JOURNAL OF VIROLOGY, Oct. 1989, p. 4249–4256
0022-538X/89/104249-08$02.00/0

Vol. 63, No. 10

Both Excision and Replication of Cloned Autonomous Parvovirus DNA Require the NS1 (*rep*) Protein

SOLON L. RHODE III

The Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68105-1065

Received 3 April 1989/Accepted 7 July 1989

When a bacterial plasmid containing the entire genome of LuIII virus except for the terminal 18 nucleotides from the right end is transfected into HeLa cells, the viral DNA is rescued and replicated, with production of infectious virus. This experimental system was used to examine the viral proteins and *cis* elements required for the excision and replication of viral DNA. The deletion of the entire NS1 gene provided a viral genome that was excised from the plasmid and replicated only when an NS1 gene was provided in *trans*. A frameshift mutation in the NS2 intron that truncates NS1 prevented excision and replication. Deletion of the left-end terminal inverted repeat or the right-end inverted repeat prevented excision of viral DNA from that end but not from the wild-type terminus. The viral terminus excised from the plasmid was protected from a processive degradation process, which began on the vector portion of the plasmid. The inhibitor of DNA polymerases α and δ, aphidicolin, blocked the excision reaction.

Parvoviruses are small DNA viruses that contain linear single-stranded DNA genomes of about 5 kilobases (kb) (reviewed in references 3, 6, and 35). The dependoviruses (adeno-associated virus [AAV]) are parvoviruses that have a nearly complete requirement for helper virus, such as adenovirus or herpesvirus, for productive infection. The nondefective or autonomous parvoviruses are widespread in nature and in many cases are pathogens with important economic consequences. They have a requirement for replicating host cells for productive infection and tend to be most pathogenic for the fetus or for renewal tissues that have high rates of replication. All of the parvoviruses show a similar overall genomic organization. Their genomes are divided into two or three genes that are collinear. By convention the left half of the genome contains the nonstructural (NS) gene and the right half contains the virion capsid protein gene (see Fig. 1, VP). Each of these genes generates two or more gene products by utilizing different splicing arrangements, different promoters and start sites, alternate translational starts, or a combination of these (3, 6, 35). Despite little homology at the nucleotide sequence level between some of the parvoviruses, all of them have a conserved domain in the NS1 protein. This is especially pronounced in an area that has homology to other proteins with ATPase activity (1, 2). This includes papovavirus large T antigens, which have been shown recently to have DNA helicase activity (30).

The NS1 or *rep* proteins of both the dependoviruses and autonomous parvoviruses have been assigned similar functions. These include their requirement for viral replicative-form (RF) DNA replication (4, 11, 16, 27, 31) and the regulation of transcription (14, 20, 22, 32). There is also evidence that the NS1 gene has apparent cytotoxic activity (13, 21). The bulk of the evidence that NS1 is required for viral DNA replication has been genetic. Plasmid clones of various parvovirus genomes have been found to require an intact NS1 gene in either *cis* or *trans* for the subsequent excision and replication of the viral sequences (11, 16, 25, 26, 31). Several temperature-sensitive mutations that map to the NS1 gene have been shown to have a phenotype of temperature-sensitive viral DNA replication (33, 37). Thus, the existence of a replication function for NS1 is firmly established, but the nature of its activity is not well defined. An important insight into the function of NS1 has been revealed by the evidence that NS1 is the protein covalently bound to the 5′ ends of viral RF DNA and progeny single strands (7, 10). This strongly suggests that NS1 may be the endonuclease that has been hypothesized to be required for the execution of the hairpin transfer reaction during viral DNA replication (6, 17). In this study, I have described an in vivo replication system that defines some of the functions that require NS1 and that will permit a detailed mapping of the *cis* elements necessary for autonomous parvovirus DNA replication.

MATERIALS AND METHODS

Cells and virus. HeLa Gey cells (ATCC CCL2.1) were used for transfections. Virus stocks were generated by propagation in simian virus 40-transformed human kidney cell line NB (28). LuIII virus was generated by transfection of HeLa cells with pGLu883 and then propagation of the culture lysate in NB cells.

DNA constructions. LuIII virus has not been fully sequenced so I will use the analogous nucleotide numbers of H-1 virus (24) to describe the corresponding positions in LuIII virus. The right end of the genome is by convention the 5′ end of the minus strand. The infectious clone of LuIII virus, pGLu883, and plasmid pLSmal3 were provided by R. Bates. pGLu883 is a genomic clone of LuIII virus in vector pUC19 constructed with *Bam*HI linkers and with the LuIII virus left end positioned proximal to the *Hin*dIII end of the polylinker (Fig. 1A). pGLu883 is missing approximately 18 nucleotides from the right end (7a). pLSmal3 is a clone of the left half of LuIII virus from a blunt left end to the *Hin*dIII site at map position 51 inserted between the *Sma*I and *Hin*dIII sites of pUC19. The orientation of pLSmal3 in the vector is opposite that of pGLu883. A number of plasmids were constructed with deletions within the right-end palindrome (Fig. 1A and B), using the *Nde*I sites in the right-end hairpin and in pUC19. The deletion in pGlu2ΔNd was constructed by first destroying the *Nde*I site in pUC19 to form pGLu2 and then deleting the right-end inverted repeat sequences between the two *Nde*I sites. The construction of pLudl, a LuIII virus clone with the right-end palindrome

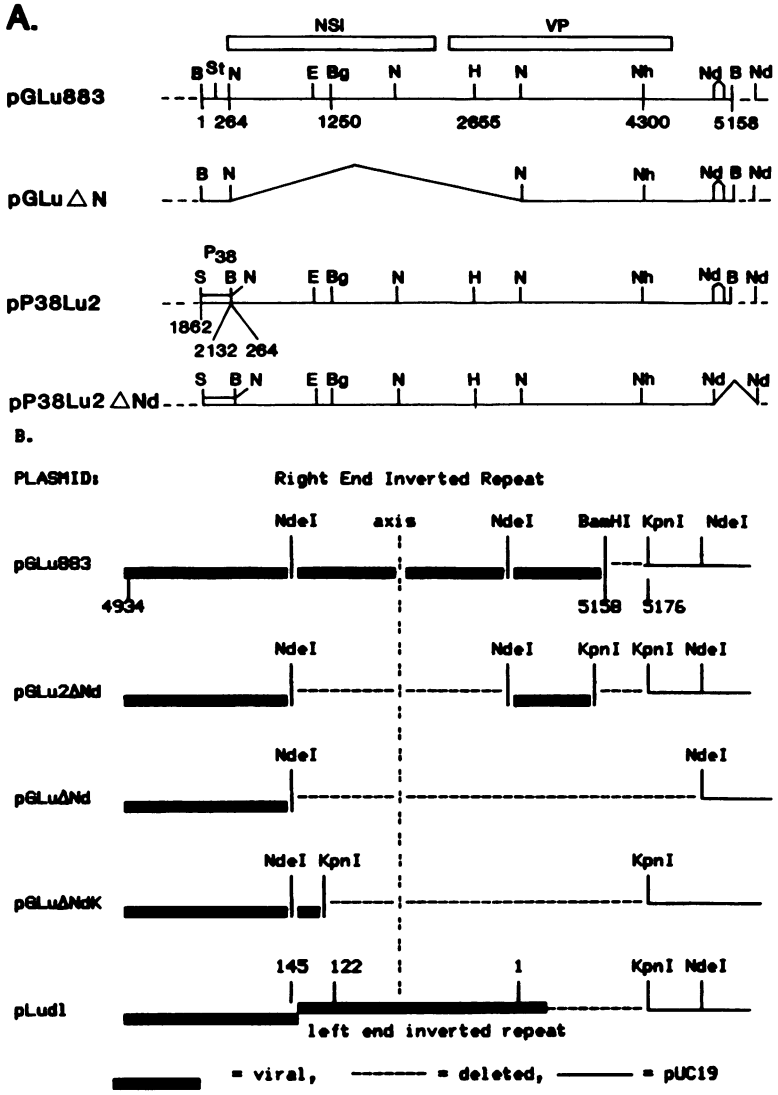

FIG. 1. (A) Diagram of the genomic clone of LuIII virus, pGLu883, and the plasmids derived from it. pGLuΔN is deleted from the *Nco*I site at nucleotide 264 (the start codon for NS1 and NS2) to an *Nco*I site at about nucleotide 3200. This deletion removes the entire NS1/NS2 gene. pP38Lu2 and pP38Lu2ΔNd have the first 264 nucleotides of viral DNA removed and replaced with the H-1 virus P38 promoter, nucleotides 1862 to 2132. pP38Lu2ΔNd was derived from pP38Lu2 by deletion of all of the sequences between the inboard *Nde*I site in the right-end hairpin and the *Nde*I site in pUC19. Abbreviations: B, *Bam*HI; St, *Sty*I; N, *Nco*I; E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; Nh, *Nhe*I; S, *Sal*I; Nd, *Nde*I. (B) Diagram of deletion mutations in the right-end inverted terminal repeat of cloned LuIII virus. The numbering is based on the corresponding numbers of the parvovirus H-1 virus (24). pGLu883 is the wild-type infectious clone of LuIII virus that is deleted by about 18 nucleotides from nucleotides 5159 to 5176. pGLu2ΔNd is deleted of the 119 base pairs between the two *Nde*I sites, and pGLuΔNd is deleted from the inboard *Nde*I site of LuIII virus to the *Nde*I site of pUC19 (161 base pairs of LuIII virus). pGLuΔNdK has the wild-type 18-base-pair sequence of LuIII virus normally at that position inserted as a synthetic sequence between the inboard *Nde*I site of pGLu883 and the *Kpn*I site of pUC19. pLudl was prepared as described in Materials and Methods.

replaced with a left-end palindrome, was prepared by inserting the first 145 nucleotides containing the left end as an *Nde*I-*Kpn*I fragment into pGLu2. This fragment was prepared by creating an *Nde*I site at nucleotide 145 in pLSmal3 by ligating an *Nde*I linker at a *Sty*I site that was blunted by treatment with T4 DNA polymerase. Plasmid pP38Lu2 consists of a genomic clone of LuIII virus with the first 262 nucleotides, that is, the left-end inverted repeat and P4 sequences, replaced by H-1 virus P38 promoter from nucleotides 1862 to 2132 as described previously for pP38NS1cat (21). pP38Lu2 has a *Bam*HI linker inserted at the *Nco*I site which initiates the coding sequences of the NS1/NS2 gene at nucleotide 264. This plasmid expresses the NS1/NS2 gene and the capsid gene from two separate copies of P38. pP38Lu2ΔNd has the deletion of right-end sequences as described for pGLuΔNd in the legend to Fig. 1. pP38Lu is the same as pP38Lu2 except that the sequences from the *Nco*I site at nucleotide 264 to the *Bgl*II site at nucleotide 1250 are from H-1 virus rather than LuIII virus (data not shown). Thus, the NS1 gene is chimeric, with the amino-terminal half from H-1 virus and the carboxy-terminal half from LuIII virus. pGLuΔN is a deletion of pGLu883 in which the sequences from the *Nco*I site at nucleotide 264 to the *Nco*I site at approximately nucleotide 3300 were re-

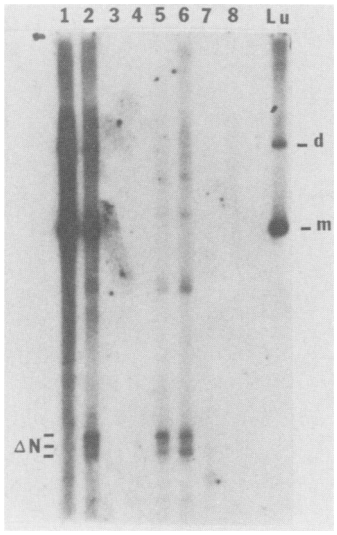

FIG. 2. Southern blot analysis after transfections of HeLa cells with pGLuΔN and various helper plasmids. Cultures in 60-mm dishes were transfected with 1 μg each of the following plasmids: 1, pGLu883; 2, pGLuΔN plus pGLu883; 3, pGLuΔN; 4, pGLuΔN plus pLuH6; 5, pGLuΔN plus pP38Lu; 6, pGLuΔN plus pSR1; 7, pLuH6; 8, pP38Lu. Lane Lu contains a sample of a LuIII virus-infected HeLa cell culture. DNA samples were collected at 48 h posttransfection, digested with *Dpn*I, and fractionated on a 1% agarose gel. The gel was probed with randomly primed *Eco*RI-restricted pGLu883 DNA. The sizes of DNA bands were measured using stained *Hin*dIII lambda DNA fragments as markers. m, Monomer LuIII virus RF DNA; d, dimer RF DNA; ΔN, various configurations of monomer RF DNA generated by pGLuΔN.

moved. This deletes the entire NS1/NS2 gene (Fig. 1A). pLuH6 has a frameshift insertion at the *Bgl*II site at nucleotide 1250 in the NS1 gene and within the NS2 intron as described for H-1 virus plasmid pH6 (22). Plasmid pSR1 is a genomic clone of H-1 virus *ts*6 that was previously described (20). This clone was made in a *recA*$^+$ host and is not infectious, presumably due to rearrangements in the terminal hairpin sequences. pSR1 expresses the NS1/NS2 gene. The *ts*6 mutation is in the capsid protein gene.

DNA transfections and replication assay. HeLa cells were transfected as described previously (22), and low-molecular-weight DNA was extracted by the method of Hirt (12) at the times indicated. The DNA was treated with proteinase K for several hours at 37°C and precipitated with isopropanol. The DNA was dissolved, treated with RNase for 1 h at 37°C, extracted once with phenol and once with phenol plus chloroform, and precipitated with 2 volumes of ethanol. Samples of DNA were treated with restriction enzymes as indicated, using the buffers recommended by the manufacturer, and analyzed by horizontal agarose gel electrophoresis. The gels were stained with ethidium bromide and the positions of the stained markers were measured. The markers were either *Hin*dIII fragments of lambda or *Hae*III fragments of φX174 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The DNA in the gels was then transferred by the rapid alkaline transfer method to Nytran membranes, using a vacuum blot apparatus (LKB Instruments, Inc., Rockville, Md.). The membranes were then hybridized to probes generated with random 6-mer oligonucleotides synthesized in my laboratory, [^{32}P]dCTP, the Klenow fragment of DNA polymerase I, and pGLu883 linearized with *Bam*HI or *Eco*RI as the template. Restriction

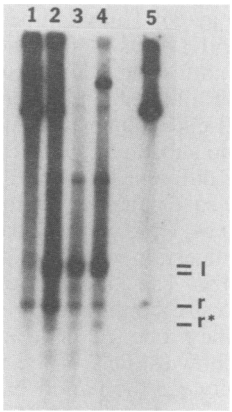

FIG. 3. Southern blot analysis of the same samples used for Fig. 2 after digestion with *Nhe*I and *Dpn*I. Lanes: 1, pGLu883; 2, pGLu883 plus pGLuΔN; 3, pP38Lu plus pGLuΔN; 4, pSR1 plus pGLuΔN; 5, LuIII virus marker. The gel was 1.5% agarose and hybridized as in Fig. 1. l, Left-end fragments of the pGLuΔN genome; r, extended right-end fragment; r*, foldback right-end fragment. The bands at 2.5 kb in lanes 3 and 4 are the dimer left-end *Nhe*I fragments of pGLuΔN.

enzyme *Dpn*I was used to distinguish DNA that was unmethylated in one or both strands and resistant to restriction. Resistance to *Dpn*I implies that the DNA had replicated. All of the plasmids were propagated in strain MV1190 [Δ(*lac-proAB*) *thi supE* Δ(*srt-recA*)*306*::Tn*10*(Tet^r) (F′ *traD36 proAB lacI*^{q}ZΔM15)] obtained from Bio-Rad Laboratories, Richmond, Calif. Enzymes were purchased from New England BioLabs, Inc., Beverly, Mass.

RESULTS

Replication requires NS1 in *trans*. Plasmid pGLuΔN was used as an NS1$^-$ and NS2$^-$ construction to test for the requirements of NS1 for replication. This plasmid has the advantage that the entire NS1 gene is deleted so that no truncated NS1 products can be made that might affect the phenotype. Cotransfection of pGLuΔN with infectious clone pGLu883 resulted in the appearance of three bands with the mobility of approximately 2 kb (Fig. 2, lane 2). This is the expected size of the LuIII virus replicon after the deletion of 3 kb. pGLuΔN did not replicate when transfected by itself (Fig. 2, lane 3) or with plasmid pLuH6, which has a frameshift insertion in the *Bgl*II site at nucleotide 1250. pLuH6 would express NS2 and a truncated NS1 protein. Replication of pGLuΔN was also supported by cotransfection with pSR1 and pP38Lu. Thus, the NS1 gene of H-1 virus or a chimeric NS1 gene that is H-1 virus from NS1 amino acid residues 1 to 329 and LuIII virus from residue 330 to the carboxy terminus support the replication of pGLuΔN. Plasmids pSR1 and pP38Lu show no evidence of replication. In addition to the bands at 1.8 to 2 kb, there are additional pGLuΔN bands at 3.8, 5.7, and 7.6 kb. These appear to represent the dimer, trimer, and tetramer of pGLuΔN. The presence of the trimer was not expected, for a trimer has not been seen with infection (see control lane Lu in Fig. 2).

pGLuΔN replicated with three different NS1 genes in *trans* so that it was of interest to look for evidence of different processing of the terminal palindromes. Samples from the DNA of lanes 1, 2, 5, 6, and Lu of Fig. 2 were restricted with *Nhe*I and *Dpn*I and analyzed by Southern

blot hybridization (Fig. 3). There is one *Nhe*I site at about nucleotide 4300 in LuIII virus so that the expected left-end fragment and right-end fragment of pGLuΔN are 1.2 and 0.9 kb, respectively. The foldback and extended configurations of the right- and left-end palindromes were visualized and there seemed to be no substantial difference in their relative proportions with the different NS1 genes. The two bands in lane 1 at about 1,300 base pairs are not the left-end fragment of wild-type LuIII virus, which is 4,300 base pairs. They may represent terminal digestion products of *Dpn*I which are known to be that size. The identities of the left-end fragments of pGLuΔN were confirmed by reprobing this blot with a left-end-specific probe (*Eco*RI fragment, sequences 1 to 1088) (data not shown). This probe hybridized to the l bands and a band between the l and r bands in lane 3 only. This band is visible in lane 3 of Fig. 3 and probably represents the left-end *Dpn*I fragment of pP38Lu. These results indicate that the excision and replication of the LuIII virus replicon are dependent on an intact NS1 gene. Since the NS1 effect acts in *trans*, this implies that it is the result of the NS1 protein.

Effects of mutations in the palindromes. A number of mutations at the right-end palindrome were constructed as shown in Fig. 1B and described in Materials and Methods. All of the plasmids shown in Fig. 1B with right-end mutations generated infectious virus after transfection of HeLa cells. The development of the cytopathic effect took longer for these constructs than for the wild-type genome, and the virus stocks produced were 10^{-1} to 10^{-2} of that of wild-type virus (data not shown). The fate of these constructs in the transient replication assay was examined in HeLa cells (Fig. 4). The DNA samples were restricted with *Bgl*II, except for pLuH6, which has no *Bgl*II site and was restricted with *Nhe*I or treated with *Dpn*I. Infectious clone pGLu883 produced three major bands (Fig. 4, lane 2), the full-length plasmid, 7.8 kb, and the two viral DNA bands at 3.9 and 1.2 kb. The *Dpn*I-treated sample in lane 3 showed replication of viral DNA at the monomer and dimer RF DNA positions as expected. The digestion with *Dpn*I in lane 3 produced a 1.2-kb band because the *Bgl*II site at nucleotide 1250 is the first *Dpn*I site in LuIII virus. The full-length plasmid band was removed by *Dpn*I; thus, plasmid DNA had not replicated. Plasmid pP38Lu2ΔNd, which expresses NS1/NS2 and supports replication of pGLuΔN (data not shown), is missing the entire left-end palindrome and 75% of the right-end palindrome. This plasmid showed no evidence of excision or replication (Fig. 4, 5, and 6). In contrast, plasmid pP38Lu2, which has an intact right-end palindrome, produced a number of *Bgl*II fragments of 3.9 kb or smaller (Fig. 4, lanes 7 and 9). *Dpn*I digestion of pP38Lu2 without restriction with *Bgl*II showed that there was evidence of DNA synthesis, with multiple bands resistant to *Dpn*I (Fig. 4, lanes 8 and 10). This result is clearer in the experiment shown in Fig. 5 (lanes 5 and 7) and described below. Plasmid pGLuΔNd has the same right-end deletion as does pP38Lu2ΔNd, but the left-end palindrome is intact. pGLuΔNd restricted with *Bgl*II produced the left-end 1.2-kb fragment and a fragment at 6.6 kb (Fig. 5, lane 11). This result indicated that excision had occurred at the left end of the viral sequences. It should be noted that the 6.6-kb fragment did not generate as much signal as did the 1.2-kb fragment, suggesting a smaller molar amount. *Dpn*I digestion of pGLuΔNd showed that a small portion of full-length plasmid was linear and resistant to *Dpn*I, implying that it had synthesized at least one strand of new DNA (Fig. 5, lane 12). Since the results with *Bgl*II indicated that excision at the left end was efficient, this implies that a portion of the plasmid that had linearized at the left end had also synthesized DNA. Plasmid pLuH6 with a mutated NS1 gene showed no evidence of excision or replication.

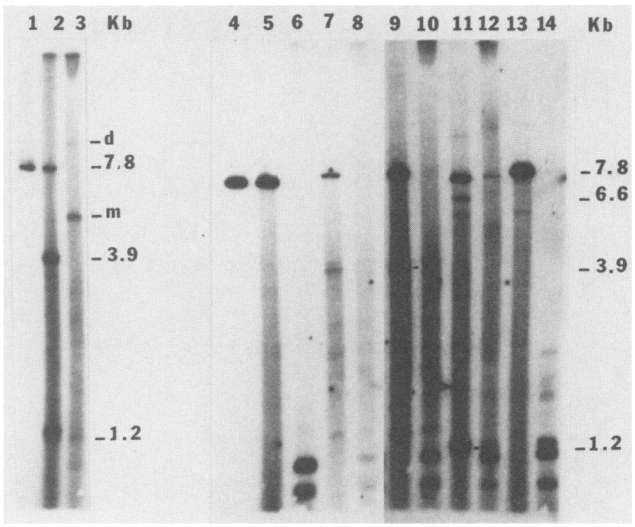

FIG. 4. Southern blot analysis after transfections with LuIII virus plasmids with modified terminal repeat sequences. HeLa cell cultures were transfected with 2 μg of each plasmid; the samples were prepared at 48 h posttransfection and analyzed as in Fig. 1. Lanes 1 and 4 contain samples of *Bgl*II-restricted plasmids pGLu883 and pP38Lu2ΔNd, respectively. The transfected samples and their respective enzyme treatments are as follows: pGLu883, lanes 2 and 3 (2, *Bgl*II; 3, *Dpn*I); pP38Lu2ΔNd, lanes 5 and 6 (5, *Bgl*II; 6, *Dpn*I); pP38Lu2, lanes 7 to 10 (7 and 9, *Bgl*II; 8 and 10, *Dpn*I); pGLuΔNd, lanes 11 and 12 (11, *Bgl*II; 12, *Dpn*I); pLuH6, lanes 13 and 14 (13, *Nhe*I; 14, *Dpn*I). Lanes 9 to 14 had a longer exposure than did lanes 4 to 8, and lanes 1 to 3 were from a separate gel. Marker positions were determined as in Fig. 1. The 3.9-kb band is the *Bgl*II fragment from nucleotide 1250 to the right end, and the 1.2-kb band is the left-end *Bgl*II fragment. Plasmids pP38Lu2ΔNd, pP38Lu2, and pGLuΔNd are $NS1^+$ and support the replication of pGLuΔN in *trans* (data not shown). d, Dimer; m, monomer.

These results show that excision occurs at either end of the viral sequences if the palindrome is intact and a portion or all of the excised molecules have synthesized at least one DNA strand. Excision did not occur without NS1. When excision occurred at only the right end (Fig. 4, lanes 7 and 9), the generation of a nested set of fragments from the *Bgl*II fragment at 3.9 kb and smaller suggested that the vector end of the DNA was not stable. These suggestions were tested further in a similar experiment with the modification that the extracts were digested with *Bgl*II, *Nhe*I, *Bgl*II and *Dpn*I, or *Nhe*I and *Dpn*I (Fig. 5). Plasmid pGLuΔNd with the intact left end produced the expected 1.2-kb left-end fragment with *Bgl*II and a small amount of the 6.6-kb fragment. This shows linearization of the plasmid at the viral left end. Resistance to cleavage with *Dpn*I showed that some of the 6.6-kb fragments had replicated and the 1.2-kb fragment was not affected since it does not have a *Dpn*I site (Fig. 5, lane 2). The signal for the 1.2-kb fragment was much greater than for the 6.6-kb fragment, even without the *Dpn*I treatment. A control experiment in which this blot was reprobed for the lambda markers showed that this was not due to inefficient transfer of the larger fragments (data not shown). *Nhe*I produced the expected band at 4.3 kb for excision at the left end and the amount of the 4.3-kb fragment was greater than the 6.6-kb fragment but less than the 1.2-kb fragment. This

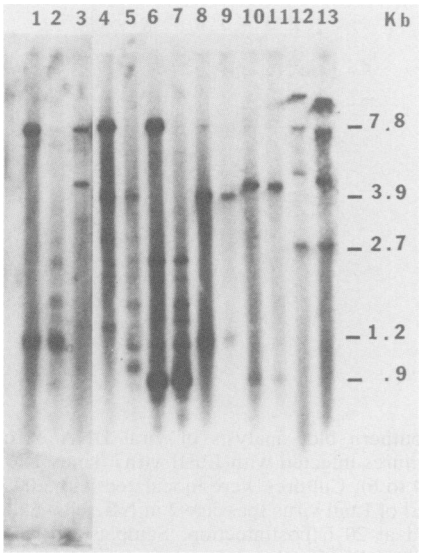

FIG. 5. Southern blot analysis after transfections with LuIII virus plasmids with modified terminal repeat sequences. Transfections and analysis are as in Fig. 4 with a 1.5% agarose gel. The samples and their enzyme treatments are as follows: pGLuΔNd, lanes 1 to 3 (1, *Bgl*II; 2, *Bgl*II plus *Dpn*I; 3, *Nhe*I); pP38Lu2, lanes 4 to 7 (4, *Bgl*II; 5, *Bgl*II plus *Dpn*I; 6, *Nhe*I; 7, *Nhe*I plus *Dpn*I); pGLu883, lanes 8 to 11 (8, *Bgl*II; 9, *Bgl*II plus *Dpn*I; 10, *Nhe*I; 11, *Nhe*I plus *Dpn*I); pLuH6 plus *Eco*RI pUC19, lane 12; pP38Lu2ΔNd plus *Eco*RI pUC19, lane 13. The right-end *Nhe*I fragment is 0.9 kb, the left-end *Nhe*I fragment is 4.2 kb, the right-end *Bgl*II fragment is 3.9 kb, and the left-end *Bgl*II fragment is 1.2 kb.

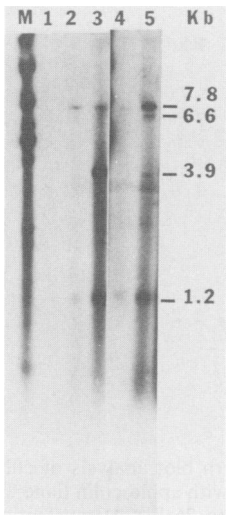

FIG. 6. Southern blot analysis of cultures transfected with LuIII virus plasmids with modified right-end terminal inverted repeats. The transfections are as in Fig. 1. Lanes: 1, pGLuΔNd; 2, pGLu2ΔNd; 3, pGLu883; 4 and 5, longer exposures of lanes 1 and 2. The samples were digested with *Bgl*II. The marker lane (M) contains a mixture of a sample of LuIII virus-infected HeLa cell DNA and the *Hin*dIII lambda DNA fragments. The full-length plasmids are about 7.8 kb, and the *Bgl*II fragments are as in Fig. 5. The agarose gel is 1%.

supports the suggestion that the vector end of the linearized plasmid is subject to a processive degradation. Plasmid pP38Lu2 with the intact right end produced the right-end fragment of 3.9 kb but not the left-end fragment of 1.2 kb with *Bgl*II (Fig. 5, lanes 4 and 5). The 3.9-kb right-end *Bgl*II fragment and smaller bands were partially resistant to *Dpn*I, indicating DNA synthesis. If there is a processive degradation from the vector end of the linearized DNA, then cutting closer to the viral end will produce a stronger signal. This is the result obtained for pP38Lu2 cut with *Nhe*I (Fig. 5, lanes 6 and 7). The right-end *Nhe*I fragment of 0.9 kb produces more signal than does the 3.9-kb right-end *Bgl*II fragment (Fig. 5, lane 6 versus lane 4). The 0.9-kb *Nhe*I fragment does not contain a *Dpn*I site, so the 0.9-kb band was not reduced by treatment with that enzyme. Infectious clone pGLu883 in lanes 8 to 11 of Fig. 5 produces the expected bands. An attempt was made to test whether NS1 induces a processive degradation activity that acts on linear DNA unless it is protected. Therefore, a linearized pUC19 DNA was cotransfected with pLuH6 ($NS1^-$) or pP38Lu2ΔNd ($NS1^+$) (Fig. 5, lanes 12 and 13). The 2.7-kb band of pUC19 produced a strong signal, suggesting no evidence of degradation comparable to that of the pUC19 DNA excised from pGLu883 in vivo (Fig. 5, lane 8 or 10).

Plasmid pGLu2ΔNd has an internal deletion within the right-end palindrome that leaves a smaller palindrome of about 60 base pairs as compared with the normal 120 base pairs. pGLu2ΔNd was tested for excision at the right end (Fig. 6). Plasmid pGLu883 with intact palindromes at both ends shows excision at both ends and has not shown any evidence of excision at one end and not the other (Fig. 6, lane 3, or Fig. 5, lane 8). In contrast, pGLu2ΔNd gives a clear band at 6.6 kb and the 3.9-kb right-end *Bgl*II fragment is reduced in amount compared with the left-end fragment. pGLuΔNd, which has a more severe deletion and has no inverted repeat sequences at the right end, produced little signal in this experiment and the 1.2-kb left-end *Bgl*II fragment is barely visible in lane 4. If there was any excision at the right end with pGLuΔNd as manifest by a 3.9-kb right-end *Bgl*II fragment, it was not detectable in any of these experiments (Fig. 6, lane 4; Fig. 4, lane 11; or Fig. 5, lane 1). This result shows that excision at the deleted right end is reduced but still occurs when part of the inverted repeat is present, but probably not when the inverted repeat is absent.

Excision requires DNA polymerase. The excision reaction was tested for a requirement for DNA polymerase activity by assessing its sensitivity to the inhibitor of DNA polymerases α and δ, aphidicolin. Cultures were transfected with pGLu883 and a sample was taken at 4 h, immediately after the glycerol shock, and at 26 h, with and without treatment with 20 μM aphidicolin from 4 to 26 h (Fig. 7A). The DNA samples were restricted with *Bgl*II and visualized by Southern blot hybridization. Aphidicolin clearly inhibited the appearance of the 3.9- and 1.2-kb *Bgl*II bands at 26 h posttransfection. However, a light band at the 6.6-kb position was apparent in the drug-treated culture (Fig. 7A, lane 2). This suggested that some excision might have occurred at the left end and that it may be more resistant to the drug than excision at the right end or have occurred before the drug was applied. The reason that the predicted 1.2-kb left-end *Bgl*II fragment is not also visible in lane 2 is not clear. If the 6.6-kb band represents excision at the left end, then plasmid pLudl should show excision at both ends in the presence of aphidicolin and both pLudl and pGLu883 should produce 4.3-kb bands when restricted with *Nhe*I instead of *Bgl*II. The results of this experiment are shown in Fig. 7B. Both pLudl and pGLu883 produced the 4.3-kb left-end fragment in the untreated culture and not in the aphidicolin-treated culture.

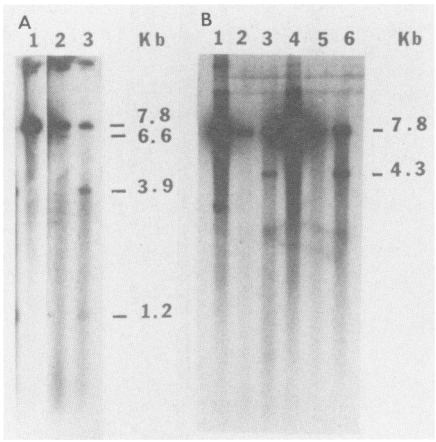

FIG. 7. (A) Southern blot analysis of cultures transfected with pGLu883 and treated with aphidicolin (lane 2) of dimethyl sulfoxide only (lane 3) from 4 to 26 h posttransfection. The samples were collected at 4 h posttransfection (lane 1) or 26 h posttransfection (lanes 2 and 3). The DNA was restricted with *Bgl*II before electrophoresis on a 1% gel. (B) Southern blot analysis as in panel A with cultures 2 and 5 treated with aphidicolin. Plasmids transfected: pGlu883, lanes 1 to 3; pLudl, lanes 4 to 6. The samples were collected at 4 h posttransfection in lanes 1 and 4 and at 26 h posttransfection in lanes 2, 3, 5, and 6. The DNA was restricted with *Nhe*I before electrophoresis on a 1% gel.

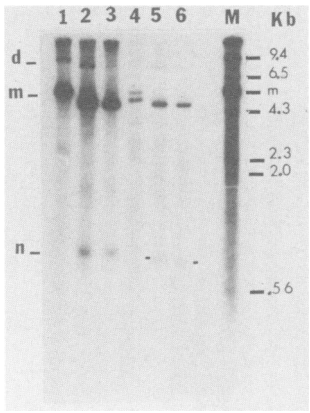

FIG. 8. Southern blot analysis of viral DNA extracted from HeLa cell cultures infected with LuIII virus (lanes 1 to 3) or Ludl virus (lanes 4 to 6). Cultures were inoculated with 200 μl of LuIII virus or 300 μl of Ludl virus (passage 2 in NB cells) and viral DNA was extracted at 20 h postinfection. Samples of the DNA were treated with the following: lanes 1 and 4, no treatment; lanes 2 and 5, *Nhe*I; lanes 3 and 6, *Nhe*I plus *Bss*HII. The gel is 1.5% agarose and the markers (M) are as in Fig. 6. d, Dimer; m, monomer; n, 0.9-kb *Nhe*I fragment.

Thus, it is clear that aphidicolin inhibited excision at both termini.

Excision is at both left-end palindromes in Ludl virus. Plasmid pLudl generates infectious virus after transfection of HeLa cells and this virus produces a complete cytopathic effect in NB cells. I have also noted that plasmids pGLu2ΔNd and pGLuΔNd can generate infectious virus as well. In the case of pGLuΔNd, there is no right-end inverted repeat sequence, so how this DNA generates infectious virus is unknown. In this experiment the structure of the right end of Ludl virus was examined to determine whether the left-end palindrome on the right end was stable or had undergone a rearrangement and whether it was used in the hairpin transfer process. Cultures were infected with LuIII or Ludl virus and viral DNA was extracted at 20 h postinfection. The right-end fragments were visualized by restriction with *Nhe*I and Southern blot hybridization (Fig. 8). The right-end *Nhe*I fragment of Ludl virus was about 40 base pairs smaller than the *Nhe*I fragment of LuIII virus, as predicted for an extended left-end hairpin. In a similar experiment the right-end foldback conformation was also visualized; it represented about 40% of the total right-end fragments and it had a mobility indicating a size of about 60 base pairs smaller than the extended conformation (data not shown). Restriction of Ludl virus with *Nhe*I and *Bss*HII, which cuts the left-end inverted repeat of LuIII virus at nucleotides 53 and 58 (7a), reduced the size of the fragment by about 60 base pairs. These results confirm that the left-end inverted repeat fused to the right end in pLudl remains on the right end of Ludl viral DNA and that the extended right-end fragment terminates at the end of the left-end inverted repeat sequence rather than the remaining portion of the right-end palindrome. If the latter occurred, the total hairpin size would be larger and *Bss*HII would reduce the fragment by 141 base pairs (62 from the right-end palindrome plus nucleotides 122 to 145 plus 58 from the left-end palindrome) rather than 58. It should also be noted that Ludl virus generates dimer RF DNA linked at the left end, despite the symmetry of its termini.

DISCUSSION

The first evidence that both the autonomous parvoviruses and the dependoviruses (AAV) required a viral protein for DNA replication consisted of the observation that defective interfering particles or deletion mutants required a helper parvovirus for DNA replication (8, 18, 19). This function, called *rep*, was required in *trans* for viral DNA replication. *rep* was mapped to the left open reading frame, which codes for the nonstructural proteins in both types of parvoviruses, by construction of mutations within infectious clones of AAV or minute virus of mice (MVM) (11, 16, 31). In addition, temperature-sensitive mutants of MVM and Kilham rat virus that have temperature-sensitive viral DNA synthesis have been isolated and the mutations are known to map to the NS1 gene (33, 37). A comparison of the amino acid sequences of a number of parvovirus nonstructural proteins has shown a highly conserved central domain that has a consensus purine triphosphate nucleotide-binding domain (1, 2). These results suggest that the role in replication for *rep* or NS1 is similar for all of the known parvoviruses.

Mutations in the *rep* gene of both AAV and MVM inhibited the subsequent excision and replication of viral sequences from plasmid clones (11, 25–27, 31). In these experiments, it was not clear whether the defect was at the excision step, replication, or both. A cell enzyme has been identified that can catalyze the excision of AAV from the plasmid, but this activity may be specific for AAV that was cloned using a C-tailing method (9). The construction of an AAV clone that allowed the viral genome to be precisely excised with restriction enzyme *Pvu*II confirmed that *rep* mutations failed to replicate viral DNA even if it was preexcised from the plasmid (25). These mutations also prevented rescue of the viral sequences from plasmid sequences. These results suggested that *rep* functions during both excision and replication. Direct evidence that NS1 acts during viral DNA synthesis is the finding that NS1 is the protein covalently bound to the 5′ termini of RF DNA and

viral DNA (7, 10). This implies that NS1 is the postulated site-specific nickase in the hairpin transfer process that occurs during viral DNA replication (6).

In this study I have shown that NS1 is required for the excision and replication of the cloned genome of the autonomous parvovirus LuIII virus. Plasmid pGLuΔN has had the entire NS1 gene deleted. The deleted viral genome in pGLuΔN was excised and replicated only if an NS1/NS2 gene was supplied in *trans*. This activity was prevented by a frameshift mutation in the NS1 gene. The LuIII virus replicon was able to replicate with the NS1 gene provided by either LuIII virus or H-1 virus or a chimeric NS1 gene that was half H-1 virus and half LuIII virus. The plasmid with the frameshift in the NS1 gene, pLuH6, showed no evidence of excision or replication. pLuH6 was shown to be capable of replication when NS1 was supplied in *trans* (data not shown). It is possible that transfected DNA enters several compartments in the cell and that excision, replication, degradation, or integration takes place in only one of these compartments, such as the nucleus. Thus, the viral genome in pLuH6 might be excised and be promptly degraded in the absence of NS1. The results presented here do show that a processive degradation does occur on the vector side of an excision event. This implies that the viral sequences, but not the vector sequences, may be protected after excision. This result is in agreement with NS1 being the terminally bound protein, which protects the RF DNA from 5′-to-3′ exonuclease activity, but not 3′-to-5′ exonuclease activity in vitro (7). Whether NS1 is terminally bound after excision or not remains to be formally demonstrated. The fact that the linearized pUC19 DNA was not destroyed (Fig. 5) is not conclusive since what remains of linearized pUC19 at 48 h posttransfection may be in a different cellular compartment. Nevertheless, plasmid pGLuΔNd in Fig. 3 and 5 has shown some survival of full-length linear plasmid that has undergone an excision event at only the left end. Because pGLuΔNd is deleted of 75% of the right-end palindrome and has no inverted repeat remaining there, it would not be expected to amplify its DNA even though the *Dpn*I resistance of a portion of the full-length linear DNA indicates that some DNA synthesis has occurred. This synthesis is presumably of one strand, using the intact viral left end as an origin. On the basis of the results with pGLuΔNd, excision of viral DNA from pLuH6 would be detectable even without replication of the excised DNA. Thus, it can be concluded that NS1 is required for the excision of viral sequences from the plasmid.

I have also tested whether DNA polymerase α or δ is required for excision by using the inhibitor aphidicolin. The results clearly indicated that the excision is blocked by 20 μM aphidicolin. We have obtained evidence that the bulk of parvovirus H-1 DNA replication in permeabilized cells is resistant to the inhibitor of polymerase α, N^2-(*p*-*n*-butylphenyl)deoxyguanidine triphosphate, implying that it is carried out by polymerase δ (S. Rhode III and J. K. Vishwanatha, unpublished data). Since the permeabilized cells can be expected to carry out only an elongation of already-initiated replicative intermediates, this result has no implications as to which aphidicolin-sensitive polymerase may be required for excision.

Previous results have indicated that the *cis* sequences required for parvovirus DNA replication are contained in or near the terminal inverted repeat sequences (29). Defective interfering particles that can be serially passaged have been described that are as small as 500 base pairs with intact terminal palindromes (8). The inverted repeats of AAV have been modified by site-specific mutagenesis and shown to be critically required for replication (5, 15). In this case the shape of the foldback structure appears to be more important than its exact sequence. In this study the deletion of the middle portion of the right-end inverted repeat in plasmid pGLu2ΔNd markedly decreased excision at the right end and decreased replication, but it did not eliminate them entirely. On the other hand, plasmid pLudl, which has the same right-end inverted repeat sequences as pGLu2ΔNd, but also has a complete copy of the left-end inverted repeat, excises and replicates with about the same efficiency as the wild-type genome in pGLu883. An important question to be addressed is why Ludl virus executes the hairpin transfer with the left-end hairpin sequence in preference to the residual right-end sequence. If it is because the nickase acts at the site most proximal to the hairpin axis of symmetry, then this will have implications for the mechanism. An interesting observation with pLudl is that it produces predominantly dimer RF DNA linked at the left end despite the symmetry of the termini. In contrast, AAV produces dimer RF DNA linked in roughly equal proportions at either end (27). Thus, Ludl virus preserves the predominance of the dimer bridge fragment at the left end seen with LuIII virus, MVM, or H-1 virus, even with the left-end palindrome on both ends. This asymmetry is not easily rationalized with our current information. It is possible that the A+T-rich region at the right end influences the conversion of the right-end foldback conformation to the extended conformation so that the right end is predominantly in the extended conformation for wild-type DNA. Sequences rich in A+T base pairs can influence the structure of adjacent inverted repeats in supercoiled plasmids (34), but whether this effect would pertain to a linear DNA is not known. Against this hypothesis is the observation that the proportion of foldback to extended configuration at the right end of Ludl virus was the same as that found at a wild-type left end. Ludl virus does not produce as much virus as does wild-type LuIII virus (unpublished data), suggesting that a defect other than DNA replication, such as packaging of viral DNA, is affected by the deletion of the right-end sequences. In support of this interpretation, I have evidence that sequences for packaging the plus strands of LuIII virus are in the right end (unpublished data).

In summary, NS1 has been found to be required for the excision and replication of cloned autonomous parvovirus DNA, in agreement with previous results with AAV. The excision reaction proceeded with similar rates at both ends when wild-type terminal hairpins were present. Excision was blocked by aphidicolin, suggesting a requirement for DNA polymerase α or δ as well. Thus, replication and excision are closely coupled in this system. After excision the viral sequences, but not the vector sequences, were protected from a processive degradation, which is presumed to have a 5′-to-3′ exonuclease component. It is likely that NS1 is terminally bound to viral sequences or the hairpin is in the foldback configuration after the excision. The mechanism of the excision reaction is probably complex. I expect that excision incorporates at least some of the steps carried out during normal viral DNA replication, hence its requirement for DNA polymerase and NS1. Whether the strong stop for polymerase previously observed in the right-end palindrome plays a role is not clear (23, 36). The apparent requirement for a potential cruciform conformation for excision suggests that a resolution of a cruciform by homologous recombination, which could release the viral sequences in a foldback configuration, might be a possible mechanism.

ACKNOWLEDGMENTS

I thank R. Bates for providing plasmids pGLu883 and pLSma13 and sequence information on LuIII virus before publication. I also thank J. Giam for reviewing the manuscript and J. deVasure for expert technical assistance.

This work was supported by grant 89-50 from the State of Nebraska Department of Health, Public Health Service grant CA36727 from the National Institutes of Health, American Cancer Society grant ACS SIG-16, and funds from the Elizabeth Bruce and Parents Memorial Endowment.

LITERATURE CITED

1. **Anton, I. A., and D. P. Lane.** 1986. Non-structural protein 1 of parvoviruses: homology to purine nucleotide using proteins and early proteins of papovaviruses. Nucleic Acids Res. **14**:7813.
2. **Astell, C. R., C. D. Mol, and W. F. Anderson.** 1987. Structural and functional homology of parvovirus and papovavirus polypeptides. J. Gen. Virol. **68**:885–893.
3. **Berns, K. I.** 1984. The parvoviruses, p. 1–406. Plenum Publishing Corp., New York.
4. **Berns, K. I., R. M. Kotin, and M. A. Labow.** 1988. Regulation of adeno-associated virus DNA replication. Biochim. Biophys. Acta **951**:425–429.
5. **Bohenzky, R. A., R. B. LeFebvre, and K. I. Berns.** 1988. Sequence and symmetry requirements within the internal palindromic sequences of the adeno-associated virus terminal repeat. Virology **166**:316–327.
6. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**: 91–174.
7. **Cotmore, S. F., and P. Tattersall.** 1988. 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. J. Virol. **62**:851–860.

7a. **Diffoot, N., B. C. Shull, K. C. Chen, E. R. Stout, M. Lederman, and R. C. Bates.** 1989. Identical ends are not required for the equal encapsidation of plus- and minus-strand parvovirus LuIII DNA. J. Virol. **63**:3180–3184.

8. **Faust, E. A., and D. C. Ward.** 1979. Incomplete genomes of the parvovirus minute virus of mice: selective conservation of genome termini, including the origin for DNA replication. J. Virol. **32**:276–292.
9. **Gottlieb, J., and N. Muzyczka.** 1988. In vitro excision of adeno-associated virus DNA from recombinant plasmids: isolation of an enzyme fraction from HeLa cells that cleaves DNA at poly(G) sequences. Mol. Cell. Biol. **6**:2513–2522.
10. **Gunther, M., and P. Tattersall.** 1988. The terminal protein of minute virus of mice is an 83 kilodalton polypeptide linked to specific forms of double-stranded and single-stranded viral DNA. FEBS Lett. **242**:22–26.
11. **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.
12. **Hirt, B.** 1963. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:266–268.
13. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7**:1320–1325.
14. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
15. **LeFebvre, R. B., S. Riva, and K. I. Berns.** 1984. Conformation takes precedence over sequence in adeno-associated virus DNA replication. Mol. Cell. Biol. **4**:1416–1419.
16. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
17. **Rhode, S. L., III.** 1977. Replication process of the parvovirus H-1. VI. Characterization of a replication terminus of H-1 replicative-form DNA. J. Virol. **21**:694–712.
18. **Rhode, S. L., III.** 1978. Defective interfering particles of the parvovirus H-1. J. Virol. **27**:347–356.
19. **Rhode, S. L., III.** 1982. Complementation for replicative form DNA replication of a deletion mutant of H-1 by various parvoviruses. J. Virol. **42**:1118–1122.
20. **Rhode, S. L., III.** 1985. *trans*-activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
21. **Rhode, S. L., III.** 1987. Construction of a genetic switch for inducible *trans*-activation of gene expression in eucaryotic cells. J. Virol. **61**:1448–1456.
22. **Rhode, S. L., III.** 1987. Characterization of the *trans*-activation-responsive element of the parvovirus H-1 P38 promoter. J. Virol. **61**:2807–2815.
23. **Rhode, S. L., III, and B. Klaassen.** 1982. DNA sequence of the 5′ terminus containing the replication origin of parvovirus replicative form DNA. J. Virol. **41**:990–999.
24. **Rhode, S. L., III, and P. Paradiso.** 1983. The parvovirus genome: nucleotide sequence of H-1 and the mapping of its genes by hybrid-arrested translation. J. Virol. **45**:173–184.
25. **Samulski, R. J., L.-S. Chang, and T. Shenk.** 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. J. Virol. **61**:3096–3101.
26. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correlation within the terminal repeats of AAV. Cell **33**:135–143.
27. **Senapathy, P., J.-D. Tratschin, and B. J. Carter.** 1984. Replication of adeno-associated virus DNA: complementation of naturally occurring *rep*$^-$ mutants by a wild-type genome or an *ori*$^-$ mutant and correction of terminal palindrome deletions. J. Mol. Biol. **179**:1–20.
28. **Shein, H. M., and J. F. Enders.** 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495–500.
29. **Shull, B. C., K. C. Chen, M. Lederman, E. R. Stout, and R. C. Bates.** 1988. Genomic clones of bovine parvovirus: construction and effect of deletions and terminal sequence inversions on infectivity. J. Virol. **62**:417–426.
30. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5**:1939–1944.
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. **Tratschin, J.-D., J. Tal, and B. J. Carter.** 1986. Negative and positive regulation in *trans* of gene expression from adeno-associated virus vectors in mammalian cells by a viral rep gene product. Mol. Cell. Biol. **6**:2884–2894.
33. **Tullis, G. E., L. Labieniec-Pintel, K. E. Clemens, and D. Pintel.** 1988. Generation and characterization of a temperature-sensitive mutation in the NS-1 gene of the autonomous parvovirus minute virus of mice. J. Virol. **62**:2736–2744.
34. **Wang, Y., and W. Sauerbier.** 1989. Flanking AT-rich sequences may lower the activation energy of cruciform extrusion in supercoiled DNA. Biochem. Biophys. Res. Commun. **158**: 423–431.
35. **Ward, D. C., and P. Tattersall.** 1978. Replication of mammalian parvoviruses, p. 1–526. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Weaver, D. T., and M. L. DePamphilis.** 1984. The role of palindromic and non-palindromic sequences in arresting DNA synthesis in vitro and in vivo. J. Mol. Biol. **180**:961–986.
37. **Wicker, R., and M. Gunther.** 1988. Isolation and characterization of thermosensitive mutants from Kilham rat virus, a rodent parvovirus. J. Gen. Virol. **69**:163–175.