Sequences Required for Coordinate Induction of Adeno-Associated Virus p19 and p40 Promoters by Rep Protein

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A series of contiguous 30-bp deletions were introduced into the regions upstream of the p19 and p40 promoters of adeno-associated virus (AAV), and the effects of these deletions on induction of AAV transcription by the *rep* gene products was evaluated. A novel complementation system was devised for supplying wild-type Rep protein when mutations disrupted the *trans* activation activity of the Rep protein. Transcription from the p40 promoter was eliminated upon deletion of the TATA sequence located between -4 and -33 from the cap site. Deletions which removed sequences from -34 to -123 bp from the p40 mRNA start site substantially reduced Rep induction of p40 transcription. p19 transcription was also undetectable when the p19 TATA sequence between -4 and -33 was deleted. In contrast to the p40 region, two types of *cis*-active sequences were found associated with the p19 promoter. Sequences between -4 and -63 bp relative to the p19 cap site were essential for Rep induction only from the p19 promoter. Deletions between -94 and -153 bp relative to the p19 cap site reduced Rep induction of both the p19 and p40 promoters coordinately. These two noncontiguous regions were separated by a 30-bp sequence that was not essential for transcription control. Further deletion analysis delineated a second *cis*-active element, associated with the p5 promoter (AAV nucleotides 191 to 320), which was also necessary for coordinate Rep activation of both the p19 and p40 promoters. Finally, the dependence of p40 transcription on the Rep-responsive elements within the p5 and p19 regions could be overcome by the presence of the AAV terminal repeats, suggesting that the terminal repeats contained redundant Rep-responsive elements. These results implied an interdependence in *cis* between the three AAV promoters and suggested a novel mechanism for coordinate regulation of gene expression in response to the *trans*-activating Rep protein. Coordinate induction appeared to be the result of a simultaneous interaction between the Rep protein and sequence elements associated with two or all three of the AAV promoters.

Gene expression in adeno-associated virus (AAV) presents an interesting problem in transcriptional control. During a productive viral infection in the presence of adenovirus, the three AAV promoters are activated simultaneously (29, 35). The coordinate induction of these promoters is mediated by the AAV *rep* gene products (29, 46). The *rep* gene (Fig. 1) codes for four overlapping polypeptides (33, 45, 50) from mRNAs transcribed from two AAV promoters located at map units 5 and 19 (p5 and p19) (16, 31, 32). An intron at the C terminus of the *rep* open reading frame allows the expression of either spliced or unspliced versions of each Rep polypeptide (31, 33). The third promoter of AAV is located within the *rep* open reading frame at map unit 40 (p40) (17, 31) and transcribes mRNAs for the capsid proteins (5, 24, 49).

Mutants carrying mutations which disrupt the *rep* open reading frame in regions which affect only the p5 Rep proteins are defective for both AAV DNA replication (19) and *trans* activation of AAV transcription (29, 46). The replication functions of the p5 Rep proteins have been partially characterized and include at least three distinct activities: binding of the AAV terminal repeats (TRs) (2, 21), which are the viral replication origins (18, 39, 42); site-specific nicking at the terminal resolution site (*trs*) (22, 44); and a DNA helicase activity (22). It is not known whether any of these activities have a role in the control of AAV

transcription. Although recent genetic and biochemical evidence indicates that the p19 Rep proteins are not required for terminal resolution (11, 43), it is not known what role, if any, they may have in transcription.

It is not known whether Rep binds specifically to DNA sequences other than the AAV TRs. Although there is evidence that the terminal repeats have enhancer function (4), DNA replication per se is not required for induction of AAV transcription (29, 46), and in the absence of the TRs, induction of AAV transcription is similar to that seen in the intact viral genome (10, 23, 29, 37, 38, 46). This implies that the TRs are not absolutely essential for the induction of AAV transcription by the Rep protein, but as yet, the specific sequence requirements for induction are unknown. Presumably, either a separate Rep-responsive element is associated with each promoter or a Rep-responsive element is situated somewhere within the genome so as to influence the three promoters coordinately.

In order to identify the Rep-responsive elements, we have constructed a set of systematic deletions to localize sequence elements which affect the levels of Rep-induced transcription from the p19 and p40 promoters. Our findings indicate that there are multiple sequence elements upstream from each promoter which contribute to the levels of transcriptional activation. Furthermore, there appear to be two types of controlling sequences which, upon deletion, reduce Rep-induced transcription. The first type of element affects transcription only from the proximal promoter. The second type reduces the ability of both p19 and p40 to respond to Rep induction.

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FIG. 1. AAV promoters and promoter deletion. (Top) Nucleotide sequences of the AAV p19 and p40 promoter regions, showing the coordinates of the 30-bp promoter deletions and the transcription start sites (arrows). Shaded regions highlight sequences that are potential transcription factor-binding sites. SRE, serum response element (13); SP1, GC box or SP1 binding site (6); CATT, C/EBP binding site (26); TATA, TFIID binding site (34); EF1A, EF1A factor-binding site (7); MLTF, major late promoter transcription factor-binding site (14, 41); ATF, cyclic AMP response element (14); AP1, Jun binding site (26); GGT, a sequence motif common to the p19 and p40 promoter regions. (Bottom) Plasmid pIM29, containing wild-type AAV sequences 191 to 4493, is shown with the Rep coding region shaded. The relative positions of the p5, p19, and p40 promoters are marked by arrows, and the intron is marked by a caret. The capsid coding region and vector sequences of pIM29 are not shown. The expanded region shows the locations of the 30-bp deletions, represented by open boxes. Numbers below the boxes denote the AAV nucleotide number which begins each deletion. These deletions are designated in the text and figures by this number prefixed by *dl*. Above the boxes, the position of each deletion relative to the proximal transcription start site is shown. Below pIM29, the 5' deletions used in this study and the AAV restriction sites used in their construction are shown. They are designated by 29d/ followed by the numbers of the AAV nucleotides deleted.

MATERIALS AND METHODS

Cells and transfections. For DNA transfections, HeLa cell monolayers were grown to 50 to 75% confluence in 100-mm dishes containing 10 ml of minimal Eagle's medium (MEM) with 10% fetal bovine serum. The cells were washed with

phosphate-buffered saline and transfected with 1 ml of MEM containing 1 mg of DEAE-dextran per ml, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4), and 10 μ g of plasmid DNA (5 μ g of test plasmid plus 5 μ g of either complementing plasmid or vector). After 20 to 30 min at room temperature, the DEAE transfection mixture was removed, and the cells were washed again with phosphate-buffered saline and incubated in 10 ml of MEM containing 2% fetal bovine serum and, for adenovirus-infected cells, adenovirus type 2 (Ad2) at a multiplicity of infection of 5.

RNA isolation and analysis. At approximately 40 h post-transfection, the cells were scraped into the medium and harvested by centrifugation. The cell pellets were resuspended in 3.5 ml of 4 M guanidinium thiocyanate lysis solution (3), and high-molecular-weight DNA was sheared by sonication. A 0.1-ml aliquot of lysate was removed for quantitation of input plasmid DNA. The remaining lysate was layered over 5.7 M CsCl, and the RNA was separated by centrifugation at $150,000 \times g$.

For primer extension assays, 10 to 20% of an ethanol suspension of the RNA from a 100-mm dish was mixed with 5'-end-labeled, gel-purified primer and centrifuged. The pellet was resuspended in 20 μ l of 34 mM Tris-HCl (pH 8.3)—6 mM MgCl₂—50 mM NaCl—5 mM dithiothreitol and then allowed to anneal for 1 h at 65°C. The annealed primers were then extended by addition of 20 μ l of reverse transcriptase mixture containing 500 μ M each deoxynucleoside triphosphate, 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 0.1 M Tris-HCl (pH 8.3), 0.15 M KCl, 6 mM MgCl₂, 20 mM dithiothreitol, and 20 U of RNasin (Promega); the reaction mix was incubated for 30 to 60 min at 45°C. The samples were then digested with RNase, extracted with phenol and chloroform, and analyzed on 6% polyacrylamide-urea gels.

The oligonucleotides used for primer extension analysis of transcription products from the p19 and p40 promoters were each 20 bases in length. The p19 primer consists of the AAV nucleotide sequence from 960 to 941, 5'-GCGCATCAGAAT TGGGATTC-3', yielding a primer extension product of 88 nucleotides. The p40 primer is from AAV nucleotides 1904 to 1885, 5'-GTCTGCGTAGTTGATCGAAG-3', and yields a primer extension product of 52 nucleotides. The oligonucleotides used to prime transcripts from the complementing plasmids, p19S and p40S, are designated P19SP and P40SP, respectively, and are complementary to the same nucleotide positions as the analogous wild-type primers. The sequence of P19SP is 5'-GGCGTCGCTGTTTGGGTTTT-3'; the sequence of P40SP is 5'-TATCGGCATAATTAATGCTC-3'. The adenovirus E1A transcript primer, used to determine the efficiency of RNA recovery, consisted of Ad5 nucleotides 593 to 574 (51), 5'-TAATAACACCTCCGTGGCAG-3', and yielded a primer extension product of 95 nucleotides. p40 transcript levels were quantitated with a scanning gas flow counter (AMBIS).

Quantitation of input plasmid DNA. Of the 100- μ l aliquot removed from the guanidinium lysate above, 5 μ l was added to 250 μ l of 0.2 M NaOH, boiled for 10 min, chilled, and neutralized with an equal volume of 2 M ammonium acetate. With a slot blot apparatus, the samples were applied to nitrocellulose filters, which were then probed with the 5'-end-labeled p40 primer used for the primer extension reactions above.

Plasmids. The mutants used in this study were derived from the plasmid pIM29, a subclone of psub201(+) (37). pIM29 consisted of the AAV sequences from an *Ava*II site at

TABLE 1. Summary of promoter mutants^a

Mutant	Oligonucleotide sequence or restriction site	Nucleotides deleted
<i>dl720</i>	AAACTGGTTCGCGGGGAACAAGGTGG	720–749
<i>dl750</i>	TGGCGCCGAGGCCCAATTACTTGC	750–779
<i>dl780</i>	TGAGTGCTACATCGAGCTCCAGTGGG	780–809
<i>dl810</i>	CAAAACCCAGCCTCAGTATTTAAGCG	810–839
<i>dl840</i>	GACTAATATGGAAGAGCGTAAACGGT	840–869
<i>dl1700</i>	ATGACTTTGGGAACCGGTGGGCAAAG	1700–1729
<i>dl1730</i>	TCAAAGACTTTTGGAGCATGAATTC	1730–1759
<i>dl1760</i>	ACGTGGTTGAGGTAGCCAAGAAAAGA	1760–1779
<i>dl1790</i>	TCAAAAAGGGTGGAGATATAAGTGAG	1790–1820
<i>dl1820</i>	CCCCCAGTGACGCGTCAGTTGCGCAG	1820–1850
<i>29dl1-320</i>	GCTCGGATGGTCTAGAATGCCGGGGTTT	1–320
<i>29dl1-687</i>	<i>SmaI</i> – <i>SstII</i>	1–687
<i>29dl1-809</i>	<i>SstI</i> – <i>SstI</i>	1–809
<i>29dl1-1044</i>	<i>BamHI</i> – <i>BamHI</i>	1–1044
<i>dl3-23</i>	<i>BstNI</i> – <i>BstNI</i>	144–1059

^a With the exception of *dl3-23*, the parental plasmid for all of the deletions was pIM29, a derivative of psub201(+) (37). Therefore, in addition to the deleted nucleotides listed, each mutant is also missing AAV nucleotides 1 to 190 and 4494 to 4680. *dl3-23* is a derivative of pSM620 (36) and is missing only the nucleotides listed.

nucleotide 191 to the *NcoI* site at 4493 cloned into the vector pBSM13+ (Stratagene) via *XbaI* linkers (Fig. 1). pIM29 contains all of the known protein-coding regions of AAV and its three promoters but lacks the 145-bp TRs, 45 bp immediately 3' to the left TR, and 42 bp immediately 5' of the right TR.

A series of five contiguous 30-bp internal deletions were made upstream from the p19 and p40 promoters (Fig. 1) by using synthetic oligonucleotides 26 bases in length and complementary to 13 bases on each side of the 30 bases to be deleted from the plasmid. The names of the deletions, the sequences of the oligonucleotides used to make them, and the nucleotide coordinates of the deleted sequences are indicated in Table 1. Each of the mutants made with these oligonucleotides is designated in this report by *dl* followed by the number corresponding to the first nucleotide of the 30 bp deleted. The mutants were constructed by annealing these oligonucleotides to single-stranded, uridine-substituted, circular molecules of pIM29 by the method of Kunkel et al. (27) and synthesis of the second strand with T4 DNA polymerase. Each of these deletions created a 10-amino-acid deletion in the Rep protein. Although the Rep protein reading frame is maintained in these constructs, two of the deletions, *dl1700* and *dl1730*, result in an additional amino acid change of Lys-460 to Asn and Phe-470 to Leu, respectively. Each oligonucleotide-directed deletion was confirmed by DNA sequencing.

In addition to the oligonucleotide-directed promoter deletions, several other deletions were constructed by conventional restriction enzyme digestion (Table 1 and Fig. 1). *29dl1-1044* was made by deleting the *BamHI* fragment of pIM29 from the polylinker to AAV nucleotide 1044; *29dl1-809* was made by deletion of the *SstI* fragment from the polylinker to AAV 809; *29dl687* was made by partial digestion of pIM29 with *SstII*, blunting the ends with T4 DNA polymerase, and ligation to the polylinker at the *SmaI* site. *29dl1-320* is a pIM29-derived clone made by oligonucleotide-directed deletion of sequences from the *XbaI* site of the polylinker to the start codon of the Rep protein at AAV

nucleotide 321. The *rep* mutant plasmid *dl3-23* was derived from pSM620 (36) and has been described previously (19). It contains both the left and right TRs and a functional capsid gene but is missing AAV nucleotides 144 to 1059, which include the two promoters for transcription of *rep* mRNA (p5 and p19) and most of the Rep protein-coding region.

Plasmids used for complementation of *rep* mutants were derived from pIM29+45, which was in turn derived from pIM29. In pIM29+45, oligonucleotide-directed insertion was used to replace the 45 bp of AAV sequence adjacent to the left TR which were missing in pIM29. The resulting plasmid contained the wild-type AAV sequence from the end of the TR at nucleotide 145 to nucleotide 4493 and retained the *XbaI* linker sites of pIM29 at 190 and 4493. During this process, a *BglII* site was inserted 5' to the beginning of the AAV sequence in place of a *BamHI* site in the polylinker of the vector. Oligonucleotide-directed mutagenesis of pIM29+45 was used to create a series of silent substitution mutations in the regions complementary to the priming sites used for primer extension assays (see Fig. 3). The mismatches prevented the transcripts from these complementing plasmids from being detected in the primer extension assays because they prevented the primer from annealing (see Fig. 3). Three complementing plasmids were made: p40S, substituted in the p40 transcript priming site; p19S, substituted in the p19 transcript priming site; and p19/40S, a combination of the two mutations above, made by replacing the *BglII*–*BamHI* fragment of p40S with that of p19S. The oligonucleotides used for the mutation of sequences in pIM29+45 complementary to the p19 and p40 priming sites are: p19, 5'-aagaga atcaAAACCCAAACAGCGACGCCCGgtgatcag-3'; p40, 5'-agacgcggaagCGAGCATTAAATTATGCCGATaggtaccaa-3'. The nucleotides in uppercase letters are within the region complementary to the primers used in the primer extension assays, and the underlined nucleotides are mismatches relative to the wild-type sequence; none of the mismatches change the protein-coding capacity of the sequence.

RESULTS

cis and trans effects of p40 promoter deletions. To identify regions of the p19 and p40 promoters that were essential for transcription and induction by the Rep protein, a series of 30-bp in-frame deletion mutations were constructed upstream of the p19 and p40 cap sites in the parental plasmid pIM29 (Fig. 1). pIM29 contained the internal *XbaI* fragment of psub201(+) cloned into the *XbaI* site of the single-stranded phage vector pBSM13+. psub201(+) was an infectious wild-type AAV variant plasmid constructed by Samulski et al. (37). The sequences of pIM29 contained virtually all of the wild-type AAV sequences (nucleotides 191 to 4493) except for the AAV TRs. The mutants constructed in pIM29 were named according to the first AAV nucleotide deleted in each mutation (Fig. 1). Each deletion removed one or more known transcriptional elements which were thought likely to be involved in control of the p19 and p40 promoters (Fig. 1, top).

The five 30-bp deletions, spanning the region from –4 to –153 relative to the p40 transcription start site, were transfected into adenovirus-infected HeLa cells, and total cellular RNA was isolated at 40 h posttransfection. p40-specific RNA levels were measured by primer extension of total cellular RNA with a 20-nucleotide primer complementary to AAV nucleotides 1904 to 1885. Since the priming site was upstream of the intron, spliced and unspliced RNAs were not distinguished.

FIG. 2. Primer extension of total cellular RNA from HeLa cells transfected with the p40 promoter deletion series. DNA (5 μ g) from pIM29 (wild type [wt]) (lane 1), p40 promoter deletion plasmids (lanes 2 to 6), or *dl3-23* (lane 7) was transfected into adenovirus-infected HeLa cells. Lanes 8 and 9 contain *dl3-23* and pIM29 plasmid transfections, respectively, in the absence of adenovirus infection (–Ad). Lane 10 contains RNA from HeLa cells infected only with adenovirus (Ad). Lane M contains labeled *Hpa*II fragments of pBR322 as molecular size markers. (Upper panel) Primers for the AAV p40 transcript and the E1A transcript of adenovirus were used to measure the amounts of p40 and E1A transcript in each RNA preparation. The E1A primer was used to monitor the efficiency of RNA isolation. *dl3-23*, in which most of the *rep* coding region is deleted, and controls not infected with adenovirus were included to show that transcription is dependent upon Rep and coinfection with adenovirus. The visible molecular size markers are 110, 90, 76, and 67 bp, respectively, from the top. (Lower panel) RNA from the same cells primed with an oligonucleotide specific for the p19 transcript. The visible molecular size markers are 90 and 76 bp, respectively, from the top. The primer extension products of the p40, p19, and E1A transcripts are 52, 88, and 95 nucleotides, respectively. The line drawing below depicts the relative positions of the p40 promoter and each 30-bp deletion in pIM29 as well as the position of the deletion in *dl3-23*; shaded boxes indicate the AAV TRs.

The p40 transcript (Fig. 2, top panel) was abundant in cells transfected with the wild-type pIM29 plasmid DNA. No p40 transcript was detectable in deletion mutants with mutations spanning the region from nucleotides 1700 to 1789 (*dl1700*, *dl1730*, and *dl1760*). Deletion of the 30 bp from nucleotides 1790 to 1819 (*dl1790*) reduced transcription from the p40 promoter approximately 10-fold, and no transcription was detected from *dl1820*, which removed the p40 TATA sequence. There was also no detectable p40 transcription in the absence of adenovirus (pIM29 – Ad lane). This was most likely an indirect effect due to a reduced level of Rep protein synthesis in the absence of adenovirus. The adenovirus E1A gene has been shown to be required for efficient AAV p5 transcription (10) as well as p19 transcription (47). In addition, other adenovirus gene products have been shown to increase AAV transcription, RNA accumulation, splicing,

and translation (8–10, 20, 23, 25, 30, 38a, 40, 52). To confirm the requirement for Rep protein in p40 transcription, we transfected the deletion plasmid *dl3-23*. *dl3-23* retained the AAV TRs and a wild-type p40 promoter but was missing the AAV sequences 144 to 1059, which included the AAV p5 and p19 promoters and much of the *rep* coding sequence (Fig. 2, diagram). As reported previously (29), p40 transcription could not be detected from *dl3-23* in either the presence or absence of coinfecting adenovirus (Fig. 2, top panel).

Because the p40 promoter was situated within the *rep* open reading frame, each of the 30-bp promoter deletion mutations also created a deletion of 10 amino acid residues in the Rep protein (Fig. 1). Since the activation of p40 in *cis* also depended on the presence of active Rep in *trans*, it was necessary to determine the effects of these deletions on the Rep protein. The *trans* activation potential of Rep protein from each of these mutants was evaluated by primer extension of transcripts from the p19 promoter. p19 could also be induced by Rep and remained wild type in *cis* in the p40 promoter deletion plasmids (Fig. 2, diagram). The 88-nucleotide primer extension product of the p19 transcript (Fig. 2, bottom panel) was evident in cells transfected with plasmids containing a wild-type *rep* gene (pIM29) and in plasmids deleted within the region from 1790 to 1849 (*dl1790* and *dl1820*). Thus, the reductions in transcript levels from the p40 promoter of *dl1790* and *dl1820* resulted from deletions of essential *cis*-active elements. The increase over the wild-type level of p19 transcripts from these mutants was reproducible and may result from decreased competition for transcription factors from the mutant p40 promoters. Cells transfected with plasmids deleted in regions between nucleotides 1700 and 1789 (*dl1700*, *dl1730*, and *dl1760*) did not accumulate p19 transcripts, indicating that the Rep protein was not functional in these mutants. Although these plasmids may have had deletions of important *cis*-active elements at p40, their effects would not be apparent in the absence of a competent *trans*-active Rep protein. In order to further characterize these mutants, we developed a novel complementation system capable of supplying wild-type Rep protein.

In devising a complementation system, we sought to maintain three parameters. First, we wished to avoid the use of a reporter gene fused to each mutant promoter in order to avoid the possible negative effects of Rep at the posttranscriptional level (48). Second, we wanted to keep the promoters in the context of the intact genome (minus the termini), since controlling elements may be remote from their associated promoters. Finally, it was necessary to distinguish between transcripts from the mutant promoters and those from the plasmid carrying the complementing *rep* gene because the Rep protein-coding sequence also carried the sequences of the p19 and p40 promoters. This was accomplished by creating a complementing plasmid, derived from pIM29+45 (see Materials and Methods and Fig. 3), in which a series of silent substitution mutations were introduced into the region used as the priming site for the primer extension assays. Directing mutations toward the third position of codons and using an alternative serine codon resulted in changes to 9 of the 20 bases in the priming site without altering the amino acid sequence of Rep. The annealing conditions for the primer extension reactions were sufficiently stringent to allow priming of transcripts only from the plasmid carrying the promoter deletion and wild-type priming site.

The effects of deletions in the p40 promoter region were measured in adenovirus-infected HeLa cells in the presence

FIG. 3. (A) Complementation system for Rep *trans* activation-defective mutants. (Top) The drawing depicts the Rep open reading frame (shaded region) containing the p19 and p40 promoters. Also shown are the three overlapping transcripts of AAV with the p19 and p40 transcripts annealed to their respective oligonucleotide primers. The vertical lines indicate base pairing; asterisks represent 5' ³²P label. The open box upstream from p19 represents a 30-bp deletion region within the p19 promoter region. Rep proteins from these mutants are defective for *trans* activation. (Bottom) This drawing depicts the constructs used to complement the promoter deletions above for Rep *trans* activation function. The X's represent mismatches in the primer annealing sites which are silent with respect to the coding capacity of the *rep* gene. This construct supplies wild-type (wt) Rep to activate the promoter deletion mutant in the plasmid depicted above; however, the transcripts from the complementing plasmid are not detected in the primer extension assay because they do not anneal to the oligonucleotide primers (wavy lines). (B) An example of the primer extension signals obtained from wild-type (wt) and complementing plasmids (taken from the experiment shown in Fig. 8). Adenovirus-infected HeLa cells were transfected with 5 μ g of either pIM29 (wt) or p19/40S. Total cellular RNA was annealed to p19 and p40 primers for 1 h at 65°C. Under these conditions, only transcripts with wt priming sites are observed. The positions of the p19 and p40 primer extension products are shown.

or absence of complementing wild-type plasmid p40S, which contained silent substitutions in the p40 priming site (Fig. 4). The primer extension analysis shown in Fig. 4 was repeated with the addition of a primer for the adenovirus E1A gene as a control for RNA recovery (data not shown). The p40 transcripts were quantitated for each transfection by measuring the 5'-end-labeled primer extension product with a scanning gas flow counter.

p40 transcription from the parental plasmid pIM29 was essentially the same in the presence and absence of complementing Rep (Fig. 4). *d11700*, which produced a *trans* activation-defective Rep protein, was induced to wild-type levels in the presence of complementing Rep, indicating that sequences within the deleted region were not essential for p40 activity in *cis*. Transcription from the p40 promoter of *d11730* was reduced by 20 to 30% compared with the wild type when complemented with wild-type Rep. The first three bases of a repeated sequence, GGTGGAG (referred to as GGT), were deleted in this mutant (Fig. 1). The GGT motif was present in two copies in the p40 promoter region, and when portions of both copies were removed by the deletion in *d11760*, induction by Rep protein was reduced approximately fourfold. Although the phenotypes of *d11730* and *d11760* suggested that the GGT sequence element may be important for Rep induction, we could not rule out the

FIG. 4. Complementation of p40 promoter deletion mutants. DNA (5 μ g) from either wild-type (wt) pIM29 or one of the p40 promoter deletion plasmids was cotransfected into adenovirus infected HeLa cells with 5 μ g of vector DNA (p40S, lanes -) or 5 μ g of complementing plasmid DNA (p40S, lanes +). The complementing plasmid contains a wild-type *rep* gene which has silent substitution mutations at the p40 priming site which prevent the oligonucleotide primer from annealing (see Materials and Methods and Fig. 3). Only transcripts from the 30-bp promoter deletion plasmids, pIM29, or *d13-23* are detected by primer extension. Lanes 1 and 2, 5 μ g of pIM29 (wt promoter) with 5 μ g of vector DNA (lane 1) or 5 μ g of p40S (lane 2); lanes 3, 5, 7, 9, and 11, 5 μ g of the indicated p40 deletion plasmid with 5 μ g of vector DNA; lanes 4, 6, 8, 10, and 12, 5 μ g of the indicated p40 deletion plasmid with 5 μ g of p40S; lanes 13 and 14, 5 μ g of *d13-23* DNA with 5 μ g of vector DNA (lane 13) or 5 μ g of p40S DNA (lane 14); lane 15, no plasmid DNA. *d13-23*, in which most of the *rep* coding region is deleted, was included to show that wild-type Rep is expressed from the complementing plasmid p40S. The position of the 52-nucleotide primer extension product of the p40 transcript is shown (P40).

possibility that other elements within this region, e.g., the major late promoter (MLTF) or ATF site (14, 41), may contribute part or all of this effect (Fig. 1).

Transcription from the p40 promoter of *d11790*, which was reduced 7- to 10-fold in the absence of complementing Rep (Fig. 2), was not increased by including the plasmid carrying the wild-type *rep* gene (Fig. 4). Therefore, the reduced level of induction from this mutant was due solely to the deletion of *cis*-active sequences and not a reduced *trans* activation capacity of the deleted Rep protein. This was consistent with the high level of induction of p19 from this plasmid in the absence of complementing Rep (Fig. 2, lower panel). Although the deletion in *d11790* removed the last two bases of one of the two GGT motifs, the GGT sequence was reformed by fusion with sequences 3 bp upstream from the TATA site. Also deleted in this mutant were a potential SP1 site (6) and a putative AP1 site (21) (Fig. 1). Transcription from the p40 promoter of *d11820* was not detectable in the presence or absence of complementing Rep. This region included the sequence TATAA, at -26 to -31 relative to the transcription start site, and presumably this is a functional TFIID binding site (34), which is required for accurate p40 transcription. It also remains a possibility that deletion of the TATAA sequence, or an as yet unidentified sequence, resulted in the displacement of the transcription start site by more than 32 bp downstream, in which case it would not be detected by primer extension.

Each of these promoter deletions produced a mutant Rep protein which might have had a negative effect on p40 promoter activity. This raised the question of whether the

phenotypes presented above resulted solely from the deletion of *cis*-active sequences or from a combination of *cis* effects and *trans*-dominant inhibition of p40 transcription by the mutant Rep proteins in the presence of wild-type complementing Rep. To address this question, RNAs from the cotransfection experiments depicted in Fig. 4 were assayed for activity of the wild-type p40 promoter on the complementing plasmid, p40S, with a primer complementary to the region which contained the silent substitutions. If any of the mutant Rep proteins from the deletion series exhibited a *trans*-dominant negative effect on transcription from the p40 promoter deletion mutant, the wild-type promoter of p40S should have been similarly affected. Primer extension analysis with the primer specific for the transcript from the p40S plasmid indicated that the level of p40 activity from the complementing plasmid in each cotransfection with a deletion mutant was equivalent to that with cotransfection with the wild-type plasmid pIM29 (data not shown). This indicated that the deleted Rep proteins did not interfere with *trans* activation by wild-type Rep protein.

Finally, transcription from the p40 promoter of *dl3-23* was found to be approximately threefold higher than that seen with pIM29 when *dl3-23* was complemented for *rep* function. *dl3-23* was capable of DNA replication because it retained the AAV TRs (19). The increased level of p40 transcription reflected the rescue and amplification of *dl3-23* DNA (2.5-fold; data not shown) following transfection, indicating that the p40 promoters of *dl3-23* and pIM29 are transcribed with nearly equal efficiencies.

***cis* and *trans* effects of p19 promoter deletions.** The p19 promoter was analyzed in the same manner as the p40 promoter, with a series of five 30-bp deletions spanning nucleotides 720 to 869 (−4 to −153 from the p19 transcription start site). The effects of these deletions were determined by transfection into adenovirus-infected HeLa cells in the presence or absence of a complementing plasmid, p19S, which had a series of silent substitution mutations at the priming site for the p19 transcript (Fig. 3). Comparison of the 88-nucleotide primer extension products of transcripts from the promoter deletion mutants (Fig. 5) revealed a more complex promoter structure than that of p40. In the presence of wild-type Rep protein, only *dl780* supported transcription at or near wild-type levels. Mutants with deletions either upstream (*dl720* and *dl750*) or downstream (*dl810*) were only weakly induced. This implied that the p19 promoter had a bipartite structure with at least two essential control regions (−94 to −153 and −4 to −63 bp) separated by a nonessential region (−64 to −93).

The mutations in both *dl720* and *dl750*, which were partially defective for activation (Fig. 5), disrupted a GC box (6) located between nucleotides 745 and 753 (Fig. 1, SP1). In addition, the mutation in *dl750* removed a GGT site and that in *dl720* removed a putative serum response (13) element (Fig. 1, SRE, and Fig. 5). The mutant *dl810*, which was defective for induction, also had a deletion in a GC box. Deletion of the region containing the sequence TATAA and sequences to within 4 bp of the initiation site (Fig. 5, *dl840*) eliminated detectable transcription from p19.

As was the case for the p40 promoter deletions, the p19 promoter mutants also produced a deleted Rep protein which may have had negative effects on p19 promoter activity. The wild-type p19 promoter of the complementing plasmid p19S was used as an internal control for *trans*-dominant negative effects with a primer specific for the transcript from this promoter. No differences were found between the levels of transcription from the p19 promoter of

FIG. 5. Effect of p19 promoter deletions on p19 transcription. DNA (5 μ g) from either pIM29 or one of the p19 deletion series plasmids was cotransfected into adenovirus-infected HeLa cells with 5 μ g of vector DNA (p19S, lanes −) or 5 μ g of complementing plasmid DNA (p19S, lanes +). The complementing plasmid encoded a wild-type (wt) Rep protein but had silent substitutions at the p19 priming site which prevented the oligonucleotide primer from annealing. Only transcripts from the promoter deletion plasmids or pIM29 were detected by primer extension. Lane 1, 5 μ g of pIM29 (wt promoter) with 5 μ g of vector DNA; lane 2, 5 μ g of p19S with 5 μ g of vector DNA; lanes 3, 5, 7, 9, and 11, 5 μ g of plasmid DNA from the indicated p19 deletion with 5 μ g of vector DNA; lanes 4, 6, 8, 10, and 12, 5 μ g of plasmid from the p19 deletion series with 5 μ g of p19S. pIM29 was omitted from the transfection shown in lane 2 to show that no transcript is detected from the complementing plasmid p19S alone. The position of the 88-nucleotide primer extension product of the p19 transcript is shown (P19). The line drawing below depicts the relative positions of the 30-bp promoter deletions and the p19 promoter.

p19S in the presence and absence of mutant Rep protein (data not shown), indicating that the mutant Rep proteins had no inhibitory effects.

Finally, it should be noted that there was a low level of transcription from plasmids which were not complemented with wild-type Rep. This might reflect constitutive activity from this promoter, or the deleted Rep proteins might have retained part of their *trans* activation potential. The former explanation was suggested by primer extension analysis of p40 transcripts from cells which were transfected with the p19 deletion series in the absence of complementing Rep. In these mutants, the p40 promoter was not *trans* activated by Rep protein (data not shown). We cannot, however, exclude the possibility that p19 and p40 are *trans* activated by separate domains within the Rep protein.

Requirement of the p19 upstream element for p40 activation. Deletion of sequences upstream of the p19 start site revealed two noncontiguous sequences required for p19 transcription. The presence of two distinct *cis*-active regions upstream of p19 contrasted with the structure of the p40 promoter, where *cis* elements appeared to be contiguous within the 124 bp immediately upstream of the p40 start site. To determine whether the p40 promoter also required noncontiguous upstream sequences, two deletions were constructed which removed the p5 promoter and part or all of

FIG. 6. Effect of deletions upstream of the p40 promoter region on p40 transcription. DNA (5 μ g) from pIM29, 29dl1-809, or 29dl1-1044 was cotransfected with either 5 μ g of vector DNA or 5 μ g of p19/40S complementing plasmid DNA. Lane 1, pIM29; lane 2, p19/40S; lanes 3 and 4, 29dl1-809 with vector DNA (lane 3) or p19/40S DNA (lane 4); lanes 5 and 6, 29dl1-1044 with vector DNA (lane 5) or p19/40S DNA (lane 6). Both the p19 and p40 primers were included in each primer extension reaction. The complementing plasmid p19/40S had silent substitutions in the primer sites of both the p19 and p40 promoters. The positions of the 88- and 52-nucleotide primer extension products of p19 and p40 transcripts, respectively, are shown. The line drawing below shows the extent of the upstream deletions relative to pIM29 and the p19 and p40 promoter regions. The open boxes show the positions of the 30-bp p19 and p40 promoter deletions.

the p19 region. 29dl1-1044 was constructed by deletion of a *Bam*HI fragment from the parental plasmid pIM29 and removed all AAV sequences 5' to nucleotide 1044, -810 bp relative to the p40 start site (Fig. 1). 29dl1-809 was constructed by deletion of an *Sst*I fragment, which removed all AAV sequences 5' to nucleotide 809 (-1045 bp and -65 bp relative to the p40 and p19 start sites, respectively). Upon transfection into adenovirus-infected HeLa cells in the presence of complementing plasmid, neither of these constructs supported wild-type induced levels of p40 transcription (Fig. 6). We concluded that p40 induction by the Rep protein required sequences located upstream from the p19 start site. In addition, we noted that 29dl1-1044 (Fig. 6) was defective for p40 transcription even though it had virtually the same deletion as *dl*3-23, a construct which expressed high levels of p40 transcripts (Fig. 4). The major difference between 29dl1-1044 and *dl*3-23 was that *dl*3-23 retained the AAV TRs (Fig. 2, diagram; Table 1). The 2.5-fold increase in copy number of the replicating *dl*3-23 template (data not shown) roughly correlated with the 3-fold increase in *dl*3-23 p40 transcript. This indicated that Rep induction of p40 of *dl*3-23 was equal to Rep induction of p40 of pIM29 when corrected for the number of template molecules. These results suggested that the AAV TRs could substitute for the *cis*-active elements missing in 29dl1-1044 relative to pIM29.

The mutant 29dl1-809 was missing the upstream elements of the p19 promoter, raising the possibility that activation of the p40 promoter was dependent in *cis* on a subset of the sequences essential for activation of p19. This possibility

FIG. 7. Effect of p19 promoter deletions on p40 transcription. pIM29 or p19 promoter deletion plasmid DNA was cotransfected into adenovirus-infected HeLa cells with 5 μ g of complementing plasmid DNA (p19/40S). p19/40S encoded a wild-type (wt) Rep protein but had silent substitutions at the priming sites for both the p19 and p40 transcripts. Only transcripts from the two promoters of the test plasmids were detected by primer extension. Lane 1, pIM29; lanes 2 through 6, plasmids from the p19 promoter deletion series. Both the p19 and p40 primers were included in the primer extension reactions. The p40 region of the gel is shown in the lower panel. The upper panel is a longer exposure of the p19 region of the same gel. The positions of the 52- and 88-nucleotide primer extension products of the p40 and p19 transcripts, respectively, are shown. The line drawing below depicts the relative positions of the p19 30-bp promoter deletions and the p19 and p40 promoters.

was tested by cotransfecting the p19 promoter deletion series with p19/40S as a source of complementing Rep protein (Fig. 7). Plasmids *dl*810 and *dl*840, which had deletions within the proximal control elements of p19 and exhibited substantially reduced levels of p19 activity, supported wild-type levels of activity from their respective p40 promoters. *dl*780, in which a region between the *cis*-active elements of p19 was deleted and which did not affect p19 transcription, also supported transcription of p40 at the same level as the parental plasmid pIM29. However, deletions in the upstream activating region of p19 (*dl*720 and *dl*750) resulted in reduced levels of transcription from p40. These reductions were similar to the reductions from p19. Thus, the p19 and p40 promoters relied on a common sequence for activation by the Rep protein, and this sequence mapped between nucleotides 720 and 779.

Second essential *cis*-active element in the p5 promoter region. In order to establish the upstream limit of the sequences required for activation of p19 and p40 in pIM29, two more deletion mutants were tested. 29dl1-687 carried a deletion to the *Sst*II site of AAV, which left 184 and 1,165 bp upstream from the p19 and p40 start sites, respectively. 29dl1-320 was deleted 5' to the translation start codon for p5 Rep protein at AAV nucleotide 321 and lacked the p5 promoter. Transfection of 29dl1-687 into adenovirus-infected HeLa cells in the presence of the complementing plasmid p19/40S resulted in low levels of *trans* activation from the p40 promoter and no detectable transcription from the p19 promoter (Fig. 8). The plasmid 29dl1-687 contained all of the sequences identified by the p19 promoter deletion series that were required for activation of p19 and p40, as

FIG. 8. Effect of deletions upstream of the p19 promoter region on p19 and p40 transcription. Primer extensions were used to measure p19 and p40 transcripts as described in the legend to Fig. 6 but with the deletion mutants *29dl1-320* and *29dl1-687*. Lane 1, pIM29; lane 2, p19/40S; lanes 3 and 4, *29dl1-320* with vector DNA (lane 3) or p19/40S (lane 4); lanes 5 and 6, *29dl1-687* with vector DNA (lane 5) or p19/40S (lane 6). The line drawing shows the extent of the upstream deletions in *29dl1-320* and *29dl1-687* relative to pIM29 and the p19 and p40 promoter regions. The open boxes show the positions of the p19 and p40 promoter deletions.

well as an additional 30 bp. This suggested that the p19 upstream activating region extended beyond the sequences present in *29dl1-687*. However, when the plasmid *29dl1-320* was transfected with complementing Rep, it also was found to be defective for *trans* activation of p19 and p40 (Fig. 8). *29dl1-320* left 552 bp upstream from the p19 promoter and therefore appeared to delineate a second region required in *cis* for activation of p19 and p40. Only 130 bp (AAV nucleotides 191 to 320) were missing from *29dl1-320* compared with the parent plasmid pIM29. These 130 bp have previously been shown to contain the sequences required for p5 transcription (10). This suggested that the p5 promoter may be similar to the p19 promoter in having an associated element which is essential in *cis* for induction of both the p19 and p40 promoters by the Rep protein.

DISCUSSION

Our analysis of deletions within the AAV p19 and p40 promoter regions reveals a complex organization of controlling elements involved in induction by the Rep protein. We have found that coordinate Rep-mediated induction of p19 and p40 is dependent on the presence of multiple *cis*-active elements. One element is located upstream from the p19 promoter and is disrupted by deletions which remove 30 bp spanning either AAV nucleotides 720 to 749 or 750 to 779. The second element maps to the region of the p5 promoter between AAV nucleotides 190 (the extent of the parent plasmid, pIM29) and 320. The presence of both regions is required for complete induction of the p19 and p40 promoters by the Rep protein. Thus, including its own proximal controlling sequences, the p40 promoter requires all three AAV promoters in *cis* for activation by the Rep protein.

Sequences essential for the coordinate induction of the p19 and p40 promoters are separated by a minimum of 400 bp (the distance between the p19 sequences deleted in *dl720* and *dl750* and the p5 region deleted in *29dl1-320*). These sequences must, in turn, control two promoters which are themselves separated by 980 bp (the distance between the p19 and p40 cap sites). In the extreme case, the distance between the *cis*-active region associated with the p5 promoter and its target at the p40 cap site is at least 1,532 bp. It is difficult to envision a mechanism which would account for these observations unless the various elements of the template molecule (p5, p19, and p40) are simultaneously juxtaposed, presumably through a protein-DNA complex. An alternative possibility is that p40 transcription could be induced as a result of a physical change in the DNA template that is due to transcription from upstream promoters. This seems less likely because the deletion in the p19 mutant *dl840*, which completely eliminates p19 transcription, has no effect on p40 transcription.

We have not, as yet, observed a reciprocal dependence of p19 induction on sequences within the p40 region. High levels of the p19 transcript are evident from p40 deletions in which Rep protein remains active in *trans* (Fig. 2, *dl1790* and *dl1820*). Further experiments have shown that p19 is transcribed at wild-type levels from all of the p40 promoter deletions when they are complemented with wild-type Rep (data not shown). It still remains possible that there are additional sequences required for coordinate Rep induction, either associated with the p40 promoter or located elsewhere in the AAV genome. Our p40 promoter deletions would not have identified a set of elements essential for p19 transcription if there were multiple, redundant elements within the p40 region.

The above observations regarding multiple, essential, *cis*-active elements apply to Rep induction on plasmids which do not contain the AAV TRs. Previous studies have implied that the AAV TRs have an enhancerlike effect on Rep-mediated activation. Beaton et al. (4) and Labow and Berns (28) have reported that the presence of a single copy of the TR, at either the left or right end of the genome, confers Rep responsiveness to the simian virus 40 early promoter when it is substituted for the AAV p5 region. A similar effect was seen in this study (Fig. 4) when the plasmid *dl3-23*, which contains the TRs, was transfected with p40S. *dl3-23* lacks both the p19 and p5 promoter regions, which are essential in *cis* for Rep inducibility in pIM29. In the presence of complementing Rep, this plasmid accumulates wild-type levels of p40 transcript (adjusted for increased template), whereas a mutant with a similar deletion in the TR-minus plasmid pIM29 (Fig. 6, *29dl1-1044*) is defective for p40 transcription. We therefore propose that the TRs contain functionally redundant *cis* elements which can substitute for the p5 and p19 promoter-associated elements needed to confer Rep responsiveness. Alternatively, the TRs may potentiate Rep induction by an entirely different mechanism, perhaps related to their ability to bind Rep protein with high affinity (2, 21).

The nature of the interaction between the Rep protein and the promoter-associated *cis* elements is not known. As yet, direct binding of Rep to specific DNA sequences outside the TRs has not been demonstrated. This suggests that activation may be mediated through additional transcription factors bound to the promoter regions. There are many potential factor-binding sites associated with these promoters (Fig. 1), the deletion of which correlates with reduced transcription from the proximal promoter. The GC boxes

associated with both p19 and p40 represent one such candidate for a role in Rep-induced transcription, and a similar sequence has been shown to be essential for activation in an autonomous parvovirus (1, 15). A second possible site for Rep interaction is the GGT motif. Although unrelated to any previously identified transcription factor-binding site, the deletion of this sequence correlates with reduced transcription from the p19 and p40 regions. We note, however, that neither the GC box nor the GGT motif is present in the p5 region of pIM29 (nucleotides 190 to 261) that was found to be required for p19 and p40 transcription. Recent studies of the p5 region have identified two sequence elements, an MLTF site and a novel E1A-responsive element, both of which are required for the induction of p5 transcription by the adenovirus E1A gene (10). It is not known whether either of these sequence elements is required for induction by the Rep protein. It is also possible that Rep interacts with the TATA element, a sequence which is common to all three promoters. A more detailed mutational analysis of all of the sequences discussed here will be necessary to determine their role in Rep induction and to determine the mechanism by which Rep recognizes and interacts with AAV *cis*-acting elements. However, whether the Rep protein interacts directly with specific sequence elements or induces transcription by interacting with cellular transcription factors, the simultaneous interaction with elements associated with three promoters represents a novel mechanism for coordinate transcriptional control in mammalian cells.

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REFERENCES

- Ahn, J. K., B. J. Gavin, G. Kumar, and D. C. Ward. 1989. Transcriptional analysis of minute virus of mice P₄ promoter mutants. *J. Virol.* **63**:5425–5439.
- Ashktorab, H., and A. Srivastava. 1989. Identification of nuclear proteins that specifically interact with the adeno-associated virus 2 inverted terminal repeat hairpin DNA. *J. Virol.* **63**:3034–3039.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology, vol. 1. Greene Publishing Associates, Brooklyn, N.Y.
- Beaton, A., P. Palumbo, and K. I. Berns. 1989. Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in *trans* by the *rep* protein. *J. Virol.* **63**:4450–4454.
- Becerra, S. P., F. Kocot, P. Fabisch, and J. A. Rose. 1988. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiations from a single transcript. *J. Virol.* **62**:2745–2754.
- Briggs, M. R., J. T. Kadonga, S. P. Bell, and R. Tjian. 1986. Purification and biochemical characterization of the promoter-specific factor, Sp1. *Science* **234**:47–52.
- Bruder, J. T., and P. Hearing. 1989. Nuclear factor EF-1A binds to the adenovirus E1A core enhancer element and to other transcriptional control regions. *Mol. Cell. Biol.* **9**:5143–5153.
- Carter, B. J., C. J. Marcus-Sekura, C. A. Laughlin, and G. Ketner. 1983. Properties of an adenovirus type 2 mutant, Add/807, having a deletion near the right-hand genome terminus: failure to help AAV replication. *Virology* **126**:505–516.
- Chang, L.-S., and T. Shenk. 1990. The adenovirus DNA binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. *J. Virol.* **64**:2103–2109.
- Chang, L.-S., Y. Shi, and T. Shenk. 1989. Adeno-associated virus p5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor. *J. Virol.* **63**:3479–3488.
- Chejanovsky, N., and B. J. Carter. 1989. Mutagenesis of an AUG codon in the adeno-associated virus *rep* gene: effects on viral DNA replication. *Virology* **173**:120–128.
- Chejanovsky, N., and B. J. Carter. 1990. Mutation of a consensus nucleotide binding site in the adeno-associated virus *rep* gene generates a dominant negative phenotype for DNA replication. *J. Virol.* **64**:1764–1770.
- Christy, B., and D. Nathans. 1989. Functional serum response elements of the growth factor-inducible gene *zif-268*. *Mol. Cell. Biol.* **9**:4889–4895.
- Flint, J., and T. Shenk. 1989. Adenovirus E1A protein: paradigm viral transactivator. *Annu. Rev. Genet.* **23**:141–161.
- Gavin, B. J., and D. C. Ward. 1990. Positive and negative regulation of the minute virus of mice P₃₈ promoter. *J. Virol.* **64**:2057–2063.
- Green, M. R., and R. G. Roeder. 1980. Transcripts of the adeno-associated virus genome: mapping of the major RNAs. *J. Virol.* **36**:79–92.
- Green, M. R., and R. G. Roeder. 1980. Definition of a novel promoter for the major adenovirus-associated virus mRNA. *Cell* **1**:231–242.
- Hauswirth, W. W., and K. I. Berns. 1977. Origin and termination of adeno-associated virus DNA replication. *Virology* **78**:488–499.
- Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka. 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. *J. Virol.* **51**:329–333.
- Huang, M.-M., and P. Hearing. 1989. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J. Virol.* **63**:2605–2615.
- Im, D.-S., and N. Muzyczka. 1989. Factors that bind to the adeno-associated virus terminal repeats. *J. Virol.* **63**:3095–3104.
- Im, D.-S., and N. Muzyczka. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**:447–457.
- Janik, J. E., M. M. Huston, K. Cho, and J. A. Rose. 1988. Efficient synthesis of adeno-associated virus proteins requires both adenovirus DNA binding protein and VA I RNA. *Virology* **168**:320–329.
- Janik, J. E., M. M. Huston, and J. A. Rose. 1984. Adeno-associated virus proteins: origin of the capsid components. *J. Virol.* **52**:591–597.
- Jay, F. T., C. A. Laughlin, and B. J. Carter. 1981. Eukaryotic translational control: adeno-associated virus protein synthesis is affected by a mutation in the adenovirus DNA-binding protein. *Proc. Natl. Acad. Sci. USA* **78**:2927–2931.
- Johnson, P. F., and S. L. McKnight. 1989. Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58**:799–839.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
- Labow, M. A., and K. I. Berns. 1988. The adeno-associated virus *rep* gene inhibits replication of an adeno-associated virus/simian virus 40 hybrid genome in Cos 7 cells. *J. Virol.* **62**:1705–1712.
- Labow, M. A., P. L. Hermonat, and K. I. Berns. 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. *J. Virol.* **60**:251–258.
- Laughlin, C. A., N. Jones, and B. J. Carter. 1982. Effect of deletions in adenovirus region 1 genes upon replication of adeno-associated virus. *J. Virol.* **41**:868–876.
- Laughlin, C. A., H. Westphal, and B. J. Carter. 1979. Spliced adenovirus-associated virus RNA. *Proc. Natl. Acad. Sci. USA* **76**:5567–5571.
- Lusby, E., and K. I. Berns. 1982. Mapping of the 5' termini of

- two adeno-associated virus 2 RNAs in the left half of the genome. *J. Virol.* **41**:518–526.
33. Mendelson, E., J. P. Trempe, and B. J. Carter. 1986. Identification of the *trans*-active Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. *J. Virol.* **60**:823–832.
 34. Nakajima, N., M. Horikoshi, and R. G. Roeder. 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. *Mol. Cell. Biol.* **8**:4028–4040.
 35. Redemann, B. E., E. Mendelson, and B. J. Carter. 1989. Adeno-associated virus Rep protein synthesis during productive infection. *J. Virol.* **63**:873–882.
 36. Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka. 1982. Cloning of AAV into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc. Natl. Acad. Sci. USA* **79**:2077–2080.
 37. Samulski, R. J., L.-S. Chang, and T. Shenk. 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. *J. Virol.* **61**:3096–3101.
 38. Samulski, R. J., L.-S. Chang, and T. Shenk. 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* **63**:3822–3828.
 - 38a. Samulski, R. J., and T. Shenk. 1988. Adenovirus E1B 55-M_r polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. *J. Virol.* **62**:206–210.
 39. Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka. 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. *Cell* **33**:135–143.
 40. Sandler, A. B., and G. Ketner. 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. *J. Virol.* **63**:624–630.
 41. Sawadogo, M., M. W. Van Dyke, P. D. Gregor, and R. Roeder. 1988. Multiple forms of the human gene-specific transcription factor USF. I. Complete purification and identification of USF from HeLa cell nuclei. *J. Biol. Chem.* **263**:11985–11993.
 42. Senepathy, P., J. Tratschin, and B. J. Carter. 1984. Replication of adeno-associated virus DNA. Complementation of naturally occurring *rep*[−] mutants by a wild-type genome or an *ori*[−] mutant and correction of terminal deletions. *J. Mol. Biol.* **179**:1–20.
 43. Snyder, R. O., D.-S. Im, and N. Muzyczka. 1990. Evidence for covalent attachment of the adeno-associated virus (AAV) Rep protein to the ends of the AAV genome. *J. Virol.* **64**:6204–6213.
 44. Snyder, R. O., R. J. Samulski, and N. Muzyczka. 1990. In vitro resolution of covalently joined AAV chromosome ends. *Cell* **60**:105–133.
 45. Srivastava, A., E. W. Lusby, and K. I. Berns. 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* **45**:555–564.
 46. Tratschin, J.-D., J. Tal, and B. J. Carter. 1986. Negative and positive regulation in *trans* of gene expression from adeno-associated virus vectors in mammalian cells by a viral Rep gene product. *Mol. Cell. Biol.* **6**:2884–2894.
 47. Tratschin, J.-D., M. H. P. West, T. Sandbank, and B. J. Carter. 1984. A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the prokaryotic gene for chloramphenicol acetyltransferase. *Mol. Cell. Biol.* **4**:2072–2081.
 48. Trempe, J. P., and B. J. Carter. 1988. Regulation of adeno-associated virus gene expression in 293 cells: control of mRNA abundance and translation. *J. Virol.* **62**:68–74.
 49. Trempe, J. P., and B. J. Carter. 1988. Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. *J. Virol.* **62**:3356–3363.
 50. Trempe, J. P., E. Mendelson, and B. J. Carter. 1987. Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in *Escherichia coli*. *Virology* **161**:18–28.
 51. Van Ormondt, H., J. Maat, A. DeWaard, and A. J. Van der Eb. 1978. The nucleotide sequence of the transforming HpaI-E fragment of adenovirus type 5. *Gene* **1**:309–328.
 52. West, M. H. P., J. P. Trempe, J.-D. Tratschin, and B. J. Carter. 1987. Gene expression in adeno-associated virus vectors: the effects of chimeric mRNA structure, helper virus, and adenovirus VAI RNA. *Virology* **160**:38–47.