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Negative and Positive Regulation in *trans* of Gene Expression from Adeno-Associated Virus Vectors in Mammalian Cells by a Viral rep Gene Product

JON DURI TRATSCHIN,† JACOV TAL,‡ AND BARRIE J. CARTER*

Laboratory of Molecular and Cellular Biology, National Intitute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, Maryland 20892

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We previously described use of the human parvovirus, adeno-associated virus (AAV), as a vector for transient expression in mammalian cells of the gene for chloramphenicol acetyltransferase (CAT). In the AAV vector, pTS1, the CAT gene is expressed under the control of the major AAV promoter p_{40}. This promoter is embedded within the carboxyl-terminal region of an open reading frame (orf-1) which codes for a protein (rep) required for AAV DNA replication. We show here that the rep product has additional *trans*-acting properties to regulate gene expression. First, deletion or frame-shift mutations in orf-1, which occurred far upstream of p_{40}, increased expression of CAT in human 293 (adenovirus-transformed) cells. This increased CAT expression was abolished when such mutant AAV vectors were transfected into 293 cells together with a second AAV vector which could supply the wild-type AAV rep product in *trans*. Thus, an AAV rep gene product was a negative regulator, in *trans*, of expression of CAT in uninfected 293 cells. In adenovirus-infected 293 cells, the function of the AAV rep product was more complex, but in some cases, it appeared to be a *trans* activator of the expression from p_{40}. In HeLa cells, only *trans* activation by rep was seen in the absence or presence of adenovirus. Neither activation nor repression by the rep product required replication per se of the AAV vector DNA. Thus, *trans*-acting negative or positive regulation of gene expression by the AAV rep gene is modulated by factors in the host cell and by the helper adenovirus.

The human parvovirus, adeno-associated virus (AAV), replicates in mammalian cells in the presence of helper functions provided by adenovirus or herpesvirus (10). When molecular clones containing the entire AAV type 2 (AAV2) genome in bacterial plasmids are transfected into mammalian cells in the presence of adenovirus, the AAV genome is excised from the plasmid and replicated to produce infectious AAV particles (38, 50, 52). This facilitated both the genetic analysis of AAV (27, 51–53, 59) and the development of AAV as a eucaryotic expression vector (26, 60, 61).

The AAV genome contains three transcription promoters, p_5, p_{19}, and p_{40}, which yield overlapping transcripts (11). Genetic analysis showed that a major AAV reading frame (orf-2) which is accessible from p_{40} transcripts codes for a major portion of the AAV capsid proteins (27, 59). Mutations in this region (*cap*$^-$) allow normal synthesis of duplex replicating form DNA but prevent synthesis of progeny single-stranded DNA and infectious particles (27, 53, 59). A second major open reading frame (orf-1) in the left half of the genome is apparently accessible from either p_5 or p_{19} transcripts and codes for one or more proteins (rep) required for AAV DNA replication (27, 53, 59). Mutations in this region (*rep*$^-$) are DNA negative but can be complemented. Deletion of both terminal palindromes results in a *cis*-dominant (*ori*$^-$) defect which reflects the presence of the AAV replication origins in the terminal repeat sequences (3, 4, 25, 51, 53). One or more of the possible rep proteins acts in *trans*, perhaps by recognizing the *cis*-acting replication origin.

Molecular clones of AAV2 have been used to construct mammalian cell expression vectors (26, 60, 61). In one class of AAV vectors, the AAV promoters functioned in the absence of helper virus, and p_{40} was an efficient promoter for expression in mammalian cells both in transient assays (61) and after stable integration into the cell genome (60). The AAV vector pTS1, which contains most of orf-1 and exhibits a *rep*$^+$ phenotype, also contains the procaryotic enzyme chloramphenicol acetyltransferase (CAT) under control of p_{40} (61). Transfection of pTS1 into two human cell lines allowed transient expression of CAT activity, and in the presence of helper adenovirus, expression was increased 7- to 10-fold (60).

To extend the genetic analysis of expression in AAV vectors, we constructed derivatives of pTS1 containing mutations in orf-1 which rendered the mutant vectors *rep*$^-$. Since the mutations were located far upstream of p_{40}, the structure of the chimeric AAV-CAT mRNA was not altered, and CAT provided a convenient assay of expression from the p_{40} transcriptional unit (18, 19, 61, 63). Studies with these pTS1 derivatives revealed two unexpected consequences of the *rep* mutations. Depending on the human cell line used and the presence or absence of helper adenovirus, the AAV rep product was either a negative or a positive *trans*-acting regulator of CAT expression.

This work provides important insights for the design of efficient AAV vectors. The results demonstrate that the AAV rep gene plays a central role in the AAV growth cycle for both DNA replication and regulation of gene expression. Thus, regulation of AAV is complex and has many features in common with a number of other mammalian viruses.

* Corresponding author.
† Present address: Institute for Hygiene and Medical Microbiology, Friedbuhlstrasse 51, CH-3010, Bern, Switzerland.
‡ Present address: Biology Department, Ben Gurion University of the Negev, Beer Sheva, 84105 Israel.

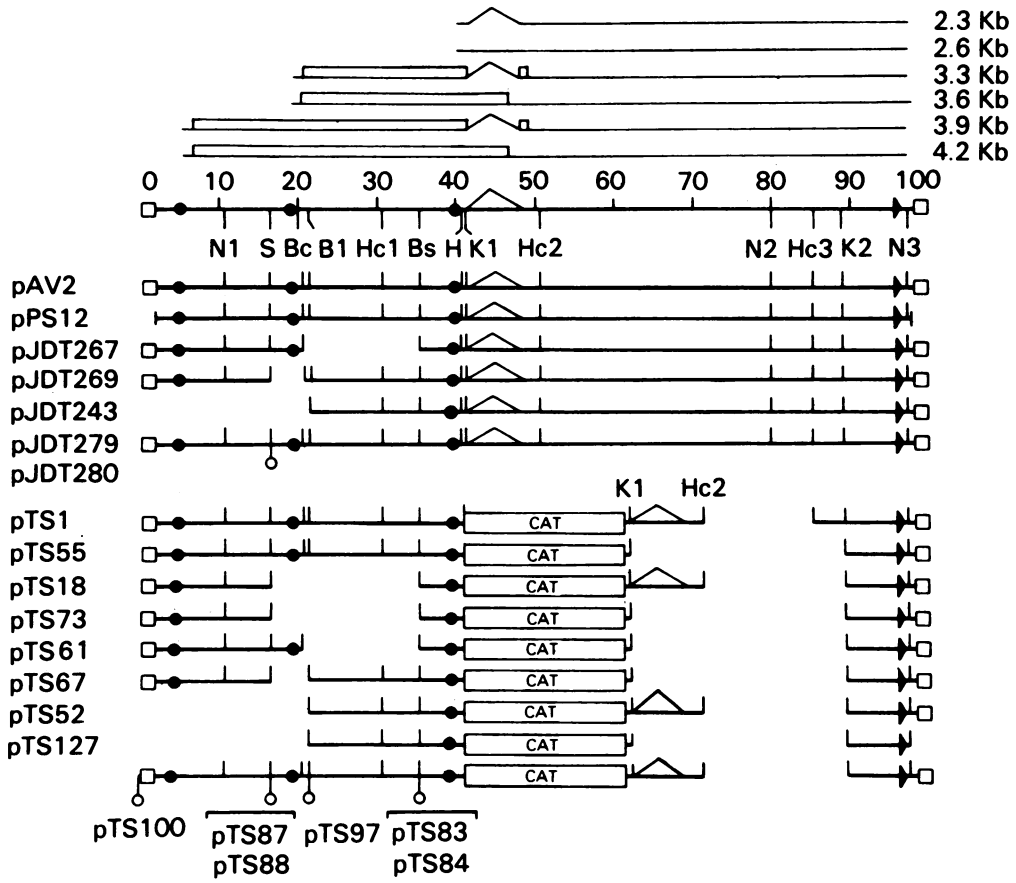

FIG. 1. Organization of the AAV genome and structure of recombinant AAV plasmids and vectors. The heavy horizontal line shows the 4,675-nucleotide long AAV genome (57) on a scale of 100 map units. Control regions: □, terminal palindrome and replication origins; ●, transcription promoters; ⌒, intron; ➡, polyadenylation site. The structures and sizes of the six known AAV mRNA species (top of figure) are shown by the thin horizontal lines (11). The direction of transcription is from left to right. The open reading frame orf-1 (rep) (▭) is indicated. Restriction sites: N, *Nco*I; S, *Sst*I; Bc, *Bcl*I; B, *Bam*HI; Hc, *Hin*cII; Bs, *Bst*EII; H, *Hin*dIII; K, *Kpn*I. For N, B, Hc, and K, the sites in AAV DNA are further designated by a number (e.g., N1, N2, N3). The structures of recombinant AAV plasmids are shown, but for clarity, the plasmid DNA is omitted. All except one of the constructs contain the AAV-derived genome inserted into a pBR322 derivative, pA11P.Xba (see Materials and Methods) via *Bgl*II linkers. pPS12 (an *ori* mutant) is contained in pBR328 at the *Bal*I site (52). pAV2 contains a wild-type AAV2 genome. The pJDT series comprises *rep*⁻ derivatives of pAV2. The region deleted is indicated by the gap. For pJDT279 and pJDT280, the 4-base deletion at the *Sst*I site is indicated by the open circle below the line. pTS1 contains a CAT-coding region (▭) under control of the AAV p_{40} promoter by insertion at the *Hin*dIII site (61). Large deletions present in derivatives of pTS1 are indicated by gaps, and small (4-nucleotide) deletions or insertions at a particular restriction site are indicated by open circles below the line. Note that in pTS100, the mutation is in the polylinker sequence of the plasmid and not in AAV.

MATERIALS AND METHODS

Growth and purification of plasmids. Construction, growth, and purification of recombinant plasmids were performed by standard methods (41) described before (59, 61). All plasmids were maintained in ampicillin-containing medium and grown in *Escherichia coli* HB101. For reproducibility in transfection experiments, care was taken to ensure high-quality DNA preparations, including twice banding in CsCl-ethidium bromide gradients. All manipulations with recombinant DNA were performed in accordance with the National Institutes of Health.

Construction of individual plasmids. pA11P.Xba is a derivative of pBR322 containing a polylinker sequence inserted between the *Eco*RI and *Bam*HI sites, thus inactivating the tetracycline resistance gene (38). pAV2 contains the entire genomic sequence of AAV2 inserted into the *Bgl*II site of pA11P.Xba via *Bgl*II molecular linkers (38). The structure of pAV2 and plasmids derived from it are shown in Fig. 1. The plasmids pJDT267 (pAV*dl*BcBs), pJDT269 (pAV*dl*SBc), and pPS12 are deletion mutants (52, 59). The genotype names shown in parentheses were used in a previous publication (59). In contrast to all of the other plasmids, pPS12 is an insertion of AAV DNA at the *Bal*I site of pBR328 (52).

pJDT243 was derived by deletion of a *Bam*HI fragment from pAV2. pJDT279 and pJDT280 are independent isolates derived by cleaving pAV2 with *Sst*I and removing the protruding 3′ 4-nucleotide-long ends with *E. coli* DNA polymerase I Klenow fragment followed by blunt-end ligations. This results in a 4-base deletion and consequently a frameshift mutation in orf-1 at the *Sst*I site.

pTS1 was derived from pAV2 by insertion of a 873-nucleotide *Hin*dIII-*Hpa*I fragment containing the CAT-coding sequences as described before (61). Note that pTS1 was previously designated as pAVHiCAT (61).

The plasmids pTS55, pTS18, pTS73, pTS61, pTS67, and pTS52 are all deletion mutants of pTS1 derived in a fashion analogous to that described for the deletion mutants of

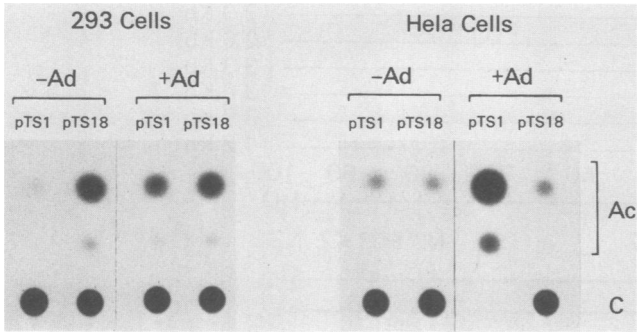

FIG. 2. Effect of a rep mutation on the expression of CAT from an AAV vector. Cultures (10^6 cells per dish) of 293 cells or HeLa cells either uninfected (−Ad) or infected with 15 PFU cell of adenovirus type 5 per cell (+Ad) were transfected with 4 μg of the rep^+ AAV vector pTS1 or the rep^- vector pTS18. Cell extracts made 40 h after transfection were assayed for CAT activity. Reaction products were analyzed on thin-layer chromatography plates by ascending chromatography and autoradiographed. Positions of [^{14}C]chloramphenicol (C) and the two acetylated products (Ac) are shown. The actual assay mixtures shown in the figure were incubated for 30 min at 37°C with 5 μl (293 cells) or 25 μl (HeLa cells) of extract. Additional CAT assays were performed on these extracts under linear conditions (see Methods). The relative levels of CAT activity (i.e., percent [^{14}C]chloramphenicol acetylated per 5 μl of extract per 30 min at 37°C) for tracks reading from left to right were: 3.0, 27.7, 15.6, 23.6, 1.9, 1.9, 19.6, and 2.4.

pAV2. The plasmids pTS83, pTS84, pTS87, pTS88, pTS97, and pTS100 are frameshift mutants constructed similarly to pJDT279 and pJDT280 by cleaving pTS1 at the *Bst*EII, *Bam*HI, or *Sst*I site and treating it with Klenow fragment before blunt-end ligation. pTS83, pTS84, pTS87, and pTS88 are independent isolates with mutations at the *Bst*EII (pTS83 and pTS84) or *Sst*I (pTS87 and pTS88) sites respectively. At the *Sst*I site this mutation results in a 4-nucleotide deletion, and at the *Bst*EII and *Bam*HI sites, the mutation results in a 4-nucleotide insertion.

The plasmid pTS127 is a pTS1 derivative which has deletions removing terminal palindrome sequences (ori^-), the inboard 20-nucleotide inverted repeats, and additional unique sequences. pTS127 was constructed as follows. In sum, the region from K1 to *Bgl*II (i.e., map units 40 to 100) in pTS52 was substituted by fragment K2/N3, which contains the AAV polyadenylation signal (Fig. 1). Specifically, pTS52 was cleaved with *Bgl*II, and the cohesive ends were filled in; the DNA was then cleaved with *Kpn*I, and the large 5.7-kilobase fragment was isolated by agarose gel electrophoresis. pJDT243 was then cleaved with *Nco*I, the cohesive ends were filled in, the DNA was cleaved with *Kpn*I, and the small *Kpn*I-*Nco*I (Fig. 1, K2 to N3) fragment was isolated and ligated to the 5.7-kilobase fragment from pTS52.

Transfection of cells and assay of CAT activity. Human 293 cells, an established line of adenovirus-transformed human embryonic kidney cells (20), or human HeLa cells were grown at 37°C in monolayer culture in 35-mm dishes in Eagle minimal essential medium supplemented with antibiotics and 10% fetal calf serum. When required cells were infected with adenovirus type 5 (15 PFU per cell) 1 h before DNA transfection. The cells (5×10^5 to 10^6 cells per 35-mm dish) were transfected with plasmid DNA using the $CaPO_4$ procedure (65) exactly as described before (61). The cell growth medium containing $CaPO_4$ was replaced with fresh medium 4 h after transfection.

CAT activity in transfected cells was determined as described previously (18, 19). However, in more recent experiments, sonication of suspended cells was replaced by freezing and thawing three times, which was more convenient for processing large numbers of samples. Cell extracts were made at 48 to 60 h after transfection. This represents the times at which maximal CAT activity was observed. At much later times (e.g., 90 h) in adenovirus-infected cells, there was usually some decrease in CAT activity. Extracts from each dish were suspended in a volume of 150 μl. CAT activity was monitored by acetylation of [^{14}C]chloramphenicol as measured by thin-layer chromatography on silica gel plates. To quantitate relative levels of CAT activity, assay mixtures contained various amounts of cell extract (0.5 to 50 μl) and were incubated at 37°C for up to 2 h to ensure that all measurements were made in the linear range. After chromatography, the thin-layer plates were analyzed by autoradiography, and then radioactivity was quantitated by liquid scintillation counting of the spots. All results are expressed as the percentage of [^{14}C]chloramphenicol which was acetylated.

DNA replication assay. The replication of AAV vectors was determined as described before (38, 59) by transfecting plasmid DNA into adenovirus-infected 293 cells. Viral DNA was extracted after 48 h and analyzed by electrophoresis in agarose gels followed by blotting, hybridizing with a nick-translated AAV [^{32}P]DNA probe and autoradiography. For AAV vectors or AAV *rep* mutants no replication of AAV DNA was detected in this procedure.

RESULTS

Structure of AAV vectors. The structures of the AAV2 genome and various AAV plasmids and vectors are summarized in Fig. 1. The AAV2 genome contains three promoters, p_5, p_{19}, and p_{40}, which each yield two transcripts differing in the presence or absence of the intron (11, 21, 39, 40, 42). All of the transcripts terminate at the same 3′ polyadenylation site (39, 57).

When recombinant plasmids such as pAV2 are transfected into adenovirus infected cells, the AAV genome is rescued from the plasmid and replicated (38, 50). Deletions in AAV DNA between map positions 40 and 90 do not prevent replicating form replication (rep^+), whereas mutations (*rep*) in orf-1 to the left of the *Hin*dIII site (map position 40) prevent replicating form DNA replication (27, 58, 59). Similarly, insertion of a foreign DNA sequence, such as CAT, as in pTS1, at the *Hin*dIII site generally permits DNA replication (60, 61). We constructed a series of derivatives of pTS1 (Fig. 1) having mutations in orf-1 far upstream of p_{40}. As expected, all of these mutants exhibited a rep^- phenotype when assayed for DNA replication in adenovirus-infected 293 cells (data not shown). Thus, for all of the constructions shown in Fig. 1, only pAV2, pTS1, pTS55, and pTS100 could replicate and were therefore rep^+. Note also that pPS12 was rep^+ in that it contained a functional rep gene and could complement a *rep* mutant. However, pPS12 cannot be replicated itself because it is an *ori* mutant in which both replication origins are deleted (52, 53).

CAT expression from a rep^- derivative of pTS1. The rep^- derivatives of pTS1 revealed unexpected properties (Fig. 2). 293 and HeLa cells were transfected with pTS1 or the rep^- derivative pTS18 in the absence or presence of adenovirus. Surprisingly, in uninfected 293 cells, the basal level of CAT expression from pTS18 was increased about ninefold over that from pTS1. As described before for pTS1 (61), in the presence of adenovirus, CAT expression was increased

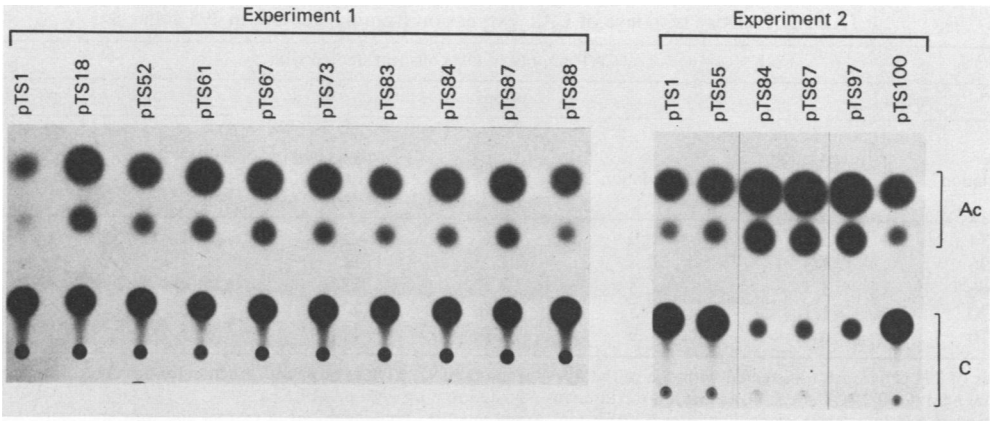

FIG. 3. Expression of CAT in uninfected 293 cells from *rep*$^-$ derivatives of pTS1. 293 cells (10^6 cells per dish) were transfected with 4 μg of the AAV vector indicated in each lane. Results from two separate experiments are shown. CAT activity was assayed at 40 h after transfection. Quantitation of these and other experiments are shown in Table 1.

5-fold in 293 cells and 10-fold in HeLa cells. In contrast, the basal level of CAT expression from pTS18 was not increased significantly by adenovirus in either cell line.

These observations suggest two consequences of the *rep* mutation. (i) The mutation removed a negative effect on p_{40} expression which otherwise was observed in the absence of adenovirus in 293 cells. (ii) The mutation removed a positive effect on the expression from p_{40} which otherwise occurred in the presence of adenovirus. We analyzed each of these effects in more detail.

Far upstream mutations increase p_{40} CAT expression in uninfected 293 cells. When 10 independent *rep*$^-$ derivatives of pTS1 (Fig. 1) were transfected into 293 cells, there was increased CAT expression as compared with that of the parent vector pTS1 (Fig. 3). Quantitation of the experiments in Fig. 3 and other experiments showed that the increase was 10- to 30-fold (Table 1). The increased CAT expression was observed for both the deletion mutants and the frameshift mutants. In contrast to the effect of the upstream mutations, the presence or absence of the AAV intron downstream of the CAT sequence had no significant effect on the level of CAT activity, regardless of whether the vector was *rep*$^+$ or *rep*$^-$ as indicated by pTS1, pTS55, pTS18, or pTS73 (Fig. 3; Table 1). Similarly, a frameshift mutation outside of the AAV sequence (pTS100) had no effect upon p_{40} CAT expression (Fig. 3; Table 1).

The AAV p_{40} transcription unit (Fig. 1) has a 5′ mRNA terminus at nucleotide 1854 and a TATA box consensus sequence at nucleotide 1824 (−30 with respect to the mRNA 5′ terminus). The *rep* mutations are all located at or between the *Sst*I site at nucleotide 811 and the *Bst*EII site at nucleotide 1700 (i.e., between nucleotides −1044 and −154 upstream of the mRNA 5′ terminus). Thus, none of these mutations removed the TATA box, and none of them should have an effect on the structure of the chimeric p_{40} transcript.

Dose response of CAT expression from AAV vectors in 293 cells. There are abundant reports of decreased expression from many eucaryotic transcription units when sequences upstream of the promoter are deleted or mutated (17, 35). Extensive activation of a transcription unit by upstream mutations is a less common event (7, 66). However, the data in Fig. 3 and Table 1 could be most readily explained by a *trans*-acting negative regulation of CAT expression by a product of orf-1. This was analyzed further in a dose-response experiment.

Individual cultures of 293 cells were transfected with various amounts of the *rep*$^+$ vector, pTS1, or one the *rep*$^-$ vectors, pTS61 or pTS67. The total amount of DNA transfected into each culture was adjusted to 5 μg of DNA with the control plasmid pA11P.Xba. In each culture, the level of CAT activity increased with increasing amounts of transfecting vector DNA (Table 2). For pTS61 and pTS67, the CAT activity increased linearly. For pTS1, the increase was not linear, and a plateau was approached at about 2 μg of vector DNA. The nonlinearity of pTS1 was revealed more clearly when the CAT activity per microgram of vector DNA was calculated (Table 2). For pTS61 or pTS67, this value was generally about 13 ± 4, but for pTS1, it decreased from 3 or 4 to 0.5.

TABLE 1. Effect of *rep* mutations on CAT expression from AAV vectors in uninfected 293 cells

Vector[a]	*rep* expression[b]	CAT activity[c]		
		Expt 1	Expt 2	Expt 3
pTS1	+	8.0	4.4	1.2
			3.1	
pTS55	+		5.6	
PTS18	−	72.0	46.0	12.0
pTS61	−	80.0		34.0
pTS67	−	70.0		32.0
pTS52	−	39.0		35.2
pTS73	−	44.0		
PTS83	−	40.0		
pTS84	−	46.0	45.0	
pTS87	−	58.0	42.0	
			39.0	
pTS88	−	41.0	33.0	
pTS97	−		39.0	
pTS100	+		5.0	

[a] Cultures of 293 cells were transfected with 4 μg of vector DNA as indicated, and cell extracts were made for assaying CAT activity at 40 h after transfection.

[b] +, *rep*$^+$; −, *rep*$^-$.

[c] Cat assays (experiments 1 through 3) were performed under linear assay conditions appropriate for each extract as described in Materials and Methods. Procedures were the same for all three experiments. All results are reported as percent acetylation of chloramphenicol by 2 μl of extract in 30 min in the standard assay. Data in experiment 1 are from the same extracts as those of experiment 1 shown in Fig. 3. Also, some of the data from experiment 2 are from the same extracts as illustrated in experiment 2 of Fig. 3. Data from two separate cultures are shown for vectors pTS1 and pTS87 in experiment 2.

TABLE 2. Dose response of CAT expression from p_{40} vectors in 293 cells

Amt of vector (μg)[a]	CAT activity of 293 cells transfected with[b]: pTS1 % Acetylation	pTS1 % Acetylation per μg of DNA	pTS61 % Acetylation	pTS61 Norm[c]	pTS61 % Acetylation per μg of DNA	pTS67 % Acetylation	pTS67 Norm[c]	pTS67 % Acetylation per μg of DNA
0.1	0.34	3.4	0.9	2.6	9	1.3	3.8	13
0.2	0.96	4.8	2.4	2.5	12	3.1	3.2	15
0.5	1.66	3.3	6.3	3.8	12	4.4	2.6	9
2.0	2.20	1.1	25.5	11.6	12	26.5	12.0	13
5.0	2.70	0.5	84.9	31.0	17	85.7	31.7	17

[a] Individual cultures of 293 cells were transfected with one of the AAV vectors, pTS1, pTS61, or pTS67. Each culture received the amount of vector DNA indicated, and the total amount of DNA in each transfection mixture was adjusted to 5 μg with pA11P.Xba.

[b] CAT activity was measured in extracts made 40 h after transfection. Assay mixtures contained 2 μl of extract and were incubated for 30 min. Results are expressed as percent [^{14}C]chloramphenicol acetylated and also as percent acetylation per microgram of DNA. The mean percent acetylation per microgram of DNA was 13 for both pTS61 and pTS67.

[c] Data for pTS61 and pTS67 were individually normalized by dividing the CAT activity (percent [^{14}C]chloramphenicol acetylated) of each by the CAT activity observed in cultures transfected by the same amount of pTS1.

For all three vectors, CAT was expressed from the same p_{40} promoter, and all cell cultures received the same amount of DNA. Thus, the nonlinearity of the dose response with pTS1 is not readily explained by saturation of DNA uptake or by saturation of the cellular transcription or translation apparatus. These results are consistent with an inhibitory effect of an orf-1 product, such that increasing amounts of pTS1 resulted in increasing amounts of the inhibitor. Consequently, the level of expression from the *rep*$^-$ vector relative to that from the *rep*$^+$ vector was much greater (30-fold) with 5 μg of vector DNA than at lower vector DNA inputs (3- to 4-fold).

An orf-1 product inhibits AAV p_{40} CAT expression in *trans* in 293 cells. A more direct test of a *trans* effect of an orf-1 product on CAT expression was performed in cotransfection complementation experiments (Fig. 4). Cultures of 293 cells were transfected with the *rep*$^+$ vector pTS1 or the *rep*$^-$ vector pTS18. Each culture was also cotransfected with up to a 10-fold molar excess of the *rep*$^+$ plasmid pAV2 or the *rep*$^-$ plasmid pJDT267. pAV2 could supply the normal orf-1

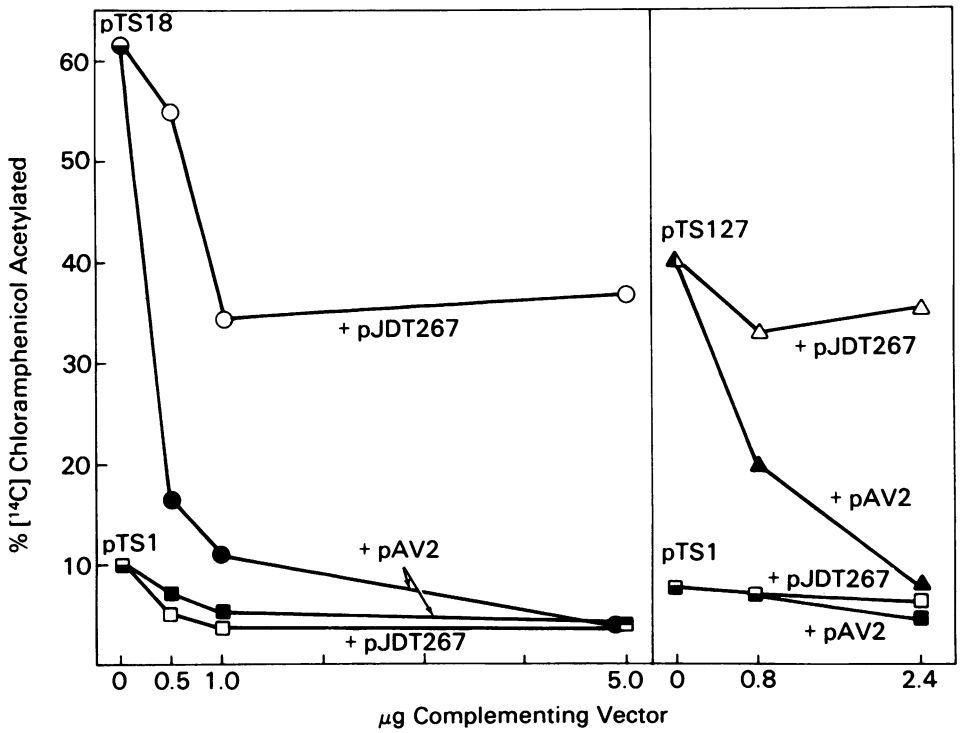

FIG. 4. *trans* effect of the AAV rep gene product on expression of CAT in uninfected 293 cells. Cultures (5×10^5 cells) were transfected with 0.5 μg of pTS1, pTS18, or pTS127 and increasing amounts of pAV2 (*rep*$^+$) or pJDT267 (*rep*$^-$). The total amount of DNA added to each culture was adjusted to 5.5 μg with pA11P.Xba DNA. CAT activity was assayed at 40 h after transfection. The CAT activity shown is that exhibited by 10 μl of cell extract in 30 min in the standard assay. The results are plotted as micrograms of complementing vector DNA and have not been adjusted for slightly different molar amounts of each plasmid. The sizes of pAV2 and pJDT267 are 8.74 and 8.0 kilobases, respectively. Also, pTS1, pTS18, and pTS127 are 8.0, 7.3, and 6.0 kilobases, respectively.

products in *trans* whereas pJDT267 could not. It is also important that pAV2 and pJDT267 each possessed all three AAV promoters and differed only with respect to deletion of part of orf-1 in pJDT267. Thus, both pAV2 and pJDT267 should be equivalent with respect to any effects of promoter competition for general transcription factors (54) due to addition of excess p_{40} (or p_5 or p_{19}). Any differences in the effects of pAV2 and pJDT267, therefore, should reflect the action of the orf-1 region.

In the experiment shown in Fig. 4, as in the previous experiments, CAT expression from pTS18 was higher than that from pTS1. However, expression from pTS18 was inhibited drastically by pAV2 (up to 94%) but only about 45% by pJDT267. The level of inhibition by pJDT267 may reflect competition for general transcription factors (54) because a similar degree of inhibition of pTS1 by about one-half was seen with either pAV2 or pJDT267. The additional inhibition of CAT expression from pTS18 by pAV2 is consistent with a specific effect of a *trans*-acting product of orf-1. In a similar cotransfection experiment (Fig. 4), CAT expression from the *ori rep* mutant pTS127 was also specifically inhibited by an orf-1 product supplied in *trans* by pAV2 but not by pJDT267.

A number of other similar cotransfection competition experiments were performed (data not shown) and can be summarized as follows. The CAT expression from other *rep* mutants such as pTS52 and pTS73 also was specifically inhibited to the level of pTS1 by pAV2 but not by pJDT267. Similarly, specific inhibition of rep^- CAT expression from pTS18 was obtained with the *ori* rep^+ mutant pPS12 but not with the *rep* frameshift mutants pJDT279 and pJDT280 or the *rep* deletion mutants pJDT269 and pJDT243.

These experiments described so far clearly show that in uninfected 293 cells, an orf-1 product has a *trans*-acting negative effect on expression from the p_{40} transcription unit. The behavior of the AAV mutants pJDT279 and pJDT280 and the CAT vectors pTS87 and pTS88 (all of which have a frameshift mutation at the *Sst*I site) suggests that a negative effect may be mediated by the product of the p_5 transcript. The experiments shown in Fig. 4 with pTS127 suggest that the *trans*-acting negative effect does not require the presence in *cis* of either the AAV replication origin or the AAV sequences upstream of the *Bst*EII site (i.e., −154 nucleotides upstream of the 5′ end of the chimeric p_{40} mRNA).

In the presence of pAV2 (Fig. 4), expression from pTS18 or pTS127 was decreased to the same level as that from pTS1 by complementation with rep. Thus, most of the increased CAT expression from *rep* mutants was accounted for by the *trans* effect of the orf-1 product. There appeared to be no significant effect in *cis* due to phenomena such as transcriptional interference or promoter occlusion (1, 13), in which promoter activity can be decreased by transcription from another active promoter located upstream. This conclusion is also supported by the data of Table 2, which show that both pTS61 (which retains p_{19}) and pTS67 (in which p_{19} is deleted) gave a linear response and that neither showed any significant evidence for an inhibitor or repression of p_{40} expression. This suggests that the p_{19} promoter in pTS61 contributes little if any *cis*-acting effects to depress expression from p_{40}. Therefore, our data do not rule out possible *cis* effects on p_{40} expression but indicate that, in uninfected 293 cells, the phenotype of the mutations described here is largely accounted for by a *trans* effect.

Effect of the *rep* mutation on expression from p_{40} in uninfected HeLa cells. In uninfected HeLa cells, the rep^- derivatives of pTS1, including the deletion mutants pTS18 and pTS73 and the frameshift mutants pTS84, 87, 88, and 97, showed no increased CAT activity (Fig. 2 and 5). Indeed, in the experiment with HeLa cells, (Fig. 5), CAT expression from the rep^- vectors was decreased about four- to fivefold relative to the rep^+ vectors, pTS1 or pTS55. This was in clear contrast to the behavior of the same vectors in uninfected 293 cells (cf. Fig. 2 and 3 and Table 1).

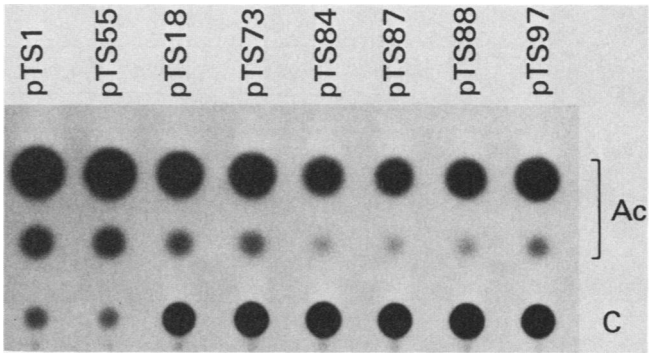

FIG. 5. Expression of CAT from rep^- derivatives of pTS1 in uninfected HeLa cells. Cells (5×10^5) were transfected with 4 μg of the indicated AAV vector, and CAT activity was assayed 40 h later. Results are shown for assay mixtures containing 50 μl of cell extract incubated for 3 h. Quantitation of CAT in the same extracts in additional assays showed the following amounts of [^{14}C]chloramphenicol acetylated (per 10 μl of extract incubated for 60 min): pTS1, 8.3; pTS55, 9.4; pTS18, 2.7; pTS73, 2.0; pTS84, 1.3; pTS87, 1.8; pTS88, 1.8; pTS97, 2.0.

These results suggest that, in uninfected HeLa cells, either the rep product was not an efficient repressor of CAT expression or the product instead was an activator. The activation hypothesis was supported by the observation (Fig. 2) that when HeLa cells were infected with adenovirus, there was no increase of CAT expression with the rep^- vector, pTS18, but a 10-fold increase with the rep^+ vector, pTS1. The complementation experiments described below show that rep was indeed an activator of p_{40} in HeLa cells.

***trans* activation of p_{40} expression in HeLa cells.** The *trans* activation hypothesis was tested in a series of complementation experiments. Uninfected or adenovirus-infected HeLa cells were transfected with a rep^+ (pTS1) or rep^- (pTS18) CAT vector and a second, complementing plasmid which was either rep^+ (pAV2 or pPS12) or rep^- (pJDT279 or pJDT269). One such complementation experiment in HeLa cells is shown in Fig. 6, and the quantitative data are summarized in Table 3. Several important conclusions can be made from these data.

(i) For pTS1 (rep^+), the complementing rep^+ or rep^- plasmids had no major effect in uninfected HeLa cells. CAT expression from pTS1 (rep^+) was increased about 10-fold by infection with adenovirus. In adenovirus-infected cells, the increase in CAT expression was somewhat lower in the presence of rep^+ or rep^- complementing plasmids. This may represent, in part, nonspecific competition for general transcription factors as noted for the experiment shown in Fig. 4 above. It may also indicate some effect of a second replicating AAV genome since the increase was greatest in the presence of pPS12, which does not replicate (53).

(ii) CAT expression from pTS18 (rep^-) was increased by the complementing rep^+ plasmids (pAV2 and pPS12). The rep^- plasmids (pJDT269 and pJDT279) had little or no effect. In uninfected cells, the increase was about 5-fold, and in adenovirus-infected cells, the increase was about 15-fold.

In summary, these complementation experiments clearly

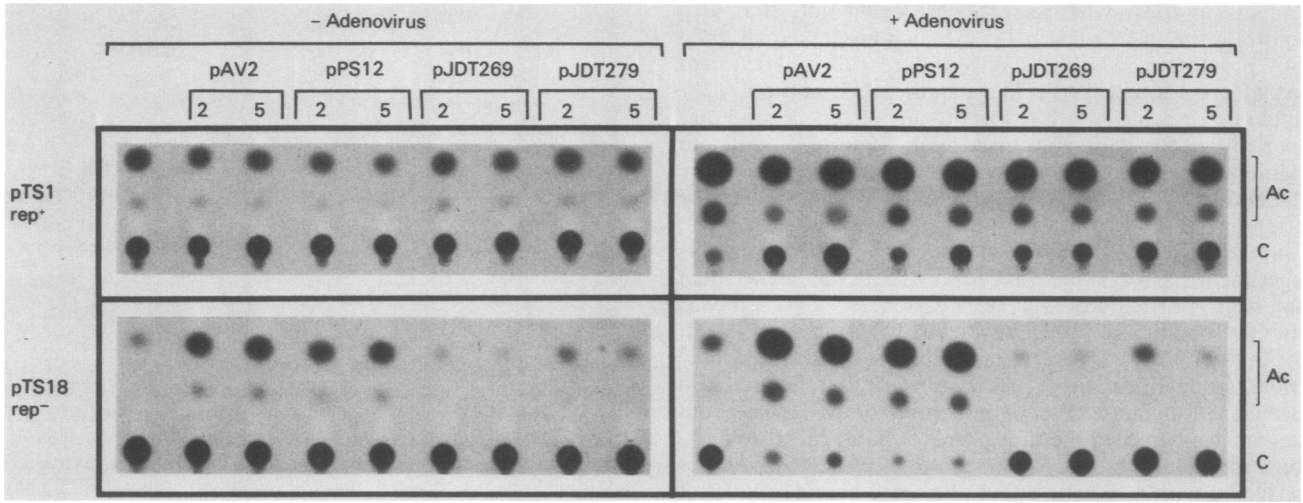

FIG. 6. *trans* effect of the AAV rep gene product in HeLa cells. Cultures (5×10^5 HeLa cells) were transfected with 2 μg of pTS1 or pTS18 together with 2 or 5 μg of complementing plasmid (pAV2, pPS12, pJDT269, or pJDT279) as indicated at the top of each lane. The total amount of DNA transfected into each culture was adjusted to 7 μg with pA11P.Xba DNA. Cultures were either mock infected (−Adenovirus) or infected with 15 PFU of adenovirus type 5 per cell (+Adenovirus) 1 h before transfection. CAT activity was assayed at 60 h after transfection. Each assay mixture shown contained 20 μl of cell extract and was incubated for 1 h. Quantitation of these assays is shown in Table 3.

show that in HeLa cells, rep was a *trans*-acting positive regulator of CAT expression. This activation did not absolutely require adenovirus and therefore occurred in the absence of replication of either the CAT vector (pTS18) or the complementing vector (pAV2 or pPS12). However, the activation was greater in the presence of adenovirus under conditions in which pTS18 could replicate when complemented either by pAV2 or pPS12 (54, 62). Note, however, that pPS12 does not replicate at all. Therefore the increased activation in the presence of adenovirus was not due simply to increased genome copies of the complementing vector. It might be accounted for either by an increased copy number or a different conformation of the pTS18 genome or also by increased expression of the rep product from the complementing genome.

***trans* activation of p_{40} expression in 293 cells.** The experiments described thus far show that the rep product was a *trans*-acting negative regulator of CAT expression in uninfected 293 cells. In contrast, in both uninfected and adenovirus-infected HeLa cells, rep was a positive regulator. However, the data in Fig. 2 suggest that, in adenovirus-infected 293 cells, the rep product may also be an activator, as shown above for HeLa cells. Thus, additional complementation experiments were performed in uninfected or adenovirus-infected 293 cells (Fig. 7; Table 3). Several conclusions can be drawn.

(i) In uninfected 293 cells (Fig. 7), expression from a rep^- vector (pTS52) was repressed in *trans* by a complementing rep^+ plasmid (pAV2 or pPS12) but not by a rep^- plasmid (pJDT269 or pJDT279). This is consistent with results already described (Fig. 2, 3, and 4).

(ii) In 293 cells complemented with a rep^- plasmid (pJDT269 or pJDT279), adenovirus infection had no major (i.e., less than twofold) effect upon expression of CAT from pTS52. When the complementing plasmid was the rep^+ pPS12, adenovirus infection resulted in a sevenfold increase in CAT expression. This suggests that the rep protein was also a *trans*-acting activator in adenovirus-infected 293 cells. In contrast, when the complementing plasmid was the rep^+ pAV2, adenovirus infection did not result in a major stimulation of CAT expression from pTS52. The apparently contradictory results with the two rep^+ plasmids, pAV2 and

TABLE 3. *trans*-acting effect of rep on expression of CAT in HeLa and 293 cells[a]

Cells	Vector	Adenovirus infection	CAT activity with the following complementing vector[b]							
			Control	pAV2		pPS12		pJDT269		pJDT279	
				2 μg	5 μg	2 μg	5 μg	2 μg	5 μg	2 μg	5 μg
HeLa	pTS1	−	9.8	8.6	7.9	7.7	5.3	10.0	8.4	9.9	7.9
		+	99.7	29.9	25.0	72.3	50.7	60.3	52.7	40.1	38.8
	pTS18	−	1.6	7.6	8.4	7.4	7.4	1.6	1.3	4.2	3.3
		+	6.1	81.3	67.6	79.9	93.7	2.3	1.7	4.7	1.7
293	PTS52	−	20.3	4.2		6.9		72.4		40.2	
		+	27.9	8.7		49.5		58.4		76.4	

[a] The data represent quantitation of the complementation experiments shown in Fig. 6 (HeLa cells) and Fig. 7 (293 cells). The detailed protocols are described in the figure legends. Cells were transfected with 2 μg of AAV-CAT vector (pTS1, pTS18, or pTS52) and 2 or 5 μg of the rep^+ (pAV2 or pPS12) or rep^- (pJDT269 or pJDT279) AAV plasmids as indicated. Cultures were either mock-infected (−) or infected with adenovirus (+). Data in the control column are from cultures in which the complementing plasmid was pA11P.Xba.

[b] The data are expressed as percent acetylation of chloramphenicol per 10 μl of cell extract per h (HeLa cells) or per 5 μl of cell extract per h (293 cells).

pPS12, may be caused by their ori phenotypes. pAV2 is ori^+ and thus replicates in adenovirus-infected cells. pPS12 lacks both replication origins and is ori^-. Similarly, pTS52 lacks one replication origin (ori_L) and is ori^-. The *ori* mutation is *cis* dominant, and *ori* mutants cannot be complemented for replication. Thus, extensive replication of pAV2 may result in a vast excess of these genomes over pTS52. If this leads to competition for the transcription apparatus, expression from pTS52 may be decreased.

(iii) The results of the experiments shown in Fig. 6 and 7 emphasize some clear differences between HeLa cells and 293 cells with respect to the function of rep. In the absence of adenovirus, 293 cells showed *trans* repression of p_{40} CAT expression, whereas HeLa cells showed *trans* activation. Apparently, in uninfected 293 cells, repression was dominant over activation, but this situation may be reversed by adenovirus infection.

DISCUSSION

The experiments described here provide evidence for both negative and positive regulation of expression of CAT inserted in the AAV p_{40} transcription unit. This regulation was mediated in *trans*, apparently by an AAV gene product, rep, coded by orf-1. Thus, the AAV rep gene had two more unexpected properties in addition to its function in AAV DNA replication.

Whether the regulation by the AAV rep product was positive or negative depended on several factors, including the host-cell type and the presence or absence of helper virus. In HeLa cells, regardless of the presence of helper virus, the rep product was a positive regulator. In contrast, rep was a negative regulator in uninfected 293 cells.

In adenovirus-infected 293 cells, the effect of the rep product was less clear. Complementation of pTS52 with the ori^- plasmid pPS12 provided clear evidence for a positive effect of rep, but complementation with the ori^+ plasmid pAV2 did not. We suggested above that this might reflect an excess of AAV genomes resulting from replication of pAV2 which could simply out-compete transcription from the lower number of nonreplicating pTS52 genomes. Therefore, we tentatively favor the notion that adenovirus infection of 293 cells alters the function of the rep protein from that of a negative effector to that of a positive effector. An alternative possibility is that some dominant effect of adenovirus, independent of rep, simply abolishes the negative effect of the rep protein. By this latter hypothesis, extensive pAV2 replication would inhibit adenovirus replication and thus inhibit any dominant adenovirus effect. Further experiments are required to resolve these points.

The basis for the different behavior of rep in HeLa cells and 293 cells remains unknown but must reflect differences in some cellular factors. The 293 cells are adenovirus-transformed and constitutively express the adenovirus 5 E1A and E1B proteins. However, neither of these classes of adenovirus proteins are necessarily involved in the regulatory phenomena we describe here because preliminary experiments (E. Mendelson and B. Carter, unpublished data) indicate similar negative regulation by rep in some other cell lines that do not contain adenovirus E1A or E1B genes. Whether any of the other adenovirus functions involved in the AAV helper effect (10) are also involved in the regulatory effects of rep remains to be determined. For instance, adenovirus VA RNA might increase translation of AAV mRNA (31), and this could account for increased CAT expression in the presence of adenovirus. However, since the structure of the chimeric AAV-CAT p_{40} mRNA is the same in rep^+ and *rep* mutants, VA RNA would not directly account for effects of rep.

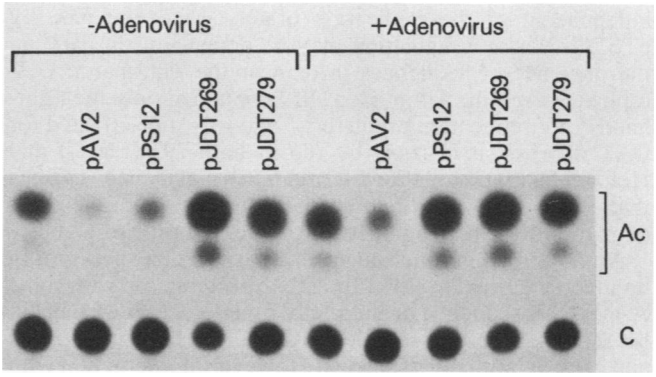

FIG. 7. *trans* effect of the AAV rep gene product in 293 cells. Individual cell cultures (5×10^5 cells) were either mock infected (−Adenovirus) or infected (+Adenovirus) with adenovirus (15 PFU per cell) and transfected with 2 μg of pTS52. The transfection mixture also contained 2 μg of complementing rep^+ (pAV2 or pPS12) or rep^- plasmid (pJDT269 or pJDT279) as indicated above each lane. The control cultures (unmarked lanes) were transfected with 2 μg of pA11P.Xba. CAT activity was assayed 48 h after transfection. Each CAT assay mixture shown contained 10 μl of cell extract and was incubated for 30 min at 37°C. Quantitation of these assays is shown in Table 3.

All of the effects of the mutations in orf-1 could be accounted for by alterations in a *trans*-acting orf-1 rep product. Alternative models in which these mutations would alter *cis*-acting regulatory sequences in orf-1 are not supported by all of our data and are explicitly argued against by the results of the complementation experiments. *Cis*-acting mechanisms for AAV gene regulation may play some role, but the major effect of the mutations that we have analyzed here is to alter the *trans*-acting regulatory effects of an orf-1 product on expression from the p_{40} transcription unit.

Orf-1 could code for several putative rep proteins (Fig. 1), and it is not known which are required for the *trans* regulation effects. However, the data obtained with pJDT279, pJDT280, pTS87, and pTS88 indicate that both *trans* effects (positive and negative) were mediated by a product from the p_5 transcription unit. All four mutants have a frameshift mutation at the *Sst*I site upstream of p_{19}, but this mutation does not affect expression from the p_{19} transcription unit (J. Tratschin, unpublished data). The role of orf-1 products from p_{19} transcripts cannot be determined readily because both p_{19} and its associated reading frame (part of orf-1) are embedded within the reading frame of the p_5 orf-1 products. In pTS1 and its derivatives, the carboxyl termini of the putative orf-1 proteins (i.e., to the right of the *Hin*dIII site) are deleted. This did not affect function of these proteins in AAV DNA replication (27, 60, 61), and apparently the carboxyl termini are also not required for the negative or positive *trans* regulation.

The AAV rep product appears to be a pleiotropic effector, and three functions can be clearly identified. First, an AAV rep gene product is required for AAV DNA replication (27, 59), perhaps by acting in *trans* to recognize the AAV DNA replication origins (27, 53, 59). Two additional functions of *rep* appear to be mediation of negative and positive control of gene expression. However, both negative and positive *trans* regulations of CAT expression were independent of AAV DNA replication per se, and the negative effect was

independent of the replication origins, as exemplified by pTS127. Positive regulation may be dependent, in part, on the presence of sequences in or near the right-hand AAV replication origin. Although pTS52 contained only the right-hand AAV replication origin (ori_R) and was still activated for CAT expression in *trans* by rep in both 293 (Fig. 7) and HeLa cells (data not shown), preliminary evidence suggests that when ori_R was deleted to generate, pTS127, *trans* activation by rep was not readily observed on HeLa cells.

An AAV rep product might decrease CAT expression in uninfected 293 cells, either directly by interaction with some AAV DNA sequence or indirectly by interaction with cellular factors. These possibilities cannot yet be distinguished and are not mutually exclusive. However, if interaction with a specific AAV DNA sequence is required, then these sequences likely lie between nucleotides −154 and +30 of the 5′ terminus of the p_{40} RNA transcript. A corollary conclusion from these studies is that these same sequences were sufficient upstream from maximum activity of the p_{40} promoter in 293 cells.

There is also evidence (48), obtained with the autonomous parvovirus, H-1, that the nonstructural (rep) protein coded by the left-hand p_4 transcription unit is a positive regulator of expression of a CAT gene inserted under control of the H-1 capsid protein promoter p_{38}. This is analogous to the positive regulation we describe here for AAV, although the mechanisms may be different. In human NB (simian virus 40 [SV40]-transformed) cells, the H-1 P_{38} promoter appears to be about 40-fold weaker than the SV40 early promoter, whereas the AAV p_{40} promoter has a strength at least equal to that of the SV40 promoter (62).

Many eucaryotic DNA viruses code for a variety of proteins which provide positive or negative control on viral gene expression and DNA replication. These include adenovirus E1A protein (2, 5, 16, 22, 23, 26, 30, 33, 34, 43, 44, 49, 59, 62, 64), the adenovirus E2A protein (15), the SV40 early protein, T antigen (6), and several herpesvirus early gene products (9, 14, 28, 30, 45, 46, 47). Also, several retroviruses including Rous sarcoma virus (8) and HTLV (55, 56) code for proteins that are *trans* activators of viral gene expression via the viral long terminal repeat sequences. Thus, the AAV rep protein(s) may be analogous to regulatory proteins of other DNA viruses in having independent pleiotropic effects on viral DNA replication and gene regulation.

In the present studies and in previous work, AAV exhibited a complex scheme of genome replication and gene expression. Elsewhere, we have argued that the expression of the two major AAV reading frames, orf-1 (rep) and orf-2 (capsid), are regulated differently at the translational level (10, 32, 60) and also with respect to transcription and the relative efficiencies of the AAV promoters p_{19} and p_{40} in response to the adenovirus E1A protein (61). Here, we have presented evidence that the AAV rep gene could differentially regulate expression from the capsid protein transcription unit. In this respect, rep and the capsid proteins may be functionally analogous to early and late gene regions, respectively. Whether there is any actual temporal regulation of the two gene regions in AAV remains to be determined. Positive regulation of the capsid protein transcription unit by rep has the obvious advantage of increasing capsid protein production coordinately with DNA replication. Negative regulation by rep might occur very early in the AAV growth cycle to provide time to amplify the replicating pool of DNA before encapsidation of progeny genomes attains maximum rates. Also, it is possible that the regulation by rep might be important during establishment or maintenance of AAV genome integration in the cell genome in the absence of helper virus or during its subsequent rescue with helper (12, 24, 29).

Finally, we note that the present work is relevant for the design of efficient AAV expression vectors (60, 61). We showed previously that for vectors such as pTS1, the AAV p_{40} promoter in 293 or HeLa cells is equal to or stronger than the SV40 early gene promoter containing a strong enhancer sequence (59). Here, we show that the intrinsic efficiency of expression from the p_{40} transcription unit may be altered significantly under different conditions of repression or activation by rep.

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