Parvovirus NS1 Stimulates P4 Expression by Interaction with the Terminal Repeats and through DNA Amplification

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Parvovirus protein NS1 is required for replication of viral DNA and plays a role in the regulation of viral gene expression. NS1 *trans*-activates the P38 promoter for capsid protein synthesis and has variable effects on other promoters. In this study, we examined the effects of NS1 on the regulation of its own promoter, P4. A number of plasmid constructions were made with the P4 promoter fused to reporter genes. The effects of NS1 on expression from the P4 promoter differed depending on the construction. Plasmids containing viral sequences which could not replicate showed a decrease in P4 expression on cotransfection with the NS1 gene. However, plasmids having replication-proficient viral sequences showed a three- to fivefold increase in P4 expression dependent on NS1. The effect on NS1 on P4 transcription was also evaluated at the steady-state RNA level. An infectious clone of the LuIII viral genome was modified to an NS1-NS2 null mutant (pLu272) that is competent for viral DNA replication by introducing a frameshift mutation at codon 5 of the NS1 open reading frame. The P4 transcripts of pLu272 are four nucleotides longer than the wild type and can therefore be resolved from the wild type by primer extension analysis. pLu272 allows comparison of the constitutive level of steady-state RNA produced by the pLu272 P4 promoter in the absence or presence of a template replication dependent on NS1 supplied in *trans*. NS1 increased P4 transcripts about six- to eightfold. Expression of P4 transcripts from clones that could not amplify depended on the presence of an intact inverted terminal repeat sequence at the left end. A clone with an intact viral left end and a defective viral right end gave an NS1-dependent threefold increase in P4 expression. Destruction of terminal hairpins at both ends resulted in no significant increase in P4 expression in the presence of NS1. Thus, the positive effect of NS1 on the steady-state levels of P4 transcripts depends on the amplification of gene copy number and the integrity of the terminal repeats.

Parvoviruses are small viruses containing a linear, single-stranded DNA genome of approximately 5 kb. The range of hosts susceptible to parvoviruses is very broad, from invertebrates to vertebrates, including humans (11, 47). Parvoviruses are divided into two groups, dependoviruses and autonomous parvoviruses. Dependoviruses, represented by adeno-associated virus (AAV), require coinfection of a helper virus, such as adenovirus or herpesvirus, to establish a lytic infection. However, recent studies have shown that a helper virus is not required in certain cellular systems (36, 37, 51, 54). Autonomous or self-replicating parvoviruses do not require coinfection of a helper virus for production of a lytic infection. Representatives of autonomous parvoviruses include rodent parvoviruses, such as minute virus of mice (MVM), H-1, rat or LuIII, canine parvovirus, porcine parvovirus, and B19 (human) (41).

The small size of the parvovirus genome greatly restricts the number of different proteins which can be encoded by the virus. The genome can basically be divided in half, with the left-handed portion coding for the nonstructural proteins and the right-hand portion coding for the viral capsid proteins. Requirements for parvovirus replication include NS1 or *rep* proteins provided by the virus (17, 24, 34, 35, 50) and S-phase specific cellular factors provided by a permissive cell host (11, 27, 28, 31, 44, 46, 53). Parvovirus replication has also been shown to be highly tropic for fetal cells (23, 40, 41, 43, 48, 49). The requirement for S-phase cells for

parvovirus replication and the fetal tropism displayed by parvoviruses make this group of viruses an intriguing research tool for the study of mammalian DNA replication and early developmental regulation.

The functions of NS1 or *rep* proteins during replication are similar in dependoviruses and autonomous parvoviruses. NS1 is required not only for viral replicative-form DNA replication but also for excision of cloned viral sequences from the plasmid (23a, 31). NS1 has also been found covalently attached to the 5' end of nearly all intracellular viral DNAs (12). This observation suggests the involvement of NS1 as the site-specific nickase required for hairpin transfer in the current model for parvovirus replication (3, 45). This has been formally confirmed with *in vitro* studies of AAV replication (19, 42). Regulation of parvoviral transcription is an important aspect of parvovirus replication because of the requirement for NS1 during replication and host cell variations in the level of NS1 expression (9, 10).

Several groups have documented that expression from the early (nonstructural genes) and late (capsid gene) parvovirus promoters is influenced by the presence of NS1. An increase in transcriptional activity from the late promoter in the presence of NS1 (*rep*) has been described for H-1 (30), MVM (16), and AAV (20, 51, 52). The effects of NS1 on P4 expression reported in the literature seem to conflict somewhat. NS1 (*rep*) has been shown to inhibit expression from the p5 and p19 promoters of AAV in the absence of helper virus (6) but stimulate p5 and p19 transcription in the presence of adenovirus infection (20). Expression from the P4 promoter of MVM increases in response to NS1 (15), but

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NS1 has no effect on expression from the P6 promoter of B19 (25).

In this report, we present data which show that the amount of P4 transcript produced by the early promoter is positively regulated by NS1 as a result of genomic amplification and NS1-dependent interactions with the left-end inverted terminal repeat.

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MATERIALS AND METHODS

Cells and virus. HeLa Gey cells (ATCC CCL2.1) and NB cells, a simian virus 40-transformed human kidney cell line, were used for transfections (39). LuIII virus was generated by transfection in HeLa cells with infectious clone plasmid pGLu883 and then propagation of the culture lysate in NB cells. pGLu883 from the laboratory of B. Bates was supplied by N. Diffoot. HaK1 cells (ATCC CCL15) were used to propagate H-1 virus (26).

Plasmid constructions and purifications. Plasmids were constructed by using standard techniques. Agarose or polyacrylamide electrophoresis was used to purify and isolate fragments before ligations. Enzymes were purchased from New England Biolabs, Inc., Beverly, Mass. Plasmids were isolated by standard lysis methods and purified on cesium chloride gradients. All of the plasmids used in the replication assay were propagated in *Escherichia coli* MV1190 [$\Delta(lac-proAB)$ *thi supE* $\Delta(srl-recA)306::Tn10(Tet^r)$ (*F'* *traD36 proAB lacI^qZAM15*)] from Bio-Rad Laboratories, Richmond, Calif.

The pP4cat clone was constructed from pSR1, a genomic clone of H-1 virus which has been described previously (Fig. 1B) (29). The pP4cat construct contains the H-1 P4 promoter, a cap site, the natural 5' untranslated leader sequence of the NS1-NS2 transcript to nucleotide 264 plus a *Bam*HI linker and 95 extra base pairs in the leader from H-1 nucleotides 2560 to 2655 (*Bcl*II-*Hind*III), and the *cat* gene (2). The *cat* gene was obtained from pH5cat (29) as a *Bcl*II-*Ava*I fragment. pP4cat is replication negative, presumably because of lack of right-end sequences and rearrangements in the left-end hairpin sequences during the original cloning of pSR1. pH5 and pH6 have been described previously (33). pH5 codes for wild-type NS1 and contains H-1 sequences 1 to 2657 and 4688 to 5176. pH6 codes for a truncated, nonfunctional NS1. The truncated protein is a result of a frameshift insertion at the *Bgl*II site at nucleotide 1250 in the NS1 gene of pH5.

The P4 promoter of pP4catB is the same as that described for pP4cat. The *cat* cassette used in this construction contained *Bam*HI linkers at both ends. Therefore, the extra 95 bp found in the leader region of the pP4cat transcript are not found in pP4catB.

pLu272 was made by restricting pGLu883 with *Mlu*I, which cleaves uniquely at nucleotide 272, blunting the ends with Klenow, and religating (Fig. 1A). This procedure resulted in a new restriction site, *Bss*HII, and a four-base insertion at bp 272. The four-base insert caused a frameshift mutation of the NS1-NS2 reading frame, which now terminates at codon 9, and increased the transcript size by four bases compared with the wild-type transcript. This plasmid contains a replication-competent LuIII genome but is a null mutant for NS1 and NS2.

pLu272 Δ Nd was constructed by restriction of pLu272

FIG. 1. (A) Diagrams of the genomic clone of LuIII virus, pGLu883, and the plasmids derived from it. pLu272 has a four-base insertion at nucleotide 272 (*Mlu*I), creating a new restriction site, *Bss*HII. This frameshift mutation on the NS1-NS2 open reading frame increases the size of the transcript by four bases compared with the wild-type transcript. pLu272 Δ tar is pLu272 deleted from the *Bgl*II site at nucleotide 1246 to the *Bst*EII site at nucleotide 1887. The deletion removes 641 bp within the NS1 gene, including the domain for the putative ATP-binding site of NS1 (4) and the *tar* region of the P38 promoter (33). pGLu Δ Nd is deleted from the inboard *Nde*I site of LuIII virus to the *Nde*I site of pUC19 (161 bp of the LuIII virus) and has previously been described (31). Abbreviations: B, *Bam*HI; Bst, *Bst*EII; St, *Sty*I; N, *Nco*I; E, *Eco*RI; Bg, *Bgl*II; H, *Hind*III; Nh, *Nhe*I; Nd, *Nde*I; M, *Mlu*I. (B) Diagrams of the P4 promoter constructs used in reporter gene assays. The cap site for H-1 P4 transcripts is nucleotide 208. pP4cat has an additional 95 bp in the leader sequence of the NS1-NS2 transcript corresponding to bp 2560 to 2655 of the H-1 genome. Abbreviations: C, *Clal*I; H, *Hind*III; S, *Sac*I; St, *Sty*I; X, *Xho*I. (C) Comparison between the wild-type (wt) P4 promoter sequence (bp 73 to 212) of H-1 and the synthetic promoter, LP4. The asterisks represent mismatches between the two sequences. Restriction sites are labeled and indicated in boldface type.

with *Nde*I, gel purification of the large fragment, and then religation with T4 DNA ligase. The resulting plasmid is deleted from the inboard *Nde*I site of the LuIII right-end terminal repeat to the *Nde*I site of pUC19. This is the same strategy used for construction of pGLu Δ Nd as described by Rhode (31). pLu272 Δ Nd is deficient in replication because of destruction of the right-end inverted repeat and is a null mutant for NS1 and NS2, as described for pLu272.

pGLu Δ BE was constructed from pGLu Δ Nd (31) by restricting the plasmid to completion by using the *Sall* and *Bss*HII restriction enzymes. The ends were blunted by using T4 DNA polymerase, and the fragment was religated. This deletion removes the first 58 bases from the parvovirus genome, destroying the left-end inverted repeat, and 13 bases of polylinker from the vector (pUC19). pGLu Δ BE is

NS1 positive but deficient in replication because of the deletions in both terminal repeats.

pLu272 Δ BE was constructed from pGLu Δ BE as described for pLu272 (see below). This plasmid is replication deficient and is a null mutant for NS1 and NS2, as described for pLu272.

pLu272 Δ tar was constructed by partial restriction of pLu272 with *Bst*EII (nucleotide 1887), followed by complete restriction with *Bgl*II (nucleotide 1246). The resulting fragment was blunted by using T4 polymerase and then religated. This deletion removes 641 bp within the NS1 gene, including the domain for the putative ATP-binding site of NS1 (4) and the *tar* region of the P38 promoter (33). Because the nucleotide sequence of the LuIII genome has not been published, these nucleotide positions are based on the corresponding nucleotide numbers in the H-1 sequence. This plasmid is replication competent and NS1 negative.

LP4cat is a clone of a synthetic H-1 P4 promoter corresponding to bp 73 to 212 (Fig. 1C). Several point mutations were included in the synthetic P4 promoter to give two unique restriction sites. Nucleotides 136 and 138 were changed to C (plus strand), resulting in a *Sac*I restriction site, while nucleotides 173 to 176 were changed from AACT to CGAG to create an *Xho*I restriction site immediately 5' to the TATA box. The synthetic promoter was synthesized by using two overlapping oligonucleotides. The oligonucleotides corresponded to a positive strand of 90 bases and a negative strand of 81 bases. Restriction sites were incorporated at the ends for cloning purposes, i.e., a *Cla*I site at the 5' end and a *Hind*III site at the 3' end of the promoter fragment. The 3' ends of the single-stranded oligonucleotides form a 13-base overlap corresponding to bp 241 to 253 of the H-1 sequence. Desalting and purification by gel electrophoresis of the single-stranded oligonucleotides were performed as suggested by Applied Biosystems Inc. Once purified, the positive and negative strands were hybridized by being heated to 65°C and slowly cooled to room temperature. DNA polymerase I (Klenow fragment) was used to convert the DNA to a double-stranded fragment, and phosphorylation was achieved by treatment with polynucleotide kinase. The synthetic promoter was cloned into the Bluescript vector (pBS-; Stratagene), which had been restricted with *Sma*I. The fragment was then subcloned into pP4cat, which had been restricted with *Cla*I and *Hind*III, replacing the wild-type P4 sequence. The synthetic promoter results in a 5' untranslated region 42 bases shorter than the natural leader of the NS1-NS2 transcript.

pLuP4cat and pLuP4luc contain the LuIII left-end hairpin and P4 promoter sequences corresponding to bp 1 to 143 (14). This represents the *Kpn*I-*Sty*I fragment from pGLu883. The remainder of the promoter, including the TATA box, corresponds to the *Sty*I-*Hind*III fragment from the synthetic promoter, LP4 (Fig. 1). The *cat* gene was cloned from LP4cat as an *Xho*I-*Bam*HI fragment, and the luciferase gene was cloned from pSVOALS' (13) as a *Hind*III-*Bam*HI fragment. pGLu Δ ND has already been described (31). pSRS405 is a replication-negative, NS1-negative derivative of pSR1 and was constructed in our laboratory by Xu Li (21).

DNA transfections and CAT assays. DNA transfections were carried out by one of two methods. HeLa Gey cells were transfected at a density of 3×10^5 to 5×10^5 cells per 60-mm-diameter dish or 10^6 cells per 100-mm dish by using the CaPO₄ precipitation method described previously (33). Samples for chloramphenicol acetyltransferase (CAT) and luciferase determinations were collected from 60-mm dishes at 48 to 72 h posttransfection. CAT assays were carried out

as previously described (30), and the plates were scraped and quantitated by scintillation counting. Luciferase assays were carried out as described by de Wet et al. (13), with a Monolight 2010 luminometer.

NB cultures were transfected by using DEAE-dextran at a final concentration of 1 mg/ml. NB cultures were transfected at the same cell density as the HeLa cells described above. Transfection conditions for a 60-mm dish were as follows. Cells were washed four times with Hanks balanced salt solution and once with Tris-buffered saline (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 50 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM Na₂HPO₄). Plasmid DNA was mixed with 100 μ l of Tris-buffered saline, DEAE-dextran, and 1.6 ml of serum-free Dulbecco's modified Eagle's medium. This mixture was added to the washed cells and incubated for 3 h at 37°C. After incubation, the cells were washed twice with Hanks balanced salt solution and fresh medium with 5% fetal bovine serum was added. For transfections using 100-mm dishes, the components of the transfection mixture were doubled.

RNA isolation and primer extension. Total RNA was isolated from 100-mm dishes by using the acid guanidinium phenol chloroform method described by Chomczynski and Sacchi (8). Total RNA was redissolved in 10 mM Tris-HCl (pH 8)–1 mM EDTA, and the concentration was determined by using a Hitachi U-2000 spectrophotometer. Primer extension experiments were done by using 15 to 50 μ g of total RNA from transfections and 1.5 to 5 μ g of total RNA from infections as previously described (5). Primers were synthesized with an Applied Biosystems 380B DNA synthesizer. H-1 P4 transcripts were detected by using a primer corresponding to bases 232 to 260 of the H-1 genome. LuIII P4 transcripts were detected with a primer corresponding to bases 281 to 311 of the LuIII genome by using sequence data provided by N. Diffoot. Samples were fractionated on an 8% polyacrylamide–7 M urea gel with 16- by 20-cm plates or 38.1- by 43-cm sequencing plates. Single-stranded synthetic oligonucleotides were end labeled with [γ -³²P]ATP and used for specific size markers.

Replication assay. HeLa Gey cells were transfected as described above, and low-molecular-weight DNA was extracted by the method of Hirt (18) at 48 h posttransfection. Treatment of the DNA samples, fractionation, Southern blot transfer, and hybridization have been previously described (31).

RESULTS

P4 expression in the presence of NS1. NS1 or *rep* protein has been shown to have a negative effect on the expression of several types of promoters, including the Rous sarcoma virus long terminal repeat, the Harvey-*ras* promoter, the simian virus 40 early gene promoter (31a, 33), and the nonstructural gene promoters of AAV, p5, and p19 (6). We too showed inhibition of the H-1 P4 promoter by NS1 but found the inhibition to be dependent upon the plasmid construction. Transfections using two different P4-controlled reporter genes, those for CAT and luciferase, in the presence or absence of a functional NS1 gene, gave conflicting results, depending on the particular P4 promoter. The experiment reported in Table 1 examines the effects of NS1 on P4 expression from plasmids which differ in their left-end sequences (functional for replication, pLuP4cat and pLuP4luc; nonfunctional for replication, pP4cat and pP4catB; absent, pLP4cat) and in the lengths of the leader sequences for the CAT transcripts (Fig. 1B and Materials and Methods).

TABLE 1. Reporter gene expression from P4 plasmids with and without NS1^a

Plasmid construct	CAT activity (% acetylation) ^b	Luciferase activity (RLU, 10 ³) ^b	Ratio ^c of CAT activities, +NS1/-NS1	Ratio ^c of luciferase activities, +NS1/-NS1	CAT/luciferase ratio
1: pP4cat, pUC19, pLuP4luc	23.06	19.3			1.20
2: pP4cat, pH5, pLuP4luc	17.72	145.3	0.90	5.9	0.12 ^d
3: pP4cat, pH6, pLuP4luc	19.62	24.8	1	1	0.79 ^e
4: pP4catB, pUC19, pLuP4luc	6.95	9.1			0.76
5: pP4catB, pH5, pLuP4luc	3.09	37.6	0.39	4.3	0.08 ^d
6: pP4catB, pH6, pLuP4luc	8.01	8.7	1	1	0.92 ^e
7: pLP4cat, pUC19, pLuP4luc	27.48	28.6			0.96
8: pLP4cat, pH5, pLuP4luc	48.95	170.7	1.50	4.7	0.29 ^d
9: pLP4cat, pH6, pLuP4luc	31.72	36.1	1	1	0.88 ^e

^a HeLa cell monolayers were transfected by calcium phosphate precipitation by using the combinations of P4 plasmids listed in the presence (pH5) or absence (pH6) of a functional NS1 gene. Plasmids were transfected by using 2 µg of each CAT plasmid, 0.5 µg of pLuP4luc, and 0.2 µg of pUC19, pH5, or pH6.

^b CAT and luciferase assays were performed at 48 h posttransfection. RLU, relative light units.

^c Values were normalized to P4 expression in the absence of a functional NS1 gene (pH6).

^d Because of the stimulation of pLuP4luc by NS1, the denominator for this ratio is large compared with CAT expression. Thus, the ratios can be compared only within the group of samples containing NS1 (pH5).

^e pH6 does not stimulate pLuP4luc expression, resulting in higher CAT/luciferase ratios than in samples with pH5. Thus, the values should be compared only within the group of samples with the nonfunctional NS1 gene.

Transfections were carried out by using an internal control, pLuP4luc. CAT expression from pP4cat or pP4catB was decreased by NS1, as shown by the ratio of CAT expression with and without NS1 (Table 1, CAT activity for constructs 2, 5, and 8). The same result was observed when these data were normalized to pLuP4luc expression with NS1 (Table 1, CAT/luciferase ratios for constructs 2, 5, and 8). However, these same plasmids showed no significant difference in expression when transfected with a nonfunctional NS1 gene (pH6) (Table 1, CAT/luciferase ratios for constructs 3, 6, and 9). In many other experiments, inhibition of pP4cat by NS1 was as high as 86% (data not shown). In contrast, the plasmid construct deleted of the left terminus, pLP4cat, was modestly stimulated by NS1. Comparisons of these normalized data can be made only within groups having NS1 or groups without NS1 because of the four- to fivefold stimulation of P4 expression from pLuP4luc by NS1 (Table 1, ratio of luciferase activities for constructs 2, 5, and 8).

The data in Table 2 demonstrated NS1-dependent stimulation when the left-end terminus was functional for DNA replication, regardless of the reporter gene used. Results obtained with pLuP4luc plus pLuP4cat showed four- to fivefold stimulation of P4 expression for both reporter genes tested in the presence of the NS1 gene at the lower dose (Table 2, construct 1). The dose response of pLuP4cat to pH5 was tested by using a range of 0 to 2 µg of pH5 to supply the NS1 gene. A four- to fivefold increase in P4 expression

was observed at every dose of pH5 (data not shown). However, pLuP4luc did show a decrease in stimulation at the highest dose of the NS1 plasmid (Table 2, construct 3). Expression of pLuP4cat was the same whether it was transfected alone or cotransfected with pH6; thus, pH6 had no inhibitory effect under these conditions (data not shown). The pLuP4cat and pLuP4luc constructs have hybrid H-1-LuIII P4 control regions. NS1 appears to be interchangeable between the two viruses for replication as well as *trans*-activation. H-1 NS1 supports replication of LuIII DNA (31), and LuIII NS1 *trans*-activates H-1 P38 (16a). The LuIII parvovirus genome has been reported to be 81% similar to the H-1 parvovirus genome (13b). Also, viruses with LuIII-H-1 chimeric NS1 genes are viable (21a). Therefore, P4 exhibited NS1-dependent stimulation when the left-end terminus was functional for DNA replication.

We tested whether the inhibition of expression by NS1 on the P4 promoter was at the RNA level by using primer extension analysis (Fig. 2). We compared expression of the NS1 gene itself using plasmids pH5 and pH6; we found no reduction in the steady-state RNA level of the P4 promoter by the functional NS1 gene in pH5 (Fig. 2). In several independent experiments, the specific extended product (53 bases) from pH5 appeared to be slightly longer than the extended product of pH6 (predicted product of 53 bases). The other extended products were the results of nonspecific priming events of cellular RNA, as they were present in the

TABLE 2. Reporter gene expression from functional left-end constructs (LuP4) with and without NS1

Plasmid construct ^a	CAT activity (% acetylation) ^b	Luciferase activity (RLU, 10 ³) ^b	Ratio ^c of CAT activities, +NS1/-NS1	Ratio ^c of luciferase activities, +NS1/-NS1
1: pLuP4luc, pLuP4cat, pH5 (0.2 µg)	65.8	126.3	4.7	4.5
2: pLuP4luc, pLuP4cat, pH6 (0.2 µg)	14.1	27.7	1.0	1.0
3: pLuP4luc, pLuP4cat, pH5 (2.0 µg)	54.2	62.6	4.0	2.3
4: pLuP4luc, pLuP4cat, pH6 (2.0 µg)	13.7	27.2	1.0	1.0

^a HeLa cell monolayers were transfected by calcium phosphate precipitation by using the combinations of P4 plasmids listed in the presence (pH5) or absence (pH6) of a functional NS1 gene. The transfections varied the amounts of the pH5 and pH6 plasmids as indicated, with the other plasmids remaining at 1 µg each.

^b CAT and luciferase assays were performed at 48 h posttransfection. RLU, relative light units.

^c Values were normalized to P4 expression in the absence of a functional NS1 gene (pH6).

FIG. 2. Primer extension analysis of P4 transcripts in the presence and absence of NS1. NB cultures were transfected by the DEAE-dextran method with 10 μ g of each plasmid, and total RNA was collected at 48 h posttransfection. pH5 represents P4 transcripts expressed in the presence of NS1. pH6 represents P4 transcripts expressed in the absence of NS1. The expected extended product is 53 bases. Lanes: 1, pH5; 2, pH6; 3, mock-transfected control.

mock-transfected control. The differences in intensity of the nonspecific bands were presumably due to either use of less total RNA or less-efficient primer extension. When P4-specific bands were normalized to nonspecific cellular bands of equal intensity, no difference in P4 expression was observed between the extended products of pH5 and pH6.

The *trans*-activator-response (*tar*) element is a *cis*-acting element located 5' of the P38 promoter. It has enhancerlike properties and, in the presence of NS1, increases expression from the P38 promoter (33). Some enhancers have the ability to act upon promoters located several hundred bases upstream (22). Because the *tar* element might activate the P4 promoter in addition to the P38 promoter, we were interested in determining how a deletion that should include the *tar* sequences would affect NS1-dependent stimulation of the P4 promoter. Primer extension was performed on transcripts isolated from NB cells transfected with pLu272 Δ tar plus NS1 (pH5) or minus a functional NS1 (pSRS405). pSRS405 and pH6 were used as null controls for NS1, and both had no effect on pLuP4cat expression (Table 1 and data not shown). The data shown in Fig. 3, lanes 1 and 2, show that expression from pLu272 Δ tar was significantly increased by NS1. Therefore, deletion of this region from LuIII, which has been sequenced and found to have a *tar* element nearly identical to that of H-1 (13a), did not prevent P4 stimulation by NS1.

Replication is responsible for increased expression from the P4 promoter. It has been shown by several investigators (12, 31; reviewed in reference 45) that NS1 is required for excision and replication of cloned parvoviral genomes. The NS1-dependent increase in expression observed from the P4 promoter of pLu272 Δ tar in Fig. 3 could be due to replication of the viral DNA. To test this hypothesis, the following experiment was carried out. Our test plasmids are based on pLu272, a null mutant for NS1 and NS2 because of the frameshift mutation at base 272 which distinguishes the mutant transcript from the wild type. The inverted terminal repeats are intact, and after cotransfection with the NS1

FIG. 3. Primer extensive analysis of the effect of a deleted *tar* element on expression of the LuP4 promoter in the presence (pH5) and absence (pSRS405) of NS1. NB cultures were transfected by the DEAE-dextran method with 10 μ g of pLu272 Δ tar and 2 μ g pH5 or pSRS405. Total RNA was collected after 48 h. Lanes: 1, pLu272 Δ tar plus pH5; 2, pLu272 Δ tar plus pSRS405.

gene, the pLu272 viral sequences were shown to be excised and replicated (data not shown). pGlu Δ Nd has been described previously (31) and is an NS1-positive, amplification-negative clone. Transfection of either pLu272 or pGlu Δ Nd alone should result in no replication. If replication results in increased expression from the P4 promoter, then supplying NS1 in *trans* to pLu272 should increase expression from the P4 promoter in plasmid pLu272. The data in Fig. 4 substantiate this hypothesis. When pLu272 was transfected into NB cells alone, a low constitutive level of expression was observed (Fig. 4, lane 5). The constitutive level of expression from the P4 promoter of pGlu Δ Nd (Rep⁻ NS1⁺) shows that the P4 promoter in this plasmid is also at a relatively low level but greater than the expression seen with pLu272 (lane 6). Represented in lane 7 were

FIG. 4. Primer extension analysis of the expression of a replication-competent plasmid (pLu272) in the presence and absence of NS1. NB cultures were infected with LuIII virus (lanes 1 to 3) or transfected by the DEAE-dextran method with 8 μ g of each plasmid (lanes 4 to 9). Total RNA was collected from infected cells at 20 h postinfection and from transfected cells at 48 h posttransfection. Lanes: M, labeled oligonucleotides corresponding to 120 and 90 bases; 1, 2, and 3, decreasing concentrations of P4 transcripts from LuIII infection; 4, mock-transfected control; 5, pLu272 alone; 6, pGlu Δ Nd alone; 7, pLu272 plus pGlu Δ Nd; 8, pLu272 plus pGlu883; 9, pGlu883 alone. wt, wild type.

plasmids pLu272 and pGLuΔNd cotransfected into NB cells. The data showed an increase in transcriptional activity from the P4 promoter of pLu272 in the presence of NS1 supplied by pGLuΔNd and a decrease in the pGLuΔNd P4 transcripts. Densitometry tracings indicated that expression from the P4 promoter of pLu272 in the presence of NS1 was sixfold greater than the expression observed from pLu272 in the absence of NS1 (Fig. 4, lanes 5 and 7). The amount of expression from pLu272 exceeded that of pGLuΔNd in lane 7 by about threefold. Excision and replication of the viral sequence leads to amplification of the genome, which could confer a competitive advantage for transcriptional factors required by the P4 promoter compared with the nonreplicating sequences. Lane 8 represents transcripts isolated from NB cells cotransfected with pLu272 and pGLu883. In this case, NS1 is supplied in *trans* to pLu272 by pGLu883. Cotransfection of these plasmids, each capable of excision and replication, resulted in equivalent expression from each P4 promoter, as indicated by the intensity of the bands in Fig. 4, lane 8. These data suggest that when both genomes are excised and amplified, the required transcriptional factors for P4 expression are shared equally between the two plasmids.

To verify that the increase in P4 expression observed from pLu272 in the presence of NS1 is due to the ability of the plasmid to replicate, several replication-deficient clones were constructed. pLu272ΔNd is an amplification-deficient plasmid because of deletion of the right inverted repeat of pLu272 (31). pLu272ΔNd retains the mutation that inactivates the NS1-NS2 gene. The inability of the plasmid to replicate in the presence of NS1 should influence the amount of expression observed from the P4 promoter. In Fig. 5a, plasmids pLu272ΔNd and pLu272 were transfected into NB cells in the presence or absence of a functional NS1 gene. Transfection of either plasmid alone resulted in an equally low basal level of expression from the P4 promoter (Fig. 5a, lanes 2 and 4). Upon cotransfection with a plasmid expressing NS1 (pGLuΔNd), the amount of expression from the P4 promoter increased. The increase of P4 expression from pLu272, a replication-competent plasmid, was 1.5 times greater than the P4 expression observed from pLu272ΔNd. Thus, the right-end terminal repeat (and replication) was important but not totally responsible for NS1-stimulated P4 expression.

The left end of pLu272ΔNd is intact and undergoes NS1-dependent excision in the plasmid (31), which could explain why pLu272ΔNd showed residual stimulation by NS1. To test this possibility, pLu272ΔBE was constructed. pLu272ΔBE has deletions in both termini and the null mutation for NS1-NS2, as described for pLu272. In Fig. 5b, plasmids pLu272ΔBE and pLu272 were transfected into NB cells in the presence or absence of the NS1 gene. As in Fig. 5a, when either plasmid was transfected alone, an equally low basal level of expression from the P4 promoter was observed (Fig. 5b, lanes 1 and 3). NS1 stimulated P4 transcription from pLu272 by 8.5-fold (Fig. 5b, lanes 3 and 4). NS1 did not increase P4 transcription from pLu272ΔBE (Fig. 5b, lanes 1 and 2). These experiments demonstrated that NS1 stimulates expression from the P4 promoter by interaction with the terminal inverted repeats.

DISCUSSION

The requirement for NS1 during parvoviral replication has been well established. NS1 contributes to excision of viral DNA from plasmid clones and subsequent replication for

FIG. 5. (a) Primer extension analysis comparing amounts of expression from the P4 promoter between a replication-competent plasmid (pLu272) and a replication-deficient (right-end deletion) plasmid (pLu272ΔNd) in the presence and absence of NS1. NB cultures were transfected by the DEAE-dextran method with 8 μg of each plasmid. Total RNA was collected at 48 h posttransfection. Lanes: 1, mock-transfected control; 2, pLu272ΔNd alone; 3, pLu272ΔNd plus pGLuΔNd; 4, pLu272 alone; 5, pLu272 plus pGLuΔNd. (b) Primer extension analysis comparing amounts of expression from the P4 promoter between a replication-competent plasmid (pLu272) and a replication-deficient (both ends deleted) plasmid (pLu272ΔBE) in the presence and absence of NS1. NB cultures were transfected, and RNA was collected as described for panel a. Lanes: 1, pLu272ΔBE alone; 2, pLu272ΔBE plus pGLuΔBE; 3, pLu272 alone; 4, pLu272 plus pGLuΔBE; 5, pGLuΔBE; 6, mock-transfected control. wt, wild type.

AAV (34, 38, 50), MVM (23a, 24), and LuIII (31). NS1 or *rep* has also been shown to be the site-specific nickase involved in hairpin transfer during parvovirus replication (12, 42).

Regulation of the transcriptional activation of the parvovirus genome is not well understood. The promoters for the capsid genes of H-1 (30), MVM (16), and AAV (20, 51) have all been shown to require NS1 for transcriptional activation. For H-1, this *trans* activation requires the *tar* element, which

has previously been described by Rhode and Richard (33). It is expected that early gene expression of parvoviruses requires cellular factors. It has been suggested that enhancer sequences are present in the terminal repeats of AAV (6). Enhancer sequences have not been found for autonomous parvoviruses upstream of the early promoter (1, 16a, 30). Gel retardation assays and footprint analysis have verified the binding of a cellular factor, presumably SP1, to the GC box of the early promoter of B19 and MVM (1, 7). In vitro transcription of the P4 promoter in which the TATA box has been deleted or replaced by linker scan mutations resulted in elimination of transcriptional activity (1).

The effects of NS1 on the transcriptional regulation of the early promoter(s) of parvoviruses seem to vary. *rep* has been found to negatively regulate the early promoters, p5 and p19, of AAV in the absence of terminal hairpin sequences and helper adenovirus (6) and, thus, in the absence of viral DNA replication. *rep* mutants had low expression from all AAV promoters independently of viral DNA replication; however, when *rep* mutants containing intact terminal repeats were complemented with *rep*-containing plasmids, p5 transcription increased (20). By using EL-4 cells and the P4 promoter from MVM with an intact left end, Doerig et al. have shown that the P4 promoter is stimulated by NS1, in agreement with our results (15). The NS gene of B19 parvovirus seems to have no effect, negative or positive, on the early promoter of B19 when transfected into HeLa cells (25).

Comparisons of *cat* expression among several P4 constructs in the presence or absence of NS1 showed variable results. P4 expression was either inhibited in the presence of NS1 (pP4cat and pP4catB) or increased (pLuP4cat and pLuP4luc). We determined that the NS1 effect on P4 promoter expression was independent of the reporter gene used by comparing P4 fused to the gene for CAT or luciferase. This suggests that the context in which the promoter sequences are placed is responsible for the varied effects of NS1 on P4 expression. The parent clone of pP4cat is pSR1, a genomic clone that supports viral DNA replication *in trans* but does not itself generate replicating viral DNA. Since pSR1 is not infectious and shows no excision of viral DNA from the plasmids (31), the terminal hairpin sequences are presumed to be rearranged, and subsequently, pP4cat has inherited these changes. Primer extension assays were used to compare pH5 (NS1⁺) and pH6 (NS1⁻) P4 mRNA levels, because pH5 and pH6 were also constructed from pSR1. The results for pH5 indicate that this P4 promoter construct was not inhibited by wild-type NS1 at the transcriptional level. NS1 may affect the selection of the transcriptional start site for the P4 transcript, since the extended product resulting from pH5 has been consistently slightly longer than the extended product from pH6. The additional leader sequence in pP4cat was a candidate for causing inhibition of pP4cat expression by NS1. We ruled this out by recloning pP4cat (pP4catB) so that only the wild-type parvovirus leader sequence remained. Inhibition of P4 expression by NS1 was also observed for pP4catB (Table 1). pP4cat and pP4catB are the only P4 constructs that have shown NS1-dependent inhibition, and the mechanism for this has not been determined. P4 expression from the clone with no left-end sequences (pLP4cat) showed slight stimulation by NS1 (Table 1). However, clones with an intact left-end inverted repeat (pLuP4cat and pLuP4luc) showed four- to fivefold stimulation of P4 activity by NS1. This four- to fivefold NS1-dependent increase was observed for the reporter genes for both CAT and luciferase. The NS1-dependent increase in P4 expression observed at the protein level was also observed

at the RNA level, as shown by the primer extension data discussed below.

In addition to demonstrating that NS1 positively regulates the P4 promoter, we also demonstrated replication-dependent stimulation of P4 transcription in LuIII parvovirus. When a cloned parvovirus genome (pLu272) is capable of viral DNA replication but deficient for NS1 expression, the level of P4 transcription is stimulated approximately six- to eightfold by supplying NS1 *in trans*. When the plasmid expressing NS1 is deficient for replication of its viral sequences because of a right-end hairpin deletion, the level of P4 expression from the replication-competent plasmid is threefold higher than the P4 transcription of the helper plasmid. When both plasmids are replication competent, i.e., the test promoter and the NS1⁺ plasmid, the cotransfection results in more-equitable expression from the two P4 promoters. When pGLuΔNd and pGLu883 were transfected alone, the amount of expression from their P4 promoters was greater than when the plasmids were cotransfected with pLu272. These results demonstrate competition for cellular factors between various P4 promoters that are sensitive to the template copy number.

The contribution of excision and/or replication to the NS1-dependent increase in P4 transcription seen for pLu272 was examined by constructing replication-deficient plasmids, pLu272ΔNd and pLu272ΔBE, from pLu272. Deletion of the right-end inverted repeat reduced but did not eliminate the NS1 response. The observed NS1-dependent increase from pLu272 was 1.5-fold greater than the increase observed from pLu272ΔNd. Rhode has previously shown that NS1 affects the fate of plasmids with the *NdeI* deletion of the right-end terminal repeat (31). By examining the replication of pGLuΔNd, the following properties were noted. (i) pGLuΔNd undergoes excision of the left-end inverted repeat only (the right-end hairpin has been destroyed). (ii) A small amount of DNA synthesis occurs, presumably of only one strand, with the intact viral left end as the origin. (iii) Transfection of pGLuΔNd generates infectious virus (not the wild type) at a very low level. The replication properties of pLu272ΔNd should be the same as pGLuΔNd when NS1 is supplied. The excision event at the viral left end could also stabilize the DNA and/or affect its nuclear localization, resulting in more template for transcription from the P4 promoter. Another possible mechanism for NS1-dependent stimulation of P4 is excision of the left-end sequences from the plasmid, followed by replication of one strand. This would generate left-end hairpin structures capable of binding NS1 in the stem region, resulting in a more active promoter (19, 42). The fact that infectious virus is produced from pGLuΔNd suggests some capability for rescue of the deleted hairpin and amplification of the genome. However, this effect should be small compared with the replication of the wild-type genome. Deletion of both terminal repeats in pLu272ΔBE eliminated NS1-dependent stimulation of P4 transcription.

These data indicate the role that NS1-dependent replication and/or excision events play in expression of the P4 promoter. Data regarding AAV p5 transcription in the presence of *rep* protein and terminal hairpin sequences also indicate a role for replication in p5 expression (20). We have found no evidence that NS1 affects the P4 promoter by interacting with any downstream sequences (exclusive of the right-end inverted repeat) in the parvovirus genome. The response to NS1 of the reporter gene constructs containing only upstream sequences was very similar to that of the genomic clones. We believe that NS1 positively regulates

the early promoter of LuIII parvovirus partly through amplification of the parvovirus genome and partly by interaction with the terminal repeats. The synthesis of parental replicative-form DNA with a covalently closed left-end hairpin results in a template for P4 transcription by cellular transcription factors. The low level of P4 transcription results in translation of NS1 and NS2. NS1 directs the parvoviral genome to engage in replication, including hairpin resolution, resulting in an increase in transcribable templates from which more NS1 mRNA can be synthesized. This replicative-form DNA has NS1 bound to the 5' termini which may contribute to transcription. Thus, a positive feedback loop is established by which genomic amplification and NS1 binding to the termini positively regulate the P4 promoter. These results suggest that reduced expression of NS1 by parvoviruses in semipermissive cells can be the result of primary defects in DNA replication rather than the cause of those deficiencies (32).

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