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Expression from the Adeno-Associated Virus p5 and p19 Promoters Is Negatively Regulated in *trans* by the *rep* Protein

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The leftward two promoters of the adeno-associated virus (AAV) 2 genome were fused to reporter genes, and the constructs were used to transfect HeLa cells. The promoters functioned constitutively but were repressed in *trans* by the AAV *rep* gene product(s). The repression was relieved by adenovirus infection. Evidence which indicated an enhancer function for the inverted terminal repeat of the AAV-2 genome was also obtained.

The genome of the human dependovirus adeno-associated virus (AAV) 2 is a linear single-stranded DNA molecule of 4,680 bases with inverted, palindromic terminal repeats of 145 bases (19). Promoters have been identified at map positions 5, 19, and 40 (6, 13). When the intact genome is introduced into continuous human cell lines in culture, transcripts from all three promoters are difficult to detect unless there is concomitant expression of genes from helper virus (either adenovirus or herpesvirus (2, 4). A fundamental question is whether the difficulty in detecting AAV-specific gene expression in the absence of helper function implies an inherent lack of ability of the promoters to function under such conditions or whether the virus itself negatively regulates its own gene expression under these conditions. Support for the latter possibility comes from earlier experiments which have demonstrated the ability of AAV *rep* gene products to inhibit the expression of genes under the control of the simian virus 40 (SV40) early promoter in transient transfection and under the control of several viral and cellular promoters in permanent transformation (11, 12, 21). More directly, the AAV promoter (p40) which controls transcription of RNA species from which the AAV coat proteins are translated has been shown to function constitutively in human HeLa cells (21).

In this paper we report experiments which demonstrate that both the AAV promoters at map positions 5 and 19 can function constitutively in HeLa cells in the absence of helper function. However, these constructs are susceptible to negative regulation by AAV *rep* gene function, and the negative regulation can be reversed by adenovirus infection. Thus, these constructs behave in a manner analogous to that of AAV. Finally, we present evidence that the inverted terminal repeats can function as enhancers.

In the presence of helper virus coinfection, AAV *rep* gene function is required in *trans* for the optimal accumulation of AAV-specific transcripts (3, 23). We wished to test whether the AAV *rep* gene product might inhibit gene expression from the p5 and p19 promoters in the absence of helper virus. To do this, we created plasmid constructs which contained either the p5 or the p19 TATA box and 60 or 30 more upstream bases, respectively, fused to the chloramphenicol acetyltransferase (CAT) gene as a reporter gene (Fig. 1). When either of these constructs was transfected into HeLa cells, CAT activity was readily detected (Fig. 2 and 3, Table 1). Hence, both promoters were able to function constitutively in HeLa cells in the absence of helper virus gene expression. Cotransfection of pSM620 (17), which contains an intact AAV genome (Fig. 1), inhibited expression of either p5 CAT or p19 CAT (Fig. 2 and 3, Table 1). Thus, in the absence of a helper virus there was expression of *rep*, but the net effect of such *rep* expression appeared to be inhibition of further expression from either p5 or p19. That the negative effects observed in cotransfection with pSM620 were due to *rep* was demonstrated by cotransfection of plasmids with mutations in either the *rep* gene or the *cap* gene (coat proteins) (Fig. 2). In the absence of adenovirus infection, AAV plasmids with an intact *rep* gene and a deletion in the *cap* gene still inhibited expression by either p5 CAT or p19 CAT, whereas a deletion or a frameshift mutation in *rep* abolished the inhibitory effect (Fig. 2). At a constant input concentration of the p5 CAT construct, increasing amounts of the *rep* construct in the cotransfection mixture led to decreased amounts of CAT activity in the absence of helper virus coinfection and also decreased the ability of helper virus coinfection at a constant multiplicity of infection to reverse the inhibitory effect (Fig. 3, Table 1). In the case of the p19 CAT construct, a similar dose-dependent inhibition of *rep* was observed, but the differences at the two higher concentrations of *rep* were minimal and there seemed to be a slight reversal of the inhibitory effect at the highest concentration of *rep* input (Fig. 3; Table 1).

In the absence of the *rep* product, adenovirus infection had a modest effect on CAT expression. Expression of p5 CAT was increased by 1.5-fold in one instance (Table 1, experiment 2). Variable effects were seen with p19 CAT: in one case expression was increased threefold (Table 1, experiment 3), but in another (Table 1, experiment 4), expression was decreased by 2.5-fold. In no case was a more marked stimulation or repression noted. By contrast, adenovirus infection had a much more marked stimulation of both p5 and p19 CAT expression in the presence of *rep* (Fig. 3; Table 1, experiments 1 and 2). In the presence of 5 μg of *rep*, p5 CAT expression was restored 50-fold to its original level (Table 1, experiment 2). The results with p19 CAT were less impressive, although in the presence of 2 μg of *rep*, adenovirus infection increased p19 CAT expression 12-fold in experiments in which CAT expression was under control of the SV40 early promoter (Table 1, experiment 1). Similar experiments were performed with SV2 CAT (Table 1, experiment 3) (5). Very modest effects of *rep* or *rep* plus adenovirus infection on SV2 CAT expression were observed. Whether the actual differences observed are significant is questionable. Although these experiments were transfec-

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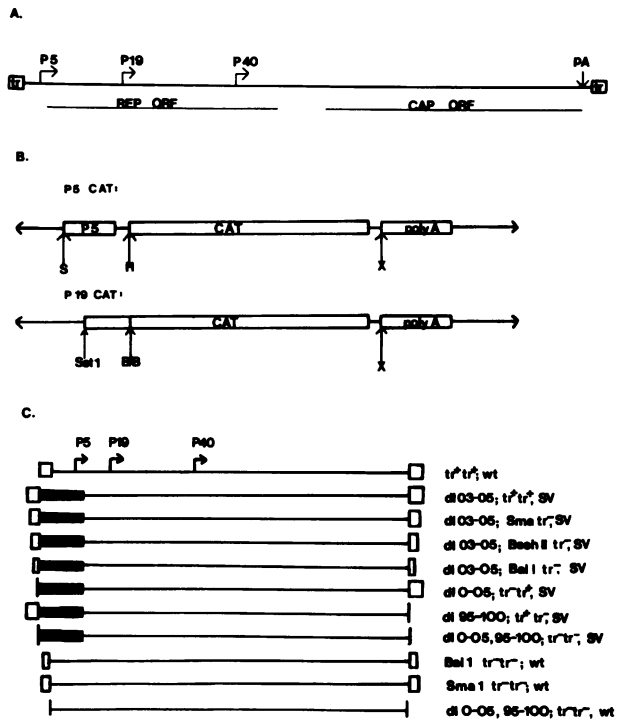

FIG. 1. Structure of wild-type AAV (A), p5 and p19 CAT constructs (B), and terminal repeat (tr) deletion/p5 replacement constructs (C). (A), trs of AAV are represented by open boxes at both ends of the genome. The three promoter regions are indicated by right-angled arrows at map positions 5, 19, and 40. The two viral open reading frames designated REP and CAP are indicated. (B) Structure of CAT gene constructs. CAT gene (Pharmacia, Inc.) was cloned into the *Hin*dIII site of pBluescript (thin lines). The p5 promoter region derived from an *Mva*I-*Fnu*DII digest (bases 144 to 310) of an AAV clone containing only the *Pst*C fragment of AAV (17) was blunted and ligated into the *Sma*I site of the Bluescript polylinker. The poly(A) site, a 235-base-pair fragment derived from a *Sna*BI-*Pst*I digestion of pSM620 (17) was blunted and inserted into the blunted *Xho*I polylinker site. For p19 CAT, an *Sst*I-*Bcl*I fragment (nt 814 to 964) isolated from pSM620 grown in *dam*-negative bacteria was ligated to *Sst*I-*Bam*HI-digested pCAT. The poly(A) site was inserted as for p5 CAT. (C) Structures of terminal repeat deletions and SV40 promoter p5 replacements. Open boxes represent terminal repeats. Wt indicates that the unaltered p5 region from AAV is present. Closed black boxes and the designation SV on the right represent replacement of the AAV p5 region (*dl*03-05) with the entire SV40 regulatory region (nt 5171-5243-270) oriented such that the SV40 early promoter drives p5 gene expression (10). Restriction enzyme designations on the right indicate partial deletion of the trs, i.e., sequences outside of those restriction sites were deleted. Deletions of 0 to 05 or 95 to 100 or both indicate complete deletions of one or both trs (1).

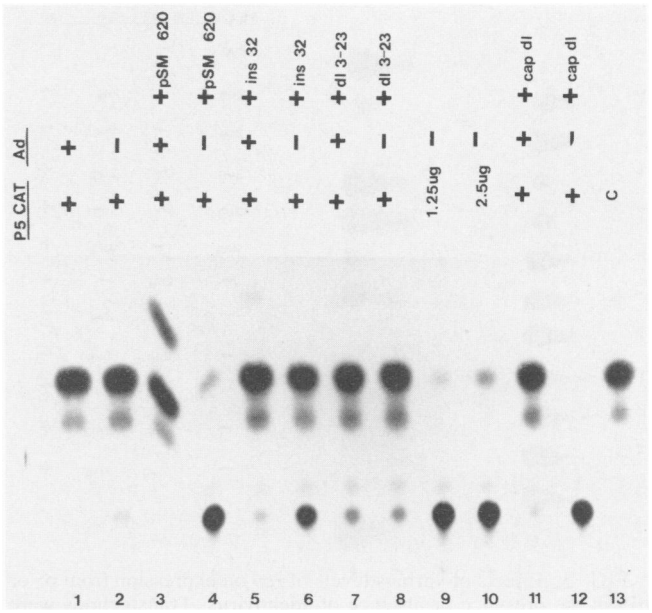

FIG. 2. The effect of *rep* or *cap* or both on expression from the p5 promoter in the presence or absence of adenovirus. HeLa monolayer cells were transfected (24) with 5 μg of p5 CAT alone (lanes 1 and 2) or p5 CAT in the presence of 5 μg of one of several AAV constructs: lanes 3 and 4, wild-type AAV; lanes 5 and 6, ins 32, a frameshift insertion which results in inactivation of *rep*, pHM326 (9); lanes 7 and 8, *dl*3-23 a deletion which results in *rep* inactivation, pHM1515 (9); and lanes 11 and 12, *cap dl* pLB101 (9), which is a deletion from map unit 63 to 86 but leaves *rep* intact. In lanes 9 and 10, 1.25 or 2.5 μg of p5 CAT, respectively, were transfected, and lane 13 represents chloramphenicol treated with a commercially available preparation of CAT enzyme (control). All transfections were equalized to 20 μg of total DNA by using high-molecular-weight *Escherichia coli* DNA as carrier. Precipitation of DNA for transfection was by the $CaPO_4$ technique (24). Transfections were harvested at 48 h; cell extracts were prepared, and CAT activity was assayed (5).

tions and thus not physiological in the same sense as virion infection, the results do mimic to a large extent those seen in virion infections (AAV plus or minus helper). The situation is rendered more complex because of the inhibitory effects of *rep* on adenovirus gene expression, which is greatly dependent on the relative multiplicities of infection of the two viruses (3). From these data we conclude: (i) the AAV p5 and p19 promoters can function constitutively in HeLa cells, and (ii) in the absence of helper virus, AAV can negatively regulate gene expression from the p5 and p19 promoters in HeLa cells.

It had been previously reported that CAT expression from the p19 promoter within the context of the AAV genome could be significantly induced by adenovirus infection (20) and that a minimal amount of constitutive CAT expression from a p19 CAT construct could be observed in HeLa cells. In the latter case, adenovirus infection increased CAT activity fourfold (22). These data are in contrast to the more limited effect of adenovirus observed with the p19 CAT construct used in these experiments and suggest additional signals for the regulation of p19 expression beyond the 30 bases upstream from the TATA box that were in our construct. From previous work in which genome replication was studied, it was shown the AAV terminal repeats also play a role as a target sequence for regulation by *rep* gene products (10). To investigate a possible regulatory role of the terminal repeats in AAV transcription, we engineered a series of constructs in which various lengths of the terminal repeat sequences were deleted and the subsequent effect on accumulation of AAV transcripts was assayed. Previously, we have reported construction of a hybrid AAV/SV40 genome in which the SV40 regulatory sequences (nucleotides [nt] 5171-5243-270) were substituted for AAV sequences from nt 144 to 264 (10). In human KB cells, the phenotype of the hybrid construct (*dl*03-05/SV40) was indistinguishable in that study from that of wild-type AAV; i.e., transcript accumulation was not detectable in the absence of adenovirus. In adenovirus-infected KB cells, the hybrid genome was

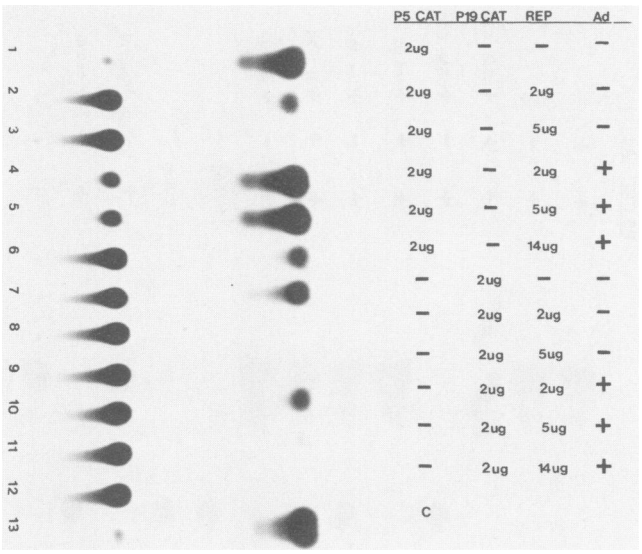

FIG. 3. Effects of various levels of *rep* on expression from p5 or p19 in the presence or absence of adenovirus. Transfections were carried out with 2 μg of p5 CAT or p19 CAT alone (lanes 1 and 7) or in the presence of 2, 5, or 14 μg of the *rep*-containing plasmid LB101 (*dl*63-86; see reference 9) in the absence (lanes 2, 3, 8, and 9) or presence (lanes 4 to 6 and lanes 10 to 12) of adenovirus. Lane 13 is a control.

biologically active and virions were produced. Thus, at the level of a full-length genome or its equivalent, the control of gene expression by the hybrid genome is indistinguishable from that of the wild type. However, removal of 125 bases from both ends of the genome (i.e., deletion of the palindromic regions of the terminal repeat) had differential effects. In the case of the wild-type AAV genome, the terminal deletions still allowed readily detectable AAV transcript accumulation in adenovirus-infected KB cells (Fig. 4). Such transcript accumulation in adenovirus-infected KB cells could barely, if at all, be detected when either 103 or 125 bases were deleted from the AAV/SV40 hybrid genome (Fig. 4) but were detectable when only 55 bases were deleted from both termini. Thus the critical region appears to lie between nt 56 and 102. However, in hybrid constructs from which either the right or the left terminal repeat had been deleted but the other terminal repeat was present, transcript accumulation was readily detected with adenovirus coinfection. Thus, some sequence within the terminal repeat was required for transcript accumulation in the presence of adenovirus, but the positive effect was position and (apparently) orientation independent. Therefore, under these conditions the terminal repeat fulfilled the criteria of an enhancer, but this effect was only observable under those assay conditions when the SV40 sequence was substituted for the p5 promoter. Within the context of the AAV genomes, the ability of AAV expression from the leftward-most promoter to respond to adenovirus helper function in a transfection assay appears to be mediated by at least two very different sequences, one corresponding to the p5 promoter (map positions 3 to 5) and the other in the terminal repeat. To detect transcription, either site appears to be sufficient. However, these data do suggest the potential for multiple layers of regulation of AAV gene expression. Regulation at posttranscriptional and translational levels have been previously described (12, 23).

We have presented data which indicate that under nonpermissive conditions AAV can negatively regulate gene expression from its own promoters. The data help to explain the unique behavior of AAV in cell culture infections and serve to illustrate the complex autoregulation of the replication of this virus. The multiple layers of regulation that are involved are also suggested by the finding that the terminal repeats can function as enhancers of transcription, in addition to their demonstrated roles in DNA replication (10, 20), integration into other DNA sequences (7, 8), and rescue from the integrated state (18). We have demonstrated previously that in monkey cos 7 cells, which constitutively synthesize the SV40 T antigen, AAV can negatively regulate the replication of an AAV/SV40 hybrid genome (10). In that case, the replication of an SV40 replicon was inhibited by the *rep* gene product in *trans* and the AAV terminal repeat was the *cis*-active target sequence. Although the latter data are indirect with respect to AAV DNA replication, we have hypothesized that under nonpermissive conditions in human cells, the AAV genome inhibits its own replication as well. Our current model is that AAV negatively regulates its own gene expression and DNA replication under nonpermissive conditions in order to enhance its ability to integrate into the cellular genome to establish a latent infection. In this way, the survival of the viral genome is optimized. This model is supported by the following observations: the plasmid pSVneo, which contains the SV40 origin of replication, replicates in cos 7 cells but does not integrate into cellular DNA to stably transform the cells to neomycin resistance (M. Labow, A. Levine, and K. I. Berns, unpublished experiments). In contrast, an AAV/SV40 hybrid plasmid in which the same SVneo sequences as in pSVneo were engineered into a

TABLE 1. Expression from both the p5 and p19 promoters

Transfection no.	Amt of plasmid transfected					Virus[a]	% Acetylation[b]
	p5 CAT	p19 CAT	SV2 CAT	pSM620	pLB101		
Expt 1							
1	2 μg					−	95
2	2 μg				2 μg	−	12
3	2 μg				5 μg	−	0.8
4	2 μg				2 μg	+	88
5	2 μg				5 μg	+	85
6	2 μg				14 μg	+	13
7		2 μg				−	26.5
8		2 μg			2 μg	−	0.8
9		2 μg			5 μg	−	3.7
10		2 μg			2 μg	+	9.9
11		2 μg			5 μg	+	2.4
12		2 μg			14 μg	+	4.1
Expt 2							
1	2 μg					−	11.9
2	2 μg					+	18.3
3	2 μg				5 μg	−	0.2
4	2 μg				5 μg	+	11
Expt 3							
1			2 μg			−	5.3
2			2 μg	2 μg		−	2.8
3			2 μg	2 μg		+	12.3
4		2 μg				+	7.8
5		2 μg				−	2.5
Expt 4							
1		2 μg				+	19.2
2		2 μg				−	47

[a] −, No adenovirus infection; +, adenovirus infection.

[b] % Acetylation was calculated by dividing the amount of ^{14}C radioactivity in the acetylated form of chloramphenicol by the sum total of radioactivity in the acetylated and nonacetylated forms.

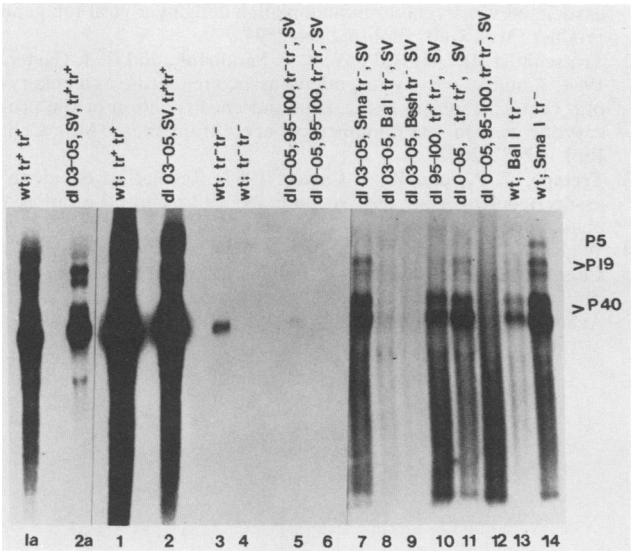

FIG. 4. Effect of terminal repeat (tr) deletions or replacement of p5 with the SV40 regulatory region on accumulation of AAV-specific transcripts. KB cells were transfected by the DEAE dextran technique (14) with 20 μg of wild-type AAV plasmid (lanes 1 and 1a [shorter exposure]) or with plasmids with a wild-type p5 region and complete deletions of the trs (nt 0 to 145) (lanes 3 and 4) deletions in as far as nt 121 (*Bal*I restriction site) (lane 13), or in as far as nt 55 (*Sma*I restriction site) (lane 14). Transfections were also carried out with plasmids in which the AAV p5 promoter region (map units 03 to 05) was deleted and replaced with the SV40 regulatory region (nt 5171-5243-270) in a construct containing complete trs, (lanes 2 and 2a), no trs, (lanes 5, 6, and 12), or deletions in as far as the *Sma*I site (lane 7), *Bal*I site (nt 121) (lane 8), or *Bss*H2 site (nt 105) (lane 9). Deletions of the right tr only or left tr only from these constructs are shown in lanes 10 and 11, respectively (1). Lanes 1, 1a, 2, 2a, 3, 5, and 7 to 14 depict transfections in which adenovirus infection of the cells was carried out 1 h prior to transfection. RNA was isolated at 48 h postinfection, electrophoresed on a 1.3% agarose-formaldehyde gel, blotted onto nylon, and probed with an AAV-specific probe (12).

deletion in the AAV cap gene did not replicate well in cos 7 cells but did integrate to stably transform the cells to neomycin resistance. Thus, at least in the case of AAV, replication of the viral genome and integration into the cellular genome seem to be mutually exclusive. Similar behavior has been observed in the case of bovine papilloma virus (16).

Parallels can be drawn between the AAV *rep* gene and the adenovirus E1A gene. Not only do products of each negatively and positively regulate gene expression, but both types of regulation may be exerted on the same gene. Similarly, products of both regulatory genes affect a wide range of heterologous promoters. In the case of AAV, there are at least four *rep* gene products with overlapping amino acid sequences (15), so that it has been difficult to sort out the specific functions of each. To make matters more difficult, the apparent phenotype of a given *rep* gene mutant is highly dependent on the cell infected. The functional diversity of the AAV *rep* gene products exceeds that of E1A products in that AAV *rep* gene products may directly affect AAV DNA replication negatively as well as positively. The pleiotropy of the *rep* gene results in a virus whose life cycle may be thought to be the paradigm of a successful parasite. When the host cell is healthy, the AAV genome establishes a latent infection with no apparent ill effect on the cell. But when the cell is exposed to toxic agents, stressed, or infected by a lytic nuclear DNA virus, the AAV genome is activated so that virions may be produced to continue the cycle in a new host.

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