Independent Cyclic AMP and E1A Induction of Adenovirus Early Region 4 Expression

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A cellular transcription factor, ATF, binds to a repeated element in the adenovirus early region 4 (E4) promoter. ATF also binds to other viral early promoter regions and to the cyclic AMP (cAMP) response elements of cellular genes. In this report, we demonstrate that a single ATF-binding site located immediately upstream of the E4 TATA box, between -62 and -46, mediates induction of E4 transcription by 8-bromoadenosine-3',5'-cyclic monophosphate or cholera toxin in the human hepatoma cell line HepG2 and rat pheochromocytoma cell line PC12. Different ATF-binding sites in the E4 control region independently conferred cAMP inducibility on the simian virus 40 early promoter in PC12 cells. Induction of E4 expression by cAMP was also observed in virus-infected HepG2 cells. Other viral early promoter regions that contain ATF-binding sites (E1A and E2A) were also induced by cAMP in infected cells. E4 expression was activated by the E1A 13S mRNA products in HepG2 cells. E1A *trans* activation appears to be distinct from the cAMP response.

Integration of an organism's responses during development or homeostasis is partly attributable to the ability of cells to communicate via production of steroid or peptide hormones. Steroid hormones, such as glucocorticoids, bind to and activate receptor molecules that then act directly on DNA and affect the transcriptional program of the cell (10, 46). Most peptide hormones, however, act by binding to cell surface receptors and triggering the synthesis of intracellular second messenger molecules, such as cyclic AMP (cAMP) (24). It has been shown for the second messenger cAMP that the binding of cAMP to the regulatory subunit of protein kinase A unleashes the phosphorylating activity of the catalytic subunit (for a review, see reference 36). It is the kinase activity of the catalytic subunit that leads to a given set of hormonal responses, which includes transcriptional changes in gene expression (4, 15).

Recently, a specific cAMP response element (CRE) has been identified in the 5'-flanking region of a number of cellular genes, e.g., somatostatin, vasoactive intestinal peptide, phosphoenolpyruvate carboxykinase (PEPCK), α -gonadotropin, tyrosine hydroxylase, and proenkephalin, that allows their expression to be induced by increased intracellular levels of cAMP (6, 8, 12, 29, 33, 38, 39, 41; for a recent review, see reference 35). Cellular CREs share properties with transcriptional enhancer elements in that CREs generally induce expression of a linked gene in an orientation- and location-independent manner. A cellular transcription factor, CRE-binding protein (CREB), specifically binds to CRE sequences (32). The *in vitro* transcriptional activity and DNA-binding properties of CREB are influenced by phosphorylation (45). Sequences similar to cellular CREs are found with the transcriptional control regions of the adenovirus early units E1A, E2, E3, and E4 (for a recent review, see reference 22).

The adenovirus E4 promoter contains multiple CRE-like sequences located upstream of the TATA box, centered around nucleotides (nt) -50, -165, -235, and -265 (Fig. 1).

Each of these copies is located in regions of the E4 promoter involved in transcriptional regulation (7, 13, 17, 25, 30). Several cellular transcription factors (ATF, E4F, and EIVF) bind to these E4 elements; ATF and EIVF bind to cellular CREs *in vitro* (7, 18, 20, 25, 26, 30, 31, 34). The relationships between CREB, ATF, E4F, and EIVF have not been determined. For clarity, we will refer to the repeated E4 CRE-like elements as ATF-binding sites. E4 transcription is activated by the E1A 13S mRNA gene products (2, 3, 23), and the ATF-binding sites have been directly implicated in this *trans* activation (7, 13, 25, 31). In this report, we demonstrate that individual E4 ATF-binding sites confer cAMP inducibility on the E4 promoter and on a heterologous promoter *in vivo* and that the E1A, E2, and E4 promoters are cAMP responsive in virus-infected cells. E1A *trans* activation of the E4 promoter can be uncoupled from the cAMP response, in assays using mutant promoter regions, indicating that E1A and cAMP act via independent mechanisms.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Plasmid pE4-CAT contains the adenovirus type 5 (Ad5) right-terminal 398 nt, containing 330 base pairs of E4 5'-flanking sequences and the 68-nt E4 mRNA leader sequence, fused to the gene encoding chloramphenicol acetyltransferase (CAT) from pSV2-CAT (14). pE4-CAT and 5'-flanking region mutants pE4-330/-104, pE4-330/-62, pE4-330/-46, and pE4-330/-11 have been described previously (30). Plasmid pSV0-CAT was derived from pSV2-CAT by removal of the enhancer region sequences between the *Sph*I site at nt 198 and the *Acc*I site at nt 520. The sequence of the E4 oligonucleotide -63/-34 is 5'-GATCCGACTTTAACCGTTACGTCATTTTTAGTCC-3'/5'-GATCCGGACTAAAAATGACGTAACGGTTAAA GTC-3'. The sequence of the 2A/2B oligonucleotide is 5'-GTCACCAGGGAGCAACTTACGGTTACCAAACAAGAGG-3'/5'-CCTCTTGTTTGGTAAACCGTAAGTTGCTCCCTG-3'. The sequence of the -280/-258 oligonucleotide is 5'-GATCGGGGTGGAGTTTGTGACGTGGCG-3'/

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FIG. 1. E4 transcriptional control region. A schematic view of the right end of the Ad5 genome containing the E4 promoter region is shown at the top. Plasmid pE4-CAT contains the right-terminal 398 nt fused to the coding sequences of the CAT gene. E4 transcription is initiated at a clustered set of sites around nt 330 (1) which is designated +1. The Ad5 inverted terminal repeat (ITR; nt 1 to 103), the repeated binding sites for transcription factor ATF located around positions -50, -165, -235, and -265, and the E4 TATA box are indicated in the figure. The oligonucleotides and DNA fragments that were used in these analyses are indicated below the promoter region schematic and span sequences from E4 -280 to -258, -257 to -228 fused to -11 to +68, and from -63 to -34. An expanded view of the nucleotide sequence around the E4 cap site is shown at the bottom. The TATA box and ATF-binding site located around -50 (31) are indicated as well as the endpoints for deletion mutants described in the text (pE4-330/-62, pE4-330/-46, and pE4-330/-11).

5'-GATCCGCCACGTCACAACTCCACCCC-3'. A DNA fragment containing the CRE sequence at E4 -235 was derived from plasmid pE4-227/-11 (30) by digestion with *Fnu*DI and *Hind*III, and a resulting fragment (-257/-228 fused to -11/+68) containing the -235 CRE in the Ad5 terminal repeat was isolated. Each of the oligonucleotides and restriction fragments were cloned into the pSV0-CAT vector by blunt-end ligation after Klenow repair. The plasmid constructions were confirmed by nucleotide sequence analysis. The E1A 13S cDNA plasmid (pE1A-13S) contains the left-terminal 1774 nt of Ad2, with a 13S cDNA substitution for the E1A splice sites, cloned in pUC18.

Cells, plasmid transfections, virus infections, and expression analyses. HepG2 cells were grown in minimal essential medium containing 10% fetal bovine serum. PC12 cells were grown in Dulbecco modified essential medium (low glucose) containing 10% heat-inactivated horse serum and 5% fetal bovine serum. DNA transfections were performed by the calcium phosphate precipitation method (43). HepG2 and PC12 cells were split the day before transfection and plated at a density of 2×10^6 cells per 100-mm-diameter plate. The cells were transfected with the amount of plasmid DNA indicated in the figure legends, supplemented to 15 μ g of DNA per 100-mm-diameter plate with HepG2 cells and to 20 μ g of DNA per 100-mm-diameter plate with PC12 cells, using salmon sperm DNA. For HepG2 cell transfections, the cells were incubated overnight with the precipitate, washed three times with Tris-buffered saline solution (TBS), and fresh medium was added that either contained or lacked inducer. For PC12 cell transfections, the cells were incubated with the precipitate for 4 h, boosted with 20% glycerol in TBS for 2 min, and washed with TBS. Fresh medium lacking inducer was added, and the cells were incubated overnight. Fresh medium was then added that either con-

tained or lacked inducer. Inducers of cAMP were included in the medium as indicated in the figure legends. 8-Bromoadenosine-3',5'-cyclic monophosphate and 3-isobutyl-1-methylxanthine (IMX) were from Sigma Chemical Co. Cholera toxin was a kind gift of Joel Levine, Department of Neurobiology and Behavior, State University of New York at Stony Brook. Total cell extracts were prepared at the times indicated in the figure legends, and CAT enzyme levels were assayed as described previously (14). Transfections were performed a minimum of four times for each experiment, using at least two different plasmid DNA stocks. The concentration of protein in each extract was determined to verify that equivalent protein yields were obtained. The results were quantitated by excision of the acetylated and nonacetylated [14 C]chloramphenicol forms from the chromatogram and direct scintillation counting.

For primer extension analyses, total cellular RNA (5) was isolated at the times indicated in the figure legends. Primer extension analyses were performed as previously described (30), using 50 μ g of total cellular RNA and with the following modifications. After reverse transcriptase extensions, the samples were precipitated with ethanol, suspended in 0.3 N NaOH, and incubated at 50°C for 1 h. The reactions were neutralized, precipitated with ethanol, and analyzed in a denaturing polyacrylamide gel.

For virus infections, HepG2 cells were split the day before infection. Cells were infected at a multiplicity of infection of 5 PFU per cell with wild-type Ad5 (wt300; 23) for 30 min, the cells were washed with TBS, and fresh medium was added that either contained or lacked 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate. Total cellular RNA (5) was prepared at 6 and 9 h after infection. Slot blot analysis was performed as described previously (42), using 4 to 8 μ g of total cellular RNA per slot and 32 P-labeled probes labeled by

random priming (11). The human β -actin clone was described previously (16). Induction of adenovirus early region expression was analyzed in three separate experiments. The construction of the recombinant adenovirus *dl310/E4-CAT* will be described elsewhere.

RESULTS

Induction of E4 transcription with cAMP. We previously reported that expression of the Ad5 E4 promoter was induced by cAMP in the human hepatoma cell line HepG2 (30). To characterize the cAMP response of the expression vector pE4-CAT further, we determined the dependency of induction on the amount of transfected DNA. HepG2 cells were transfected with various amounts of pE4-CAT and then treated for 6 to 7 h with 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate. The level of induction of E4 expression was determined by assaying CAT enzyme activity in cell extracts. Induction of E4 expression with cAMP was dependent on the amount of DNA transfected; induction ratios increased an average of 1.7- to 3.6-fold as the amount of plasmid DNA transfected was decreased from 5 to 0.2 μ g per plate (Fig. 2A). The basal level of E4 expression decreased linearly as the concentration of transfected DNA was decreased (data not shown). The maximal level of induction was similar whether 8-bromoadenosine-3',5'-cyclic monophosphate or cholera toxin (10 ng/ml [40]) was used (Fig. 2B), indicating that the response was truly a result of increased intracellular levels of cAMP. Varying the cell density or the concentration of serum or glucose in the medium before or during induction or inclusion of 0.5 mM IMX (a phosphodiesterase inhibitor) during cAMP treatment did not have an effect on the level of induction of pE4-CAT expression (data not shown). The stimulation of E4 expression by cAMP as assayed by CAT enzyme activity was transient; increased E4 expression was observed between 6 and 8 h, but not at 4 or 10 h, after addition of the inducer (data not shown).

To map the 5' ends of the transcripts initiated from pE4-CAT, HepG2 cells were transfected with pE4-CAT, and total cellular RNA was analyzed by primer extension, using a CAT-specific oligonucleotide. These products were compared with those detected at early times after infection of HeLa cells with a hybrid virus (*dl310/E4-CAT*) in which the right terminus of the viral genome was replaced by the E4-CAT fusion gene. The authentic E4 cap sites were used in HepG2 cells during transient expression of pE4-CAT (Fig. 3A). Next, to determine whether the increase in CAT enzyme activity after cAMP treatment reflected a change in the levels of E4-CAT transcripts, total cellular RNA was prepared from HepG2 cells that had been cotransfected with pE4-CAT and an SV40-CAT vector and induced with cholera toxin for 3 or 5 h. Primer extension analysis of these RNA samples revealed that cAMP induced the levels of E4-CAT transcripts approximately threefold as early as 3 h after addition of the inducer (Fig. 3B). The level of induction of E4 expression by cAMP was comparable in both the CAT assays and primer extension analyses. Plasmid pSV0-CAT was included as an internal standard in this experiment. This derivative of pSV2CAT (14) lacks the SV40 enhancer sequences between the *Sph*I site at nt 198 and the *Acc*I site at nt 520, thus deleting the cAMP-responsive AP2-binding sites in the SV40 enhancer region (21). The levels of pSV0-CAT transcripts were not altered by cAMP induction (Fig. 3B).

Definition of sequences mediating the cAMP response. Earlier studies established that ATF binding to the E4 promoter

FIG. 2. E4 expression is induced by cAMP. (A) HepG2 cells were transfected with pE4-CAT at the concentrations of DNA indicated in the figure (per 100-mm-diameter plate), supplemented to 15 μ g of DNA per plate, using high-molecular-weight salmon sperm DNA. After incubation overnight with the calcium phosphate precipitate, the cells were washed and fresh medium was added that either lacked inducer (–) or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate (+). Total cell extracts were prepared 6 to 7 h after addition of the fresh medium, and CAT enzyme levels were determined. The level of CAT activity in cultures that lacked inducer was taken as 1.0 for each concentration of input plasmid DNA, and the fold induction of CAT activity in cultures treated with cAMP was calculated. The mean value and standard deviation for the results obtained from multiple experiments with each concentration of plasmid DNA is shown. (B) HepG2 cells were transfected with pE4-CAT as described above, using 0.1 μ g of plasmid DNA per plate. After transfection, fresh medium was added that lacked inducer (–) or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate (cA) or 10 ng of cholera toxin (Ch.Tox., C.T.) per ml. Induction of pE4-CAT expression is expressed as described above. The right half of the panel shows representative data from one experiment.

was specifically competed by cellular CREs (7, 30, 31). To determine whether the sequences containing the ATF-binding sites were mediating the cAMP response in vivo, the cAMP response of the following E4 deletion mutant plasmids was tested after transfection into HepG2 cells: plasmid pE4330/104 contains the ATF-binding site located immediately upstream of the TATA box and approximately 50 base pairs of additional 5'-flanking sequences, but it lacks the other three upstream ATF-binding sites (including the two ATF-binding sites in the inverted terminal repeat); plasmid pE4330/62 retains the ATF-binding site located at 50 and the TATA box; plasmid pE4330/46 lacks the ATF-binding site but retains the TATA box; plasmid pE4330/11 lacks both of these elements (Fig. 4).

A plasmid with a deletion of E4 5'-flanking sequences to –104 was induced fourfold by cAMP, demonstrating that the three ATF sites upstream of –104 are not essential for induction. Deletion of sequences to –104 reduced E4 basal

FIG. 3. Levels of E4 mRNAs are induced by cAMP. (A) HepG2 cells were transfected with pE4-CAT (5 μ g per plate), and total cellular RNA was isolated 24 h after transfection. HeLa cells were infected with the recombinant virus *d/310/E4-CAT*, and total cellular RNA was isolated 9 h after infection. The 5' ends of E4 transcripts were analyzed by primer extension, using a CAT-specific oligonucleotide. Lanes: M, 32 P-labeled marker DNAs; MOCK, products of primer extension using RNAs isolated from mock-infected HeLa (left lane) and mock-transfected HepG2 (right lane) cells; 310/E4-CAT, products of primer extension using RNA isolated from HeLa cells infected with the *d/310/E4-CAT* recombinant adenovirus; pE4-CAT, products of primer extension obtained using RNA isolated from HepG2 cells transfected with plasmid pE4-CAT. The major upper bands represent bona fide E4 transcripts that initiate at a clustered set of start sites around E4 nt 330 (1). A minor initiation site was also detected located around E4 +40 (lower band). (B) HepG2 cells were cotransfected with plasmids pE4-CAT (0.2 μ g per plate) and pSV0-CAT (5 μ g per plate). After transfection, fresh medium was added that lacked inducer (-) or contained 10 ng of cholera toxin per ml (+). Total cellular RNA was isolated at 3 and 5 h after addition of the fresh medium, and pE4-CAT and pSV0-CAT transcripts were analyzed by primer extension, using a CAT-specific oligonucleotide. pE4-CAT-specific 5' ends and pSV0-CAT-specific 5' ends are indicated. The lower portion at the right of the figure shows a longer exposure of the region of the gel containing the pSV0-CAT-specific 5' ends.

expression two- to fourfold (Fig. 4). The additional deletion of sequences to -62 reduced E4 basal expression an additional 10- to 20-fold (data not shown). Induction of expression from the -62 mutant plasmid was not observed when low concentrations of DNA were transfected, as was evident with the wild type (pE4-CAT) and -104 deletion mutant plasmids (data not shown). Instead, cAMP induction with this plasmid became evident as the amount of plasmid DNA transfected was increased; a cAMP induction level that was comparable with that of the wild-type plasmid was observed when 40 μ g of plasmid DNA was transfected per plate (Fig. 4). We do not understand this phenomenon, but it may reflect the titration of a negative regulator of the truncated E4 promoter region. Induction of E4 transcription by cAMP was lost when the region between -62 and -46 was deleted.

FIG. 4. Induction of E4 expression by cAMP and E1A. HepG2 cells were transfected with pE4-CAT (0.2 μ g per plate) or E4 mutant plasmids pE4-330/-104 (-104; 0.2 μ g per plate), pE4-330/-62 (-62; 40 μ g per plate), pE4-330/-46 (-46; 40 μ g per plate), and pE4-330/-11 (-11; 40 μ g per plate) or cotransfected with pE4-CAT or mutant plasmids (at the concentrations given above) and pE1A-13S (0.1 μ g per plate for pE4-CAT and pE4-330/-104; 2.0 μ g per plate for pE4-330/-62, pE4-330/-46, and pE4-330/-11). After transfection, fresh medium was added that lacked inducer (-) or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate (cA). Fresh medium lacking inducer was added to cultures cotransfected with pE1A-13S. Total cell extracts were prepared 6 to 7 h after addition of fresh medium, and CAT enzyme levels were determined. The data are presented as described for Fig. 2.

This region contains the TATA-proximal ATF-binding site (Fig. 1). The mutant plasmids pE4-330/-46 and pE4-330/-11 did not respond to cAMP under any of the transfection conditions tested (data not shown).

E4 ATF-binding sites confer cAMP inducibility on a heterologous promoter. Several E4 ATF-binding sites were tested for their ability to confer cAMP inducibility on a heterologous promoter. Monomers of two synthetic oligonucleotides (-63/-34 and -280/-258 [Fig. 1]) containing the E4 ATF-binding sites located at -50 and -265, respectively, were inserted upstream of the SP1 binding sites in pSV0-CAT at nt 198. The oligonucleotides were inserted in opposite (-63/-34) and direct (-280/-258) orientations relative to their context in the E4 promoter region. A DNA fragment of the Ad5 inverted terminal repeat containing the ATF-binding site located at -235 (-257/-228) was also inserted at this site in pSV0-CAT in direct orientation; this copy was separated from the SV40 promoter by an additional 80 nt. As a control for specificity and possible spacing effects of the insertions, an oligonucleotide of nonspecific sequence (2A/2B), but of similar length to the E4 oligonucleotides, was inserted upstream of the SV40 promoter. We previously demonstrated that the -63/-34 oligonucleotide competed efficiently for ATF binding to the E4 promoter region, while the 2A/2B oligonucleotide did not compete for binding (30).

Expression from the SV40-based constructs was not inducible by cAMP after transfection into HepG2 cells (data not shown). We reasoned that a factor(s) required to mediate a cAMP response from the SV40 promoter in conjunction with the CRE sequence might be lacking in these cells. Therefore, we tested cAMP inducibility of the plasmids in the rat pheochromocytoma cell line PC12 which had previ-

FIG. 5. The E4 promoter contains multiple CREs. PC12 cells were transfected with pE4-CAT (2.0 μ g per plate) or pSV0-CAT derivatives (10 μ g per plate) that contain the E4 oligonucleotides (shown in Fig. 1) or a control oligonucleotide, 2A/2B. After transfection, the cells were incubated overnight in fresh medium lacking inducer. Fresh medium was then added that lacked inducer (–) or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate (+). Total cell extracts were prepared 24 h later, and CAT enzyme levels were determined. The data are presented as described for Fig. 2.

ously been used to test similar expression plasmids containing the somatostatin CRE (33). As observed in HepG2 cells, cAMP induction of pE4-CAT expression was dependent on the amount of DNA transfected with increased induction at lower amounts of input plasmid DNA (average induction, threefold, was detected by using 2 μ g of plasmid DNA per plate; Fig. 5 and data not shown). Expression from each plasmid that contained an E4 CRE-like sequence was induced by cAMP an average of threefold, similar to the level of induction observed with the intact E4 promoter in pE4-CAT (Fig. 5). Insertion of the control oligonucleotide, 2A/2B, had no effect on levels of CAT expression (Fig. 5). Insertion of an oligonucleotide into this vector that contains the CRE of the PEPCK gene resulted in a twofold stimulation by cAMP (data not shown); this level of induction is similar to level of induction that the PEPCK CRE confers on the thymidine kinase promoter (38). We conclude from these results that the E4 ATF-binding sites behave as CREs. These results are consistent with those recently reported that describe cAMP induction of the adenovirus E2A and E3 promoters in a transient expression assay in PC12 cells (37) and the E1A and E4 promoters during viral infection of S49 cells (9).

Induction of adenovirus early gene expression in infected HepG2 cells. Our results led us to test the effect of cAMP induction on adenovirus early gene expression in infected cells. CRE-like sequences are found in the adenovirus early region E1A, E2, E3, and E4 transcriptional control regions (7, 18, 20, 25, 26, 30, 31, 37). HepG2 cells were infected at a low multiplicity (5 PFU/cell) with wild-type Ad5. Medium was then added that either lacked or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate. Total cellular RNA was isolated at 6 and 9 h after infection, and early region expression was measured by slot blot analysis using

FIG. 6. Induction of adenovirus early region expression by cAMP. HepG2 cells were infected with wild-type Ad5 virus at 5 PFU per cell for 30 min. After infection, fresh medium was added that lacked inducer (–) or contained 1 mM 8-bromoadenosine-3',5'-cyclic monophosphate (+). Total cellular RNA was isolated at 6 and 9 h after infection. Specific transcripts were analyzed by slot blot analysis using probes for adenovirus early regions E1A, E1B, E2, E3, and E4 and the cellular β -actin gene.

probes for early regions E1A, E1B, E2, E3, and E4. A cellular β -actin probe was used as an internal standard. The results of these analyses are shown in Fig. 6. Consistent with the results presented above, E4 expression was induced by cAMP at early times after infection. The level of induction varied between experiments from three- to sixfold. Expression from regions E1A and E2 was also induced between two- and fourfold; induction of E2 transcription, however, was delayed relative to E1A and E4. In contrast, expression from Ad5 regions E1B and E3 and the cellular β -actin gene was not induced by cAMP treatment. These results are consistent with the results obtained in transient expression analyses and confirm that the E4 promoter is inducible by cAMP in vivo.

Role of CRE in E1A *trans* activation. Optimal E4 expression in HeLa cells depends on *trans* activation by the E1A 13S mRNA gene products (2, 3, 23). Previous analyses suggested that the E4 ATF-binding sites located at –235 and –165 were important for E1A *trans* activation in 293 and HeLa cells (13, 25). On the other hand, in other studies (7), it was determined that sequences between –104 and –46 were sufficient for E1A stimulation of the E4 promoter in HeLa cells. We tested E1A induction of E4 transcription in HepG2 cells by cotransfecting pE4-CAT and the E4 mutant plasmids described above with a plasmid that expresses the E1A 13S mRNA gene products (Fig. 4).

The wild-type E4 promoter region was induced six- to sevenfold by the E1A 13S mRNA gene products. This level of E4 induction is below the level commonly reported in HeLa cells (7, 13, 25) and most likely reflects a cell-type-specific effect. E1A induction was lost with the mutant plasmid containing a deletion to –104; the –104 plasmid, however, was fully responsive to cAMP. When tested using transfection conditions under which a cAMP response was observed, the –62 mutant plasmid was E1A responsive. This level of E1A induction was still observed with deletion of 5'-flanking sequences to –11, although the –11 mutant plasmid was not cAMP responsive. A CAT plasmid lacking the E4 promoter was not responsive to E1A (data not shown). These analyses demonstrate that two separate regions respond to the E1A products under certain conditions of transfection. These two regions are distinct from the region that is required for cAMP induction. These results suggest that the mechanisms of cAMP and E1A induction of E4 expression are independent. This idea is supported by the observation that cAMP and E1A had an additive effect on the induction of pE4-CAT in HepG2 cells (data not shown), consistent with the similar result obtained with cAMP induction of E4 expression in virus-infected HepG2 cells (Fig. 6).

DISCUSSION

Our results demonstrate that transcription from the adenovirus E4 promoter is induced by increased intracellular levels of cAMP, both in a transient assay using plasmid DNA (Fig. 2, 3, and 4) and in virus-infected cells (Fig. 6). The E4 cAMP response is dependent on a repeated sequence element that closely matches the consensus CRE (5'-TGACGTCA-3') derived from the analyses of a number of cellular genes (35). Each of the E4 CRE-like sequences tested (located at -50, -235, and -265) was able to confer cAMP inducibility on the SV40 promoter *in vivo* (Fig. 5). As reported for cellular cAMP-induced genes, cAMP induction of E4 expression was both rapid (detected within 3 h after addition of the inducer [Fig. 3]) and transient (data not shown). Genetic analyses of cAMP-inducible cellular promoters and heterologous promoter regions linked to synthetic CREs have shown that duplication and multimerization of CREs increases the response of a promoter to cAMP (6, 12, 39, 45). In the context of the E4 promoter, one CRE copy (located at -50) was sufficient to confer the level of induction observed, using the wild-type promoter in a transient expression assay (pE4-CAT versus pE4-330/-104 [Fig. 4]), demonstrating that the multiple CREs in the E4 promoter region do not have an additive effect. The induction of the E4 promoter by cAMP (three- to fourfold) is similar to the response of many cellular genes to cAMP. The induction ratios reported for cellular genes (e.g., proenkephalin, somatostatin, PEPCK, α -gonadotropin, tyrosine hydroxylase, and vasoactive intestinal peptide) generally range from 2- to 10-fold, depending on the promoter tested, the cell line used for the analysis, and the procedure used to increase intracellular cAMP levels (6, 8, 12, 29, 33, 38, 39, 41). Relative to the HepG2 cell line, the α -gonadotropin promoter was induced eightfold by cAMP (8).

During adenovirus infection of HepG2 cells, the cAMP induction of E4 expression was similar (Fig. 6) (three- to sixfold) with that observed in transient expression analyses using pE4-CAT. Two other viral transcription units that contain ATF-binding sites (E1A and E2) were also induced by cAMP, yet expression from the E3 promoter was not affected by cAMP even though an ATF-binding site is located in the E3 promoter region (20, 26). It was recently reported (37) that the E2A and E3 promoters were both induced by cAMP in a transient expression assay in PC12 cells. The lack of induction of E3 transcription in HepG2 cells in our experiments may reflect a cell-type-specific effect similar to that observed using the SV40-CAT vector in cAMP induction experiments in HepG2 and PC12 cells (data not shown and Fig. 5). It was also recently reported (9) that transcription of regions E1A, E1B, and E4 was induced by cAMP in virus-infected S49 cells. Our results for the induction of E1A and E4 expression (Fig. 6) are consistent with these analyses. We did not, however, observe an induction of E1B expression in HepG2 cells (Fig. 6). Induction of E1B transcription in S49 cells may have been mediated by transcription factor AP2 (9). If this is the case, then the unresponsiveness of E1B to cAMP in our experiments is not surprising, since HepG2 cells lack detectable AP2 activity (21).

The multiple ATF-binding sites in the E4 promoter have been proposed to have a specific role in mediating the stimulatory effect of the E1A gene products in 293 and HeLa cells (7, 13, 25, 31). Hence, the function of the E1A proteins may be to activate protein kinase A or CREB, in the absence of cAMP. We have not detected induction of E4 expression

by cAMP, however, in either 293 or HeLa cells (data not shown). E1A-mediated *trans* activation of the E4 promoter in HepG2 cells was mediated by two regions in the E4 promoter: one region located upstream of -104 and a second region located downstream of -11 (Fig. 4). Induction of E4 expression by cAMP in these cells, however, was mediated by the -62 to -46 region. These results suggest that E1A *trans* activation and cAMP induction of E4 transcription are mediated by independent pathways. The results obtained with cAMP induction in virus-infected HepG2 cells also support this idea, since cAMP had a stimulatory effect on E4 expression in the presence of the E1A products. If E1A and cAMP activated E4 transcription by a common mechanism, an additive effect of these two agents would not be expected. It is unlikely that induction of E4 expression in these experiments was due to increased E1A levels, since neither E1B nor E3 transcription was affected. Similarly, cAMP and E1A had an additive effect on the induction of E4 expression in HepG2 cells transfected with pE4-CAT (data not shown). The bimodal nature of the response of E4 promoter to E1A may reflect the specific combination of *cis*-acting elements as seen with E1A *trans* activation of the E2 promoter (48) and dexamethasone induction of the PEPCK promoter (38).

Engel et al. (9) have reported synergism between E1A and cAMP in a mouse lymphoma cell line (S49), implying different but interactive pathways of activation. Sassone-Corsi (37) concluded that a single pathway conveyed both responses in a rat pheochromocytoma cell line (PC12). In a human hepatoma cell line (HepG2), we find evidence suggesting that the E1A and cAMP responses are neither synergistic nor interrelated but that they are independent. These contrasting findings may be due to differences in the cell types and species of origin, to the assays employed (virus infection versus DNA transfection), and to the different promoter regions analyzed.

A plasmid containing a deletion to E4 -11 was stimulated by E1A even though this plasmid only contains E4 sequences from nt -10 to +68 (Fig. 4). Properly initiated transcripts from plasmid pE4-330/-11 were also detected in *in vitro* transcription reactions (30), suggesting the presence of an unidentified element(s) in this downstream region. Proper selection of initiation sites in the absence of a functional TATA box has also been observed *in vivo* with the E1B (44), E2 (47), E3 (27), and major late promoters (19). It has also been reported (48) that an element involved in E1A *trans* activation of the E2A promoter lies between nt -13 and +62, and others (28) noted a similar requirement for sequences in the major late promoter between the cap site and +33. Thus it is possible that an element of similar function, and a target of E1A *trans* activation, may be present in the E4 promoter downstream of the TATA box. In 293 and HeLa cells, E4 promoter sequences between -100 and -200 are also the targets for E1A *trans* activation (13, 25). This result is consistent with the fact that E1A *trans* activation in HepG2 cells was more efficient with the wild-type E4 promoter region compared with truncated promoter regions (Fig. 4).

Finally, we note that the region between -104 and -62 has contrasting functions in HeLa and HepG2 cells. In HeLa cells, these sequences had no effect on basal (E1A⁻) E4 promoter activity, yet they were important for E1A *trans* activation (7). On the other hand, in HepG2 cells, deletion of nt -104 to -62 resulted in a 10- to 20-fold decrease in basal E4 expression. The complex organization of the E4 control region may reflect the evolution of the promoter to respond

to different viral and cellular stimuli and to utilize the transcription factors that are available in different cell types.

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