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Positive Regulation of *jun*/AP-1 by E1A

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Proteins encoded by the adenovirus E1A oncogene are capable of positive and negative transcriptional regulation of both viral and cellular genes. E1A regulatory function is commonly thought to involve modifications of specific cellular factors that interact with responsive promoters. In this report we present evidence that E1A induces the activity of the *jun*/AP-1 transcription factor in three different cell types: P19, JEG-3, and HeLa. AP-1 binds to 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive elements (TREs); therefore, E1A might modulate a specific signal transduction pathway normally induced by activation of the protein kinase C. Binding of *jun*/AP-1 to a TRE is induced in all cell types studied when E1A is expressed. We observe that the expression of endogenous c-*jun* and *jun* B genes is induced by E1A, which directly transactivates the promoters of c-*fos*, c-*jun*, and *jun* B. Similar inducibility is obtained by treatment with retinoic acid and differentiation of P19 embryonal carcinoma cells. The E1A 13S product transactivates TRE sequences and cooperates with c-*jun* in the transcriptional stimulation. The 12S E1A product does not activate a TRE sequence, but cotransfection with c-*jun* circumvents this lack of stimulation. Coexpression of c-*fos* and E1A 12S, however, blocks the transactivation by c-*jun*, suggesting an important role for *fos* in determining the dominance of the 12S or 13S protein.

The E1A oncogene products of adenovirus have been implicated in the transcriptional activation of the other early viral genes during infection (6, 41). Some cellular genes, such as β-tubulin, heat shock, c-*fos*, and c-*myc* oncogenes, are also transcriptionally activated by the E1A proteins (42, 74, 82). In addition to the transactivation properties, E1A proteins can specifically repress the activity of several viral and cellular transcriptional enhancers (10, 34, 84). These pleiotropic characteristics of the E1A products suggest that their role in cellular metabolism during adenovirus infection must be complex.

The E1A proteins have immortalizing properties as well as the ability to transform primary cells in conjunction with other oncogenes, such as E1B (the natural adenovirus complementary oncogene) and *ras* (69). Two mRNA forms, 12S and 13S, are produced by the E1A transcription unit early in infection. Comparison of several adenovirus serotypes shows that the E1A gene products contain three highly conserved regions. Two of these regions occur in both the 12S and 13S products, whereas the third consists essentially of the region unique to the 13S product. Mutational analysis indicated that the E1A transforming activity requires the 12S product, which is also responsible for the enhancer-repressor function (53, 61). Additional evidence suggests that both products may be more potent in inducing complete transformation. The 13S product, on the other hand, appears to be principally responsible for the transactivation function (53). The mechanism by which the E1A products elicit their functions is not totally understood but appears to involve a response of distinct cellular factors (5, 8, 38, 68, 81, 87). The search for specific sequences which might be targets of the E1A transactivation has been complicated by the fact that E1A proteins do not appear to directly bind DNA (12). Recently, however, it has been found that the sequence 5′-ACGTCA-3′, which occurs in most of the viral early promoters and binds transcription factor ATF, is one of the elements that can mediate E1A transactivation (33, 39, 40, 52, 55, 73). The identical sequence is found in several cellular genes whose expression is regulated by the level of cyclic AMP (cAMP) (7, 14, 19, 20, 23, 24, 60, 78, 80); it binds a nuclear protein termed cAMP-responsive element-binding protein (CREB). Moreover, some early viral promoters have been found to be cAMP inducible (48, 73).

A 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) represents a promoter target of the protein kinase C (PKC) signal transduction pathway and binds the nuclear factor AP-1 (2, 49). Interestingly, the sequence of a TRE differs from that of a cAMP-responsive element (CRE) only for one nucleotide (TRE is TGACTCA; CRE is TGACGTCA), and both elements have a homologous palindromic sequence. Furthermore, it has been shown that crosstalk in signal transduction is detectable at the transcriptional level since a CRE can be activated by *jun*/AP-1 (37, 77). AP-1 is a family of nuclear proteins which are likely to be encoded by several members of the *jun* oncogene family (9, 36, 71). *jun* and *fos* oncoproteins cooperate in binding to the TRE via formation of heterodimers which require intact leucine zipper domains of both proteins (29, 32, 43, 65, 76, 79, 83).

The effects of E1A on c-*fos* expression and activation of CRE sequences led us to study the possible modulation of *jun*/AP-1 activity by the E1A proteins. In addition, although they are apparently final elements of two distinct signal transduction pathways, members of both CREB and AP-1 families are similar and immunologically related (31). Therefore, we are interested in whether E1A alters the PKC-modulated factor *jun*/AP-1.

In this report we show that expression of E1A in several cell types increases the binding of *fos* and *jun* proteins to a

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TRE sequence. In P19 embryonal carcinoma cells a similar effect is observed upon treatment with retinoic acid (RA), which induces differentiation. In the same cells both E1A and RA activate c-*jun*, *jun* B, and c-*fos* gene transcription to a similar extent. Cotransfection experiments of E1A with c-*jun* and *jun* B promoter-reporter plasmids indicate that the effect is due to direct transactivation. E1A efficiently transactivates various TRE sequences which are inserted in a heterologous promoter environment. The transactivation is dependent on the 13S product and is enhanced by TPA treatment of the cells and cotransfection with a *jun* expression vector. The 12S product does not activate the TRE, but it does not block activation by *jun* unless the *fos* protein is coexpressed.

MATERIALS AND METHODS

Cells and plasmids. P19 embryonal carcinoma, P19 E1A5, MES-1, MES-1 E1A5, END-2, and END-2 E1A5 cells were cultured as described earlier (64). JEG-3 human choriocarcinoma cells and HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. As probes for hybridization studies we used a 1.0-kb *Pst*I mouse c-*jun* genomic fragment homologous to v-*jun* (18), a 1.5-kb *Eco*RI cDNA fragment of mouse *jun* B (71), a 0.8-kb *Pst*I fragment of v-*fos* (16), and a 1.4-kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH [26]). Chloramphenicol acetyltransferase (CAT) reporter plasmids have already been described (30). The thymidine kinase (TK) promoter from herpesvirus (positions −109 to +57) was fused to the CAT structural gene and used as a background for the analysis of TRE sequences in the transfection experiments. The expression vectors for E1A, c-*jun*, and c-*fos* are described elsewhere (50, 76).

Soft agar growth assay. P19 EC, P19 E1A, MES-1, MES-1 E1A, END-2, and END-2 E1A cells were plated at a density of 10^4 cells per 60-mm dish in DF-Bic–15% fetal calf serum with 0.375% agar onto a base layer of 0.5% agar. When indicated, retinoic acid was added at 10^{-6} M. Colonies larger than eight cells were scored after 14 days by counting 20 random fields corresponding to 2.3% of the total dish area. Data are expressed as the percentage of cells plated that were able to form colonies.

Oligonucleotides. In our study several oligonucleotides were used for binding analysis and transactivation experiments; their sequences are as follows: human collagenase TRE, 5′-AGCTTGATGAGTCAGCCG-3′; human metallothionein IIA TRE, 5′-GATCCGGCTGACTCATCA-3′; mouse c-*jun* TRE, 5′-AAGCTTGCATGACATCAGACAG-3′; human c-*fos* TRE, 5′-CATCTGCGTCAGCAGGTT-3′.

RNA isolation and Northern (RNA) blotting. Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride method (13). Total RNA (15 μg) was denatured for 10 min at 68°C in 50% (vol/vol) formamide–2.2 M formaldehyde–20 mM morpholinepropanesulfonic acid (MOPS; pH 7.0)–5 mM sodium acetate–1 mM EDTA, separated through 0.8% agarose–2.2 M formaldehyde gels, and subsequently transferred to nitrocellulose filters (BA 85; Schleicher & Schuell) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was immobilized by baking at 80°C for 2 h under vacuum. Hybridization took place in 50% formamide–5× SSC–50 mM sodium phosphate (pH 6.8)–10 mM EDTA–0.1% sodium dodecyl sulfate–0.1 mg of sonicated salmon sperm DNA per ml–2× Denhardt solution (1× Denhardt solution contains 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) at 42°C overnight. ^{32}P-labeled probes were generated by using a multiprime DNA labeling kit (Amersham). After hybridization and washing, filters were exposed to Kodak XAR-5 film at −70°C with intensifying screens.

DNA transfection and transient-expression assays. P19 EC cells were plated in DF-Bic–7.5% fetal calf serum at 3×10^5 cells per 50-mm tissue culture dish 24 h prior to transfection. At 2 h before transfection, the dishes received fresh medium. Cells were incubated for 16 to 20 h with calcium phosphate-precipitated DNAs (10 to 20 μg of plasmid per 50-mm dish), and then fresh medium was added. At 16 to 24 h later, the cells were harvested and then CAT activity was measured. pSV2Apap (1 μg) (35) was always included to serve as an internal control to correct for possible variations in the transfection efficiency between different cell types. Placental alkaline phosphatase assays were performed as described previously (35). Transfections with JEG-3 and HeLa cells were performed as already described (78). CAT activity was determined as described previously (30) and was quantitated by liquid scintillation counting of ^{14}C spots on thin-layer chromatography plates.

Adenovirus infection. Wild-type adenovirus infections were performed at a multiplicity of infection of 10. At 2 h after infection, extracts were prepared as described below. For infection with the adenovirus *dl*312 mutant (41), a multiplicity of infection of 100 was used and extracts were prepared after 24 h (28).

Gel mobility shift assay. Oligonucleotides containing AP-1-binding sites were synthesized and cloned into pGEM plasmids (Promega). The oligonucleotides were excised from the plasmids and isolated from a 10% (wt/vol) polyacrylamide gel. The cohesive ends of fragments were labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP (5,000 Ci/mol) by using the Klenow fragment of DNA polymerase I. Labeled DNA fragments were separated from unincorporated nucleotides by gel filtration, using Sephadex G-50 spin columns (Pharmacia) equilibrated in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–150 mM sodium chloride.

Nuclear extracts were isolated by the technique of Dignam et al. (21) or by the miniscale method described by Lee et al. (47). Extracts from adenovirus type 5 (Ad5)-infected cells were prepared as already described (68). The protein concentration was determined by the Bio-Rad protein assay, performed as specified by the manufacturer. The electrophoretic mobility shift assay that we used is based on the procedures described by Fried and Crothers (27) with slight modifications. Binding took place in 20 μl of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–50 mM sodium chloride–2 mM magnesium chloride–10% (vol/vol) glycerol–1 mM dithiothreitol–0.1 mg of poly(dI-dC) per ml–10 ng of pUC8 plasmid DNA per ml–0.02% (vol/vol) Nonidet P-40. Then 10 μg of nuclear extract was incubated with 0.1 to 0.5 ng of ^{32}P-labeled DNA fragment at room temperature for 20 min. To stop the reaction, 4 μl of 0.2% bromophenol blue, 0.2% xylene xyanol-F, and 25% Ficoll were added; the mixture was immediately loaded onto a 5% polyacrylamide gel containing 50 mM Tris hydrochloride (pH 8.5), 192 mM glycine, and 1 mM EDTA. Electrophoresis was carried out at 2 V/cm until the samples had entered the gel and was then continued at 7 V/cm for 3 to 4 h. The gel was fixed in 10% (vol/vol) methanol–10% (vol/vol) acetic acid for 20 min, dried, and visualized by autoradiography.

Antibodies to *fos* and *jun*. Affinity-purified antibodies to the M2 peptide of mouse c-*fos* have been described previously (17). Anti-*jun* Pep-1 peptide antiserum was a kind gift of T. J. Bos and P. Vogt (9). Inhibition of DNA-protein complex

binding with antibodies was performed by adding the antisera to the extracts or the purified proteins and incubating the mixture for periods from 2 h to overnight at 4°C. No disruption of the nucleoprotein complex binding was observed when a fivefold excess of either M2 or N peptide was added together with the antibody to the extract or to the purified proteins.

RESULTS

Induction of *fos/jun* binding by TPA, retinoic acid, adenovirus infection, and E1A. Specific binding to a TRE sequence is induced upon treatment of several cell types with TPA (2, 67, 75). The induction is thought to involve the increased number of *fos* and *jun* products available, since both genes are rapidly inducible by TPA and other agents (44, 46, 72, 86) and/or an increased DNA-binding potential of the preexisting *fos* and *jun* oncoproteins. *fos* protein is in fact phosphorylated whereas *jun* protein is dephosphorylated upon TPA treatment of cultured cells and subsequent induction of the PKC signal transduction pathway (3, 10a). In HeLa cells there is an important enhancement of *fos-jun* binding to a collagenase TRE (2) after 1 h of TPA treatment (Fig. 1A, compare lanes 1 and 2). Interestingly, nuclear extracts from Ad5-infected HeLa cells also contain an augmented TRE-binding activity (lane 3). In control extracts from mock-infected cells, no increased binding was detectable (lane 2). Similar results were obtained by using a TRE from the human metallothionein II promoter (49) (data not shown). To ensure that the observed binding was due to enhanced AP-1 activity in the extract, we treated the extract with *jun*/AP-1-specific antibody (Pep-1 [9]). As expected, inhibition of AP-1 binding was observed (lane 5), and because the binding activity of *jun*/AP-1 is modulated by the *fos* product ($p55^{fos}$; for a review, see reference 15), treatment of the extract with anti-*fos* antibody (M2Ab [17]) also produced an inhibition of TRE binding (lane 4). No disruption of the nucleoprotein complex binding was observed when a fivefold excess of M2 peptide was added to the extract together with the anti-*fos* M2 antibody (lane 6). As previously observed (76), no disruption of the nucleoprotein complex binding was obtained when preimmune Pep-1 (lane 7) serum was used. Infection with the E1A-deficient mutant *dl*312 (41) does not lead to enhanced TRE binding (Fig. 1B, compare lanes 1 and 2). The enhanced TRE binding found after wild-type adenovirus infection is therefore probably caused by the products of the adenovirus E1A gene, since under the infection conditions used, all other adenovirus early genes in the *dl*312 mutant are expressed at levels comparable to those found after wild-type infection (28).

To explore the possible role played by the products of nuclear oncogenes in the differentiation process, we next tested the well-characterized P19 embryonal carcinoma (EC) cell line (57). Undifferentiated P19 EC cells are tumorigenic cells, which exhibit all the characteristics of transformed cells, including anchorage-independent growth and proliferation in the absence of growth factors. Differentiation of EC cells in response to chemical agents (e.g., RA or dimethyl sulfoxide) is accompanied by loss of tumorigenicity and the transformed phenotype. Nuclear extracts from undifferentiated P19 cells were prepared and compared with extracts from differentiated P19 cells obtained after 5 days of RA treatment. There is a much higher level of TRE-binding proteins in extracts from RA-treated cells than in undifferentiated P19 cells (Fig. 2B; Table 1). To investigate whether E1A could have an effect on AP-1-binding activity, we

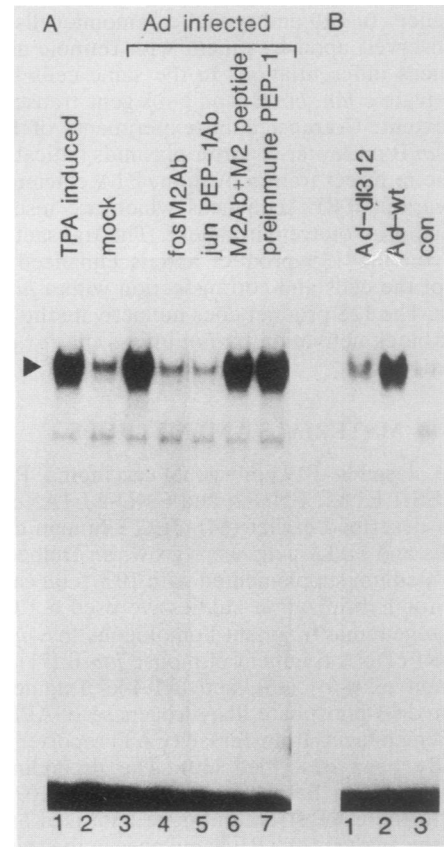

FIG. 1. Increase of specific TRE binding by the E1A product. (A) Gel retardation analysis with HeLa cell nuclear extracts from uninduced (mock, lane 2) and TPA induced (1 h) (lane 1) cells. Infection of HeLa cells with Ad5 (68) increases binding to the TRE (lane 3). Binding inhibition was obtained by preincubating the nuclear extracts from Ad5-infected cells with specific M2 anti-*fos* (lane 4) and anti-*jun* Pep-1 (lane 5) antibodies. Addition of a fivefold excess of M2 *fos* peptide to the immunoreaction blocked the inhibitory function of the antibody (lane 6). Preimmune serum of the anti-*jun* Pep-1 antibody showed no effect (lane 7). (B) Gel retardation analysis with HeLa cell nuclear extracts from uninfected (lane con), Ad5-infected (lane Ad-wt), or Ad5 E1A-deficient mutant-infected (lane Ad-dl312) cells. Infection and preparation of nuclear extracts were performed as described in Materials and Methods. The observed increase in binding to the TRE is E1A specific, since infection with the Ad-*dl*312 mutant does not enhance TRE binding.

established a number of P19 cell lines stably transfected with the E1A gene from Ad5 (P19 clones 15, 16, and 20 [Table 1]). These cell lines expressed E1A mRNA (Fig. 2A) and the corresponding E1A proteins (Table 1) (61a). Nuclear extracts from all the E1A-expressing P19 clonal cell lines contain an AP-1-binding activity comparable to that of the RA-treated P19 cells, which is about 20-fold higher than that of undifferentiated cells (Fig. 2B). These results indicate that E1A mimics the positive effect of RA on AP-1-binding activity. Interestingly, E1A expression in P19 cells leads to loss of the transformed phenotype in all three cell lines as judged by growth in soft agar (Table 1). Since RA treatment has the same effect on P19 cells (Table 1), this indicates that expression of E1A, like RA treatment, might lead to differentiation of these cells. To test whether the observed enhancement of AP-1-binding activity by E1A is limited to undifferentiated P19 EC cells, we used two proliferating

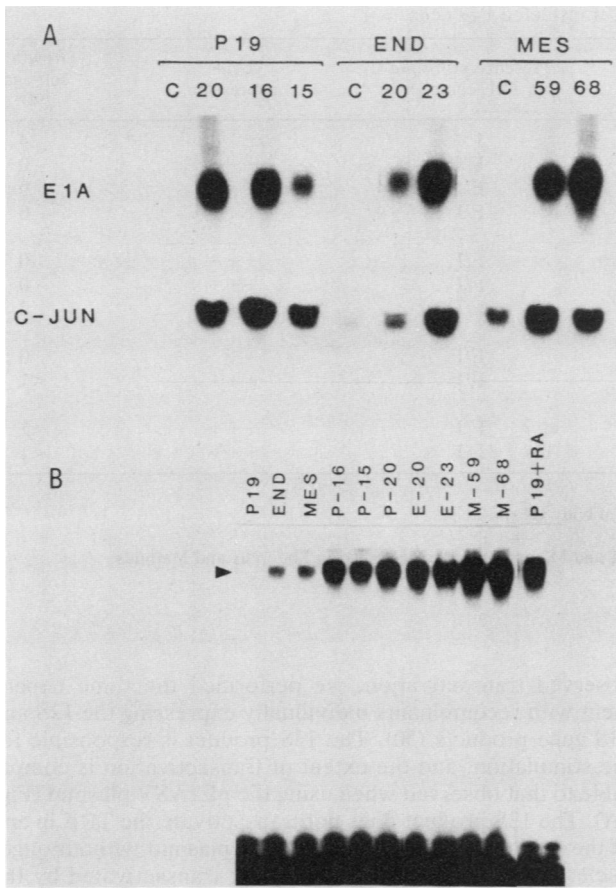

FIG. 2. E1A enhances c-*jun* expression and TRE binding in P19 EC and its differentiated derivatives. (A) Northern analysis of P19 EC, END-2, and MES-1 cells and E1A-transfected clones. Hybridization with an E1A-specific probe shows that all the transfected cell lines, unlike the wild-type cells (lanes marked C), express high levels of E1A mRNA. Hybridization with a c-*jun*-specific probe shows that in all E1A-expressing cell lines the level of c-*jun* mRNA is strongly enhanced. (B) Gel shift analysis of nuclear extracts from the E1A-expressing cell lines with the collagenase TRE as a probe. All E1A-expressing cells contain elevated levels of proteins that bind to the TRE. A similar rise is observed after treatment of P19 cells for 5 days with RA (lane P19 + RA).

differentiated clonal derivatives of P19 cells, MES-1 and END-2. MES-1 is a mesodermlike cell line isolated from P19 EC cells aggregated in the presence of dimethyl sulfoxide (63), whereas END-2 is an endodermlike cell isolated from P19 cells aggregated in the presence of RA (64). Both cell lines have lost the transformed properties of P19 EC cells. Nuclear extracts from MES-1 and END-2 cells naturally contain a fivefold-higher level of AP-1-binding activity than those from P19 cells do (Fig. 2B). In two MES-1 cell lines stably transfected with the Ad5 E1A gene (MES clones 59 and 68), which express high levels of E1A mRNA (Fig. 2A), there is a fivefold increase in TRE binding with respect to MES-1 cells (Fig. 2B). In addition, two stably transfected END-2 cell lines, END clones 20 and 23, both express high levels of E1A mRNA (Fig. 2A) as well as strongly enhanced levels of TRE-binding proteins (Fig. 2B). Binding inhibition by specific antibodies indicated that both *fos* and *jun* products are responsible for the increased binding in extracts from E1A-expressing P19, MES-1, and END-2 cells (Table 1). These results show that E1A enhances specific binding properties of *fos* and *jun* proteins to a TRE sequence both in undifferentiated and in differentiated cells and that this enhancement does not result indirectly from an E1A-induced change in the differentiation state of the cells. It is noteworthy that the E1A effect is on the number of nucleoprotein complexes binding to TRE oligoprobes; there is no detectable change in the mobility of the retarded band (Fig. 2B). This strongly suggests that the E1A proteins do not directly participate in the formation of a stable complex.

E1A transactivates c-*jun* and *jun* B genes. To further investigate the nature of the increased TRE binding, we examined the effect of Ad5 E1A on the expression of the c-*jun* gene, whose product is a major component of transcription factor AP-1 (9). c-*jun* mRNA levels are strongly increased in all three E1A-expressing P19 clones compared with the level in normal P19 EC cells (Fig. 2A). This enhancement is similar to the increase in the level of c-*jun* mRNA after differentiation with RA for 5 days (Table 1; Fig. 3, compare lanes 1, 3, and 5). In addition, in both the E1A-expressing MES-1 and END-2 cell lines, c-*jun* mRNA levels are significantly higher than in the wild-type MES-1 and END-2 cells (Fig. 2A; Table 1). These results indicate that E1A strongly enhances c-*jun* mRNA expression both in undifferentiated and in differentiated cell lines (summarized in Table 1). To study whether E1A had a similar effect on two other components of AP-1, *jun* B and c-*fos*, we studied their expression in a P19 E1A-expressing cell line (clone 20). E1A induced a smaller increase in the constitutive expression of *jun* B (Fig. 3, compare lanes 3 and 5), a gene whose expression cannot be detected in RA-differentiated P19 cells (lane 1). Surprisingly, constitutive c-*fos* expression was not enhanced by E1A (lane 5). In differentiated MES-1 cells, similar effects of E1A were found (data not shown).

Since c-*jun* is positively autoregulated by its own gene product, which binds to a TRE in the 5′-flanking sequences of its gene (1), we investigated the effects of TPA on the expression of c-*jun*, *jun* B, and c-*fos*. In undifferentiated EC cells these genes were not induced by TPA (Fig. 3, lane 4), whereas in E1A-expressing P19 cells, TPA treatment caused two-, five-, and eightfold induction of c-*jun*, *jun* B, and c-*fos* expression, respectively (lane 6). These levels of induction are comparable to those observed in TPA-stimulated RA-differentiated P19 cells, although *jun* B is induced to a somewhat lesser extent in these cells (lane 2).

Next we analyzed the possibility that E1A directly transactivates the promoters of c-*fos*, c-*jun*, and *jun* B. Promoter transactivation has already been shown for c-*fos* (74). Here we used a c-*jun* promoter region between positions −1800 and +554, a *jun* B promoter region from −858 to +245, and a c-*fos* promoter fragment from −358 to +8. These elements were linked to the bacterial CAT reporter gene (Fig. 4, bottom). Cotransfection of P19 cells with these reporter plasmids and expression vectors coding for c-*jun* protein (76) or the E1A products 12S and 13S (50) was performed. The results (Fig. 4; Table 2) clearly show that the c-*jun* and E1A 13S proteins transactivate all three promoters, whereas the E1A 12S product is unable to do so. These results also confirm that c-*jun* positively autoregulates the c-*jun* promoter (1) and extend this function to both *jun* B and c-*fos* promoters.

Activation of a TRE by the E1A 13S product. Since E1A enhances binding of the *fos*/*jun* complex to a TRE sequence (Fig. 1) and stimulates c-*jun* and *jun* B gene transcription (Fig. 2 and 3), we wanted to analyze whether it could directly activate a TRE. Cotransfections were performed in three

TABLE 1. Properties of E1A-transfected P19 cells

Cell	E1A expression		TRE binding[c]	Presence of *fos*/*jun* in TRE complex[d]	c-*jun* expression	Growth in soft agar (%)[e]
	mRNA[a]	Protein[b]				
P19-EC	−	−	±	J	±	24
P19-RA	−	−	+++	F/J	+++	0
END	−	−	+	F/J	±	0
MES	−	−	+	F/J	+	0
P19 clone 15	++	±	+++	F/J	+++	0
P19 clone 16	+++	+	+++	F/J	+++	0
P19 clone 20	+++	+	+++	F/J	+++	2
END clone 20	++	+	+++	F/J	++	0
END clone 23	+++	+	+++	F/J	+++	<1
MES clone 59	++	+	+++	F/J	+++	<1
MES clone 68	+++	+	+++	F/J	+++	<1

[a] E1A mRNA expression was determined by Northern blotting.
[b] E1A protein expression was determined by immunofluorescence with the M73 antibody (61a).
[c] TRE binding was performed with a collagenase TRE.
[d] The presence of *fos* and *jun* in the TRE complex was determined by using Pep-1 and M2 antibodies as described in Materials and Methods.
[e] Growth in soft agar was determined as described in Materials and Methods.

different cell types, HeLa, P19, and JEG-3. The recombinant TRE/TK-CAT contains the human metallothionein IIA TPA-responsive element linked to the herpesvirus TK promoter (−109 to +57) and the reporter CAT gene. When TRE/TK-CAT was cotransfected with the c-*jun* expression vector pSVc-*jun*, the expected activation was detected in all cell types tested (Fig. 5A), albeit to different degrees. Cotransfection of TRE/TK-CAT with an E1A expression vector shows a transcriptional induction which is comparable to the one obtained with pSVc-*jun* in the three cell types studied. As mentioned above, two mRNAs are produced early during the infection by the E1A transcription unit. To determine whether one or both E1A products are responsible for the observed transactivation, we performed the same experiment with recombinants individually expressing the 12S and 13S gene products (50). The 13S product is responsible for the stimulation, and the extent of transactivation is comparable to that observed when using the pE1ASV plasmid (Fig. 5A). The 12S product does not transactivate the TRE in any of the cell types tested. The reporter plasmid without oligonucleotide insertion (TK-CAT) is not transactivated by the 13S product in HeLa (Fig. 5C) or JEG-3 (not shown) cells. Moreover, specific mutations in the TRE sequence which abolish TPA inducibility (2) also block transactivation by the 13S product (Fig. 5C, TRE*-TK).

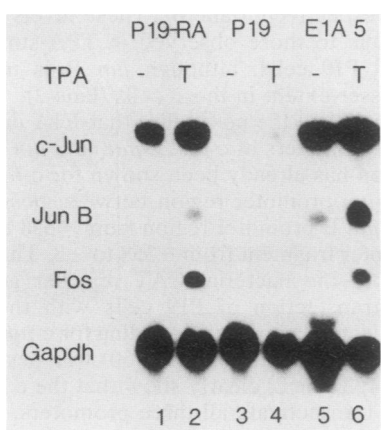

FIG. 3. Additional effects of E1A and TPA on c-*jun*, *jun* B, and c-*fos* expression. The figure shows a Northern blot analysis of RNA from P19 EC cells treated for 5 days with 10^{-6} M RA (lanes P19-RA) and 100 ng of TPA per ml, compared with RNA from P19 clone 20 cells stably transfected with Ad5 E1A (lanes E1A5). Transcripts from c-*jun*, c-*fos*, and *jun* B genes were analyzed with the specific probes described in Materials and Methods. The same Northern blot was also hybridized with a GAPDH-specific probe to ensure that the same RNA amounts were loaded on the gel.

The AP-1 recognition site (TRE) mediates the transcriptional response to the phorbol ester TPA (2, 49). We studied whether cotransfection of *jun* and E1A expression vectors and TPA treatments might indicate cooperativity between these factors. We observed that TPA enhances the transactivation function of both *jun* and E1A in HeLa cells (Fig. 5B) when cotransfected separately or together. Activation by coexpressed *jun* and E1A is stronger than activation by each of them separately, but there is no real additive or synergistic effect. Since both c-*fos* and c-*jun* promoters contain TRE-like sequences (1, 86) and both promoters are transactivated by E1A (Fig. 4), we next tested the TRE sequences from c-*fos* and c-*jun* promoters for E1A transactivation. The two elements, synthetically obtained as oligonucleotides, were inserted into the same position as the metallothionein TRE in TRE/TK-CAT (Fig. 6). Cotransfection experiments with E1A and c-*jun* expression vectors in JEG-3 cells indicate that all three TRE sequences are activated (Fig. 6), albeit to different degrees. In particular, the c-*fos* AP-1-like site, which has been shown to be induced by TPA (25), is only weakly induced. Similar results were obtained with HeLa cells (data not shown). The difference in inducibility of the c-*jun* and the c-*fos* TRE sequences is presently unclear, since both these TRE sequences have been shown to bind similar protein complexes to those bound by the metallothionein TRE (1, 25).

Opposite functions of *fos* and *jun* in conjunction with the 12S product. The E1A 12S product is not able to transacti-

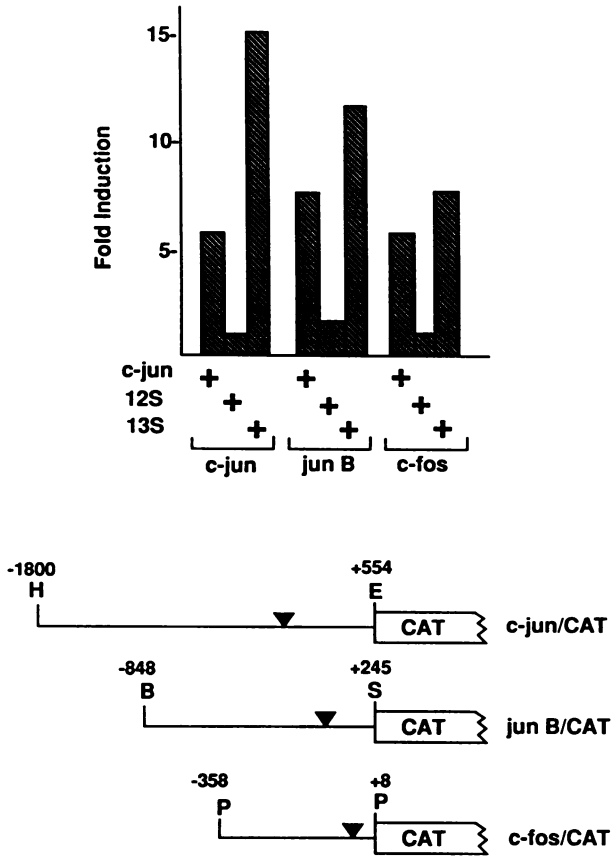

FIG. 4. E1A transactivates c-*jun*, *jun* B, and c-*fos* promoters. The figure shows direct transactivation of c-*jun*, *jun* B, and c-*fos* promoters by E1A and c-*jun* products in cotransfection experiments in P19 EC cells. The expression vectors used code for the mouse c-*jun* protein (c-*jun*) and the individual E1A early proteins (indicated as 12S and 13S). In cotransfection assays a 2:1 ratio of reporter plasmid to expression vector was used. The proto-oncogene promoters used in this study are depicted in the lower panel. The c-*jun* promoter region encompasses sequences from positions −1800 to +554; the *jun* B promoter contains sequences from −848 to +245; and the c-*fos* promoter contains sequences from −358 to +8. All promoters were linked at the same position of the structural gene for the bacterial CAT.

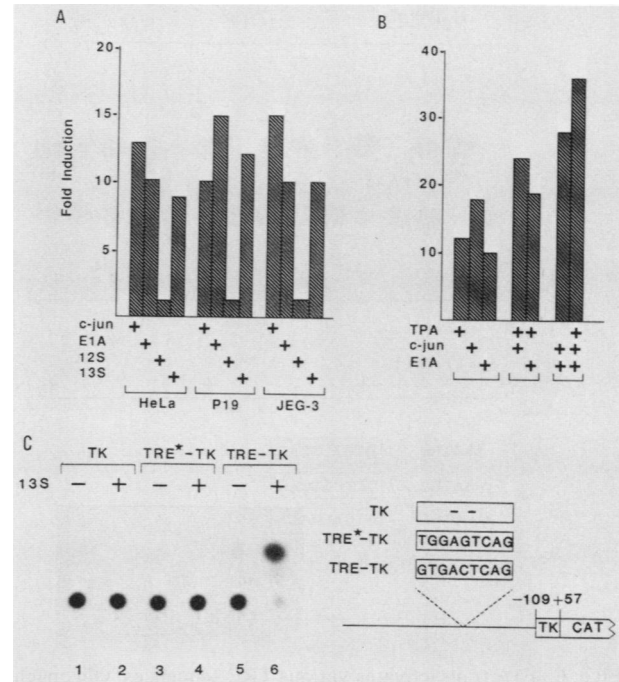

FIG. 5. The 13S E1A product transactivates a TRE in several cell types. (A) Results of cotransfection of a TRE/TK-CAT reporter plasmid bearing the metallothionein II AP-1 site and expression vectors coding for c-*jun* protein, the 12S E1A product, the 13S E1A product, or both E1A early proteins (E1A). The 13S protein is responsible for the induction, whereas the 12S product has no effect. The values are the average of several experiments with each cell type. Fold induction is relative to the basal level of CAT activity in each cell type. In each experiment a 2:1 ratio of reporter plasmid to transactivator was used. (B) Additional activations by c-*jun*, E1A, and TPA. Several experiments conducted with HeLa cells used the TRE/TK-CAT reporter plasmid containing the metallothionein II AP-1 site. Fold induction is relative to the basal-level transcription of the reporter plasmid. TPA, c-*jun*, and E1A give somewhat additional inductions. (C) E1A 13S does not activate a mutant TRE. Cotransfection in HeLa cells of E1A 13S with TK-CAT, wild-type metallothionein TRE-TK-CAT, or mutant TRE*-TK-CAT, which fails to bind AP-1 (2), shows that E1A 13S only transactivates the wild-type TRE. This experiment was repeated several times, and a representative result is shown.

vate the TRE (Fig. 4). Also, the enhancer-repressor function of E1A has been associated with the 12S product (10, 53). In relation to the different activities of the two E1A proteins, we next studied the coexpression of *jun* with the two individual E1A mRNAs. Cotransfection of pSVc-*jun* with pE1A 13S resulted in an activation similar to the one obtained with pE1ASV and c-*jun*. Thus, the 13S product appears to be dominant in the TRE *trans*-regulation. Transcriptional activation by *jun*/AP-1 does not appear to be negatively affected by the 12S product.

TABLE 2. Activation of *jun* and *fos* promoters by E1A

Reporter	Activator construct[a]:				
	Control	c-*jun*	E1ASV	13S	12S
c-*jun*/CAT	3.1 ± 0.6	18 ± 3	46 ± 5	48 ± 4	3.5 ± 0.5
jun B/CAT	1.6 ± 0.5	12 ± 2	18 ± 3	20 ± 4	2.5 ± 0.6
c-*fos*/CAT	1.3 ± 0.4	8 ± 2	9 ± 3	10 ± 3	1.7 ± 0.4

[a] Activation data are indicated as percent acetylation and were determined by liquid scintillation counting of ^{14}C spots on a thin-layer chromatography plate. All transfections were performed with P19 EC cells. Results are means ± standard deviations of four independent experiments.

TRE sequences have been shown to bind heterodimers of the *fos* and *jun*/AP-1 oncoproteins. Because of the pleiotropic characteristics of the *fos* protein, we next studied its effect in this system. In both JEG-3 and HeLa cells, expression of *fos* only slightly induced transcription from the TRE, although *fos* enhances transactivation by *jun* when they are cotransfected (Fig. 7). Experiments in which the E1A and *fos* expression vectors were cotransfected indicated that *fos* does not affect the transactivation obtained with pE1ASV and pE1A 13S. Remarkably, however, when E1A 12S was coexpressed with both *fos* and *jun*, no significant transactivation of the TRE was observed. Thus, the 12S protein appears to block *jun* transactivation only in the presence of *fos*.

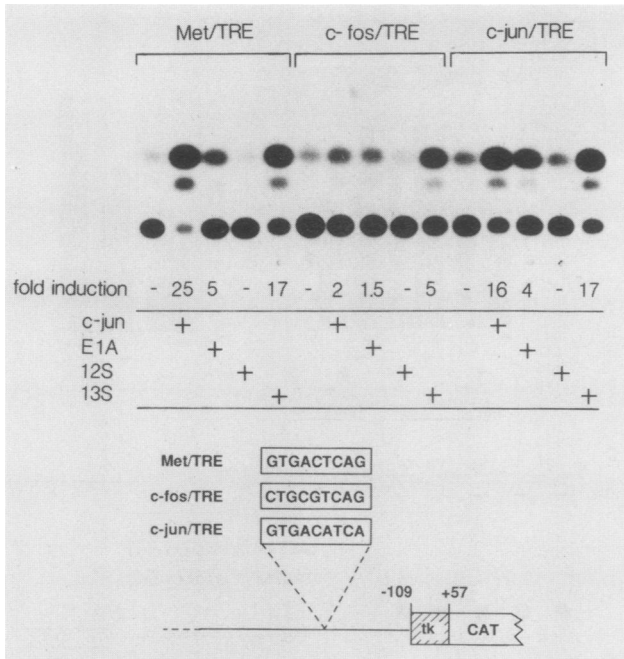

FIG. 6. E1A transactivates various TRE sequences. Oligonucleotides containing putative AP-1 sites from the human metallothionein II promoter (49) (lanes Met/TRE), from the c-*fos* promoter (25) (lanes c-*fos*/TRE), and from the c-*jun* promoter (1) (lanes c-*jun*/TRE) were inserted upstream from the herpesvirus TK promoter element (positions −109 to +57) fused to the CAT gene. These TRE reporter plasmids were tested for transactivation by pE1ASV, pE1A 12S, pE1A 13S (8), and c-*jun* in cotransfection experiments in JEG-3 cells. Transactivation with pE1ASV, pE1A 13S (E1A and 13S in the figure), and c-*jun*, but not with pE1A 12S (12S in the figure), was obtained with all TRE sequences, albeit to various extents. Induction values are the average of two experiments.

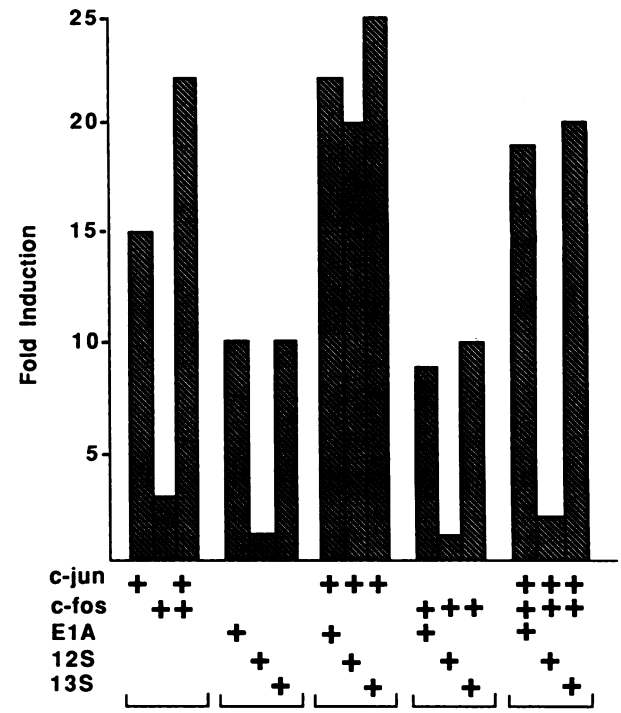

FIG. 7. Opposite functions of c-*jun* and c-*fos* in conjunction with the E1A products 12S and 13S. Cotransfection experiments were performed with JEG-3 cells by using combinations of expression vectors for c-*jun*, c-*fos*, and the E1A proteins. The experiments were performed by using the TRE/TK-CAT plasmid with the metallothionein II AP-1 site. The 12S product represses c-*jun*-induced TRE transactivation only when c-*fos* is coexpressed. Fold induction is relative to the basal transcription level of the reporter plasmid in these cells.

DISCUSSION

The adenovirus E1A gene products have been shown to possess both transcriptional activator as well as transcriptional repressor activity on a variety of viral and cellular promoters and enhancers (6, 10, 34, 41, 50, 84). These pleiotropic regulatory activities are mediated by two distinct protein products, encoded by the 12S and 13S mRNAs of the E1A transcription unit. Previous investigations have shown that the effect of E1A on promoter activation is mediated by the 13S gene product, while the 12S gene product is involved in enhancer repression (for reviews, see references 5 and 61).

E1A induces *jun*/AP-1. In this report, we show that expression of E1A results in increased binding of nuclear factors to the TRE enhancer. This increase in TRE binding was observed in HeLa and JEG-3 cells following infection with Ad5, but not following infection with the Ad5 E1A-deficient mutant *dl*312, and in P19 EC cells and its differentiated derivatives MES-1 and END-2 stably transfected with the Ad5 E1A transcription unit. In all these cell types, both *fos* and *jun* proteins were present in the TRE-binding complex. The observed increase in TRE binding is at least partly due to an increased expression of c-*jun* mRNA, which, unlike the *jun* B and c-*fos* genes, is expressed at greatly elevated levels in the two cell lines stably transfected by E1A. However, both *jun* B and c-*fos* are rapidly induced by TPA in cell lines expressing E1A, indicating that the E1A gene products do not interfere with the transcriptional activation of these genes upon activation of the PKC by TPA. Similar results on transactivation of c-*jun* by E1A and TPA were obtained with HeLa cells. These results show that the effects of E1A on TRE binding and TRE-dependent transcription result at least partly from an increased abundance of c-*jun* mRNA and protein. Our observed effects of E1A on TRE binding by *jun*/AP-1 are further supported by a recent report showing that adenovirus infection of S49 cells leads to a higher inducibility of AP-1-binding activity and c-*fos* and *jun* B mRNA by cAMP, implicating E1A in the positive regulation of *jun*/AP-1 (62).

Previously, it was shown that the c-*jun* gene is regulated by its own gene product, owing to the presence of an AP-1-binding site in its 5′-flanking sequences (1). This raises the question of which mechanism underlies the effect of E1A on the expression of c-*jun*. It was suggested previously that activation of CREB/ATF by E1A is mediated by specific protein phosphorylation, analogous to the effect of cAMP-dependent protein kinase II on CREB (22, 73). Similarly, E1A may induce posttranslational modifications; hence, phosphorylation of *jun* may increase its binding activity and thus autocatalytically increase c-*jun* expression. Alternatively, c-*jun* expression may normally be repressed at its autoregulatory site, for example by *jun* D, which is constitutively expressed in most cells (17a, 70). By a similar mechanism, the E1A activator function may inactivate the putative repressor by posttranslational modification, thereby

allowing autocatalytic activation of the c-*jun* gene by its own gene product. A third model was recently suggested by Green and co-workers, who presented evidence that a specific member of the ATF transcription factor family, ATF-2, might function as a promoter-bound receptor for E1A, thereby positioning the activation domain of E1A at the promoter (54, 56). It remains to be determined, however, whether ATF-2 binds to the c-*jun* promoter.

12S-13S dominance switching is dependent on *fos*. Our data show that E1A transactivates TRE sequences from the metallothionein, c-*fos*, and c-*jun* promoters in three different cell lines and confirm that the TRE activator function of E1A is encoded by the 13S transcription unit. The 12S gene product was found to be unable to activate TRE dependent transcription, in agreement with the inability of this gene product to activate transcription from viral or cellular promoters (53, 61). Similar effects of E1A on TRE transactivation in HeLa and 293 cells were recently shown by Buckbinder et al. (11). Although the 12S gene product by itself is unable to activate TRE-dependent transcription, it does not inhibit c-*jun*-induced TRE activation in a cotransfection assay. An important finding of our investigations, however, is that expression of the 12S transcription unit will result in reversal of the activation of TRE by c-*jun* when c-*fos* is coexpressed. This effect has been observed in the three cell lines HeLa, JEG-3, and P19 EC. What could be the mechanism of the c-*fos*-dependent repression by E1A 12S? *fos-jun* heterodimers have been shown to exhibit increased binding to the TRE enhancer associated with increased transcription from the TRE (32, 43, 65, 76). One possibility, therefore, is that the 12S gene product somehow interferes with the transcription-activating function of *fos*. On the other hand, the 12S product could be affecting the DNA-binding properties of the *fos-jun* complex. Further experiments are necessary to clarify the observed dependence on *fos* of TRE repression by the 12S product.

Transformation and differentiation by E1A. A cellular effect of E1A is the immortalization of cells in culture and transformation in combination with either *ras* or E1B (69). The immortalizing effect is attributed to the 12S gene product which is also responsible for enhancer repression (10, 53, 61). Mutant *ras* proteins have been shown to activate TRE-dependent transcription (75). Therefore, opposite effects on TRE activation are apparently involved in full transformation. We note, however, another interesting correlation between induction of *jun*/AP-1 by E1A and cellular differentiation. Previously it was shown that cellular differentiation is induced by *ras* and nerve growth factor in PC12 cells (4, 45, 51, 66, 75) and by TPA in HL60 cells (58). Differentiation is preceded by increased expression of TRE binding proteins. In this regard, it is interesting that transfection of E1A in P19 EC cells induces differentiation, resembling the effects of RA on these cells (61a). Similar effects have been observed by transfection of E1A in F9 cells, although conflicting data have been reported on the role of either the 12S or 13S products in the differentiation inducing event (59, 85). Recent studies indicate that high levels of c-*jun* expression are somehow sufficient for differentiation to occur in P19 EC cells (18), indicating that the observed effects of E1A on cellular differentiation are likely to be mediated by the 13S gene product. Further analysis of the role of *jun* and *fos* proteins will be required to untangle the complex interplay between these factors and E1A in transformation and differentiation.

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