JOURNAL OF VIROLOGY, Sept. 1987, p. 2807–2815
0022-538X/87/092807-09$02.00/0

Vol. 61, No. 9

Characterization of the *trans*-Activation-Responsive Element of the Parvovirus H-1 P38 Promoter

SOLON L. RHODE III* AND SANDRA M. RICHARD

The Eppley Institute and Department of Pathology and Microbiology, The University of Nebraska Medical Center, Omaha, Nebraska 68105

Received 12 January 1987/Accepted 20 May 1987

The parvovirus early protein NS1 positively regulates the expression of the P38 promoter for the viral capsid protein gene. We have examined the *trans*-activation of P38 by NS1 by using fusions of P38 to the reporter gene, chloramphenicol acetyltransferase (*cat*). Maximal *trans*-activation requires a small 5′ *cis* element (tar) between −137 and −116. The tar element has activity in both orientations when 5′ to the P38 promoter, but no activity has been detected 3′ to the promoter. The wild-type P38 has a biphasic response to NS1 depending on the dosage of the NS1-expressing plasmid. Promoters lacking the tar also have a biphasic response that is reduced about 10-fold, and they can be inhibited by larger doses of the NS1 plasmid. Heterologous promoters from other viruses and the Harvey-*ras* oncogene promoter are inhibited by NS1. Truncated and internally deleted versions of NS1 lose the *trans*-activation, but some of them retain the inhibitory properties. Thus *trans*-activation can be uncoupled from inhibition. The tar element has shown no activity with the heterologous simian virus 40 early promoter. In contrast, the P38 promoter responds to a heterologous enhancer, but the enhanced promoter loses activity to *trans*-activation by NS1. In summary, the P38 tar element has some of the properties of an enhancer with a high preference for a 5′ position and a stringent requirement for the P38 promoter.

The rodent autonomous parvoviruses have two overlapping tandemly arranged transcription units with promoters at map positions 4 and 38 (2, 3, 35, 37, 40). The P4 promoter generates two types of transcripts that are separately processed by splicing to translate the nonstructural proteins NS1 and NS2 (8, 9, 17, 23, 30) (Fig. 1). Similarly, the P38 promoter produces transcripts that are alternatively spliced to translate the capsid proteins VP1 and VP2 (17, 30–32). Kinetic analyses of the appearance of NS1 and the coat protein genes indicate that the NS1 gene is expressed early and the coat protein genes are late genes, as commonly found with other viruses (28). In a previous study of the regulation of the P38 promoter, we have found that it is constitutively very weak and is *trans*-activated by the NS1 protein (35).

Enhancers are *cis*-acting control elements that positively regulate transcription in response to cellular or viral proteins (for reviews, see reference 12 and 19). The emerging consensus for the definition of an enhancer includes the properties that it can act over long distances (several kilobases) and independently of orientation. The proteins that act upon enhancers and promoters may exhibit developmental regulation and species specificity (12, 22, 38). Enhancers and other promoter elements, such as the SP1 binding sites, appear to have some promoter activity on their own, but they function best in combination with other promoter elements (18, 26, 47, 51). The mechanism of action of promoter-associated transcription factors is not known.

In the work described in this report the location of the sequences (tar) required in *cis* for the *trans*-activation of the parvovirus P38 promoter were mapped and the responses of this promoter to a heterologous enhancer were examined. Evidence is presented that the tar element has many of the properties of a classical enhancer. The tar element has activity in both orientations. It has an unusually stringent promoter specificity and has some activity in the absence of the proximal promoter elements. The inhibition of expression of P38 or heterologous promoters by NS1 and truncated or deleted mutants of NS1 will also be described.

* Corresponding author.

MATERIALS AND METHODS

Cells and virus. The simian virus 40 (SV40)-transformed human kidney cell line NB (42) and HeLa Gey cells (ATCC CCL2.1) were grown in monolayer culture in Eagle minimal essential medium with 10% fetal calf serum. A stock of H-1 virus with a titer of 3×10^8 PFU/ml was prepared in HAK cells (ATCC CCL15) from a virus stock that was plaque purified three consecutive times.

DNA constructions. The plasmids used in this study are listed in Table 1, and a diagram of H-1 sequences with the relevant restriction enzyme sites is shown in Fig. 1. The constructions were made by standard recombinant DNA methods, and the natural restriction sites were used except when noted. For example, pH9cat, which is a deletion of H-1 sequences from the *Bgl*II site at nucleotide 1250 to the *Mbo*I site at nucleotide 1862, was made by restricting pH5cat with *Bgl*II and *Xba*I (nucleotide 2086) and gel purifying the two fragments. The 845-base-pair (bp) fragment was restricted with *Mbo*I, and the 230-bp *Mbo*I-*Xba*I fragment from 1862 to 2091 was purified and ligated to the vector fragment. The *Bgl*II-*Mbo*I fusion regenerated a *Bgl*II site. Other deletions were made at the appropriate restriction sites; when the ends did not match, they were filled in by treatment with T4 polymerase. The plasmids were then religated.

The pH3ΔX1 and pH3ΔX2 deletions were made by restricting pH3cat with *Xba*I, treating with BAL 31 nuclease, blunting the ends with T4 polymerase, ligating to *Bgl*II linkers, restricting with *Bgl*II, and religating. The deletion positions of pH3ΔX1 and pH3ΔX2 have been estimated by restriction mapping. The SV40 enhancer was obtained as a *Bam*HI fragment from the plasmid pACT provided by M. Botchan. The synthetic oligonucleotides were prepared on

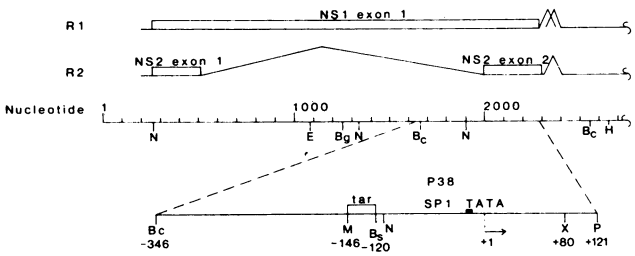

FIG. 1. Diagram of the P38 region. The various restriction sites used in the construction of plasmids listed in Table 1 are indicated. The start point of transcription for P38, +1, is positioned at nucleotide 2008 for diagrammatic purposes. Symbols: N, *Nco*I; E, *Eco*RI; Bg, *Bgl*II; Bc, *Bcl*I; H, *Hin*dIII; M, *Mbo*I; Bs, *Bst*EII; P, *Pst*I; X, *Xba*I; P4, the P4 promoter; P38, the P38 promoter; TATA, the promoter consensus TATA sequence.

the Applied Biosystems 380B synthesizer and purified by acrylamide gel electrophoresis. Enzymes were purchased from New England BioLabs, Inc., and used in the buffers recommended by the manufacturer.

DNA transfections and transient assay. The DNA transfections were as previously described; we generally used 2 μg of plasmid with the P38 promoter-*cat* fusion per 60-mm dish and 20 μg of calf thymus DNA per ml as a carrier (49). The samples for chloramphenicol acetyltransferase (CAT) determinations were collected at the times indicated in the text. The assays were carried out as previously described and quantitated by either laser densitometry or scintillation counting (35).

RESULTS

Mapping of the p38 tar. A variety of deletion mutations were constructed in plasmids that contained a fusion of P38 to the *cat* reporter gene, and expression of the cat gene was measured in transient assays in either NB or HeLa cells. The pH2 plasmids are fused to the *cat* gene at +127, and the pH1, pH3, and pH9 plasmids are fused at the +646 position. The small overlapping introns of H-1 lie in the leader between +127 and +646 along with the first translated exon of VP1. An additional intron, the small t intron of SV40, is supplied on the 3′ side of the *cat* gene. The relative expression of the test promoter in the presence or absence of NS1, which was supplied by cotransfection with a plasmid producing NS1 (and NS2), was determined. In experiments described below

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

No.	Plasmid	Vector[b]	H-1 sequences (nucleotide no.)	Source	*cat* gene
1	pSR1	a	1–5176	H-1 RF DNA[c]	None
2	pH5	a	1–2657, 4688–5176	pSR1	None
3	pH1cat	b	1662–2133	pSR1	2138
4	pH2cat	b	1088–2138	pSR1	2138
5	pH3cat	b	1662–2658	pSR1	2658
6	pH5cat	b	1–2658	pSR1	2658
7	pH9cat	b	1–1256, 1862–2658	del. pH5cat	2658
8	pH2-120	b	1088–1250, 1888–2138	del. pH2cat	2138
9	pH2-108	b	1080–1333, 1900–2138	del. pH2cat	2138
10	pH3ΔX/H	b	1662–2087	del. pH3cat	2087
11	pH3ΔBS/X	b	1662–1892, 2087–2658	del. pH3cat	2658
12	pH5catΔX/H	b	1–1892, 2087–2658	del. pH5cat	2658
13	pH5catΔBs/X	b	1–1892, (Bg), 2087–2658	del. pH5cat	2658
14	pH3ΔX1	b	1662–1988, (Bg), 2270–2658	del. pH3cat	2658
15	pH3ΔX2	b	1662–1993, (Bg), 2113–2658	del. pH3cat	2658
16	pH3ΔBs	b	1662–1887, 1893–2658	del. pH3cat	2658
17	pH5Δ1	a	1–262, 1900–2658, 4688–5176	del. pH5	None
18	pH5Δ2	a	1–262, 1333–2655, 4688–5176	del. pH5	None
19	pH5Δ3	a	1–1333, 1900–2655, 4688–5176	del. pH5	None
20	pH6cat	b	1–1250, (GATC), 1251–2658	pH5cat	2658
21	pH6	a	1–1250, (GATC), 1251–2657, 4688–5176	ins. pH5	None
22	pH8	a	1–2091, (CTAG), 2092–2657, 4688–5176	ins. pH5	None
23	pH2catΔtar	b	1088–1250, 1900–2138	pH2cat	2138
24	pH9SVecat	b	1–1256, (SVe), 1862–2658	ins. pH9cat	2658
25	pH3ΔX2SVp	b	1662–1993, (Bg), (SVp)	ins. pH3ΔX2	SV40 5171
26	ptarSVp	b	1662–1892, (SVp)	ins. pH3ΔBs/X	SV40 5171
27	ptar2cat1	b	1662–1892, 1862–2658	7 and 13	2658
28	ptar2cat2	b	1662–1988, 1862–2658	14 and 13	2658
29	pStarBg	b	1088–1250, (tarBa), 1900–2138	ins. 23	2138
30	pStarIBg	b	1088–1250, (tarBa), 1900–2138	inv.ins. 23	2138
31	pStarBa	b	1088–1250, 1900–2138, cat, (tarBa)	ins. 23	2138
32	pH3Sy	b	1662–1900, synthetic P38	pH3cat	+48

[a] The table lists the plasmids, the vector used, and the H-1 sequences included, the source of the H-1 sequences used ion the construction, and the position the *cat* gene is fused to the H-1 sequences. The H-1 sequences are listed in order from the left end (5′ end of the plus strand) by the nucleotide number of the reported sequence (37). The gaps represent deletions (del.). Insertions are indicated in parentheses: (Bg), *Bgl*II linker; (SVe), SV40 enhancer; (SVp), SV40 200-base-pair promoter from pB10cat (21), (tarBa), synthetic tar (Fig. 2). inv. ins. denotes an inverted insertion. The restriction sites in H-1 sequences used in the construction of these plasmids are as follows (cf. Fig. 1): *Eco*RI, 1088; *Nco*I, 262, 1333, 1900; *Bcl*I, 1662; *Bgl*II, 1250; *Mbo*I, 1862; *Bst*EII, 1888, *Xba*I, 2087; *Pst*I, 2133; *Hin*dIII, 2658; and *Hpa*I, 4688. Note that the table may list the 5′ or 3′ side of the restriction endonuclease cleavage site. The reporter gene cassette, *cat* gene, the SV40 small t splice fragment, and the SV40 poly(A)$^+$ *Bam*HI-*Bcl*I fragment are fused to the promoter sequences at the H-1 or SV40 nucleotide position shown. The plasmid pH3Sy, (no. 32) contains a synthetic P38 with new restriction sites that is similar to wt P38 up to the +1 position (F.-X. Chen, S. Rhode, and S. Richard, manuscript in preparation). The plasmids pSR1, pH2cat, pH3cat, pH5cat, and pH6cat were described previously (35).

[b] a, pUC9; b, pBR327.

[c] RF, Replicative form.

TABLE 2. Effects of 5′ and 3′ deletions on P38 *cat* expression[a]

Plasmid	Deletion (5′ − 3′)[b]	%aC −NS1	%aC +NS1	+NS1/−NS1
pH2cat	None	0.20	52	260
pH3cat	None	0.38	21	54
pH9cat	−757/−147	1.12	81	72
pH2-120	−757/−121	0.5	17	35
pH2-108	−675/−108	0.12	1	11
pH3ΔX/H	+80/+650	0.17	21	121
pH3ΔX1	−19/+261	0.19	16	85
pH3catΔBs/X	−116/+78	0.09	2	20

[a] Transfections were done with 2 μg of each plasmid in HeLa cells, and NS1 was supplied with pH5 at 0.2 μg per 60-mm petri dish. The extractions were made at 72 h posttransfection. The chromatograms were quantitated by scintillation spectrometry, and the values shown are the percentage of 3-acetylchloramphenicol (aC) of the total 3-acetylchloramphenicol plus chloramphenicol.

[b] As described in the text and Fig. 1.

and in Fig. 3, we determined that when 2 μg of test plasmid was transfected per 60-mm dish, the response to NS1 *trans*-activation was near maximal when 0.2 μg of the plasmid containing NS1 was included in the cotransfection. The deleted constructs were tested with the near-optimal level of the NS1 gene; in other experiments 1 or 2 μg of plasmid per dish was used (see Table 4) (data not shown). The results of the most relevant of these constructions are summarized in Table 2. The constitutive expression levels vary over a considerable range, but they are all low. We have found that some of these differences are consistent; for example, the plasmid pH3ΔBs/X is always low in expression. Nevertheless, a comparison of one plasmid with another would be more significant if it were made with an internal standard. Deletions on the 5′ side of P38 had the greatest effects on *trans*-activation when they extended past the *Mbo*I site at −146. The deletion that extended to the *Bst*EII site at −120 (pH2-120) has shown a reduced *trans*-activation in both HeLa and NB cells in many experiments (Table 2; also see Table 4). The 5′ deletion that extended to the *Nco*I site at −108 (pH2-108) diminished the level of *trans*-activated expression further under these conditions (see below). At higher levels of the NS1 plasmid, pH5, pH2-108 was unchanged or inhibited (see Table 4) (data not shown).

The deletions generated on the 3′ side of the promoter did not destroy the *trans*-activation even when they extended to the −116 position of the *Bst*EII site. The pH3ΔBs/X and pH5ΔBs/X (data not shown) constructions deleted from the *Bst*EII site to the *Xba*I site (−116/+79) were remarkable in that they retained *trans*-activation activity even though the TATA box, cap site, and SP1 binding site had been deleted. The constitutive activity of these constructs was reduced about four- to sixfold in various experiments, but the relative *trans*-activation by NS1 remained significant. These results show that a major portion of the tar sequences map between −146 and −116. A residual amount of the response seems to result from sequences 3′ to −116.

Deletions on a finer scale were constructed by synthesizing deleted tar elements in vitro. Two such tar elements are illustrated in Fig. 2. The H-1tard5/9 fragment is shortened by 5 base pairs on the 5′ side, nucleotide pairs −142 to −138, and the H-1tard22/24 fragment is shortened by 3 base pairs, −123 to −121, 5′ to the *Bst*EII site. They were cloned into the *Bgl*II-*Bst*EII sites of pH9cat, in which they replaced the wild-type (wt) tar. Both plasmids continued to show *trans*-activation with no significant change in the +NS1/−NS1

```
                Sau3A                          BstE11
H-1 tar:        GATCTGTGCTTGGTTGGTGAAGAATG
                    ACACGAACCAACCACTTCTTACCAATGG

              BamH1                      BstE11  BamH1
H-1tarBa:     GATCCTGTGCTTGGTTGGTGAAGAATGGTTACCG
                  GACACGAACCAACCACTTCTTACCAATGGCCATG

                Sau3A                          BstE11
H-1tard5/9:     GATC-----TTGGTTGGTGAAGAATG
                         AACCAACCACTTCTTACCAATG

                Sau3A                          BstE11
H-1tard22/24: GATCTGTGCTTGGTTGGTGAA---TG
                  ACACGAACCAACCACTT---ACCAATG
```

FIG. 2. Sequences of the synthetic tar elements. The G of the Sau3A site is −146, so that the fragments include the region from −146 to −114. H-1tard5/9 has residues −142 to −138 deleted, and H-1tard22/24 has residues −123 to −121 deleted. The upper line has the 5′ terminus to the left.

ratio (Table 3). The deletion at −142 to −138 results in a net change of sequence of only 3 base pairs in the area deleted: the wt sequence is TGTGC, and after the deletion this position becomes AGATC. The results indicate that the TGTG that has been changed to AGAT is not an essential part of the tar active site. The GAA deletion at −123 to −121 alters the distance between sequences 5′ to it that appear to have activity and those 3′. More detailed study is required to find whether this mutant has a subtle deficiency.

Position effects of the tar. To test whether the tar can function bidirectionally or from the 3′ side of a P38-driven gene, we constructed the plasmid pH2catΔtar (Table 1, plasmid no. 23). This plasmid has a deletion from the *Bgl*II site at nucleotide 1250 to the *Nco*I site at nucleotide 1900 (−108), and a *Bgl*II linker was placed at the site of the deletion. The synthetic tar element, H-1tarBa (Fig. 2), was cloned at the *Bgl*II site 5′ to the promoter in both orientations, giving the plasmids pStarBg and pStarIBg, or into the *Bam*HI site 3′ to the *cat* gene, giving the plasmid pStarBa. The tar at the *Bam*HI site is oriented toward the P38 promoter; thus it is inverted with respect to the direction of transcription. The expression of cat by these plasmids with and without *trans*-activation by NS1 in NB cells is shown in Table 4. The NS1 gene supplied by plasmid pH5 was used at the higher doses of 1 or 2 μg per dish in this experiment. pH2catΔtar, with the tar element deleted, was not *trans*-activated by NS1, which is similar to the results that we obtained with pH2-108 at the higher levels of the NS1 plasmid. Both orientations of the tar in pStarBg and pStarIBg were stimulated by NS1 to about the same level. Compared with the native P38 plasmid in pH1cat or the synthetic P38 (pH3Sy), the response was weaker, 11- to 12-fold versus 28- to 30-fold. It is not clear how significant

TABLE 3. Effects of deletions of −142 to −138 and −125 to −123 on the tar[a]

Plasmid	%aC −NS1[b]	%aC +NS1[b]	+NS1/−NS1[b]
pH9cat (wt tar)	1.6 (2.2)	12.8 (77.0)	8 (35)
pHtar5/9	1.1 (0.6)	12.3 (14.3)	11 (24)
pHtar22/24	0.6	4.6	7

[a] Transfections were done in HeLa cells as for Table 2, except that pH5 was supplied at 2 μg per dish and the extractions were made at 48 h posttransfection. The plasmid pHtar5/9 has −142 to −138 deleted, and pHtar22/24 has −125 to −123 deleted by using the synthetic tar elements shown in Fig. 2.

[b] Numbers in parentheses are from another independent experiment. %aC, Percentage of 3-acetylchloramphenicol.

TABLE 4. Effects of position and orientation of the synthetic tar element on P38 expression of *cat*[a]

Plasmid	%aC −NS1[b]	%aC +NS1[b]	+NS1/−NS1
pH2catΔtar	0.9	1.0	1.1
pStarBg	1.0	11.4	11.4
pStarIBg	0.6	7.3	12.1
pH2-120	1.4	4.9	3.5
pH2-108	1.2	1.7	1.4
pH1cat	0.5	15.1	30.0
pH3Sy	0.5	14.0	28.0
pStarBa	0.2	0.2[c]	1.0

[a] Transfections were done with 2 μg of each plasmid per 60-mm dish of NB cells, and cells were extracted at 72 h posttransfection. The cultures were treated with 5 mM sodium butyrate for 16 h after the glycerol shock. The CAT assay was quantitated by scintillation spectrometry.
[b] %aC, Percentage of 3-acetylchloramphenicol.
[c] pStarBa was cotransfected with 1 μg of pH5.

this difference is. The lower constitutive expression of pH1cat greatly affects the ratio, and a variation in the level of expression of NS1 can markedly affect the magnitude of the *trans*-activation (see below). It is clear that the insertion of the tar element increases the *trans*-activation compared with that in pH2catΔtar.

The synthetic tar was constructed so that the distance relationships of the wt tar were preserved in the normal orientation. Thus the pStarBg construction is equivalent to a wt P38 promoter with a *Bgl*II linker insertion 5′ to the *Nco*I site at −108. The pStarBa plasmid with the tar inserted into the *Bam*HI site 3′ to the *cat* gene had no detectable response to NS1.

Effect of the amount of NS1 gene on *trans*-activation. When high doses of the NS1 gene or NS1 genes with alternative promoters were used in the *trans*-activation assays, a paradoxical decrease in expression of the P38 reporter gene was observed (data not shown). A hypothesis to explain such variations in *trans*-activation under different conditions with various NS1 gene constructions or various doses of the NS1 gene is that the dose response of P38 to NS1 is biphasic. The P38 promoters with the tar deleted had residual positive activity to NS1 at near-optimal dose levels, but not at higher doses (Tables 2 and 4). The different experiments may vary in their efficiency of transfection, resulting in variations in the NS1 concentrations attained.

To test the hypothesis that the NS1 gene has a biphasic effect on mutant P38 constructs, transfections with various amounts of plasmid pH5 and constant amounts of the target plasmids were carried out. The target plasmids were the wt P38 in pH2cat, pH2catΔtar, which is deleted to −108, pP38-120, which is deleted to −120, and the heterologous promoter in pRSVcat. The pP38-120 plasmid usually has a greater response to NS1 than does pH2catΔtar. The results are summarized in Fig. 3. The wt P38 showed the highest level of stimulation by NS1 (50-fold) at the lowest dose (0.2 μg per dish). At higher amounts of pH5 the expression showed diminishing *trans*-activation (Fig. 3A). The Rous sarcoma virus (RSV) long terminal repeat (LTR) had no positive response to NS1 at any dosage and was inhibited in a dose-dependent manner. Both mutant P38 plasmids clearly showed a biphasic response qualitatively similar to that of wt P38 but with about a 10-fold-lower magnitude (Fig. 3B). The absolute amount of CAT produced was higher for the −120 construct than for the −108 construct at each dose of pH5. These results suggest that the deletions to −120 or −108 did not remove all of the sequences that respond specifically to NS1 or that NS1 has a nonspecific stimulatory activity at low levels. At the higher dose levels of the NS1 plasmid, the plasmids with the tar element deleted were inhibited. The failure of the RSV promoter to respond positively at the low dose would argue for the former interpretation.

To assign the inhibitory effect to NS1 and not some other property of the pH5 plasmid, it is necessary to have a control. We show below that many mutants of NS1 are also inhibitory. The most likely mutant to use as a control is the one with the frameshift mutation in the NS1 gene generated by filling in the *Bgl*II site at nucleotide 1250, as in pH6cat (36). pH6cat responded to NS1 as well as pH2cat, implying that the truncated NS1 generated by pH6cat was not inhib-

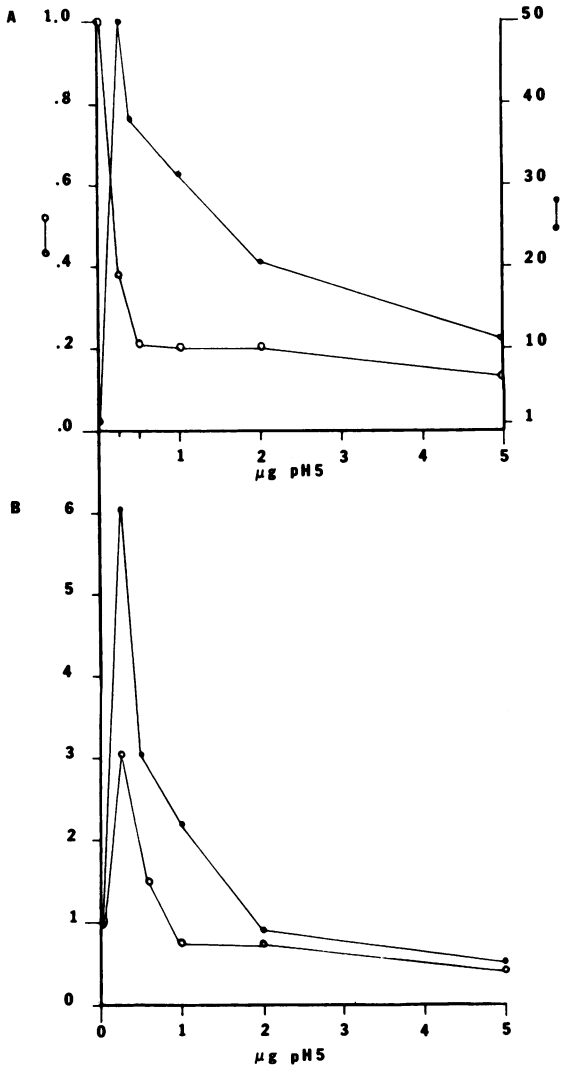

FIG. 3. The relative expression of wt P38, deletion mutants of P38, and the RSV LTR (10) fused to the *cat* gene as a function of dosage of the NS1-NS2-producing plasmid pH5. (A) Symbols: ●, pH2cat (wt P38); ○, pRSVcat. (B) Symbols: ●, pH2catΔtar (5′ deletion to −108 + *Bgl*II linker); ○, pH2-120 (5′ deletion to −120). Test plasmid (2 μg) was cotransfected into HeLa cells with the indicated amounts of pH5, and the expression of *cat* was determined at 72 h posttransfection. The constitutive expression levels of each plasmid in percent acetylation of the chloramphenicol were as follows: pH2cat, 0.4; pRSVcat, 35.0; pH2catΔtar, 0.8; pH2-120, 2.1. The relative values shown were normalized to the respective constitutive value.

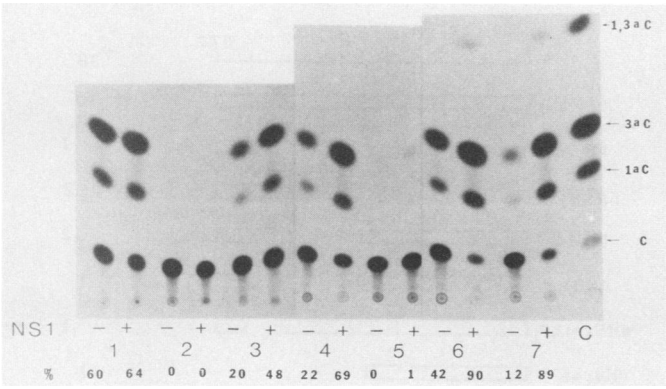

FIG. 4. Autoradiogram of a CAT assay testing the expression of P38 constructs in the presence and absence of pH5, which contains the NS1-NS2 gene and has the VP2 gene deleted. Transfections were done in NB cells with 2 μg of each plasmid per 60-mm dish, and the extracts were made 48 h posttransfection. Shown below each lane is the percent acetylation as measured by densitometry. Lanes: 1, pH9SVecat; 2, ptarSVp; 3, pH3ΔBs; 4, pH3ΔX2; 5, pH3ΔX2SVp; 6, ptar2cat1; 7, ptar2cat2.

itory. This mutation is within the intron of NS2 and should not affect the expression of NS2 directly. The pH6cat mutation was transferred into the plasmid pH5, giving the plasmid pH6. Using this plasmid, we tested the effect of the truncation of NS1 in *trans*. Cotransfection of pH2cat and pRSVcat with 1 μg of pH6 in HeLa cells produced slight increases in the expression of both plasmids (data not shown). This confirms that the inhibitory effect of pH5 is due to the expression of NS1 and can be destroyed by a frameshift mutation at nucleotide 1250.

Enhancement of P38. When the complete P38 promoter was placed 3′ to the heterologous SV40 enhancer, which is inserted in the *Bgl*II site in pH9cat at position −146, its expression was constitutively activated and it exhibits no significant *trans*-activation by NS1 (Fig. 4, lane 1). Similarly, insertion of the SV40 enhancer 3′ to the *cat* gene at the *Bam*HI site also constitutively activated the expression of *cat*, and the construct showed little further stimulation by NS1 (data not shown). It should be noted that although NS1 did not stimulate the enhanced P38 it did not inhibit it at this dose of pH5, as in the case of the SV40 early promoter (35) or the RSV LTR (Fig. 3). The insertion of the SV40 promoter, without its enhancer 3′ to the tar element, resulted in undetectable constitutive or *trans*-activated activity (Fig. 4, lane 2). This construction is comparable to the insertion of the tar element into pA10cat as a test for enhancer activity (21). A similar result was obtained with the plasmid pH3ΔX2SVp (lane 5), in which the SV40 promoter was fused to the P38 promoter at position −15 (approximate position determined by restriction mapping). The control plasmid, pH3ΔX2 (lane 4), has positions −15 to +105 deleted and was used to construct the fusion to the SV40 promoter in lane 5. When pH3ΔX2 was used to insert a tandem repeat of the P38 promoter by fusing the −15 *Bgl*II site to the −146 *Bgl*II site of pH9cat (ptar2cat1) (lane 6), the constitutive expression was increased and the promoter remained highly inducible by NS1. In this construction, it is unlikely that transcripts initiated by the 5′ copy of the promoter are able to express *cat*, because at the *Nco*I site at −108 in the 3′ copy of the promoter, there is a consensus ATG start codon in an open reading frame. Translation of this open reading frame would block translation of the *cat* gene. The plasmid in lane 3 was mutagenized with mung bean nuclease to destroy the *Bst*EII site, and it continues to be *trans*-activated by NS1.

When the tar element was directly repeated without an intervening promoter, plasmid ptar2cat2 (lane 7), the constitutive expression was not increased and the *trans*-activation was approximately the same as with a single tar element (data not shown). The enzyme levels in the +NS1 samples for lanes 6 and 7 exceed the linearity of the assay, but this does not affect our conclusions.

In summary, P38 responded to activation by a heterologous enhancer positioned either 5′ or 3′ to the P38 *cat* gene cassette by increasing its constitutive expression and becoming more refractory to *trans*-activation by NS1. P38 can also be activated constitutively by tandem duplication of the complete P38 promoter, but in this case it continues to respond well to *trans*-activation. The tar element showed no enhancer activity when tested in the 5′ position to the SV40 early promoter or when tandemly duplicated in the P38 promoter.

Inhibition of expression by NS1. Cotransfection of several heterologous promoters with plasmids expressing NS1 has shown an inhibition of expression (Fig. 3) (35). Additional tests of heterologous promoters were made, and the results are summarized in Table 5. NS1 was inhibitory for an SV40-enhanced P38 construct, the Harvey-*ras* promoter, RSV LTR, human immunodeficiency virus (HIV) I LTR, and the P38 with the tar element deleted (Fig. 3).

A search for functional domains in NS1 was made by constructing plasmids with in-frame deletions or truncations of the NS1 gene. The constitutive activity of the P38-driven *cat* gene in plasmid pH3cat was tested at various doses of three such mutant NS1 plasmids (Fig. 5). pH5Δ2 and pH5Δ3 were highly inhibitory to P38, and pH5Δ1 was less inhibitory. Because all of these plasmids contain P38 sequences, it was possible that the promoter sequences were binding a rate-limiting factor, such as SP1. Cotransfection with a clone of P38 from −346 to +127 in pBR327 was not inhibitory even at 10 μg, ruling out this possibility. The relative activities of these NS1 mutants and several others on P38 activity are

TABLE 5. NS1-induced inhibition of heterologous promoters and an enhanced P38 promoter[a]

Plasmid	%aC −NS1[b]	%aC +NS1[b]	+NS1/−NS1
ptar2cat1	45.4	93.9	2.1
pSVtar2cat1	66.0	45.5	0.7
prascat	2.6	0.1	0.04
pU3RIII + ptar2tatIII	65.3	22.6	0.3
pP38tar3 + ptar2tatIII	5.1	12.8	2.5
pRSVcat	49.3	5.5	0.1

[a] The transfections were carried out in NB cells, and extracts were made at 48 h posttransfection. NS1 was supplied by cotransfection with 2 μg of the plasmid pH5. The assays were quantitated by densitrometry of the autoradiogram. The plasmid constructions are described in Table 1 in reference to Fig. 1. The plasmid prascat contains the promoter of the Harvey-*ras* gene from the *Bam*HI site of the pEJ clone to the *Xma*III site at nucleotide 596 fused to the *cat* gene (33, 43). The pU3RIII plasmid contains the HIV I LTR (39), and the ptar2tatIII contains the HIV tatIII gene as a *Sal*I-*Bam*HI fragment fused to the tandemly repeated P38 promoter from the plasmid ptar2cat1. In ptar2tatIII the tatIII expression would be increased by NS1. The plasmid pP38tar3 contains a fusion of the P38 promoter from pH3ΔX2 at the *Bgl*III site (−15) to a synthetic HIV tar3 element that extends from −13 to the end of the tandem repeat at +49 with sequences for *Bgl*II and *Hin*dIII ligation on the 5′ and 3′ ends, respectively. The expression of this construct is positively regulated by tatIII (data not shown) and is included here as a control for pH5 + ptar2tatIII. pRSVcat contains the RSV LTR fused to the *cat* gene (10).

[b] %aC, percentage of 3-acetylchloramphenicol.

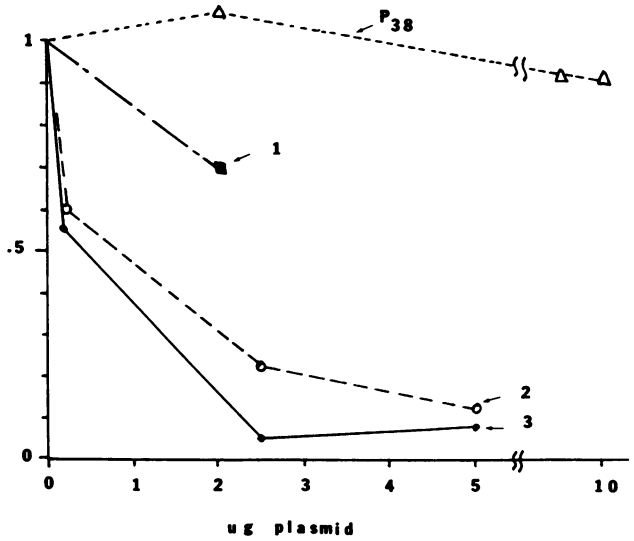

FIG. 5. Relative expression of pH3cat in the presence of various amounts of the following plasmids: pP38, the P38 promoter from −346 to +127 (as in pH3cat) cloned into pBR322; pH5Δ1 (line 1); pH5Δ2 (line 2); and pH5Δ3 (line 3). The transfections were made in NB cells, and the extracts were collected at 48 h posttransfection.

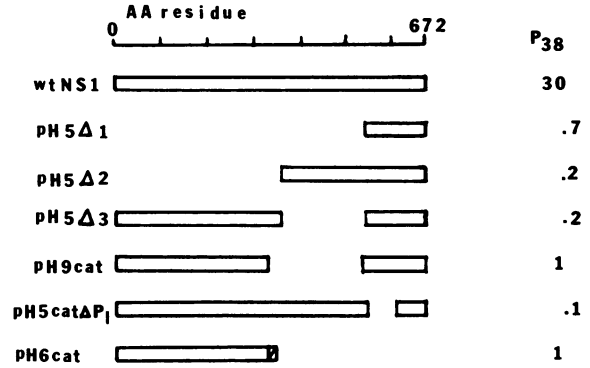

FIG. 6. Diagram of the exon regions expressed by truncated and internally deleted versions of the NS1 gene. The wt NS1 was supplied by pH5. The constructions used restriction site ligations that preserved the open reading frame of the NS1 gene (Table 1). The plasmids pH5Δ1, pH5Δ2, and pH5Δ3 were made with *Nco*I sites. In the right-hand column are the relative expression values of 2 μg of the P38cat gene in the presence of wt or mutant NS1 generated by 2 μg of the corresponding plasmid. The P38 test plasmid was pH3cat, and these values were derived from transfections of NB cells.

summarized in Fig. 6. All of the mutants (except pH8 [discussed above and not shown here]) have lost their *trans*-activation activity, and most of them show some level of inhibitory activity.

DISCUSSION

Parvoviruses have two (or more) nonstructural gene products, which are called NS1 and NS2 for the autonomous parvoviruses H-1, minute virus of mice, canine parvovirus, and other closely related viruses (7, 9, 37). The NS1 protein has functions required for viral DNA replication for both the autonomous parvoviruses and the adeno-associated viruses (AAV) (14, 25, 45) and regulates the transcription of viral genes (20, 35, 46). In the work described in this report we have shown by deletion mapping that the *cis* element sufficient for NS1 *trans*-activation (tar) of the coat protein P38 promoter and necessary for a maximal *trans*-activation is an upstream element that lies within the region −137 to −116. The exact boundaries of the tar element are uncertain. The constructs with 3′ deletions to −116 retained significant *trans*-activation activity relative to the constitutive level. On the other hand, the linker insertion from −109 to −115 in a construct with a synthetic tar element produced a modest reduction in the level of *trans*-activation. This suggests that the sequences between −116 and −108 may have a role in the *trans*-activation.

Inspection of the sequences in the H-1 and canine parvovirus tar elements reveals a significant homology to the CCAAT-binding protein (CBP) DNA-binding site in the murine sarcoma virus LTR (11), which is in the opposite orientation (Fig. 7). In that study, evidence was presented that this CBP binding site was also present in the opposite orientation in the herpes simplex virus *tk* gene promoter. A CBP binding site has been identified in the α2(I) and α1(III) collagen gene promoters (13), the ovalbumin gene (29), and the adenovirus type 2 major late promoter (27). In most promoters, the CCAAT box is positioned at −80, whereas the parvovirus tar is at −120. The connection between the CCAAT box and the tar is not clear, but the homology suggests a possible relationship between NS1 and CBP. A more detailed analysis of this region by site-specific mutagenesis is in progress to clarify the composition of the tar element.

The H-1 tar element has some properties that are similar to those of classical enhancer elements. The inversion of the synthetic tar reduced, but did not destroy, the activity for *trans*-activation. This inversion altered the distance relationships of the tar to the other promoter elements, including the ACCA at the *Nco*I site. This could have been unfavorable for maximal activity, but there was no difference between the two orientations of the tar. The placement of the tar 3′ to the test gene did not show any activity. The H-1 tar has activity in the absence of the other 3′ elements of its homologous promoter. This latter property has similarity to that of the A element in the 72-bp enhancer of SV40, which does not absolutely require a promoter (44). The finding of no activity on certain heterologous promoters may reflect a property that some of the more classical enhancers have as well. Evidence was recently reported that some of the cell type specificity that enhancers have shown may be the result of a specificity of enhancer-promoter combinations that vary in activity depending on the cell environment in which they are tested (4, 38). Unlike the tar elements described for human T-cell lymphotropic virus types I and II or the glucocorticoid receptor element of mouse mammary tumor virus, which function at a distance, bidirectionally, and with heterologous promoters, the parvovirus tar element has shown no activity on the SV40 early promoter. Another unusual property of the P38 tar is that it is not synergistic with heterologous enhancers. In contrast, the P38 promoter

```
H1 TAR:     TGTGCTTGGTTGGTG-AAGAATGGTTACCAATCTACCATGG
              ** ****** **  **  * ***
MSV LTR:    ACTGATTGGTTAGTTCAAATAAGGTACA
              **  ********  *** *****
CPV TAR:    TGTGCATGGTTAGTG-AAACAAGGTTATGAATCAACCATGG
```

FIG. 7. Comparison between the H-1 P38 and CPV tar sequences and the CCAAT binding protein sequence in the MSV LTR (11). Positions of identity are indicated by asterisks.

shows increased constitutive activity when it is tandemly duplicated, in conjunction with no diminution of its ability to be *trans*-activated by NS1. The tandem duplication of specific domains within enhancers has been reported to increase enhancer activity in other cases (15, 43, 48, 51). These domains have been shown to be DNA-binding sites for proteins that activate transcription (50). Apparently the activity of such a protein-DNA complex can be increased by duplication of the binding site through some type of cooperative activity. The factors binding the A and B domain of the SV40 enhancer have been recently characterized and named AP1 and AP2, respectively (R. Tjian, American Society of Virology Annual Meeting, 1986). Interestingly, Tjian also reported that the basal-level enhancer 2 in the metallothionein promoter contains repeats of the AP2 binding site. Thus this may represent a natural example of activity generated by repeats of the binding site of a single factor. The increased constitutive activity of the tandemly repeated P38 promoter could be the result of the additional SP1 binding site (6, 16, 24) or the duplication of the tar element. Our tests of these two possibilities do not substantiate either possibility. The tandem duplications of either the tar element or the SP1 binding site (S. Rhode, unpublished data) did not show an effect of this magnitude. However, the configurations of these duplications were not the same as in the tandemly repeated promoter.

Whether the parvovirus P38 tar binds NS1 directly or a cellular protein, such as CBP, remains to be determined. If NS1 binds the tar element, then footprinting analysis will greatly aid the definition of the tar element. A region of the NS1 gene, just 5′ to the tar element, codes for a large domain within NS1 that is highly conserved in all of the parvoviruses for which sequence data are available (41). This domain also has homology to the ATPase domains of the large T antigens of papovaviruses and a number of procaryotic proteins that have ATPase activity including RecA and DnaA of *Escherichia coli* (C. Astelle, Am. Soc. Virol. 1986, 1). It seems likely that an ATPase activity will be found in NS1.

NS1 inhibits all of the promoters that we have tested that do not have the tar element. This includes the heterologous promoters SV40 early, RSV LTR, HIV I LTR, Harvey-*ras*, and the H-1 P4 promoter (N. Hanson and S. Rhode, unpublished data). In these tests the plasmids were introduced to cells simultaneously. Therefore we do not know whether the inhibition is for the initiation of transcription or its maintenance. The wt P38 and P38 mutant promoters that have the tar element deleted show a biphasic response to NS1 that is similar, but the response of the mutant promoters is greatly reduced in magnitude. They continue to show some *trans*-activation at low levels of the NS1-producing plasmid, but are inhibited at levels that would significantly stimulate the wt P38. The cause of the inhibition is not clear. It does not seem to be the result of competing sequences, since cotransfection of pH3cat and increasing amounts of a clone containing P38 did not inhibit the constitutive expression. The plasmid pH6, with a truncated NS1 and a wt NS2, was not inhibitory. This indicates that the inhibition can be attributed to NS1 and not NS2 or some other effect. Several truncated or deleted versions of NS1 that would not produce NS2 were also inhibitory to the constitutive level of P38. All of the inhibitory constructs retained the carboxy-terminal domain of NS1. pH9cat may be an exception, since it has the carboxy-terminal domain of NS1 but its P38 expression appeared to be unaffected at the constitutive level and it responded well to wt NS1 in *trans*. All of the mutants with mutations in the NS1 gene, except pH8, lost all *trans*-activation of P38. Thus the inhibitory property can be dissociated from the *trans*-activation function of NS1 and from any NS2 effect. Plasmids that generate only NS1 or NS2 will be needed to sort out the effects of each protein. The finding that NS1 can produce opposite effects on transcription depending on the gene dosage must be considered in the study of this protein. The Rep protein of AAV, which is analogous to NS1, also has been shown to have positive and negative effects on AAV transcription (20, 46). Unlike NS1, the Rep protein was a negative regulator of AAV P40 in some cells (human 293 cells) (46). In adenovirus-infected 293 cells and in HeLa cells, Rep *trans*-activated AAV P40. It is possible that these complicated opposing effects of Rep are a result of different concentrations of Rep protein or different ratios of Rep to a cellular factor. These paradoxical effects of NS1 and AAV Rep are similar to the positive and negative effects reported for the adenovirus E1A gene products (5).

For the H-1 P4 promoter, the tar is a downstream element at a distance of about 1.7 kilobases. At a similar distance downstream from the P38 promoter, the tar had no positive *trans*-activation effect on P38. It seems likely that P4 is regulated by a constitutively active enhancer and downregulated by NS1 in the same manner as the RSV LTR. Thus we predict that NS1 autoregulates its own synthesis to achieve concentrations that are near optimal for the *trans*-activation of the P38 promoter. During infection, the inhibition of P4 by NS1 would have to be an inhibition of maintenance of P4 transcription, since P4 transcription must be initiated to produce NS1. In contrast to our studies with transfected plasmids, the copy number of viral DNA rapidly increases during infection. The viral genes and *cis* elements that may be protein-binding sites increase to large copy numbers. Therefore the extrapolation of results obtained with a static system to the more dynamic system of productive infection should be made with caution.

The parvovirus early protein NS1 has complex roles during virus replication. Four likely phenotypes for NS1 have been identified: requirement for viral DNA replication (1, 14, 25, 34, 45), inhibition of cell DNA replication (Rhode, unpublished observations), positive regulation of the capsid protein gene promoter, and negative regulation of the P4 promoter (NS1/NS2 gene) and heterologous promoters. Mutant NS1 genes have been used to dissociate some of these functions from each other. The inhibition of P4 and heterologous promoters does not require an NS1 that functions in the *trans*-activation of P38. We have detected no inhibition of transcription for NS2, which should be expressed by pH6cat or pH6 at wt levels or possibly increased levels, under the conditions used here and previously (35). A function for NS2 has not yet been identified.

ACKNOWLEDGMENTS

We thank R. Weinberg for plasmid pEJ, B. Howard for pRSVcat, C. Rosen for pU3RIII, K. Sakai for a genomic clone of HIV I, and M. Botchan for pACT. We are very grateful for the expert technical assistance of Jane deVasure.

This work was supported by Public Health Service grants CA37481 and CA36727 from the National Institutes of Health and grant DMB-8444778 from the National Science Foundation.

LITERATURE CITED

1. **Anton, I. A., and D. P. Lane.** 1986. Non-structural protein 1 of parvoviruses: homology to purine nucleotide using proteins and early proteins of papovaviruses. Nucleic Acids Res. **14**:7813.

2. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–669.
3. **Astell, C. R., M. Thomson, 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.
4. **Berg, P. E., Z. Popovic, and W. F. Anderson.** 1984. Promoter dependence of enhancer activity. Mol. Cell. Biol. **4**:1664–1668.
5. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
6. **Briggs, M. R., J. T. Kadonaga, S. P. Bell, and R. Tjian.** 1986. Purification and biochemical characterization of the promoter-specific transcription factor SP1. Science **234**:47–52.
7. **Carlson, J., K. Rushlow, I. Maxwell, F. Maxwell, S. Winston, and W. Hahn.** 1986. Cloning and sequence of DNA encoding structural proteins of the autonomous parvovirus feline panleukopenia virus. J. Virol. **55**:574–582.
8. **Cotmore, S., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
9. **Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two non-structural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
10. **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.
11. **Graves, B. J., P. F. Johnson, and S. L. McKnight.** 1986. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell **44**:565–576.
12. **Gruss, P.** 1984. Magic enhancers. DNA **3**:1–5.
13. **Hatamochi, A., B. Paterson, and B. de Crombrugghe.** 1986. Differential binding of a CCAAT DNA binding factor to the promoters of the mouse α2(I) and α1(III) collagen genes. J. Biol. Chem. **261**:11310–11314.
14. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. **51**:329–339.
15. **Herr, W. and Y. Gluzman.** 1985. Duplications of a mutated simian virus 40 enhancer restore its activity. Nature (London) **313**:711–714.
16. **Jones, K. A., J. T. Kadonaga, P. A. Luciw, and R. Tjian.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, SP1. Science **232**:755–760.
17. **Jongeneel, C. V., R. Sahli, G. K. McMaster, and B. Hirt.** 1986. A precise map of the splice junctions in the RNAs of the minute virus of mice, an autonomous parvovirus. J. Virol. **59**:564–573.
18. **Keller, J. M., and M. C. Alwine.** 1985. Analysis of an activatable promoter: sequences in the simian virus 40 late promoter required for T-antigen-mediated *trans*-activation. Mol. Cell. Biol. **5**:1859–1869.
19. **Khoury, G., and P. Gruss.** 1983. Enhancer elements. Cell **33**: 313–314.
20. **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.
21. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
22. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1984. Host specificity of enhancement of gene expression by activator elements, p. 325–330. *In* M. L. Pearson and N. L. Sternberg (ed.), Gene transfer and cancer. Raven Press, New York.
23. **Lebovitz, R. M., and R. G. Roeder.** 1986. Parvovirus H-1 expression: mapping of the abundant cytoplasmic transcripts and identification of promoter sites and overlapping transcription units. J. Virol. **58**:271–280.
24. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46**:795–805.
25. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
26. **Mishoe, H. A., J. N. Brady, M. Radonovich, and N. P. Salzman.** 1984. Simian virus 40 guanine-cytosine-rich sequences function as independent transcriptional control elements in vitro. Mol. Cell. Biol. **4**:2911–2920.
27. **Miyamoto, N. G., V. Moncollin, J. M. Egly, and P. Chambon.** 1985. Specific interaction between a transcriptional factor and the upstream element of the adenovirus-2 major late promoter. EMBO J. **4**:3563–3570.
28. **Molitor, T. W., H. S. Joo, and M. S. Collett.** 1985. Identification and characterization of a porcine parvovirus nonstructural polypeptide. J. Virol. **55**:554–559.
29. **Pastorcic, M., H. Wang, A. Elbrecht, S. Y. Tsai, M.-J. Tsal, and B. W. O'Malley.** 1986. Control of transcription initiation in vitro requires binding of a transcription factor to the distal promoter of the ovalbumin gene. Mol. Cell. Biol. **6**:2784–2791.
30. **Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward.** 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. **11**:1019–1038.
31. **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.
32. **Pintel, L. L., and D. Pintel.** 1986. The minute virus of mice P_{39} transcription unit can encode both capsid proteins. J. Virol. **57**: 1163–1167.
33. **Reddy, E. P.** 1983. Nucleotide sequence analysis of the T24 human bladder carcinoma oncogene. Science **220**:1061–1063.
34. **Rhode, S. L., III.** 1982. Complementation for replicative-form DNA replication of a deletion mutant of H-1 by various parvoviruses. J. Virol. **42**:1118–1122.
35. **Rhode, S. L., III.** 1985. Nucleotide sequence of the coat protein of canine parvovirus. J. Virol. **54**:630–633.
36. **Rhode, S. L., III.** 1985. *trans*-Activation of parvovirus P38 promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
37. **Rhode, S. L., III, and P. R. Paradiso.** 1983. Parvovirus genome: nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J. Virol. **45**:173–184.
38. **Robbins, P. D., D. C. Rio, and M. R. Botchan.** 1986. *trans*-Activation of the simian virus 40 enhancer. Mol. Cell. Biol. **6**: 1283–1295.
39. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
40. **Sahli, R., G. K. McMaster, and B. Hirt.** 1985. DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice. Nucleic Acids Res. **13**:3617–3633.
41. **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 an aplastic crisis. J. Virol. **58**:921–936.
42. **Shein, J. 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.
43. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29**: 161–169.
44. **Takahashi, K., M. Vigneron, H. Matthes, A. Wildeman, M. Zenke, and P. Chambon.** 1986. Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature (London) **319**:121–126.
45. **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.

46. **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.
47. **Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon.** 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell **32**:503–514.
48. **Weber, F., J. DeVilliers, and W. Schaffner.** 1984. An SV40 "enhancer trap" incorporates exogenous enhancers or generates enhancers from its own sequences. Cell **36**:983–992.
49. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaut, and L. Chasin.** 1979. Biochemical transfer of a single copy eukaryotic gene using total cellular DNA as donor. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
50. **Wildeman, A. G., M. Zenke, C. Schatz, M. Wintzerith, T. Grundstrom, H. Matthes, K. Takahashi, and P. Chambon.** 1986. Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. **6**:2098–2105.
51. **Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.