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Cellular Transcription Factor Binds to Adenovirus Early Region Promoters and to a Cyclic AMP Response Element

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We have analyzed the sequences that regulate the transcription of adenovirus type 5 early region 4 (E4). A region located immediately upstream of the E4 TATA box is required for efficient E4 transcription in vitro and in vivo. A cellular transcription factor, termed ETF-A, binds to this region. ETF-A also binds to additional sites in the E4 5′-flanking region, including the adenovirus terminal repeat, as well as to the adenovirus early region 2 promoter region and the adeno-associated virus early promoter region. A repeated sequence motif is found in each binding domain. The same factor binds to a region upstream of a cellular gene that contains a cyclic AMP response element. Consistent with this result, E4 expression is induced in vivo by cyclic AMP. Two other regions further upstream of the E4 initiation site also contribute to efficient E4 expression. These domains are functionally redundant and contain binding sites for ETF-A. One domain is the adenovirus terminal repeat, which has strong promoter activity in vitro and in vivo.

The structure of eucaryotic promoters recognized by RNA polymerase II is complex. Many RNA polymerase II regulatory regions contain multiple *cis*-acting elements that regulate the expression of a gene (see references 7, 27, and 30 for reviews). Frequently, specific regulatory sequences are repeated. Further, similar or distinct elements within a transcriptional control region are often functionally redundant. The mechanisms by which *cis*-acting elements control gene expression have been the focus of intensive study. Viral and cellular genes have been shown to be regulated in species- and tissue-specific, developmentally specific, and temporal manners (23, 27, 30), as well as to respond to gene products that activate or repress transcription (2) and to specific environmental stimuli such as hormones (47) and heat shock (37). The level of transcription of specific genes appears to depend, at least partly, on the abundance and activity of *trans*-acting factors that bind to specific regulatory elements (7, 27, 30).

This study is focused on the adenovirus type 5 (Ad5) early region 4 (E4) transcriptional control region. The expression of E4 is regulated in both a positive and a negative manner after infection with adenovirus. The E1A gene products act early after infection to increase the rate of E4 transcription in vivo (3, 22, 35). Later in infection, around the onset of viral DNA replication, transcription of E4 is specifically repressed by the early region 2 (E2) DNA-binding protein (36). Specific repression of E4 transcription also has been observed in vitro with purified DNA-binding protein (15). An E1A-inducible enhancer region is located in the E4 5′-flanking sequences (9, 25). E1A-inducible control elements also have been identified upstream of the E1A (24), E1B (46), and E2A (19, 20) transcription initiation sites. The E1A gene products appear to activate several of these promoter regions by different mechanisms.

We report here an analysis of the sequences that regulate E4 expression in vivo and in vitro. Our results demonstrate that multiple elements located within the E4 5′-flanking sequences regulate the expression of this early transcription unit. One element is located immediately upstream of the E4 TATA box, between nucleotides (nt) −62 and −46 relative to the E4 cap site at +1, and is required for efficient E4 expression in vivo and in vitro. A cellular transcription factor binds to a site within this region as well as to three additional sites within the E4 5′-flanking sequences. A repeated sequence motif is located within each binding domain. The same factor also binds to other viral early regulatory regions. We have termed this factor ETF-A, for viral early region transcription factor A. ETF-A binds to a region upstream of the cellular phosphoenolpyruvate carboxykinase (PEPCK) gene that contains a cyclic AMP (cAMP) response element (CRE; 39; P. Quinn and D. Granner, personal communication). Consistent with this result, E4 expression is induced in vivo by cAMP. In addition to the promoter element at nt −62 to −46, two other regions further upstream of the E4 cap site contribute to efficient E4 transcription. These domains are functionally redundant and contain binding sites for ETF-A.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Plasmid pE4-CAT contains the right-terminal 398 nt of the Ad5 genome, containing 330 base pairs (bp) of E4 5′-flanking sequences and the 68-nt E4 mRNA leader, fused to the gene encoding chloramphenicol acetyltransferase (CAT) from pSV2-CAT (10). Starting in a clockwise direction from the pBR322 *Eco*RI site, pE4-CAT contains 29 bp of pBR322 sequences, Ad5 sequences from nt 1 through 398, the *Hin*dIII-*Bam*HI fragment from pSV2-CAT (containing the CAT-coding sequences and a simian virus 40 splice site and polyadenylation signal [10]), and pBR322 sequences from the *Pvu*II site at nt 2067 to the *Eco*RI site at nt 4362. pE4-CAT was constructed by the following procedure. Plasmid pXba-C (containing the Ad5 *Xba*C fragment, 84.3 to 100 map units, in pBR322 [12]) was digested with *Sma*I (at Ad5 nt 575) and then with exonuclease III and S1 nuclease to remove Ad5 sequences toward the E4 leader. Following a T4 DNA polymerase repair reaction, *Hin*dIII linkers were ligated to the termini. The DNA was digested with *Eco*RI, repaired, and then digested with *Hin*dIII. DNA fragments corresponding in size to the right-terminal 400 bp of Ad5 were isolated and ligated into

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the large *Sph*I (repaired with T4 DNA polymerase)-*Hin*dIII fragment of pSV2-CAT (10). A clone (pE4-SV2CAT-26) that contains the Ad5 right-terminal 398 nt fused to the CAT gene was identified by nucleotide sequence analysis. A unique *Bgl*II linker was introduced at the Ad5 *Hph*I site at nt 104, and an *Xba*I linker was introduced at the *Bam*HI site at the 3′ end of the CAT gene (10). Finally, the *Cla*I (5 bp outside the Ad5 terminal repeat [12])-*Xba*I fragment from this plasmid was ligated into pBR322 between the *Cla*I and *Pvu*II (converted to *Xba*I) sites. This final plasmid was termed pE4-CAT.

Unidirectional deletions (see Fig. 1) were generated by standard procedures with the *Bgl*II site of pE4-CAT to generate mutants in the ITR$^+$ series (see below). *Bam*HI linkers were introduced at the junction of each deletion. The endpoints of each deletion were determined by nucleotide sequence analysis. Deletion mutants in an ITR$^-$ background (lacking sequences between Ad5 nt 1 and 103) were generated from pE4-CAT, and the ITR$^+$ mutants were generated by removing plasmid sequences between the *Cla*I and *Bgl*II sites of pE4-CAT and the *Cla*I and *Bam*HI sites of individual mutant clones.

Plasmid pE1B-4 contains Ad5 left-terminal sequences from the *Xba*I site at nt 1339 to the *Hin*dIII site at nt 2805 cloned in pBR322 between the *Hin*dIII and *Pvu*II (converted to *Xba*I) sites. Plasmid pE2-2 contains Ad5 E2 control region sequences from the *Bss*HI site at E2 nt −14 to the *Nar*I site at E2 nt −98 cloned into the *Sma*I site of pUC9. Plasmid pGM620-C contains the left-terminal 513 bp of the adeno-associated virus (AAV) genome cloned into pBR322 (kindly provided by J. Gottlieb and N. Muzyczka, Department of Microbiology, State University of New York at Stony Brook, Stony Brook). Plasmids pPL150 and pPL15 contain sequences from the rat PEPCK gene from positions −107 to +69 and −74 to +69, respectively, fused to the CAT gene. These plasmids were constructed and kindly provided by P. Quinn and D. Granner (Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tenn.). PEPCK fragments corresponding to positions −107 to +69 and −74 to +69 were released from these plasmids by digestion with *Hin*dIII and *Bgl*II. The sequence of the E4 oligonucleotide from nt −34 to −63 is 5′-GATCCGACTTT AACCGTTACGTCATTTTTTAGTCC - 3′:5′-GATCCGGA CTAAAAAATGACGTAACGGTTAAAGTC-3′. The sequence of the 2A/2B oligonucleotide is 5′-GTCACCAGGG AGCAACTTACGGTTTACCAAACAAGAGG-3′:5′-CCTC TTGTTTGGTAAACCGTAAGTTGCTCCCTG-3′. The 2A/2B oligonucleotide was kindly provided by R. Kuhn and E. Wimmer (Department of Microbiology, State University of New York at Stony Brook).

Cells and transfections. 293 cells (11) were grown in Dulbecco modified Eagle medium containing 10% calf serum. Suspension cultures of HeLa cells were grown in Spinner minimum essential medium containing 7% calf serum. HepG2 cells (American Type Culture Collection, Rockville, Md.) were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. DNA transfections were performed by the calcium phosphate precipitation method described by Wigler et al. (45). 293 cells were transfected with 5 μg of plasmid DNA plus 10 μg of high-molecular-weight DNA per 100-mm dish (10^6 cells per dish). After incubation with the calcium phosphate precipitate overnight, the cells were washed and fresh medium was added. Total cell extracts were prepared 48 h after the addition of fresh medium. HepG2 cells were transfected with 2 μg of plasmid DNA plus 10 μg of high-molecular-weight DNA per 100-mm dish. After incubation with the calcium phosphate precipitate for 4 h, the cells were boosted with Tris-buffered saline containing 20% glycerol for 1 min and then washed. The cells were incubated in medium containing 0.5% fetal bovine serum for 16 h and then in medium with or without 1 mM cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate; Sigma Chemical Co., St. Louis, Mo.) for 8 h. Cell extracts were then prepared. CAT assays were performed as described previously (10). Transfections were performed at least six separate times with at least two different plasmid DNA preparations for 293 cells and three times for HepG2 cells. The concentration of protein in each extract was determined to verify that equivalent protein yields were obtained. The results were quantitated by excision of the acetylated and nonacetylated [^{14}C]chloramphenicol forms from the chromatogram and direct counting with a liquid scintillation counter.

In vitro transcription and DNase I footprint analysis. Whole-cell transcription extracts were prepared from uninfected HeLa cells as described previously (14). In vitro transcription reactions were performed as described previously (14) with 300 ng of E4 template DNA, 100 ng of E1B template DNA, and 10.5 μl of whole-cell extract (6 mg of protein per ml) per 25-μl reaction volume. Transcription templates were (i) pE4-CAT and mutant derivatives linearized with *Xba*I and (ii) pE1B-4 linearized with *Hin*dIII. The reactions were terminated and processed as described previously (21). Runoff transcription products were separated in a 6% formaldehyde–1.5% agarose gel, and the gel was dried and exposed to X-ray film for autoradiography. Primer extension analyses were performed as described previously (21). In vitro transcription reactions were performed with at least two different preparations of plasmid DNAs and three different whole-cell extracts. The results were quantitated by densitometric scanning of the autoradiographs from six to eight independent experiments.

DNase I footprint analyses were performed as described previously (21) with the following modifications. Polyvinyl alcohol was omitted from the binding buffer and the reaction was not diluted with 5 mM $CaCl_2$–10 mM $MgCl_2$ prior to the addition of DNase I (which was diluted in buffer containing $CaCl_2$ instead). For footprinting reactions, 1 to 2 ng of ^{32}P-labeled DNA (labeled with Klenow DNA polymerase and ^{32}P-labeled deoxynucleoside triphosphates) and 2 μg of nonspecific competitor DNA [poly(dA)-oligo(dT) or poly(dC)-oligo(dG) (6)] were incubated with various amounts of HeLa whole-cell extract. Concentrations of extract and competitor DNAs are given in the figure legends.

RESULTS

Analysis of E4 promoter function in vitro. Deletion mutations in the Ad5 E4 transcriptional control region were constructed in a recombinant plasmid, pE4-CAT. This plasmid contains E4 sequences from the right terminus of the viral genome to the end of the E4 mRNA leader sequence (Ad5 nt 398) fused to the gene encoding CAT (10). A schematic view of the E4 promoter region is shown at the top of Fig. 1. Two series of mutants were constructed for these analyses. The first set of mutants carried deletions that begin upstream of the Ad5 inverted terminal repeat (ITR) (nt −330 to −228) and progress toward the initiation site (Fig. 1, ITR$^-$ series). The second set of mutants carried deletions that begin downstream of the Ad5 ITR (nt −227) and progress toward the initiation site (Fig. 1, ITR$^+$ series). Both sets of mutants had the same 3′ endpoints for their deletions.

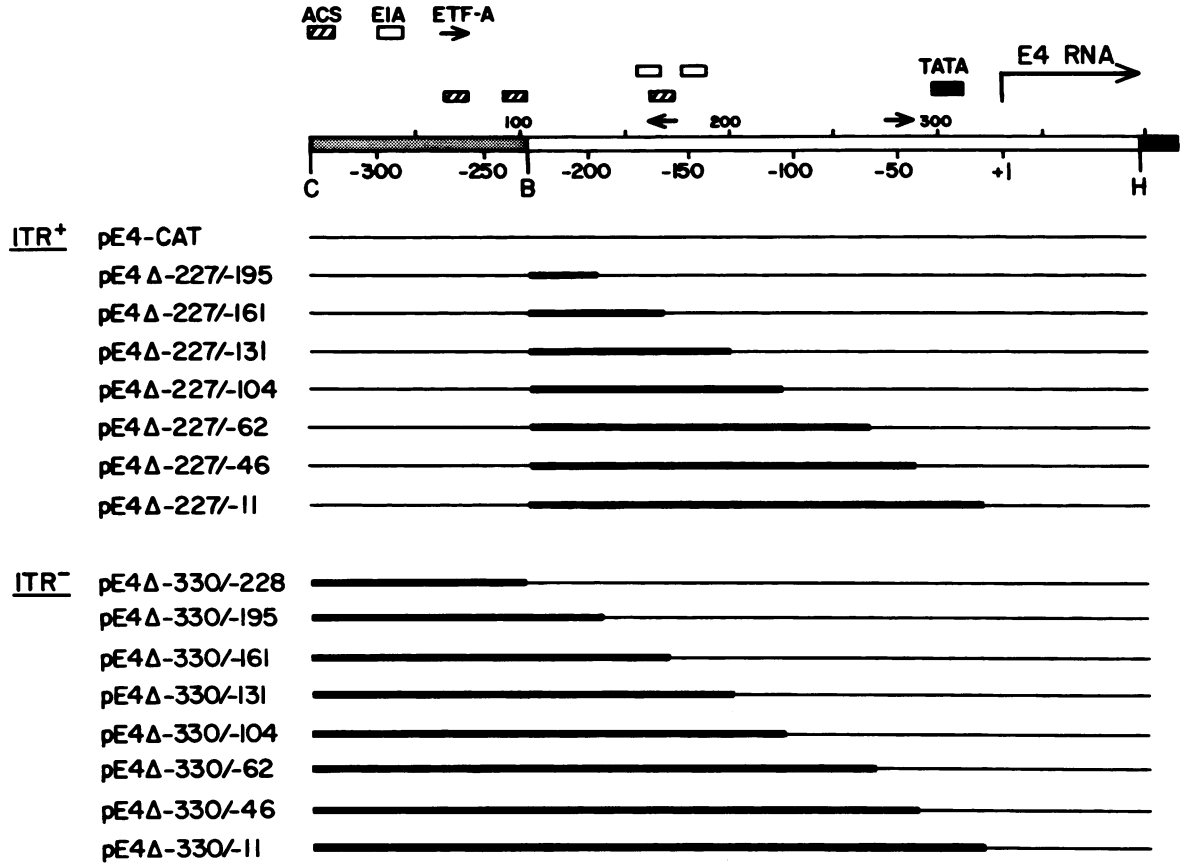

FIG. 1. E4 transcriptional control region and deletion mutants. The top part of the figure represents a schematic view of the right end of the Ad5 genome. Numbers above the lines represent Ad5 nucleotides from the right terminus; numbers below the lines represent nucleotides upstream of the E4 cap site at +1. E4 transcription is initiated at a clustered set of cap sites around nt 330 (1); the downstream-most site at nt 331 is taken as E4 +1. Four sequence motifs are indicated: the hatched boxes refer to an ACS defined by Stillman et al. (43) and referred to as the ACS repeat in the text; the open boxes refer to sequences perfectly homologous to the Ad5 E1A core enhancer element I sequence (17); the dark box refers to the E4 TATA box (1); and the bold arrows refer to the E4 10-bp repeat described in the text. The stippled region between the lines represents the Ad5 ITR between nt 1 and 103. Unique restriction sites in the pE4-CAT vector are as follows: C, pBR322 *Cla*I site; B, *Bgl*II linker; H, *Hin*dIII linker. Sequences for the CAT gene (10) are located to the right of the *Hin*dIII site at Ad5 nt 398. The ITR$^+$ series of mutants contained deletions that begin on the downstream side of the ITR (nt 104) and progress toward the E4 initiation site at +1. The ITR$^-$ series of mutants contained deletions that begin on the upstream side of the ITR (at the *Cla*I site) and progress toward the E4 initiation site. The numbers adjacent to each mutant represent the upstream and downstream endpoints of the deletion.

Mutant plasmids were used as templates for in vitro transcription with a whole-cell extract (28) from uninfected HeLa cells, and runoff products were analyzed by gel electrophoresis (Fig. 2). A plasmid containing the Ad5 E1B promoter region was used as an internal standard. In control titration experiments we found that the E4 and E1B promoter regions did not compete with each other for transcription in vitro (data not shown). Identical results were obtained with the E4 mutant templates in the absence or presence of an internal standard (data not shown). In an ITR$^-$ background (Fig. 2A), a gradual decrease in E4 transcription was observed as the region from nt −227 to −131 was deleted. Transcription from the −131 mutant template was reduced fivefold relative to that in the parental plasmid. A reproducible, although modest, increase was detected as the deletions progressed toward the TATA box (Fig. 2A, −104 and −62). A second fivefold decrease in E4 transcription was observed when the region between nt −62 and −46 was deleted. This reduction was as dramatic as that which occurred when the TATA box region was removed (Fig. 2A, −11).

In contrast, in an ITR$^+$ background (Fig. 2B), deletion of sequences from nt −227 to −46 did not impair E4 transcription in vitro. In fact, as the deletions progressed toward the E4 TATA box, E4 transcription was increased. In plasmids in which the ITR was brought close to the TATA sequence, a 5- to 10-fold increase in E4 transcription was observed (Fig. 2B, −62 and −46). Deletion of the TATA box region resulted in a 10- to 20-fold decrease in E4 transcription (Fig. 2B, −11), demonstrating that most of the ITR-induced transcription was TATA box dependent. Since both series of mutants (ITR$^+$ and ITR$^-$) have identical flanking plasmid sequences, the sequences responsible for the induction of E4 transcription observed with mutants in an ITR$^+$ background must be localized within the terminal repeat. The ITR increased transcription in vitro of the E4 promoter fivefold (Fig. 2A and B, pE4-CAT versus pE4Δ−330/−228). From these analyses, we conclude that the E4 promoter contains a number of regions that are required for efficient transcription in vitro: the region from nt −227 to −131, the sequences between nt −62 and −46, and the TATA box region (nt −46 to −11). In addition, the Ad5 terminal repeat contains

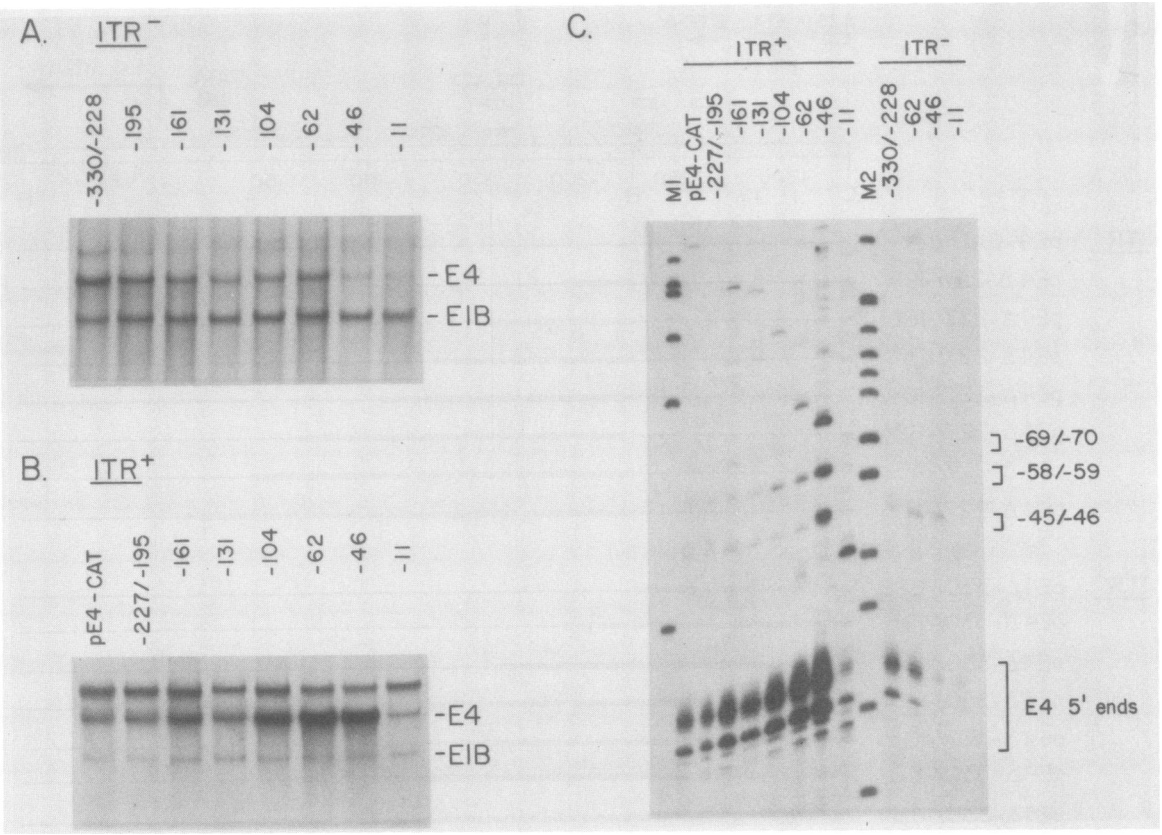

FIG. 2. In vitro transcription analysis of E4 mutant plasmids. (A and B) Linearized plasmids were used as templates for in vitro transcription with an uninfected HeLa whole-cell extract (28). ^{32}P-labeled runoff products were separated in a 1.5% agarose–6% formaldehyde gel and visualized by autoradiography. Transcription reactions were performed in the presence of an internal standard containing the Ad5 E1B promoter region. The E4 and E1B runoff products are indicated; the expected E4 runoff product is approximately 1,700 nt in length, and the expected E1B runoff product is approximately 1,100 nt in length. The numbers above the gel indicate the plasmids (Fig. 1) used in the analysis. Mutant plasmids in the ITR$^-$ (A) and ITR$^+$ (B) series were analyzed. In panel B, the runoff product migrating above the E4 transcript corresponds to a product promoted by the ITR that is transcribed from the opposite strand of the plasmid DNA. (C) In vitro transcription reactions were performed with mutant plasmids in the ITR$^-$ and ITR$^+$ series, and sites of initiation of transcription were determined by primer extension analysis. The products were electrophoresed in an 8% polyacrylamide–8.3 M urea gel and visualized by autoradiography. The set of wild-type E4 initiation sites is indicated by the large bracket (E4 5′ ends). Several additional sites of initiation further upstream are indicated. M1 and M2 are ^{32}P-labeled markers from pBR322.

activation sequences that can functionally substitute for several of these elements.

Primer extension analysis was used to map the 5′ ends of E4 transcripts initiated in vitro (Fig. 2C). As previously described (1), a clustered set of E4 cap sites was detected with the wild-type E4 promoter (Fig. 2C, pE4-CAT, E4 5′ ends). In addition, several minor transcripts were observed with sites of initiation upstream of the E4 TATA box region (Fig. 2C, pE4-CAT, −45/−46, −58/−59, and −69/−70). The results with the E4 mutants were analogous to those observed in the runoff assays (Fig. 2A and B) and demonstrated that the wild-type E4 initiation sites were used predominantly with mutant templates in both the ITR$^-$ and ITR$^+$ series. An increased level of transcripts initiating upstream of the TATA box was observed with the mutant carrying a deletion to nt −46 including several initiation sites within the ITR itself (Fig. 2C, −227/−46). Interestingly, in both the ITR$^-$ and ITR$^+$ backgrounds, transcripts initiating at the wild-type E4 sites were detected with mutants that contained a deletion of the TATA box region (Fig. 2C, −227/−11 and −330/−11).

Analysis of E4 promoter function in vivo. Both series of mutant plasmids were tested for E4 transcriptional activity in vivo. Since the E1A gene products are required as *trans* activators for optimal E4 transcription (3, 22), the expression of these plasmids was tested in 293 cells, a cell line that expresses the E1A gene products (11). The level of E4 expression in the various mutants was assayed by monitoring the level of CAT activity after transfection (Fig. 3). Results similar to those observed for E4 transcription in vitro were obtained with the mutant plasmids in vivo. In an ITR$^-$ background, a gradual decrease in E4 expression was observed as the sequences from nt −227 to −131 were deleted (Fig. 3, ITR$^-$ set). E4 expression in the −131 mutant plasmid was reduced sixfold relative to that in the parental plasmid. A fourfold decrease in E4 transcription was observed with a deletion from nt −104 to −62, and an additional fivefold decrease in E4 transcription was detected with a deletion between nt −62 and −46. The level of E4 expression observed in the −46 mutant was comparable to the level obtained with the mutant carrying a deletion of the TATA box region (Fig. 3, ITR$^-$ set, −11).

As was observed with transcription in vitro, the Ad5 ITR could functionally substitute for several of the control regions. In an ITR$^+$ background, a deletion to nt −62 reduced E4 expression less than twofold (Fig. 3, ITR$^+$ set). A

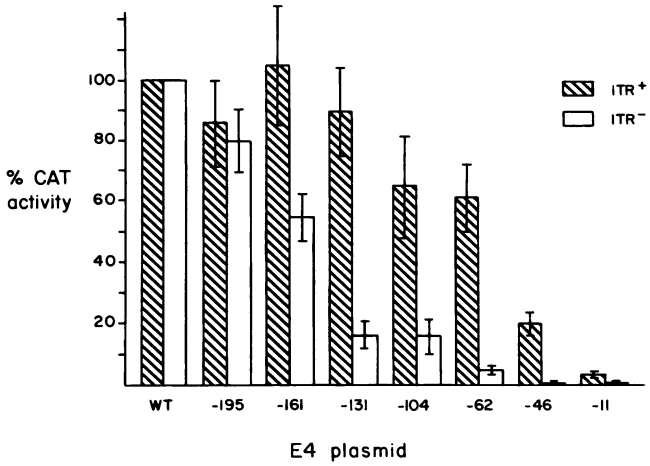

FIG. 3. Transient expression analyses of mutant plasmids in 293 cells. 293 cells were transfected with wild-type (WT) and mutant plasmids, and levels of CAT activity in cell extracts prepared 48 h after transfection were determined. The results are presented for each mutant plasmid as the percent CAT activity of the wild-type parental plasmid. Percent wild-type expression was determined for each mutant in each experiment, and the mean was determined. Standard deviations are indicated by vertical lines in each bar. The numbers below the histogram refer to the 3′ endpoint of each deletion.

threefold decrease was observed with a deletion to nt −46, and an additional fivefold decrease in E4 expression was observed with a deletion of the TATA box region (Fig. 3, ITR$^+$ set, −11). Thus, the major difference between in vitro and in vivo transcription analyses with mutants in the ITR$^+$ background was that E4 expression was not increased above wild-type levels in vivo as the ITR was brought close to the E4 TATA box region. In addition, ITR-dependent transcription in vivo was stimulated by the region from nt −62 to −46; this was not the case with transcription in vitro. The terminal repeat in its normal location increased E4 expression only slightly (twofold; pE4-CAT versus pE4Δ−330/−228; data not shown). We conclude from these analyses that E4 transcription in vivo is dependent on regulatory sequences located from nt −227 to −131 and from nt −104 to −46. As with transcription in vitro, the ITR can functionally substitute for upstream regulatory regions in a TATA box-dependent fashion. In both ITR$^+$ and ITR$^-$ backgrounds, the region between nt −62 and −46 is an important component of the E4 promoter.

Binding of cellular factor ETF-A to E4 control sequences. Because we observed efficient E4 transcription in vitro that was dependent on elements within the 5′-flanking sequences, the HeLa whole-cell extract was used to detect cellular factors that bind to these regions. Fragments of the E4 control region were incubated with increasing concentrations of HeLa cell extract, and regions of protection were detected by DNase I footprint analysis. Footprint analyses were performed with E4 probes ^{32}P labeled at nt −228 (Fig. 4A) and at nt +70 (data not shown). Two regions of protection were observed (Fig. 4A): nt −58 to −41 (region I) and nt −173 to −156 (region II). The region from nt −58 to −41 corresponds almost precisely to the TATA-proximal element required for efficient E4 transcription in vitro and in vivo (Fig. 2 and 3). A 10-bp perfect repeat is located within the boundaries of each region of protection (5′-CGTTACGTCA-3′; bold arrows in Fig. 1; see Fig. 8 and 9 for sequences). The repeated sequence is present in an inverted orientation at nt −160 relative to the sequence at nt −45 (Fig. 8).

To test if the same factor binds to each of these regions, we performed competition experiments (Fig. 4B and C). Competition for binding to both regions I and II was observed with an E4 fragment that contains the downstream 10-bp repeat but not the upstream 10-bp repeat (Fig. 4B, E4 −130/+70). A competitor fragment lacking both 10-bp repeats did not compete for binding to either region I or region II (Fig. 4B, E4 −45/+70). An oligonucleotide corresponding to E4 sequences between nt −34 and −63 (region I) was synthesized. This oligonucleotide specifically competed for binding to both regions I and II (Fig. 4C, E4 −34/−63), while a nonspecific oligonucleotide did not (Fig. 4C, 2A/2B). These analyses suggested that the same factor binds to both regions I and II and that the binding site may include sequences in the 10-bp repeat.

A 7-bp repeated sequence (5′-GTGACGT-3′) is conserved in the terminal repeats of a number of adenovirus serotypes, including Ad5 (43; adenovirus conserved sequence [ACS]; see Fig. 1, 8, and 9). This repeat shares a 6- of 7-bp homology with the 10-bp repeat located in regions I and II (see Fig. 9). We tested if the factor described above could bind to the ACS repeats. An E4 fragment ^{32}P-labeled adjacent to the Ad5 ITR was incubated with the HeLa cell extract, and regions of protection were detected by DNAse I footprint analysis (Fig. 5A). Five regions of protection were observed: over regions I and II, the nuclear factor I-binding site (34), and each of the ACS repeats (Fig. 5A). The E4 fragment that includes region I specifically competed for binding to the two ACS repeats and to regions I and II but not for binding to the nuclear factor I site (Fig. 5A, E4 −130/+70). The E4 fragment that lacks both 10-bp repeats did not compete for binding to these sites (Fig. 5A, E4 −45/+70). These results suggested that the same factor that binds to regions I and II also binds to the ACS repeats within the ITR.

A number of other sequences within the Ad5 genome are similar to the E4 10-bp repeat. A sequence in the E2 promoter (33) shares homology with this element (see Fig. 9). Protection over this region in the E2 promoter was observed in binding assays with the HeLa cell extract (Fig. 5B, lane marked + extract, −62 to −82). This region of protection was specifically competed for by the E4 fragment that contains the downstream copy of the 10-bp repeat (Fig. 5B, E4 −130/+70) but not by the control fragment (Fig. 5B, E4 −45/+70), suggesting that the same factor that binds to the E4 5′-flanking sequences also binds to the E2 control region.

Three sequence motifs similar to the E4 10-bp repeat are also located in the left terminus of the AAV genome upstream of the early promoter (P5) cap site (41). An AAV fragment containing these sequences specifically competed for binding of the factor to E4 regions I and II (Fig. 6A, AAV 185), while an adjacent (nonspecific) AAV fragment did not (Fig. 6A, AAV 232). Because the same factor appears to interact with several adenovirus early region promoters as well as the AAV early region promoter, we have named this factor ETF-A, for viral early region transcription factor A.

CRE in the E4 promoter region. The E4 10-bp repeat is very similar to the consensus sequence derived for the CRE of a number of cellular genes (5, 32, 39, 40) (see Fig. 9). A DNA fragment from the PEPCK gene containing a functional CRE (39; P. Quinn and D. Granner, personal communication) specifically competed for binding of ETF-A to E4 regions I and II (Fig. 6B, PEPCK −107). In contrast, a PEPCK fragment lacking the CRE did not compete for

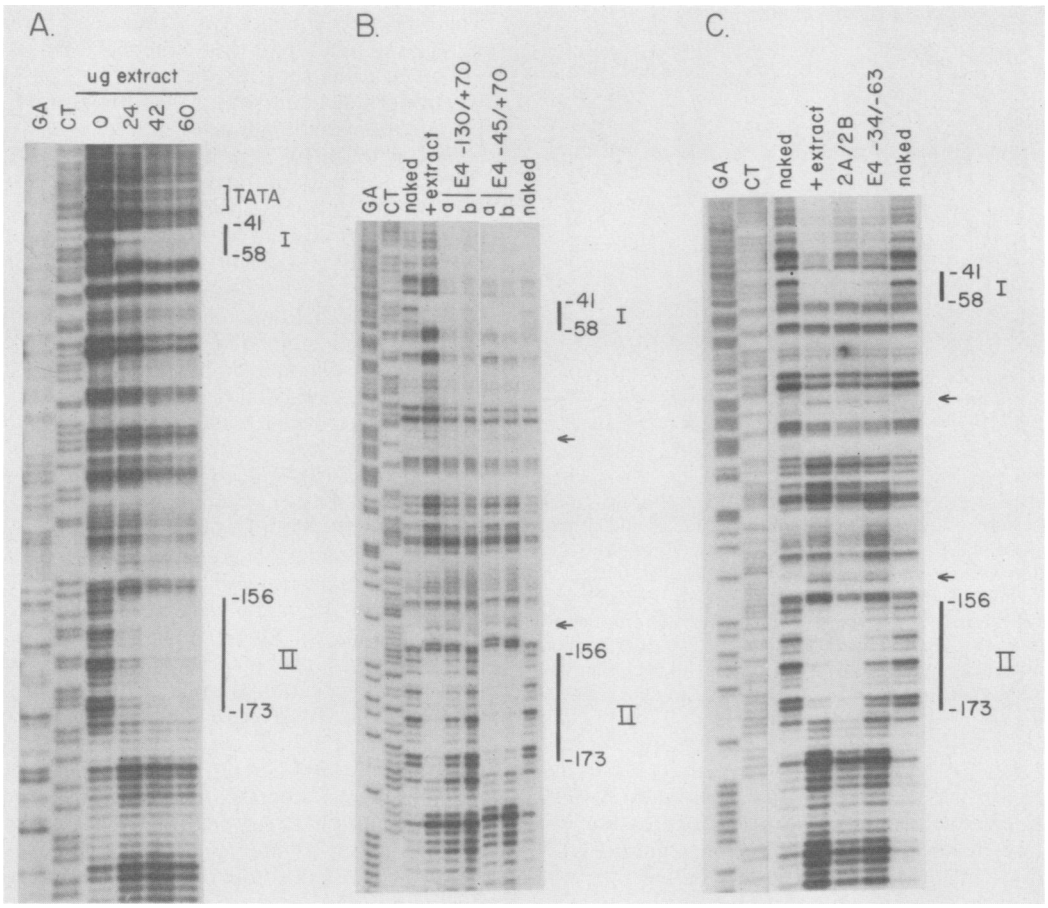

FIG. 4. Binding of a factor in the HeLa cell extract to two sites in the E4 control region. (A) A fragment of the E4 control region, ^{32}P labeled at the *Bgl*II site of pE4-CAT (Fig. 1), was incubated with increasing concentrations of HeLa cell extract (24, 42, and 60 μg of extract per 50-μl binding reaction). The lane marked 0 refers to control naked DNA (no extract). Following incubation, the reaction mixtures were digested briefly with DNase I. The products were electrophoresed in an 8% polyacrylamide–8.3 M urea gel and visualized by autoradiography. Regions of protection are indicated by bars: region I from nt −58 to −41 and region II from nt −173 to −156. The region homologous to the TATA box is indicated. GA and CT refer to sequencing ladders (29) of a heterologous DNA fragment. (B and C) Binding reactions and DNase I footprint analyses were performed as described above. The lanes marked naked refer to ^{32}P-labeled DNA incubated without extract; the lanes marked + extract refer to ^{32}P-labeled DNA incubated with 60 μg of HeLa cell extract per 50-μl binding reaction. Region I (nt −58 to −41) and region II (nt −173 to −156) DNase footprints are indicated adjacent to the gels. DNase I-hypersensitive sites generated after incubation with extract are indicated by arrows. GA and CT refer to sequencing ladders of the homologous DNA fragment. In panel B, the DNA competitors used were E4 −130/+70 (the *Bam*HI-*Hin*dIII fragment of pE4Δ−330/−131) and E4 −45/+70 (the *Bam*HI-*Hin*dIII fragment of pE4Δ−330/−46). Competitors were included in the binding reactions at 200-fold (a) and 450-fold (b) molar excesses over the ^{32}P-labeled DNA. In panel C, the DNA competitors used were 2A/2B (a nonspecific 38-bp oligonucleotide corresponding to a region in the poliovirus genome) and E4 −34/−63 (a specific 35-bp oligonucleotide corresponding to sequences from E4 nt −34 to −63). Oligonucleotides were included in the binding reactions at a 500-fold molar excess over the ^{32}P-labeled DNA.

binding of ETF-A to E4 sequences (Fig. 6B, PEPCK −74). The PEPCK fragment containing the CRE (PEPCK −107) also competed for binding of ETF-A to both ACS repeats in the ITR, while the control fragment (PEPCK −74) did not (data not shown).

These results prompted us to test if E4 expression was induced in vivo by cAMP. The human liver cell line HepG2 was used for these analyses. The E4 promoter is expressed efficiently in HepG2 cells in the absence of the E1A gene products. These analyses were performed in the absence of E1A induction to specifically monitor the effect of cAMP on E4 expression. HepG2 cells were transfected with the wild-type E4 plasmid, pE4-CAT, and a control plasmid, pSV2-CAT (containing the simian virus 40 promoter and enhancer region [10]). Following transfection, the cells were incubated in medium containing a low concentration of serum for 16 h, followed by incubation in medium containing cAMP for 8 h. Cell extracts were then prepared and tested for CAT activity. The results of these analyses are shown in Fig. 7. The expression of pE4-CAT was induced after treatment of cells with cAMP (three- to sixfold in different experiments), while the expression of pSV2-CAT was not induced and was even decreased in some experiments. We concluded from these analyses that the cellular factor that binds to the E4 promoter, ETF-A, also binds to a CRE. In agreement with this conclusion, the expression of the E4 promoter is induced in vivo by cAMP.

DISCUSSION

Our results demonstrate that the E4 control region contains multiple transcription elements that are, at least partially, functionally redundant. The nucleotide sequence of the E4 5′-flanking region is shown in Fig. 8. In vitro and in

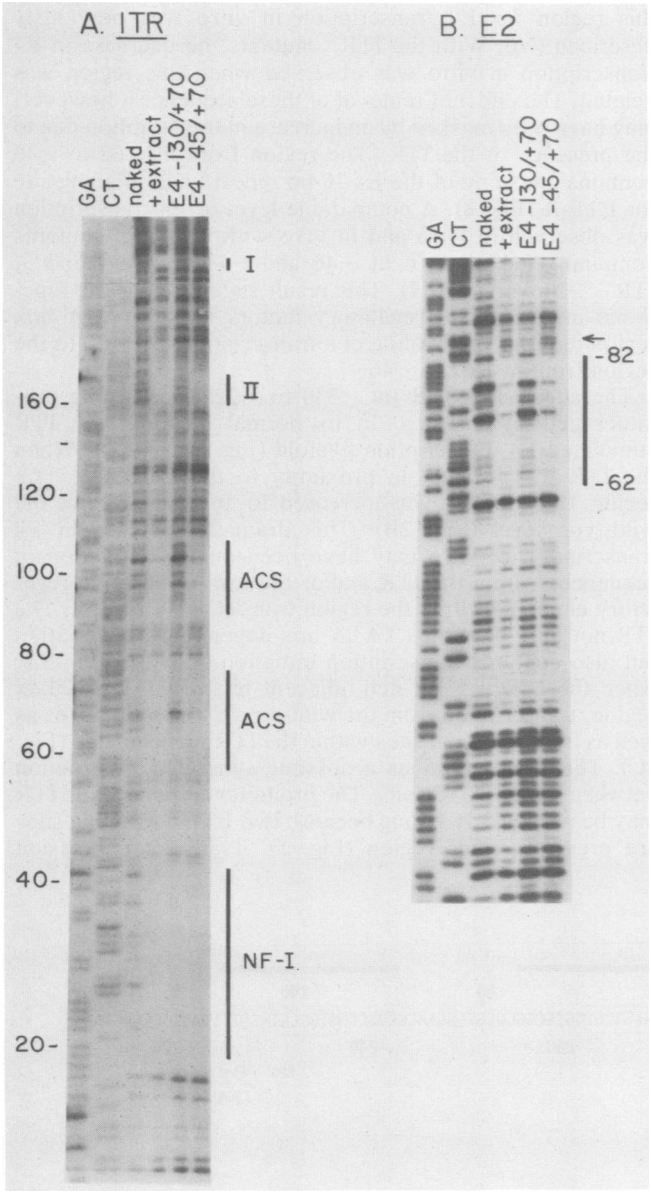

FIG. 5. Binding of factors in a HeLa cell extract to the Ad5 ITR and E2 control region. A DNA fragment corresponding to the Ad5 ITR (A) was ^{32}P labeled adjacent to nt 1 at the *Eco*RI site of pE4-CAT (21 bp to the left of the *Cla*I site in Fig. 1), and a DNA fragment corresponding to the E2 control region (B) was ^{32}P labeled adjacent to E2 nt −14 (pUC9 *Eco*RI site of pE2-2). Binding reactions and DNase I footprint analyses were performed as described in the legend to Fig. 4. The lanes marked naked refer to ^{32}P-labeled DNA incubated without extract; the lanes marked + extract refer to ^{32}P-labeled DNA incubated with 60 μg of HeLa cell extract per 50-μl binding reaction. GA and CT refer to homologous sequencing ladders. Competitors E4 −130/+70 and E4 −45/+70 were described in the legend to Fig. 4 and were included in the binding reactions at a 450-fold molar excess over the ^{32}P-labeled DNA in panels A and B. The arrow in panel B indicates a DNase I-hypersensitive site generated after binding with extract.

vivo analyses indicated that at least four regions contribute to E4 transcription; these include the ITR (nt, −330 to −228), sequences from nt −227 to −131 and nt −62 to −46, and the TATA box region. The transcription factor described in these studies, ETF-A, binds to three of these

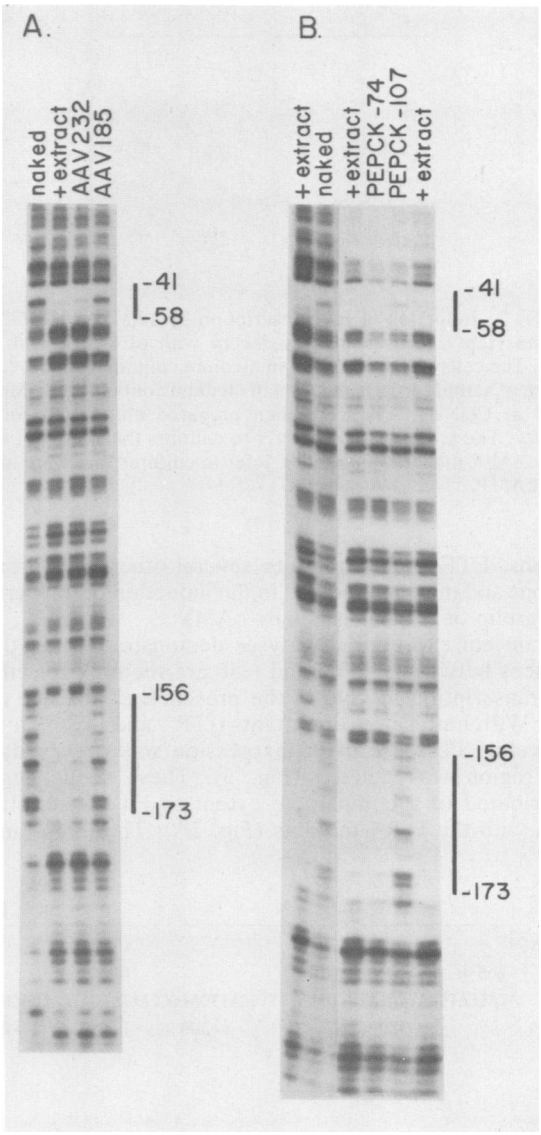

FIG. 6. Competition for binding to the E4 control region with AAV DNA and the CRE of the PEPCK gene. A DNA fragment containing the E4 control region was ^{32}P labeled at the *Bgl*II site of pE4-CAT (Fig. 1). Binding reactions and DNase I footprint analyses were performed as described in the legend to Fig. 4. The lanes marked naked refer to ^{32}P-labeled DNA incubated without extract; the lanes marked + extract refer to ^{32}P-labeled DNA incubated with 60 μg of HeLa cell extract per 50-μl binding reaction. (A) DNA competitors were from a clone (pGM620-C) containing the left end of the AAV genome. AAV 185 is a 185-bp fragment from the left terminus of the AAV genome (*Ava*I-*Ava*I, nt 78 to 263) that contains three sequences that share homology with the E4 10-bp repeat (see Fig. 8); AAV 232 is an adjacent, internal 232-bp AAV fragment (*Ava*I-*Pst*I, nt 264 to 496) that lacks homology with the E4 10-bp repeat and was used as a control competitor. AAV competitors were included in the binding reactions at a 200-fold molar excess over the ^{32}P-labeled DNA. (B) DNA competitors were from the rat PEPCK gene. PEPCK −107 contains sequences from nt −107 to +69 in the PEPCK gene. This fragment contains a functional CRE located around position −90 (39; P. Quinn and D. Granner, personal communication). PEPCK −74 contains sequences from nt −74 to +69 in the PEPCK gene. This fragments lacks the CRE described above. PEPCK competitors were included in the binding reactions at a 250-fold molar excess over the ^{32}P-labeled DNA.

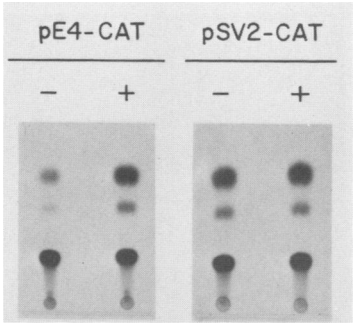

FIG. 7. Induction of E4 transcription by cAMP in HepG2 cells. Human HepG2 cells were transfected with pE4-CAT and pSV2-CAT. The cells were incubated in medium containing a low concentration of serum for 16 h and then in medium containing 1 mM cAMP for 8 h. Cell extracts were then prepared and tested for CAT activity. The lanes marked − refer to cultures that were not treated with cAMP; the lanes marked + refer to cultures that were induced with cAMP.

regions. ETF-A also binds to several other viral promoter regions and may be involved in the induction of transcription of a group of cellular genes by cAMP.

Transient expression analyses demonstrated that the sequences between nt −62 and −46 are required for efficient E4 transcription in vivo in the presence or absence of the ITR. With both series of mutants (ITR$^-$ and ITR$^+$), a three- to fivefold decrease in E4 expression was observed when this region was deleted (Fig. 3). These sequences also contributed to a comparable extent to E4 transcription in vitro with the ITR$^-$ mutants (Fig. 2A). The importance of this region for E4 transcription in vitro was previously described (16). With the ITR$^+$ mutants, no decrease in E4 transcription in vitro was observed when this region was deleted. The effect of removal of these sequences, however, may have been masked by an increase in transcription due to the presence of the ITR. The region from nt −62 to −46 contains one copy of the E4 10-bp repeat and a binding site for ETF-A (Fig. 8). A comparable level of E4 transcription was observed in vitro and in vivo with the ITR$^-$ mutants containing deletions to nt −46 and −11 (Fig. 2A and 3, ITR$^-$, −46 versus −11). This result suggests that the functional interaction of regulatory factors at the TATA box region requires the binding of a transcription factor(s) to the region from nt −62 to −46.

The adenovirus ITR (nt −330 to −228) has strong promoter activity in vitro. In its normal location, the ITR stimulated E4 transcription fivefold (Fig. 2A and B). When the ITR was brought in proximity to the E4 TATA box region, transcription was increased 10- to 20-fold above the wild-type level (Fig. 2B). This dramatic increase in E4 transcription in vitro may have been due to activation of sequences within the ITR and/or the loss of negative regulatory elements within the region from nt −131 to −62. The ITR not only promotes TATA box-dependent transcription but also contains transcription initiation activity (Fig. 2C); when the ITR was located adjacent to the E4 TATA box region, transcription from the wild-type E4 initiation sites as well as from initiation sites within the ITR was elevated (Fig. 2C). This result suggests a possible synergistic interaction between these two regions. The promoter activity of the ITR may be particularly strong because two ETF-A-binding sites are present in this region (Fig. 5). Transient expression

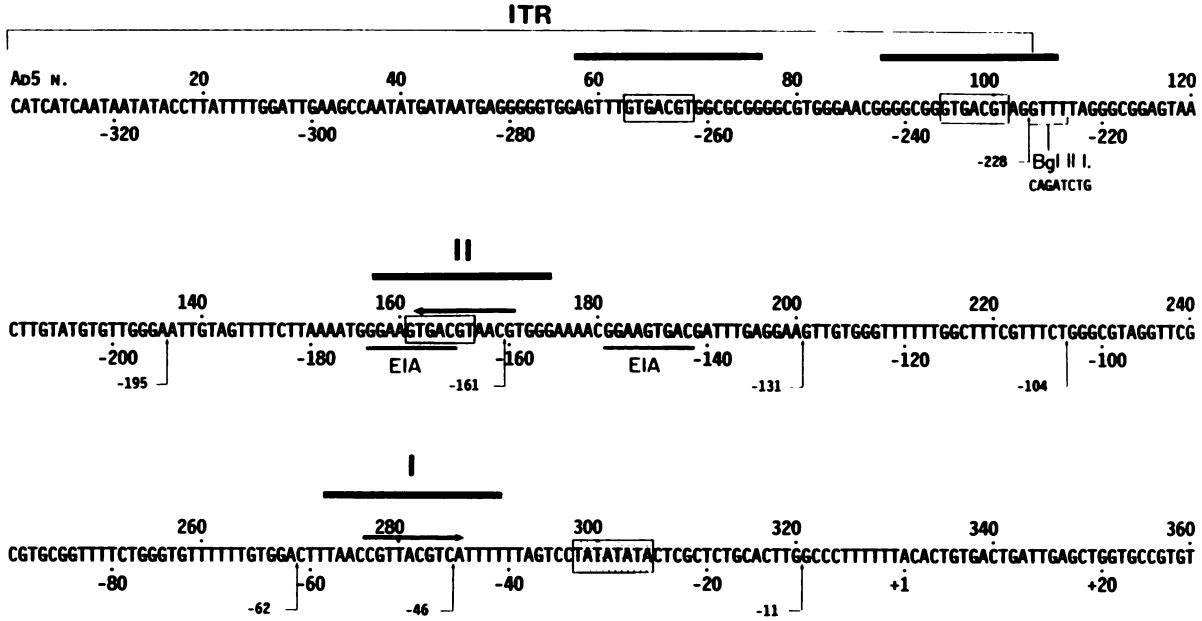

FIG. 8. Nucleotide sequence of the E4 control region and Ad5 ITR. The nucleotide sequence of the right-terminal 360 bp of the Ad5 genome (42) is shown. The numbers above the sequence refer to nucleotides from the right terminus. The numbers below the sequence are relative to the E4 initiation site at +1 (nt 331). The Ad5 ITR (nt 1 to 103, ITR) is indicated by a bracket above the sequence. A *Bgl*II linker was inserted at an *Hph*I site at nt 104; 4 nt were removed in this process, and an 8-bp linker was inserted as indicated below the sequence. The E4 TATA box is indicated by a stippled box. The E4 10-bp repeat is indicated by bold arrows above the sequence at nt −45 and −160. The 7-bp ACS repeats are indicated by open boxes in the sequence at nt −164, −230, and −262. Areas homologous to the E1A core enhancer element I sequence (17) are underlined. The 3′ endpoints of deletion mutations in the E4 control region are indicated by small arrows below the sequence. The four regions of protection conferred by the binding of ETF-A are indicated by solid bars above the sequence and correspond to regions I and II (nt −58 to −41 and nt −173 to −156, respectively) and to two sites in the ITR over the ACS repeats.

analyses demonstrated that the ITR also has promoter activity in vivo (Fig. 3), although at a reduced level as compared with that in vitro. Transcription activity of the ITR recently was recently reported (13). We do not know at this point what role the transcription activity of the ITR may have in the viral life cycle.

The region between nt −227 and −131 is required for efficient E4 transcription in vitro and in vivo (Fig. 2 and 3). This region contains a number of interesting sequence motifs (Fig. 8), including one copy of the E4 10-bp repeat at nt −160, one copy of the ACS repeat at nt −164, and two areas of perfect homology to the E1A core enhancer element I sequence (17) at nt −166 and −142. Gilardi and Perricaudet (8, 9) identified two functionally redundant regions that are required for efficient E4 transcription in vivo and are located from nt −239 to −218 and from nt −179 to −158. Each of these regions contains a binding site for ETF-A (Fig. 8). Our in vivo analyses demonstrated that the ITR (nt −330 to −228) can functionally substitute for the region from nt −227 to −131 (Fig. 3), a fact which is consistent with these results. Lee and Green (25) observed a dramatic decrease in E4 transcription in vivo and in vitro when the sequences between nt −200 and −138 were deleted. Our results with mutations in this region (Fig. 2 and 3) are in agreement with these analyses. The E4F-1 transcription factor identified by these authors (also termed ATF [26]) binds to regions I and II and is likely the same factor as ETF-A.

Each of the regions that contains an ETF-A-binding site is important for E4 transcription in vitro and in vivo (Fig. 2 and 3). These results suggest that the binding of ETF-A may mediate the transcription activation function of these regions. This idea is supported by the fact that the concentration of protein (extract) used for in vitro transcription reactions resulted in saturation of the ETF-A-binding sites on the template DNA (Fig. 5 and data not shown). Our results and those previously reported (8, 9, 13, 16, 25) are consistent with the idea that each ETF-A-binding region contributes to E4 transcription activation in an additive fashion. The control elements in the ITR and the region from nt −227 to −131 are functionally redundant when analyzed in the context of specific deletion mutants (Fig. 2B and 3). These regions also can exert an additive effect on E4 transcription, since the ITR in its normal location stimulates E4 transcription in vitro and in vivo.

ETF-A binds to the adenovirus E2 promoter region (Fig. 5) described by Murthy et al. (33) and is likely the same factor as the HeLa cell factor, ATF, described by Lee et al. (26), that binds to this region and to the early region 3 promoter. ETF-A also binds to the left terminus of the AAV genome in the early P5 promoter region and to a region upstream of the PEPCK gene that contains a CRE (Fig. 6). Consistent with the latter result, Hurst and Jones (18) recently demonstrated that ATF binds to the CRE of the somatostatin gene. Each of these binding regions contains a sequence motif that is similar to the E4 10-bp repeat (Fig. 9). We have derived a consensus binding sequence for ETF-A from these data (Fig. 9). Two sequences with a 7- of 8-bp homology to this consensus sequence are also located in the Ad5 E1A and early region 3 promoter regions (Fig. 9). This sequence is nearly identical (7 out of 8 bp homology) to the consensus CRE sequence defined by several groups (5, 32, 39, 40). The strong similarity between the E4 10-bp repeat and the CRE core sequence led us to test if the E4 promoter was induced by cAMP. We found that E4 expression was induced three- to sixfold by cAMP in HepG2 cells, while the expression of a plasmid containing the simian virus 40 early

E4 −160 REPEAT	(O.)	5′- TTCCCACGTTACGTCACTTCCC -3′	
E4 −45 REPEAT	(D.)	5′- TTTAACCGTTACGTCATTTTTT -3′	
ITR N. 63 ACS	(O.)	5′- GCCCCGCGCCACGTCACAAACT -3′	(8/10)
ITR N. 95 ACS	(O.)	5′- CCTAAAACCTACGTCACCCGCC -3′	(7/10)
E2 −70	(O.)	5′- CGCGAAAACTACGTCATCTCCA -3′	(7/10)
AAV N. 160	(O.)	5′- GACGTAATTCACGTCACGACTC -3′	(7/10)
AAV N. 170	(D.)	5′- GACGTGAATTACGTCATAGGGT -3′	(8/10)
AAV N. 210	(D.)	5′- ATTAGAGGTCACGTGAGTGTTT -3′	(7/10)
PEPCK −80	(D.)	5′- CCGGCCCCTTACGTCAGAGGCG -3′	(9/10)
CONSENSUS:	(D.)	5′- $\frac{TT}{CC}$ACGTCA -3′	
	(O.)	5′- TGACGT$\frac{AA}{GG}$ -3′	
E1A −35	(O.)	5′- AATACACTACACGTCAGCTGAC -3′	(7/8)
E3 −60	(D.)	5′- CGGGCGGCTTTCGTCACAGGGT -3′	(7/8)
CREB CORE:		5′- TGACGTCA -3′	(7/8)

FIG. 9. Sequences of ETF-A-binding sites. The two copies of the E4 10-bp repeat are shown at the top of the figure with flanking sequences (the 10-bp repeat is underlined). (O.) (opposite) and (D.) (direct) refer to the orientation of the sequence relative to the site of initiation of transcription of each gene. Sequences similar to the E4 10-bp repeat are found in the Ad5 ITR (ACS sequences at nt 63 and 95; Fig. 7), within the E2 promoter defined by Murthy et al. (33), at the left terminus of the AAV genome (41), and upstream of the PEPCK gene (39; P. Quinn and D. Granner, personal communication). The numbers of nucleotides that are homologous to the E4 10-bp repeat are indicated in parentheses. A consensus sequence for the putative ETF-A-binding site, based on sequence comparisons, is shown in both orientations. The core consensus sequence for the CRE-binding protein (CREB core) is taken from Montminy and Bilezikjian (31). Similar sequences are located upstream of the E1A (44) and early region 3 (E3) (4) initiation sites.

promoter and enhancer region was not induced (Fig. 7). This result is consistent with the results of competition experiments that demonstrated that the CRE region of the PEPCK gene competed for ETF-A binding to the E4 control region and suggests that ETF-A may be involved in the cAMP response of a group of cellular genes. The protein that binds to the CRE of the somatostatin gene (CREB [31]) may be the same protein as ETF-A. Alternatively, two different proteins with overlapping binding domains may bind to the E4 10-bp repeat. Evidence for this possibility was recently provided by Raychaudhuri et al. (38), who identified an E1A-inducible factor that binds to regions I and II described above yet is distinct from ETF-A and ATF, since it does not bind to other adenovirus early region promoters. The complex organization of the Ad5 E4 transcriptional control region may reflect the evolution of this promoter to respond to different environmental stimuli during infection and to utilize the transcription factors that are available in different cell types.

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