JOURNAL OF VIROLOGY, Oct. 1990, p. 4654–4660
0022-538X/90/104654-07$02.00/0

Vol. 64, No. 10

Mutation of Lysine 405 to Serine in the Parvovirus H-1 NS1 Abolishes Its Functions for Viral DNA Replication, Late Promoter *trans* Activation, and Cytotoxicity

XU LI AND SOLON L. RHODE III*

Department of Pathology and Microbiology and the Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198

Received 4 May 1990/Accepted 26 June 1990

A consensus sequence in parvovirus nonstructural protein NS1 has been predicted to be an ATP-binding domain associated with an ATPase and a DNA helicase activity. To investigate the function of NS1 in viral gene expression, a site-directed mutagenesis converting NS1 lysine 405 to serine in parvovirus H-1 was carried out by the polymerase chain reaction. As shown previously, a parvovirus genome containing a deleted NS1 gene was excised from a bacterial plasmid and replicated when a wild-type NS1 gene was provided in *trans* but failed to be excised and replicate when the mutant NS1 gene was supplied. Interestingly, the serine 405 mutation totally lost the activity of *trans* activation on the virus late promoter (P38) in a chloramphenicol acetyltransferase (CAT) assay and it lost evidence for cytotoxicity in two tumor cell lines (HeLa Gey and NB324K). The serine 405 NS1 protein was translocated normally to the nucleus. These results suggest that the NS1 lysine 405 of H-1 in its putative purine nucleotide-binding site is essential for viral DNA replication and that this domain may be involved in the regulation of the P38 promoter by an unknown mechanism. The loss of NS1 cytotoxicity on tumor cells suggests that NS1 expression is the major cause of cell killing by parvoviruses, which may facilitate further study of the mechanism of oncosuppression by parvoviruses.

Parvoviruses are a large family of animal viruses which infect species from humans to insects. Those which infect vertebrates are divided into two subgroups. Adeno-associated viruses (AAVs) are defective and need a helper virus for their replication, such as adenovirus or herpesvirus, whereas autonomous parvoviruses are capable of productive replication by themselves. Parvoviruses are small, and they all contain a linear single-stranded DNA genome of about 5 kilobases (kb) with a similar genomic organization. There are two large open reading frames in the genome; the left-hand open reading frame, driven by the P4 promoter, encodes two nonstructural proteins, NS1 and NS2, while the right-hand open reading frame, controlled by the P38 promoter, encodes viral capsid proteins VP1 and VP2 (reviewed in references 8 and 22).

Many functions for the *rep* proteins or NS1 of both AAV and autonomous parvoviruses have been proposed. NS1 (Rep) has been found to be essential for viral replicative-form DNA replication (2, 27, 30, 36), necessary for the regulation of P4 and P38 promoters (19, 24, 25, 37), and involved in the cytotoxic activity of the virus (4, 18, 26). How NS1 exerts its functions during the viral infection cycle is becoming apparent. In minute virus of mice, NS1 was found to attach covalently to the 5′ termini of duplex replicative-form DNA and all newly synthesized progeny single strands, indicating that it may have a site-specific nickase activity during the hairpin transfer (9). In AAV, Rep protein has been observed to have a specific nuclease and helicase activity in viral DNA replication (15, 16, 32). An A-type consensus sequence [(G/A)XXXXGK(S/T)] for purine nucleotide binding was found among the NS1 proteins of parvoviruses, the large T antigens of polyomaviruses, and the E1 proteins of papillomaviruses (1). This consensus is predicted to be an ATP- or GTP-binding site associated with an ATPase and a possible DNA helicase activity.

In this study, we have generated a mutation at this site by converting lysine to serine at codon 405 in the parvovirus H-1 NS1 gene to test the hypothesis that this ATP-binding domain plays an important role in viral DNA replication. We report here that one amino acid substitution at the A-type consensus sequence of NS1 abolishes most of its known functions.

MATERIALS AND METHODS

Cell lines and virus. The simian virus 40-transformed human newborn kidney cell line NB324K (31) and HeLa Gey (ATCC CCL2.1) were grown in monolayer culture in Eagle minimal essential medium (EMEM) with 5 to 10% fetal calf serum. H-1 virus was propagated in NB324K cells.

DNA construction. The plasmids used in this study are listed in Table 1.

Site-directed mutagenesis by PCR. Four primers (primers 3 and 4 are mutagenic) were made on an Applied Biosystems model 380B DNA synthesizer and desalted over a C-18 Sep-Pak cartridge column (Millipore): primer 1, GCCAA CTTTTCCATGGCTAGCACC-3′ (plus strand); primer 2, TCCTTTTTGATCAATGCGTATGGT-3′ (minus strand); primer 3, AGCACAGGGAGCTCTATTATTGCACAA-3′ (plus strand); and primer 4, CGGTCGTGTCCCTCGAGAT AATAACGTG-3′ (minus strand). Primer 1 contains an *Nco*I-*Nhe*I site at nucleotides 1333 to 1338 and primer 4, with the mutation, contains a *Sac*I site at nucleotide 1480 (underlined sequences). Primers 1 and 4 generate a mutated fragment from 1333 to 1480. Primers 2 and 3 contain a *Bcl*I site (nucleotide 1662) and a *Sac*I site at the mutation and generate a mutated fragment from 1480 to 1662. The polymerase chain reaction (PCR) was performed in an automatic thermal cycler (Perkin-Elmer Cetus) for 25 cycles. Each cycle consists of 1 min of denaturation at 94°C, 2 min of

* Corresponding author.

TABLE 1. H-1- and LuIII-derived plasmids used in this study[a]

Plasmid	Origin and sequence	Expression	Reference(s)
pSR1	nt 1–5176 of H-1 RF DNA	wt NS1 and NS2	24, 25
pSRK405	Identical to pSR1, converting serine 405 to lysine	wt NS1 and NS2	This work
pSRS405	Identical to pSRK405 except for mutation of lysine 405 to serine	Mutant NS1 and wt NS2	This work (Fig. 1A)
pH3CAT	P38 promoter (nt 1662–2658 of pSR1) fused to *cat* gene	CAT	25
pGLuΔN	Deletion of *Nco*I fragment (nt 264–3300) of pGLu883, genomic clone of LuIII	No NS1/NS2 expression	27
pP38Lu2ΔNd	Deletion of right-end inverted sequences of pP38Lu2, genomic clone of LuIII	wt NS1/NS2 under control of H-1 P38 instead of LuIII P4 promoter	27

[a] wt, Wild type; RF, replicative form; nt, nucleotide number from 5′ end of the plus strand.

annealing, and 3 min of extension at 72°C. The annealing temperature was calculated by using a computer program for virology and molecular biology (5). A 20-ng amount of *Bgl*II-*Xba*I fragment (nucleotides 1247 to 2091 of H-1) was used as the template, and 100 pmol of each primer was used for the reaction in a volume of 100 μl with the reaction buffer recommended by the manufacturer. The products of the PCRs were restricted with *Nhe*I and *Sac*I or *Bcl*I and *Sac*I and analyzed on a 4% NuSieve agarose gel (FMC Corp.). The restricted DNA fragments were excised from the gel and recovered by electroelution in dialysis tubing. Then the fragments were cloned into the plasmid pSR1 to generate plasmid pSRS405 (Table 1) by a three-fragment ligation. The mutation was confirmed by restriction endonuclease mapping and DNA sequencing (Sequenase, Version 2.0; United States Biochemical Corporation). The procedure is described in the legend to Fig. 1. As a final control, the wild-type *Nhe*I-*Bcl*I (1338 to 1662) fragment was used for marker rescue by cloning it into pSRS405 to make pSRK405.

DNA transfection. Cells were seeded in 60-mm petri dishes with 3×10^5 cells per dish. The plasmids were transfected by either the calcium phosphate precipitation method as previously described (26) or the DEAE-dextran method (13). Cotransfections were done with the selectable marker gene for aminoglycoside phosphotransferase (*neo*) in the plasmid pSV2neo (34). HeLa Gey and NB324K cells were selected for *neo* gene expression by exposure to G418 at 1.25 mg/ml for 10 to 14 days, and G418-resistant clones were counted for measuring NS1/NS2 cytotoxicity.

Transient-expression assays. The expression of chloramphenicol acetyltransferase (CAT) was assayed as previously described (23, 24) and quantified by scintillation counting.

Immunofluorescent staining. The rabbit anti-NS1 antibody was provided by S. Cotmore, and the staining procedure was described previously (26).

Replication assay. The plasmid DNA was extracted by the method of Hirt and analyzed by Southern blot as previously described (27).

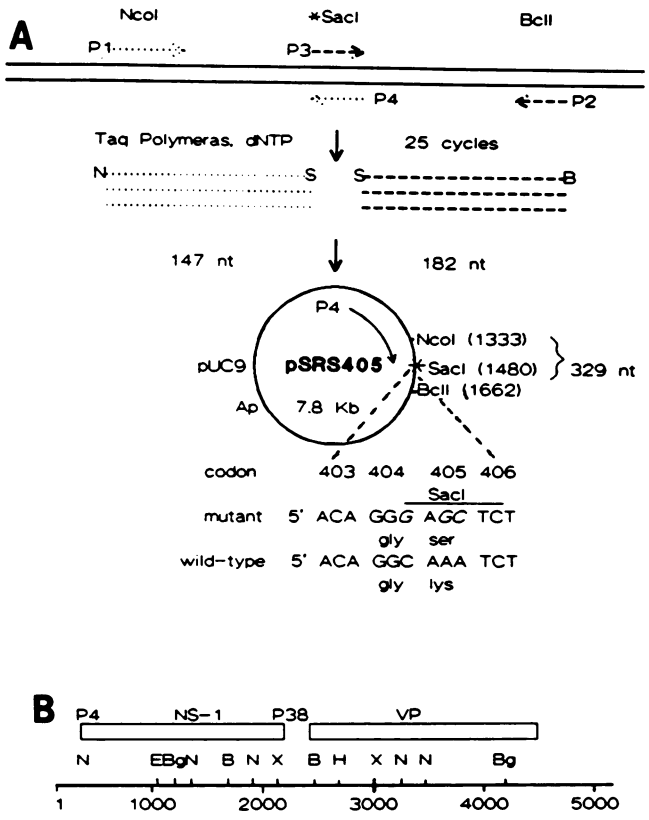

FIG. 1. (A) Site-directed mutagenesis of parvovirus H-1 at codon 405 of the NS1 gene by PCR. The sequences of the four primers are given in the text. nt, Nucleotide; P4, P4 promoter of H-1; italic letters, mutated nucleotides. Three nucleotides were mutated. As a result, lysine 405 was changed to serine 405 and a new *Sac*I site was generated. The restriction sites in the diagram are not drawn to scale, but their nucleotide positions in the genome are given. (B) Genomic organization of parvovirus H-1. The NS2 exons and the splicing sites are not shown (for details, see references 22 and 26). Restriction sites: B, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; X, *Xba*I; S, *Sac*I; Ap, β-lactamase gene.

RESULTS

Mutation of lysine 405 to serine in the A-type consensus sequence of NS1. The genomic map of H-1 is shown in Fig. 1B. H-1 contains a single-stranded DNA genome of 5,176 nucleotides. NS1 is composed of 672 amino acids. The A-type consensus sequence is located between codons 399 and 412 of NS1. By changing AAA to AGC at codon 405, lysine was substituted by serine. This was combined with a silent mutation at codon 404 (GGC to GGG) so that a new restriction site, *Sac*I, at nucleotide 1480 was generated (Fig. 1A). The mutation was confirmed by restriction endonuclease mapping and by complete DNA sequencing of 329 nucleotides of PCR product (*Nco*I [1333]-*Bcl*I [1662]) to exclude the possibilities of genome rearrangement during ligation or errors by *Thermus aquaticus* polymerase during PCR (data not shown).

Nuclear translocation of the mutant NS1. To investigate whether the mutant NS1 gene is expressed and the NS1 is transported to the nucleus, an indirect immunofluorescent

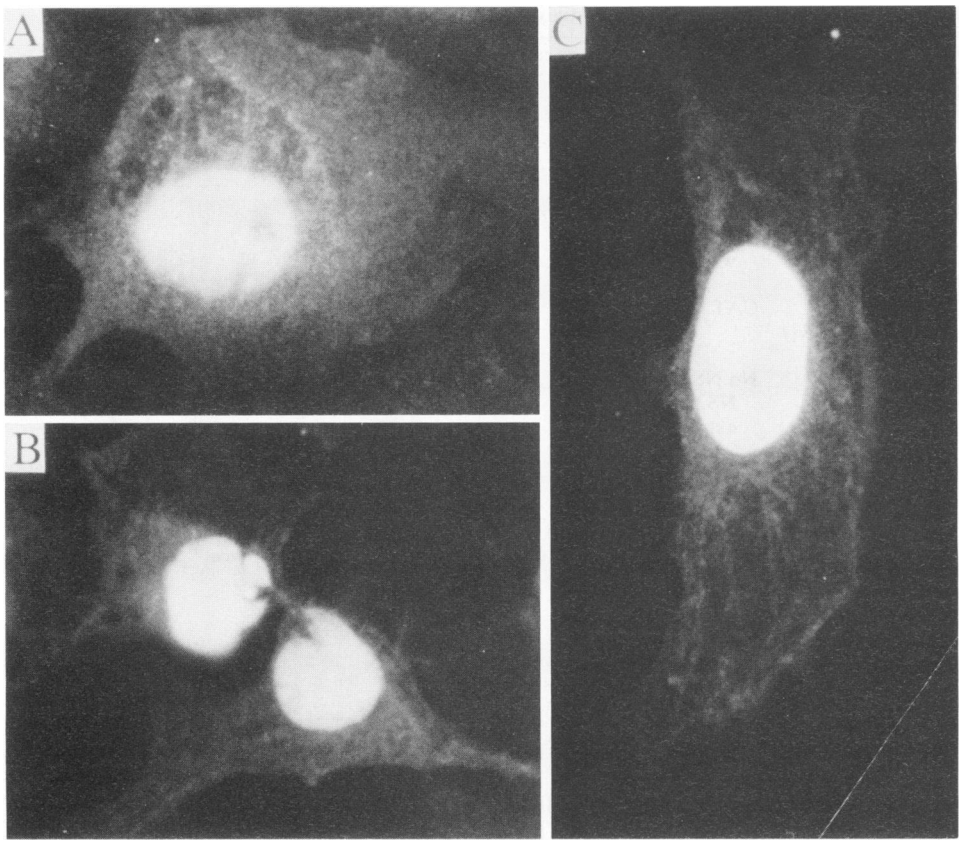

FIG. 2. Indirect immunofluorescent staining for NS1 with anti-NS1 rabbit antiserum. NB324K cells were transfected by the DEAE-dextran method without glycerol shock and/or chloroquine treatment. Cultures were fixed for staining at 48 h posttransfection or 20 h postinfection. (A) NB324K cells transfected by pSRK405. (B) NB324K cells transfected by pSRS405. (C) NB324K cells infected by H-1 virus. All of them showed nuclear localization of NS1. Magnification, ×400.

staining with rabbit anti-NS1 serum was carried out after transfection with pSRS405. Wild-type NS1 was introduced into cells either by transfection with pSRK405 or by infection with H-1 virus. As shown in Fig. 2, pSRS405 expressed NS1 that was translocated into the nucleus normally in NB324K cells after transfection. The two nuclei positive for NS1S405 in Fig. 2B appeared to be daughter cells that retained a thin nuclear bridge. This would suggest that the mutant protein did not inhibit mitosis. The efficiency of transfection was fairly low for both plasmids; about 30 positive cells were examined for each per cover slip. Thus, the two plasmids transfected and expressed NS1 at about the same frequency, and the fluorescence intensity of the positive cells appeared to be equal.

Ser-405 mutation of NS1 inhibits viral DNA excision and/or replication. To assess the effect of the mutation in the NS1 putative ATP-binding site on viral DNA excision and replication, we used an assay which demonstrates NS1-dependent replication of a test genome as described previously (27). In this system, a LuIII genome in the plasmid pGluΔN did not replicate when transfected by itself, since the entire NS1/NS2 gene was deleted (Fig. 3, lane 1). Neither pP38Lu2ΔNd, derived from a genomic clone of LuIII, nor pSRK405, a genomic clone of H-1, replicated their own genomes, but they expressed wild-type NS1/NS2 genes and served as helper plasmids for replication of pGluΔN. As shown in Fig. 3, lanes 2 and 4, the 1.8- to 2-kb viral genome of pGluΔN was excised from pUC19 and replicated when wild-type NS1 was supplied in *trans*. When the mutant NS1 gene was provided, there was no evidence of excision and replication (lane 3), indicating that the Ser-405 mutation inactivated NS1 function for excision and directly or indirectly abolished the viral DNA replication.

In a recent study of a similar mutation in AAV, it was reported that the mutant Rep protein showed a *trans*-dominant inhibition of viral DNA replication (6). We tested for this effect with pSRS405. When an equal amount of wild-type pSRK405 and the mutant pSRS405 DNA was transfected, the amplification of LuΔN was reduced to 46% compared with pSRK405 alone (Fig. 4, lanes 4 and 2). The replication of LuΔN was further decreased to 11% of the wild-type level when the amount of pSRS405 was increased to twice that of pSRK405 (Fig. 4, lanes 5 and 2) and totally abolished when the amount of pSRS405 was increased to fourfold that of pSRK405 (Fig. 4, lane 6). In contrast, fourfold more of the wild type than of the mutant only increased the DNA amplification of LuΔN to 58% of the control and could not overcome the inhibition of viral DNA replication by the mutant NS1 (Fig. 4, lanes 7 and 2). The results indicate that the Ser-405 mutant in NS1 is dominant for the inhibition of viral DNA excision and replication, which is consistent with the report on AAV (6).

Ser-405 mutation of NS1 blocks its *trans* activation of the P38 promoter. pH3CAT, which contains the P38 promoter of H-1 fused to the reporter gene for CAT (*cat*), was used to measure the stimulation of P38 by NS1 in transient-expres-

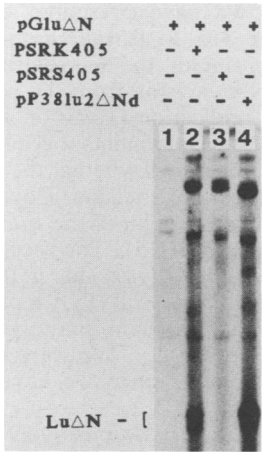

FIG. 3. Southern blot analysis of parvovirus DNA excision and replication. HeLa cells in 60-mm dishes were transfected with 2 μg of each plasmid DNA by the calcium phosphate precipitation method. DNA samples were collected at 48 h posttransfection and fractionated on a 1.4% agarose gel. After transfer onto a Nytran nylon membrane (Schleicher & Schuell), an *Eco*RI-restricted pGlu883 DNA (27) was labeled with [α-^{32}P]dCTP by the random primer method and used to probe the membrane. The plasmids used in each lane are indicated on the top. The sizes of the DNA bands were measured by using stained *Hin*dIII-digested lambda DNA fragments as markers. LuΔN, Various configurations of monomer replicative-form DNA generated by pGluΔN. The higher-molecular-weight bands shown in the gel represent different forms of plasmid DNA used in this experiment.

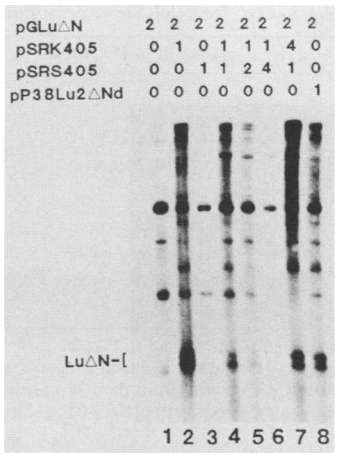

FIG. 4. Inhibition of parvovirus DNA replication by the mutant NS1. The transfection of HeLa cells and the Southern blot analysis were performed as described in the text and the legend to Fig. 3. The amounts of plasmid DNA for each transfection are indicated on the top of the figure (in micrograms). pUC18 plasmid DNA was used to adjust the total amount of plasmid DNA to 7 μg for each treatment. LuΔN, Various configurations of monomer replicative-form DNA generated by pGluΔN. The LuΔN bands in each lane were scanned in a Betascope 603 blot analyser (Betagen), and the data are given in the text.

sion assays. When pH3CAT was cotransfected with pSRK405 expressing wild-type NS1, the CAT activity increased 22.7-fold (Fig. 5, lane 2) compared with pH3CAT alone (Fig. 5, lane 1). However, when pH3CAT was cotransfected with pSRS405 expressing the mutant NS1, no stimulation was seen (Fig. 5, lane 3).

To address the question of whether the mutant phenotype is dominant, a mixture of an equal amount of pSRK405 and pSRS405 plasmid DNAs was transfected, and the CAT activity was tested. No inhibition was observed when both the wild-type and mutant NS1 were present, indicating that the wild-type phenotype seems to be dominant (Table 2). As the amount of pSRS405 DNA was increased to 5 μg per dish for transfection, the CAT activity was inhibited compared with the basal level, perhaps by depression of the uptake of pH3CAT (Table 2). The marker rescue plasmid pSRK405 *trans* activated P38 to the same level as pSR1, indicating no hidden mutations in portions of the gene that were not sequenced (Table 2).

Ser-405 mutation of NS1 eliminates its cytotoxicity in NB and HeLa cells. To determine whether expression of the Ser-405 mutant NS1 still had cytotoxic effects, cotransfections of pSRS405 or pSRK405 with pSV2neo into HeLa and NB cells were carried out, and stable transformation to G418 resistance was measured. We and others have found that wild-type NS1 inhibits transformation to G418 resistance (4, 18, 26). Cells that constitutively express the NS1/NS2 gene have never been isolated. The cytotoxicity of NS1 can be measured by the reduction of colonies compared with the culture transfected with pSV2neo alone. A typical experiment is shown in Fig. 6. No reduction in G418-resistant colonies was seen in the HeLa cells transfected by equal amounts of pSRS405 and pSV2neo, whereas the number of colonies was reduced by >90% in the cells transfected by pSRK405 and pSV2neo. We repeated the same experiment several times in both NB324K and HeLa Gey cells, and very similar data were obtained. We also used the human immunodeficiency virus long terminal repeat to replace the H-1 P4 promoter for expressing the NS1 genes (both Ser-405 and Lys-405) and cotransfected with an RSVTAT plasmid expressing the human immunodeficiency virus *tat* gene in a similar experiment with identical results (data not shown).

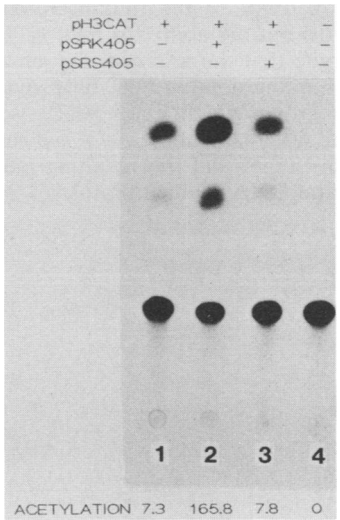

FIG. 5. *trans* activation of H-1 P38 promoter in HeLa cells. Cells (3×10^5 per 60-mm dish) were seeded, and 2 μg of pH3cat with 0.2 μg of each test plasmid DNA (shown on the top of the figure) were transfected by the calcium phosphate precipitation method. Cells were harvested at 48 h posttransfection, and CAT activity was determined. A 20-μl amount of CAT extract was used for each lane except for lane 2 (5 μl). The percent acetylation is shown on the bottom of the figure; the result for lane 2 was multiplied by 4.

TABLE 2. CAT assay[a] for NS1 Ser-405 mutant in HeLa cells

Plasmids	Amount of DNA (μg)	Acetylation (%)
pH3CAT[b]	2.0	6.4
+ pSR1	0.2	108.4
+ pSR1	0.4	87.7
+ pSR1 + pSRS405	0.2 each	70.5
+ pSRS405	0.2	4.5
	0.4	7.1
	1.0	4.7
	2.5	3.2
	5.0	1.6
+ pSRK405[c]	0.2	74.0

[a] The assay is described in the legend to Fig. 4.
[b] pH3CAT (2 μg) was included in each transfection, and pH3CAT alone gave the basal level of CAT activity from the P38 promoter.
[c] pSRK405 was generated by converting Ser-405 to Lys-405 in the NS1 gene (see text).

DISCUSSION

A purine NTPase-associated helicase activity has been found to play an important role in genome replication, recombination and repair, transcription, and mRNA translation in *Escherichia coli*, *Saccharomyces cerevisiae*, insects, mammals, and viruses (11, 14, 20, 33). Most, if not all, of the established or putative helicases from different species have been found to share a type A consensus sequence [(G/A)XXXXGK(S/T) or GXGK(S/T)], which binds and hydrolyzes ATP or GTP (1, 12).

The consensus topography in this purine NTP-binding domain has been proposed from the X-ray data and computer analysis. The motif is composed of the β-strand-β-turn-α-helix. The A-type consensus sequence is located in the center of the structure, and the lysine residue is presumed to couple to 2,3′-dialdehyde of ATP by its amino group (3, 7). This model is supported by the mutation of lysine 48 to arginine in the *S. cerevisiae RAD3* gene, which is one of the essential genes for excision repair of DNA damage (35). Their results showed that the ATPase and DNA helicase activity of the mutant RAD3 protein was abolished in vitro but the ability to bind ATP was retained. The authors argue that the arginine may change the configuration of the binding domain but still provide a positively charged amino group which binds ATP. Very recently, a similar mutation was made in the AAV *rep* gene by changing lysine 340 to histidine, and the resulting mutant showed a deficiency of viral DNA replication (6).

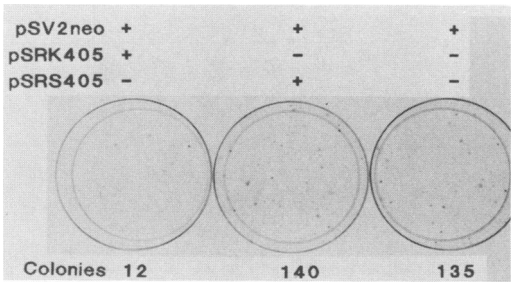

FIG. 6. Cytotoxic activity of NS1 Ser-405 mutant in HeLa cells. Cells (3×10^5) were seeded in a 60-mm dish, and 1 μg of each plasmid DNA was used for transfection by the calcium phosphate precipitation method. Cultures were transferred into 100-mm dishes at 48 h posttransfection and maintained in EMEM containing 1.25 mg of G418 per ml for 10 to 14 days. After the colonies were formed, cells were fixed with cold methanol, stained with Giemsa, and counted.

In this article, we have presented evidence that the mutation of lysine 405 to serine in the putative purine nucleotide-binding site of the parvovirus H-1 NS1 gene abolished viral DNA excision from the plasmid and subsequent viral DNA replication. Excision alone would have been detected in these experiments, because *Dpn*I treatment was not used. It is not known whether the mutant NS1 could support replication but not excision. However, we consider that possibility unlikely in light of the *trans*-dominant inhibitory effect of the Ser-405 NS1. The results further support the hypothesis that the lysine residue in the A-type consensus sequence is essential in viral DNA replication, indicating that this ATP-binding domain may be associated with ATPase and helicase activity. We were unable to test the helicase activity directly, since we have not yet purified wild-type or mutant NS1.

Considering that there is no positively charged group in the serine residue, we predict that this mutant will lose all of the ATP-binding, ATPase, and helicase activity. NS1 must be transported into the nucleus to regulate viral DNA replication. We have shown that the Ser-405 mutant is translocated into the nucleus normally, and therefore the replication defect observed was not due to altered nuclear transportation. Because of the inefficiency of transfection, we have not evaluated the stability or steady-state level of the mutant NS1 protein. The *trans*-dominant effect on DNA replication and the intensity of the immunofluorescence suggest that the lack of function of the mutant protein is not due to inadequate protein concentration.

To our surprise, the Ser-405 mutation also abolished P38 promoter *trans* activation and cytotoxicity. The mechanism for *trans* activation of P38 is still unknown. Our previous studies showed that maximal *trans* activation requires a small 5′ *cis* element (*tar*) between −137 and −116 of the P38 promoter (25). We have evidence that NS1 does not bind to *tar* directly, suggesting that *trans* activation is mediated by unidentified cellular factors (M.-L. Gu and S. L. Rhode III, unpublished data). The interaction between NS1 and these cellular factors may play an essential role in the stimulation of P38. The Ser-405 mutation may alter the conformation of NS1, especially in a specific domain, which in turn interferes with the interaction between NS1 and cellular proteins that bind to *tar*. Another possibility is that the regulation of P38 depends on the NTPase or helicase activity of NS1. It has been found that large T antigen, a major regulatory protein in simian virus 40, has both intrinsic non-ATP NTPase activity that is stimulated by single-stranded RNA and ATPase activity stimulated by single-stranded DNA (29). Interestingly, a monoclonal antibody (PAb204) inhibited both the DNA helicase and the RNA helicase reaction, indicating that they may share the same active center, including their nucleotide-binding site (29). There is no evidence, however, that NS1 of parvoviruses is capable of binding to and unwinding RNA to regulate viral transcription.

Since *tar* is important for the *trans* activation of P38 by NS1, but the *tar* sequence is not included in the P38 RNA transcripts, the hypothesis that P38 is regulated through a putative RNA helicase activity of NS1 seems to be improbable. It should be noted that the *trans*-dominant inhibition of Ser-405 NS1 on viral DNA replication was not seen with the *trans* activation of P38.

One of the peculiar properties of parvoviruses is their oncosuppressive activity. Animals persistently infected with parvoviruses have been found to be significantly protected against spontaneous tumorigenesis. Parvoviruses also efficiently suppress the experimental induction of tumors in

their host (reviewed in reference 28). The mechanism of oncosuppression by parvoviruses is unknown. One explanation is that neoplastic cells provide a suitable environment for the proliferation of parvoviruses and therefore are more susceptible to virus-induced killing (28). It has been reported that the inhibition of tumorigenesis in animals could be achieved by both complete virion and deleted viral DNA, suggesting that certain regions of the viral genome may be involved in this activity (10). Our data and the results of others also indicated that the expression of nonstructural proteins of parvoviruses drastically reduced the yield of stable transformants after cell transfection with selectable marker genes (18, 26).

The cytotoxic effect of nonstructural proteins is probably mediated through the mechanism of inhibition of heterologous gene expression and/or cell DNA synthesis (18, 26). In our study, the loss of cytotoxicity in the Ser-405 mutant was evidence that NS1 is the major component of parvoviruses for cell killing. Another nonstructural protein, NS2, seems to be indirectly involved in the cytotoxic activity, since the Ser-405 mutation should produce normal NS2, and no toxic effect was observed (Fig. 5). A recent report has shown that a truncated NS2 mutant in parvovirus minute virus of mice, with most of its C-terminal sequences from exon 2 and exon 3 removed, reduced the inhibitory action of NS1 on stable transformation of NB-E cells to geneticin resistance, suggesting that NS2 may act synergistically in the cytotoxic activity (4). This result agrees with our recent findings that the contribution of NS2 to cytotoxicity may be mediated by the enhancement of NS1 expression (X. Li and S. Rhode, unpublished data). Evidence has been reported that the viral capsid proteins are not cytotoxic. Mouse cell lines that constitutively express minute virus of mice capsid proteins VP1 and VP2 were isolated after transformation by chimeric bovine papillomavirus-minute virus of mice vectors without cytopathogenesis (17, 21). Further study of the mechanism of cytotoxicity of NS1 may facilitate the understanding of parvoviral oncosuppression at the molecular level.

ACKNOWLEDGMENTS

We thank Mary Wichman for synthesizing the primers for PCR, Carol Stessman for technical assistance, and S. Cotmore for antisera.

This work was supported by Public Health Service grants AI25552 (S.R.) and CA36727 from the National Institutes of Health, grants from the American Cancer Society (ACS MV-479 and 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. **Berns, K. I., R. M. Kotin, and M. A. Labow.** 1988. Regulation of adeno-associated virus DNA replication. Biochim. Biophys. Acta **951**:425–429.
3. **Bradley, M. K., T. F. Smith, R. H. Lathrop, D. M. Livingston, and T. A. Webster.** 1987. Consensus topography in the ATP binding site of the simian virus 40 and polyomavirus large tumor antigens. Proc. Natl. Acad. Sci. USA **84**:4026–4030.
4. **Brandenburger, A., D. Legendre, B. Avalosse, and J. Rommelaere.** 1990. NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virology **174**: 576–584.
5. **Campione-Piccardo, J.** 1986. Software library with applications in virology and molecular biology. J. Virol. Methods **13**: 191–195.
6. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide-binding site in the adeno-associated virus *rep* gene generates a dominant negative phenotype for DNA replication. J. Virol. **64**:1764–1770.
7. **Clertant, P., and F. Cuzin.** 1982. Covalent affinity labeling by periodate-oxidized [α-^{32}P]ATP of the large-T proteins of polyoma and SV40 viruses. J. Biol. Chem. **257**:6300–6305.
8. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**: 91–174.
9. **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.
10. **de la Maza, L., and B. J. Carter.** 1981. Inhibition of adenovirus oncogenicity in hamsters by adeno-associated virus DNA. J. Natl. Cancer Inst. **67**:1323–1326.
11. **Geider, K., and H. Hoffman-Berling.** 1981. Proteins controlling the helical structure of DNA. Annu. Rev. Biochem. **50**:233–260.
12. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. **17**:4713–4730.
13. **Gorman, C.** 1990. High-efficiency gene transfer into mammalian cells, p. 143–165. *In* D. M. Glover (ed.), DNA cloning, vol. II. IRL Press, Washington, D.C.
14. **Hirling, H., M. Scheffner, T. Restle, and H. Stahl.** 1989. RNA helicase activity associated with the human p68 protein. Nature (London) **339**:562–564.
15. **Im, D.-S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63**:3095–3104.
16. **Im, D.-S., and N. Muzyczka.** 1990. The AAV origin binding protein rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61**:447–457.
17. **Labieniec-Pintel, L., and D. Pintel.** 1986. The minute virus of mice P39 transcription unit can encode both capsid proteins. J. Virol. **57**:1163–1167.
18. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adenoassociated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7**:1320–1325.
19. **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.
20. **Muskavitch, K. M. T., and S. Linn.** 1981. RecBC-like enzymes exonuclease V deoxyribonucleases, p. 233–250. *In* P. D. Boyer (ed.), The enzymes, vol. 14. Academic Press, Inc., New York.
21. **Pintel, D., M. J. Merchlinsky, and D. C. Ward.** 1984. Expression of minute virus of mice structural proteins in murine cell lines transformed by bovine papillomavirus-minute virus of mice plasmid chimera. J. Virol. **52**:320–327.
22. **Rhode, S. L., and P. Iversen.** 1990. Parvovirus genomes: DNA sequences, p. 31–57. *In* P. Tijissen (ed.), Handbook of parvoviruses, vol. I. CRC Press, Inc., Boca Raton, Fla.
23. **Rhode, S. L., III.** 1976. Replication process of the parvovirus H-1. V. Isolation and characterization of temperature-sensitive mutants defective in progeny DNA synthesis. J. Virol. **17**: 659–667.
24. **Rhode, S. L., III.** 1985. *trans*-Activation of the parvovirus late promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
25. **Rhode, S. L., III.** 1987. Characterization of the *trans*-activation-responsive element (*tar*) of the parvovirus H-1 P38 promoter. J. Virol. **61**:2807–2815.
26. **Rhode, S. L., III.** 1987. Construction of a genetic switch for an inducible *trans*-activation of gene expression in eucaryotic cells. J. Virol. **61**:1448–1456.
27. **Rhode, S. L., III.** 1989. Both excision and replication of cloned autonomous parvovirus DNA require the NS1 (*rep*) protein. J. Virol. **63**:4249–4256.
28. **Rommelaere, J., and P. Tattersall.** 1990. Oncosuppression by parvoviruses, p. 41–57. *In* P. Tijissen (ed.), Handbook of parvoviruses, vol. II. CRC Press, Inc., Boca Raton, Fla.
29. **Scheffner, M., R. Knippers, and H. Stahl.** 1989. RNA unwinding activity of SV40 large T antigen. Cell **57**:955–963.
30. **Senapathy, P., J.-D. Tratschin, and B. J. Carter.** 1984. Replication of adeno-associated virus DNA: complementation of natu-

rally occurring *rep*$^-$ mutants by a wild-type genome or an *ori*$^-$ mutant and correction of terminal palindrome deletions. J. Mol. Biol. **179**:1–20.

31. **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.
32. **Snyder, R. O., R. J. Samulski, and N. Muzyczka.** 1990. In vitro resolution of covalently joined AAV chromosome ends. Cell **60**:105–113.
33. **Sopta, M., Z. F. Burton, and J. Greenblatt.** 1989. Structure and associated DNA-helicase activity of a general transcription initiation factor that binds to RNA polymerase II. Nature (London) **341**:410–414.
34. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early promoter. J. Mol. Appl. Genet. **1**:327–341.
35. **Sung, P., D. Higgins, L. Prakash, and S. Prakash.** 1988. Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. EMBO J. **7**:3263–3269.
36. **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.
37. **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.