JOURNAL OF VIROLOGY, Oct. 1992, p. 5705–5713
0022-538X/92/105705-09$02.00/0

Vol. 66, No. 10

Terminal Regions of the NS-1 Protein of the Parvovirus Minute Virus of Mice Are Involved in Cytotoxicity and Promoter *trans* Inhibition

DANIEL LEGENDRE[1]* AND JEAN ROMMELAERE[1,2]

Laboratoire de Biophysique et de Radiobiologie, Département de Biologie Moléculaire, Université Libre de Bruxelles, 67 rue des Chevaux, B 1640 Rhode St Genèse, Belgium,[1] and Unité d'Oncologie Moléculaire, Institut Pasteur de Lille, Institut National de la Santé et de la Recherche Médicale U186 and Centre National de la Recherche Scientifique URA 1160, 59019 Lille Cédex, France[2]

Received 22 April 1992/Accepted 23 June 1992

The nonstructural (NS) transcription unit of minute virus of mice (MVMp) encodes proteins that are involved in viral DNA replication and in the regulation of homologous and heterologous promoters. Moreover, it has been shown that NS-protein accumulation is toxic for transformed cells. With the aim of identifying the NS-protein function(s) responsible for cytotoxicity, point mutations and deletions were introduced in the NS-protein-coding sequence of MVMp. This strategy indicated that in transformed human NBE cells, the NS-1 protein is indispensable for MVMp DNA replication, *trans* activation of the late parvoviral promoter P38, *trans* inhibition of the long terminal repeat promoter of the Rous sarcoma virus, and cytotoxicity. Moreover, some mutations led to the dissociation of the replicative and regulatory functions of the NS-1 protein and showed that cytotoxicity correlated with the latter, more particularly with the capacity to *trans* inhibit the heterologous promoter. The NS-1 sequences required for cytotoxicity were found to be restricted to the amino- and carboxy-terminal portions of the protein. Although the cytotoxicities of NS-1 extremities were weak when the extremities were tested separately, the cytotoxicities were comparable to that of the full protein when the extremities were fused. Interestingly, an overall negative charge can be predicted from the NS-1 sequence over about 100 amino acids at both ends. The conservation of this charge distribution among the NS proteins of different parvoviruses suggests that NS-1 may bear some similarities to acidic transcriptional activators.

Minute virus of mice (MVMp) and the closely related parvovirus H-1 encode two nonstructural (NS) proteins, NS-1 (83 kDa) and NS-2 (25 kDa), that are phosphorylated and that show nuclear or both cytoplasmic and nuclear localization, respectively (8–10, 12). NS-1 is necessary for viral DNA replication and participates in the regulation of viral gene expression. This protein *trans* activates the P38 promoter driving the structural transcription unit (14, 34, 36) and exerts both positive and negative controls over the activity of its own promoter (P4) (13, 19, 36). An inhibitory effect on the p38 promoter was also observed at high NS-1 concentrations (36). The NS-1 protein of MVMp fulfills two of the biochemical expectations of parvoviral DNA replication models, i.e., ATPase and ATP-dependent helicase activities (46). Moreover, the homologous rep68 protein of parvovirus adeno-associated virus type 2 (AAV-2) was shown to interact with the viral-DNA terminal repeats and to have helicase and strand- and site-specific endonuclease functions that are both ATP dependent (22–24). The role of the NS-2 protein during the viral life cycle is more elusive. This protein seems to be required in some cells for efficient viral DNA and protein synthesis and for virus production (27, 29).

The NS proteins of parvoviruses also appear to interfere with a series of nonparvoviral processes, including gene expression programmed by some heterologous promoters (25, 36), in vitro neoplastic transformation (20), and stable cell transformation with exogenous DNA (4, 25, 30, 35). These data have suggested that the NS-protein gene products encoded by various parvoviruses (H-1 virus, MVMp, and B19 virus) have cytotoxic activities. This hypothesis has recently been confirmed by means of neoplastic cellular clones that have incorporated the NS-protein transcription unit of MVMp placed under the control of a hormone-dependent promoter. Indeed, these cells proved to be killed upon induction of the NS-protein genes (5). Neoplastic transformation of host cells is often accompanied by an increase in the cytopathic effect of parvoviruses, raising the possibility that the antitumoral activity of these agents in vivo involves an oncolytic component (37). The mechanism by which neoplastic transformation enhances the severity of parvoviral attack is presently unclear. A possible reason for this increased severity may be the greater capacity of a number of transformed cells for NS protein production compared with the capacities of the parental normal cells (37, 45). In addition, a direct or indirect effect of transformation on the cytotoxic potency of NS proteins deserves to be considered.

Several recent reports indicate that the NS and rep proteins of H-1 virus, MVMp, and AAV-2 comprise at least partly distinct functional domains that can be dissociated by mutagenesis. With regard to NS-protein-mediated negative and positive *trans* regulation of promoter activity, the former effect proved to be separable from the latter. Thus, deletion mutants of H-1 virus encoding C-terminal portions of NS-1 are unable to *trans* activate the parvoviral P38 promoter and, in fact, repress it to a significant extent (36). Likewise, the replicational functions of NS proteins appear to involve more than one determinant. Indeed, AAV-2 (6) and H-1 virus (26) mutants with mutations in the putative ATP-binding–ATPase site of the respective rep and NS proteins

* Corresponding author.

are deficient in DNA replication but inhibit in *trans* the amplification of wild-type genomes. Given that the mechanism of NS-protein-induced killing is not known, it seems interesting to use a similar mutational mapping and search for a possible colocalization of the NS protein region(s) involved in this and other known molecular activities. Consistently, the failure to isolate stable cell transformants producing truncated NS-1 proteins of H-1 virus (35) suggests that the whole polypeptide is not necessary for cytotoxicity. Altogether, these considerations prompted us to generate a set of NS-1 mutants of parvovirus MVMp and test them for the various NS protein properties outlined above after transfection of neoplastic human cells. This investigation led, in particular, to the identification of minimal deletion mutants whose cytotoxicities and promoter-inhibiting activities are segregated from other NS protein functions.

MATERIALS AND METHODS

Construction of molecular clones of NS-1 mutants of MVMp (Fig. 1). Plasmid pMM984 is an infectious molecular clone of the parvovirus MVMp (28). This plasmid contains the whole MVMp genome cloned into the *Bam*HI site of the bacterial plasmid pBR322. Point mutations (mutants pULB3240, pULB3241, pULB3242, pULB3243, and pULB3244) were introduced by oligonucleotide site-directed mutagenesis using single-stranded DNA template from plasmid pULB3221. This plasmid contains the 2.4-kb *Eco*RI fragment of pMM984 (nucleotides 1084 to 3518 of MVMp) cloned into the *Eco*RI site of the M13 vector mp11 (4). The mutated 2.4-kb fragment was transferred into plasmid pULB3239, a pMM984 derivative lacking the *Eco*RI site of pBR322 (deletion of the *Aat*II-*Cla*I fragment). Plasmids pMMBal17, pMMBal20, pMMBal03, pMMBal16, pMMBal02, pMMBal13, pMM Bal30, and pMMBal31 were generated by a limited digestion with *Bal* 31 nuclease from the single *Eco*RI site (nucleotide [nt] 1084 of MVMp) of the plasmid pULB3255. This plasmid was derived from plasmid pULB3239 by excision of the *Bgl*II-*Bgl*II fragment in the capsid coding region (nt 3450 to 4209 of MVMp). Plasmids pMMBal02ΔS, pMMBal13ΔS, and pMMBal30ΔS are identical to plasmids pMMBal02, pMMBal13, and pMMBal30, respectively, except for a deletion around the NS-2 splice acceptor site (nt 1890 to 2070). This deletion was generated by digestion with *Bst*EII and *Xho*I, blunt ending, and religation. Plasmid pULB3245 contains a *Nco*I-*Nco*I deletion (nt 259 to 1897 of MVMp) in the NS-1-coding sequence. The genuine ATG initiation codon is conserved, and the C-terminal part of the NS-1 protein is expressed in phase. Plasmid pULB3246 was constructed from pMM984 by inverting the *Eco*RV-*Eco*RV fragment which contains the P4 promoter and the NS-1–NS-2 initiation codon. Moreover, all listed mutants contain a spontaneous deletion in the 5′ palindrome of MVMp and, in consequence, cannot be amplified (3). Plasmid pMM984Δ, which contains this spontaneous deletion, was used as wild-type NS-protein-producing plasmid. Introduced mutations were verified by sequencing with the Sequenase kit from USB (Cleveland, Ohio).

Cells. The simian virus 40-transformed human cell line NBE (newborn kidney cells) (40) was grown in minimal essential medium supplemented with 5% fetal calf serum.

DNA transfection. NBE cells (1.5×10^5 cells per 6-cm-diameter dish) were transfected according to the Ca-phosphate precipitation method described previously (4). Stable transformation experiments were performed with plasmids pSV2*neo* (42) and pULB3236 (pP38*neo*) (4), which contain the *neo* gene under control of the early and late (P38) promoters of simian virus 40 and MVMp, respectively. Geneticin (G418; GIBCO) was added 2 days after transfection at a final concentration of 0.7 mg/ml. Fourteen days after transfection, surviving cell colonies were stained and counted. Transient-expression assays were performed with plasmids pRSV*cat* (16) and pULB3562 (pP38*cat*) (4), which carry the reporter gene *cat* (chloramphenicol acetyltransferase [CAT] gene) under the control of the long terminal repeat (LTR) early and late (P38) promoters of Rous sarcoma virus (RSV) and MVMp, respectively. Cells were recovered 48 h after transfection, and CAT activity was determined as described by Gorman et al. (17).

Viral DNA replication. The replication probe was MVMp DNA from plasmid pULB3262, which has a large internal deletion (*Nco*I-*Nco*I, nt 259 to 3122) in the NS protein and capsid genes and cannot be excised and amplified unless functional NS-1 protein is provided in *trans*. Intracellular MVMp DNA was extracted 48 h after transfection by the modified Hirt sodium dodecyl sulfate (SDS)–high-salt lysis procedure (21), as previously described (38). Viral-DNA replicative forms were analyzed by agarose gel electrophoresis followed by blotting to Zeta Probe membranes (Bio-Rad) and hybridization to an MVMp-specific DNA probe (fragment *Xba*I-*Bam*HI, nt 4339 to 5151) that was ^{32}P labeled by the random priming procedure (15). Prehybridization and hybridization were done in a solution containing 1.5× SSPE (1× SSPE is 180 mM NaCl, 10 mM Na_2HPO_4, and 1 mM EDTA), 1% SDS, 0.5% nonfat dry milk, and 0.1 mg of denatured salmon sperm DNA per ml at 65°C. Blots were washed successively in 2× SSC–0.1% SDS (1× SSC is 150 mM NaCl and 15 mM trisodium citrate), 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS for 30 min per wash at 65°C each time.

RESULTS

Three point mutations (pULB3240, pULB3241, and pULB3242) were introduced in a region that shows a very significant homology among the NS proteins of different parvoviruses and also with the T antigens of polyomaviruses and simian virus 40. This region includes the consensus sequence [(G/A)X_4GK(T/S)] common to purine nucleotide-binding proteins (1). These three point mutations affect only the NS-1-coding sequence. We have previously shown that an internal deletion in the NS-1-coding sequence which preserves the phasing of 5′- and 3′-end fragments (pULB 3201) produces a shortened protein that can have an important toxic effect (4). In order to determine the role of each extremity of the NS-1 protein in the cytotoxicity of the protein, we generated a series of NS-1 deletion mutants expressing either amino-terminal (mutants pMMBal17, pM MBal20, pMMBal03, pMMBal16, pMMBal02, pMMBal13, and pMMBal30) or carboxy-terminal (mutant pULB3245) fragments of this polypeptide. On the other hand, two stop signals were introduced in the reading frame encoding the COOH part of the NS-1 protein without affecting the amino acid sequence of NS-2 (pULB3243 and pULB3244). An internal deletion mutant of NS-1 that kept the 5′ and 3′ ends in phase was also generated (pMMBal31). Plasmid pULB 3246, which contains the entire sequence of MVM(p) but cannot express NS proteins as a result of an internal inversion, was used as a negative control in transfections.

***trans* inhibition of the RSV LTR.** The RSV LTR was chosen as a model heterologous promoter that can be inhibited by the NS proteins of MVMp (4). As illustrated in

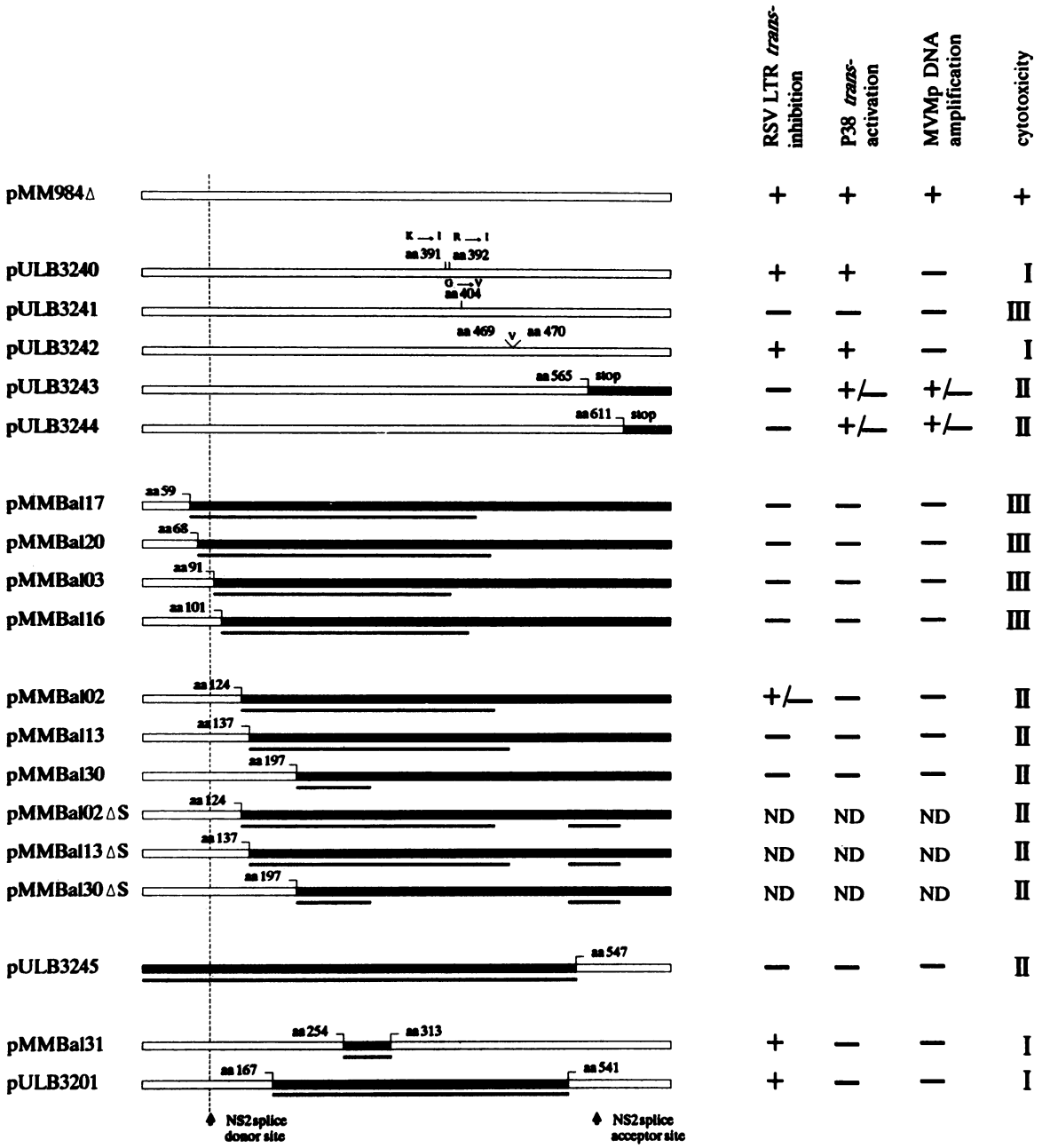

FIG. 1. Structures of pMM984Δ-derived mutants. Open boxes represent the expressed NS-1 protein sequences, and underlying lines correspond to deletions in the NS-protein-encoding DNA sequence. Amino acid substitutions are indicated by the one-letter code: I, isoleucine; G, glycine; K, lysine; R, arginine; V, valine. The corresponding mutations in the MVMp DNA sequence are as follows: pULB3240, nt 1432 (AAA to ATA) and 1435 (AGA to ATA); pULB3241, nt 1471 and 1472 (GGC to GTG); pULB3242, insertion of one GTG codon between nt 1667 and nt 1668; pULB3243, nt 1956 (GAG to TAG, stop codon); pULB3244, nt 2095 (TGG to TAG, stop codon). The last mutation had no effect on the NS-2 amino acid sequence. The *Bal* 31 frameshift mutants encoded truncated NS-1 proteins terminating in 7 (pMMBal17), 3 (pMMBal20), 34 (pMMBal03), 5 (pMMBal16), 5 (pMMBal02), 16 (pMMBal13), and 17 (pMMBal30) amino acids read in frame 1 (pMMBal20 and pMMBal13) or frame 2 (other mutants) instead of frame 3 (NS-1). Arrows at the bottom indicate the positions of the major splices that generate the NS-2-encoding transcript. For the NS-protein functions listed on the right-hand side, see the main text: +, strong; +/−, weak; −, undetectable; ND, not determined; I, II, III, groups of high, moderate, and insignificant toxicities, respectively. aa, amino acid.

Fig. 2 and summarized in Fig. 1, the extent of this inhibition was evaluated by quantifying CAT activity 48 h after cell transfection with the reporter plasmid pRSV*cat* in the presence or absence of either pMM984Δ or a mutated derivative thereof. No competition between cotransfected promoters was detected in the absence of NS proteins (inversion mutant pULB3246). The level of RSV LTR repression achieved by wild-type NS proteins (pMM984Δ) was about

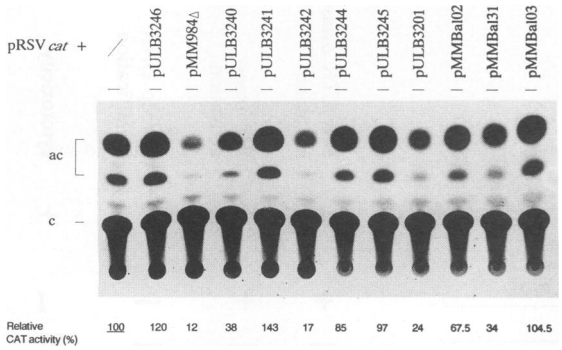

FIG. 2. Effects of pMM984Δ and mutated derivatives on the activity of the RSV LTR. Cultures (1.5×10^5 NBE cells) were cotransfected with 1 μg of pRSV*cat* and a threefold molar excess of pULB3246 (negative control), pMM984Δ, or the indicated mutated derivatives. The autoradiogram of a representative CAT assay is shown. c, ^{14}C-labeled chloramphenicol; ac, acetylated derivatives. CAT activities are given at the bottom relative to the value achieved by pRSV*cat* alone, which is considered to be 100 (underlined).

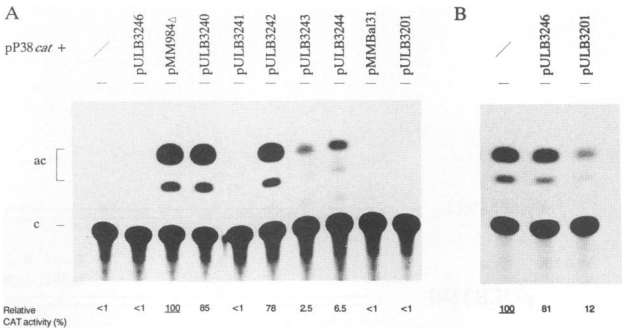

FIG. 3. Effects of pMM984Δ and mutated derivatives on the activity of the late P38 promoter of MVMp. NBE cultures (1.5×10^5 cells) were cotransfected with 1 μg of pP38*cat* and a threefold molar excess of pULB3246 (negative control), pMM984Δ, or the indicated mutated derivatives. Autoradiograms of two representative CAT assays are shown, illustrating P38 transactivation (A) and inhibition (B) in the presence of NS proteins. The CAT reaction was run for 1 h (A) or 12 h (B). c, ^{14}C-labeled chloramphenicol; ac, acetylated derivatives. CAT activities are given at the bottom relative to the value achieved by pP38*cat* plus pMM984Δ (A) or pP38*cat* alone (B), which is considered to be 100 (underlined).

90%. Mutants that expressed only an N-terminal (pMM Bal03 and other relevant clones not shown) or a C-terminal (pULB3245) fragment of NS-1 did not significantly alter RSV LTR-driven expression, except for mutant pMMBal02, which had a weak inhibitory effect (about 30%). In contrast, NS-1 mutants that retained both extremities (pULB3240, pULB3242, pULB3201, and pMMBal31) repressed the RSV LTR almost as efficiently as did those with the wild-type protein. This synergy was particularly obvious for the protein encoded by pULB3201 that is characterized by an extensive internal deletion of 374 amino acids.

In the cell line studied, the NS-2 protein appears to be dispensable for RSV LTR inhibition. Indeed, a mutant molecular clone expressing truncated NS-2 but normal NS-1 protein interferes with RSV LTR-driven expression just as the wild-type plasmid does (4). Conversely, plasmid pULB3241, which contains a point mutation in the NS-1 coding sequence but is unaffected for NS-2, did not inhibit the RSV LTR (Fig. 2).

***trans* activation of the MVMp P38 promoter.** Given that the late P38 promoter of MVMp is positively regulated by NS-1 (14), the *trans*-activation function of this protein was tested by transient-expression assays after cell cotransfection with the reporter plasmid pULB3562 (pP38*cat*) containing the *cat* gene under P38 control and with NS-1-producing vectors. As shown in Fig. 3A, a marked activation of P38-driven expression was observed in the presence of wild-type NS proteins (pMM984Δ) compared with that in the presence of the nonproducing control (pULB3246). Point mutants pULB 3240 and pULB3242 were proficient in P38 induction. These mutations lie in the above-mentioned homology region of NS-1, indicating that there is no strict requirement for the conservation of this domain with respect to P38 *trans* activation. This is consistent with a recent report showing that an in-frame insertion of 12 amino acids between residues 467 and 468 failed to block the capacity of the NS-1 protein for *trans* activating P38 (41). Nevertheless, a point mutation in the ATPase site included in the conserved region (pULB3241) abolished P38 induction. NS-1 mutants truncated in the C-terminal part of the protein (pULB3243 and pULB3244) transactivated the P38 promoter but to a much lower extent than did the wild-type protein. All the other mutants studied were deficient for P38 induction (Fig. 1). With a few exceptions (pMMBal17, pMMBal20, and pULB3245), these inactive mutants had an intact NS-2 sequence, indicating that NS-1 is requisite to *trans* activation, as is the case for *trans* inhibition.

NS-1 mutants deficient in P38 activation include two molecular clones (pMMBal31 and pULB3201) that were able to repress the RSV LTR promoter. The question therefore arose whether the lack of *trans*-activating function would unmask in these mutants some capacity of NS-1 for *trans* inhibiting the parvoviral P38 promoter. This possibility was tested and verified for pULB3201, which not only failed to induce P38 (Fig. 3A) but actually repressed P38-driven expression under conditions in which this promoter was activated by the wild-type protein (Fig. 3B). These data confirm that the inhibitory effect of the NS-1 protein can be attributed to the amino- and carboxy-terminal extremities of the protein and suggest a similar mechanism of inhibition for the RSV LTR and P38 promoter.

MVMp DNA replication. NS-1 protein is required for the replication of the parvoviral genome in NBE cells, whereas the NS-2 protein appears to be dispensable (29). We tested the replicative function of NS-1 through its ability to trigger in *trans* MVMp DNA replication from the NS-deleted pULB3262 molecular clone. It should be stated that all NS-1-expressing plasmids were deficient in DNA replication because of a deletion in the 5′-terminal palindromic sequence. This complementation assay, which used a single target for replication, avoids possible *cis* effects of the NS-1 mutations tested and of the size of the probe on the rate of DNA amplification.

As illustrated in Fig. 4A, two of the NS-1 mutants tested, pULB3243 and pULB3244, which are truncated in the C-terminal part of the protein, drove MVMp DNA amplification, albeit at a lower level than did the wild-type NS-1 protein (pMM984Δ). This result indicates that the 100 or so C-terminal amino acids are not absolutely necessary to the replicative function of the protein. The other mutants studied were unable to excise and/or replicate the MVMp DNA (data not shown and Fig. 1). This result is not surprising, since many of these mutants contain large deletions and it is known that several NS-1 activities are required for parvovi-

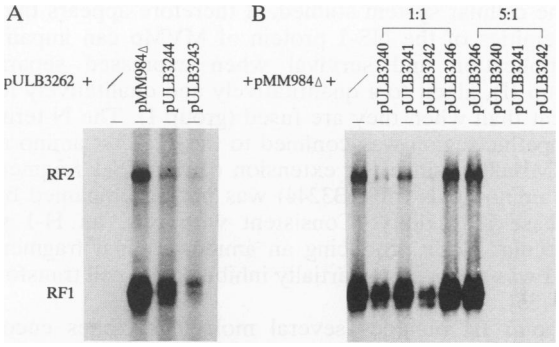

FIG. 4. Effects of wild-type or mutated NS proteins on MVMp DNA replication. The NS-defective pULB3262 replication probe was complemented with wild-type (pMM984Δ) or the indicated mutant NS-1 molecular clones. (A) NBE cultures were transfected with 1 μg of pULB3262 per 1.5×10^5 cells alone or together with a threefold molar excess of NS-protein-producing plasmids. (B) Cultures were cotransfected with pULB3262 and pMM984Δ as for panel A with or without the indicated mutants supplemented at a 1:1 or 5:1 ratio relative to pMM984Δ. All transfection mixtures were adjusted to 11 μg of total DNA with salmon sperm DNA. Intracellular MVMp DNA was extracted and analyzed by agarose gel electrophoresis, Southern blotting using a ^{32}P-labeled MVMp DNA probe, and autoradiography. RF1 and RF2 are monomer- and dimer-length replicative forms, respectively.

ral DNA amplification (26, 46). Moreover, many of the introduced mutations hit the conserved region putatively involved in the purine nucleotide binding-hydrolysis that is essential to DNA replication (6, 26), in particular to the nickase and helicase activities of NS/rep proteins (23, 46).

It was recently reported that replication-defective NS/rep mutants with mutations in the conserved region inhibited wild-type protein-driven parvoviral DNA replication (6, 26). Consistently, three MVMp molecular clones mutated in this region (pULB3240, pULB3241, and pULB3242) proved to be dominant, as indicated by the fact that they suppressed the replication of the MVMp DNA probe in cells cotransfected with a wild-type NS-1-producing plasmid. As shown in Fig. 4B, a strong or even complete inhibition of replication was observed with 1:1 and 5:1 ratios, respectively, of mutated to wild-type NS-1-expressing plasmids. These data suggest that the NS-1 mutants in question, although enzymatically inactive, retained the ability to interact with their DNA target or with a cofactor(s) essential to DNA replication. This residual capacity was apparently lost by the other replication-defective deletion mutants tested, since they failed to compete with the wild-type protein for its replicational function (data not shown).

Cytotoxicity. The toxicities of NS proteins were indirectly evaluated by measuring the abilities of the proteins to inhibit stable DNA transformation of cells after cotransfection with NS-protein-producing and selectable (pSV2*neo*) plasmids. The comparison of the number of geneticin-resistant (G418^r) colonies induced by pSV2*neo* in the presence or absence of NS proteins gives an estimate of the cytotoxicities of these products. Indeed, it has been shown that the reduction of G418^r colony formation observed under these conditions is unlikely to result from an interference of NS proteins with the G418 selection procedure, including the expression and integration of pSV2*neo* (4). Consistently, cells that have incorporated the NS-protein transcription unit under control of a hormone-dependent promoter are killed upon induction of NS-protein expression (5).

TABLE 1. Transformation to geneticin resistance inhibition by defective derivatives of pMM984Δ

Plasmid used in conjunction with pSV2*neo*[a]	No.[b] of G418^r colonies
None	453
pULB3246	475
pMM984Δ	2
Group I	
pULB3240	62
pULB3242	20
pMMBal31	51
pULB3201	29
Group II	
pULB3243	162
pULB3244	155
pMMBal02	75
pMMBal13	131
pMMBal30	166
pMMBal02ΔS	102
pMMBal13ΔS	128
pMMBal30ΔS	132
pULB3245	146
Group III	
pULB3241	380
pMMBal17	362
pMMBal20	530
pMMBal03	411
pMMBal16	475

[a] Plasmids pULB3246 (nonproducing control), pMM984Δ, and mutated derivatives (Fig. 1) were cotransfected with pSV2*neo* (3:1), which contains the neomycin resistance gene under the control of the simian virus 40 early region promoter.

[b] Results are the averages of three independent experiments with three plates per experiment.

In keeping with our previous observations (4), wild-type NS proteins (pMM984Δ) drastically reduced the appearance of transformed cell colonies (Table 1 and Fig. 5). This inhibition was not achieved by the nonproducing control (pULB3246) and has been shown to absolutely require the NS-1 protein (4). The NS-1 mutants could be arranged according to cytotoxicity in three groups, referred to as I, II, and III in Fig. 1.

Mutants from group I (pULB3240, pULB3242, pULB 3201, and pMMBal31) were highly toxic, as is apparent from their abilities to reduce the number of G418^r transformants by about 90% (Table 1 and Fig. 5). Since mutants pULB3240 and pULB3242 *trans* activated the P38 promoter of MVMp as wild-type NS-1 did (Fig. 3A), we attempted to obtain G418^r clones by cell cotransfection with these mutants and the selectable plasmid pP38*neo*, which contains the *neo* gene under P38 control. Given that the activity of induced P38 is similar to that of the simian virus 40 early promoter, numerous G418^r clones would be expected if these two mutants were innocuous. In two independent experiments in which about 150 G418^r colonies per plate were obtained in the presence of pSV2*neo*, only two or three cell transformants arose in cultures inoculated with pP38*neo*, whether or not it was combined with pULB3240 or pULB3242. Since the NS-1 proteins encoded by the latter two plasmids *trans* activate the P38 promoter programming the *neo* gene, their failure to enhance the rate of stable transformation argues for the toxicity of these products. Interestingly, the four

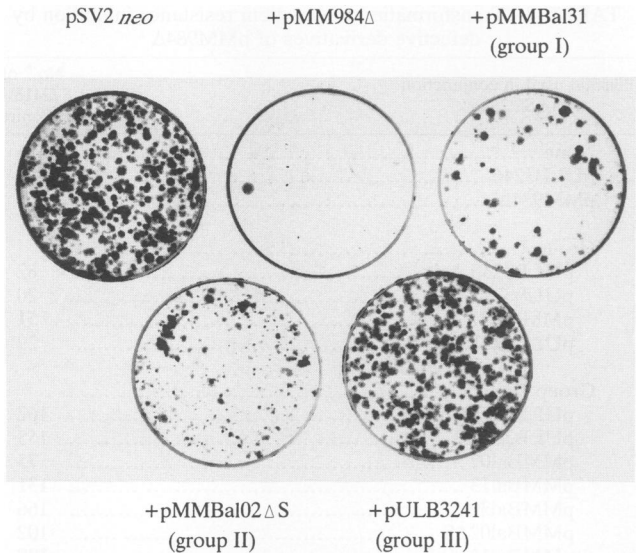

FIG. 5. Inhibition of DNA transformation of NBE cells by NS-protein-producing plasmids. Cultures (1.5×10^5 cells) were transfected with 1 μg of the selectable plasmid pSV2*neo* alone or together with a threefold molar excess of MVMp molecular clones producing wild-type (pMM984Δ) or mutated (pMMBal31, pMM Bal02ΔS, and pULB3241) NS-1 protein. Geneticin-resistant colonies were fixed and stained 2 weeks after transfection.

group I mutants were most potent with respect not only to their cytotoxicities but also to their capacities for promoter *trans* inhibition (Fig. 2), pointing to a possible correlation of these properties, for both of which the retention of the N- and C-terminal sequences of NS-1 appeared to be sufficient (Fig. 1). In contrast, group I included mutants that were deficient in promoter *trans* activation (pMMBal31 and pULB3201) and/or MVMp DNA replication (all four clones), indicating that the latter functions were not required for NS-1 cytotoxicity.

Mutants from group II expressed either an N-terminal (pMMBal02, pMMBal13, pMMBal30, pULB3243, and pULB3244) or a C-terminal (pULB3245) fragment of NS-1 (Fig. 1) and also inhibited the development of G418^r colonies very significantly. Yet, they could be distinguished from group I mutants by a lower level of stable transformation inhibition (about 70%; Table 1) and, in particular, the persistence of many isolated cells or microcolonies surviving the selection condition (Fig. 5). These observations suggest that the inhibitory action of group II mutants resulted, at least in part, from a cytostatic rather than a cytocidal effect on G418^r transformants. In parallel to their reduced toxic potentials, group II mutants had little promoter *trans*-inhibiting activity compared with group I (Fig. 1). Indeed, only the pMMBal02 clone, which is the most toxic member of group II (Table 1), achieved a weak but reproducible repression of RSV LTR-driven gene expression (Fig. 2), confirming the above-noticed correlation of cytotoxic and *trans*-inhibiting properties of NS-1. It should also be stated that a deletion around the NS-2 splice acceptor site (pMM Bal02ΔS, pMMBal13ΔS, and pMMBal30ΔS; Fig. 1) did not alter the capacities of corresponding mutants for disturbing hosts cells (Table 1). Together with the lack of an NS-2 reading frame in pULB3245, these data indicate that residual NS-1 sequences rather than the NS-2 protein of MVMp are responsible for the cytotoxic properties of group II mutants in the cellular system studied. It therefore appears that the extremities of the NS-1 protein of MVMp can impair cell multiplication and survival when expressed separately (group II), albeit to a quantitatively and qualitatively lower extent than when they are fused (group I). The N-terminal cytopathic region was confined to the 124 first amino acids (pMMBal02), since the extension of the NS-1 fragment to 611 amino acids (pULB3244) was not accompanied by an increase in toxicity. Consistent with this, an H-1 virus molecular clone producing an amino-terminal fragment of NS-1 was reported to partially inhibit stable cell transformation (35).

Group III includes several molecular clones encoding shortened N-terminal fragments of NS-1 (pMMBal17, pMM Bal20, pMMBal03, and pMMBal16) and one with a mutation in the putative ATPase site (pULB3241) (Fig. 1). These mutants had no significant cytopathic effect, as assessed by the fact that they induced no or little reduction of G418^r colony formation (Table 1). The innocuousness of pMM Bal16 (group III) contrasts with the toxicity of pMMBal02 (group II): the latter differs from the former by only 23 additional amino acids on the COOH side of the N-terminal fragment of NS-1 (Fig. 1). Therefore, at least some of these amino acids appear to be crucial for constituting a cytopathic region. On the other hand, the inability of pULB3241 to suppress cell colony formation, like its above-mentioned failure to *trans* inhibit the RSV LTR, is surprising, since this clone is point mutated in a region that is not present in the highly inhibitory and toxic mutant pULB3201 (Fig. 1). Mutant pULB3241 was deficient in all measured activities, like a recently described H-1 virus substitution mutant mutated in the neighboring amino acid (26). It should be stated that pULB3241 and wild-type plasmids produced similar amounts of NS-1 proteins (data not shown). The inactivity of pULB3241 indicates that the middle portion of the NS-1 molecule is somehow important to cytotoxicity. This is in agreement with the fact that the toxic effects of other internal mutants (pULB3240, pULB3242, pMMBal31, and pULB3201), significant though they might be (about 90% inhibition of colony formation), were lower than the activity of the wild-type clone (more than 99% inhibition). Since they affected to some extent all NS-protein properties tested, the mutations introduced into the central region of the NS-protein molecule may reduce cytotoxicity in an indirect way by modifying the overall structure of the protein and in particular by preventing its terminal sequences from adopting an optimal cytotoxic conformation. It cannot be ruled out, however, that these mutations had a direct deleterious effect on an additional cytotoxic domain that is located in the middle portion of the protein and predominates over the terminal sequences in so far as the toxicity of the whole protein is concerned. Incidentally, the innocuousness of pULB3241 confirms the need of NS-1 for cytotoxicity, since NS-2 coding sequences in this mutant are unaffected.

DISCUSSION

Identification of NS-1 cytotoxic sequences. The mechanism by which NS proteins exert their cytopathic effect is not known. One ultimate target of NS-protein products may be cellular DNA, given the known regulatory activities of these polypeptides at the level of the viral genome (13, 14, 36). NS proteins control both the replication and the expression of parvoviral DNA through processes that were each shown to involve multiple functions. Indeed, some NS-protein mutants deficient in these activities proved to have dominant

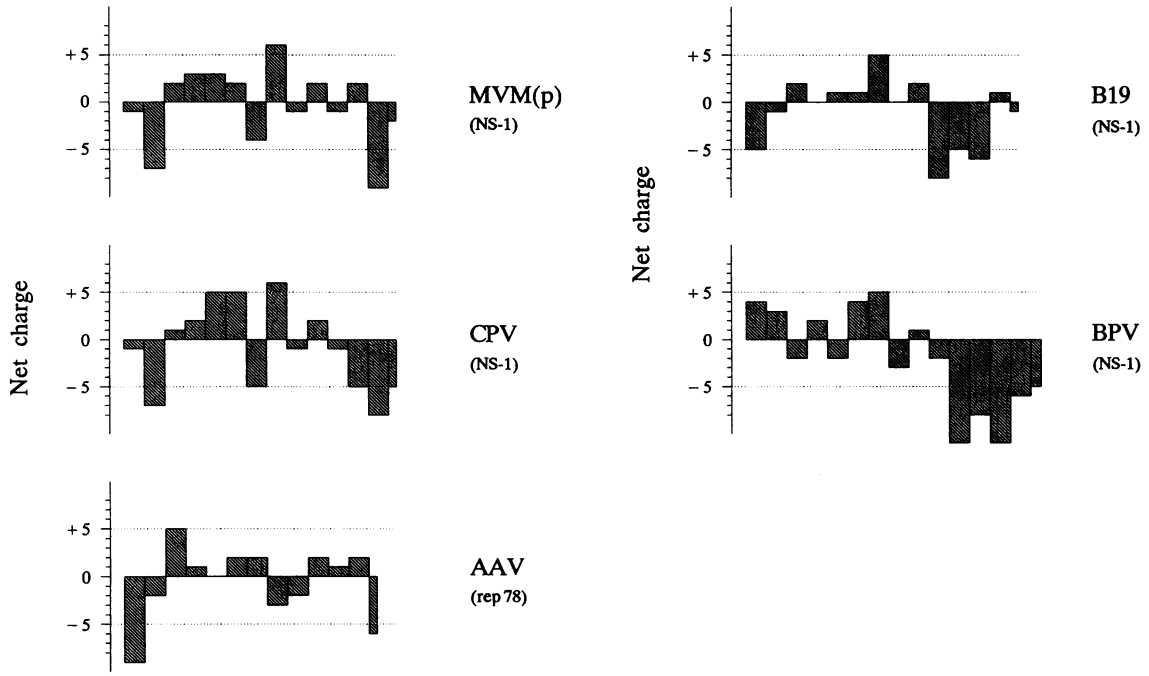

FIG. 6. Distribution of net charge (over successive 50 residues) along the NS-1/rep proteins of MVM(p) (2), canine parvovirus (CPV) (33), AAV (43), B19 virus (39), and bovine parvovirus (BPV) (7).

negative and *trans*-inhibitory effects on viral DNA replication (26) and promoter activity (36), respectively. The point mutations and deletions described in this paper also led to the separation of these various NS-protein functions and indicated that the cytotoxic and promoter-repressing abilities cosegregated and could be mapped to the N- and C-terminal portions of the NS-1 polypeptide.

(i) NS-1 versus NS-2 proteins. The NS-protein transcription unit comprises two overlapping genes encoding NS-1 and NS-2 polypeptides (9). The point mutations and some of the deletions created in this work were chosen so as to specifically alter NS-1 while keeping NS-2 unaffected, taking advantage of a major splice in the NS-2-encoding transcript and of the different reading frames used for synthesis of the C-terminal part of each protein. The data presented here confirm that the NS-1 polypeptide is necessary and sufficient for cytopathogenicity in the human NBE cell line. Thus, the capacity for inhibiting the formation of transformed cell colonies was abolished by some NS-1-specific mutations but retained by certain mutants deficient in NS-2 production. However, these results do not rule out the possibility that the NS-2 protein reinforces the cytotoxic effect of NS-1, as suggested by previous reports from our (4) and other (26) laboratories.

(ii) Segregation of NS-1 functions. The multiplicity of functions involved in the replicational activity of the NS-1 protein is apparent from its various enzymatic properties (ATPase, helicase, and nickase) (46) and the dominant negative character of some replication-deficient mutants (26). The present genetic analysis confirmed the latter feature but failed to correlate NS-1 cytotoxicity with the capacity for either inducing or inhibiting parvoviral DNA replication. Thus, replication-deficient mutants that either had or lacked the ability to interfere with wild-type NS-driven replication included molecular clones that still suppressed transformed-cell clonogenicity. Conversely, some nontoxic mutants remained dominant negative with respect to DNA amplification. As in the case of replication, certain NS-1 mutants that became deficient in promoter *trans* activation were proficient in the inhibition of DNA transformation of NBE cells, indicating that toxicity could also be dissociated from this activating property. Yet, in contrast to replication-deficient mutants, only those *trans*-activation-deficient mutants which retained the capacity for promoter *trans* inhibition were found to be strongly cytotoxic. *trans*-inhibitory and cytopathic effects cosegregated from other NS-1 functions in mutants that kept only the N- and C-terminal portions of the protein. Although they were moderately cytotoxic when taken separately, the two extremities of NS-1 had to be combined to achieve a marked promoter repression and a toxic effect comparable to that of the full protein. The assignment of the latter two NS-protein activities to similar amino acid sequences may possibly be indicative of a functional relationship; i.e., one component of NS-protein cytopathogenicity may involve the disregulation of essential cellular gene expression.

Model of NS-1-mediated promoter *trans* inhibition. (i) Structural features. The NS-1 terminal regions that are necessary and sufficient together for efficient promoter *trans* inhibition are characteristically acidic, as is apparent from the charge distribution over the whole polypeptide (Fig. 6). The negatively charged segments flanking the NS-1 protein are actually those fused in the strong inhibitory mutant pULB3201. Moreover, the negative charges of NS-1 extremities may be further increased by phosphorylation. Indeed, NS-1 is phosphorylated on serine residues (31) that are particularly abundant in these regions (2). The potential importance of these acidic domains is supported by conservation of the negative charge (Fig. 6) and high serine content (2, 7, 33, 39, 43) of the amino and/or carboxy termini of various parvovirus NS proteins that otherwise show little sequence homology.

(ii) Relationship with *trans* activation. Two functional features of NS-protein-mediated promoter *trans* inhibition are worth noting. First, under conditions in which the protein has lost its *trans*-activating capacity through mutagenesis (e.g., P38 repression by pULB3201) or is produced in excess (36), NS-1 can inhibit a promoter that it normally stimulates. Second, all the NS-1 mutants so far identified as being deficient in promoter *trans* inhibition are also incapable of *trans* activation, suggesting that the former activity may involve a function necessary to the latter. As a minimal although not unique model reconciling these observations, *trans* activation may be assumed to require several NS-protein functions (allowing, in particular, the interaction of the protein with target promoters and cellular regulatory factors), some but not all of which are inoperative in the context of certain promoters, at high protein concentrations, or following mutagenesis. Accordingly, promoter *trans* inhibition would result from the fact that NS-1 proteins sequester some transcription factors or lock responsive promoters in inactive complexes. However, the possibility that independent mechanisms are responsible for promoter repression and activation cannot be ruled out.

(iii) *trans*-inhibitory function. The NS-1 function causing promoter *trans* inhibition remains to be determined. Although covalent binding of NS-1 to the 5′ ends of viral DNA strands occurs during the process of parvoviral DNA replication (11), there is no evidence of a direct interaction of this protein with promoter sequences (18). It seems more likely that the *trans*-inhibitory function of NS-1 allows its association with other proteins that participate in either promoter recognition or activation. These target proteins may be general transcription factors, owing to the variety of heterologous and homologous promoters that can be repressed by NS-1 (36). In this respect, an intriguing parallel can be drawn between the above-mentioned features of NS-protein-mediated promoter repression and the reported properties of some acidic transcriptional regulators, like the herpes simplex virus VP16 protein and the yeast GAL4 activator. These factors (for a review, see reference 32) are modular proteins that can bind to DNA either directly or through a protein intermediate and that contain an acidic activating domain whose potency can be increased by phosphorylation. Moreover, these acidic regulators appear to interact with a component(s) of the transcription complex (32, 44) and modulate a large number of promoters positively (in the presence of corresponding DNA responsive elements) and/or negatively (in the absence or even, at high protein concentrations, in the presence of such elements). The structural and functional similarities of NS-1 and acidic transcriptional regulators suggest that the parvoviral product may be a member of this family. It would be interesting to test this possibility by determining whether the fused NS-1 terminal sequences can be substituted for the activating regions of known acidic regulators, given that these regions are readily interchangeable within the family. The negative charges of both NS-1 extremities may cooperate to provide the protein with the capacity for binding one or several factors essential for the onset of transcription. Modifying the net charge of one and/or the other end of NS-1 by mutagenesis would also be informative about the influence of acidic domains on the *trans*-regulatory and cytotoxic activities of the protein. This possibility is supported by a recent report (41) showing that the *trans*-activating function of NS-1 was blocked by the insertion of positively charged amino acids but not of an acidic residue in the C-terminal portion of the protein.

ACKNOWLEDGMENTS

We thank D. C. Ward and P. Tattersall (New Haven, Conn.) for plasmid pMM984. We also thank A. Brandenburger for helpful discussions.

The work was supported by the Commission of the European Communities and the Caisse Générale d'Epargne et de Retraite.

REFERENCES

1. **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.
2. **Astell, C. R., M. Thomas, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
3. **Boissy, R., and C. R. Astell.** 1985. An *Escherichia coli recBCs-bcBrecF* host permits the deletion-resistant propagation of plasmid clones containing the 5′-terminal palindrome of minute virus of mice. Gene **35**:179–185.
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 MVM(p). Virology **174**: 576–584.
5. **Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere.** 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J. **9**:2989–2995.
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. **Chen, K. C., B. C. Shull, E. A. Moses, M. Lederman, E. R. Stout, and R. C. Bates.** 1986. Complete nucleotide sequence and genome organization of bovine parvovirus. J. Virol. **60**:1085–1097.
8. **Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two nonstructural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
9. **Cotmore, S. F., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
10. **Cotmore, S. F., and P. Tattersall.** 1986. The NS-1 polypeptide of the autonomous parvovirus MVM is a nuclear phosphoprotein. Virus Res. **4**:243–250.
11. **Cotmore, S. F., and P. Tattersall.** 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. J. Virol. **63**:3902–3911.
12. **Cotmore, S. F., and P. Tattersall.** 1990. Alternate splicing in a parvoviral gene links a common amino-terminal sequence to downstream domains which confer radically different localization and turnover characteristics. Virology **177**:477–487.
13. **Doerig, C., B. Hirt, J.-P. Antonietti, and P. Beard.** 1990. Nonstructural protein of parvovirus B19 and minute virus of mice controls transcription. J. Virol. **64**:387–396.
14. **Doerig, C., B. Hirt, P. Beard, and J.-P. Antonietti.** 1988. Minute virus of mice non-structural protein NS-1 is necessary and sufficient for *trans*-activation of the viral P39 promoter. J. Gen. Virol. **69**:2563–2573.
15. **Feinberg, A. P., and B. Vogelstein.** 1982. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132**:6–13.
16. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Gu, M.-L., F. Chen, and S. L. Rhode.** 1992. Parvovirus H-1 P38 promoter requires the *trans*-activation region (tar), an SP1 site, and a TATA box for full activity. Virology **187**:10–17.
19. **Hanson, N. D., and S. L. Rhode III.** 1991. Parvovirus NS1

stimulates P4 expression by interaction with the terminal repeats and through DNA amplification. J. Virol. **65**:4325–4333.
20. **Hermonat, P. L.** 1989. The adeno-associated virus rep78 gene inhibits cellular transformation induced by bovine papillomavirus. Virology **172**:253–261.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:363–369.
22. **Im, D.-S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63**:3095–3104.
23. **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.
24. **Im, D.-S., and N. Muzyczka.** 1992. Partial purification of adeno-associated virus Rep78, Rep52, and Rep40 and their biochemical characterization. J. Virol. **66**:1119–1128.
25. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adeno-associated gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7**:1320–1325.
26. **Li, X., and S. L. Rhode III.** 1990. 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. J. Virol. **64**:4654–4660.
27. **Li, X., and S. L. Rhode III.** 1991. Nonstructural protein NS2 of parvovirus H-1 is required for efficient viral protein synthesis and virus production in rat cells *in vivo* and *in vitro*. Virology **184**:117–130.
28. **Merchlinsky, M. J., P. 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.
29. **Naeger, L. K., J. Carter, and D. J. Pintel.** 1990. The small nonstructural protein (NS2) of the parvovirus minute virus of mice is required for efficient DNA replication and infectious virus production in a cell-type-specific manner. J. Virol. **64**: 6166–6175.
30. **Ozawa, K., J. Ayub, S. Kajigaya, T. Shimada, and N. Young.** 1988. The gene encoding the nonstructural protein of B19 (human) parvovirus may be lethal in transfected cells. J. Virol. **62**:2884–2889.
31. **Paradiso, P. R.** 1984. Identification of multiple forms of the noncapsid parvovirus protein NCVP1 in H-1 parvovirus-infected cells. J. Virol. **52**:82–87.
32. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335**:683–689.
33. **Reed, A. P., E. V. Jones, and T. J. Miller.** 1988. Nucleotide sequence and genome organization of canine parvovirus. J. Virol. **62**:266–276.
34. **Rhode, S. L., III.** 1985. *trans*-activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
35. **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.
36. **Rhode, S. L., III, and S. M. Richard.** 1987. Characterization of the *trans*-activation-responsive element of the parvovirus H-1 P38 promoter. J. Virol. **61**:2807–2815.
37. **Rommelaere, J., and P. Tattersall.** 1989. Oncosuppression by parvoviruses, p. 41–57. *In* P. Tijssen (ed.), Handbook of parvoviruses, vol. 2, CRC Press, Inc., Boca Raton, Fla.
38. **Russell, S. J., A. Brandenburger, C. L. Flemming, M. K. L. Collins, and J. Rommelaere.** 1992. Transformation-dependent expression of interleukin genes delivered by a recombinant parvovirus. J. Virol. **66**:2821–2828.
39. **Shade, R. O., M. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell.** 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J. Virol. **58**:921–936.
40. **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.
41. **Skiadopoulos, M. H., R. Salvino, W. L. Leong, and E. A. Faust.** 1992. Characterization of linker insertion and point mutations in the NS-1 gene of minute virus of mice: effects on DNA replication and transcriptional activation functions of NS-1. Virology **188**:122–134.
42. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
43. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45**:555–564.
44. **Strulh, K.** 1991. Acid connections. Curr. Biol. **1**:188–191.
45. **Van Hille, B., N. Duponchel, N. Salomé, N. Spruyt, S. F. Cotmore, J. J. Cornelis, and J. Rommelaere.** 1989. Limitations to the expression of parvoviral nonstructural proteins may determine the extent of sensitization of EJ ras-transformed rat fibroblasts to minute virus of mice. Virology **171**:89–97.
46. **Wilson, G. M., H. K. Jindal, D. E. Yeung, W. Chen, and C. R. Astell.** 1991. Expression of minute virus of mice nonstructural protein in insect cells: purification and identification of ATPase and helicase activities. Virology **185**:90–98.