MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5138–5149
0270-7306/90/105138-12$02.00/0

Vol. 10, No. 10

E4F and ATF, Two Transcription Factors That Recognize the Same Site, Can Be Distinguished Both Physically and Functionally: a Role for E4F in E1A *trans* Activation

ROBERT J. ROONEY, PRADIP RAYCHAUDHURI, AND JOSEPH R. NEVINS*

Department of Microbiology and Immunology, Howard Hughes Medical Institute, Duke University Medical Center, P.O. Box 3054, Durham, North Carolina 27710

Received 26 April 1990/Accepted 5 July 1990

Previous experiments have identified an element in the adenovirus E4 promoter that is critical for E1A-dependent *trans* activation and that can confer inducibility to a heterologous promoter. This DNA element is a recognition site for multiple nuclear factors, including ATF, which is likely a family of DNA-binding factors with similar DNA recognition properties. However, ATF activity was found not to be altered in any demonstrable way as a result of adenovirus infection. In contrast, another factor that recognizes this element, termed E4F, was found at only very low levels in uninfected cells but was increased markedly upon adenovirus infection, as measured in DNA-binding assays. Although both the ATF activity and the E4F activity recognized and bound to the same two sites in the E4 promoter, they differed in their sequence recognition of these sites. Furthermore, E4F bound only to a small subset of the ATF recognition sites; for instance, E4F did not recognize the ATF sites in the E2 or E3 promoters. Various E4F and ATF binding sites were inserted into an expression vector and tested by cotransfection assays for responsiveness to E1A. We found that a sequence capable of binding E4F could confer E1A inducibility. In contrast, a sequence that could bind ATF but not E4F did not confer E1A inducibility. We also found that E4F formed a stable complex with the E4 promoter, whereas the ATF DNA complex was unstable and rapidly dissociated. We conclude that the DNA-binding specificity of E4F as well as the alterations in DNA-binding activity of F4F closely correlates with E1A stimulation of the E4 promoter.

It is now evident that the control of transcriptional activity from eucaryotic polymerase II promoters is brought about through the interaction of sequence-specific DNA-binding proteins with upstream regulatory elements. In a number of cases, proteins that appear to be rate-limiting factors, as defined by changes in DNA-binding activity or transcriptional activity, have been identified (for reviews, see references 32 and 33). An often found complication, however, is the observation that a regulatory site in the promoter may bind multiple proteins, many or all of which are capable of stimulating transcription in in vitro assays. Thus, in the absence of other information, it becomes difficult to identify one particular factor as the component that regulates transcription of the gene inside the cell. Certainly, the definitive answer to this question would be a genetic approach; that is, once the gene for the transcription factor is cloned, one might engineer a mutation of that gene to then ask whether that mutation impairs transcription from the promoter that binds that particular transcription factor. This may be a feasible approach in simple organisms such as yeasts, but in higher eucaryotic cells it is not practical at this time. Alternative approaches include analysis of the various proteins that interact with regulatory sites to identify properties that correlate with transcription of the gene. The most easily assayed property is DNA binding; indeed, in several cases in which multiple proteins have been found to interact with a regulatory element, one particular protein shows evidence of involvement in the control, that is, changes in DNA-binding activity that correlate with transcription of the gene. For instance, at least two DNA-binding proteins, OTF1 and OTF2 (also termed Oct1 and Oct2), recognize and interact with the octamer element found in the promoter and enhancer of the immunoglobulin heavy-chain locus (7, 23, 38, 45, 49, 50). However, only one of these proteins, OTF2, is found exclusively in B cells where the gene is transcribed (24, 49), and thus one presumes that this protein is responsible for the B-cell-specific transcription of the gene.

Another indication of complexity of transcription factor interactions can be found in the early adenovirus genes that are *trans* activated by the E1A protein (2, 37). For example, previous studies have identified a repeated sequence element in the E4 promoter that is critical for transcription and for E1A-dependent *trans* activation (26). This sequence element is the binding site for multiple transcription factors that can be assayed in extracts of human cells (4, 26, 27, 41). Thus, identification of the factor responsible for E1A control of E4 promoter activity is obviously complicated by the fact that multiple proteins interact with the regulatory site.

One of the activities that interacts with the E4 promoter, termed ATF, is abundant in extracts of mammalian cells and appears to recognize and interact with a variety of both viral and cellular promoters (27, 30). The ATF factor is in fact a family of transcription factors that recognize common sequence elements (13) and includes the cyclic AMP (cAMP) regulatory protein termed CREB (12, 16, 34, 54). No differences have been noted in either the characteristics of binding or the amount of binding activity of ATF after adenovirus infection (8, 19, 31, 48). An activity termed $E_{iv}F$ has also been shown to interact with this site, but again, there is no indication of an alteration as a function of E1A (4). In contrast, one other factor that interacts with the E4 regulatory site, termed E4F, does change markedly upon adenovirus infection (41). There is a significant stimulation of E4F

* Corresponding author.

DNA-binding activity after adenovirus infection that is dependent on E1A function, and the kinetics of E4F activation parallels the kinetics of E4 transcription activation. Thus, the interaction of E4F with the E4 promoter correlates with the E1A-dependent *trans* activation of E4 transcription. We have now directed further studies toward a careful characterization of the various activities and an analysis of the roles of the factors in mediating E1A-dependent *trans* activation.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in suspension culture in minimal essential medium (Joklik) containing 5% calf serum. The procedures for growth and purification of adenovirus type 5 (Ad5) and the methods for infection of cultures have been described elsewhere (36).

DNA probes and plasmids. E4wt is a synthetic double-stranded (ds) oligonucleotide containing the E4F/ATF sequence between −42 and −56 of the E4 promoter, cloned into the *Bam*HI site of pUC19 and excised as a 76-base-pair *Eco*RI-*Hin*dIII fragment. Point mutations in E4wt (as described in the text) were synthesized to create E4pm1, E4pm2, E4pm3, and E4pm4, which were also cloned into the *Bam*HI site of pUC19 and excised as *Eco*RI-*Hin*dIII fragments.

E2ATF is a ds synthetic oligonucleotide containing the ATF sequence between −69 and −79 of the E2 promoter, cloned into the *Bam*HI site of pUC19 and excised as a *Eco*RI-*Hin*dIII fragment (41).

E3ATF is a synthetic ds oligonucleotide containing the ATF sequence between −36 and −65 of the E4 promoter, cloned into the *Eco*RI site of pBluescript (Stratagene) and excised as a *Bam*HI-*Hin*dIII fragment.

E3AP1 is a synthetic ds oligonucleotide containing the AP1 sequence between −82 and −105 of the E3 promoter, cloned into the *Eco*RI site of pBluescript and excised as a *Bam*HI-*Hin*dIII fragment.

SP1 is a synthetic ds oligonucleotide containing the SP1 sequence between −31 and −54 of the E1B promoter, cloned into the *Sal*I-*Hin*dIII sites of pUC19 and excised as a *Eco*RI-*Hin*dIII fragment.

The expression plasmids pRE4wt, pRE4pm1, pRE4pm2, pRE4pm4, pRE2ATF, and pRE3ATF each contain a tandem repeat of the respective E4F/ATF sequence (described above), cloned into pBluescript directly upstream of a fragment containing the simian virus 40 TATA sequence and the E3 cap site linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. The tandem E4F/ATF sites are spaced 20 base pairs apart and are in the same orientation, relative to the TATA box, as the E4F/ATF site at −45 in the E4 promoter. Plasmid pE1A was used for the expression of E1A in cotransfections (20).

Plasmids pE4 and pE4-90 contain sequences between +32 and +224 and between +32 and −90, respectively, of the E4 promoter, cloned into pUC13 as described previously (41).

Extract preparation. Nuclear extracts were prepared from HeLa cells or Ad5-infected HeLa cells as described previously (5, 22). Whole-cell extracts were prepared from Ad5-infected HeLa cells as described previously (41).

Purification of E4F and ATF. ATF was purified from nuclear extracts of HeLa cells or Ad5-infected HeLa cells according to a procedure adapted from Hai et al. (12). Extracts were applied to heparin-agarose columns (5 mg of protein per ml of bed resin; Sigma Chemical Co.) and washed with buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 0.2 mM dithiothreitol [DTT], 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM NaF, 0.5 mM Na_3VO_4, 10% glycerol) plus 0.1 M KCl. The bulk of ATF activity was step eluted with buffer A plus 0.3 M KCl. After dialysis against buffer B (20 mM Tris hydrochloride [pH 7.5], 0.2 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 10% glycerol) plus 0.1 M KCl, the heparin-agarose fractions containing ATF were applied to a phosphocellulose column (Whatman P11; 20 mg of protein per ml of bed resin), washed with buffer B plus 0.15 M KCl, and step eluted with buffer B plus 0.6 M KCl. The phosphocellulose-purified ATF was dialyzed against buffer B plus 0.1 M KCl, applied to a DEAE-Sephacel column (5 mg of protein per ml of bed resin; Pharmacia), and washed with buffer B plus 0.1 M KCl. ATF activity was collected in the flowthrough fractions, adjusted to 0.1% Nonidet P-40, and twice loaded onto a DNA-Sepharose affinity column containing multimers of the E4F/ATF binding site (sequences between −43 and −54 in the E4 promoter), prepared as described previously (21). After successive washes with buffer C (20 mM HEPES [pH 7.9], 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40) plus 0.1 M KCl and buffer C plus 0.3 M KCl, ATF activity was eluted with buffer C plus 1 M KCl. This material was dialyzed against buffer C to 0.1 M KCl and subjected to a second round of DNA-Sepharose affinity chromatography. All chromatographic steps were monitored for ATF activity by the gel retardation assay.

E4F was purified from whole-cell extracts of Ad5-infected HeLa cells as previously described (40). Briefly, extracts were applied to heparin-agarose columns, washed successively with buffer A plus 0.1 M KCl and buffer A plus 0.25 M KCl, and eluted with a 0.25 to 0.75 M KCl linear gradient. E4F activity (eluting at ~0.32 M KCl) was dialyzed against buffer B plus 0.1 M KCl, applied to a DEAE-Sephadex column (Pharmacia), and eluted with a 0.1 to 0.4 M KCl gradient. E4F activity (eluting at ~0.15 M KCl) was diluted with buffer B to 0.1 M KCl, applied to a carboxymethyl (CM)-Sepharose column (Pharmacia), and eluted with a 0.1 to 0.4 M KCl gradient. The CM-Sepharose-purified E4F (eluting at ~0.2 M KCl) was adjusted to 0.1 M KCl, 0.1% Nonidet P-40, and 1 mM $MgCl_2$ (E4F requires Mg^{2+} to bind to DNA) and twice loaded onto a DNA-Sepharose affinity column containing multimers of the E4F/ATF binding site. This column was washed successively with buffer D (buffer C plus 1 mM $MgCl_2$) plus 0.1 M KCl and buffer D plus 0.3 M KCl, and then E4F activity was eluted with buffer D plus 1 M KCl. All steps were monitored for E4F activity by the gel retardation assay.

Gel retardation assays. Binding assays for E4F contained 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM DTT, 4% Ficoll, and 40 to 60 mM KCl, with 1 ng of 3'-end-labeled E4wt probe, 0.5 to 3 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia), and 1 to 5 μl of column fractions in a total volume of 30 μl. Each reaction was initiated by addition of protein and incubated for 30 min at room temperature, and then 10 μl was analyzed in a 4% polyacrylamide gel as described previously (41).

Binding assays for ATF were as described above except that poly(dI) · poly(dC) (Pharmacia) was used as nonspecific competitor DNA instead of the alternating copolymer.

Binding assays for E4F in crude nuclear extracts used E4pm2 as the labeled probe and contained 8 μg of poly(dI-dC) · poly(dI-dC) and 40 μg of protein per 30-μl reaction.

Specific competitor DNA fragments were added to E4F or ATF binding reactions before the introduction of protein in the amounts specified in the text. Binding reactions were

then analyzed as described above, and the levels of competition were measured by the loss of specific complexes.

Antibody supershifting of DNA-protein complexes was performed by adding 1 μl of antiserum 1025 or 1056 (see below for description of antisera) to E4F or ATF binding reactions 30 min after the start of the reaction and further incubating the mixtures for 1 h on ice. Complexes were analyzed as described above.

Dissociation rate analysis. Binding reactions (scaled up threefold) containing the E4wt probe and either affinity-purified E4F or ATF were incubated for 40 min to allow DNA protein complexes to form. Time course experiments showed that this incubation period was more than sufficient to allow the binding reactions to reach equilibrium (data not shown). After incubation, a 500-fold molar excess of unlabeled E4wt DNA was added to the reaction; 10-μl samples were removed at the times indicated and immediately loaded onto a 4% polyacrylamide gel running at 150 V. Five minutes after the last sample was loaded, the voltage was increased to 280 V and the gel was run to completion.

DNase I footprint assays. Saturating amounts (20 to 30 μl) of affinity-purified E4F or ATF (dialyzed to 0.1 M KCl in buffer C) were incubated with 0.2 ng of the *Eco*RI-*Hin*dIII fragment from plasmid pE4, 3′ end labeled at the *Eco*RI site (noncoding strand), in a final volume of 50 μl containing 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.5 mM DTT, and 40 to 60 mM KCl. After a 45-min incubation, an equal volume of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added, followed by the addition of 5 ng of freshly diluted DNase I (Boehringer Mannheim Biochemicals). The digestions were carried out for 60 s, stopped, and processed as described previously (40).

Methylation interference assays. The *Eco*RI-*Hin*dIII fragment from plasmid pE4-90 was 3′ end labeled at the *Eco*RI site (noncoding strand) or the *Hin*dIII site (coding strand), partially methylated with dimethyl sulfate, and used as a probe in binding reactions containing affinity-purified E4F or ATF. DNA-protein complexes were separated from free DNA by gel retardation, eluted from the gel and cleaved with piperidine, and then analyzed in an 8% sequencing gel (3, 41).

UV cross-linking. To synthesize the E4F/ATF binding-site probe, a 32-base oligonucleotide template, containing the sequence between −34 and −65 of the E4 promoter, was annealed to an oligonucleotide primer complementary to the final (3′) 12 bases of the template and filled in with dATP, [^{32}P]dCTP, [^{32}P]dGTP, and bromo-dUTP (Pharmacia) by Klenow enzyme. Binding reactions (scaled up fivefold) containing CM-Sepharose-purified E4F (50 μl) or DEAE-Sephacel-purified ATF (30 μl) were incubated at room temperature for 60 min, irradiated (on ice) with UV light at an intensity of 1,400 μW/cm^2, treated with nuclease, and terminated with sodium dodecyl sulfate (SDS) sample buffer as described previously (3, 55). The photoaffinity-labeled adducts were analyzed in a 10% SDS-polyacrylamide gel. E4F and ATF labeling was specifically competed for by addition of a 100-fold molar excess of E4wt DNA. The nonspecific competitor was a 100-fold molar excess of SP1 DNA.

Antisera. Affinity-purified rabbit antisera against CREB/ATF (1025 and 1056) were a gift of A. Merino and D. Reinberg (University of Medicine and Dentistry of New Jersey). Antiserum 1025 was raised against a synthetic peptide containing the sequence between amino acid residues 125 and 155 of the CREB protein (10); antiserum 1056 was raised against a synthetic peptide containing the sequence between amino acid residues 283 and 309 of the CREB protein (10).

Transfection assays. Vero cells (American Type Culture Collection) were maintained as monolayers in Dulbecco modified Eagle media containing 10% fetal calf serum. JEG-3 cells (American Type Culture Collection) were maintained as monolayers in minimal essential medium (Mediatech) containing 10% fetal calf serum. For E1A *trans* activation assays, Vero cells were plated overnight in Dulbecco modified Eagle medium containing 1% fetal calf serum and grown to 70 to 80% confluence. Each 60-mm dish was cotransfected with 3 μg of target DNA and 12 μg of either pE1A or pUC19 DNA by the calcium phosphate procedure (52). DNA precipitates were removed after 12 to 16 h, and the cells were replenished with fresh medium containing 1% fetal calf serum. Cells were harvested 24 h later, and CAT assays were performed as described previously (11). For cAMP induction assays, JEG-3 cells, at 40 to 60% confluence in 100-mm dishes, were transfected with 2.5 μg of target DNA and 15 μg of pUC19 DNA. DNA precipitates were removed after 18 to 20 h, and the cells were replenished with fresh medium. After 12 h, dibutyryl cAMP (Sigma) was added to the cells at a final concentration of 1 mM. Cells were harvested 12 h later and assayed for CAT activity. The protein concentration of all cell supernatants was determined by dye-binding assay (Bio-Rad Laboratories).

RESULTS

Role of ATF recognition sites in E1A control. The analysis of sequences and factors responsible for mediating E1A-dependent *trans* activation of transcription has led to the conclusion that multiple sites and factors must be targeted. There is strong evidence for a role for the E2F factor (22, 23, 55), the TFIIIC factor (17, 56), and a TATAA-specific factor (46, 53). In addition, a number of reports have implicated the ATF recognition elements as targets for E1A control. Mutagenesis of the ATF sites in a number of E1A-responsive promoters results in the loss of activity or serious impairment of those promoters. However, it is important in this regard to distinguish between a factor that is necessary for the transcription of a responsive gene and a factor that is the actual target for activation; i.e., the rate-limiting component. For instance, a deletion of the ATF site in the E2 promoter was shown to eliminate promoter activity in the presence of E1A, leading to the conclusion that the ATF site mediated E1A responsiveness (44). However, since in this experiment the deletion also eliminated basal activity, one cannot distinguish between an essential promoter element and a target for regulation. Indeed, two other studies have shown that although alteration or deletion of the E2 ATF site does lower promoter activity, E1A inducibility remains (20, 31). Likewise, deletions of the ATF sites in the E3 promoter (8, 28) and the c-*fos* promoter (47) reduce the activity of those promoters but do not eliminate E1A responsiveness. Thus, the ATF sites in the E2 promoter, the E3 promoter, and the c-*fos* promoter are certainly important for full transcriptional activity, but the evidence that these sites are the actual targets for *trans* activation is lacking. In contrast, there is good evidence to support the conclusion that the ATF sites in the E4 promoter are targets of E1A control. In particular, if a fragment containing the E4 ATF site located at −165 or a multimer of the proximal ATF element (located at −50) is placed upstream of a promoter containing no other E1A-responsive elements, then E1A activation is conferred (25, 26). To our knowledge, however, this is the only example of

TABLE 1. Sequence comparison of ATF recognition sites[a]

Promoter	Sequence
HTLV-I LTR	C G T T G A C G A C A A C C
	C C C T G A C G T G T C C C
	C T C T G A C G T C T C C C
HTLV-II LTR	C T C T G A C G A T T A C C
	C C C T G A C G T C C C T C
BLV LTR	T G C T G A C G G C A G C T
	A G C T G A C G T C T C T G
Somatostain	C T C T G A C G T C A G C C
Fibronectin	C C G T G A C G T C A C C C
c-*fos*	C C G T G A C G T T T A C A
	T G C T G A C G C A G A T G
Enkephalin	A G C T G A C G C A G G C C
hCG	C C A T G A C G T C A A T T
	C C A T G A C G T C A A T T
TH	C T T T G A C G T C A G C C
PEPCK	C T C T G A C G T A A G G G
VIP	C T G T G A C G T C T T T C
E1A	T T G T G A C G T G G C G C
	G G G T G A C G T A G T A G
	A A G T G A C G T T T T T G
E2A	A G A T G A C G T A G T T T
E3	C T G T G A C G A A A G C C
E4	A A G T G A C G A T T T G A
	G G G T G A C G T A G G T T
	T T G T G A C G T G G C G C
E4F binding site	A A A T G A C G T A A C G G
E4F binding site	A A G T G A C G T A A C G T
ATF consensus	T G A C G T
E4F consensus	T G A C G T A A C

[a] From reference 51. HTLV-I and -II, Human T-cell leukemia virus types I and II; LTR, long terminal repeat; BLV, bovine leukemia virus; hCG, human chorionic gonadotropin; TH, tyrosine hydroxylase; PEPCK, phosphoenolpyruvate carboxykinase; VIP, human vasoactive intestinal polypeptide.

an ATF element that can confer E1A inducibility. In every other case, the role of an ATF site in activation cannot be distinguished from a role in providing promoter activity.

These ATF sites in the E4 promoter are unique in another way. The proximal site located at −50 and the distal site located at −165 are identical in sequence and differ from all other ATF recognition sites, both in adenovirus promoters as well as in cell promoters. The two E4 sites possess the sequence TGACGTAAC (Table 1). Although the TGACGT component is found in other ATF sites, the AAC component is not. Given that our previous experiments have shown that it is these two sites that are uniquely recognized by the E4F factor (41) and the fact that the E4F factor is stimulated in an adenovirus infection, we have now further investigated the properties of E4F and ATF recognition of these sequences.

Purification and analysis of ATF and E4F. Previous studies have described the purification of both the E4F transcription factor and the ATF activities. Purification of E4F activity results in the isolation of a single 50-kilodalton (kDa) polypeptide. This protein has binding properties identical to those assayed in crude extracts and can stimulate E4 transcription in vitro (40). Previous studies in other laboratories have described the ATF activity as consisting of polypeptides ranging in molecular size from 43 to 47 kDa and possessing transcriptional activity in vitro (12, 19). Recently, cDNA clones encoding eight different ATF proteins (although not including CREB) were isolated (13). Although it is unclear how many or to what extent these different factors are expressed in any one cell type, the majority of ATF activity in HeLa cells appears to be isolated by using a purification procedure described by Hai et al. (12). Thus, using this approach, we have purified the ATF activity to facilitate a direct comparison of E4F and ATF. The significant differences in the purification of the two activities are chromatography patterns on DEAE columns, in which E4F binds and ATF does not, as well as heparin-agarose, in which the two activities elute at different salt concentrations. Distinct DNA-protein complexes, as judged by gel retardation assays, were formed with the ATF/E4F probe in the purified E4F and ATF preparations (Fig. 1A). A difference in the mobility of the DNA-protein complexes formed with the two factors was clearly evident in this analysis such that the ATF complex migrated somewhat slower in the gel than did the E4F complex. A molecular weight difference in the two polypeptides was also evident after UV cross-linking of each factor to the DNA recognition site. UV cross-linking of the ATF preparation labeled a protein of 47 kDa, whereas UV cross-linking of the E4F factor labeled a 50-kDa polypeptide (Fig. 1B). Although the two proteins are close in molecular weight, it is clear from this analysis that they do differ.

Finally, we also used two antisera raised against peptides derived from the CREB sequence to examine the relationship between ATF and E4F. One of the antisera (1025) detected a factor in the ATF-DNA complex, as judged by the reduced mobility of the complex in the gel retardation assay in the presence of the antibody (Fig. 1C). In contrast, this antiserum did not alter the mobility of the E4F-DNA complex. We conclude from these analyses that the activities termed ATF and E4F are distinct DNA-binding proteins as defined by differences in molecular weight, purification properties, and antibody recognition. We do not wish to exclude the possibility that E4F is a member of the ATF family; indeed, one of the ATF clones exhibits binding properties similar to those of E4F (13). We do suggest, however, that E4F is distinct from the major ATF binding activity detected in HeLa cell extracts.

The DNA-binding characteristics of E4F and ATF differ. Our previous experiments suggested differences in the DNA recognition properties of the two factors, since E4F appeared to bind only to two of the three ATF sites in the E4 promoter. This specificity is shown by a DNA-binding competition assay in Fig. 2. Although ATF bound to sites in the E2, E3, and E4 promoters, E4F bound only to the E4 promoter. The differences in the DNA recognition properties of the two factors are also clearly evident from DNase footprinting assays using a probe containing the entire E4 promoter (Fig. 3A). The ATF factor protected three regions of the promoter, including sequence between −45 and −52 (site I), −138 and −145 (site II), and −162 and −169 (site III). These are the sites previously characterized as ATF recognition sites within the E4 promoter (26). In contrast, the purified E4F factor protected only sites I and III, and this did not change upon addition of more protein. Sites I and III are the same two sequences previously characterized as the E4F recognition sites (40, 41). Thus, under the same assay conditions of DNA binding and DNase protection, there are apparent differences in the binding specificities of the two factors.

Methylation interference analysis of E4F and ATF binding to the E4 site I demonstrated very clearly that the recognition specificities of the two factors overlap but differ. On the noncoding strand of the DNA, both factors were inhibited by methylation of the G residues at −46 and −49 (Fig. 3B). Methylation of the G residue at −48 on the coding strand also prevented binding of each factor. However, methylation of the G residue at −53 prevented binding of the E4F factor

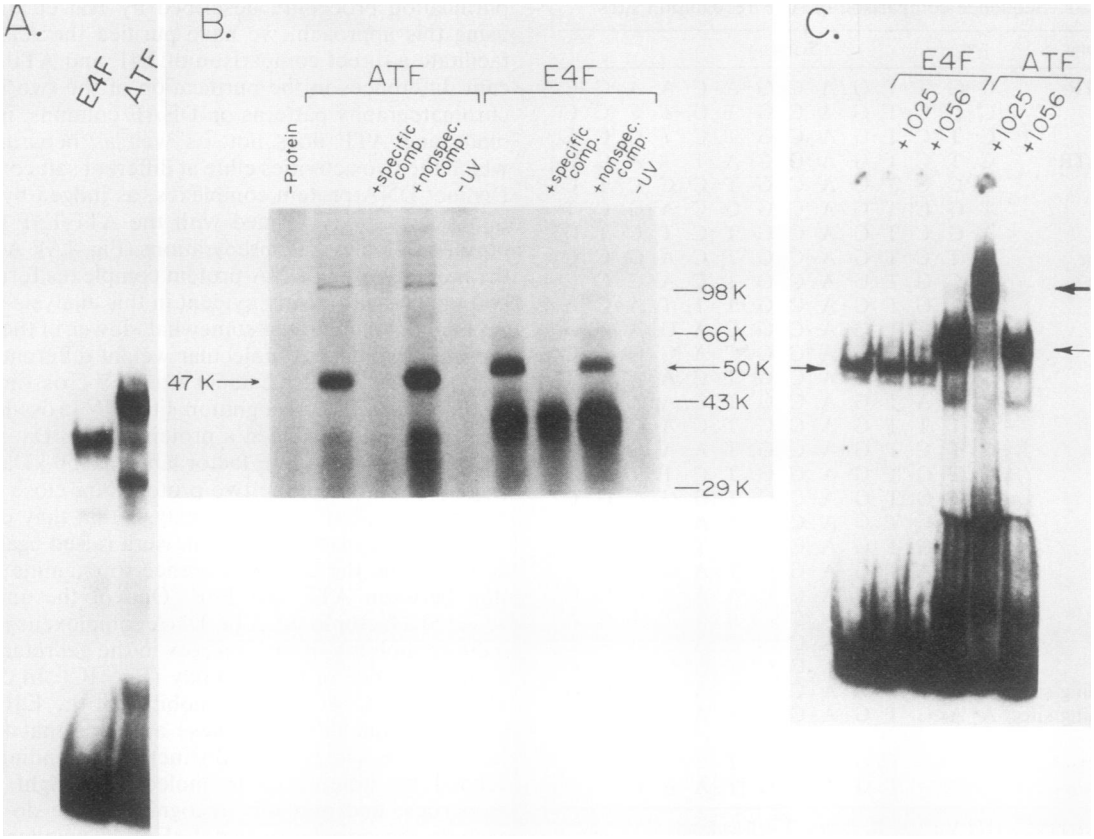

FIG. 1. Demonstration that ATF and E4F are distinct DNA-binding proteins. (A) Gel retardation assays. Affinity-purified ATF and E4F, prepared as described in Materials and Methods, were assayed for DNA binding to the E4wt probe by gel retardation assay. (B) Labeling of ATF and E4F by UV cross-linking. Partially purified preparations of ATF and E4F were incubated with labeled probe, cross-linked by UV irradiation, digested with nuclease, and then analyzed by SDS-gel electrophoresis. Binding reactions were also carried out in the presence of an excess of cold competitor DNA, either the homologous sequence (E4wt) or a nonspecific sequence (Sp1). Lane 1 depicts a sample treated without protein. Molecular sizes are indicated in kilodaltons (K). (C) Effect of the ATF/CREB-specific antisera on mobility of ATF-DNA and E4F-DNA complexes. Affinity-purified ATF or E4F was incubated with labeled probe, 1 μl of antiserum 1025 or 1056 was added, and then the samples were analyzed by gel retardation. The larger arrow indicates the ATF complex reduced in mobility by the antibody. Smaller arrows indicate the normal positions of the E4F and ATF complexes.

but did not alter binding of ATF. This result clearly defines a difference in the DNA sequences recognized by the two factors, since E4F recognition extended 3′ to that of ATF recognition. This finding is also consistent with the distribution of E4F sites in the adenovirus genome. The only two E4F sites in the viral genome, sites I and III in the E4 promoter, have a common sequence of TGACGTAAC. Notably, it is the methylation of the G residue opposite the 3′ C that prevents binding of E4F but not ATF.

Finally, we also generated a series of single-base substitutions in the E4F/ATF recognition sequence in an attempt to further distinguish binding characteristics of the two factors. Oligonucleotides that differed at four positions in the recognition sequence were synthesized (Fig. 4A) and used as competitor DNAs in binding assays for E4F and ATF. Mutant pm1 did not bind either factor, and mutant pm3 did not alter binding of either factor (Fig. 4B and C). In contrast, mutant pm4 eliminated binding of E4F but did not affect binding of ATF. This result is consistent with the methylation interference assays of Fig. 3, that demonstrated a recognition of the additional 3′ sequence by E4F but not ATF, as well as the DNase footprinting data indicating that the site not containing the AAC extension (site II) was not protected by E4F. The pm2 mutation clearly eliminated ATF binding and reduced E4F binding, although E4F was not as severely affected as ATF binding. Quantitative analysis of the competitions indicated that the pm2 mutation reduced E4F binding threefold as compared with the wild-type sequence.

It would appear from the binding analyses of these mutants as well as the natural sequences in the E4 promoter that the recognition sequences for the two activities (ATF and E4F) overlap but clearly differ. Most importantly, the only two locations of this more complex sequence in the adenovirus genome are the two sites in the E4 promoter.

The E4F recognition sequence, but not other ATF recognition sequences, confers E1A inducibility. Previous experiments have shown that the E4F/ATF site I from the E4 promoter not only is necessary for full E1A-induced activity but also can confer E1A inducibility to a nonresponsive promoter (25, 26). This finding has been interpreted as meaning that ATF is a mediator of E1A *trans* activation. However, this is the only ATF element that has been assayed in this way, and thus this conclusion is compromised by the fact that multiple factors recognize and bind to this sequence in the E4 promoter. We have now made use of the differences in sequence recognition of the two factors as detailed in previous sections to define the factor involved in

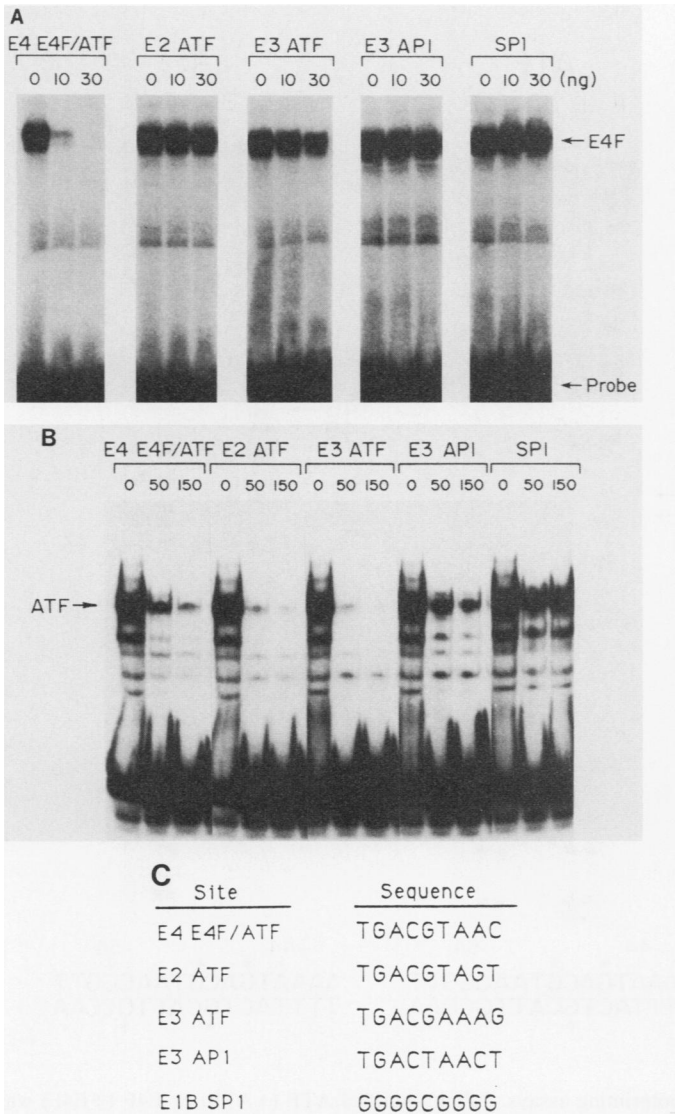

FIG. 2. Promoter specificity of ATF and E4F. Affinity-purified preparations of ATF and E4F were assayed by gel retardation binding assays using the E4wt probe. Competitions were carried out by using DNA fragments containing promoter sequences from the indicated genes, descriptions of which are in Materials and Methods. Each reaction contained 1 ng of labeled probe and the indicated amounts of competitor DNA. (A) Competition of E4F binding; (B) competition of ATF binding; (C) sequence comparison of competitor binding sites. Depicted is the sequence of the ATF/E4F consensus at each site. The actual competitor DNA also contained flanking promoter sequence as detailed in Materials and Methods.

control. That is, if the ATF factor was indeed responsible for E1A control, then any of the ATF sites might be expected to confer inducibility. If, however, E4F was the target of E1A *trans* activation, then only the E4F sites, which are a limited subset of the ATF sites, would confer control. We have addressed this question by constructing simple promoters containing either ATF sites or E4F sites upstream of a TATA element. In this case, we used the TATTTAT sequence from the early simian virus 40 promoter since previous experiments have shown that this element is not responsive to E1A (46). No other promoter elements are present in these constructs. This strategy thus isolates the E4F/ATF elements as the only potential targets of E1A *trans* activation.

As a first test, we constructed promoters containing duplicated ATF recognition sites derived from the E2 promoter, the E3 promoter, and the E4 promoter and used them to measure induction by E1A. Each promoter will bind the ATF factor, but only the promoter containing the E4 sequences will bind the E4F factor. Each construct was transfected into Vero cells with or without pE1A, a plasmid expressing the E1A gene. The promoter containing the E4F/ATF site from the E4 promoter was induced by cotransfection with E1A (Fig. 5A). In contrast, neither the E2 nor the E3 ATF site was capable of conferring E1A inducibility. Thus, a promoter that can bind E4F (and ATF as well) is E1A inducible, whereas a promoter that can only bind ATF is not. That these promoter constructs containing the E2 or E3 ATF site were indeed functional was indicated by the finding that each was inducible by cAMP (Fig. 5B), consistent with the fact that each does bind ATF.

As another test, we constructed promoters with the E4F/ATF point mutants depicted in Fig. 4A. Again, each site was duplicated upstream of the simian virus 40 TATTTAT element and assayed by cotransfection with an E1A-expressing plasmid. The wild-type construct was clearly inducible, whereas, as expected from the binding assays, the pm1 mutant was not induced (Fig. 5C). The pm2 mutation, which eliminates ATF binding and reduces E4F binding, also did not allow an E1A activation. Finally, the pm4 mutant, which does not bind E4F but does bind ATF, was not induced by E1A. Thus, a promoter that can bind E4F (and ATF) is E1A inducible, whereas a promoter than can bind only ATF is not induced. An assay of each of these promoter constructs for cAMP inducibility is shown in Fig. 5D. As expected, the construct containing the wild-type E4F/ATF sites was inducible, whereas the pm1 mutant, which does not bind either ATF or E4F, was not. The pm2 mutant also did not respond, consistent with the failure of this mutant to bind ATF. The pm4 mutant, which was not inducible by E1A, is responsive to cAMP, demonstrating that this construct is functional.

From these results, we make the following conclusions. A tandem pair of elements to which both E4F and ATF can bind (E4) can confer E1A inducibility. A pair of sites that do not allow E4F to bind but do allow ATF binding (E2, E3, pm4) does not confer E1A inducibility. The pm2 mutant, which is deficient for ATF binding but does bind E4F at a reduced level, was also nonresponsive. Although more complex explanations are possible, we believe the most likely reason for this result is the reduced binding efficiency of E4F.

E4F DNA-binding activity is induced by viral infection. Our previous results have suggested an increase in E4F DNA-binding activity as a result of adenovirus infection (41). However, this conclusion was limited by the fact that these initial assays used fractionated extracts for the assays, since it proved to be impossible to detect E4F binding in crude nuclear extracts because of the presence of high levels of ATF. Thus, it is possible that E4F binding is not induced but rather that E4F is altered such that it simply fractionates differently. The finding that the pm2 mutation did not bind ATF but did bind E4F at a reduced level (Fig. 4) provided a mechanism to distinguish these possibilities. That is, we could assay for E4F in crude extracts by using the pm2 probe and avoid the interference from ATF binding. This assumption was valid, since no specific binding activity was detected in nuclear extracts of mock-infected cells with the pm2 probe (Fig. 6). In contrast, a specific complex was

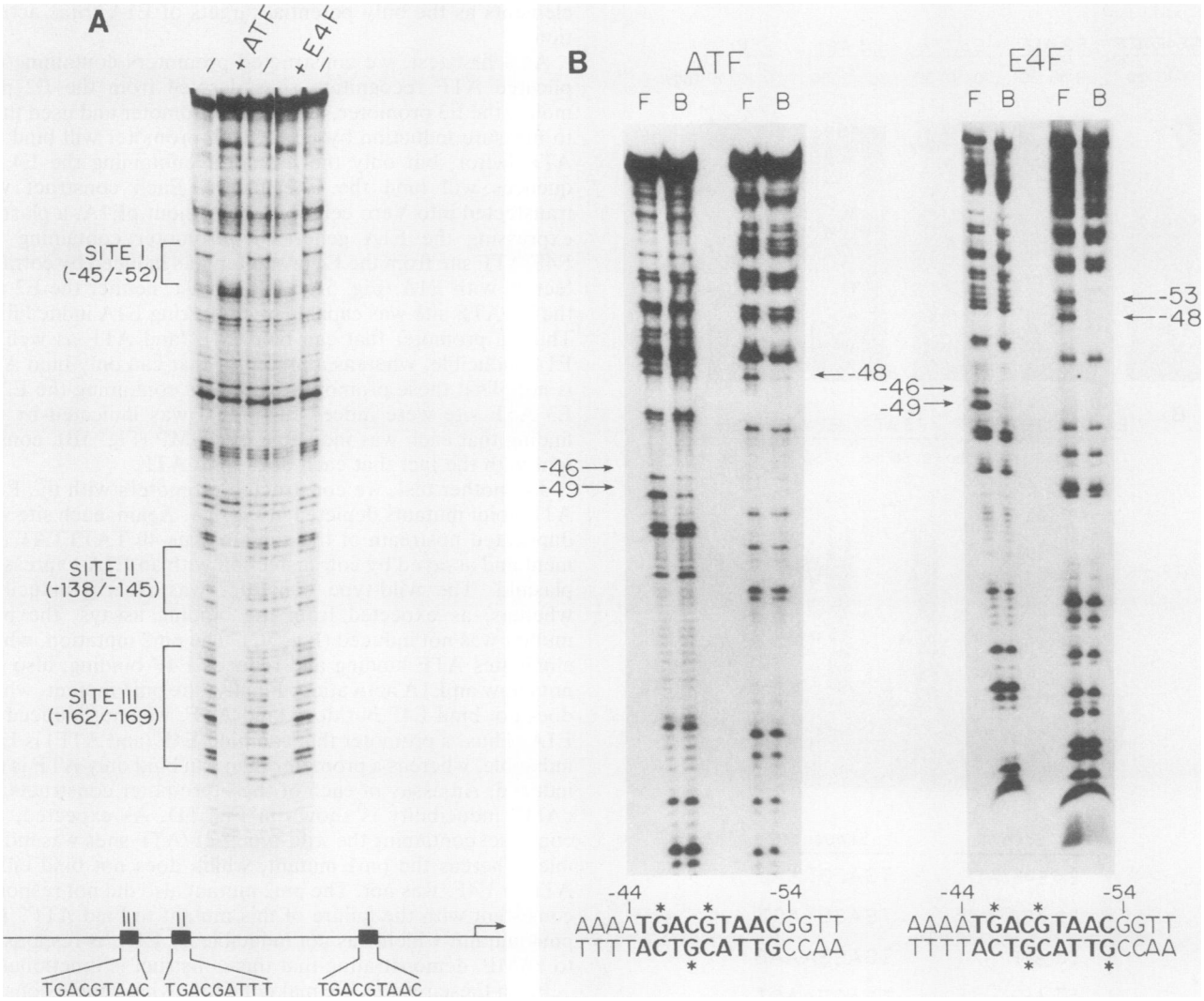

FIG. 3. DNA-binding specificity of ATF and E4F. (A) DNase footprinting assays. Affinity-purified ATF (+ATF) or E4F (+E4F) was bound to a probe containing E4 promoter sequences between −224 and +32 and then digested with DNase I as described in Materials and Methods. The protected sites in the E4 promoter are depicted at the bottom; ATF protects all three sites, whereas E4F protects only sites I and III. (B) Methylation interference assays. Partially methylated probe containing E4 promoter sequences between −90 and +32 was bound to affinity-purified ATF or E4F, separated from unbound probe by gel retardation, and processed as described in Materials and Methods. Analyses of both DNA strands are shown. Lanes B, Bound DNA; lanes F, free DNA. Methylated G residues in the E4F/ATF binding site that inhibit protein binding (arrows) are depicted at the bottom.

detected with the pm2 probe in the extract of adenovirus-infected cells. We therefore conclude that adenovirus infection does indeed result in a stimulation of the level of active E4F.

E4F forms a stable complex on the promoter. Although these results demonstrate a clear difference between the E4F factor and the ATF factor and provide strong evidence that the regulation of E4F is important for the E1A response, it remains that both factors can bind to the E4F and ATF sites in the E4 promoter and that both factors are present in the infected cell at the time of activation of E4 transcription. Thus, even with the activation of E4F, how would this factor occupy the promoter sites rather than the ATF factor? Moreover, the ATF factor appears to be a rather abundant nuclear protein and clearly has the capacity to stimulate transcription in vitro (12). What then would be the advantage in the induction of the E4F factor? An apparent answer to this enigma is provided by an analysis of the stability of DNA protein complexes formed by ATF and E4F as measured by dissociation rates. This is a simple and straightforward analysis whereby complexes are formed and allowed to equilibrate, and then an excess of cold probe DNA is added. Samples are taken at various times thereafter, and the remaining complexes are measured by gel retardation. The ATF complexes were unstable, since there was near-complete loss of complexes after 5 min (Fig. 7). In contrast, the E4F-DNA complex was very stable, showing little evidence of dissociation even after 40 min. Thus, even in the presence of an abundance of the ATF factor, it can readily be seen that the E4F factor would quickly occupy the E4F-specific sites in the E4 promoter, since once the factor binds, it would not readily dissociate.

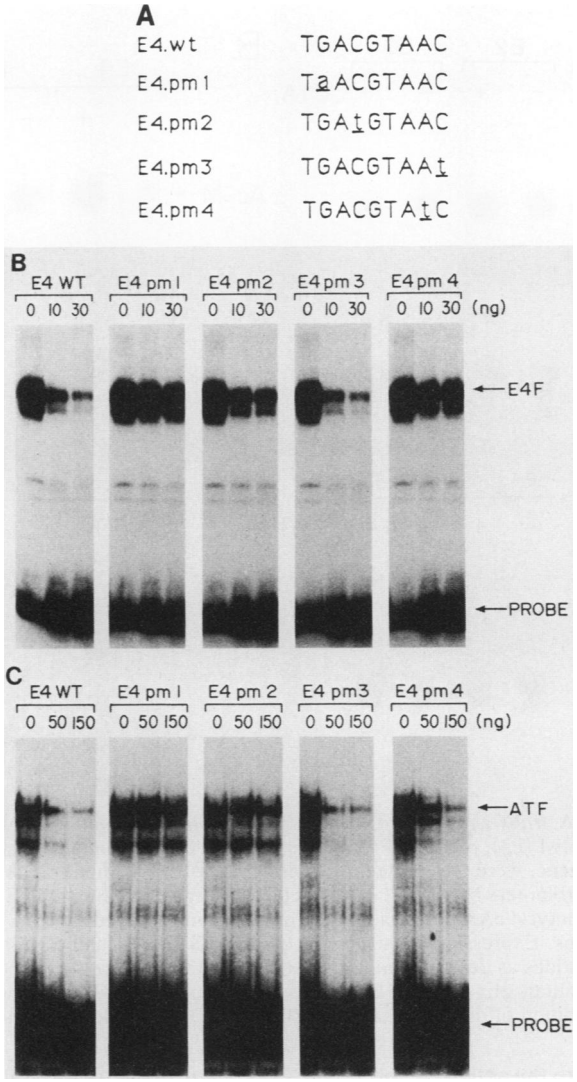

FIG. 4. DNA recognition specificity of ATF and E4F. (A) Point mutations in the E4 site I E4F/ATF sequence. Synthetic oligonucleotides containing single-point mutations in the E4 E4F/ATF site I sequence were cloned into pUC19, excised, and used as competitor DNAs in gel retardation assays. Only the ATF/E4F consensus sequence is shown. E4.wt, Wild-type E4 sequence; E4.pm1, E4.pm2, E4.pm3, and E4.pm4, point mutants. Mutations are underlined. (B and C) DNA-binding competition assays. Affinity-purified E4F (B) or ATF (C) was bound to the E4wt probe in the presence of the wild-type or mutant competitor DNAs listed in panel A. Competition was assayed by gel retardation of remaining DNA-protein complexes. Each reaction contained 1 ng of probe and the indicated amount of competitor DNA.

DISCUSSION

The study of E1A-dependent *trans* activation of transcription has proved invaluable as a system for the analysis of mechanisms of transcriptional regulation in eucaryotic cells. Activation of the various viral and cellular promoters by E1A involves the use of cellular transcription factors that are undoubtedly important components of various cellular transcriptional control pathways. Thus, an understanding of the mechanism by which this well-defined viral regulatory protein alters the activity of a cellular transcription factor will likely help to elucidate mechanisms of cellular transcription control. However, as is the case for most transcriptional control regions, the viral and cellular promoters that are subject to E1A *trans* activation utilize multiple transcription factors for full and efficient transcription. Given the complexity of factors that interact with promoters of polymerase II-transcribed genes, it is crucial to identify the rate-limiting factors that are the targets of the activation process, since it is likely that many of the factors that interact with upstream sequence elements are not regulated. Although such factors are important for promoter function, they only complicate the task of defining a regulatory circuit. This becomes an even more difficult problem when multiple factors recognize and bind to the same regulatory sequence.

We believe that the data presented here and in previous studies (40, 41) provide strong evidence for a role for the E4F factor in E1A-dependent *trans* activation of E4 gene transcription. Specifically, on the basis of the observations that E4F activation is E1A-dependent, that the kinetics of E4F activation parallel the activation of E4 transcription during adenovirus infection, that E4F does stimulate transcription from the E4 promoter in vitro, and that the only ATF sites unambiguously shown to confer E1A inducibility, the E4 ATF sites I and III, are the ATF sites to which E4F binding specificity is limited, we argue that the E4F transcription factor is a likely target of the E1A *trans* activation

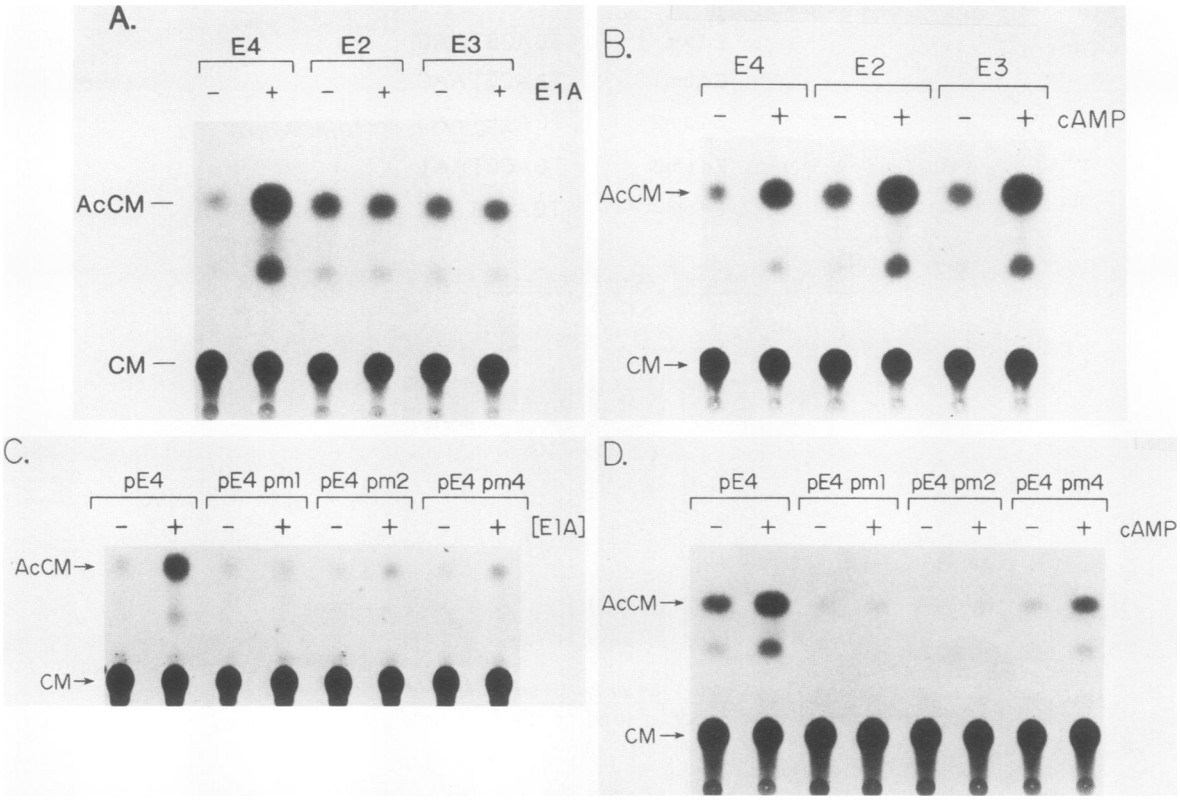

FIG. 5. Sequence specificity of E1A *trans* activation. (A) *trans* activation of promoters containing ATF sites from early adenovirus promoters. Expression constructs pRE4wt (E4), pRE2ATF (E2), and pRE3ATF (E3), containing tandem repeats of the E4, E2, and E3 ATF sites, respectively, linked to the CAT gene, were transfected into Vero cells with or without pE1A. Extracts were prepared and assayed for CAT activity. (B) Activation of ATF promoters by cAMP. JEG-3 cells were transfected with pRE4wt (E4), pRE2ATF (E2), and pRE3ATF (E3) and then induced by addition of dibutyryl cAMP. Extracts were prepared and assayed for CAT activity. (C) *trans* activation of promoters containing the E4F/ATF point mutations. Expression constructs pRE4pm1, pRE4pm2, and pRE4pm4, containing tandem repeats of the E4 E4F/ATF site point mutant oligonucleotides as described in Materials and Methods, were transfected into Vero cells with or without pE1A. (D) Activation of E4F/ATF site point mutants by cAMP. JEG-3 cells were transfected with pRE4wt or point mutants pRE4pm1, pRE4pm2, and pRE4pm4 and then induced by addition of dibutyryl cAMP. AcCM, Acetylated chloramphenicol; CM, chloramphenicol.

pathway and therefore contributes to the activation of the E4 promoter. This is not to say that E4F is the only factor targeted in E1A-dependent activation of E4 transcription. Indeed, a recent report suggests that sequences near the transcription initiation site may contribute to *trans* activation of E4 transcription (25). Regardless of the final complexity, we believe that the assays do clearly identify E4F as a target in the process of E1A *trans* activation.

The E1A protein itself is not a promoter-specific DNA-binding protein (6) and thus most likely must exert its effect indirectly by modulating the activity of preexisting cellular transcription factors. It is possible that the E1A protein could form a heteromeric protein complex with a promoter-specific DNA-binding protein, as has been suggested by recent experiments (29), but there is no direct evidence for such. In the case of E4F, the effect of E1A is an activation of DNA-binding activity which may involve a change in phosphorylation of the factor. Recent experiments have shown that E4F activity is lost upon phosphatase digestion and that this activity can be restored by incubation of the phosphatase-inactivated factor with an extract of Ad5-infected cells (40). In contrast, an extract of mock-infected cells does not reactivate E4F. Since there is no reason to believe that E1A is a kinase, these results suggest that a cellular kinase is involved and thus may be a target for E1A action.

A complete understanding of the involvement of E4F in E1A *trans* activation ultimately requires the isolation of the gene encoding E4F and the generation of antibodies against the protein. Regarding this, a recent publication (13) reported the isolation and characterization of cDNA clones encoding eight different ATF or ATF-like proteins. We note that one of these clones, ATF-3, encodes a protein with binding characteristics similar to those of E4F. Binding of the ATF-3 protein to the E4 ATF site I involves the same DNA contacts as does E4F binding, as judged by dimethyl sulfate methylation interference analysis. If this clone does prove to encode E4F, this would suggest a relationship between E4F and ATF that goes beyond DNA sequence recognition, since the ATF-3 clone is related by primary and secondary structure to the other members of the ATF family. Of course, this is only speculation at the moment, and further analyses of this protein are needed to determine the relationship to E4F. Regardless of whether E4F is related to the ATF family by amino acid sequence, it clearly is related by DNA sequence recognition, and thus the important question remains as to the strategy of the virus, in the form of E1A control, in targeting one particular member of a family of factors. If each of these factors can interact with the sites in the E4 promoter and each can stimulate transcription, then why should one be targeted by E1A over the others?

In this regard, an analysis of the activation of the E2F

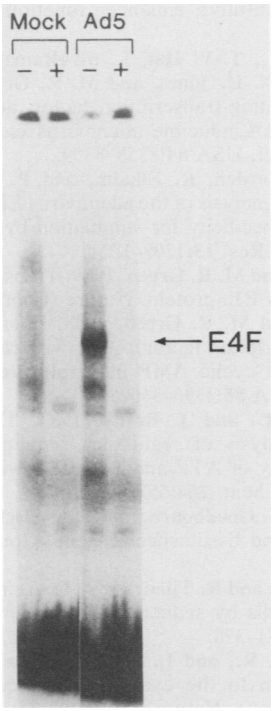

FIG. 6. Activation of E4F DNA-binding activity by adenovirus infection. Extracts were prepared from mock-infected HeLa cells or adenovirus-infected HeLa cells and assayed for E4F DNA-binding by gel retardation using the pm2 mutant probe. Assays were carried out in the absence (−) or presence (+) or cold competitor DNA (E4wt).

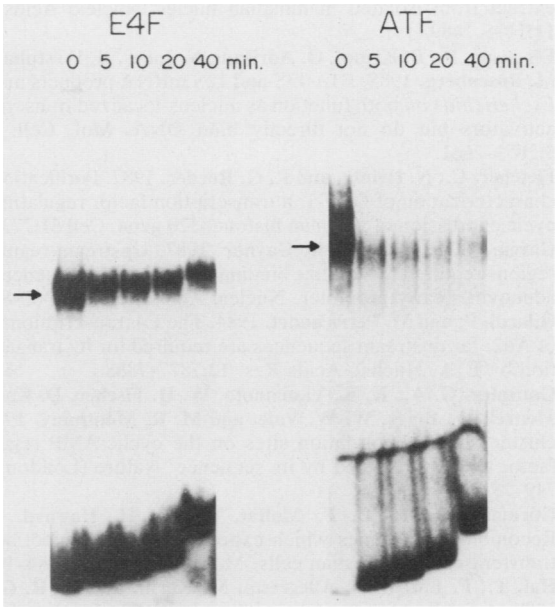

FIG. 7. Demonstration that E4F forms a stable DNA complex, whereas ATF binding is unstable. DNA-protein complexes were formed with either affinity-purified E4F or affinity-purified ATF and the E4wt probe. After equilibration, a large excess of cold competitor DNA (E4wt) was added, and samples were removed at the indicated times and loaded onto a native acrylamide gel that was already running.

factor has provided an interesting parallel to the activation of E4F as we describe here and may provide an explanation. Recent experiments have demonstrated a complexity to the E2F activation process whereby two events appear to contribute to the final activated form of the factor. There is an increase in the DNA-binding activity of the E2F factor that may involve the same mechanism as the activation of E4F (39). Activation of the DNA-binding activity of both factors requires E1A function and may involve phosphorylation (1, 40). There is a further change in E2F that allows the factor to bind to adjacent promoter sites in a cooperative fashion to form a stable complex (14, 39). This change in E2F binding is the result of an interaction with a 19-kDa product of the E4 gene with the E2F factor (18, 35). Although the E2F activation process is more complicated than the activation of E4F in that two events are required, the final result is the same: an increase in the level of active factor that forms a stable promoter complex. Interestingly, the E1A-dependent activation of the polymerase III TFIIIC transcription factor may be similar. Two forms of a TFIIIC-DNA complex can be detected in extracts of uninfected cells. Upon adenovirus infection, there is an increase in the more slowly migrating form of the complex. This complex is also more stable, at least as measured by resistance to increasing ionic strength (17). Thus, in two cases (E2F and E4F), and possibly a third (TFIIIC), activation of transcription factors involved in early adenovirus transcription results in the formation of a factor that can generate a stable complex with the DNA. Perhaps this is one strategy of the virus to ensure high levels of transcription during the lytic infection. In the case of E4F, one could imagine that this particular factor possesses the unique ability, among factors that recognize the E4 ATF sequence, to form a stable DNA complex. If, however, the factor is present normally in the cell only in very low amounts, as indicated by our assay, we suggest that adenovirus has taken advantage of this circumstance by achieving an increase in the concentration of the active factor, as a result of E1A action, so as to efficiently drive factor onto the E4 promoter.

ACKNOWLEDGMENTS

R.J.R. was an National Institutes of Health postdoctoral fellow. This work was supported by Public Health Service grant GM26765 from the National Institutes of Health.

We thank Laszlo Jakoi and Lynne Dengler for supplying cell extracts. We also thank Danny Reinberg for the gift of the CREB-specific antisera and Keith Parker for the JEG-3 cells.

LITERATURE CITED

1. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. Proc. Natl. Acad. Sci. USA **86**:4352–4356.
2. **Berk, A. J.** 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20**:45–79.
3. **Chodosh, L. A., R. W. Carthew, and P. A. Sharp.** 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol. **6**:4723–4733.
4. **Cortes, P., L. Buckbinder, M. A. Leza, N. Rak, P. Hearing, A. Merino, and D. Reinberg.** 1988. EivF, a factor required for transcription of the adenovirus EIV promoter, binds to an element involved in E1a-dependent activation and cAMP induction. Genes Dev. **2**:975–990.
5. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by polymerase II in a soluble

extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475–1489.
6. **Ferguson, B., B. Krippl, O. Adrisani, N. Jones, H. Westphal, and M. Rosenberg.** 1985. E1A 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind DNA. Mol. Cell. Biol. 5:2653–2661.
7. **Fletcher, C., N. Heintz, and R. G. Roeder.** 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell 51:773–781.
8. **Garcia, J., F. Wu, and R. Gaynor.** 1987. Upstream regulatory regions required to stabilize binding to the TATA sequence in an adenovirus early promoter. Nucleic Acids Res. 15:8367–8385.
9. **Gilardi, P. and M. Perricaudet.** 1984. The E4 transcriptional unit of Ad2: far upstream sequences are required for its transactivation by E1A. Nucleic Acids Res. 12:7877–7888.
10. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs, W. W. Vale, and M. R. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic-AMP regulated factor CREB predicted by its sequence. Nature (London) 337: 749–752.
11. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
12. **Hai, T., F. Liu, E. A. Allegretto, M. Karin, and M. R. Green.** 1988. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. Genes Dev. 2:1216–1226.
13. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. 3:2083–2090.
14. **Hardy, S., D. A. Engel, and T. Shenk.** 1989. An adenovirus early region 4 gene product is required for induction of the infection-specific form of cellular E2F activity. Genes Dev. 3:1062–1074.
15. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Hoeffler, J. R., T. E. Meyer, Y. Yun, J. L. Jameson, and J. F. Habener.** 1988. Cyclic AMP-responsive DNA binding protein: structure based on a cloned placental cDNA. Science 242:1430–1433.
17. **Hoeffler, W. K., R. Kovelman, and R. G. Roeder.** 1988. Activation of transcription factor IIIC by the adenovirus E1A protein. Cell 53:907–920.
18. **Huang, M.-M., and P. Hearing.** 1989. The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. Genes Dev. 3:1699–1710.
19. **Hurst, H. C., and N. C. Jones.** 1987. The identification of factors that interact with the E1A-inducible adenovirus E3 promoter. Genes Dev. 1:1132–1146.
20. **Imperiale, M. J., R. P. Hart, and J. R. Nevins.** 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A-induced transcription. Proc. Natl. Acad. Sci. USA 82:381–385.
21. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA 83:5889–5893.
22. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A *trans*-activation. Cell 45:219–228.
23. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1987. Role of the promoter-specific factor E2F in E1A-mediated coordinate gene control. Proc. Natl. Acad. Sci. USA 84:2180–2184.
24. **Landolfi, N. F., J. D. Capra, and P. W. Tucker.** 1986. Interaction of cell-type-specific nuclear proteins with immunoglobulin V_H promoter region sequences. Nature (London) 323:548–551.
25. **Lee, K. A. W., J. S. Fink, R. H. Goodman, and M. R. Green.** 1989. Distinguishable promoter elements are involved in transcriptional activation by E1A and cyclic AMP. Mol. Cell. Biol. 9:4390–4397.
26. **Lee, K. A. W., and M. R. Green.** 1987. A cellular transcription factor E4F1 interacts with an E1A-inducible enhancer and mediates constitutive enhancer function in vitro. EMBO J. 6:1345–1353.
27. **Lee, K. A. W., T.-Y. Hai, L. SivaRaman, B. Thimmappaya, H. C. Hurst, N. C. Jones, and M. R. Green.** 1987. A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. Proc. Natl. Acad. Sci. USA 84:8355–8359.
28. **Leff, T., J. Corden, R. Elkaim, and P. Sassone-Corsi.** 1985. Transcription analysis of the adenovirus EIII promoter: absence of sequence specificity for stimulation by E1A gene products. Nucleic Acids Res. 13:1209–1221.
29. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) 338:39–44.
30. **Lin, Y.-S., and M. R. Green.** 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1A and cyclic AMP inducible promoters. Proc. Natl. Acad. Sci. USA 85:3396–3400.
31. **Loeken, M. R., and J. Brady.** 1989. The adenovirus EIIA enhancer: analysis of regulatory sequences and changes in binding activity of ATF and EIIF following adenovirus infection. J. Biol. Chem. 264:6572–6579.
32. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science 236: 1237–1245.
33. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371–378.
34. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) 328:175–178.
35. **Neill, S. D., C. Hemstrom, A. Virtanen, and J. R. Nevins.** 1990. An adenovirus E4 gene product *trans*-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F. Proc. Natl. Acad. Sci. USA 87:2018–2012.
36. **Nevins, J. R.** 1980. Definition and mapping of adenovirus-2 nuclear transcription. Methods Enzymol. 65:768–785.
37. **Nevins, J. R.** 1989. Mechanisms of viral mediated *trans*-activation. Adv. Virus Res. 37:35–83.
38. **O'Neill, E. A., and T. J. Kelly.** 1988. Purification and characterization of nuclear factor III (origin recognition protein C), a sequence-specific DNA binding protein required for efficient initiation of adenovirus DNA replication. J. Biol. Chem. 263: 931–937.
39. **Raychaudhuri, P., S. Bagchi, S. D. Neill, and J. R. Nevins.** 1990. Activation of the E2F transcription factor in adenovirus-infected cells involves E1A-dependent stimulation of DNA-binding activity and induction of cooperative binding mediated by an E4 gene product. J. Virol. 64:2702–2710.
40. **Raychaudhuri, P., S. Bagchi, and J. R. Nevins.** 1989. DNA-binding activity of the adenovirus-induced E4F transcription factor is regulated by phosphorylation. Genes Dev. 3:620–627.
41. **Raychaudhuri, P., R. Rooney, and J. R. Nevins.** 1987. Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. EMBO J. 6:4073–4081.
42. **Reichel, R., I. Kovesdi, and J. R. Nevins.** 1988. Activation of a pre-existing cellular factor as the basis for adenovirus E1A-mediated transcription control. Proc. Natl. Acad. Sci. USA 85:387–390.
43. **Reichel, R., S. Neill, I. Kovesdi, M. C. Simon, P. Raychaudhuri, and J. R. Nevins.** 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for *trans*-activation of E2 transcription and for E2F activation. J. Virol. 63:3643–3650.
44. **Sassone-Corsi, P.** 1988. Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A *trans*-activation. Proc. Natl. Acad. Sci. USA 85:7192–7196.
45. **Scheidereit, C., A. Heguy, and R. G. Roeder.** 1987. Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter in vitro. Cell 51:783–793.
46. **Simon, M. C., T. B. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz.** 1989. Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A

regulation. Cell **52**:723–729.
47. **Simon, M. C., R. J. Rooney, T. M. Fisch, N. Heintz, and J. R. Nevins.** 1990. E1A-dependent *trans*-activation of the c-*fos* promoter requires the TATAA sequence. Proc. Natl. Acad. Sci. USA **87**:513–517.
48. **SivaRaman, L., S. Subramanian, and B. Thimmappaya.** 1986. Identification of a factor in HeLa cells specific for an upstream transcriptional control sequence of an E1A-inducible adenovirus promoter and its relative abundance in infected and uninfected cells. Proc. Natl. Acad. Sci. USA **83**:5914–5918.
49. **Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore.** 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. Nature (London) **323**: 640–643.
50. **Sturm, R., T. Baumruker, B. R. Franza, Jr., and W. Herr.** 1987. A 100-kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer. Genes Dev. **1**:1147–1160.
51. **Tan, T.-H., R. Jia, and R. G. Roeder.** 1989. Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator *tax*. J. Virol. **63**:3761–3768.
52. **Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel.** 1977. Transfer of purified herpesvirus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
53. **Wu, L., D. S. E. Rosser, M. C. Schmidt, and A. Berk.** 1987. A TATA box implicated in E1A transcriptional activation of a simple adenovirus-2 promoter. Nature (London) **326**:512–515.
54. **Yamamoto, K. K., G. A. Gonzales, W. H. Biggs, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.
55. **Yee, A. S., P. Raychaudhuri, L. Jakoi, and J. R. Nevins.** 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. Mol. Cell. Biol. **9**:578–585.
56. **Yoshinaga, S., S. Dean, M. Han, and A. J. Berk.** 1986. Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. EMBO J. **5**:343–354.