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A Genome-Linked Copy of the NS-1 Polypeptide Is Located on the Outside of Infectious Parvovirus Particles

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The 5′ ends of all newly synthesized single-stranded (s1) DNA genomes of the autonomous parvovirus minute virus of mice are covalently linked to the major virally coded nonstructural protein NS-1, but later in infection this association is disrupted, giving rise to an abbreviated form of single-stranded DNA designated s2. Both s1 and s2 forms are encapsidated and migrate in velocity gradients as 110S particles, and, as such, both appear to be infectious. Most virions are released from A9 cells as s1 particles, but the NS-1 molecules are located on the outside of the virion where they are accessible to both antibodies and enzymes. These polypeptides are cleaved from the encapsidated DNA by nucleolytic or proteolytic digestion, which can occur either in the culture medium or upon subsequent entry into further host cells. Since the s1 to s2 cleavage can be minimized by blocking viral reentry, it is likely that most of the processing occurs after entry into the host cell. Incoming virus is rapidly converted to the s2 form when it is used to infect new host cells, but in vitro removal of the NS-1 molecules with proteases or nucleases fails to influence the infectivity of s1 particles under normal culture conditions. Limited proteolysis of s1 particles with trypsin demonstrates that NS-1 is linked to the DNA via its amino-terminal domain. Analysis of the 5′ ends of s1 and s2 forms indicates that there are approximately 24 externally located nucleotides linking the NS-1 molecules to the 5.1-kilobase nuclease-resistant DNA core of the virion.

Parvoviruses are isometric, nonenveloped particles, 20 to 25 nm in diameter, comprising approximately 60 capsid polypeptides and a single-stranded DNA genome of about 5,000 nucleotides (16). Polyamines, which are thought to assist in stabilizing the DNA by charge neutralization, have been reported in virions of the densoviruses, the subgroup of insect parvoviruses (11), but have yet to be identified in either the autonomous or dependovirus groups of vertebrate viruses, and previous studies have failed to detect particle-associated lipids, carbohydrates, cellular or virally coded enzymes, or low-molecular-weight histone-type proteins (15). However, Muller and Siegl (12, 13) have shown that newly synthesized and encapsidated DNA of the autonomous virus LuIII, prepared in an in vitro replication system, is unable to penetrate a neutral agarose gel within 10 min of its synthesis but that at later times this aberrant property is lost. This suggests that newly packaged progeny DNA may be complexed with a protein of unknown function and origin but that, at least in vitro, this association is rapidly disrupted upon maturation of the particle.

Similarly, we have previously shown that in vivo, all newly synthesized single-stranded DNA molecules of the autonomous parvovirus minute virus of mice (MVM) are covalently associated with the major virally coded nonstructural protein NS-1 (9) and that later in infection this association is disrupted, giving rise to a second form of single-stranded DNA which has a slightly faster mobility than its protein-complexed precursor when electrophoresed in sodium dodecyl sulfate (SDS)-agarose gels (5). We have designated these forms s1 (protein associated) and s2 (protein free), respectively. In this report we demonstrate that s1 forms are encapsidated and released from the infected cell as infectious particles, and then we explore the nature, origin, and significance of the s1-s2 cleavage.

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MATERIALS AND METHODS

Cells and viruses. The prototype strain of MVM was grown in the mouse L-cell derivative A9 ouab^{r}11 as previously described (19). Cells were synchronized by using a double-block schedule described elsewhere (8). Briefly, cells were allowed to accumulate in G_0 by starving them of isoleucine for 48 h and were then released from the isoleucine block while simultaneously being exposed to aphidicolin, an inhibitor of both alpha and delta DNA polymerases. Under this regimen, cells leave G_0 somewhat synchronously in the 20 h after application of the polymerase inhibitor 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 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 $^{32}PO_4$ at 0.5 mCi/ml in Dulbecco modified Eagle minimal medium containing 1/10 of the normal concentration of unlabeled phosphate and 5% dialyzed fetal calf serum, generally starting 6 h after release from the aphidicolin block and terminating with virus collection at 25 h postrelease, unless other times are specified in the text. Viral reentry was blocked by adding *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, Mo.) at 0.1 mg/ml or rabbit anti-MVM capsid serum at 1% to the culture medium 6 h postrelease at the time of isotope addition. Culture medium was gently removed from the cells and was centrifuged at 12,000 × *g* for 5 min to remove cell debris. Cells were then scraped from the plates, washed in cold in phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma), and collected by centrifugation. For gradient fractionation and some analyses, cell pellets were extracted with TE 8.7 (0.05 M Tris–0.005 M EDTA, pH 8.7), by freezing

and thawing 10^6 cells three times in 0.25 ml of buffer and removing cell debris by centrifugation at 12,000 × *g* for 20 min. For analysis of total cell-associated virus, cells were extracted in a similar volume of TE 8.7 containing 2% SDS and 0.02 M EDTA by heating at 60°C for 20 min and then at 70°C for 10 min. Reinfection experiments were carried out with virus from the medium of cultures labeled and harvested as described above. Culture supernatants were concentrated 10×, and much of the free isotope was removed by centrifugation in a Centricon 30 microconcentrator (Amicon Corp., Danvers, Mass.), with several changes of TE 8.7. Residual isotope was removed by repeated centrifugation through Sephadex G-50 columns (Pharmacia Diagnostics, Piscataway, N.J.). Cells were collected in G_0 by isoleucine starvation and then were exposed to aphidicolin in the presence of ^{32}P-labeled virus at approximately 20 PFU per cell. Neuraminidase at 0.1 mg/ml was also added to one of the cultures at this point. Cells and medium were collected after 3 h of incubation at 37°C.

Glycerol gradients. Cell extracts (0.25 ml) in TE 8.7 were layered onto 12-ml 10 to 30% glycerol gradients made up in the same buffer (and with a 0.1-ml 60% metrizamide cushion at the bottom of the tube) and were centrifuged in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 35,000 rpm for 150 min at 5°C. After centrifugation, 36 fractions of approximately 0.3 ml each were collected by pumping from the bottom of the tube.

Antisera. Antisera used in these experiments have been described in detail elsewhere (7, 9). Briefly, antiserum A is a rabbit serum raised against a gel-purified bacterial fusion protein which contains 22 amino acids of the *cro* protein specified by phage lambda and the 84-amino-acid amino-terminal peptide shared by NS-1 and NS-2. Antiserum B is directed against a similar *cro* fusion in which the MVM sequence is derived from the middle of the NS-1 molecule (nucleotides 1110 to 1638), and antiserum C is a rabbit serum directed against a synthetic peptide of 16 amino acids derived from the extreme carboxy terminus of NS-1 and conjugated to hemocyanin (9). Rabbit anti-NS-2 antibodies were raised against a fusion protein (7) in which the MVM sequence was derived from the C-terminal region of NS-2 (nucleotides 2075 to 2291). Rabbit anti-MVM capsid serum was raised against gradient-purified MVM empty capsids.

Enzyme digests. Medium-derived virus was digested with tosyl-amide phenylethyl chloromethyl ketone-treated trypsin (Sigma) in 0.025 M Tris hydrochloride, pH 8.0, at 0.0025 mg/ml (limited proteolysis) or 0.025 mg/ml (extensive proteolysis) for 30 min at 37°C, and the reaction was stopped by addition of 0.025 M EGTA (ethylene glycol-bis(β-aminoethyl ether-*N*,*N*,*N*′,*N*′-tetraacetic acid). Similar reaction and stop conditions were used for micrococcal nuclease (Pharmacia, Inc.), except that the reaction mixtures contained 0.005 M $CaCl_2$ and the enzyme was present at 0.1 mg/ml. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used at 0.05 mg/ml in the presence of 0.5% SDS, and no attempt was made to stop the reaction.

Immunoprecipitations. Immunoprecipitation was carried out on native or disrupted material by using Formalin-fixed *Staphylococcus aureus* (Boehringer) as previously described (6, 9). Briefly, all samples were precipitated and washed in the presence of 0.01 M Tris hydrochloride (pH 8.0)–0.15 M NaCl–0.01 M EDTA–1% Nonidet P-40 (Sigma), but disrupted samples were pretreated with 2% SDS at 60°C for 20 min and then at 70°C for 10 min in the above buffer prior to use in the precipitation reactions. For immunoprecipitations used in the infectivity study, buffers were similar, except that all detergent was omitted from the extraction and precipitation steps in the protocol, although it was still used in the washing steps.

Primer extension analysis of the 5′ ends. Normal and micrococcal nuclease-digested virus from culture medium, 110S particles from glycerol gradients, and material from the 20S region of these gradients (a mixture of replicative-form (RF) DNA and low-velocity complexes) were digested with proteinase K (0.1 mg/ml in the presence of 0.5% SDS and 0.02 M EDTA) for 1 h at 37°C, extracted two times with phenol-chloroform, and ethanol precipitated. Samples were then digested with the restriction endonuclease *Rsa*I (New England BioLabs, Inc., Beverly, Mass.), which cuts between residues 5010 and 5011 and 5082 and 5083 in the 5′ hairpin of viral DNA, releasing a short 5′ fragment (extending from nucleotide 5083 to the end of the DNA at about nucleotide 5149), a long 3′ predominantly single-stranded fragment (5,010 nucleotides), and a 72-nucleotide internal palindromic fragment. After phenol extraction and ethanol precipitation, these samples were denatured with alkali and annealed to a ^{32}P-labeled 18-nucleotide primer complementary to the sequence between nucleotides 5088 and 5105 in the viral strand. This oligonucleotide primed synthesis of fragments complementary to the 5′ end of the viral DNA, but since it could also anneal to nucleotides 5005 to 4985 in the complementary strand, with RF molecules it could also prime synthesis of much longer fragments stretching back to an *Rsa*I site nearer the 3′ (left-hand) end of the genome. Primer extensions were carried out by using avian myeloblastosis virus reverse transcriptase (Promega Biotec, Madison, Wis.) or a modified form of bacteriophage T7 polymerase marketed under the name of Sequenase (United States Biochemical Corp.) in appropriate buffers and at appropriate temperatures as specified by their manufacturers. All extensions were carried out in a mixture of all four deoxynucleotide triphosphates at 0.08 M each. The MVM sequence through the region of the 5′ cleavage sites was obtained by sequencing a plasmid clone of viral DNA which terminated rightwards at the *Hha*I site at nucleotide 5069. This clone contains only a single copy of the primer binding site, between nucleotides 5005 and 4985, from which the primer directs synthesis of sequences leftward from the right-hand palindrome. It was sequenced with Sequenase according to the instructions supplied by the manufacturer.

RESULTS

Two forms of progeny single-stranded DNA are encapsidated. In order to establish whether or not both s1 and s2 forms of MVM progeny single-stranded DNA were encapsidated, we prepared TE 8.7 extracts from synchronized MVM-infected A9 cells labeled with $^{32}P_i$ for 2 h between 8 and 10 h after release into S phase and harvested after the end of a single round of viral infection (25 h after the start of S phase). These extracts were subjected to velocity sedimentation through glycerol gradients. SDS-agarose gel analysis of individual fractions from such a gradient is shown in Fig. 1. Both s1 and s2 forms of single-stranded DNA cosedimented in these gradients with the major 110S peak of hemagglutinating activity and infectivity, thus confirming that both of these forms were particle associated. However, these two forms did sediment at slightly different rates, with s1 forms predominating at the trailing edge of the peak and s2 forms predominating at the front.

Origin of the s2 form. Although the cells used in these experiments were rigorously synchronized and a consider-

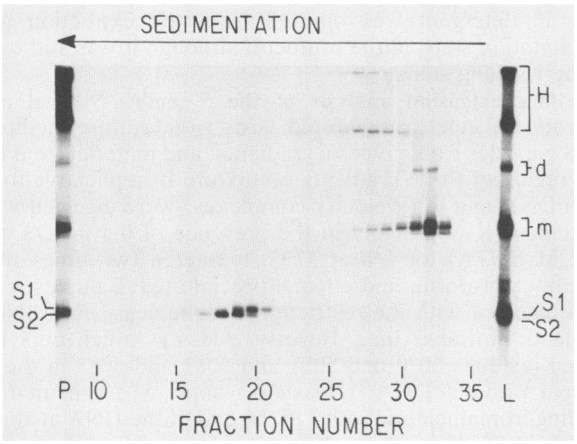

FIG. 1. Autoradiograph of a 1% agarose-SDS gel showing fractions through a glycerol gradient velocity centrifugation analysis of an extract from synchronized MVM-infected A9 cells. The cells had been labeled with $^{32}PO_4$ from 8 to 10 h after release into S phase and were harvested 15 h later (25 h after the start of DNA replication). A sample of material loaded on the gradient is shown in lane L and comprises host DNA (H), various species of dimer (d) and monomer (m) duplex replicative-form viral DNA, and progeny single-stranded viral DNA (s1 and s2). Aggregated material pelleting at the bottom of the centrifuge tube is shown in lane P.

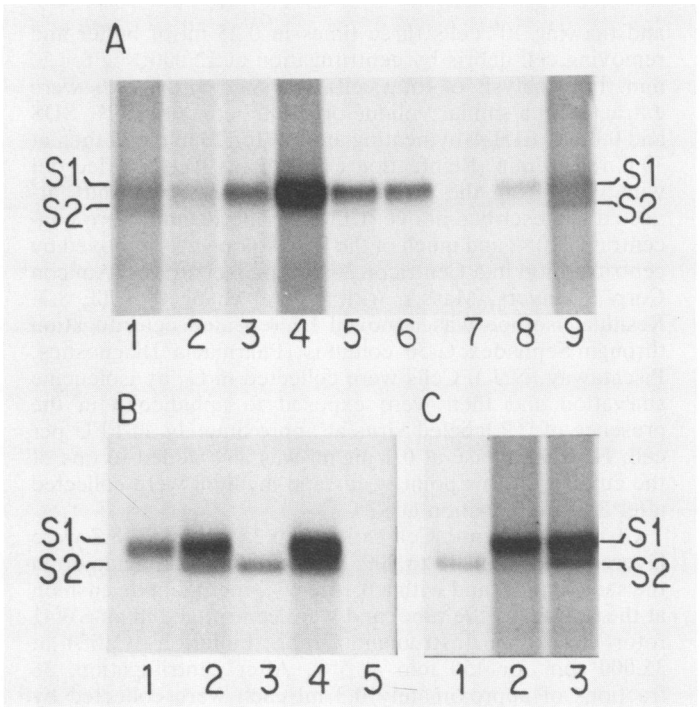

FIG. 2. Autoradiographs of 1% agarose-SDS gels showing $^{32}PO_4$-labeled single-stranded progeny viral DNA, labeled continuously from 6 h and harvested 29 h after the start of DNA replication. (A) Total DNA samples extracted from infected cell pellets by heating in buffered SDS (lanes 1 through 4 and 9) and virus obtained from the surrounding medium (lanes 5 through 8). Cell pellets obtained from normal infections contained both s1 and s2 forms of single-stranded DNA (lanes 1 and 9), but cells cultured in the presence of neuraminidase (lane 2) or anti-MVM capsid antiserum (lanes 3 and 4) contained only the uncleaved s1 form. Culture medium surrounding the control, MVM-infected cells (lane 5), the neuraminidase-treated cells (lane 6), or the antibody-treated cells (lanes 7 and 8) contained virions in which the DNA was predominantly in the s1 form, although traces of s2 were also present. Cell and medium samples shown in lanes 3, 5, 6, and 7 were each derived from approximately 2.8×10^3 cells, whereas samples in lanes 1, 2, 4, 8, and 9 were derived from five times more cells (approximately 1.4×10^4 cells). (B) State of viral DNA in $^{32}PO_4$-labeled virions used to reinfect synchronized A9 cells (lane 1) and in virions recovered from the culture medium (lanes 2 and 4) and cell pellets (lanes 3 and 5) of either untreated (lanes 2 and 3) or neuraminidase-treated (lanes 4 and 5) cells 3 h after addition of the virus shown in lane 1. Medium-derived virus samples electrophoresed on this gel correspond to 0.625% of the total culture supernatant, while cell pellet samples represent 12.5% of the cells, indicating that approximately 1% of the applied virus was cell associated at this time. (C) DNA of virions extracted into the supernatant by resuspending cells, infected and labeled as in panel A, at 1.4×10^6/ml in 50 mm Tris hydrochloride–1 mm EDTA, pH 8.7, and freezing and thawing the sample three times (lane 1); material remaining in an equivalent sample of the pellet after this treatment is shown in lane 2 (after solubilization in SDS), and total cell-associated DNA, obtained by heating an equivalent, unextracted MVM-infected cell pellet in buffered SDS, is seen in lane 3.

able effort was made to keep cell culture conditions as constant as possible from one experiment to the next, the relative proportions of s1 and s2 forms present in cell extracts obtained at identical times late in infection varied significantly between experiments. Since the s2 form only appeared late in infection, it was possible that this form might be generated during viral reentry, rather than in the original infected cell. To explore this possibility, we carried out the experiment illustrated in Fig. 2. Infected cells were labeled with $^{32}P_i$ in a continuous pulse between 6 and 29 h after the onset of DNA replication in the presence or absence of reagents known to prevent viral reentry, and both cell pellets and culture medium were harvested at the end of the experiment. Figure 2A, lane 1 shows the two forms of single-stranded DNA present in a total SDS extract of cells cultured in the absence of inhibitor. In contrast, cells cultured in the presence of the receptor-destroying enzyme neuraminidase (18; Figure 2A, lane 2) or neutralizing anti-MVM capsid antibody (Fig. 2A, lanes 3 and 4) contained only the NS-1-associated s1 form. This suggests that s2 is generated at some point after the virus is released from the parent cell. Analysis of the culture medium from these various infections (lanes 5 through 8) tends to support this conclusion, since the s1 form invariably predominates in these samples. However, a small proportion (5 to 10%) of the single-stranded DNA in such samples appeared as the s2 form (most easily seen in lanes 5 and 6), suggesting that the s1-s2 conversion might occur in the medium surrounding the infected cell. In similar experiments carried out with synchronized MVM-infected BALB/c 3T3 cells, a far greater proportion of the released virus contained s2-like forms of viral DNA, perhaps indicating that the extracellular environment surrounding different cell types may influence the extent of this conversion (data not shown).

Virus labeled with $^{32}P_i$, obtained from the culture medium of a normal A9 infection (Fig. 2A, lane 5; Fig. 2B, lane 1), was used to reinfect A9 cells according to the standard synchronization and infection procedure. For this, the virus was mixed with fresh culture medium containing aphidicolin, an inhibitor of both alpha and delta DNA polymerases, and was applied to cells which had been accumulated in G_0 following isoleucine deprivation. Virus recovered from the culture medium after incubation with the cells for 3 h contained substantially more of the s2 form (Fig. 2B, lane 2), perhaps indicating that the exact phase of cell growth can influence the extent of s1-s2 conversion in the surrounding

medium. Almost all of the virus recovered from the cell pellet at this time was in the s2 form (Fig. 2B, lane 3). At present we do not know whether all of the s1-s2 conversion occurs outside the host cells, with these preconverted forms being taken up preferentially by subsequent host cells, or whether a significant proportion of the conversion occurs upon viral reentry into a second host cell, presumably by cleavage in the acidified endosome-lysosome network during transport to the nucleus. Experiments are in progress to distinguish between these two possibilities. Since most of the cell-associated virus was cleaved in the untreated culture (Fig. 2B, lane 3), it was theoretically possible that the s2 form of virus found in the medium surrounding these cells (Fig. 2B, lane 2) represented particles which had transiently cycled through the host cells and had been cleaved in the cell cytoplasm. However, cells cultured in the presence of neuraminidase do not take up virus (Fig. 2B, lane 5), but the medium virus retrieved from this infection still showed signs of conversion (Fig. 2B, lane 4). We conclude from these experiments that the s2 form of virion DNA is not generated in the parent cell but that the cleavage occurs sometime after release from the cell, either in the extracellular compartment or following viral reentry into a new host cell. Clearly the whole process of viral release, maturation, and reentry is a much more extensive topic than can be addressed in the context of this report. However, it is perhaps pertinent to point out here that in studies in which MVM-infected A9 cells were labeled with [^{35}S]methionine between 6 and 25 h after release into S phase, the virus harvested from the culture medium, predominantly s1 forms, was made up of approximately 80% full particles and only about 20% empty particles. The capsids of these full virions contained only virion polypeptides VP-1 and VP-2, although VP-3 forms could be generated by cleavage with trypsin in vitro. In contrast, 95% of the particles released from the cell pellet by extraction in TE 8.7 were empty, and such full particles as were released contained all three virion polypeptides (data not shown).

Quantitation of the radioactivity associated with the viral DNA bands indicates that in synchronized infections of A9 cells cultured in either the absence or presence of neuraminidase and harvested 29 h after release into S phase, approximately 95% of the full viral particles were present in the culture medium, leaving approximately 5% associated with the cell pellet (Fig. 2A). In contrast, cells incubated in the presence of anticapsid serum synthesized approximately the same amount of single-stranded DNA, but 95% of this remained cell associated and only 5% was released into the medium. The basis for this major difference in progeny particle distribution remains to be explored.

Infectivity titers tend to support this quantitation, but there is an additional complication. As seen in Fig. 2C, when cells containing both s1 and s2 forms of viral DNA (lane 3) were extracted by the usual procedure for liberating infectious MVM virus (that is, freeze-thawing three times in TE 8.7 and removing the cell debris by centrifugation), the supernatant was massively enriched for the s2 forms of virus (Fig. 2C, lane 1), leaving the pellet fraction enriched for s1 forms (Fig. 2C, lane 2). Although the extent of this enrichment varies somewhat from experiment to experiment, perhaps reflecting the exact numbers of cells involved, this observation probably means that most cell-derived virus preparations are highly enriched for cleaved forms of the viral protein-DNA complex. It also explains why we were unable to release significant titers of infectious virus from the antibody- or neuraminidase-treated cell pellets. Obviously,

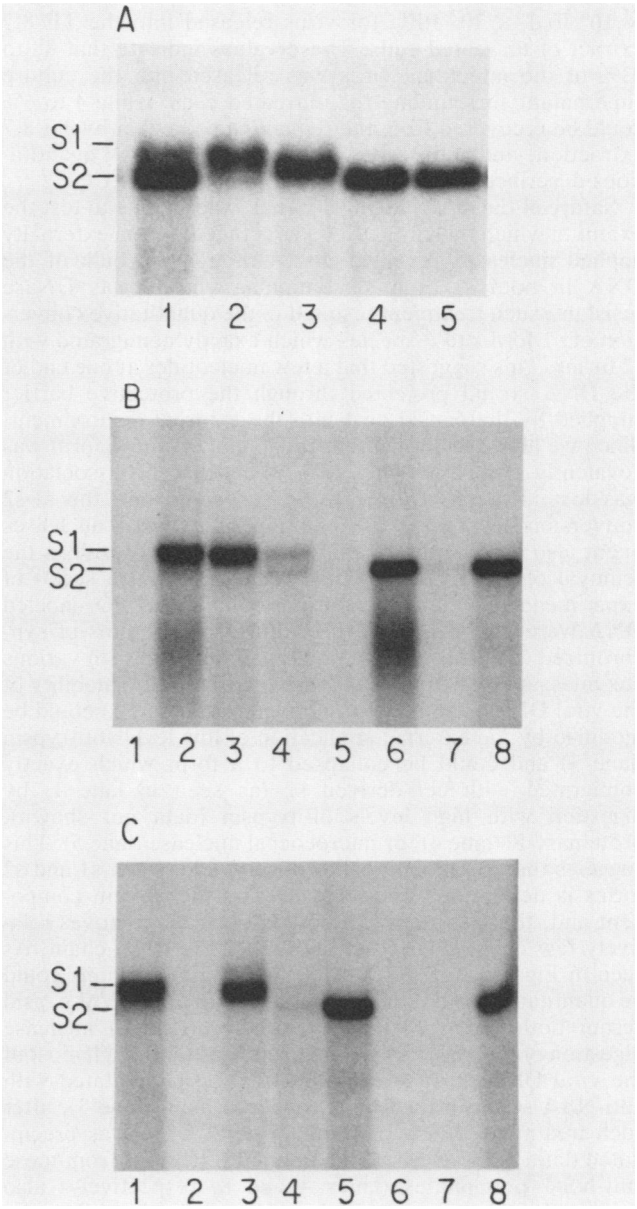

FIG. 3. Autoradiographs of 1% agarose-SDS gels showing $^{32}PO_4$-labeled progeny single-stranded DNA released from virions harvested from infected cell cultures 29 h after the start of DNA replication. (A) Viral DNA in virions released from infected cell pellets by TE 8.7 extraction (lane 1) and in virions recovered from the culture medium (lane 2). These medium-derived virions were then treated with low levels of trypsin (lane 3), proteinase K (lane 4), or micrococcal nuclease (lane 5). (B and C) Results of immunoprecipitating intact and SDS-disrupted virions, respectively. Unbound material from immunoprecipitations with anticapsid polypeptide antibody is shown in lanes 1 and 5, with their corresponding precipitates in lanes 2 and 6, respectively. Likewise, lanes 4 and 8 show material unbound by an antiserum directed against the extreme N-terminal region of NS-1, with their corresponding bound material run in lanes 3 and 7, respectively.

we were also unable to titer the virus released in the presence of neutralizing antibody. The infectivity titers obtained per 10^6 cells were 1.5×10^8 PFU for virus released into the medium of untreated cells, 1.4×10^8 PFU for virus released into medium of neuraminidase-treated cells, and 6

$\times 10^6$ to 1×10^7 PFU for virus released into the TE 8.7 extract of untreated cells. These values indicate that 88 to 93% of the infectious virus was released into the culture supernatant surrounding the untreated cells, while 4 to 6% could be recovered from the remaining cell pellet by TE 8.7 extraction, and thus generally support the DNA quantitations described above.

Nature of the s1-s2 cleavage. Initial experiments to test the extent to which the viral DNA was protected from externally applied nucleases revealed that, although the bulk of the DNA in both s1- and s2-containing virions was DNase resistant, such treatment resulted in the quantitative conversion of s1 forms to a species which exactly comigrated with s2 forms. This suggested that a few nucleotides at one end of the DNA strand projected through the protective barrier supplied by the capsid coat into the external environment. Since we also had shown that the 5′ end of the s1 form was covalently associated with NS-1 (9) but that this association was lost in the s2 form (5), we suspected that the s1-s2 conversion seen upon the addition of external nucleases might also involve the 5′ end of the DNA and result in the removal of NS-1. To test this, we carried out a series of experiments in which intact virions containing $^{32}PO_4$-labeled DNA were obtained from the culture supernatants of synchronized MVM-infected A9 cells and digested with various enzymes. As seen in Fig. 3A, the electrophoretic mobility of the viral DNA from medium-derived virus (lane 2) could be modified by the external application of low levels of trypsin (lane 3) and could be collapsed to a form which exactly comigrated with cell-derived s2 (as seen in lane 1) by digestion with high levels of trypsin (data not shown), proteinase K (lane 4), or micrococcal nuclease (lane 5). This suggests that the mobility shift observed between s1 and s2 forms is determined predominantly by the protein component and, thus, that the nucleolytic cleavage removes relatively few nucleotides from the end of the DNA chain. As seen in Fig. 3B, the DNA in medium-derived virions could be quantitatively immunoprecipitated with anti-MVM capsid serum both before (lane 2) and after (lane 6) nuclease digestion of the intact particles. In contrast, although most of the viral DNA in intact virions could be precipitated with anti-NS-1 serum prior to nuclease treatment (lane 3), after such treatment almost none of the viral DNA was precipitated (lane 7). Analysis of the unbound fractions from these anti-NS-1 precipitates (lanes 4 and 8, respectively) also reveals that a major component of untreated medium-derived virus which failed to precipitate was in the s2 form, while all of the unprecipitated material in the nuclease-digested preparation was s2-like. This analysis demonstrates that in the intact virion the DNA-associated NS-1 molecules are accessible to antibodies, that nuclease digestion of the intact particle removes NS-1 from the end of the viral strand, and that NS-1 is linked stably to the virion only through this stretch of nucleotides. Control experiments in which the medium virus was treated or not treated with micrococcal nuclease and the capsids then disrupted in SDS prior to immunoprecipitation are shown in Fig. 3C. Under these conditions, anticapsid serum totally failed to immunoprecipitate the viral DNA (lanes 2 and 6), while anti-NS-1 serum precipitated the viral DNA from untreated (lane 3) but not from nuclease-digested virions (lane 7).

The observation that low levels of externally applied trypsin modified the mobility of s1 DNA but did not reduce it as markedly as proteinase K suggested that after such treatment domains of the protein adjacent to the DNA-protein linkage site would be left associated with the viral DNA, while more distant domains might be lost. To explore this possibility, we carried out the studies shown in Fig. 4. Intact virions containing $^{32}PO_4$-labeled s1 DNA were obtained from the culture supernatant of infected cells and were left undigested or digested with low levels of trypsin before being disrupted with SDS and the DNA was immunoprecipitated with antisera directed against different domains of the 672-amino-acid NS-1 molecule. DNA from the untreated virus preparations was precipitated almost quantitatively with serum A, directed against the 84-amino-acid peptide at the extreme amino terminus of NS-1 (Fig. 4A, lane 3; cf. unbound material in lane 4), and with serum B, directed against residues 283 to 459 (lane 5; cf. unbound in lane 6), but relatively poorly with serum C, directed against the 16 amino acids at the extreme carboxy terminus of the protein (lane 7). Reprecipitation of the unbound material with this same serum (C) brought down little additional viral DNA (lane 8), suggesting that the problem was not simply one of antibody titer but that the unprecipitated DNA (seen in lane 9) simply did not carry the determinant. If this unbound material was then precipitated with serum A, most of the remaining viral DNA could still be immunoprecipi-

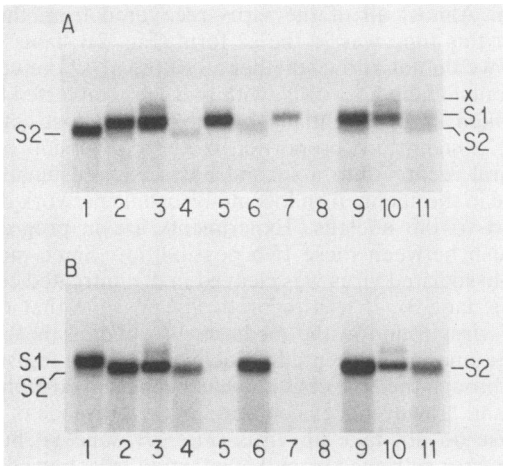

FIG. 4. Autoradiographs of 1% agarose-SDS gels showing $^{32}PO_4$-labeled DNA released from virions harvested from the culture medium of infected cells both before (A, lane 2; and B, lane 1) and after (A, lane 1; and B, lane 2) digestion of the intact particle with trypsin. Subsequent lanes in panels A and B are arranged in exactly the same order, but the samples immunoprecipitated in panel A contain untreated virus, while those in panel B contain trypsin-treated virus. In each case, lane 3 contains viral DNA immunoprecipitated with antiserum A, directed against the extreme amino-terminal region of NS-1, while lane 4 contains the unbound material from this precipitate; lane 5 contains DNA precipitated with antiserum B, directed against residues 283 through 459 in NS-1 (counting from the amino terminus of this 672-amino-acid protein), while lane 6 contains the unbound fraction; lane 7 contains material immunoprecipitated with antiserum C, directed against the extreme carboxy-terminal 16 amino acids of NS-1; lane 8 contains material precipitated, during a second round of immunoprecipitation, from the unbound fraction of the sample seen in lane 7 by using the same anti-carboxy-terminal antibody; lane 8 contains the unbound material from this double precipitation. Samples equivalent to those seen in lanes 8, that is, unbound after repeated precipitation with antiserum C, were then reprecipitated with serum A, directed against the amino-terminal region of NS-1, giving the precipitates seen in lanes 10 and the unbound fractions shown in lanes 11. Two forms of viral DNA are designated s1 (NS-1 associated) and s2 (protein free) as before, and an additional form generated in the presence of serum A, but of unknown structure, is designated X.

tated, indicating that the input DNA had not lost the covalently attached protein during the various incubation steps involved in these procedures. This suggests that much of the medium virus has already lost the extreme carboxy-terminal peptide of NS-1 and thus that the DNA-protein linkage cannot involve this domain. Unbound samples from these various precipitations (lanes 4, 6, and 9) also show a progressive increase in apparent molecular weight as domains nearer to the carboxy terminus are precipitated, suggesting that the linkage may involve a site near the amino terminus of the protein. This conclusion is strongly supported by the immunoprecipitations of trypsin-digested virions seen in Fig. 4B. After trypsin treatment, some of the fastest migrating DNA cannot be precipitated with any anti-NS-1 serum (Fig. 4B, lanes 4 and 11). However, serum A, directed against the amino terminus of NS-1, still precipitates most of the proteolysed DNA (Fig. 4B, lane 3; cf. unbound in lane 4), but serum B, directed against a domain in the middle of the protein (Fig. 4B, lane 5; cf. with unbound in lane 6), and serum C, directed against the carboxy terminus of NS-1 (lanes 7 and 8; cf. unbound in lane 9), precipitate very little DNA. Once again, much of the unbound DNA remaining after repeated precipitation with serum C can still be precipitated with serum A (lane 10; cf. unbound in lane 11). These data suggest that NS-1 is linked to the viral DNA at a site within the most amino-terminal domain of the protein.

Mapping the 5′ deletion in s2. We used primer extension analysis to compare the length of the 5′ end of virion DNA recovered either from infected cells or culture medium and to compare it to viral DNA from intact culture medium-derived virus which had been exposed to externally applied micrococcal nuclease. The selected 18-mer oligonucleotide primer annealed to the proteinase K-digested viral DNA between residues 5088 and 5105 in the published sequence (2) and primed synthesis toward the 5′ end of the viral strand. Since this site lies in the terminal palindrome, a second binding site for the primer is present on the complementary strand of duplex forms of viral DNA between nucleotides 5005 and 4985. In order to provide sequence information about the exact endpoints of the viral DNAs, we cloned MVM sequences between the *Eco*RI site at nucleotide 3522 and the *Hha*I site at nucleotide 5069 into the plasmid vector pUC18 (20) to yield the plasmid pSEQ 5′, as described in Materials and Methods. This fragment contains a single primer binding site on the complementary strand (5005 to 4985) and primes synthesis leftwards, towards the viral-coding region, through the sequence which is complementary to the endpoints of the viral strands. These sequences are shown alongside the primer extension products of the various viral species in Fig. 5A. Given that in single-stranded DNA this primer binds to a single site, it would yield an extension product of 62 nucleotides if the viral DNA extended to the reported 5′ end of viral DNA and one of 80 nucleotides if the product extended to the reported 5′ end of replicative form DNA. The observed cut sites were somewhat unexpected (Fig. 6). With a duplex template (as in RF DNA), additional primer extension products from the primer binding site on the plus strand (extending to an upstream *Rsa*I site at nucleotide 4663) would be much larger (up to 342 nucleotides) than those resolved in the gels shown in Fig. 5 and so do not complicate interpretation of the results. We expected the terminal (5′) nucleotide of the NS-1-linked species to carry whatever remained of the DNA-protein linkage after digestion with proteinase K and were uncertain as to whether or not such a peptide-linked nucleotide could be copied by any DNA polymerase. In the

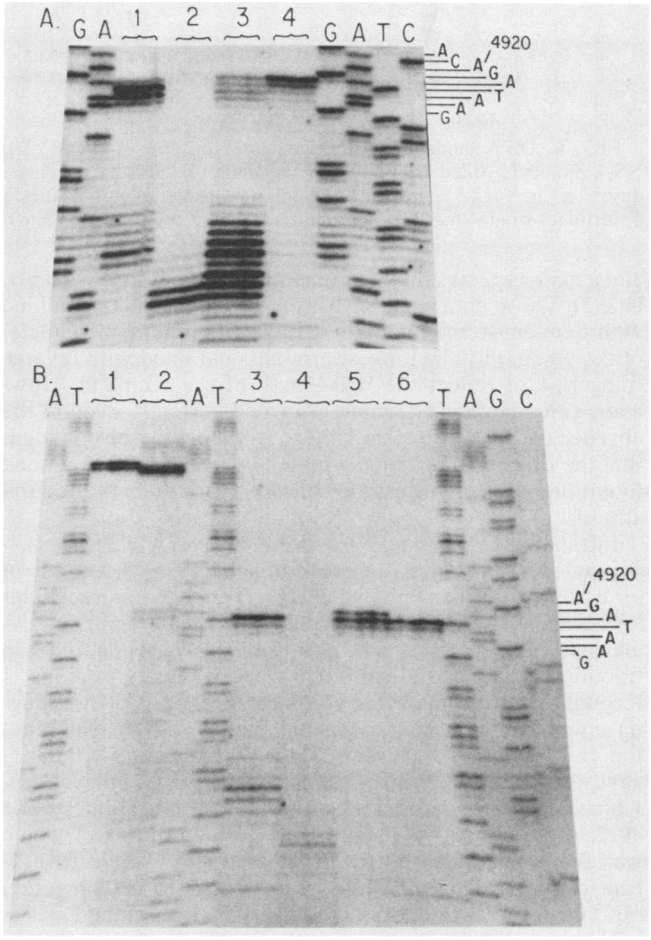

FIG. 5. Autoradiographs of denaturing 8 M urea–10% acrylamide gels comparing the dideoxy sequence of the inboard arm of the 5′ palindrome of MVM minus strand DNA with the extension products synthesized from the 5′ ends of various viral DNA species, with the same ^{32}P-labeled primer. (A) Extension products obtained by using Sequenase with the following substrates: substrate A, single-stranded DNA from medium-derived virions (lane 1); substrate B, DNA obtained from medium-derived virions which had been digested with micrococcal nuclease prior to detergent disruption (lane 2); substrate C, cell-derived virion DNA from the total 110S region of a glycerol gradient (lane 3); and substrate D, viral DNA from the top of such a gradient, predominantly RF DNA species (lane 4). Sequencing tracks are marked G, A, T, and C as appropriate. (B) Extension products obtained by using avian myeloblastosis virus reverse transcriptase with the following substrates: substrate A (lane 3); substrate B (lane 4); substrate D (lane 6); substrate E, pSEQ 5′ cut with the restriction endonuclease *Dra*I (lane 2). For comparison, the extension products obtained with Sequenase from substrates E and D are shown in lanes 1 and 5, respectively. Sequencing tracks are marked as for panel A.

event, the termination point of the two polymerases selected for study, Sequenase and reverse transcriptase, proved even more complex. As a control substrate for these reactions, we used the cloned DNA pSEQ 5′, described above, cut between nucleotides 4898 and 4899 with the restriction endonuclease *Dra*I. With this substrate, Sequenase gave two practically equimolar extension products, one of which was the expected length and one which was one nucleotide longer (Fig. 5B, lane 1), and reverse transcriptase gave a minor product which comigrated with the expected product

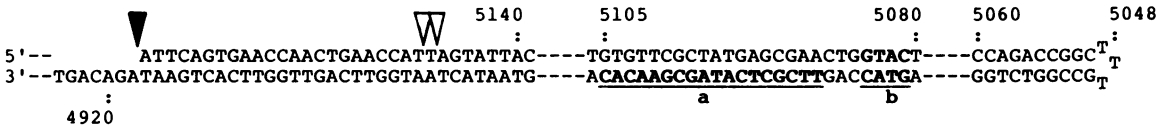

FIG. 6. DNA sequence of the right-hand terminus of the MVM(p) virion DNA, showing the point of attachment of the externally located NS-1 molecule (filled triangle) and the major 5′ ends of nuclease-treated virion DNA (open triangles), as determined in the experiments described in Fig. 5. The sequence of the 18-mer oligonucleotide used in those experiments to prime synthesis leftwards on the small 5′-terminal single-stranded fragment is denoted by a; the sequence released by cleavage of viral DNA at the *Rsa*I site is denoted by b.

and a major species that was one nucleotide shorter (Fig. 5B, lane 2). These data suggest that extension products obtained with Sequenase may contain a single supernumerary nucleotide, while only the longest products obtained with reverse transcriptase reflect the real length of the template. However, since neither enzyme performed perfectly even in the absence of a DNA-protein linkage, we can only be confident that the extension products obtained with viral DNA extend to within one or two residues of the real 5′ ends of the viral strands.

Intracellular DNA was fractionated over glycerol gradients (as seen in Fig. 1) in order to separate two sources of viral DNA for sequence analysis. Packaged intracellular virus was obtained from the pooled 110S peak region (equivalent to Fig. 1, fractions 17 through 21), while the top fractions from the gradient (equivalent to Fig. 1, fractions 31 through 35) were taken as a control sample. When Sequenase was used in primer extension studies with this material, the control samples (Fig. 5A, lane 4, and Fig. 5B, lane 5) gave two major extension products, one of 84 nucleotides, which terminated coincident with the A residue at nucleotide 4922 in the control sequence, and a second product which was one nucleotide shorter (83 nucleotides), while reverse transcriptase gave a single major product of 83 residues (Fig. 5B, lane 6). This suggests that during replication the DNA is probably cut between the first thymidine and the adenine residues in the sequence 5′ TATT 3′, leaving the NS-1 molecules attached to the DNA via the adenine residue at position 5170, as diagrammed in Fig. 6. Gel-purified RF DNA from asynchronous cultures gave similar major extension products (data not shown), although these preparations also contained a significant proportion of molecules which were 27 nucleotides shorter than the products described above but which had the same 5′ trinucleotide sequence (5′ ATT), suggesting an additional upstream in vivo cleavage site. Cleavage at this second site is also seen in a small proportion of the molecules analyzed in this study, giving the primer extension products seen near the bottom of lanes 3 through 6 in Fig. 5B. These additional cleavage sites are currently the subject of further study. In contrast, 110S virus extracted from the infected cells gave a whole range of extension products with either Sequenase (Fig. 5A, lane 3) or reverse transcriptase (not shown) which varied substantially from one preparation of virus to another and which ranged from some rather minor species which coelectrophoresed with the RF extension products (83 or 84 nucleotides long) to major forms which were up to 24 nucleotides shorter than the RF products. Virus obtained from the culture medium gave a much less heterogeneous product (Fig. 5A, lane 1; Fig. 5B, lane 3), with a large proportion of the extension products comigrating with those of RF DNA, although some shorter forms were also present. However, digesting this virus with micrococcal nuclease prior to disrupting the capsid structure gave a fairly uniform population of viral strands which gave major extension products with Sequenase of 60 and 59 nucleotides (Fig. 5A, lane 2) and a major product of 59 nucleotides with reverse transcriptase (Fig. 5B, lane 4). This suggests that most of the virus released from infected cells into the culture medium has the same 5′ end as RF DNA but that approximately 24 nucleotides at the 5′ end are accessible to exogenous nucleases, while the rest of the viral strand is sequestered within the protective capsid coat. When virions are allowed to reinfect the host cells, 110S virus is obtained which exhibits a whole range of 5′ ends from the full-length RF-like form down to the most extensively nuclease-treated form, presumably depending on the extent to which they have been exposed to secreted or cellular nucleases. Since neither polymerase performed perfectly on the control DNA and because we cannot be certain whether or not they could copy a peptide-linked nucleotide, we cannot definitively locate the in vivo cleavage sites but suggest that RF DNA is probably 5,170 nucleotides long, with cleavage occurring between the first and second residues in the sequence 5′ TATT 3′, as illustrated in Fig. 6.

Infectivity of virions containing either s1 or s2 DNA. The particle-to-infectivity ratio of MVM virions is rather high, and thus it was necessary to determine whether or not particles carrying the 5′ NS-1 linkage were actually infectious or simply an uninfectious but abundant by-product of the infectious process. Initial attempts to investigate this by using neutralization assays with anti-NS-1 sera failed to demonstrate any plaque-neutralizing activity for these sera. However, to test this possibility further, NS-1-associated

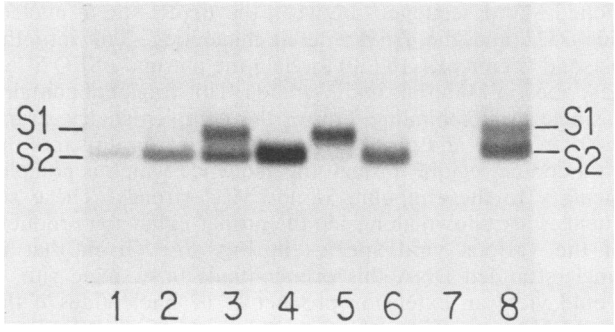

FIG. 7. Autoradiograph of a 1% agarose-SDS gel showing $^{32}PO_4$-labeled DNA from intact (i.e., nondisrupted) medium-derived virus before (lane 3) and after (lane 4) digestion with high levels of trypsin. When intact virions were immunoprecipitated with anti-NS-1 serum (serum B in this case), those containing the s1 form of single-stranded DNA bound to the *S. aureus* immunoabsorbant (lane 5), while most of the virions containing s2 forms were left in the unbound fraction (lane 6). Much of the virus shown pelleted in lane 5 could then be removed from the immunoabsorbant by digestion with trypsin, which also converted it to an s2-like form (lane 2), but this digestion usually failed to go to completion and so some virions still remained associated with the immunoabsorbant (lane 1). In contrast, immunoprecipitates obtained with a control serum (anti-NS-2) contained very few virions (lane 7), and most of the viral DNA remained in the unbound fraction (lane 8).

TABLE 1. Infectivity of NS-1-associated virions[a]

Serum	PFU[b]		% Viral DNA[c]	
	Unbound fraction	Trypsin-released fraction	Unbound fraction	Trypsin-released fraction
Serum A (anti-NS-1)	5.7×10^5 (59.4)	3.9×10^5 (40.6)	41	59
Serum B (anti-NS-1)	7.5×10^5 (78.1)	2.8×10^5 (29.2)	62	38
Anti-NS-2 (control)	8.7×10^5 (90.6)	0.28×10^5 (2.9)	96	4

[a] Aliquots of a preparation of $^{32}PO_4$-labeled virions partially purified from medium, each containing 9.6×10^5 PFU, were complexed with antibody and adsorbed onto fixed *S. aureus*. After washing, bound virus was released by trypsin treatment as described in Materials and Methods, and its infectivity was measured by plaque assay as described elsewhere (19).

[b] Figures in parentheses indicate the percentage of the input infectivity recovered in each fraction (these do not add up to 100% in all cases because of inaccuracies inherent in the plaque assays).

[c] Single-stranded DNA associated with unbound and released fractions was quantitated by autoradiography and densitometric scanning following SDS-agarose gel electrophoresis as described in Materials and Methods.

virions were affinity purified, and their infectivity was assayed directly. The medium-derived virus used for one such experiment, documented in Fig. 7, was somewhat exceptional in that it contained approximately equimolar amounts of both the s1 and s2 DNA forms. Nonetheless, the infectivity data obtained (presented in Table 1) closely reflect those obtained in similar experiments with more typical medium-derived virus preparations. This particular study is presented here because removal of the NS-1-associated virus was practically quantitative and so facilitates comparison of approximate DNA concentrations and infectivities. When medium-derived virus (Fig. 7, lane 3) was immunoprecipitated with antiserum directed against NS-1, all the s1 DNA forms bound antibody and could be collected on Formalin-fixed *S. aureus* (lane 5), while the s2 forms remained predominantly in the unbound fraction (lane 6). The amount of the input infectivity removed from the sample by this procedure was in quite good agreement with the amount of DNA removed from the same sample, indicating that the specific infectivities of the two forms of virion are equivalent (Table 1). Furthermore, much of the bound virus, both in terms of DNA and infectivity, could then be released from the immunoprecipitates with high levels of trypsin (Fig. 6, lane 2), which digested away the NS-1, converting the DNA to an s2-like form and also destroyed any potentially neutralizing antibody.

These data suggest that s2 virions are infectious in their own right, while s1 virions are either infectious or are capable of giving rise to infectious virus following tryptic digestion. To explore this further and to ask whether or not the presence of NS-1 or the 5′ end of the DNA actually influenced the infectivity of the virus, we digested medium-derived virus with trypsin, to remove a large proportion of the NS-1 molecule but leave the nucleotide chain intact, or micrococcal nuclease, to remove both NS-1 and the exposed oligonucleotide. When plaqued on A9 monolayers, these preparations gave essentially equal infectivity titers, which were 1.8×10^8 PFU/ml for untreated culture medium virus, 1.6×10^8 PFU/ml for trypsin-digested medium virus, and 1.6×10^8 PFU/ml for nuclease-digested medium virus.

Thus, removal or fragmentation of NS-1 has no detectable effect on the infectivity of these virus preparations in culture, suggesting that whatever function is served by NS-1 and the terminal nucleotides in the s1 form, it must operate prior to release of virus from the original parent cell.

DISCUSSION

We have shown that viral single-stranded DNA becomes encapsidated while still bearing a 5′ oligonucleotide sequence which is covalently linked to the amino-terminal region of an NS-1 polypeptide. Although more than 99.5% of the packaged DNA in such particles is protected from the external environment by capsid proteins, NS-1 and a stretch of approximately 24 nucleotides at the 5′ end of the DNA strand project through the capsid structure, as depicted in Fig. 8, and so remain accessible to proteases and nucleases in the external medium. Such virions are released from the host cell into the surrounding medium and appear to be infectious, although at present it is not clear whether they need to be cleaved, either extracellularly or intracellularly, to s2 forms before this infectivity is manifest. However, NS-1 and the 5′ 24 nucleotides of the DNA strand are apparently not required for subsequent steps in the infectious cycle, since they can be removed in vitro without influencing the infectious virus titer. Newly infected cells accumulate s2 virions almost exclusively, either by preferentially importing s2 forms or, perhaps more likely, by

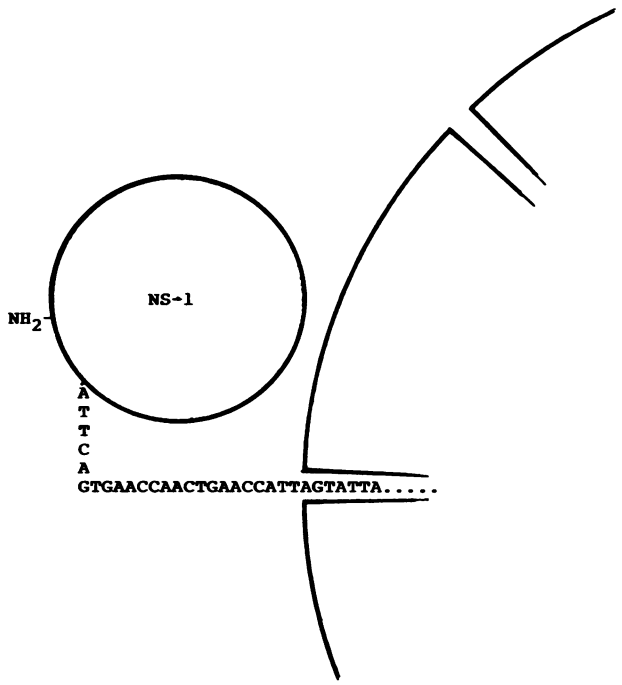

FIG. 8. A diagrammatic representation of the short, externally located tether sequence which links the nuclease-resistant 5.1-kilobase internal viral core DNA to the NS-1 molecule located on the outside surface of the mature virion.

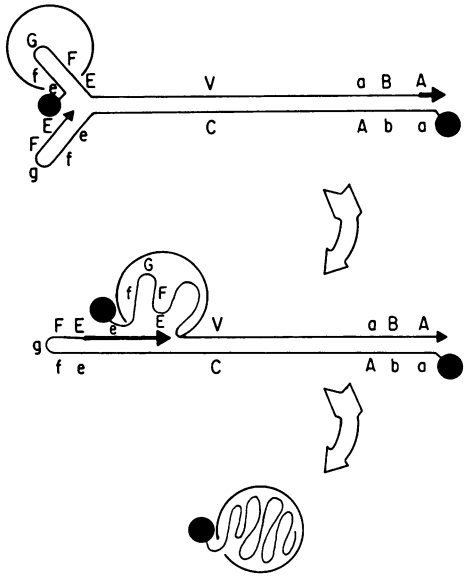

FIG. 9. Updated version of the last two steps of the modified rolling hairpin model for parvoviral DNA replication, described in detail in the text. Upper- and lowercase letters denote complementary sequence blocks; A and a and B and b represent the sequences within the left-hand (viral 3′) palindrome and E and e, F and f, and G and g represent sequences within the right-hand (viral 5′) palindrome. Closed circles denote terminal NS-1 molecules; viral sense strand sequences are denoted V; complementary strand template sequences are denoted C.

exposing the virions to lysosomal enzymes as they pass through a presumed endocytotic pathway en route to the nucleus. Thus it would seem likely that the substrate for DNA replication in the newly infected cell is an NS-1 minus s2 form.

Sequence studies had previously suggested that the 5′ end of MVM virion DNA was approximately 18 nucleotides shorter than that of RF DNA (1, 3). This led us to suggest that viral strands might be synthesized from an 18-base primer which was cleaved from the nascent strand by an intranuclear, site-specific nuclease, leaving the primer permanently associated with the complementary strand of RF DNA (8). Satisfying though such a mechanism would have been, it must now be discarded, since RF DNA and newly packaged viral DNA clearly have the same 5′ sequence and the additional 5′ nucleotides are not excised until after release of the virus. Similarly, previous studies had shown that the 5′ ends of a large proportion of packaged DNA strands are covalently associated with protein (3, 4), and we have shown that this protein is NS-1 and that it is associated with the 5′ ends of all newly synthesized RF and single-stranded DNA (9). At the time, this finding was somewhat surprising since it suggested that if, as we suspected, NS-1 became attached to the DNA while carrying out a site-specific cleavage reaction, its presence on the supposedly truncated viral strands would indicate that it could cut the same DNA molecule successively at two different sites. However, the present studies show that NS-1 is predominantly attached to a single site on viral DNA.

Since virions do not appear to need the NS-1 complex in order to infect a new host cell, we must presume that any function it may serve operates prior to release from the original infected cell. In the autonomous parvoviruses, single-strand DNA synthesis is concomitant with packaging (14), and since displacement synthesis would peel off the viral DNA in a 5′ to 3′ direction, we can assume that packaging also occurs in this direction, as depicted in Fig. 9. Thus the initial interaction between the viral strand and the preformed capsid could well involve the NS-1 molecule.

Whether NS-1 directs packaging or not, it clearly must complicate packaging, since there is no evidence that NS-1 ever enters the capsid shell. Thus the 5′ end of the DNA is not simply pushed into the empty capsid, with the rest of the strand following behind as it is displaced. Instead, an NS-1-associated loop of viral DNA might be displaced from its parental strand, and the loop then inserted into an opening in the adjacent capsid. It is tempting to speculate that such a mechanism might use the natural loop of DNA created by the whole palindromic sequence, as suggested in Fig. 9.

Figure 9 shows an updated version of the last two steps of the modified rolling hairpin model for parvoviral DNA replication recently proposed by us (8). Taking account of the 5′ attachment of NS-1 to single-stranded viral DNA reported in reference 9 and the data presented in this paper, we now propose that packaging occurs by the invagination of the right-hand hairpin, represented here by the sequence blocks fGF, into preformed capsids. These hairpins would be generated by the "cruciforming out" of terminal palindromes, as originally proposed for the hairpin transfer aspect of the rolling hairpin model (8), and their association with the packaging apparatus might perhaps be catalyzed by the presence of NS-1 molecules on their 5′ ends. Further displacement synthesis of viral sequences on the complementary strand template would occur coordinately with further packaging of the displaced viral strand (14), finally yielding a completely packaged progeny genome, linked through the short, externally located tether sequence, denoted e, to the terminal NS-1 molecule.

MVM predominantly packages the minus DNA strand, but there is a significant error rate, such that approximately 1% of virions contain the plus strand (10). Since MVM has unique termini, it is difficult to see how a packaging mechanism which recognizes a specific DNA sequence exclusively could be so error prone. However, the 5′ ends of both the plus and minus strands carry NS-1 molecules (Fig. 9), and if packaging was directed by an initial interaction between NS-1 and the preformed capsid, followed and stabilized by an interaction between the capsid and a specific DNA sequence, such errors would be more easily explained.

NS-1 molecules situated on the outside of the virion might equally well serve other roles in the viral life cycle. At present it is not clear that all progeny virus is released from the parental cell by cell death and lysis, since there is also evidence for a noncytotoxic viral export pathway (R. Moir and P. Tattersall, unpublished results). If so, the NS-1 molecules would be well situated to direct transport of the virus out of the nucleus and subsequently to the cell surface. Additional support for such a mechanism comes from the observations reported here that most of the full particles, but very few of the empty particles, are released into the culture medium and that the presence of antiviral antibody, but not neuraminidase, in the extracellular environment blocks release of virus from infected cells. If this occurs as a result of the active transport of full, but not empty, virions out of the cell, the mechanism responsible would need to be able to differentiate between the two species of particle and thus it could not involve the surface receptors which are responsible for virus uptake, since these appear to bind both species equally well (17).

Thus, while there is as yet no proof that particle-associ-

ated NS-1 molecules influence packaging or virion maturation, their presence on the outside of the particle makes this possible and suggests additional roles for this interesting polyfunctional viral protein.

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