Adenovirus Early Region 3 Promoter Regulation by E1A/E1B Is Independent of Alterations in DNA Binding and Gene Activation of CREB/ATF and AP1

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Transcription of the adenovirus early region 3 promoter is strongly induced by the adenovirus E1A protein. Previous DNase I footprinting has indicated that four regions in this promoter serve as binding sites for HeLa nuclear proteins. These include binding sites for NF-1 (site IV), AP1 (site III), CREB/activating transcription factor (ATF) (site II), and TATA (site I). To determine the relative importance of these sites in both the *in vivo* and *in vitro* transcriptional regulation of the E3 promoter, oligonucleotide-directed mutagenesis of these sites was performed. Each of these constructs was assayed by transfection onto HeLa cells in the presence of either *dl434*, an E1A/E1B deletion mutant, or wild-type adenovirus. Mutations of either the ATF- or AP1-binding sites but not the TATA- and NF1-binding sites resulted in severe decreases in both basal and E1A/E1B-induced transcriptional levels. These constructs were also assayed in *in vitro* transcription assays with cellular extracts prepared from *dl434*-infected or wild-type-adenovirus-infected HeLa cells. The wild-type E3 promoter was transcribed approximately 30 times more efficiently in extracts containing the E1A/E1B proteins compared with extracts lacking these proteins. Mutations of either the TATA element, the ATF site, or the AP1-binding site decreased both basal and E1A/E1B-induced transcriptional levels. Gel retardation analysis using these extracts indicated that the binding to ATF, AP1, or NF1 oligonucleotides was not altered in the presence of the E1A/E1B proteins compared with extracts lacking these proteins. Northern (RNA) blot analysis of *c-jun* and CREB RNA prepared from wild-type adenovirus and *dl434*-infected cells indicated that the levels of these RNAs were not altered by the E1A/E1B proteins. Immunoprecipitation of AP1 and CREB from both *dl434*- and wild-type-adenovirus-infected cells indicated that the amounts of these proteins were not significantly altered. These results suggest that E1A/E1B-induced activation of the E3 promoter does not involve activation of transcription factor genes nor a change in the DNA binding activity of important promoter-binding components. Our results are consistent with a model in which the E1A/E1B proteins either directly or indirectly alter the interactions of factors that bind to the basal E3 promoter transcription complex, thereby inducing transcription.

Regulation of both viral and cellular promoters involves the interplay of multiple promoter elements which serve as the binding sites for cellular transcription factors (for a review, see reference 46). These elements have been found to be required for basal promoter function and induction by exogenous substrates, such as phorbol esters and serum, and by viral transactivator proteins, such as the adenovirus E1A proteins. The mechanisms by which these substrates and transactivators induce transcription is not known. Potential mechanisms include interacting directly with DNA-binding proteins, altering the state of phosphorylation of transcription factors, modifying non-DNA-binding components which interact with the transcription complex, and affecting the rate of assembly of stable transcription complexes.

One of the best-studied viral transactivator proteins is the adenovirus E1A protein (5, 6, 34). The E1A protein is capable of activating the transcription of a variety of cellular and viral class II and III genes newly introduced into the cell by transfection and infection; however, it activates relatively few endogenous cellular genes (for a review, see reference 5). It has been shown that a promoter element which serves as the binding site for a factor known as activating transcrip-

tion factor (ATF) is found upstream of all early adenovirus promoters except the E1B promoter (5, 15, 18, 24, 25, 31, 32, 37–40, 42, 44, 50, 54, 56, 62, 65). The ATF site has been shown to be required for efficient transactivation by E1A. A similar binding element is found upstream of cellular cyclic AMP responsive promoters and binds a protein which is known as CREB (28, 47). The observations that the ATF element mediates both cyclic AMP and E1A induction of adenovirus early promoters and that CREB and ATF are of similar molecular mass (43 to 47 kilodaltons) and give similar gel-retarded species suggest that CREB and ATF may be related or identical (12, 24, 31, 41, 54). However, several different cellular proteins may bind to the ATF motif (9).

The ATF site is not the only promoter element which mediates E1A activation. The TATA element is critical for E1A activation of a number of promoters, including the human immunodeficiency virus (HIV) long terminal repeat (LTR) and the E1B and heat shock promoters (35, 55, 61, 62). However, detailed mutagenesis of the hsp70 promoter (61) and the HIV LTR (35) have indicated that other components of the basal promoter complex, in addition to the TATA element, are required for complete E1A/E1B activation. Studies with a variety of adenovirus early promoters have indicated that no unique promoter element is responsible for E1A-mediated activation (7, 15, 32, 56). Instead,

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multiple promoter elements can contribute, and the exact nature of E1A/E1B-mediated transactivation may be gene specific.

The mechanism by which E1A stimulates transcription is an area of intense investigation. Several studies have suggested that the E1A protein is capable of modulating the activity of cellular transcription factors (TF) (3, 29, 52, 64). E1A is capable of increasing the binding activity of TF III C, which regulates transcription by RNA polymerase III (29, 64). Activation of TF III C by E1A is associated with altered phosphorylation of this factor (29). E1A has also been shown to induce a cellular kinase which increases the phosphorylation of two factors, E2F and E4F, resulting in an increase in their DNA-binding activity (3, 52, 64). Studies with an E1A-gal4 fusion protein have shown that this hybrid protein was capable of stimulating transcription of promoters containing gal4-binding sites (43). This result suggested that E1A could directly interact with other transcription factors. Thus, E1A may activate transcription by multiple mechanisms.

In addition to E1A, other adenovirus early proteins are also involved in the activation of early adenovirus promoters. The adenovirus E4 protein has been shown to activate E2F-binding and E2 transcriptional activation (19, 53). The adenovirus E1B region has been shown to activate both transfected cellular genes and adenovirus early promoters (4, 8, 27, 33, 59). Thus, E1A activation can be augmented by other adenovirus early regions.

One of the promoters that is strongly induced by E1A is the adenovirus early region 3 promoter. Previous studies of this promoter have revealed that it serves as the binding site for four cellular proteins (15, 31). These include nuclear factor 1 (site IV), AP1 (site III), ATF (site II), and TATA (site I). Deletion and insertion mutagenesis of this promoter revealed that both the ATF- and AP1-binding sites are critical for E1A activation (15). In this report, we have examined the role of these transcriptional regulatory elements on basal and E1A/E1B-induced levels of transcription from the E3 promoter both in vivo and in vitro. Our results suggest that E1A/E1B activation likely involves either direct or indirect interactions with multiple components of the E3 basal promoter complex and does not involve an increase in the binding or gene activation of these factors.

MATERIALS AND METHODS

Cells and virus stocks. Wild-type adenovirus type 5 (Ad5) was grown in HeLa suspension cultures maintained in minimal essential medium (MEM) plus 5% newborn calf serum, and titers were determined by plaque formation on 293 cells (22). Ad5 *dl434* (23) and Ad5 *dl312* (34) were grown on monolayers of the complementing 293 cell line, and titers were determined by plaque assay on this cell line. HeLa monolayers were maintained on complete Iscoves medium with 5% newborn calf serum containing penicillin and streptomycin.

Transfections and chloramphenicol acetyltransferase (CAT) assays. HeLa plates were split on the day before transfection so that each 100-mm plate was 50 to 70% confluent at the time of transfection. Cells were infected with either wild-type adenovirus or *dl434* at a multiplicity of infection of 20. At 1 h postinfection, fresh medium with cytosine arabinoside (ara-C) (20 µg/ml) was added. At 4 h postinfection, medium was removed and 5 µg of each E3 CAT construct was transfected onto identically prepared plates. Fresh medium containing ara-C was added (17), the medium was changed 12 h later, the plates were harvested at 26 h posttransfection,

and CAT assays were performed as described previously (21).

Plasmids. The *EcoRI-SacI* (−236 to +31)-cut E3 promoter was cloned into *SmaI-SacI* mp18 (15). Oligonucleotides complementary to regions IV, III, II, and I containing specified point mutations were synthesized (courtesy of T. Sutherland, Molecular Biology Institute and Jonsson Cancer Center) and used for oligonucleotide-directed mutagenesis according to the protocol of the manufacturer (Amersham Corp., Arlington Heights, Ill.). Each mutant was verified by dideoxy sequencing. The replicative form DNA from each isolate was prepared, the E3 upstream region was isolated by cutting with *HincII* and *SacI*, and the resulting fragment was then cloned into an *NruI-SacI*-cut Rous sarcoma virus *cat* derivative in which the *HindIII* site was converted into a *SacI* site by the addition of linkers. The fragments were also cloned into the *HincII-SacI* site of pUC19 for use in DNase I footprinting (14).

RNA preparation and Northern (RNA) blot analysis. HeLa spinner cells infected with either wild-type adenovirus or *dl434* were harvested for cytoplasmic RNA at 0, 6, and 30 h postinfection as described previously (16). Poly(A) RNA was selected by using oligo(dT) columns, and 20 µg of each RNA sample was electrophoresed on a 0.8% agarose-formamide gel. RNA was transferred to nitrocellulose filters as described previously (20). Samples were denatured at 65°C for 10 min, size separated by an agarose-formaldehyde gel (1% agarose [Bethesda Research Laboratories, Gaithersburg, Md.], 50 mM sodium acetate, 10 mM disodium EDTA, 200 mM MOPS [morpholinepropanesulfonic acid], 2.2 M formaldehyde) and transferred to a nylon membrane filter (ICN Biomedicals Inc., Irvine, Calif.). Filters were dried, baked at 80°C in vacuo for 2 h, and then prehybridized for 16 to 24 h. Filters were probed with random primed fragments (13) prepared from either the full-length 2.3-kilobase (kb) human *c-jun* cDNA (2), a 2.4-kb fragment from the human CREB cDNA (28), or a 700-base-pair (bp) fragment from the β -actin gene in the plasmid pH FBA-3' (51). Hybridizations with ³²P-labeled DNAs (10⁶ cpm/ml) were performed at 42°C for 16 to 24 h in a solution containing 50% (vol/vol) formamide, 2× standard saline citrate (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 5× Denhardt solution (1× Denhardt solution is 0.02% ficoll–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin), 0.01% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10% (vol/vol) dextran sulfate (Sigma) (*M_w*, 500,000), and 100 µg of salmon sperm DNA (Sigma Chemical Co., St. Louis, Mo.) per ml. Filters were washed to a stringency of 0.1× SSC–1% SDS at 65°C and exposed for 24 to 28 h at −70°C to Kodak XAR-5 film.

DNase I footprinting. HeLa cell nuclear extract (11) was loaded onto a heparin-agarose column, washed with 5 column volumes of this same buffer, and eluted with 0.4 M KCl (15). The extract was dialyzed into 20 mM Tris (pH 7.9)–100 mM KCl–0.2 mM dithiothreitol (DTT)–0.2 mM phenylmethylsulfonyl fluoride and used in DNase I-footprinting assays (14). DNase I-footprinting studies were done with a clone containing the E3 upstream region (−236 to +31) cloned into *SmaI-SacI*-cut pUC19. The coding strand was labeled with T4 kinase and [γ -³²P]ATP at the *HindIII* site in the polylinker, and a 400-bp fragment was isolated by electroelution after being cut with *PvuII*. Fragments for footprinting each of the point mutants were labeled and isolated in the same manner. From 1 to 5 ng of end-labeled probe was added to each 50-µl reaction mix with 100 µg of HeLa extract, poly(dI-dC) (3 µg), and final concentrations of 10 mM Tris (pH 7.6), 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 5%

(vol/vol) glycerol. The DNA and extract were allowed to bind for 30 min at room temperature, the reaction volume was increased to 100 μ l, and final concentrations of DNase I (0.4 to 3.0 μ g/ml), 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$ were added. The reaction was stopped after 30 s with phenol-chloroform, ethanol precipitated, and loaded on a 10% polyacrylamide-8 M urea sequencing gel. All gels were then subjected to autoradiography.

Gel retardations. HeLa spinner cells (8×10^8 cells) were either uninfected or were infected with *dl434* or wild-type adenovirus at a multiplicity of infection of 20 in the presence of 20 mg of ara-C per ml added every 8 h and were harvested after 30 h. Cells were washed with phosphate buffered saline, and nuclear extracts were prepared as described previously except (11) that $MgCl_2$ was not present in the buffers. Gel retardations with oligonucleotides complementary to the NF1, AP1, and CREB E3 sites (31) were then performed. Double-stranded oligonucleotides complementary to the NF1 (TAGTTGGCCCGCTTCCCTGGTGTA)-, AP1 (GAAGTTCAGATGACTAACA)-, and ATF (GGCGGCTTTCGTCACAGGGTG)-binding sites of the E3 promoter (15, 31) were purified and labeled with T4 polynucleotide kinase, and 10,000 cpm was used in gel retardations with from 1 to 5 μ g of HeLa cell nuclear extract as described previously (31). Oligonucleotide competitions were performed with a 50-fold excess of either wild-type double-stranded oligonucleotides or a similar excess of mutated oligonucleotides for NF1 (TAGTTAACCCGGTGCAGTGGTGTA)-, AP1 (GAAGTTCAGATCTCTAACA)-, or ATF (GGCGTTCGTCACAGGGTG)-binding sites of the E3 promoter.

In vitro transcription. Transcription reactions (50 μ l) contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 500 μ M each ribonucleoside triphosphate, 500 ng of supercoiled template DNA, and 200 μ g of *dl*- or wild-type-Ad5-infected HeLa cell nuclear extract (approximately 20 μ l). Reactions were incubated for 60 min at 30°C and terminated by the addition of 150 μ l of stop buffer (200 mM NaCl, 20 mM EDTA, 1% SDS, 250 μ g of tRNA per ml). Each reaction was extracted twice with phenol-chloroform and precipitated with 0.3 M sodium acetate-ethyl alcohol. The pellets were rinsed with 70% ethyl alcohol and dried. Primer extension was performed by a modification of a previously described protocol. Each pellet was suspended in 8 μ l of 10 mM Tris hydrochloride (pH 7.9), 1 mM EDTA containing approximately 50 fmol of 5'-end-labeled primer (24-nucleotide primer hybridizing to sequences 4945 to 4968 of Rous sarcoma virus CAT). Two microliters of 10 mM Tris (pH 7.9), 1 mM EDTA, and 1.25 M KCl was added, and the primer was annealed to the RNA for 60 min at 63°C. A 25- μ l portion of a buffer containing 20 mM Tris (pH 7.9), 10 mM $MgCl_2$, 5 mM DTT, 500 μ M deoxynucleoside triphosphates, and 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) was added, and the primer extension reaction was incubated for 90 min at 37°C. Each reaction was extracted with phenol-chloroform, precipitated with 0.3 M sodium acetate-ethyl alcohol, suspended in 10 μ l of loading buffer (80% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue, $1 \times$ TBE), and loaded onto a 1-mm-thick 8 M urea-8% acrylamide gel. Dried gels were subjected to autoradiography overnight at -70°C with an intensifying screen. Densitometry was performed using an Ultrascan XL densitometer (LKB Instruments, Inc., Rockville, Md.).

In vivo labeling of cells with [35 S]methionine and [32 P]

phosphate. HeLa spinner cells infected with either *dl434* or wild-type adenovirus in the presence of 20 μ g of ara-C per ml were harvested at 26 h postinfection. Cells (2×10^5) to be labeled with [35 S]methionine were washed four times in MEM without methionine and serum. The cells were suspended in MEM containing 500 μ Ci of [35 S]methionine (ICN) per ml and 8% dialyzed newborn calf serum, and the infections were allowed to proceed for 4 h before harvesting. Labeling of cells with [32 P]phosphate was as described for [35 S]methionine, except that MEM without phosphate was used and the cells were suspended in MEM supplemented with 500 μ Ci of carrier-free [32 P]phosphate (Amersham) per ml. After being harvested, nuclear extracts were prepared from the [35 S]methionine and [32 P]phosphate-labeled cells by a previously described procedure (36).

Antisera and radioimmunoprecipitations. To prepare anti-CREB antiserum, a full-length CREB cDNA was cut with *PvuII* and *NsiI*, and the 850-bp fragment was end-filled and fused to *trpE* in a path2 vector which had been cut with *SmaI*. To prepare anti-Jun antiserum, a full-length *c-jun* cDNA was cut with *NaeI* and *HindIII*, and the 1-kb fragment was fused to *trpE* in a path2 vector which had been cut with *SmaI* and *HindIII*. Induction and preparation of *trpE*-CREB and *trpE*-*c-jun* fusion proteins and injection schedules were performed as previously described (57). Immunoprecipitations were done with 75 μ g of [35 S]methionine- or [32 P]phosphate-labeled nuclear extracts and either 10 μ l of control preimmune serum, 10 μ l of CREB/ATF, or 10 μ l of *c-jun*-specific antiserum. Antigen-antibody complexes were collected by the addition of protein A-Sepharose (Sigma), and the immunocomplexes were washed in radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.9], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and prepared for SDS-polyacrylamide gel electrophoresis analysis as described previously (5). Immunoprecipitated complexes were resolved in 12.5% gels which were then dried and subjected to autoradiography.

RESULTS

Oligonucleotide mutagenesis of the E3 promoter. Oligonucleotide-directed mutations of the E3 promoter were constructed that interrupted previously defined binding sites between -179 and -156 for NF-1 (site IV), between -103 and -83 for AP1 (site III), between -67 and -47 for ATF (site II), and between -37 and -16 for TATA (site I) (15, 31). The sequence of the E3 promoter, the positions of the binding motifs, and the nucleotides altered by oligonucleotide mutagenesis are indicated in Fig. 1A. DNase I footprinting of the E3 promoter mutations was performed by using HeLa extract. Mutations of sites II (lane 4), III (lane 5), and IV (lane 6), respectively, eliminated protection of these sites (Fig. 1B). Mutation of the TATA sequence appeared to somewhat decrease but did not eliminate protection of site I, indicating that other potential binding motifs in this region are involved in DNase I protection (Fig. 1B, lane 3). Other upstream mutations also had minor effects on binding of site I, although the degree of difference varied between experiments.

ATF and AP1 regulatory domains are critical for basal and E1A/E1B-induced gene expression of the E3 promoter. It has been demonstrated that, in addition to the E1A proteins, the E1B proteins and/or a *cis*-acting sequence with the E1B promoter are important for the complete activation of adenovirus early promoters and transfected viral and cellular genes (4, 8, 27, 33, 59). In order to investigate whether the

FIG. 1. Sequence of the E3 promoter. (A) Nucleotide sequence of the E3 promoter from -237 to $+1$. Boxes (sites I to IV) indicate regions of protection by DNase I footprinting with HeLa extract. The changes introduced by oligonucleotide-directed mutagenesis are indicated by underlined and substituted sequences. A schematic of the cellular proteins binding to the E3 promoter with HeLa extract is shown below. (B) DNase I footprinting of the E3 wild-type promoter in the absence (lane 1) or presence (lane 2) of HeLa cell nuclear extract and E3 site I (lane 3), site II (lane 4), site III (lane 5), or site IV (lane 6) mutants in the presence of $100\ \mu\text{g}$ of partially purified HeLa cell nuclear extract.

E1B region was required for full induction of the E3 promoter, the wild-type E3 CAT plasmid was transfected onto HeLa cells infected with either wild-type adenovirus, *dl312* (an E1A deletion mutant [34]), or *dl434*, and E1A/E1B deletion mutant (23). Infection with wild-type adenovirus resulted in a 12-fold activation of a wild-type E3 CAT activity compared with infection with *dl434* (Fig. 2A). However, infection with *dl312* consistently resulted in a two- to threefold activation of E3 CAT compared with *dl434* infection, indicating a role for the E1B region in addition to that of E1A in E3 activation (Fig. 2A). The level of expression in mock-infected HeLa cells did not differ from that seen in *dl434*-infected cells (data not shown). Quantitation of CAT assays from three independent experiments is shown in Table 1. In the remainder of the experiments, only wild-type adenovirus and *dl434* were used.

Oligonucleotide-directed mutations in each of the E3-binding domains (I, II, III, IV, and a double mutation in sites II and III) were fused to the CAT gene. Previous S1 analysis of RNA prepared from transfections using the E3 CAT construct confirmed that CAT activity accurately reflected steady-state mRNA levels (15). Each E3 CAT construct was transfected onto HeLa cells previously infected with either wild-type adenovirus or *dl434*. All E3 CAT constructs were activated by the E1A/E1B proteins (Fig. 2B). Mutations of site II (ATF) or site III (AP1) resulted in significant decreases in both basal and E1A/E1B-induced transcription levels (Fig. 2B). Mutations of both of these sites (site II and site III mutations) resulted in even further decreases in E1A/E1B-induced activation (Fig. 2B). These results suggest that both ATF- and AP1-binding sites are important for complete E1A/E1B activation. Mutations of site I (TATA) or

site IV (NF-1) had minimal effects on basal and E1A/E1B activation (Fig. 2B). The level of CAT conversion and fold induction for three independent experiments are shown in Table 2. No mutations were found that affected only E1A/E1B-induced levels of transcription without affecting basal transcription. This suggests that the same elements are required for both basal and E1A/E1B-induced levels of transcription.

Promoter regulatory region mutations alter in vitro transcription of the E3 promoter. We next wished to determine the effect of each of these E3 mutations on in vitro transcription from the E3 promoter. The E3 CAT constructs were transcribed in vitro by using uninfected HeLa extract and assayed by primer extension. Transcription of the E3 CAT construct in vitro resulted in a 115-bp E3-specific primer-extended product (Fig. 3A, lane 1). This product was sensitive to levels of alpha amanatin ($2\ \mu\text{g}/\text{ml}$), which inhibits RNA polymerase II-mediated transcription (Fig. 3A, lane 2).

FIG. 2. Activation of the E3 promoter by E1A/E1B. (A) The E3 CAT plasmid was transfected into HeLa cells after infection with either wild-type (WT) adenovirus, *dl434*, or *dl312*, and CAT activity was determined. (B) The E3 CAT plasmid or E3 CAT promoter mutants of sites I to IV were transfected onto HeLa cells infected with either wild-type adenovirus or *dl434* and harvested for determination of CAT activity.

Each of the mutant E3 constructs was transcribed in vitro and, as a control, an HIV LTR CAT construct, which gave a 145-bp primer-extended species, was included in each transcription reaction (Fig. 3B). Mutation of site IV had little effect on the in vitro transcription of the E3 promoter. Mutants of either site II or III decreased E3 transcription, while a double mutant of both of these sites resulted in a much larger decrease in in vitro transcription (Fig. 3B). Mutation of site I also resulted in a large decrease in the transcription of the E3 promoter (Fig. 3B). This large effect of the TATA mutant in vitro compared with the minor effect seen in vivo indicates the increased importance of this element in vitro compared with in vivo (26, 39).

E1A/E1B proteins increase the activity of the E3 promoter in vitro. To determine whether the in vivo activation of the E3 promoter in the presence of E1A/E1B would be reflected in vitro, nuclear extracts were prepared from wild-type adenovirus-infected and *dl434*-infected HeLa cells. These extracts were prepared at 30 h postinfection, at the time high levels of E1A/E1B proteins were present (17), and at the same time at which transfections with the E3 constructs were assayed. Similar studies with the HIV LTR have indicated that extracts containing the E1A/E1B proteins strongly induce in vitro transcription from this promoter and a variety of other promoters which are E1A inducible in transient assays of gene expression (35).

Transcription from the wild-type E3 promoter was induced approximately 30-fold in wild-type adenovirus-infected extracts compared with *dl434*-infected extracts as

TABLE 1. CAT activity of the E3 promoter in the presence of adenovirus infection^a

Virus	% CAT conversion	Relative CAT activity
None (mock infection)		0.05–0.015
Wild type	30.8	1.00
<i>dl434</i>	3.1	0.08–0.14
<i>dl312</i>	7.3	0.23–0.30

^a Both unacetylated and acetylated [¹⁴C]chloramphenicol were determined by scintillation counting of CAT assays to determine the percent CAT conversion. The percent CAT conversion was calculated for the experiment depicted in Fig. 2A. The CAT activity was determined relative to that of wild-type infection (1.00) for mock-infected, *dl434*-infected, and *dl312*-infected HeLa cells in three independent experiments.

TABLE 2. CAT activity of E3 regulatory region mutations

Construct	% CAT conversion ^a with infection by:		Fold activation ^b
	Wild-type adenovirus	<i>dl434</i>	
ΔI	16.4	2.4	7–10
ΔII	0.9	0.1	6–12
ΔIII	4.6	0.5	9–11
ΔII/III	0.1	0.1	ND ^c
ΔIV	27.5	2.6	7–10
Wild type	36.8	4.1	9–13

^a Both unacetylated and acetylated [¹⁴C]chloramphenicol were determined by scintillation counting of CAT assays in the presence and absence of the E1A/E1B gene products to determine the percent CAT conversion (calculated for the experiment depicted in Fig. 2B).

^b The fold activation was determined by calculating the ratio of CAT activity in the presence of wild-type adenovirus infection compared with that in the presence of *dl434* for three independent experiments, and the ranges of activation in these experiments are indicated.

^c ND indicates that the fold activation could not be determined.

determined by densitometry (Fig. 4). Mutations of site IV did not significantly alter activation of the E3 promoter (Fig. 4). Mutations of site III slightly decreased E1A/E1B-induced levels of transcription, while mutations of either site I or II had marked effects on E1A/E1B-induced levels of transcription (Fig. 4). A double mutation of sites II and III also resulted in a large decrease in E1A/E1B-induced levels of transcription (Fig. 4). Thus, mutations of sites I, II, and III significantly affected both E1A/E1B-induced levels of transcription and basal activity of the E3 promoter in vitro.

E1A/E1B proteins do not alter the binding to E3 regulatory regions. Oligonucleotides complementary to E3 regulatory regions were synthesized and used in gel retardation assays with uninfected HeLa extract. Gel retardation assays revealed specific binding to site II (lane 2), III (lane 6), and IV

FIG. 3. In vitro transcription of the E3 promoter in uninfected HeLa extract. (A) The E3 CAT plasmid was transcribed in vitro with 50 μg of HeLa extract in either the absence (lane 1) or presence (lane 2) of 2 μg of alpha amanatin per μl (lane 2). Molecular weight markers (M) are shown in the left lane, and the position of the 115-bp E3 primer-extended product is indicated. (B) The wild-type (WT) E3 CAT plasmid and E3 CAT mutants (ΔI, ΔII, ΔIII, ΔIV, and ΔII/III) plus the HIV LTR CAT plasmid were transcribed in vitro with 50 μg of HeLa extract. The positions of both 115-bp E3 and the 145-bp HIV primer-extended products are indicated.

FIG. 4. In vitro transcriptional activation by the E1A/E1B protein. Wild type (wt) and ΔI , ΔII , $\Delta II/III$, ΔIII , and ΔIV E3 CAT plasmids were transcribed in vitro with 25 μ g of nuclear extract prepared from either *dl434*-infected HeLa cells (odd-numbered lanes) or wild-type adenovirus-infected HeLa cells (even-numbered lanes). The position of the 115-bp E3 specific primer-extended product is indicated.

(lane 10) oligonucleotides (Fig. 5A). This specificity was indicated by competition by a 50-fold excess of specific competitor oligonucleotides (Fig. 5A, lanes 4, 8, and 12) and a lack of competition by mutated oligonucleotides (Fig. 5A, lanes 3, 7, and 11). However, we were unable to obtain specific binding to the TATA element with gel retardation assays (data not shown).

It was important to determine whether the increased transcriptional activity found in extracts containing the E1A/E1B proteins resulted from an increased binding activity to E3 regulatory regions. Gel retardations were then performed with increasing amounts of either wild-type-adenovirus-infected or *dl434*-infected HeLa cell nuclear extracts (Fig. 5B). No major changes in either the number or intensity of gel-retarded species were detectable with either the site II, III, or IV oligonucleotides (Fig. 5B). Thus, even though the transcriptional activities of the extracts containing the E1A/E1B proteins were higher than those of extracts lacking these proteins, no changes in binding activity to E3 regulatory region oligonucleotides were found. This suggests that transcriptional activity of the E1A/E1B proteins involves properties other than effects on DNA binding to isolated binding motifs of the E3 promoter. However, this does not rule out potential interactions of E1A/E1B proteins with multiple components of the E3 basal promoter complex.

E1A/E1B proteins do not increase the expression of *c-jun* and CREB genes. Even though the binding activity of ATF and AP1 proteins to the site II and site III oligonucleotides did not appear to be altered, we next examined whether E1A/E1B proteins could be responsible for increasing the level of these transcription factor mRNAs. Since CREB and ATF proteins appear to be related or identical, we used the cDNA encoding human CREB (28) along with probes specific for the human *c-jun* cDNA (2) and β -actin cDNA (51) to probe Northern blots of RNA prepared from wild-type-adenovirus-infected or *dl434*-infected HeLa cells. Cytoplasmic RNA was prepared from HeLa cells at the time of viral infection (Fig. 6, lane 1) and at 6 and 30 h postinfection for both *dl434* (Fig. 6, lanes 2 and 4) and wild-type adenovirus (Fig. 6, lanes 3 and 5) (at the time nuclear extracts were prepared). There was no change in the quantity of CREB RNA during either *dl434* or wild-type adenovirus infection. Several RNA species hybridized to the labeled CREB probe under high-stringency hybridization, suggesting that multiple RNA species can encode CREB or related proteins (9). The amount of the 2.3-kb *c-jun* mRNA was also unchanged during viral infection (Fig. 6B). An actin probe revealed that equivalent levels of the 2.1-kb actin mRNA were present in each of the virus-infected RNA samples (51) (Fig. 6C).

FIG. 5. Gel retardations of site II, III, and IV E3 regulatory regions with either mock-infected, wild-type-adenovirus-infected, or *dl434*-infected HeLa extract. (A) Oligonucleotides complementary to E3 regulatory regions (II, III, and IV) were incubated either in the absence of extract (lanes 1, 5, and 9) or with 5 μ g of uninfected HeLa cell nuclear extract (lanes 2, 6, and 10) in the presence of a 50-fold excess of mutant oligonucleotides (ΔII to ΔIV) (lanes 3, 7, and 11, respectively) or in the presence of a 50-fold excess of wild-type oligonucleotides (II to IV) (lanes 4, 8, and 12, respectively). (B) Oligonucleotides complementary to E3 regulatory regions (II, III, and IV) were incubated either in the absence of extract (lane 1) or in the presence of 1, 3, or 5 μ g of HeLa cell nuclear extract (lanes 2, 3, and 4) prepared from either *dl434*-infected or wild-type (WT)-adenovirus-infected HeLa cells.

To extend the studies of *c-jun* and CREB RNA levels, immunoprecipitation of both of these proteins was performed. Both *dl434* and wild-type-adenovirus-infected cells were labeled with either [35 S]methionine and [32 P] from 26 to 30 h postinfection and immunoprecipitated with polyclonal rabbit antiserum to either *c-jun* or CREB. Minimal differences in the levels of AP1 were seen in the presence of *dl434* (Fig. 7A, lane 3) and wild-type adenovirus infection (lane 5) with [35 S]methionine labeling. Preimmune antiserum did not precipitate species corresponding to AP1 (Fig. 7A, lanes 4 and 6). In vitro-translated *c-jun* migrated identically to the immunoprecipitated cellular proteins. Similar results were obtained by using this antiserum to immunoprecipitate [32 P]-labeled *dl434*- (Fig. 7B, lane 3) and wild-type-adenovirus-infected cells (Fig. 7B, lane 4). Thus, the level of AP1 did not appear to be altered by the E1A/E1B proteins.

Immunoprecipitation of CREB from [35 S]methionine-la-

FIG. 6. Northern analysis of CREB and *c-jun* RNA in wild-type adenovirus-infected and *dl434*-infected HeLa cells. Cytoplasmic RNA was harvested from either mock-infected HeLa cells (lane 1), *dl434*-infected HeLa cells at 6 and 30 h postinfection (lanes 2 and 4), or wild-type-adenovirus-infected HeLa cells at 6 and 30 h postinfection (lanes 3 and 5). Twenty micrograms of poly (A) mRNA was loaded in each lane, and the filter was sequentially probed with random-primed fragments for CREB (A), *c-jun* (B), and β -actin (C). RNA markers of 7.0 and 2.3 kb are indicated.

beled cells did not result in detectable CREB species (data not shown). The reasons for this remain unclear. However, CREB was readily detected in immunoprecipitation from $^{32}\text{P}_i$ -labeled cells. There were minimal differences in the level of CREB from *dl434*-infected (Fig. 7C, lane 4) and wild-type-adenovirus-infected cells (lane 5). This immunoprecipitated species had a mobility identical to that of in vitro-translated CREB (Fig. 7C, lane 1) and was not detected with preimmune serum (Fig. 7C, lanes 2 and 3). The presence of a lower-molecular-weight species was also detected by using specific antibody to CREB. These results suggest that the levels of AP1 and CREB are not altered by the E1A/E1B proteins and that no stable high-molecular-weight complexes of E1A with either AP1 or CREB were detectable.

DISCUSSION

In this study, we have investigated the sequence elements required for adenovirus-induced activation of the E3 promoter. Our results demonstrate that both the ATF- and AP1-binding sites are critical for E1A/E1B activation of the

E3 promoter in vivo, while these same binding sites, in addition to the TATA element, were required for in vitro activation. Mutations that reduced E1A/E1B-induced expression also resulted in a proportional decrease in basal levels of transcription, thereby maintaining a constant fold induction. Thus, the same sequence elements which confer basal expression are necessary for E1A/E1B-induced levels of transcription. However, since cytosine arabinoside does not completely inhibit late viral gene expression, we cannot conclusively rule out that late viral proteins or other early proteins may also be involved in the transcription induction by wild-type adenovirus. There was no change in the DNA-binding activity to ATF-, AP1-, and NF1-binding sites in extracts prepared from wild-type-adenovirus-infected cells compared with that in extracts prepared from *dl434*-infected cells. No increase in the level of CREB and *c-jun* mRNA or protein was detected during the course of adenovirus infection. However, we cannot rule out that E1A may activate other members of the *jun* family, such as *jun B* or *jun D* (49), or other members of the CREB gene family, such as CREB1 (45). Recently, it was demonstrated that E1A in combination with cyclic AMP can increase the levels of *jun B* RNA (48). Thus, E1A in combination with other cellular activators may modulate the mRNA levels of certain transcription factor genes. Our results would be consistent with a model of E1A/E1B activation of the E3 promoter by either a direct or indirect effect on multiple factors binding to the E3 basal promoter complex.

Previous studies with adenovirus promoters have indicated that early promoters, with the exception of E1B, have a binding site for a factor known as ATF (5, 15, 18, 24, 25, 31, 37–39, 41, 42, 44, 50, 54, 56, 65). The ATF factor has been purified and found to have a molecular mass of from 43 to 47 kilodaltons (31). This is the same molecular mass as the CREB protein which binds to cyclic AMP response elements in a number of genes (28, 47, 63). Since both CREB- and ATF-binding sites give similar gel retardation patterns with cellular extract, these proteins may be related or identical (24, 37, 41). Several studies demonstrated that both CREB- and ATF-binding sites are inducible by cyclic AMP and

FIG. 7. Immunoprecipitation of CREB and AP1 in wild-type-adenovirus-infected and *dl434*-infected HeLa cells. HeLa cells infected with either *dl434* or wild-type adenovirus were labeled from 26 to 30 h postinfection with ^{35}S methionine (A) or $^{32}\text{P}_i$ (B and C). Nuclear extracts were prepared for immunoprecipitation with either preimmune serum or antiserum to *c-jun* (A and B) or CREB (C), and electrophoresis on 8% SDS-polyacrylamide gels was performed. (A) Lane 1, in vitro translated *c-jun*; lanes 2 and 3, immunoprecipitation of *dl434*-infected extract with preimmune serum and *c-jun* antiserum, respectively; lanes 4 and 5, immunoprecipitation of wild-type-adenovirus-infected extract with preimmune serum and *c-jun* antiserum, respectively. (B) Lane 1, in vitro translated *c-jun*; lanes 2 and 3, *dl434*- and wild-type-infected extracts immunoprecipitated with preimmune serum; lanes 4 and 5, the same extracts as in lanes 2 and 3, respectively, immunoprecipitated with *c-jun* antiserum. (C) Lane 1, in vitro-translated CREB; lanes 2 and 3, *dl434*-infected and wild-type-infected extracts immunoprecipitated with preimmune serum, respectively; lanes 4 and 5, the same extracts as in lanes 2 and 3, respectively, immunoprecipitated with CREB antiserum. Kd, Kilodaltons. The arrow indicates the position of either the CREB or AP1 species.

E1A, though the magnitude of this induction may be promoter specific (12, 24, 42). In addition, the mechanism of E1A and cyclic AMP induction may differ (12, 37, 42). Mutagenesis of ATF sites in a number of adenovirus early promoters has indicated that although this site is important for basal and E1A-induced transcriptional levels, it is not uniquely required for this induction (15, 18, 39, 50, 65). The DNA-binding activity of this factor does not appear to be altered by adenovirus infection (15, 32, 44, 56). In addition, promoters lacking this binding site, such as the E1B promoter, are still E1A inducible (50, 62). Thus, although the ATF site is a critical promoter element for most early adenovirus genes, it is not absolutely required for E1A induction.

Another promoter element which has been shown to be important for E1A induction is the TATA element (40, 55, 61, 62). Mutations of this element have been shown to markedly decrease E1A activation of the E1B (62) and hsp70 (61) promoters and the HIV LTR (35). The results of our *in vivo* analysis of the E3 promoter indicate that the binding motifs for ATF and AP1 are critical for E1A/E1B activation. Unlike the E1B and hsp70 promoters and the HIV LTR, mutation of the E3 TATA sequence had little effect on basal and E1A/E1B-induced transcriptional levels *in vivo*. Furthermore, deletion of the E3 TATA region also had minimal effects on E1A activation of the E3 promoter (15, 39). The differences in the requirement of the TATA motif for transactivation by E1A may be due to variations in the primary sequence of the TATA element or the context of the TATA box with respect to other binding sites (30, 55). Different TATA elements might be recognized by different TATA-binding proteins or, alternatively, a single TATA-binding protein might bind distinct TATA elements differently, thus responding differently to E1A/E1B (55). In contrast to the lack of dependence on the TATA element *in vivo*, there was a strong dependence on this element *in vitro* for basal and E1A/E1B-induced transcription levels, perhaps due to increased TATA factor binding *in vitro* (39, 40). This strong dependence on the TATA element *in vitro* has been demonstrated with E3 and other promoters (26, 39, 40).

The mechanism by which the E1A/E1B proteins activate transcription remains open to question. The binding activities of the ATF, AP1, and NF1 regions were not altered by E1A/E1B, even though these extracts were increased in transcriptional activity by *in vitro* transcription performed with the E3 promoter. Furthermore, the CREB and *c-jun* genes were not activated, as determined by Northern analysis. Thus, two models for E1A/E1B activation of the E3 promoter seem plausible. First, E1A might modulate the phosphorylation state of the E3-binding transcription factors, either directly or indirectly. This model is supported by reports that E1A can affect the phosphorylation of three factors: E2F, E4F, and TF III-C (3, 29, 52). E1A is able to induce a cellular kinase which is capable of phosphorylating E2F and E4F (3, 52). This increased phosphorylation is associated with increased binding of these factors to the E2 and E4 promoters, respectively (3, 52). E1A has also been shown to activate RNA polymerase III transcription by activating the binding of the factor TF III-C, which is also associated with increased phosphorylation of this factor (29, 64).

A second potential model for E1A activation is a direct interaction with the factors constituting the basal promoter complex. Studies with E1A-gal4 fusions demonstrated that E1A-gal4 would strongly activate only in the presence of promoters containing gal4-binding sites (43). This suggests

that E1A can interact directly with the promoter structure. E1A contains a zinc finger-like structure in the transactivating region which could facilitate this interaction (10). Another transactivator, VP16, has been shown to directly interact with the transcription factor oct-1 (58). However, no changes in gel retardations were seen in extracts containing E1A/E1B proteins, indicating that the E1A/E1B proteins did not form a detectable stable interaction with DNA-binding proteins. Neither were detectable stable complexes of E1A with either CREB or AP1 detected by immunoprecipitation. Such complexes have been noted between E1A and a number of cellular proteins, such as the retinoblastoma protein (60). However, more than one component of the E3 basal promoter complex may be required for stable interaction with E1A. A mechanism by which E1A either directly or indirectly interacts with multiple promoter-specific factors could also be responsible for E1A activation. However, these two mechanisms are not mutually exclusive, and both mechanisms of activation may come into play, thereby explaining the diverse promoters activated by E1A.

In addition to E1A, other early adenovirus genes also play a role in activation of early adenovirus promoters. The E4 gene product has been shown to be important in E2F activation (53). The E4 product itself can activate the E2 early promoter, though the presence of the E1A protein greatly enhances this activation (19, 53). The E1B 19-kilodalton protein has also been shown to be important in activating a variety of transfected genes by increasing the stability of transfected DNA (27). However, other studies have indicated that the 19-kilodalton protein will activate early adenovirus genes, likely by a direct effect of this protein on gene expression (59). In addition, *cis*-acting sequences in the E1B promoter gene are also capable of increasing E1A gene expression (33). Thus, at least two other early adenovirus genes play a role in activation of adenovirus promoters, and a complex interplay of viral encoded factors are likely to be involved in adenovirus transcriptional activation. The isolation and characterization of cellular transcription factors will be required to elucidate the mechanisms by which E1A and other viral regulatory proteins activate the early adenovirus promoter.

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ADDENDUM IN PROOF

Recently, it has been demonstrated that the 19-kilodalton E1B protein increased the stability of transfected plasmids (C. H. Herrman and M. B. Mathews, *Mol. Cell. Biol.* 9:5412-5423). Our experiments indicated that there were no differences in the stability of DNA templates added to *dl434*- and wild-type adenovirus-infected extracts used in *in vitro* transcription assays. Thus, the differences in *in vitro* transcription (Fig. 4) were not due to changes in the stability of the DNA templates in these extracts. Also, the multiple RNA species seen with the CREB cDNA probe in Northern analysis (Fig. 6A) are likely due to the multiple members of the ATF family (*Genes Dev.* 3:2083-2090).

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