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Positive and Negative Autoregulation of the Adeno-Associated Virus Type 2 Genome

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The defective human parvovirus, adeno-associated virus (AAV), requires multiple functions provided by a coinfecting helper virus for viral replication. In addition, it has recently been shown that at least one AAV gene is also required for AAV DNA replication. In this paper, we investigate the autoregulation of the AAV genome by analyzing the expression of mutant AAV genomes upon transfection into adenovirus-infected human cells. Evidence is presented which indicates that the AAV genome regulates its own gene expression in at least two ways. First, either the AAV p5 gene or both the p5 and p19 genes appear to encode a *trans* activator of AAV transcription. Frameshift mutations within the p5 or p19 gene severely inhibited the synthesis and accumulation of all AAV transcripts. The defective accumulation of transcripts could be complemented in *trans*, in a manner independent of DNA replication, by cotransfection with a capsid deletion mutant. Second, evidence is presented which suggests that the p5 and p19 genes contain negative *cis*-active regulatory elements. Deletion of sequences within the p5 and p19 genes enhanced the accumulation of the p5 transcript in *cis* upon complementation with an AAV capsid deletion mutant, whereas certain deletions enhanced p40 RNA accumulation in the absence of *trans* activation by the p5 gene.

The defective human parvovirus, adeno-associated virus (AAV) type 2, is dependent on functions provided by a coinfecting helper virus, either adenovirus or herpes simplex virus, for its own replication (1, 9; for reviews, see references 12 and 14). The helper virus supplies functions which are required for all aspects of AAV replication, including RNA transcription, DNA replication, and protein synthesis. Although very little is known about the role of specific herpes simplex virus helper genes, several different adenovirus genes are known to affect AAV replication. The E1a and E1b genes are both required for optimal helper function, and indeed, E1a appears to activate transcription of at least one AAV gene (25, 30, 42, 54). Several adenovirus genes, including the VAI and E2a genes, appear to positively regulate the translation of the AAV structural proteins (24, 25, 27). Finally, the E4 gene is required for AAV DNA replication (41).

Studies on the physical and genetic structure of the AAV genome and AAV RNAs have yielded a large amount of information on the replication and organization of the AAV genome. AAV contains a linear, single-stranded genome of 4.7 kilobases (kb) which has been completely sequenced (5, 7, 34, 35, 50). The genome is flanked by 145-base terminal repeats (TRs) which are required as origins and primers for DNA replication (21, 34; for a review, see reference 6).

Three sets of polyadenylated AAV transcripts have been identified (Fig. 1) (14, 18, 19, 33). The RNAs have 5′ ends at 5, 19, or 40 map units (m.u.) and appear to be produced by three separate promoters, p5, p19, and p40, as suggested by the presence of TATA boxes approximately 30 bases upstream from each RNA start site (17, 35). Since the RNAs differ only at the 5′ end, they are named the p5, p19, and p40 transcripts. The RNAs have coterminal 3′ ends at 96 m.u. (50). Each transcript exists in unspliced and spliced forms of 4.2 and 3.9 kb, 3.6 and 3.3 kb, and 2.6 and 2.3 kb for the p5, p19, and p40 transcripts, respectively. The 2.3-kb spliced p40 transcript comprises approximately 90% of the AAV-specific RNA in helper virus-infected cells.

Physical and genetic analyses have divided the AAV genome into two parts. The AAV DNA sequence contains a large open reading frame (ORF) within the left half of the genome and another large ORF within the right half of the genome (35, 50). The nucleotide sequence of the ORF within the right half predicts a protein of size and composition corresponding to the most abundant AAV capsid protein. There are at least three coat proteins with overlapping amino acid sequences encoded within the ORF that is within the right half (10, 28, 43). All three of these coat proteins are produced by in vitro translation of spliced 2.3-kb p40 RNAs and appear to be produced by alternative RNA splicing and by the use of alternative, nonmethionine, translation start codons upstream of and in frame with the large ORF contained in the p40 transcript (2, 23, 27). Although the larger p5 and p19 RNAs also contain the ORF within the right half, they do not produce capsid proteins when translated in vitro (27). The large ORF within the left half is contained uniquely within the p5 and p19 transcripts. Recently, E. D. Sebring and J. A. Rose (European Molecular Biology Organization Workshop on Parvoviruses, 1985) reported that in vitro translation of the p5 and p19 messages does produce proteins of a size similar to that predicted by the ORFs within the left half.

The study of the genetics of AAV has been greatly facilitated by the cloning of the AAV genome in an infectious form into bacterial plasmid vectors (32, 44). When such a clone (e.g., pSM620) is transfected into adenovirus-infected human cells, the AAV genome is rescued from the bacterial plasmid, and infectious AAV is produced. This has allowed both the targeting of mutations anywhere within the AAV genome and the analysis of the effects of such mutations by transfection. These experiments also have divided the AAV genome into two functional domains. Mutations of the ORF within the right half of the genome inhibit or abolish the functioning and synthesis of the AAV capsid proteins (23,

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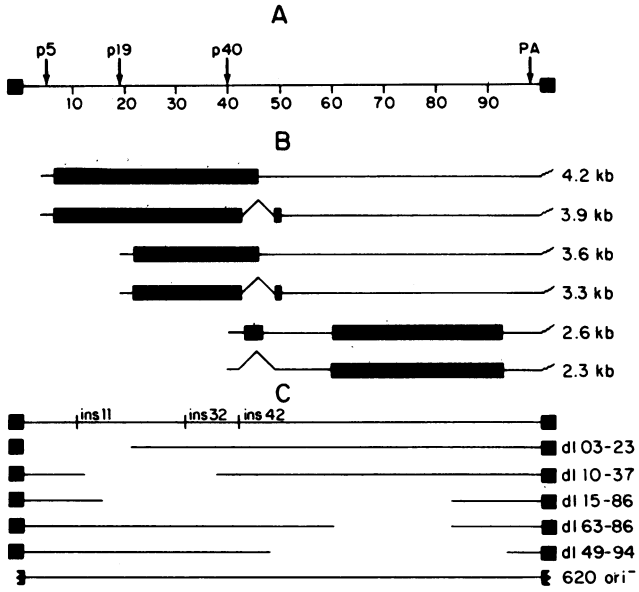

FIG. 1. Structure and genetic organization of wild-type and mutant AAV genomes. (A) The 4.7-kb AAV genome is shown schematically divided into 100 m.u. The 145-base TRs are represented as small black boxes. The locations of the three AAV transcription promoters, p5, p19, and p40, as well as the 3' polyadenylation site (PA) for all AAV RNAs, are indicated by arrows. (B) The structure of the known AAV mRNAs are shown and are aligned with the genome representation in panel A. ORFs present within these messages are depicted by black rectangles within the RNA. The common intron removed is represented by the carets. The sizes of the transcripts (not including the polyadenylation tail) are indicated on the right side of the figure. The polyadenylation tail is indicated by angled curvy lines at the end of the RNAs. (C) The structures of the mutant AAV genomes used in this study are shown and are aligned and drawn as described above. The first structure is a composite which illustrates the three 8-bp insertion mutants used in this study. The boundaries of deletion mutations are shown by gaps in the genomes of the deletion mutants. The deletion mutation of 620*ori*⁻ is illustrated by jagged truncated black boxes.

53). As expected, certain specific mutations in the right half of the genome affect only a subset of the capsid proteins (26).

A second domain is characterized by mutations of the ORF within the left half of the genome. Mutations of this type render AAV genomes defective for DNA replication (13, 23, 31, 53). The replication of the mutant genomes is complemented in *trans* when cotransfected with genomes containing an intact ORF within the left half. Whereas much of the ORF within the left half is carried in both the p5 and p19 transcripts, mutation at 11 m.u., within the ORF unique to the p5 transcript, abolishes DNA replication (23). Thus, the p5 gene is referred to as a *rep* gene. Although the p5 *rep* gene appears to encode at least one protein (*rep* protein) essential for AAV DNA replication, no critical or unique function has yet been assigned to the p19 gene. Proteins have recently been identified immunologically from AAV-infected cells which correspond to translation products of the unspliced species of the p5 and p19 transcripts (E. Mendelson, J. Trempe, and B. J. Carter, personal communication). Thus, at least five proteins encoded by the small AAV genome have been identified, and there is evidence for additional AAV-encoded proteins as well (10).

As described above, all aspects of AAV macromolecular synthesis are regulated by helper virus functions and undoubtedly are also affected by cellular gene products. The data given above suggest a passive role of the AAV genome with regard to its own regulation. However, the complex organization of AAV described suggests that AAV-encoded proteins may perform a variety of functions. In this paper, we document that the AAV genome also regulates its own expression in at least two ways. First, a product encoded by the p5 gene is required for *trans* activation of AAV transcription; second, sequences within the p5 and p19 genes appear to negatively regulate AAV gene expression in *cis*.

MATERIALS AND METHODS

Cells. Human KB and HeLa cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, 2 mM glutamine, and 500 U of each of penicillin and streptomycin per ml.

Enzymes. Restriction enzymes, T4 ligase, and the Klenow fragment of *Escherichia coli* DNA polymerase I were purchased from International Biotechnologies, Inc., and Bethesda Research Laboratories, Inc., and the enzyme reactions were performed as specified by the suppliers.

Construction of AAV mutants. The infectious AAV clone pSM620 (44) was digested with *Sma*I, and the large AAV fragment was cloned into the *Eco*RV site of pBR322 to create 620*ori*⁻. This plasmid was completely digested with *Apa*I to yield *dl*63-86*ori*⁻. The large *Sma*I fragments of *ins*11, *ins*32, *ins*42, *dl*03-23, and *dl*10-37 (23) were also cloned into the *Eco*RV site of pBR322 to create *ori*⁻ *rep*⁻ double mutants. *dl*15-86 was constructed by partial digestion of pSM620 with *Nco*I and subsequent religation.

DNA transfection and virus infection. Cells were transfected by the DEAE-dextran method as described previously (37). Briefly, 80% confluent dishes (150-mm diameter) of KB cells were washed once with phosphate-buffered saline, and a DNA-dextran mix was added and left on the cells at room temperature for 30 min. DNA-dextran consisted of 20 μg of each plasmid (including cotransfections) and 500 μg of DEAE-dextran (molecular weight, 10^6; Pharmacia) per ml of Dulbecco modified Eagle medium in a 3-ml solution. After transfection, the cells were treated with 50 μM chloraquine diphosphate for 3 h in Dulbecco modified Eagle medium and simultaneously infected with adenovirus type 2 (Ad2) at a multiplicity of infection of 10 PFU per cell, after which the medium was replaced with 20 ml of fresh medium.

Cells used for nuclear runoff transcription (NRT) analysis were transfected by the calcium phosphate coprecipitation method (56). Transfections were done without carrier DNA by using 20 μg of plasmids per ml, in 2-ml precipitates, added to the 20 ml of medium in dishes (150-mm diameter) of 50% confluent HeLa cells. The next day, the medium and precipitate were removed, the cells were treated with 12% glycerol in phosphate-buffered saline for 1 min, and 20 ml of fresh medium was added. After 4 h, the cells were infected with Ad2 at a multiplicity of infection of 10.

RNA extraction and analysis. At 30 h postinfection, cells were harvested by scraping, washed with cold phosphate-buffered saline, and swelled on ice. The cells were lysed by Dounce homogenization, and the nuclei were removed by centrifugation. RNA was then extracted from the homogenate as previously described (35). RNA concentrations were determined spectrophotometrically and by band intensities of rRNAs after electrophoresis and UV illumination. RNA was fractionated by electrophoresis in 1.2% formaldehyde

gels, blotted onto nitrocellulose as described previously (36), and then hybridized to AAV virion DNA labeled with ^{32}P by the primer extension method of Feinberg and Vogelstein (16). Probes were typically labeled to specific activities of 2×10^9 cpm/μg of DNA. Certain blots were probed with a fragment of the AAV genome as described in the text.

NRT analyses. Transfected cells were harvested 24 h postinfection, and nuclei were isolated as described above, except that dithiothreitol was added to 1 mM. Nuclei were prepared, and transcription was carried out as described previously (15), except that nuclei from 5×10^7 cells were labeled in 300-μl reactions containing 600 μCi of [^{32}P]UTP (ICN Pharmaceuticals, Inc.; specific activity, 3,000 Ci/mM). Nuclei were lysed, and labeled RNA was prepared as described previously (38). Equal counts of labeled RNA for each experiment (approximately 5×10^7 cpm) were then hybridized to Southern blots (49) of restriction enzyme-digested Ad5 DNAs (kindly supplied by R. J. Samulski) and AAV DNAs (as described in Results).

RESULTS

Accumulation of AAV mRNAs by two types of *rep* mutants. To determine whether an AAV p5 or p19 gene product regulated AAV gene expression, we first compared the accumulation of RNAs by two types of replication-defective AAV genomes: those that had *trans*-active mutations in the ORF within the left half (*rep* gene) or those that had *cis*-active mutations in the origins of DNA replication. The structures of the mutant AAV genomes used in these experiments are shown in Fig. 1. The insertion mutants *ins*11 and *ins*32 contain 8-base-pair (bp) frameshift mutations at the indicated map positions. The construction and characterization of these insertion (*ins*) mutants have been previously reported (23). Both of these mutants are characterized as *rep* mutants since they are completely defective for DNA replication, due to mutation of the p5 and p19 ORFs. The 620*ori* mutant contains all of the internal AAV genes, but its replication is blocked in *cis* by irreparable deletions in the origins of DNA replication (45). Although the replication of 620*ori*$^-$ was blocked, the plasmid genome was able to complement the replication of the *rep* mutants described above (unpublished data), as expected from other studies (47). The 620*ori* mutant was used as a control rather than pSM620, the parental plasmid of *ins*11 and *ins*32, since pSM620 is infectious and therefore produces a greatly amplified number of potential transcription templates due to the rescue and replication of the AAV genome.

The accumulation of RNAs by these AAV genomes was tested by analyzing the RNAs they produced after transfection into Ad2-infected KB cells. Total RNA was isolated from the transfected cells at 30 h posttransfection. The RNA was fractionated and analyzed by blotting and hybridization to radiolabeled AAV DNA, as described in Materials and Methods. The results are shown in Fig. 2. The 620*ori* mutant (lane 1) produced readily detectable amounts of all of the AAV RNAs in the expected proportions except the 3.9-kb spliced p5 transcript, which was not resolved in these studies. The most abundant RNA accumulated was the spliced 2.3-kb p40 transcript. The larger p19 and p5 transcripts were accumulated in much lower amounts than was the p40 message. This steady-state pattern of transcripts was identical to that produced by either wild-type AAV (18, 33) or infectious clones of AAV (data not shown). Thus, it appears that neither a functional origin of replication nor DNA replication was required for efficient expression of the

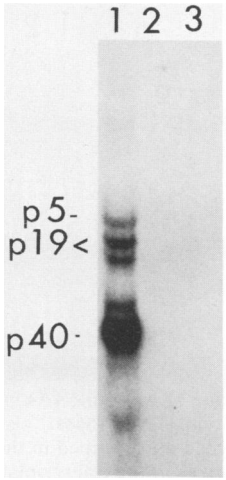

FIG. 2. Accumulation of AAV mRNAs by two types of *rep* mutants. KB cells were transfected with 620*ori*$^-$ (lane 1), *ins*32 (lane 2), or *ins*11 (lane 3) and infected with Ad2 as described in the text. At 30 h postinfection, cytoplasmic RNAs were isolated, and 20 μg of RNA (Materials and Methods) from each experiment was analyzed on a formaldehyde-agarose gel and subsequently analyzed by blotting and hybridization to ^{32}P-labeled AAV virion DNA. The sizes of the RNAs are as shown in Fig. 1, and indicated here are the 4.2-kb unspliced p5 transcript, the 3.3-kb spliced (bottom of caret) and 3.6-kb unspliced (top of caret) p19 transcripts, and the 2.3-kb spliced p40 transcript. The 2.6-kb unspliced p40 RNA is directly above the 2.3-kb RNA.

AAV genes. These results were similar to those of Janik et al. (26). However, the insertion mutants accumulated RNAs in greatly reduced amounts. Both insertion mutants accumulated barely detectable levels of RNAs only after longer exposures of the autoradiogram (data not shown).

Accumulation of AAV RNAs by *rep*$^-$ *ori*$^-$ double mutants. The AAV genomes used in the experiment described above contained either of two different *rep* mutations. *ins* mutants contained intact TRs and defective *rep* genes, whereas 620*ori* mutants contained only deleted TRs. Thus, the difference in the accumulation of transcripts by these mutants might be explained by either the loss of a positive *trans* activator of expression in the *ins* mutants or the removal of a *cis*-active repressor of expression in the TRs of 620*ori*$^-$. To test this, we analyzed the accumulation of transcripts from a series of mutant AAV genomes, all of which contain the same origin (*ori*) mutations as 620*ori*$^-$. The AAV RNAs produced by these genomes upon transfection into Ad2-infected cells are shown in Fig. 3A. Again, both *rep* insertion mutants, *ins*11*ori*$^-$ (lane 2) and *ins*32*ori*$^-$ (lane 3), produced very low levels of transcripts compared with 620*ori*$^-$ (lane 1). In addition, an *ori*$^-$ capsid gene mutant, *dl*63-86*ori*$^-$, accumulated normal amounts and proportions of its deleted transcripts (lane 4).

Although most mutations within the *rep* gene abolished AAV DNA replication in addition to greatly inhibiting p40 expression, a mutation within the carboxy-terminal portions of the AAV *rep* coding region (*ins*42) was reported to allow a very low, but detectable, level of DNA replication (0.1 to 1%) (23). The carrier of this mutation, however, accumulated large amounts of mRNAs relative to that of completely defective *rep* mutants (data not shown). To determine whether this was due to either an AAV gene product retaining a positive regulatory function or a *cis*-active positive regulatory effect of DNA replication on gene expres-

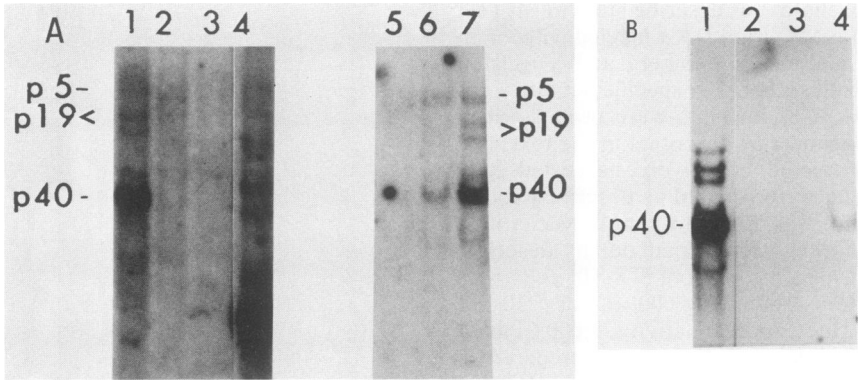

FIG. 3. Accumulation of AAV mRNAs by *ori*⁻ AAV genomes. (A) KB cells were transfected with 620*ori*⁻ (lanes 1 and 7), *ins*11*ori*⁻ (lane 2), *ins*32*ori*⁻ (lanes 3 and 5), *dl*63-86*ori*⁻ (lane 4), or *ins*42*ori*⁻ (lane 6) and were infected with Ad2. A 20-μg amount of RNA from each transfection was analyzed as described in the legend to Fig. 2. The RNAs indicated are of wild-type size and are the same as shown in the previous figures. The sizes of the transcripts of *dl*63-86*ori*⁻ are 1.2 and 1.5 kb (spliced and unspliced p40 RNAs), 2.2 and 2.5 kb (spliced and unspliced p19 RNAs), and 3.1 kb (unspliced p5 RNA). (B) This experiment is the same as in panel A, except that the cells were transfected with 620*ori*⁻ (lane 1), *ins*32*ori*⁻ (lane 2), *dl*10-37*ori*⁻ (lane 3), or *dl*03-23*ori*⁻ (lane 4).

sion, an *ori*⁻ replication-defective version of *ins*42 was constructed, and its expression was tested as described above. The *ins*42*ori* mutant (lane 6) was only partially defective for positive regulation and accumulated about fivefold less transcripts than did 620*ori*⁻ (lane 7) but clearly higher levels than did *ins*32*ori*⁻ (lane 5) (Fig. 3A). Thus, *ins*42*ori*⁻ appeared to be more defective for DNA replication than for gene expression.

We also compared the accumulation of AAV RNAs by *ori*⁻ genomes which have deletions on the left side of the genome. We constructed *ori*⁻ versions of *dl*03-23 and *dl*10-37 (23), as described above. A Northern blot of the RNAs produced by these plasmids, as well as by 620*ori*⁻ and *ins*32*ori*⁻, is shown in Fig. 3B. The mutants with deletions on the left side of the genome accumulated much lower amounts of transcripts than did 620*ori*⁻, as expected due to their defective p5 and p19 genes. Unexpectedly, *dl*03-23*ori*⁻ produced significantly higher levels of p40 RNA than did the other mutants with deletions or insertions on the left side. This finding was reproduced several times by using both *ori*⁻ and *ori*⁺ plasmids (data not shown).

Transcription of *rep* mutants in isolated nuclei. The experiments described above indicated that the mutants with deletions on the left side were defective for gene expression and suggested that an AAV gene product(s) acts as a positive regulator of AAV gene expression. To determine whether this positive regulatory effect was at the transcriptional or posttranscriptional level, we measured the rate of transcription of transfected AAV genomes in isolated nuclei (Fig. 4). Nuclei from Ad2-infected HeLa cells transfected with the indicated AAV plasmids were isolated and incubated as described in Materials and Methods to allow runoff elongation of preinitiated transcriptional complexes. Radiolabeled transcripts were isolated and hybridized to a Southern blot of 620*ori*⁻ DNA which was digested with *Pst*I and *Hin*dIII; this produced three large fragments (lane 1). Fragment a (3.8 kb) contained pBR322 sequences in addition to 400 bp of AAV DNA (m.u. 91 to 98) at the 3′ end of the AAV transcriptional units. Fragment b (2.4 kb) consisted only of AAV DNA from m.u. 42 to 91. Because of the relatively small amount of AAV DNA in fragment a, it should have bound much less AAV RNA than did fragment b. However, both fragments a and b hybridized to all complete AAV RNAs. Fragment c (1.35 kb) contained AAV DNA from 11 to 40 m.u. and hybridized to the p5 and p19 transcripts only. A small fourth fragment, d, containing AAV DNA from 3 to 11 m.u. and pBR322 DNA, is also indicated, although not well resolved in this experiment. Equal counts of labeled RNA from cells transfected with 620*ori*⁻ (panel A), *ins*32*ori*⁻ (panel B), or no plasmid (panel C) were hybridized to separate blots. Each blot also contained *Pst*I digests of cloned Ad5 DNA, either pXHOC (E1a and E1b sequences, lane 2) or pXBAC (E4 sequences, lane 3), to confirm that each experiment contained equal amounts of labeled nuclear transcripts. Although a certain amount of hybridization signal to the pBR322 DNA was observed in all experiments (lane 1, fragment a), hybridization signal to the AAV-specific fragments was only observed when labeled nuclear RNA

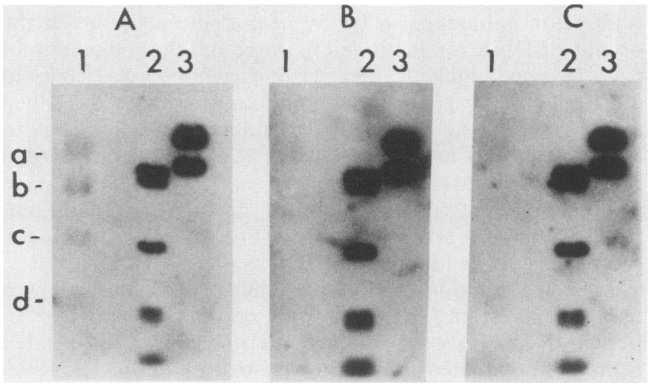

FIG. 4. NRT analysis of AAV genomes. HeLa cells were transfected with 620*ori*⁻ (A), *ins*32*ori*⁻ (B), or no plasmid (C) and then were infected with Ad2 as described in the text. Nuclei were isolated at 24 h postinfection, and transcription reactions were carried out as described in the text. The labeled RNA was then hybridized to Southern blots of AAV and adenovirus DNAs. Lane 1 contains *Pst*I- and *Hin*dIII-digested 620*ori*⁻ DNA, which produced three large fragments: a 3.6-kb fragment which consisted of pBR322 sequences and AAV DNA from 91 to 98 m.u. (a), a 2.4-kb fragment which consisted of AAV DNA from 42 to 91 m.u. (b), a 1.4-kb fragment which consisted of AAV DNA from 11 to 40 m.u. (c), and a 500-base fragment which consisted of AAV DNA from 3 to 11 m.u. and pBR322 sequences (d). Lanes 2 and 3 contain *Pst*I adenovirus type 5 E1a and E1b DNA from pXHOC and E4 DNA from pXBAC, respectively.

from 620*ori*$^-$-transfected cells (panel A) was used. No AAV-specific transcription from *ins*32*ori*$^-$ was detected in isolated nuclei (panel B) in this experiment. Although we attempted to use equal amounts of labeled nuclear RNA in each of these experiments, the level of adenovirus transcription in the *ins*32*ori*$^-$ experiment appears to be approximately two-fold higher than that of the 620*ori*$^-$ experiment, as indicated by the hybridization to adenovirus DNA. We do not consider this to be a consequence of AAV gene expression, because, due to the low efficiency of DNA transfection, only a small fraction of the cells received AAV plasmid DNA, whereas nearly all of the cells were infected by adenovirus. Increased hybridization to the pBR322 sequences in the 620*ori*$^-$ experiment appeared to be partly due to readthrough of AAV-specific transcription into the pBR322 vector because this hybridization was reduced when transcription was measured from cells infected with AAV virus (data not shown).

Complementation and expression of p5 and p19 mutants. The experiments described above suggested that at least one p5 *rep* gene product positively regulates AAV transcription. If this was true, then the defect should be complementable in *trans*. To test this, we conducted cotransfection experiments with *ins*32*ori*$^-$ and a large capsid deletion mutant, *dl*49-94 (23), as shown in Fig. 5. The RNAs produced by these plasmids were analyzed as described in the legend to Fig. 2, except that the Northern blot was probed with a 52- to 91-m.u. fragment of the AAV genome so that the capsid mutant transcripts were not detected (lane 4). Without complementation, *ins*32*ori*$^-$ (lane 2) produced no detectable transcripts in this assay. However, when complemented by cotransfection, the 2.3-kb p40 transcript of *ins*32*ori*$^-$ was readily detected (lane 3). The p5 and p19 RNAs were also detected in much lower amounts than was the p40 RNA after very long exposures of the autoradiogram (data not shown).

We also examined the accumulation of transcripts by replicating *ori*$^+$ mutants with deletions on the left side of the genome upon complementation with capsid deletion mutants having deletions on the right side. Capsid deletion mutants *dl*58-87 and *dl*63-86 (Fig. 6, lanes 1 and 2) produced readily detectable amounts of their deleted transcripts, whereas the *rep* deletion mutants did not (lanes 3 and 6). Cotransfection

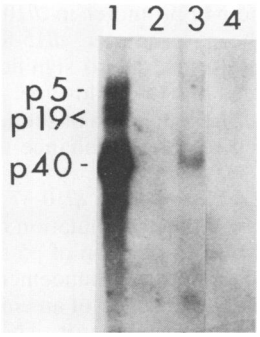

FIG. 5. Complementation of expression by cotransfection with a capsid deletion mutant. KB cells were transfected with 620*ori*$^-$ (lane 1), *ins*32*ori*$^-$ (lane 2), *ins*32*ori*$^-$ and *dl*49-94 (lane 3), or *dl*49-94 (lane 4). RNA was isolated and prepared as described in the legend to Fig. 2. The Northern blot was analyzed as described in the text, except that the blot was probed with a fragment of ^{32}P-labeled AAV DNA from 52 to 91 m.u. The RNA species indicated is the wild-type-size spliced p40 RNA. The sizes of the 620*ori*$^-$ RNAs are as indicated in Fig. 1 and 2.

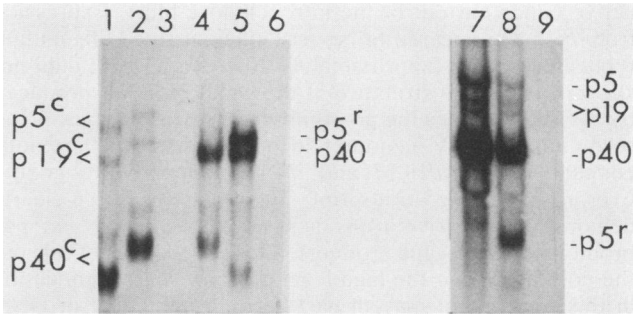

FIG. 6. Complementation of mutants with deletions in the left half of the genome. KB cells were transfected with *dl*58-87 (lane 1), *dl*63-86 (lane 2), *dl*03-23 (lane 3), *dl*63-86 and *dl*03-23 (lane 4), *dl*58-87 and *dl*10-37 (lane 5), *dl*10-37 (lane 6), pSM620 (lane 7), pSM620 and *dl*15-86 (lane 8), or *dl*15-86 (lane 9), and RNA was analyzed as described in the legend to Fig. 2. The locations of specific transcripts originating from the capsid deletion mutants are indicated by a superscript c. Only the spliced p40 and unspliced p19 and p5 transcripts of the capsid deletion mutants are indicated. The bottom of the caret points to the *dl*58-87 transcripts, and the top of the caret points to the *dl*63-86 transcripts. The wild-type-size transcripts of pSM620 are indicated without superscripts as in Fig. 2. The sizes of the transcripts produced by *dl*63-86 are the same as for *dl*63-86*ori*$^-$, as described in the legend to Fig. 3. The transcripts produced by *dl*58-87 are approximately 300 bases smaller than that produced by *dl*63-86. The truncated p5 transcripts of the *rep* gene deletion mutants are indicated by a superscript r.

of *dl*03-23 with *dl*63-86 resulted in the accumulation of large amounts of 2.3-kb p40 transcripts (lane 4), which must have been produced from *dl*03-23 since the capsid mutant does not produce a 2.3-kb message. Alternatively, the 2.3-kb RNA may be a product of a recombinant genome. This alternative seems unlikely because of the high levels of transcripts detected, the low level (approximately 1%) of observed recombination between cotransfected AAV genomes (23, 46), and the experiments described below. The deletion mutant *dl*10-37 also accumulated high levels of 2.3-kb p40 RNA when complemented by *dl*58-87 (lane 5). However, *dl*10-37 also contains a p5 promoter, and indeed when complemented, *dl*10-37 accumulated a 2.6-kb transcript, which corresponded in size to the spliced version of the truncated p5 transcript, in amounts equal to that of the p40 transcript. Normally, the p5 RNA is accumulated at 5 to 10% of the level of the p40 message. Thus, it appeared that *dl*10-37 overproduced its p5 RNA. This transcript does appear to be a p5 transcript since it was not produced by *dl*03-23, which contains the same p40 transcription unit as does *dl*10-37. Also, the highly abundant message hybridized to sequences upstream of p40 (data not shown).

To determine whether the increased accumulation of the p5 transcript of *dl*10-37 required regulatory elements within or near the promoter for the highly expressed p40 transcript, a deletion mutant, *dl*15-86, containing only the putative p5 promoter, was constructed. RNAs accumulated by this plasmid upon transfection with and without pSM620 (to supply p5 and p19 *rep* gene products) are also shown in Fig. 6. Although a very low level of the *dl*15-86 900-bp p5 transcript was detected without complementation after longer exposures of the blot, a high level was detected upon cotransfection with pSM620 (lane 8). The *dl*15-86 transcript was accumulated in molar amounts approximately equal to that of the p40 transcript and 16-fold greater than that of the p5 transcript of pSM620 (determined by counting the radio-

active bands cut out of the nitrocellulose blot). Expression from *dl*15-86 appeared to be very efficient since this mutant replicated poorly (approximately 10% of pSM620; data not shown). Also, the structure of the small p5 RNA produced by *dl*15-86 excludes the possibility that it was produced by a recombinant AAV genome, in accord with the previous conclusion that *dl*10-37 and *dl*15-86 appeared to greatly overproduce the p5 transcript. The results in Fig. 6 clearly demonstrate positive *trans* activation of the p5 and p40 promoters by *rep* gene products, although we cannot rule out the possibility that the high level of transcript accumulation in this experiment may in part be a dosage effect of DNA replication.

DISCUSSION

Analysis of in vitro-generated mutant AAV genomes has demonstrated that at least one protein encoded in the large reading frame contained specifically within the p5 transcript is essential for AAV DNA replication. The presence of p19 transcripts which contain smaller versions of the p5 *rep* ORF has suggested that these RNAs encode one or more additional essential proteins. In this paper, we show that at least one p5 gene product or both p5 and p19 gene products are also required for *trans*-active positive regulation of AAV gene expression. Mutations which destroyed either the p5 ORF or both the p5 and p19 ORFs greatly reduced the accumulation of the AAV transcripts in adenovirus-infected cells. Activation of gene expression appeared to be independent of an intact TR and DNA replication since the effect of a mutation was the same in either an ori^- or an ori^+ plasmid. Although the ori^- deletions used in this study were sufficient to block DNA replication, the internal 60 bp of the TR were retained in the ori^- genomes and therefore might still have regulatory functions.

When the transcriptional activities of AAV genomes were compared by measuring the rates of transcription in isolated nuclei (NRT assay) from cells transfected with either a *rep* gene mutant or a rep^+ ori^- plasmid, only the rep^+ plasmid produced readily detectable labeled AAV RNA. Thus, *rep* gene mutants were defective for transcription. Because of the DNA probes used in this experiment, it was not possible to determine the relative rates of p5 and p19 transcription. Although studies of the steady-state levels of AAV RNAs showed a 10-fold excess of the p40 transcripts (e.g., Fig. 2 and 3), the NRT assay detected only an approximately 2-fold-increased hybridization to the right-side-specific fragment b compared with the left-side-specific fragment c. This suggests an approximately equal rate of synthesis for all of the AAV genes. This ratio of transcription did not appear to be due to an artifact of expression from a nonreplicating plasmid since 620ori^- accumulated cytoplasmic RNAs in the expected proportions, and NRT assay using wild-type AAV showed similar results (data not shown). These data suggest that the levels of the p5 and p19 transcripts accumulated may also be controlled by either transcription attenuation or differential message stability. Preliminary results, however, indicated that the cytoplasmic AAV transcripts are equally stable, with half-lives of at least 4 h (data not shown), which is consistent with earlier studies which examined the half-life of total AAV RNA (11). However, it is not known at this time whether the stabilities of the nuclear AAV transcripts differ.

The defective gene expression of *ins*32ori^- was complementable in *trans*. The 2.3-kb p40 transcript of the insertion mutant was only detected upon cotransfection with a capsid deletion mutant (Fig. 5). The larger p5 and p19 RNAs of *ins*32ori^- were also detected upon longer exposures of the blot (data not shown). The level of RNAs accumulated by *ins*32ori^- upon complementation was significantly lower than that accumulated by 620ori^-. It is unclear at this time why this complementation experiment was so inefficient. This complementation experiment was done by using *ori* mutants with mutations on the left side of the genome so that induction of expression was assayed directly without an increase in template copy number due to rescue and replication of the defective AAV genome. An ori^+ capsid deletion mutant was used as complementor so as to maximize the production of the p5 and p19 gene products. However, for unknown reasons, the expression of the replicating capsid mutant appeared to be greatly favored over that of the nonreplicating *ori* mutants (data not shown). As discussed below, the expression of ori^+ mutants with deletions on the left side of the genome (Fig. 6) was also complementable and was also much more efficient.

The data presented above demonstrate that at least one AAV gene product encoded in the p5 and p19 genes is required for optimal transcription of the AAV promoters. Although the complementation experiment did not show clear evidence for the activation of the p19 promoter, the transcript accumulation by the insertion mutants with mutations on the left side of the genome, as shown in Fig. 2 and 3, suggests that the p19 promoter is also most active when intact AAV p5 and p19 genes are present. These experiments do not indicate whether the AAV gene products act directly or indirectly to enhance transcription. The answer to this question awaits the isolation and characterization of the p5 and p19 gene products.

Although the p5 gene product is associated with positive regulation of AAV transcription, several experiments indicate the p5 gene itself is negatively regulated. The p5 and p19 transcripts of genomes containing an intact left side are accumulated at 5 to 10% of the level of the p40 transcripts. However, upon complementation, *dl*10-37 produced two equally abundant RNAs, a 2.3-kb p40 transcript and a 2.6-kb truncated p5 transcript. This relative overproduction of the p5 transcript was not seen when a simple *rep* insertion mutant was used (Fig. 5). Thus, the overproduction of the truncated p5 transcript does not appear to be an artifact of the complementation assay. Similarly, the high level of p5 expression did not appear to be due to the proximity of the p5 promoter to the p40 promoter in *dl*10-37 since a plasmid containing only the p5 promoter, *dl*15-86, also produced a highly truncated p5 transcript to significantly higher levels than were produced by the wild-type AAV genome contained in pSM620. Thus, deletion of sequences between m.u. 15 and 37 appeared to greatly enhance the accumulation of the p5 transcript.

The sequences removed from *dl*10-37 and *dl*15-86 appear to function to depress the accumulation of the p5 transcript. Alternatively, the overexpression of p5 may result from the mutations causing an active enhancement of p5 expression (e.g., the fortuitous construction of an enhancer), rather than by removal of a negative regulator. The former possibility appears unlikely since both *dl*10-37 and *dl*15-86 have similar phenotypes. The experiments presented here have not determined the level at which p5 gene expression is affected by the deletions on the left side of the genome. The relative accumulation of p5 may be controlled at the level of message stability, attenuation, or initiation of transcription. Interestingly, transcription of the p4 gene of minute virus of mice, an autonomous parvovirus, appears to be regulated by attenu-

ation (3) in addition to being necessary for *trans* activation of p38 transcription (40). The NRT data discussed above may also indicate attenuation in AAV transcription. At this time, it is also not known whether a cellular, adenoviral, or AAV-encoded protein mediates the negative regulation of p5, though recent evidence suggests a role of the adenovirus E1a gene in the repression of AAV transcription. Several investigators have recently identified a transcription repressor activity of an adenovirus E1a gene product on both the E1a promoter and heterologous promoters (48, 55). Another interesting possibility is that the p5 gene product negatively autoregulates its own expression. This is speculatively appealing in that other viral transcriptional *trans* activators, including the simian virus 40 T antigen, the 289-amino-acid E1a gene product, and the herpes simplex virus type 1 ICP-4 product, have negative autoregulatory functions (4, 20, 22, 29, 39, 48, 51, 52).

The fact that the p5 *rep* gene product has now been shown to be required for the efficient synthesis of both AAV DNA and RNA does suggest that the *rep* gene products are multifunctional, possibly in a manner analogous to the simian virus 40 T antigen (52). The p5 gene product and the simian virus 40 T antigen appear to have several similar functions. Both proteins are required for viral DNA replication while also functioning to activate transcription of their structural protein genes (8, 20, 52). Alternatively, the p5 or p19 gene products might function only to regulate transcription, turning on helper virus or cellular genes needed for AAV DNA replication. However, the phenotype of *ins*42 suggests that the p5 and p19 gene products have separate functional domains analogous to the simian virus 40 T antigen. Although *ins*42 is severely defective for DNA replication (23), the mutant still appears to positively regulate transcription almost as efficiently as wild type. The mutation of 42 m.u. is contained within the known AAV intron which could code for approximately 100 amino acids at the carboxy-terminal end of the *rep* proteins.

In summary, AAV is structurally among the simplest of eucaryotic DNA viruses. AAV is also completely dependent upon helper virus functions for its replication. Despite the structural simplicity and helper dependence of AAV, the AAV genome autoregulates its own DNA replication and gene expression through both *cis*- and *trans*-active regulatory elements. Thus, AAV illustrates that, no matter how simple a eucaryotic virus may appear to be, there are several regulatory functions that all viruses may share. All are autonomous replicons that must have an origin of replication. All DNA viruses that have been analyzed in detail also encode proteins required in *trans* for DNA replication. A third common feature appears to be an ability to also autoregulate and enhance the expression of the replicating genes through *cis*-active sequences and *trans*-active positive regulators. These elements are essential when one considers the competition between viral transcriptional promoters and the vast excess of cellular gene promoters for limited transcriptional machinery.

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