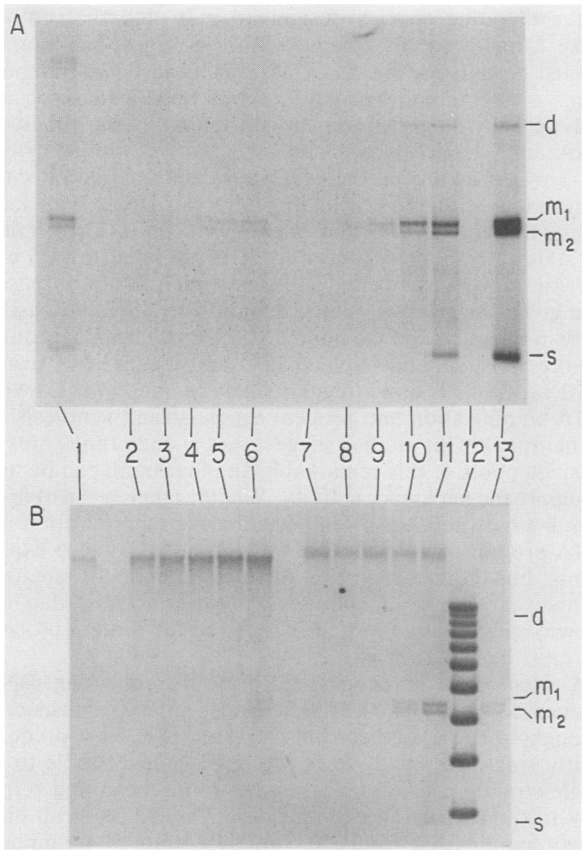

FIG. 7. Autoradiograph of a 1% agarose–SDS gel of DNA extracted from MVM-infected A9 cells. Lanes 1 and 13 show the total extract and an anti-NS-1 immunoprecipitate, respectively, from infected cells labeled with $^{32}P_i$ 10 to 12 h after release from aphidicolin. Lanes 2 through 6 show equal cell equivalents of total extracts from cells labeled with [^{35}S]methionine for 1 h (lane 2) or from pulse-chase samples labeled with [^{35}S]methionine for 15 min and then transferred into unlabeled medium for 45 min (lane 3), 90 min (lane 4), 3 h (lane 5), or 6.5 h (lane 6), each regime starting 6.5 h after release from aphidicolin. Immunoprecipitates of five cell equivalents of each of these [^{35}S]methionine-labeled extracts with anti-NS-1 serum (serum B) are shown in lanes 7 through 11 in the same sequence. Letter designations are as in Fig. 5. Lane 12 contains molecular weight standards (a 1-kilobase ladder series obtained from Bethesda Research Laboratories, Gaithersburg, Md.). (B) Negative image of the gel in A, stained with ethidium bromide before autoradiography.

appeared. This band exactly comigrated with labeled NS-1 from the included fractions of this column (lanes 4 through 10). An additional trace band at 65 kDa also became apparent in the nuclease-treated void fraction upon prolonged exposure (data not shown). This band exactly comigrated with a minor species seen in the included fractions which is known to be a carboxy terminally truncated form of NS-1. The material applied to the gel in lanes 1 and 2 represents 5% of the total recovered in the column void fractions, whereas the material in lanes 3 through 11 represents 5% of each alternate column fraction through the NS-1-specific region of the column run. Densitometry of the 83-kDa species in these lanes suggests that at the time of harvest (11 h after the start of the S phase and 4 h after the removal of a 1-h [^{35}S] methionine pulse in this experiment) approximately 1% of the NS-1 synthesized between 6 and 7 h after the start of the S phase had become covalently associated with viral DNA.

DISCUSSION

Genetic origin of MVM terminal protein. In this study we show that full-length copies of the MVM-encoded NS-1 polypeptide (83 kDa) are covalently attached to the 5′ termini of extended-form conformers of both the viral and complementary strands of all newly replicated duplex RF DNA and to the 5′ termini of all newly synthesized progeny single strands. We show that a number of different and highly specific anti-NS-1 sera, raised against a number of different bacterially or chemically synthesized peptides and directed against different protein domains spanning the entire length of the NS-1 molecule, almost quantitatively precipitate newly synthesized forms of viral DNA, and that precipitation of viral DNA by one of these antibodies, directed against a 16-amino-acid peptide from the extreme carboxy terminus of NS-1, can be quantitatively inhibited by the synthetic peptide originally used to invoke the antibody. This control confirms that viral DNA is being coprecipitated by antibodies directed specifically against the NS-1 sequences rather than against any minor contaminant which might, somehow, have been recognized by all of the immunized rabbits. After nuclease treatment, purified, metabolically labeled protein-DNA complexes released a single, major polypeptide species of 83 kDa which exactly comigrated on SDS-acrylamide gels with authentic NS-1.

These are somewhat surprising observations since previous studies had described an MVM terminal protein of 60 to 65 kDa (2, 8) which was antigenically distinct from any of the known virally coded polypeptides (8). Why our results should be so dramatically different remains unclear, but perhaps one reason for the discrepancy lies in the way the DNA complexes were treated in the two studies. Since our procedure effectively removes these complexes from the bulk of cellular proteins and DNA in a single-step immunoprecipitation, we are able to lyse our cells directly into high concentrations of SDS and EDTA, thus minimizing the probability of enzyme-mediated degradation. As a result,

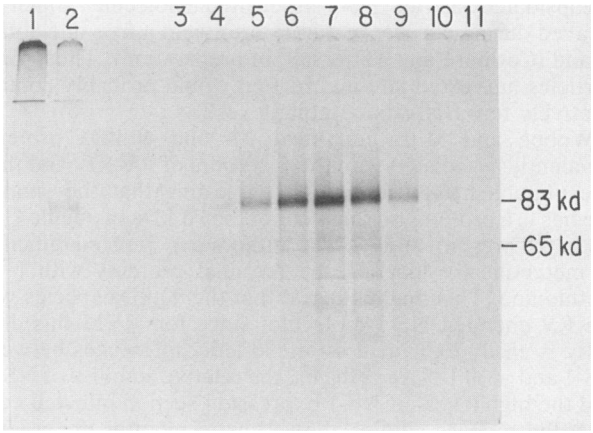

FIG. 8. Autoradiograph of a 10% SDS–polyacrylamide gel showing Sepharose CL4B column fractions of pooled anti-NS-1 immunoprecipitates prepared from extracts of MVM-infected cells. The cells were labeled with [^{35}S]methionine for 1 h starting 6.5 h after the start of the S phase and then chased in medium containing cold methionine for 4 h before harvest. Material in the column void volume (4 ml) was pooled, and 5% samples were electrophoresed before (lane 1) or after (lane 2) digestion with pancreatic DNase. Samples (5%) from alternate fractions through the NS-1 peak included in the column are shown in lanes 3 through 11.

our analyses probably describe the situation in the infected cell rather closely. The 60- to 65-kDa form of terminal protein described previously could simply be a specific proteolytic cleavage product of NS-1, especially since a carboxy-terminally truncated form of NS-1 in this size range is known to be present in infected cells (11), but it remains difficult to explain the immunochemical discrepancies. However, in a study of the proteins associated with RF DNA from the related parvovirus RV (32), Wobbe and Mitra described a number of other proteins which are tightly bound to RV RF DNA and copurify with it through a number of substantially denaturing purification steps. One particular group of these proteins, with molecular weights of around 66,000, 64,000, and 55,000, were thought to be of cellular origin, since similar proteins from uninfected cell extracts became associated with protease-digested RF DNA if mixed with it before purification (32). These molecules, which Wobbe and Mitra suggest may be related to the proteins of the nuclear lamina, are obvious candidates for the polypeptide previously designated as the MVM terminal protein, especially since immunoblots with the anti-terminal protein antiserum clearly identify multiple proteins migrating in the 60-kDa region of the gel (8).

Quantitation and kinetics of complex formation. In our hands, all newly replicated RF and single-stranded DNA molecules were found to be covalently complexed with NS-1, whereas previous reports suggest that only RF DNA is protein associated (24), or that only a somewhat variable proportion of the single-stranded DNA is protein associated (2, 8). This apparent discrepancy probably reflects the fact that in the studies presented here we analyze pulse-labeled, newly synthesized DNA in synchronized cells at specific points after the beginning of the S phase, whereas previous studies dealt with DNA harvested in bulk from asynchronous cultures many hours or days after infection was initiated. Additional studies have shown that some of the protein-associated DNA forms identified here progress late in infection to free DNA forms (Cotmore and Tattersall, in preparation). Significantly, this is most apparent for single-strand progeny DNA, which is synthesized and becomes encapsidated as an NS-1-associated molecule which is cleaved during particle maturation to yield a free nucleotide strand (Cotmore and Tattersall, in preparation). Thus, virus particles harvested late in infection would probably contain relatively few DNA–NS-1 complexes.

Wobbe and Mitra identified 90- and 40-kDa proteins covalently associated with the 5′ termini of RV RF, and they cite unpublished observations suggesting that the smaller species is a proteolytic fragment of the 90-kDa molecule (32). As in the present study, these authors experienced difficulty in metabolically labeling the terminal proteins with [^{35}S] methionine. This might suggest that the 90-kDa species was the RV-encoded NS-1 molecule, since for MVM this difficulty is easily explained by the kinetic differences between NS-1 and viral DNA synthesis, the relative stability of NS-1, and the high levels of NS-1 expression seen in infected cells (11). Pulse-chase studies allowed us to monitor the gradual transfer of protein from preexisting nuclear pools of NS-1 on to the newly synthesized viral DNA and showed that NS-1 molecules labeled at the time of peak NS-1 synthesis (around 6 to 7 h after the start of the S phase) could remain in the nucleus for several hours before becoming associated with viral DNA. Earlier analyses with $^{32}P_i$-labeled complexes (Fig. 1) had established that newly synthesized monomer RF DNA could be resolved into two distinct forms on SDS-agarose gels (a major species designated m1 and a faster-migrating species designated m2), and that both of these forms were associated with NS-1. [^{35}S]methionine-labeled protein became associated with these two monomer forms almost simultaneously but was only seen associated with progeny single strands at substantially later times. As DNA amplification proceeded, progressively more labeled NS-1 was drawn from the presynthesized pool and became complexed with the expanding pool of DNA. However, during the time span of our experiment (up to 13.25 h after the start of the S phase) only 1 to 2% of the labeled NS-1 ever became protein associated, a figure which is not unreasonable given the relatively large amounts of NS-1 synthesized early in infection and the limited number of terminal proteins required to supply one molecule per unit-length DNA strand. NS-1 synthesis is down-regulated late in infection (11) when DNA amplification and progeny single-strand synthesis are at their peak. Our studies suggest that at such times presynthesized pools of this remarkably stable protein can be used to supply the necessary activity. Whether there is an obligate time lag between NS-1 synthesis and its incorporation into DNA-protein complexes is not apparent from these experiments, but the incorporation of trace amounts of label into complexes within an hour of protein synthesis (data not shown) suggests that any time required for protein processing must be rather brief.

A previously unrecognized form of replicating DNA, designated band x in the illustrations, appears consistently throughout these studies. This material is at least predominantly single stranded, since the band is susceptible to the single-strand-specific nuclease from mung bean and refractory to the restriction endonuclease *Eco*RI. As in all other newly synthesized forms of viral DNA, its 5′ terminus is associated with NS-1, but its exact significance in the replication scheme remains to be examined. Its appearance in these studies may well simply reflect the power of SDS-agarose gels in resolving slightly different replicative intermediates.

Potential roles for NS-1 in MVM DNA replication. The role of covalently bound NS-1 molecules in MVM DNA replication has yet to be explored experimentally, but certain predictions can be made based on our knowledge of intracellular RFs of MVM DNA and the roles of such virally coded proteins in other eucaryotic and procaryotic virus-host systems. In other systems such proteins are generally implicated either in priming complementary strand synthesis, as in the replication of adenovirus and bacteriophage ϕ29 (7, 19, 22, 23, 26, 28), or as the mediators of site-specific nicking, as in bacteriophage ϕX174 (12, 13) and hepatitis B virus (20). MVM is unlikely to require protein-mediated priming for complementary strand synthesis since the genome is provided with palindromic termini which spontaneously fold into hairpin duplexes in vitro (6), thus providing the base-paired 3′-hydroxyl necessary for initiation of DNA synthesis. However, all models of MVM DNA replication require at least one site-specific nickase, and it now seems highly likely that NS-1 provides such a function.

Although the terminal palindromes account for less than 6% of the viral genome, they contain all the *cis*-acting information necessary for viral DNA replication and encapsidation (14); thus models of parvoviral DNA replication have focused on explaining their regeneration (1, 2, 5, 27, 30). A rolling-hairpin system of self-priming at the genomic termini (30) coupled with a hairpin transfer mechanism which copies the palindromic sequence at the 5′ end of one strand on to the 3′ end of its complementary strand, forms the basis of most models, but in recent years a more exact

knowledge the DNA sequences present in the viral termini has allowed novel predictions to be made concerning the nature of the enzymes used to regenerate these structures (1, 2). Briefly, the termini of all autonomous parvoviruses analyzed to date are imperfect palindromes, containing a few asymmetrical nucleotides which are mispaired in the hairpin form. In MVM the right- and left-hand palindromes are different sizes (200 and 116 nucleotides, respectively) and have quite different primary sequences (3). The presence of the mismatched residues allows the identification of two sequence orientations, termed "flip" and "flop," in the right-hand termini of both viral and RF molecules of MVM, whereas only a single sequence is present at the left end of either molecule (1, 2). This, therefore, implies that the mechanism used to cut and replicate the 5′ (right-hand) end of the DNA must be different from, and less conservative than, that used to copy the left-hand (3′) end. Since the right-hand end of RF DNA is also at least 18 nucleotides longer than that of viral DNA (1, 2), the nicking site and possibly also the enzymes involved in generating the 5′ end of viral and RF DNA could be different. Similar length differences are not seen at the 3′ (left-hand) end of viral and RF DNA. If covalently attached copies of NS-1 molecules do provide fossil evidence for a site-specific nickase function, this implies that NS-1 can find specific binding and cutting sequences which are common to both rather disparate palindromes but which are only presented in a single orientation at 3′ terminus, thus forcing incorporation of a palindrome with a unique sequence onto the 3′ end of all viral sense strands. Astell et al. (1, 2) provide an elegant model to account for this phenomenon, in which the sequences required for binding (but not those required for cutting) at the 3′ end of the viral DNA are only available in one strand of the 3′-to-3′ (head-to-head) duplex dimer intermediate. The authors propose that at this site the terminal protein could function in a manner analogous to that described for the gene A protein of ϕX174 (12, 13), nicking the duplex at a single site and, in so doing, becoming covalently attached to the 5′ end of the molecule, while leaving a free 3′ hydroxyl for extension by a DNA polymerase. As the free 3′ end is extended, the strand carrying the terminal protein then becomes displaced along the DNA until it encounters a second copy of its cutting site in the opposite strand of the duplex. The protein cuts at this site and ligates the new 3′-hydroxyl to the 5′ end of the displaced strand, while transferring itself to the new 5′ end. Generation of new 5′ (right-hand) termini could proceed via a similar mechanism, except that since both the binding and cutting sequences are available in the turnaround form of the palindrome, simple hairpin transfer mechanisms generate two sequence orientations at the 5′ end. Knowing that NS-1 is the candidate molecule for this postulated topoisomerase will allow us to use genetic and biochemical means to explore the validity of this proposal. Other parvoviruses, such as the adeno-associated viruses, have palindromic sequences in both the flip and flop orientations at both genomic termini (4), and it would seem unnecessarily complex to invoke this type of enzymology for the generation of their termini. Nevertheless, the adeno-associated viruses encode nonstructural proteins which are essential for DNA replication (16, 31) and which share a region of close protein sequence homology with the NS-1 protein of MVM through a region which is thought to encode a purine triphosphate binding site (11, 25). Thus, it will be of great interest to see whether the nonstructural proteins function differently in the replication of these two viral groups.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-19973 and CA-16038 from the National Institutes of Health.

LITERATURE CITED

1. **Astell, C. R., M. B. Chow, and D. C. Ward.** 1985. Sequence analysis of the termini of virion and replicative forms of minute virus of mice DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. J. Virol. **54**:171–177.
2. **Astell, C. R., M. Thomson, M. B. Chow, and D. C. Ward.** 1983. Structure and replication of minute virus of mice DNA. Cold Spring Harbor Symp. Quant. Biol. **47**:751–762.
3. **Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of Minute Virus of Mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
4. **Berns, K. I. (ed.).** 1984. The parvoviruses. Plenum Publishing Corp., New York.
5. **Berns, K. I., and W. W. Hauswirth.** 1984. Adeno-associated virus DNA structure and replication, p. 1–13. *In* K. I. Berns (ed.), The parvoviruses. Plenum Publishing Corp., New York.
6. **Bourguignon, G. J., P. J. Tattersall, and D. C. Ward.** 1976. DNA of minute virus of mice: self-priming, non-permuted, single-stranded genome with a 5′ terminal hairpin duplex. J. Virol. **20**:290–306.
7. **Challberg, M. D., S. V. Desiderio, and T. J. Kelly.** 1980. Adenovirus DNA replication in vitro: characterization of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. USA **77**:5105–5109.
8. **Chow, M., J. W. Bodnar, M. Polvino-Bodnar, and D. C. Ward.** 1986. Identification and characterization of a protein covalently bound to DNA of minute virus of mice. J. Virol. **57**:1094–1104.
9. **Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two nonstructural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
10. **Cotmore, S. F., and P. Tattersall.** 1986. Organization of the nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
11. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**:91–174.
12. **Eisenberg, S., J. Griffith, and A. Kornberg.** 1977. ϕX174 cistron A protein is a multifunctional enzyme in DNA replication. Proc. Natl. Acad. Sci. USA **74**:3198–3202.
13. **Eisenberg, S., and A. Kornberg.** 1979. Purification and characterization of ϕX174 gene A protein. A multifunctional enzyme of duplex DNA replication. J. Biol. Chem. **254**:5328–5332.
14. **Faust, E. A., and D. C. Ward.** 1979. Incomplete genomes of the parvovirus minute virus of mice: selective conservation of genome termini. J. Virol. **32**:276–292.
15. **Hayward, G. S., H. Bujard, and M. Gunther.** 1978. Three distinct replicative forms of Kilham-rat-virus DNA, p. 327–340. *In* D. C. Ward and P. Tattersall (ed.), Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. **51**:329–339.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Mellado, R. P., M. A. Penalva, M. R. Inciarte, and M. Salas.** 1980. The protein covalently linked to the 5′ terminus of the DNAs of *Bacillus subtilis* phage ϕ29 is involved in the initiation of DNA replication. Virology **104**:84–96.
20. **Molnar-Kimber, K. L., J. Summers, J. M. Taylor, and W. S. Mason.** 1983. Protein covalently bound to minus-strand DNA intermediates of duck hepatitis B virus. J. Virol. **45**:165–172.
21. **Muller, D.-E., and G. Siegl.** 1983. Maturation of parvovirus

LuIII in a subcellular system. I. Optimal conditions for in vitro synthesis and encapsidation of viral DNA. J. Gen. Virol. **64:** 1043–1054.

22. **Prieto, I., J. M. Lazaro, J. A. Garcia, J. M. Hermoso, and M. Salas.** 1984. Purification of a functional form of the terminal protein of Bacillus subtilis phage ϕ29. Proc. Natl. Acad. Sci. USA **81:**1639–1643.
23. **Rekosh, D. M. K., W. C. Russell, A. J. D. Bellett, and A. J. Robinson.** 1977. Identification of a protein linked to the ends of adenovirus DNA. Cell **11:**283–295.
24. **Revie, D., B. Y. Tseng, R. H. Graftsom, and M. Goulian.** 1979. Covalent association of protein with replicative form DNA of parvovirus H1. Proc. Natl. Acad. Sci. USA **76:**5539–5543.
25. **Shade, R. O., M. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell.** 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from an aplastic crisis. J. Virol. **58:**921–936.
26. **Shih, M., K. Watabe, and J. Ito.** 1982. In vitro complex formation between bacteriophage ϕ29 terminal protein and deoxynucleotide. Biochem. Biophys. Res. Commun. **105:**1031–1036.
27. **Straus, S. E., E. Sebring, and J. Rose.** 1976. Concatamers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. Proc. Natl. Acad. Sci. USA **73:**742–746.
28. **Tamanoi, F., and B. W. Stillman.** 1982. Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. USA **79:**2221–2225.
29. **Tattersall, P., and J. Bratton.** 1983. Reciprocal productive and restrictive virus-cell interactions of immunosuppressive and prototype strains of minute virus of mice. J. Virol. **46:**944–955.
30. **Tattersall, P., and D. C. Ward.** 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature (London) **263:**106–109.
31. **Tratschin, J. D., I. L. Miller, and B. J. Carter.** 1984. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. J. Virol. **51:**611–619.
32. **Wobbe, C. R., and S. Mitra.** 1985. Proteins tightly associated with the termini of replicative form DNA of Kilham rat virus, an autonomous parvovirus. Proc. Natl. Acad. Sci. USA **82:**8335–8339.