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Adeno-Associated Virus Gene Expression Inhibits Cellular Transformation by Heterologous Genes

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In this paper we report that adeno-associated virus (AAV) genomes inhibit stable transformation by several dominant selectable marker genes upon cotransfection into mouse tissue culture cells. Cotransfection of AAV genomes also inhibited the expression of pSV2*cat* in transient assays. In both cases, the inhibitory effect was independent of AAV DNA replication but required the AAV p5 and p19 genes, which encode proteins required for AAV DNA replication and regulation of AAV gene expression. Finally, addition of a cloned E4 gene in the transfection experiments partially blocked the AAV-mediated inhibitory activities.

Adeno-associated virus type 2 (AAV2) is a defective human parvovirus (dependovirus) which requires coinfection with either adenovirus (Ad) or herpesvirus for its own replication (1, 4, 5, 19). Detailed genetic studies with Ad as the helper have shown that all aspects of AAV macromolecular synthesis are regulated by Ad functions. Ad early region E1A, E1B, E2A, and VA RNAs are required for maximal AAV gene expression (20, 21, 23, 26, 33, 34). AAV DNA synthesis also has been reported specifically to require expression of the E4 region (33). In addition to regulation by the helper virus, and undoubtedly by cellular functions as well, the AAV genome also autoregulates its own gene expression and DNA replication. The left side of the AAV genome contains a large open reading frame (ORF) between map positions 5 and 44 (29, 41) which is transcribed from two promoters, p5 and p19, located at map positions 5 and 19, respectively (Fig. 1) (15, 27, 29). Polypeptides of approximately 70 and 50 kilodaltons would be predicted from the nucleotide sequences of the unspliced forms of the p5 and p19 RNAs and have been observed recently in infected-cell extracts (31). The p5 and p19 genes were originally characterized as *rep* genes since frameshift mutations within this region abolished AAV DNA replication (17, 43). Mutations within the p5 and p19 ORFs also inhibit expression and transcription of AAV genomes in helper virus-infected cells, in a manner independent of DNA replication (25, 44). Both types of mutants can be complemented in *trans*. AAV structural proteins are encoded within a large ORF on the right side of the genome and are translated from a transcript with a promoter at map position 40 (14, 22, 23). Recent studies have also indicated that a product encoded within the p5 ORF can negatively regulate expression of the p40 transcription unit in human 293 cells (44).

The regulation observed in cells coinfected with Ad and AAV is not unidirectional. AAV coinfection inhibits Ad DNA replication and virus production (6, 7). AAV coinfection also inhibits the ability of Ad both to transform cells and to cause tumors when injected into susceptible animals (24, 30). The tumorigenicity of Ad-transformed cells is also greatly decreased after AAV infection (32). In this case, the accumulation of the Ad5 E1B 55-kilodalton tumor antigen was observed to decrease in parallel to the decrease in tumorigenicity. Thus, AAV appeared to negatively regulate expression of the integrated Ad genome. Similarly, AAV infection inhibits the oncogenicity of herpes simplex virus-transformed cell lines (3, 8, 9). In these studies virion AAV was used; thus, the specific regions of the AAV genome responsible for the inhibitory activities could not be rigorously identified nor could the functions of the AAV genome be assayed for in the complete absence of helper virus functions.

In this paper we present evidence that the AAV genome also negatively regulates the activity of several different genes on separate DNA molecules in both stable transformation and transient gene expression assays in the apparent absence of helper virus functions. Further, these inhibitory activities were absolutely dependent on the AAV p5 and p19 genes and appeared to be modulated by the E4 gene of Ad. These data serve to illustrate the complex matrix of regulatory interactions that occur during viral infection.

MATERIALS AND METHODS

Cell culture. B78H1 cells, a mouse melanoma cell line (12), were maintained as monolayer cultures in minimal essential medium containing 5% calf serum, 5% fetal calf serum, 1% glutamine, and the following antibiotics: penicillin, streptomycin, gentamicin, and amphotericin B (Fungizone) in concentrations suggested by the manufacturer (GIBCO Laboratories, Grand Island, N.Y.). Mouse L thymidine kinase-negative (L*tk*$^-$) cells and HeLa cells were maintained similarly except that Dulbecco modified Eagle medium was used.

Plasmid DNAs. Constructs used in this study are described in Fig. 1 and in the text. The construction of all the cloned AAV genomes used has been previously described (17, 18, 25, 35). p*tk* (HSV thymidine kinase gene) (45), pXBAC (E4 gene) (16), pSV2*cat* (11), and pMMT*neo* and pBPV/MMT*neo* (28) plasmids were generous gifts of M. Chao, R. J. Samulski, E. Rifkin, and P. Howley, respectively. The *neo* vector, pGC*cos3neo*, has been described previously (12) and was derived from pSV2*neo* (40), differing mainly by the insertion of a lambda *cos* sequence outside of the *neo* gene.

Transfection of DNA. All DNAs were transfected by the calcium phosphate coprecipitation method (13, 47). Cells used for stable transformation were seeded at a density of 1.25×10^5 cells or 3×10^5 cells per 100-mm dish for either B78H1 or L*tk*$^-$ cells, respectively. Calcium phosphate coprecipitates were placed dropwise onto the culture medium the next day. Each plate received a 1-ml precipitate

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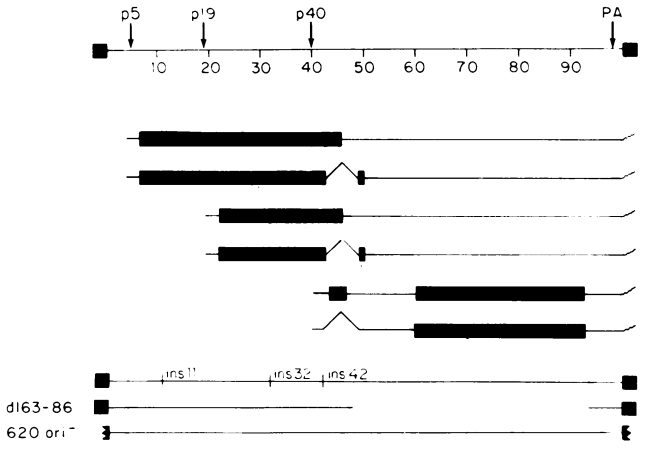

FIG. 1. Structure and organization of wild-type and mutant AAV genomes. The first line represents the 4.7-kilobase AAV genome divided into 100 map units. The terminal repeats are represented as small black boxes. The locations of the three AAV transcription promoters, p5, p19, and p40, as well as the common polyadenylation site (PA) are indicated by arrows. The AAV RNAs are illustrated below the genetic map. ORFs present within the RNAs are represented as long black boxes. The carets within the RNAs represent the common AAV intron, and the wavy lines represent the poly(A) tails. The structures of cloned mutant genomes are represented below the six RNAs. The first line is a composite which illustrated the positions of 8-base frameshift mutations present within different insertion mutants. The second line represents the deletion, indicated by the gap, present within the AAV mutant *dl*63-86. The deletion mutations of the terminal repeats of 620*ori*$^-$ are represented by truncated black boxes.

containing 0.1 or 0.05 μg of either a Neo^r or *tk* gene, respectively, variable amounts of cloned AAV genomes as indicated in the text, and carrier DNA (calf thymus DNA; Boeringer Manheim Biochemicals, Indianapolis, Ind.) so that the total DNA in each transfection was 25 μg. Some transfections also contained pXBAC or pFB69 as indicated in the text and tables. Culture medium and precipitates were removed the next day, replaced with fresh medium, and replaced with selective medium at 48 h posttransfection. Selective medium for neomicin-resistant (Neo^r) colonies consisted of 1 mg of G-418 per ml in normal growth medium. TK$^+$ cells were selected in hypoxanthine-aminopterin-thymidine medium as previously described (47). Cultures were grown on selective medium for 10 to 18 days, after which the resultant colonies were fixed with 10% formaldehyde and stained with 1% cresyl violet. The number of colonies per dish was determined visually.

Coprecipitates for transient expression assays were similarly prepared except that 0.2 μg of pSV2*cat*, 5 μg of AAV- and E4-containing plasmids, and carrier DNA for a total of 25 μg of DNA per transfection were used. Also, 30% confluent cultures of L*tk*$^-$ cells were used for transfections. At 4 h posttransfection, the precipitates were removed, and the cells were glycerol shocked and then placed in medium containing 5 mM sodium butyrate as described previously (10).

CAT assays. Chloramphenicol acetyl transferase (CAT) assays were carried out as previously described (11). Cell extracts were made by freeze-thawing the cells from a 100-mm dish three times in 100 μl of 0.25 M Tris hydrochloride (pH 7.9). One-third of each extract and 0.25 μCi of [^{14}C]chloramphenicol (New England Nuclear Corp., Boston, Mass.) were used in each assay. Acetylated [^{14}C]chloramphenicol was resolved by silica thin-layer chromatography (Eastman Kodak Co., Rochester, N.Y.), and the percentage of acetylation was determined by counting of spots cut from the thin-layer chromatography plates.

RESULTS

AAV-mediated inhibition of transformation. Our initial experiments were performed in an attempt to transform cells to produce the AAV *rep* gene products constitutively, using cotransfection and coselection with a marker gene. In this first experiment, pGC*cos*3*neo* (0.1 μg) (which contains the *neo* gene under the control of the simian virus 40 [SV40] early promoter) was transfected alone or cotransfected with various cloned AAV genomes (5 μg). An excess of AAV DNA was used to ensure that every cell that received the *neo* gene also received AAV sequences. Cells were selected in the presence of G-418 for 10 to 18 days and finally fixed and stained. The plates of such an experiment are shown in Fig. 2. Transfection of the *neo* vector alone gave rise to approximately 200 Neo^r colonies (Fig. 2A). However, cotransfection with pSM620, a plasmid containing the entire AAV genome, inhibited the number of Neo^r colonies formed by >99% (Fig. 2B). Further analysis showed that the rare colonies arising after cotransfection of pGC*cos*3*neo* and the AAV plasmid did not retain integrated AAV DNA (data not shown). To determine whether a specific AAV function or sequence was involved in the inhibition, we also cotransfected cloned mutant AAV genomes with pGC*cos*3*neo*. Cotransfection with a cloned AAV genome containing a large deletion mutation within the capsid gene, *dl*63-86 (Fig. 2C), or with a genome containing deletion mutations in both terminal repeat sequences, 620*ori*$^-$ (Fig. 2D), also inhibited transformation. However, cotransfection with a mutant containing a frameshift insertion at map position 32 within the p5 and p19 ORFs, *ins*32*ori*$^-$ (Fig. 2F), had no effect on transformation. Cotransfection of *ins*11 (Fig. 2E) also inhibited transformation but to a lesser extent than by genomes containing a completely intact left-side ORF, indicating that although the p19 gene itself was inhibitory to transformation, the presence of the p5 gene enhanced the inhibition. However, in other experiments in a different cell line, *ins*11 failed to affect transformation significantly (see below). From these data we conclude that one or both *rep* genes are required for the inhibitory effect. Also, because the *ori*$^-$ mutants are replication defective owing to deletions in the terminal repetitions required for AAV DNA replication (2, 36, 38, 42), replication of the AAV genome is not required for inhibition. (This control assumes more significance with the recent report of AAV DNA replication in the absence of helper [37]).

Specificity of AAV-mediated inhibition of transformation. The next question we asked was whether the inhibitory effects of the AAV *rep* genes were specific for either the *neo* gene or the SV40 early promoter. Table 1 contains the results of several experiments in both the B78H1 and L*tk*$^-$ cell lines with various cloned AAV mutants and various selectable markers. Cotransfection with 620*ori*$^-$ or *dl*63-86 inhibited transformation by pGC*cos*3*neo* in all experiments, while *ins*32*ori*$^-$ and *ins*42*ori*$^-$ did not. The AAV mutant *ins*11 inhibited transformation in two experiments shown (Table 1, A and B) but not in a third experiment (Table 1, E). The reason for this variability is not known at this time, but it should be noted that experiments A and B were in B78H1 cells and experiment E was in L*tk*$^-$ cells. It should also be

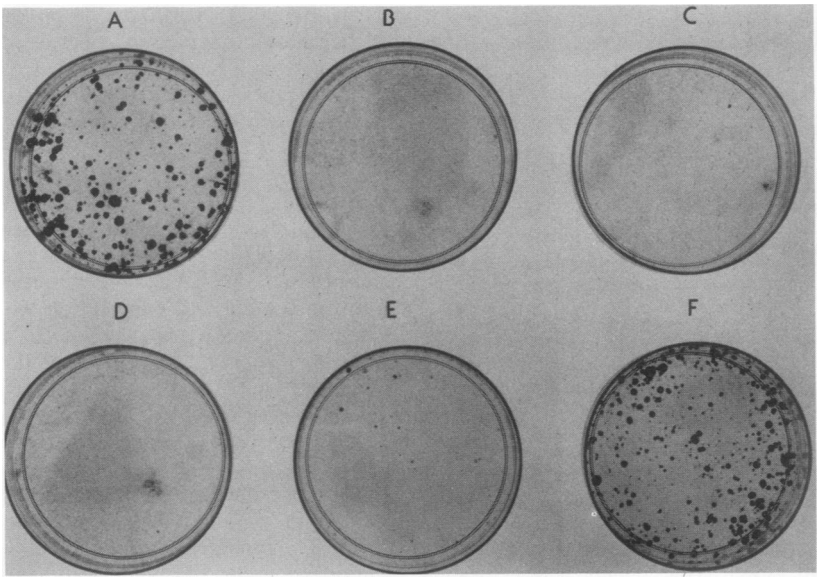

FIG. 2. In this experiment, plates containing 1.25×10^5 B78H1 cells were transfected with 0.1 μg of pGC*cos3neo* DNA alone (A) or with 5 μg of pSM620 (B), 620*ori*$^-$ (C), *dl*63-86 (D), *ins*11 (E), or *ins*32*ori*$^-$(F). All transfections contained high-molecular-weight carrier DNA such that 25 μg of total DNA was used in each transfection. Neo^r colonies were selected as described in Materials and Methods.

noted that the inhibition exhibited by *ins*11 in experiments A and B was not as great as that observed for 620*ori*$^-$.

AAV DNA also inhibited transformation by both p*tk* (containing the herpes simplex virus *tk* gene under the control of its endogenous promoter) (Table 1, F and G) and the *neo* gene under the control of the mouse metallothionein promoter (Table 1, H and I) whether the gene was present alone in a plasmid vector (pMMT*neo*) or inserted into a bovine papilloma virus vector (pBPV/MMT*neo*). This inhibition was again dependent on intact AAV p5 and p19 genes as *ins*32*ori*$^-$ had no effect on transformation. Thus, the inhibitory effect did not appear to be specific for a single selectable gene or promoter.

Lack of *rep* gene lethality. A possible reason for the inhibitory effects of vectors containing intact AAV *rep* genes could have been that *rep* gene expression was lethal for transfected cells. Several experiments were done to assess this possibility with the plasmid *dl*52-91/*neo*, in which the *neo* gene under the control of the SV40 early promoter has been inserted into a deletion in the AAV coat protein gene (Table 1, J and K) (18). All three AAV promoters are retained in this construct as well as intact p5 and p19 genes. This plasmid was able to transform L*tk*$^-$ cells with the same efficiency as pGC*cos3neo* alone. In cotransfections of *dl*52-91/*neo* and 620*ori*$^-$ the number of colonies observed was approximately 20 times greater than that observed for cotransfections of pGC*cos3neo* and 620*ori*$^-$. Thus, lethality of transfection of 620*ori*$^-$ cannot account for the extent of inhibition of pGC*cos3neo* transformation. The inhibition that was observed with *dl*52-91/*neo* appears more likely to have been predominantly of a competitive nature because cotransfection with *ins*32*ori*$^-$ caused nearly half the reduction in colonies observed with 620*ori*$^-$ (note that a 20/1 620*ori*$^-$/*dl*52-91/*neo* input ratio was used).

Dose-dependent inhibition of transformation. In the above experiments a large excess of AAV to *neo* DNA was used. We also did cotransfections with various amounts of AAV or *neo* DNA (Table 2). In this experiment either B78H1 or L*tk*$^-$

TABLE 1. Inhibition of stable transformation by AAV genomes

Expt	Cell line	Marker DNA[a]	No. of colonies with the following AAV DNA[b]:				
			None	620*ori*$^-$	*ins*11$^-$	*ins*32*ori*$^-$	*ins*42*ori*$^-$	*dl*63-86
A	B78H1	pGC*cos3neo*	268	1	21	312	ND[c]	1
B	B78H1	pGC*cos3neo*	252	0	ND	82	ND	0
C	B78H1	pGC*cos3neo*	800	17	220	800	ND	ND
D	L*tk*$^-$	pGC*cos3neo*	220	6	ND	162	118	ND
E	L*tk*$^-$	pGC*cos3neo*	252	1	104	225	124	ND
F	L*tk*$^-$	p*tk*	115	1	ND	500	ND	ND
G	L*tk*$^-$	p*tk*	104	1	ND	138	ND	ND
H	L*tk*$^-$	pBPV/MMT*neo*	88	3	ND	108	ND	ND
I	L*tk*$^-$	pMMT*neo*	224	4	ND	230	ND	ND
J	L*tk*$^-$	*dl*52-91/*neo*	220	51	ND	132	ND	ND
K	L*tk*$^-$	*dl*52-91/*neo*	360	55	ND	114	ND	ND

[a] Transfections with pGC*cos3neo*, p*tk*, pBPV/MMT*neo*, and pMMT*neo* or *dl*52-91/*neo* contained 0.1, 0.05, 0.1, or 0.2 μg of each marker gene, respectively.
[b] The numbers shown are for individual plates and represent the total number of colonies on single dishes containing under 200 colonies and an approximate number for dishes containing over 200 colonies. Transfections containing AAV DNA received 5 μg of supercoiled AAV-containing plasmids.
[c] ND, Not done.

TABLE 2. Dose dependence of AAV and *neo* DNA on transformation

Expt	Cell line	No. of Neo^r colonies[a] with 620*ori*$^-$ DNA (μg/transfection):			
		0	0.2	1.0	5.0
A[b]	B78H1	1,000	76	31	1
B[b]	L*tk*$^-$	420	87	17	14
C[c]	L*tk*$^-$				
	0.1 μg	480			6
	1.0 μg	600			39
	5.0 μg	2,000			240

[a] The numbers shown are reported as in Table 1.
[b] In these two experiments 0.1 μg of pGC*cos3neo* was transfected with the indicated amounts of 620*ori*$^-$.
[c] In this experiment variable amounts of pGC*cos3neo* were transfected with or without 620*ori*$^-$ (5 μg).

cells were used as recipients. Cotransfection with 0.2 μg of 620*ori*$^-$ (1:1 molar ratio) resulted in 80 to 90% inhibition of transformation, while larger amounts of AAV DNA inhibited transformation to greater extents. Similar results were obtained with either cell line. We also varied the amount of *neo* transfected using a constant amount of AAV DNA (5 μg). While the number of colonies formed increased with increasing amounts of transfected *neo* DNA, the presence of the AAV DNA still inhibited the total amount of transformation. Interestingly, a similar amount of inhibition was seen at 1:1 molar ratios of AAV to *neo* DNA whether 0.1 or 5 μg of each DNA was used.

Effect of E4 gene on inhibitory action of AAV. The above experiments showed that at least one AAV *rep* gene product appeared to be produced in the apparent absence of helper virus functions which resulted in a large inhibition of transformation by various marker genes. Since helper virus genes are known to affect both AAV replication and expression of the *rep* genes, we asked whether several of the early Ad genes affected the inhibitory activity described here. The E1A gene of Ad is known to activate expression of at least the AAV p19 gene (45). However, experiments done to examine the effect of the E1A gene on AAV-mediated inhibition would be difficult to interpret owing to the inhibitory action of the E1A gene products on the expression of heterologous genes including the SV40 early gene (46). In fact, the addition of a plasmid containing the E1A gene of Ad5 to cotransfections inhibited transformation by pGC*cos3neo* alone or in the presence of AAV DNA (data not shown). In the latter case the inhibitory effect was additive.

We also examined the effect of the E4 gene on the inhibition of transformation. The E4 gene of Ad is especially interesting in that it appears to be the only helper virus gene specifically necessary for AAV DNA replication (33). Although cotransfection of pXBAC (E4 gene) with pGC*cos3neo* had no effect on transformation (data not shown), the addition of a cloned E4 gene to 620*ori*$^-$–pGC*cos3neo* cotransfections resulted in a significant increase in Neo^r colony formation (Table 3). Control experiments did not reveal any increase in transformation by pGC*cos3neo* upon addition of heterologous plasmids such as pFB69, a derivative of pBR322. To demonstrate this effect the ratio of E4/AAV plasmids used was 2 to 10/1. Ratios lower than 2/1 were not effective (data not shown).

TABLE 3. Effect of cloned E4 sequences on AAV-mediated inhibition of *neo* transformation

Expt	No. of colonies with the following cotransfected DNA[a]:			
	None	620*ori*$^-$	620*ori*$^-$ + pXBAC	620*ori*$^-$ + pFB69
A	460	14	170	
B	200	6	112	
C	372	8	48	0

[a] The numbers of colonies per dish are reported as in Table 1. In this experiment, 0.1 μg of pGC*cos3neo* DNA was transfected alone or with additional plasmid DNAs as described in Materials and Methods except that in experiments A and B, 3 μg of 620*ori*$^-$ and 6 μg of pXBAC were used. In experiment C, 1 μg of 620*ori*$^-$ and 10 μg of pXBAC DNAs were used.

TABLE 4. Inhibition of pSV2*cat* expression by cotransfected AAV DNA

Expt	CAT activity with the following cotransfected DNA[a]:			
	None	*ins*32*ori*$^-$	620*ori*$^-$	620*ori*$^-$ + E4
A	46 (1)	58 (1.2)	15 (0.32)	26 (0.56)
B	41 (1)	38 (0.93)	4 (0.10)	14 (0.34)
C	42 (1)	32 (0.76)	7 (0.17)	ND[b]

[a] CAT activity was assayed as described in Materials and Methods. The percentage of choloramphenicol converted to acetylated forms is listed along with the fraction of activity compared with transfections with pSV2*cat* alone, as shown in parentheses. pSV2*cat* (0.2 μg) was transfected alone or with various plasmid DNAs (5 μg each) as described in Materials and Methods.
[b] ND, Not determined.

Inhibition of transient gene expression. Several mechanisms could account for the ability of the AAV *rep* genes to inhibit transformation by the tested markers. One explanation, in accord with the known role of the *rep* gene products as regulators of gene expression, would be an ability to regulate negatively the expression of the cotransfected marker genes. To test whether the AAV genome could inhibit the expression of a heterologous gene, we analyzed the effect of cotransfection of AAV DNA on the expression of pSV2*cat* (11) which contains the CAT gene under the control of the SV40 early promoter. pSV2*cat* was transfected alone or cotransfected with either 620*ori*$^-$ or *ins*32*ori*$^-$ into L*tk*$^-$ cells as described above. At 40 to 48 h after transfection, extracts from the cells were tested for the amount of CAT activity. Cotransfection of pSV2*cat* with 620*ori*$^-$ resulted in a 70 to 90% reduction in the amount of *cat* activity produced (Table 4), while cotransfection with the AAV mutant *ins*32*ori*$^-$ had no effect on CAT production. Some inhibition of CAT production was also observed in human HeLa and KB cells (data not shown). Thus, it appears that the AAV p5 and p19 genes can inhibit the expression of a heterologous gene. The addition of cloned E4 DNA in these transient assays again partially blocked the effect of the AAV p5 and p19 genes on CAT expression.

DISCUSSION

In this paper we described experiments that demonstrate the ability of the AAV genome to inhibit stable transformation by several markers introduced into cells by cotransfection. Although replication of the AAV genome was not required, the ORF on the left side of the genome had to be intact for the inhibition to be fully effective. Because small frameshift mutations within critical regions of the p5 and p19 ORFs blocked the inhibitory effect, we conclude that the inhibition was mediated by expression of one or more of the gene products encoded by this region of the AAV genome.

This is the first instance in which it has been possible to attribute a biological function to the p19 gene. In several experiments involving transfection of B78H1 murine melanoma cells, the *ins*11 mutant demonstrated a significant

inhibitory effect (albeit less than that shown by wild-type genomes). Because this mutation would prematurely terminate the p5 gene product(s) but not the p19 gene product(s) an inhibitory function can be attributed to the latter. Since the p5 and p19 reading frames overlap in phase it has not yet been possible to mutate selectively the p19 gene. Previously these reading frames have been implicated in autoregulation of AAV gene expression and AAV DNA replication (17, 24, 41, 42). In both cases the *ins*11 mutant is defective, i.e., the p5 product is required for both so that the requirement for the p19 product could not be determined. Interestingly, the *ins*11 mutant was reproducibly less effective in inhibiting transformation of L*tk*$^-$ murine cells in cotransfections, so that the p5 gene product appears to be more critical in these cells than in the B78H1 cells.

In addition to autoregulation, the AAV genome has previously been demonstrated to inhibit Ad plaque formation, DNA replication, and the expression of the major product of the Ad early region 1B, the 55-kilodalton tumor antigen (6, 7, 32). In this paper we showed that the AAV genome can inhibit transformation by genes under the control of other viral promoters (SV40 early promoter and herpes simplex virus *tk* promoter) and of a murine promoter (the inducible metallothionein promoter). Thus, the inhibitory effect was rather general.

The inhibition appears to be at the level of gene expression. The ability of the plasmid *dl*52-91/*neo* to transform L*tk*$^-$ cells successfully, even though this vector contains intact p5 and p19 genes, argues strongly against the inhibition being due to a lethal effect of p5 or p19 expression or both. Supporting this conclusion was the observation that a 20-fold excess of 620*ori*$^-$ reduced the number of colonies by only a factor of 2 over the reduction seen with *ins*32*ori*$^-$. This was in marked contrast to the 620*ori*$^-$/*ins*32*ori*$^-$ ratio of >100 observed in other transformation experiments. The *dl*52-91/*neo* results would also argue against an inhibition of marker stabilization (presumably integration) as the major mechanism of inhibition. Why *neo* transformation is not as greatly inhibited in this instance is not clear, but the p40 promoter is retained in *dl*52-91/*neo*, and it is possible that *neo* expression is off of the p40 promoter rather than the SV40 promoter. Both earlier experiments and the experiments with the pSV2*cat* vector reported in this paper would support the notion that the p19 or p5 gene product(s) or both can inhibit gene expression regulated by a number of different kinds of promoters. As with the inhibition of stable transformation, it was clearly demonstrated that the AAV inhibition of transient pSV2*cat* expression was dependent on an intact *rep* region. However, it is important to note that the level at which the inhibition observed in the CAT assays occurred was not determined. Such inhibition could have been at the level of transcription or at a subsequent step. This distinction is of interest for several reasons. Labow et al. (25) have noted that transactivation of AAV gene expression by the *rep* genes is at the level of initiation of transcription. *Cis*-active negative regulation was more likely at the elongation step (25). In an earlier study by Ostrove et al. (32) the inhibition of Ad E1B expression in transformed hamster cells as well as the inhibition of AAV p40 expression observed in 293 cells (J. Trempe, E. Mendelson, and B. Carter, personal communication) appeared to be posttranscriptional. The very recent work of Mendelson et al. (31) has shown that both p5 and p19 proteins are nuclear but that the p19 protein(s) is predominantly cytoplasmic, which is consistent with activities at multiple levels in the control of gene expression. These authors also detected p5 and p19 expression at the protein level after both transfection and viral infection in the absence of helper.

Ad E1A expression transactivates AAV gene expression but has been reported to have negative effects on a variety of non-AAV promoters (39, 46). When plasmids containing E1A were cotransfected with AAV plasmids the inhibitory effects on selectable markers were qualitatively the same as observed with either E1A or AAV alone. However, very different results were obtained when AAV was cotransfected with the E4-containing plasmid pXBAC. In these experiments, a marked reduction in inhibition was consistently noted. Further, preliminary results suggest that an E4 gene product(s) was required since the effect was lost if the cotransfected E4 gene was first disrupted by restriction digestion (data not shown). Whether the effect of the added E4 gene was a consequence of decreased AAV *rep* expression, complex formation between *rep* and E4 gene products, or some other process is currently the subject of study in our laboratory.

The results reported in this paper indicate extensive interaction of AAV with both Ad and cellular gene expression. Using AAV as a general probe of viral or host transcription controls should provide valuable insight into general mechanisms functioning in the regulation of eucaryotic gene expression.

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