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A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters

(cellular transcription factor/DNA-binding proteins)

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ABSTRACT **We have examined the relationship between sequence-specific DNA-binding proteins that activate transcription of E1A-inducible adenovirus early promoters. Factors previously referred to as E4F1 and E2A-EF bind to the *E4* and *E2A* promoters, respectively. We demonstrate here that E4F1 and E2A-EF have identical DNA-binding specificity. Moreover, E4F1 and E2A-EF both activate transcription of the *E4* and *E2A* promoters *in vitro*. These findings demonstrate that E4F1 and E2A-EF are the same factor, which we have designated activating transcription factor, or ATF. In addition to the *E4* and *E2A* promoters, ATF binds to an important functional element of the E1A-inducible *E3* promoter. Interaction of a common activator protein, ATF, with multiple E1A-inducible early viral promoters, suggests a significant role for ATF in E1A-mediated transcriptional activation.**

The *E1A* gene of adenovirus produces closely related 13S and 12S mRNAs that encode nuclear-localized phosphoproteins with diverse transcriptional regulatory properties (1–3). The E1A 13S gene product coordinately activates a set of viral early genes (*E1A*, *E1B*, *E2A*, *E3*, and *E4*) during a productive infection of permissive human cells (4–7). The E1A 12S gene product encodes a transcriptional repression function that appears to act through transcriptional enhancer elements (8–10). In addition to regulating viral transcription, E1A activates or represses transcription of a limited number of cellular genes (11, 12), and activates polymerase III-dependent promoters (for review, see ref. 7).

Despite numerous studies, several factors have contributed to the difficulties in elucidating the mechanism(s) by which E1A activates transcription of early viral genes. (*i*) Unlike many other transcriptional regulatory proteins, E1A is not a sequence-specific DNA-binding protein (13), implying that E1A acts indirectly via interaction with cellular transcription components. (*ii*) Extensive mutagenesis studies have failed to identify common promoter target sequences that signal induction by E1A (see ref. 7). (*iii*) The biochemical activities and cellular components (transcription factors) required for transcriptional activation by E1A protein are largely unidentified.

Several groups have recently identified cellular factors that interact with E1A-inducible viral promoters (14–19). In one case, a protein factor that interacts with the *E2A* promoter (E2F) is markedly increased in DNA-binding activity (or amount) by E1A (15, 20). This suggests that activation of E2F is required for E1A-mediated activation of the *E2A* promoter. However, E2F protein does not interact with all early viral promoters (21), and consequently, activation of E2F cannot entirely account for coordinate activation of early viral genes. Other observations support this view. For the *E4* and *E3* promoters, there is currently no evidence that E1A acts by increasing the DNA-binding activity of factors required for *E4* and *E3* transcription (18, 19). For the *E1B* promoter (22) and the cellular β-globin promoter (23), the "TATA" box has been implicated as an E1A-responsive promoter element. Thus activation of a variety of E1A-inducible promoters appears to involve different cellular factors and may occur through divergent pathways, ultimately linked by E1A.

Two independent studies have identified additional factors that interact with early viral promoters. A factor referred to as E4F1 binds to the *E4* promoter and also interacts with the *E1A*, *E2A*, and *E3* promoters (18). Similarly, a factor referred to as E2A-EF binds to the *E2A*, *E1A*, *E3*, and *E4* promoters (17). We show here that E4F1 and E2A-EF have the same DNA-binding specificity and that both factors activate transcription of the *E4* and *E2A* promoters *in vitro*. These results demonstrate that E4F1 and E2A-EF are the same factor, which we refer to as ATF, for activating transcription factor. In addition to the *E4* and *E2A* promoters, ATF interacts with an important functional element of the adenovirus *E3* promoter. The interaction of ATF with multiple E1A-inducible promoters suggests a significant role for ATF in E1A-mediated transcriptional activation.

MATERIALS AND METHODS

Plasmids. pE4WT contains the adenovirus type 5 genome between map units 100 and 89, including the entire *E4* gene cloned into pBR322 between the *Eco*RI and *Pvu* II sites. βΔ128 contains the entire human β-globin gene including 128 base pairs (bp) of 5′ flanking sequences cloned between the *Eco*RI and *Pvu* II sites of pBR322. pBR730 contains adenovirus type 5 sequences between map units 59.5 and 75.9 (containing the *E2A* promoter) cloned between the *Eco*RI and *Bam*HI sites of pBR322.

***In Vitro* Transcription.** Nuclear extracts were prepared from HeLa cells as previously described (18, 24). Transcription reactions and competition assays were done as described (18). RNA was prepared and analyzed by primer extension as described (10).

DNase I "Footprinting" Experiments. The 3′ ^{32}P end-labeled probes were prepared by isolating a DNA fragment containing *E4* promoter sequences between −138 and +250 and labeling at position −138 with reverse transcriptase. Footprinting assays were done as described (18). Competitor DNA and labeled probe (≈1 ng per reaction) were added simultaneously to the binding reactions. DNase I digestions were terminated by the addition of 3 vol of 2× proteinase K buffer (25) containing an additional 10 mM EDTA followed by proteinase K treatment, purification of labeled DNA, and fractionation on denaturing polyacrylamide gels as described (18).

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Abbreviations: Me_2SO_4, dimethyl sulfate; E2F, *E2* promoter binding factor; ATF, activating transcription factor; . . .F, factor.

Gel Retardation Assays. Probes used for gel retardation assays are described in the figure legends. Labeled probes were prepared by end labeling gel-purified DNA fragments or synthetic double-stranded oligonucleotides. Gel retardation assays were done as described (17) using either crude nuclear extract from uninfected HeLa cells or a nuclear protein fraction obtained as follows. Crude nuclear extract was loaded onto a phosphocellulose (P11) column at 40 mM KCl followed by step elution with 250 mM and 600 mM KCl. The 600 mM fraction (containing most of the binding activity) was dialyzed against buffer D (24) containing 100 mM KCl and then loaded onto a DEAE-Sephacel column equilibrated in buffer D. The flow-through fraction contained most of the ATF-binding activity, and we refer to this fraction as DEAE-fractionated nuclear extract.

Dimethyl Sulfate (Me_2SO_4) Footprinting Experiments. Binding reactions using 10 μg of protein from DEAE-fractionated nuclear extract were done as for DNase I footprinting experiments. Me_2SO_4 was diluted 10-fold in water, and 1 μl of diluted solution was added to a 20-μl binding reaction. After incubation at room temperature for 4 min, reactions were stopped by addition of 50 μl of stop solution (26), 180 μl of water, and 750 μl of ethanol, precooled on dry ice. DNA was precipitated by centrifugation for 15 min at 4°C in a Microfuge; the pellet was resuspended in 200 μl of 0.3 M sodium acetate (pH 7) and ethanol-precipitated once more. The pellet was resuspended in 100 μl of 1 M piperidine and incubated at 90°C for 30 min. Samples were lyophilized three times and then fractionated on denaturing polyacrylamide gels.

RESULTS

A Related Protein Factor Binds to the Adenovirus *E4*, *E3*, and *E2A* Promoters. Using a DNase I footprinting assay we

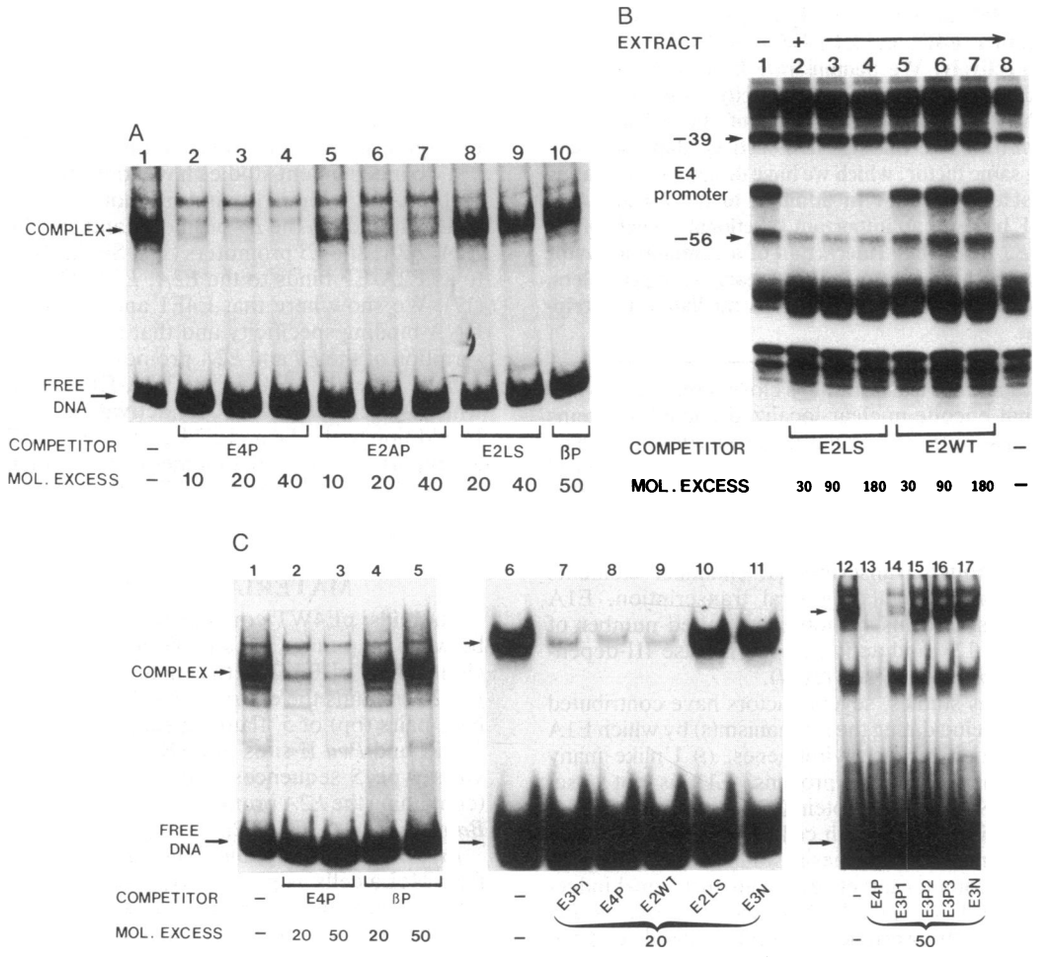

FIG. 1. A related factor binds to the *E4*, *E3*, and *E2A* promoters. Labeled DNA probes and unlabeled competitor DNA fragments were as follows: E4P (containing an E4F1 binding site), 27-bp synthetic oligonucleotide (oligo) containing positions −37 to −63 of the *E4* promoter; E2AP (containing an E2A-EF binding site), 27-bp oligo containing positions −63 to −85 of the *E2A* promoter; E3P1 (containing an E3F2 binding site), 24-bp oligo containing positions −44 to −67 of the *E3* promoter; E3P2 (containing an E3F3 binding site), 22-bp oligo containing positions −82 to −103 of the *E3* promoter; E3P3 (containing an E3F4 binding site), 24-bp oligo containing positions −157 to −180 of the *E3* promoter; E3N, "nonsense" 22-bp oligo (containing no factor-binding sites) containing positions −107 to −128 of the *E3* promoter; E2WT, 81-bp DNA fragment containing positions −17 to −98 of the *E2A* promoter; E2LS, equivalent to E2WT but lacking positions −74 to −85 of the *E2A* promoter (17); βP, 24-bp oligo containing sequences from the human β-globin pre-mRNA (5′ GCCCT CTATTTTCCCACCCTTAGG 3′). (*A*) E4F1 was detected by a retardation assay using crude nuclear extract. E4P contained within an ≈100-bp DNA fragment from the polylinker region of pGEM3 was the labeled probe. Excess of unlabeled competitor DNA is indicated at bottom. Lane 1, no competitor DNA; lanes 2–4, E4P as competitor DNA; lanes 5–7, E2AP as competitor DNA; lanes 8 and 9, E2LS as competitor DNA; lane 10, βP as competitor DNA. (*B*) E4F1 was assayed in the crude nuclear extract by DNase I footprinting as described, in the presence of excess unlabeled competitor DNA as indicated at bottom. Lane 1, no nuclear extract; lanes 2–4, 10 μl of nuclear extract and increasing amounts of E2LS as competitor; lanes 5–7, 10 μl of nuclear extract and increasing amount of E2WT as competitor; lane 8, 10 μl of nuclear extract in the absence of competitor. (*C*) E2A-EF, E3F2, and E4F1 were assayed by gel retardation assays using E2WT, E3P1, and E4P, respectively, as labeled probes, in the presence of excess unlabeled competitor DNAs as indicated at the bottom. Lanes 1–5, E2WT as probe; lanes 6–11, E3P1 as probe; lanes 12–17, E4P as probe.

have recently detected a cellular factor (E4F1) that binds to multiple sites in the adenovirus *E4* promoter and activates transcription (18). Based on sequences present within E4F1 binding sites of the *E4* promoter (18) we inferred a consensus E4F1 binding site (ACGTA/CAC). Significantly, related sequences are present in functionally important regions of the adenovirus *E2A* (27) (ACGTCAT, positions −71 to −77) and *E3* (28) (TCGTCAC, positions −59 to −53) promoters, and factors have been shown to bind to these regions. A protein factor called E2A-EF binds to the *E2A* promoter between positions −82 to −66 (17), and a protein factor called E3F2 binds to the *E3* promoter between positions −68 to −44 (19).

To determine the relationship between E4F1, E2A-EF, and E3F2 we first performed a series of competition studies (Fig. 1). Using a gel retardation assay for E4F1, we examined the ability of various *E2A* promoter fragments to compete for E4F1 binding (Fig. 1*A*). A 20-fold molar excess of a 27-bp synthetic oligonucleotide containing an E2A-EF-binding site (E2AP) efficiently competes for E4F1 binding, whereas a 20-fold molar excess of an *E2A* promoter fragment (E2LS), containing a mutated E2A-EF-binding site, fails to compete. [E2LS contains a linker scan mutation in which positions −74 to −85 of the *E2A* promoter are replaced by linker DNA (17). E2LS does not bind E2A-EF (17) and is impaired for *E2A* transcription (27).] These data indicate that E4F1 and E2A-EF are closely related. To demonstrate that E4F1 as detected by DNase I footprinting is related to E2A-EF, we performed competition studies using the DNase I footprinting assay for E4F1 (Fig. 1*B*). As previously shown, E4F1 binds to the region between −39 and −56 of the *E4* promoter (18). Binding of E4F1 is efficiently inhibited by a 30-fold molar excess of E2WT DNA, but this binding is not inhibited by a 180-fold molar excess of E2LS promoter fragment.

We next examined the ability of E4P to compete for binding of E2A-EF to the *E2A* promoter (Fig. 1*C*). Using a gel-retardation assay for E2A-EF (Fig. 1*C*, lanes 1–5), a 20-fold molar excess of E4P DNA efficiently competes for binding of E2A-EF, whereas a 50-fold excess of βP DNA fails to compete. We also assayed E3F2 (19) using a gel retardation assay (Fig. 1*C*, lanes 6–11) and found that E3F2 binding to the *E3* promoter is efficiently competed against by E4P and E2WT DNA fragments but not by the E2LS fragment, which lacks the E2A-EF binding site. Finally we assayed E4F1 by using E4P-labeled DNA fragment as probe (Fig. 1*C*, lanes 12–17) and found that an oligonucleotide (E3P1) containing an E3F2-binding site competes for E4F1 binding. In contrast, oligonucleotides containing binding sites for other protein factors that interact with the *E3* promoter [E3P2 and E3P3 oligonucleotides contain binding sites for E3F3 and E3F4 proteins, respectively, and E3N, the nonsense oligonucleotide, contains *E3* promoter sequences that do not bind any factors (19)] do not compete for E4F1 binding. Thus E4F1 is related to E3F2, but the former protein factor is not related to E3F3 or E3F4. We therefore conclude that the DNA-binding specificities of E4F1, E2A-EF, and E3F2 are closely related.

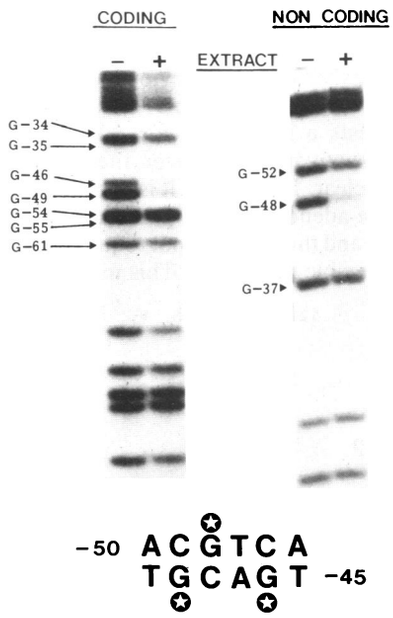

FIG. 2. Methylation protection by E4F1. Nuclear extract (DEAE-fractionated) was incubated with 3′ end-labeled probe from the *E4* promoter region under footprinting conditions as described. Guanosine residues and their positions with respect to the *E4* transcription start site are indicated to the left, for both the coding and noncoding strands. Guanosine residues that are protected by E4F1 are indicated by stars within the E4F1 binding site at the bottom.

VIRAL PROMOTERS	SEQUENCE	POSITION
Adenovirus 5 E4	A C G T C A	−50/−45
	A C G T C A	−164/−170
	T C G T C A	−140/−146
Adenovirus 5 E2A	A C G T C A	−71/−76
Adenovirus 5 E3	T C G T C A	−59/−54
HTLV-II LTR	A C G T C A	−174/−180
BLV LTR	A C G T C A	−156/−161
Intracisternal A partical	A C G T C A	−96/−101
CELLULAR PROMOTERS		
c-fos	A C G T C A	−61/−66
Hsp 70	T C G T C A	−31/−36
Somatostatin	A C G T C A	−43/−48
Tyrosine hydroxylase	A C G T C A	−43/−49
Alpha-gonadotropin	A C G T C A	−122/−117
VIP	A C G T C A	−71/−76
Fibronectin	A C G T C A	−171/−166
ATF CONSENSUS	A/T C G T C A	

FIG. 3. ATF binding sites in other viral and cellular transcriptional control regions. The consensus binding site is derived from the ATF-binding sites described in this study, by taking into account the sequences surrounding the three important guanosine contacts between ATF and DNA. Protected guanosine residues (determined by methylation protection or interference assays) are indicated by closed circles. Note that in some cases the consensus sequence is on the coding strand and in other cases on the noncoding strand, consistent with the ability of ATF to function bidirectionally (18). We previously showed that ATF interacts with the adenovirus *E1A* promoter (18) and noted the position of putative ATF binding sites within the *E1A* promoter region (GCGTAAC, −249 to −243; ACGTAAA, −121 to −127; ACGTCAG, −38 to −44). We have not determined whether ATF interacts with these sequences within the *E1A* promoter. HTLV, human T-cell leukemia virus; LTR, long terminal repeat; BLV, bovine leukemia virus; Hsp, heat shock protein; VIP, vasoactive intestinal peptide.

Protein Factors E4F1, E2A-EF, and E3F2 Bind to Identical Sequences. To determine some of the important DNA–protein contacts in the E4F1-binding site between positions −56 and −39 of the *E4* promoter, we performed methylation protection experiments for both coding and noncoding strands (Fig. 2). Unprotected DNA, or DNA protected by factors present in DEAE-fractionated nuclear extract, was treated with Me_2SO_4, isolated, cleaved with piperidine, and analyzed on denaturing gels. The guanosine residues present at positions −46 and −49 of the coding strand and position −48 of the noncoding strand are protected from methylation by E4F1. We also determined the pattern of methylation protection for the high-affinity E4F1 site in the *E4* enhancer (between positions −170 and −164) and obtained the same result (data not shown, see Fig. 3). A summary of the methylation-protection data for the analyzed E4F1-binding site is indicated schematically at the bottom of Fig. 2. Significantly, the three guanosine residues in the E4F1 consensus sequence that contact E4F1 are also important contacts in the binding of E2A-EF (17, 20) and E3F2 (19) to their respective promoters (Fig. 3). Thus from all the above data, we conclude that E4F1, E2A-EF, and E3F2 have essentially identical DNA-binding specificities.

Factors E4F1 and E2A-EF Activate Transcription of the *E4* and *E2A* Promoters *in Vitro*. We have previously shown that E4F1 is required for efficient transcription of the *E4* promoter *in vitro* in nuclear extracts from uninfected HeLa cells (18); this conclusion is based on the ability of E4P DNA to specifically compete for binding of E4F1 and inhibit *E4* transcription, as shown below. Using this assay we tested whether E4F1 and E2A-EF are functionally equivalent (Fig. 4). In control experiments a 75-fold molar excess of E4P DNA almost abolishes *E4* transcription, whereas the same amount of an unrelated double-stranded oligonucleotide (βP) has no effect (Fig. 4, lanes 3–5). In addition, inhibition of *E4* transcription is promoter specific [as previously shown (18)] because E4P DNA does not inhibit β-globin transcription even at 180-fold molar excess (Fig. 4, lanes 1 and 2). We examined whether E4F1 is required for transcription of the *E2A* promoter *in vitro*. As previously shown for the adenovirus type 2 *E2A* promoter (29, 30) the adenovirus type 5 *E2A* promoter is accurately transcribed *in vitro* in the crude nuclear extract, although less efficiently than the *E4* promoter. Significantly, *E2A* transcription is inhibited by the same concentrations of E4P required to inhibit *E4* transcription [Fig. 4, lanes 6–8 (E4) and lanes 9–11 (*E2A*)]. Thus E4F1 is required for *E2A* transcription *in vitro*. In agreement with this conclusion, the E4F1 binding site in the *E2A* promoter maps precisely to a cis-acting promoter element previously defined *in vitro* for the adenovirus type 2 *E2A* promoter (30). We next examined whether *E2A* promoter fragments containing or lacking an E2A-EF binding site could inhibit *E4* transcription. A 40-fold molar excess of E2WT DNA strongly inhibited *E4* transcription, whereas the same amount of E2LS DNA had a slight stimulatory effect (Fig. 4, lanes 12–16). We conclude that E2A-EF is able to activate the *E4* promoter. Thus, by the criteria of transcriptional activation of common promoters, E4F1 and E2A-EF are functionally equivalent. Together with their identical DNA-binding specificity, these data demonstrate that E4F1 and E2A-EF are the same factor.

DISCUSSION

We have shown that a cellular factor, ATF, interacts with the E1A-inducible adenovirus *E4*, *E2A*, and *E3* promoters. This factor corresponds to E4F1, E2A-EF, or E3F2 described in previous studies on the individual early viral promoters (17–19). Our data cannot rule out the possibility that multiple factors with identical DNA-binding specificities bind to ATF consensus sequences, as appears to be true for factors binding to the octamer motif (31). However, because the *E2A*, *E3*, and *E4* promoters are physically linked and coordinately activated within the same cell, we favor the interpretation that E4F1, E2A-EF, and E3F2 represent a single factor, which we refer to as ATF.

Several pieces of evidence indicate that ATF is required for transcriptional activation of the *E2A*, *E3*, and *E4* promoters. First, in each case ATF binds to DNA sequences that are critical for functioning of the corresponding promoters *in vivo* (18, 27, 28, 32, 33). Second, our previous data (18) and the transcription competition experiments reported here, demonstrate that binding of ATF to the *E2* and *E4* promoters is required for transcription *in vitro*. Although related by the presence of ATF-binding sites, the *E2A*, *E3*, and *E4* promoters are otherwise dissimilar and interact with distinct transcription factors (Fig. 5). For the *E4* promoter, multiple binding sites for ATF and the "TATA" box might provide full promoter function (18), whereas for the *E2A* and *E3* promoters ATF must functionally interact with additional factors to promote transcription.

The finding that ATF binds to multiple E1A-inducible promoters, together with our previous observation that ATF interacts with an E1A-inducible enhancer element (18), strongly suggests a direct role for ATF in E1A-mediated transcriptional activation. However, the nature of such a role is currently unclear. In addition, it is pertinent that ATF does not bind to the adenovirus *E1B* promoter (17) or major late promoter (14), and thus, ATF is not required for transcription of all E1A-inducible promoters. This might reflect the exist-

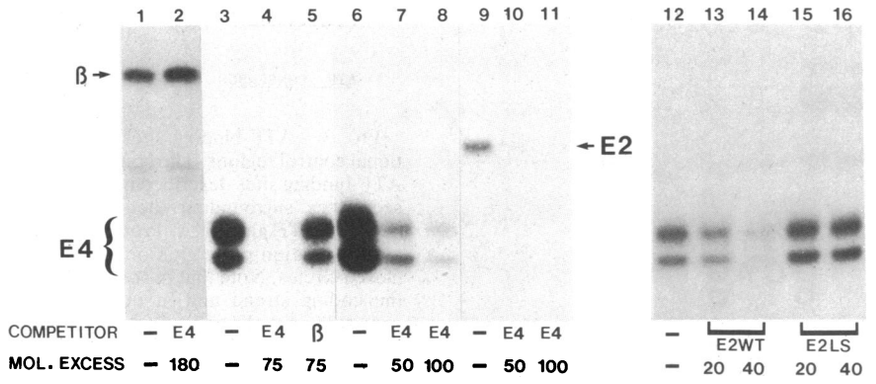

FIG. 4. E4F1 and E2A-EF activate transcription *in vitro*. *In vitro* transcription reactions were done using crude nuclear extract from uninfected HeLa cells as described (18). In each case 800 ng of circular DNA template was used to program transcription. Primer extension products representing correctly initiated RNAs (β, β-globin) are indicated at the side. DNA competitor fragments and the molar excess of competitor DNA are indicated at bottom. Transcription templates were as follows. Lanes 1 and 2, βΔ128 (containing the human β-globin promoter); lanes 3–8 and lanes 12–16, pE4WT (containing the *E4* promoter); lanes 9–11, pBR730 (containing the *E2A* promoter).

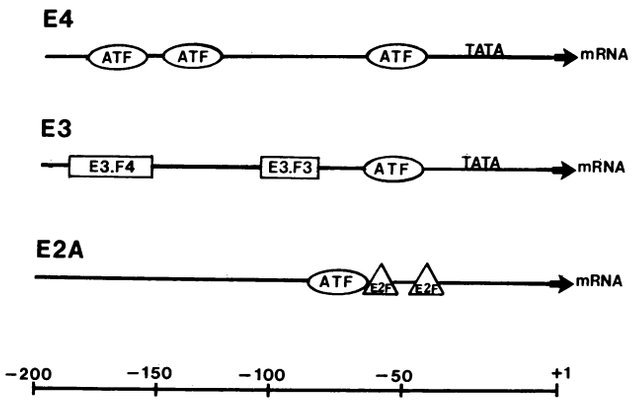

FIG. 5. Positions of ATF-binding sites in adenovirus early promoters. The figure schematically represents the *E4*, *E3*, and *E2A* promoters, emphasizing the known factor-binding sites; this scheme does not necessarily provide a complete description for any of the promoters, but it is intended to summarize existing data. Positions of factor-binding sites with respect to the transcription start site (+1) are indicated at bottom. Binding sites for the *E2A* [E2F (15, 20, 21) and E2A-EF (17, 20)], *E3* [E3F3 and E3F4 (19)], and *E4* (E4F1 (18)] promoters have been described previously. In addition, all factor-binding sites indicated coincide with functionally important elements of the corresponding promoters (15, 18, 21, 27, 28, 30, 32–34).

ence of multiple pathways involved in E1A-mediated transcriptional activation—some requiring ATF and others not. Certainly the fact that E1A has diverse activating effects on cellular and viral transcription is consistent with the notion that E1A acts through multiple pathways. E1A does not increase the amount or DNA-binding activity of ATF, which is abundant in nuclear extracts from uninfected cells (17–19). Possibly, E1A directly modifies ATF and increases its transcriptional activity without affecting its DNA-binding properties. An alternative possibility is that ATF is indirectly activated via interaction with another (as yet unidentified) factor. In this regard it is interesting that ATF does not appear to interact with the *E2A* promoter *in vivo* in the absence of E1A (35). Thus ATF binding to the *E2A* promoter is E1A-dependent *in vivo* but E1A-independent *in vitro*, suggesting that additional factors influence ATF binding to the *E2A* promoter *in vivo*.

Because ATF is a cellular factor, ATF is almost certainly involved in the transcriptional activation of specific cellular genes. Accordingly, ATF-binding sites are present in a limited number of cellular promoters (Fig. 3). The c-*fos* promoter contains an ATF consensus sequence within an important promoter element and is bound by a nuclear factor that makes the essential guanosine contacts characteristic of ATF (36). Thus, ATF is probably involved in transcription of the c-*fos* gene. ATF consensus binding sites are also closely associated with cAMP-inducible elements within cAMP-inducible promoters (37, 38). It will be of interest to determine whether ATF binds to these promoters and whether the spectrum of genes activated by E1A and cAMP overlap.

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