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Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A trans-activation

(transcriptional regulation/nuclear factor/cAMP-dependent protein kinase/signal transduction)

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ABSTRACT **Early in adenovirus infection, the E1A (early region 1A) oncogene products trans-activate the other early viral transcription units, as well as some cellular promoters. The mechanism by which E1A elicits its activity is still unknown. In this report, I show that the adenovirus E2a and E3 promoters are cAMP inducible in rat pheochromocytoma PC12 cells and that this activation requires the presence of the cAMP-dependent protein kinase II. Using deletion mutants of the E2a promoter, it was found that the sequence TACGTCAT located between positions −70 and −77 is involved in both the cAMP response and the E1A trans-activation. Also, in the mutant PC12 cell line A126-2B, which lacks the cAMP-dependent protein kinase II, E1A is still able to activate E2a and E3 promoters. This suggests that E1A products may circumvent the lack of the kinase by activating an alternative signal transduction pathway, which could mimic the effect of agonists of adenylate cyclase. I propose that E1A is capable of modifying by phosphorylation, either directly or indirectly, the transcription factor that binds the ACGTCA motif. Such a factor, termed ATF (adenovirus transcription factor), has already been characterized and appears to have strong similarities to the transcriptional factor CREB (cAMP responsive element binding protein), which binds homologous sequences in cAMP responsive genes, such as somatostatin and c-*fos*.**

Eukaryotic RNA polymerase II promoters are composed of a complex array of cis-acting genetic elements that regulate basal, induced, and repressed transcriptional rates (1, 2). The interaction of specific trans-acting nuclear factors with various cis-regulatory elements dictates the control of tissue-specific, hormone-induced, viral-induced, and growth–differentiation-related gene expression.

Adenovirus constitutes an excellent model system to study the regulation of eukaryotic gene expression. During infection, the various adenoviral transcription units are coordinately regulated. The E1A (early region 1A) oncogene products, in particular, are implicated in the transcriptional activation of the other early viral genes (3, 4), as well as some cellular genes (5, 6) and oncogenes (7). In addition to the trans-activation properties, E1A can specifically repress the activity of several viral and cellular transcriptional enhancers in a cell-specific manner (8–12). Two mRNA forms, 12S and 13S, are produced by the E1A transcription unit early in infection. Mutational analysis indicates that the 12S product (a 243-amino acid protein) is required for the transforming activity and the enhancer–repressor function (13, 14). The 13S product, a 289-amino acid phosphoprotein, is responsible for the transcriptional activation function (13, 14). The mechanism by which the E1A 13S product functions is still unclear. Viral infection may increase the synthesis of host transcription factors or modify the preexisting ones present in the nucleus. Alternatively, the transcription factors that are sequestered by the host promoters may, upon infection, be diverted to viral early promoters by an as yet unidentified mechanism.

Several recent reports indicate that the levels of promoter-specific host factors increase considerably upon viral infection (15, 16). In some cases, the cis elements required for the binding of the host nuclear factor(s) involved in the E1A trans-activation have been identified.

For some genes, the E1A trans-activation appears to require an intact "TATA box" and, consequently, it has been postulated that the E1A protein might interact with the TATA box binding protein (18, 19). A transcriptional factor recently identified, termed ATF (adenovirus transcription factor) (20, 21), also appears to be involved in E1A trans-activation. ATF binds to the consensus motif ACGTCA, which is present upstream from most of the early adenovirus promoters. This sequence is also identical to the cAMP responsive element (CRE) found in most cAMP-regulated promoters and that binds the nuclear factor CREB (22). In Table 1, a comparison of putative ATF and CREB binding sites is shown.

In this paper, I demonstrate that the adenovirus early E2a and E3 promoters are cAMP inducible in rat pheochromocytoma PC12 cells and that such activation requires the presence of the cAMP-dependent protein kinase II. The sequence required for the cAMP response also appears to be involved in the E1A trans-activation. However, in mutant PC12 cells lacking the cAMP-dependent protein kinase II, E1A is still able to activate both the E2a and the E3 promoters. It is proposed that E1A is capable of modifying, either directly or indirectly, ATF, which is presumed to be identical to CREB. It is proposed that this modification is very likely to be phosphorylation.

MATERIALS AND METHODS

Plasmids. The recombinants used in this study have already been described. pE1ASV (23) is a pBR322-based plasmid containing the entire adenovirus type 2 (Ad2) E1A transcription unit (0–4.5 map units) linked to the simian virus 40 poly(A) addition signal (coordinates 2469–2604); pE1A12S and pE1A13S (24) are equivalent to pE1ASV except that the E1A coding sequence has been replaced by the cDNAs corresponding to the 12S and 13S mRNAs. pE3CAT (25) contains sequences between −487 and +65 of the Ad5 E3 promoter linked to the bacterial chloramphenicol acetyltransferase (CAT) gene (gift from N. Jones). The plasmids pEC-97, pEC-79, pEC-70, and pEC-59 are deletion mutants of the Ad2 E2a promoter containing, respectively, fragments from positions −97, −79, −70, and −59 to +40, respectively, linked to the CAT gene (26) (gift from J. Nevins). The

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Abbreviations: Ad2, adenovirus serotype 2; Ad5, adenovirus serotype 5; ATF, adenovirus transcription factor; CRE, cAMP responsive element; CREB, CRE binding protein; CAT, chloramphenicol acetyltransferase; α-CG, human glycoprotein α-subunit gene.

Table 1. Cellular and viral promoter sequences constituting potential binding sites for nuclear factors ATF and CREB

Gene	Sequence	Position
Somatostatin	A C G T C A	−41
c-*fos*	A C G T C A	−66
Proenkephalin	G C G T C A	−89
HSP70	T C G T C A	−38
Tyrosine hydroxylase	A C G T C A	−49
Fibronectin	A C G T C A	−166
PEPCK	A C G T C A	−83
VIP	A C G T C A	−69
Parathyroid hormone	A C G T C A	−68
α-Chorionic gonadotropin	A C G T C A	−93
Ad2 E2a	A C G T C A	−76
Ad5 E3	T C G T C A	−54
Ad5 E4	A C G T C A	−45
Ad5 E4	A C G T C A	−170
HTLV-II LTR	A C G T C A	−180
BLV LTR	A C G T C A	−161
Cytomegalovirus enhancer	A C G T C A	−115
Intracisternal A particle	A C G T C A	−101
Consensus	A C G T C A	

HSP70, heat shock protein 70; PEPCK, phospho*enol*pyruvate carboxylase; VIP, vasoactive intestinal particle; HTLV-II, human T-cell leukemia virus type II; BLV, bovine leukemia virus; LTR, long terminal repeat.

plasmids pUCSom (gift from M. Montminy) and pUCα-CG (gift from P. Mellon) contain oligodeoxynucleotides of the CRE elements from the rat somatostatin gene (22) (positions −32 to −61) and from the human glycoprotein hormone α-subunit gene (27) (α-CG, positions −152 to −100), respectively, cloned in the polylinker of pUC19.

Tissue Culture and DNA-Mediated Transfection. PC12 pheochromocytoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% horse serum. The PC12 mutant cell line A126-2B (28) (gift from J. Wagner) was grown in the same conditions. Cells at 70% confluence were transfected by the calcium phosphate coprecipitation technique (23) and exposed to the precipitate for 12 hr. After washing with phosphate-buffered saline, fresh medium was added and the forskolin treatment was extended for 4–8 hr, when required, at a final concentration of 20 μM. When <20–30 μg of specific DNA was used per 10-cm culture dish, pUC19 plasmid DNA was added to give 20–30 μg of total DNA. After DNA transfection, the cells were harvested and CAT assays were performed as described (29).

***In Vitro* Transcription Assay.** *In vitro* transcription of the E3 promoter was performed as described (30, 31), with the following modifications: (*i*) a HeLa cell nuclear extract was used (32); (*ii*) the E3 template was derived from the pE3CAT recombinant by purifying a 550-base-pair *Eco*RI fragment. In a run-off assay, this template produces a transcript of 315 nucleotides. The RNA synthesized in the presence of [α-^{32}P]GTP was phenol/chloroform-extracted, ethanol-precipitated, and loaded on a denaturing 8 M urea/6% polyacrylamide gel.

RESULTS

The E2a and E3 Promoters Are cAMP Inducible. To test whether the adenovirus early promoters were inducible by cAMP, by virtue of the presence of putative CRE sites, recombinants containing the E2a and E3 promoters linked to the CAT gene were transfected into PC12 cells. The E2a promoter region used in this experiment contains sequences between position −97 to +40; this fragment is known to contain all sequences required for both basal and E1A-induced expression (26). The putative E2a CRE motif is

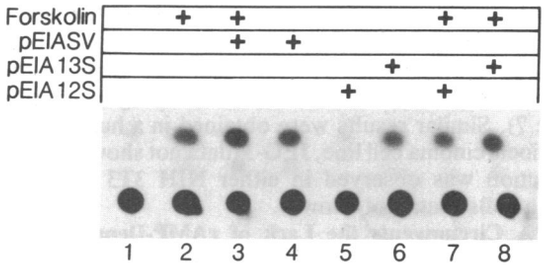

FIG. 1. Activation of the E3 promoter by E1A products and cAMP. PC12 cells were transfected with 10 μg of pE3CAT. The basal level promoter activity is shown in lane 1; CAT activity after forskolin treatment or cotransfection (with 10 μg) of the different E1A plasmids is shown in the indicated lanes. Induction by forskolin is slightly enhanced by cotransfection with functional E1A trans-activator plasmids pE1ASV and pE1A13S (lanes 3 and 8; see text). The CAT assay (29) monitors acetylation of [^{14}C]chloramphenicol and its products are separated by thin-layer chromatography and visualized by autoradiography.

located between positions −70 and −77 (see Table 1). The E3 promoter region used in these experiments contains sequences between −487 and +65; this region also contains all sequences required for basal and E1A-induced expression (25). The putative E3 CRE site is located between position −60 and −53 (see Table 1). Forskolin was used to induce the cAMP response in the transfected PC12 cells. As shown in Fig. 1, the E3 promoter is induced by forskolin, producing an increase in CAT activity of 8- to 10-fold (compare lanes 1 and 2; see also Fig. 2, lanes 9 and 10). The E2a promoter responded in a similar way (Fig. 2, lanes 1 and 2). Under the same conditions, the two promoters were inducible by the E1A products (Fig. 1, compare lanes 1 and 4; Fig. 2, compare lanes 1 and 4). As expected (13), the 13S product is responsible for the induction (Fig. 1, compare lanes 1 and 6), while the 12S product is not capable of stimulating E3 transcription

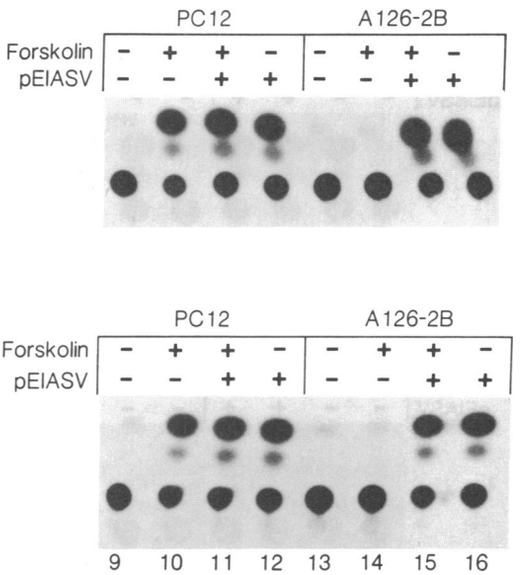

FIG. 2. Lack of cAMP-dependent protein kinase II impairs cAMP inducibility, but not E1A trans-activation. Activity of the E2a (lanes 1–8) and E3 (lanes 9–16) promoters in both PC12 and A126-2B cells. Basal promoter activities are indicated in lanes 1, 5, 9, and 13. Transcriptional inductions using forskolin and cotransfection with pE1ASV are shown. In PC12 cells, both E2a and E3 promoters are induced by either forskolin or pE1ASV (lanes 1–4 and 9–12); in PC12 mutants (A126-2B) lacking the cAMP-dependent protein kinase, there is no forskolin effect, whereas the E1A trans-activation function is maintained (lanes 5–8 and 13–16).

(Fig. 1, lanes 1 and 5). A weak increase in induction was observed when forskolin treatment was coupled to expression of a cotransfected 13S producing recombinant (lanes 3 and 8). The 12S product failed in enhancing forskolin effect (lane 7). Similar results were obtained in a human placental choriocarcinoma cell line, JEG-3 (data not shown). No cAMP induction was observed in either NIH 3T3 fibroblasts or HeLa cells (data not shown).

E1A Circumvents the Lack of cAMP-Dependent Protein Kinase in A126-2B Cells. A126-2B cells are PC12 mutants that have been shown to be specifically deficient in the regulatory subunit of the cAMP-dependent protein kinase II activity (28). As already shown for the rat somatostatin gene (33) and the human c-*fos* gene (34), there is no cAMP induction in this cell type. As shown in Fig. 2, both the E2a (compare lanes 5 and 6) and the E3 promoters (compare lanes 13 and 14) are not induced by forskolin in A126-2B cells. Interestingly, the E1A products still trans-activate both E2a and E3 promoters in these cells (lanes 8 and 16). The weak increase in induction observed using both E1A and forskolin in PC12 cells is not present in A126-2B cells (lanes 7 and 15).

E2a Promoter Sequences Involved in E1A and cAMP Induction Overlap. The region between −70 and −79 of the E2a promoter has been described to be required for full E1A induction (26). To establish whether the same promoter element is involved in both cAMP and E1A regulation in PC12 cells, several deletion mutants of the E2a promoter linked to the CAT gene were tested. The deletion end points, as well as the position of the putative CRE, are indicated in Fig. 3 (*Upper*). The results show that both E1A and cAMP inductions involve the sequence ACGTCA (see Fig. 3 *Lower*).

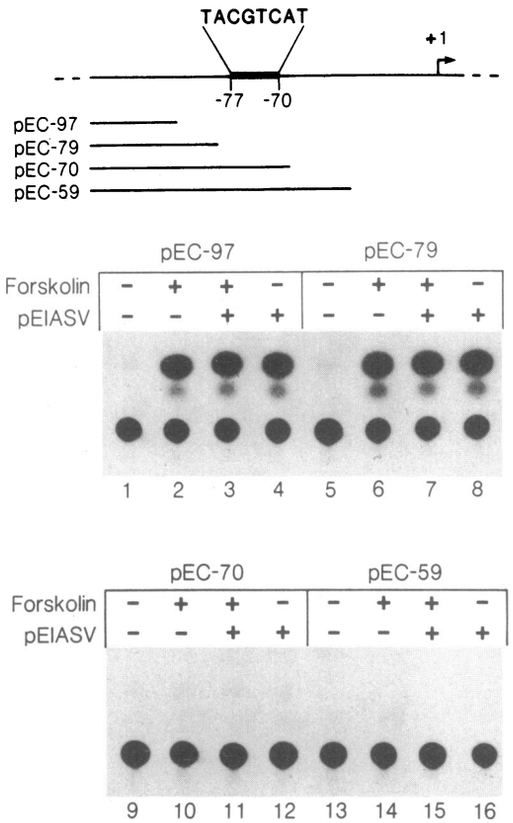

FIG. 3. The same sequence is required for both cAMP and E1A inductions of the E2a promoter. (*Upper*) Position of the TACGTCAT sequence in the E2a promoter and the end points of the deletion mutants. E2a deletion mutants that contain the −77/−70 sequence (pEC-97 and pEC-79) are induced by both forskolin treatment and cotransfection with pE1ASV (lanes 1–8). Deletion mutants that are lacking the −77/−70 element (pEC-70 and pEC-59) are not inducible by either forskolin treatment or pE1ASV cotransfection (lanes 9–16).

Both Basal and Induced Expression Competed with Heterologous CREs. To investigate whether the nuclear factor involved in the E1A and cAMP inductions of the early adenovirus promoters is related to CREB, both *in vivo* and *in vitro* competition experiments were performed. Fig. 4*A* shows that E1A-induced transcription from both E2a and E3 promoters was substantially decreased by cotransfection of increasing amounts of the pUCSom competitor plasmid bearing the somatostatin CRE oligonucleotide. A similar effect was observed with recombinant pUCα-CG, which contains the α-CG CRE oligonucleotide (data not shown). When similar experiments were performed on the E2a or E3 cAMP-induced promoters, analogous results were obtained (Fig. 4*B*). When a control competitor plasmid was used, little or no competition was observed (Fig. 4). Interestingly, the basal transcription of both E2a and E3 promoters was also decreased in competition experiments using either the somatostatin or the α-CG plasmids (Fig. 4*C*). This observation is supported by previous data (36, 37) indicating that the sequence ACGTCA binds a trans-acting factor required for basal level transcription. To further investigate this possibility, *in vitro* transcription experiments using the E3 promoter

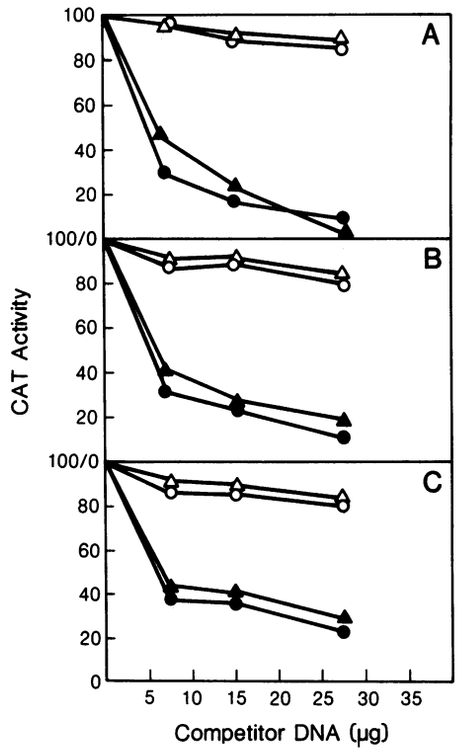

FIG. 4. Competition experiments in PC12 cells with heterologous CRE sequences. E1A-induced expression (*A*), cAMP-induced expression (*B*), and basal expression level (*C*) were analyzed. The activity of the E2a (triangles) and E3 (circles) promoters was determined by densitometric scanning of several autoradiograms. Competition with increasing amounts of pUCSOM (solid symbols) shows a decrease in E1A-induced, cAMP-induced, and basal expression of both E2a and E3 promoters. When a control competitor plasmid (pUL-DSE, bearing the dyad symmetry element of the c-*fos* promoter; ref. 35) was used, no or little competition was observed (open symbols). The total DNA transfected was always 30 μg per 10-cm plate. Similar results were obtained with the pUCα-CG competitor (data not shown). The maximum molar ratio of competitor CRE sequences to either E2a or E3 CRE sequences was ≈15-fold. The CAT activity values are in reference to either noncompeted induced levels (*A* and *B*) or the noncompeted basal levels (*C*), which are considered to be 100% CAT activity.

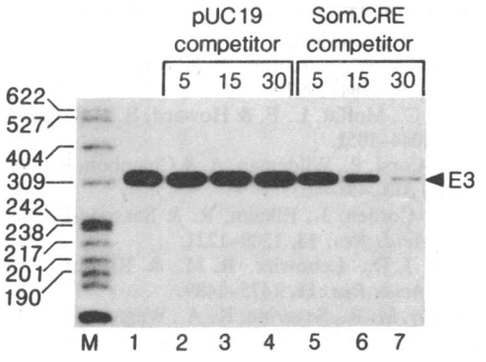

FIG. 5. Competition of *in vitro* transcription of the E3 promoter. A 550-base-pair purified fragment containing 235 base pairs of the E3 promoter was incubated in an *in vitro* transcription reaction using a HeLa cell nuclear extract (see *Materials and Methods* and refs. 30–32). The expected transcript is 315 nucleotides long (arrowhead). Competition with 5, 15, or 30 molar excess of competitor fragment is indicated. The somatostatin CRE competitor is a 127-base-pair fragment containing the CRE from positions −61 to −32 of the gene (33). The nonspecific competitor is a 97-base-pair fragment from pUC19. Lane M, end-labeled *Msp* I-digested pBR322.

were performed. Fig. 5 shows that a fragment containing the somatostatin CRE is able to compete a nuclear factor required for the basal *in vitro* transcription of the E3 promoter in a nuclear HeLa cell extract (lanes 5–7). As control, a fragment derived from pUC19 DNA was used as competitor (lanes 2–4), showing no detectable decrease in transcription activity. The CRE-containing fragment showed no competitor activity in a control experiment using the rabbit β-globin promoter (data not shown).

DISCUSSION

It is the general consensus that the E1A trans-activation phenomenon requires preexisting cellular factors that might be modified upon adenovirus infection. In this paper, I show that a cellular factor that binds to the sequence ACGTCA is likely to be involved in E1A trans-activation. This factor, previously identified as ATF (20, 21), has similar characteristics to CREB, which has been purified by affinity chromatography using the somatostatin CRE (22). The affinity-purified CREB protein binds to the E2a −70/−77 promoter sequence in a "footprinting" assay (P.S.-C. and M. Montminy, unpublished data). Thus, ATF and CREB appear to be strongly related, if not identical, proteins. These findings led to the experiments (Figs. 1–3) that demonstrated the cAMP responsiveness of the E2a and E3 promoters. Both deletion analysis (Fig. 3) and competition experiments (Fig. 4) show that the same sequence is involved in either E1A trans-activation or cAMP induction. The same sequence is also required for basal levels of transcription (Figs. 4*C* and 5). This indicates that both the E1A and cAMP inductions involve the modification of the ATF (or CREB) preexisting factor. Recent reports show the requirement of an unaltered TATA sequence to obtain a full E1A trans-activation (18, 19). The requirements of an ATF (or CREB) promoter element reported in this paper, as well as in other studies (20, 21), does not necessarily constitute a discrepancy. It seems plausible, in fact, that E1A trans-activation requires both the ATF and the TATA factors binding in concert on the promoter to form a more stable transcriptional complex. This hypothesis is strengthened by the observation that the TATA factor binds to the Ad5 E4 promoter more efficiently when it is in the presence of ATF, suggesting cooperative interaction of the two proteins in the transcriptional complex (17, 38). Thus, the E1A protein appears to function by interacting with more than one factor and may be inducing modifications that will increase the transcriptional complex stability and thus produce a higher RNA initiation rate. Results reported in this paper suggest that the E1A products are able to modify either directly or indirectly the endogenous CREB (or ATF). The modification is likely to be phosphorylation since E1A is able to circumvent the lack of cAMP-dependent protein kinase II in the activation of the CREB sites of the E2a and E3 promoters. Interestingly, among the cellular proteins that have been found to be associated with E1A after adenovirus infection, there is a CREB-like factor (39). It is noteworthy that some of the cellular genes that have been reported to be transcriptionally activated by E1A, such as c-*fos* (7) and *HSP70* (6), contain CRE sequences in the promoter region (see Table 1). However, it should be noted that transcription from a recombinant bearing the α-CG CRE oligonucleotide (27) linked to the herpes thymidine kinase promoter is not trans-activated by the E1A products (data not shown). This also indicates that other unidentified components may be required for E1A function. The same site of the E2a promoter acts as the target of both E1A and cAMP induction (Fig. 3). It is likely that the same transcriptional factor is also required for the two kinds of activation. The preexisting cellular ATF (or CREB) is also responsible for the basal level of transcription from both the E2a and the E3 promoters (Figs. 4*C* and 5). An intracellular alternative pathway of ATF activation must be operating in the PC12 mutant cell line. It is possible that E1A activates a different signal transduction mechanism that could mimic the effect of the agonists of adenylate cyclase. Alternatively, the E1A protein may be associated with some other cellular factor and could have some kinase activity. The cAMP inducibility of the early adenovirus promoters could possibly be important with respect to the infective cycle of the virus. It is conceivable that E1A-deficient viruses might not show the classical delayed infection kinetics in some endocrine responsive systems. Further investigations are required to explore these possibilities. It is conceivable that by using adenovirus as a model system we will be able to gain further insight into the molecular links between signal transduction and transcriptional activation.

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