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Distinguishable Promoter Elements Are Involved in Transcriptional Activation by E1a and Cyclic AMP

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The sequence motif CGTCA is critical for binding of a group of cellular transcription factors (ATF, CREB, E4F, and EivF) and for activation of certain E1a-inducible and cyclic AMP (cAMP)-inducible promoters. We have tested different promoter elements containing the CGTCA motif (referred to here as ATF-binding sites) for the ability to function as E1a or cAMP response elements. The adenovirus E4 promoter and the cellular vasoactive intestinal peptide (VIP) promoter responded differently to E1a and cAMP, demonstrating that the activating potential of ATF-binding sites within these promoters is not equivalent. While particular ATF-binding sites were sufficient for the activity of both the E4 (E1a inducibility) and VIP (cAMP inducibility) enhancers, these two enhancers had contrasting effects on E1a- and cAMP-inducible transcription. Thus, the relationship between E1a- and cAMP-inducible transcription is not simply explained by the action of these two inducers through the same promoter elements.

Transcriptional activation of eucaryotic genes often allows specific gene expression in response to a variety of physiological and viral inducing agents. Activation occurs, in part, through binding of cellular transcription factors to specific sites within the affected promoter (for reviews, see references 13, 33, 34, and 42) and through protein-protein interactions between activators (35, 38, 51). In this way genes can be targeted for activation according to the particular array of factor-binding sites present in their control regions. It is now apparent that this simple view is unable to explain certain aspects of structure-function relationships for inducible promoters. Of salient interest is the fact that multiple factors (or promoter elements) with similar or identical DNA-binding specificity (or sequence) have different activation potential (12, 16, 19, 25, 41, 45, 48). The subtle mechanisms underlying control of the above factors are central to understanding differential gene activation.

Promoter elements containing the sequence motif CGTCA (referred to throughout this paper as ATF-binding sites) have been shown to be critical for E1a inducibility of certain adenovirus early viral promoters (3, 7, 22, 26, 27, 44) and for cyclic AMP (cAMP)-mediated activation of many neuropeptide promoters (5, 9, 11, 15, 28, 36, 37, 43, 47). As is the case with other eucaryotic transcriptional control elements (see above), a complex set of activator proteins bind directly to ATF sites (7, 10, 19, 22, 31, 36). Polypeptides of ~43 and 47 kilodaltons (kDa) (referred to as activating transcription factor 43 [ATF-43] and ATF-47, respectively [19, 22]) and polypeptides of ~65 and 72 kDa (referred to as EivF [7]) bind to the adenovirus early-promoter ATF sites. In addition, a ~43-kDa polypeptide designated CREB (cAMP response element-binding protein) binds to the ATF site in the somatostatin promoter (36). Of the above polypeptides, CREB (which is likely to correspond to ATF-43) has been directly implicated in activation of the somatostatin promoter by cAMP (36, 50), and ATF (19) and EivF (7) have been shown to function as transcriptional activators in vitro. Beyond this, the functional relationship between polypeptides that bind to ATF sites is unknown. The inability to distinguish most (but not all [7, 39]) ATF-binding sites containing the invariant CGTCA motif (2, 7, 20, 23, 27, 31) together with the effect of mutations on binding of nuclear factors (2, 22) demonstrate that the CGTCA motif is the major determinant of ATF-binding sites. However, the ability of ATF sites to function as transcriptional elements is influenced by sequences in proximity to the CGTCA motif (10, 36). These observations raise the possibility that transcriptional elements containing ATF-binding sites (as defined by binding assays) might not be functionally equivalent.

Our aim here was to determine the relationship between promoter elements required for E1a and cAMP inducibility. The adenovirus E4 promoter and the human vasoactive intestinal polypeptide (VIP) promoter provide an ideal starting point for this analysis. Two ATF sites of the E4 promoter are important for activity of an E1a-inducible enhancer (referred to here as the E4 enhancer [17, 26]), and similarly, two ATF sites form a cAMP-inducible enhancer (referred to here as the VIP enhancer or VIP CRE [VIP cAMP response element]) within the VIP promoter (15, 31, 47). In contrast to previous studies, we found that the requirements for E1a- and cAMP-inducible transcription do not necessarily correspond. Despite their structural relatedness, the E4 and VIP promoters respond very differently to E1a and cAMP, as do their associated enhancers. Moreover, the ability of the VIP enhancer to mediate an E1a response is highly promoter specific. These results demonstrate that at least two factors can influence the activating potential of ATF-binding sites. First, ATF-binding sites can differ among themselves. Second, the activity of particular ATF sites can be modulated via interaction with additional promoter elements.

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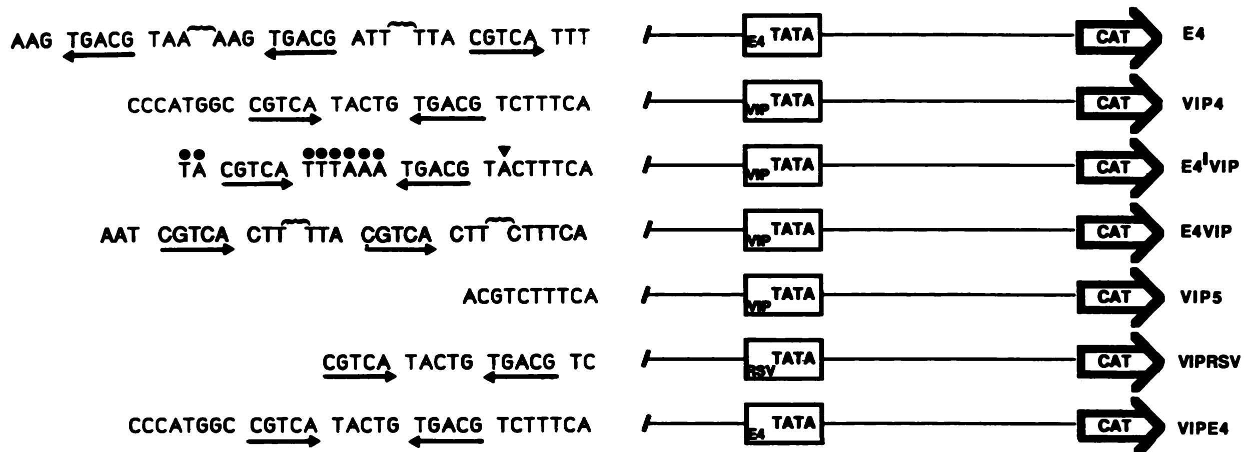

FIG. 1. Structure of hybrid promoters. The figure shows the general structure of all hybrid promoters and details of the ATF sites (CGTCA motif and flanking sequences) present within each. The solid arrows indicate the orientation of each ATF site. The CGTCA motif and flanking sequences are separated by a gap. Symbols: horizontal bracket, sequences of varying length that are not included in the schematic; ●, bases that differ from the VIP CRE sequence; ▼, insertion of an additional base. The origin of ATF sites is as follows. For pE4VIP, the VIP CRE was replaced by the E4 enhancer containing ATF sites at positions −164 and −140 (26, 27). For pE4'VIP, the VIP CRE was replaced with a 20-base-pair inverted duplication of sequences between positions −51 and −42 of the E4 promoter.

MATERIALS AND METHODS

Plasmids and constructions. The general structure of recombinant plasmids used is schematized in Fig. 1. Plasmids pRSVCAT (18), pVIP4, pVIP5, pVIP32CAT, pVIP17CAT (47), and pE4Δ240 (26) have been described elsewhere. Briefly, pVIP4 contains VIP promoter sequences between −94 and +146, fused to the chloramphenicol acetyltransferase (CAT) coding sequences at position +146. pVIP4 thus contains the VIP CRE (47). pVIP5 contains VIP promoter sequences between −76 and +146 and therefore lacks the CRE. pVIP17CAT contains the CRE (VIP promoter sequences between −86 and −70) fused to a truncated Rous sarcoma virus (RSV) promoter linked to the *cat* gene. pE4Δ240 contains the entire adenovirus type 5 E4 gene and approximately 240 base pairs (bp) of 5'-flanking sequence, cloned between the *Eco*RI and *Pvu*II sites of pBR322.

All new recombinants were constructed according to standard protocols (32). pE4CAT contains E4 promoter sequences (positions −240 and +35) fused to *cat* at position +35 relative to the E4 transcription start site. pE4CAT was obtained by cloning a *Taq*I fragment containing the E4 sequences between the *Hin*dIII (5025) and *Nde*I (578) sites of pRSVCAT. E4Δ38 contains the entire adenovirus type 5 E4 gene and 38 base pairs of 5'-flanking sequence, cloned between the *Eco*RI and *Pvu*II sites of pBR322. pE4Δ38CAT was obtained by cloning a *Taq*I fragment from pE4Δ38 (containing E4 sequences from −38 to +35) between the *Hin*dIII (5025) and *Nde*I (578) sites of pRSVCAT. pE4VIP contains the E4 enhancer (positions −200 to −100 of the E4 promoter [26]) cloned between the *Aat*II sites of pVIP5, with the E4 enhancer in the inverse orientation compared with the wild-type E4 promoter. pE4'VIP contains a 20-base-pair perfect inverted duplication of the sequence between positions −51 and −42 of the E4 promoter (the ATF core consensus sequence, CGTCA, is present at positions −49 to −45 [27]), cloned between the *Aat*II sites of pVIP5. pVIPE4 contains sequences between positions −94 to −61 of the VIP promoter (the CRE) fused to pE4Δ38 at position −38 of the E4 promoter. pVIPE4 was constructed by cloning a 170-base-pair *Pvu*II-*Bgl*II fragment from pVIP32CAT, containing the CRE, into the *Eco*RI site of pE4Δ38 so that position −61 of the VIP CRE is proximal to the E4 TATA box. pVIPE4CAT is analogous to pVIPE4, with CAT coding sequences replacing the E4 transcribed region at position +35 relative to the E4 transcription start site. pVIP'E4CAT was derived from pVIPE4CAT by partial digestion with *Aat*II followed by religation.

Cells, viruses, and transfections. (i) cAMP induction. PC12 cells grown in Dulbecco medium with 5% horse serum and 10% fetal calf serum were plated at 2×10^6 per 100-mm dish. The medium was changed 20 h later, and calcium phosphate-DNA precipitates (49) containing 20 μg of DNA per 100-mm dish were added 3 h later. Sixteen hours later, the cells were glycerol shocked, and each plate was divided into two new plates. One plate was treated with a final concentration of 10 μM forskolin. Cells were harvested for CAT assays 24 to 48 h after addition of forskolin. Experiments involving induction of viral genes were performed as follows. PC12 cells at 4×10^6 cells per 100-mm dish were infected as previously described for other cell types (30) at a multiplicity described in the figure legend. At 20 h postinfection, cells were induced with forskolin followed by RNA analysis (using primer extension) at the times indicated in the figure legend. Transfection experiments in HeLa cells were done as follows. Cells were transfected with calcium phosphate-DNA precipitates. Then 16 h later, cells were induced by addition of 200 μM chlorophenylthio-cAMP (cpt-cAMP), followed by RNA analysis at the times indicated in the figure legends. A PC12 cell line containing a stably integrated VIP-*cat* (VIPCAT) fusion gene was obtained by cotransfection of PC12 cells with pVIP25CAT (47) and pRSVneo, followed by selection in G418.

(ii) E1a induction. E1a inducibility was assayed by using a cotransfection assay described by many groups. Briefly, cells were transfected with the test promoter attached to the bacterial *cat* gene in the presence and absence of an E1a-expressing plasmid (pH3G [26]). DNA precipitates were left on the cells for 16 h, followed by addition of fresh medium and further incubation for 16 to 24 h. CAT activity was then assayed as described elsewhere (18). For quantitation of results, percent conversion of unacetylated to acetylated chloramphenicol under linear assay conditions was determined by excision of spots from the thin-layer chromatography (TLC) plate and quantitation of radioactivity with a

liquid scintillation counter. Each experiment was performed at least twice (three times for the data presented in Fig. 5) with different DNA preparations.

Assay of CAT activity and RNA analysis. Assays of CAT activity and RNA analysis by primer extension were done as described previously (15, 26, 30, 47).

RESULTS

The E4 promoter is not strongly induced by cAMP. For ease of discussion, we will refer to all promoter elements containing the CGTCA motif as ATF sites, without implying that all ATF sites are identical or that they act through the same transcription factors. Because ATF sites are required for E1a inducibility of the adenovirus E4 promoter (26) and for cAMP inducibility of the VIP promoter (15, 31, 47), we examined whether E1a- and cAMP-mediated activation can be signalled by these elements alone. Initially, we tested the E4 promoter for cAMP inducibility in PC12 cells, a cell line derived from a rat pheochromocytoma that has been used extensively for the study of cAMP-inducible transcription (47). For comparison, we tested E1a inducibility of the E4 promoter, cAMP inducibility of the adenovirus E2A promoter (present with the E4 promoter on the viral chromosome), and cAMP inducibility of a stably integrated VIP-*cat* gene, as a control for cAMP inducibility. RNA analysis was done by primer extension as described previously (30).

PC12 cells were infected with wild-type adenovirus (plus E1a) and adenovirus *dl*1500 (minus E1a, *dl*1500 produces only the 12S E1a mRNA and is defective for activation of early viral genes [30]). Transcription of the E4 promoter was highly efficient but E1a dependent (compare wild type and *dl*1500, Fig. 2A), indicating that the factors required for E1a-inducible transcription are functional in PC12 cells. In the absence of E1a (*dl*1500, Fig. 2B), forskolin (a postreceptor activator of adenyl cyclase) strongly activated the endogenous VIP CRE (35-fold, as determined by CAT activity) and the E2A promoter (>30-fold) but had no detectable effect on the E4 promoter. The uninduced levels of E4 and E2A RNAs were comparable under these conditions (Fig. 2A) but were often below the limit of detection in particular experiments (Fig. 2B). In agreement with these results, it has recently been reported that the E2A promoter is cAMP inducible in PC12 cells (40). Thus, in contrast to the VIP and E2A promoters, the E4 promoter is not substantially activated by cAMP in PC12 cells. This suggests that the ATF sites of the E4 and VIP promoters are somehow different (either within themselves or through interaction with additional promoter elements) in their ability to mediate transcriptional activation by cAMP.

The VIP CRE functions in HeLa cells but is insufficient for E1a inducibility. The activity of cAMP response elements (CREs) containing ATF sites is sometimes modulated by cell-specific factors or cell type-specific enhancer elements (1, 8, 9, 11, 21, 43). To enable a comparison of cAMP and E1a inducibility in the same cell type, we tested the E4 and VIP enhancers for E1a and cAMP inducibility in HeLa cells.

To raise cAMP levels in HeLa cells, we used highly stable analog cpt-cAMP to directly increase intracellular cAMP levels, because forskolin alone does not increase cAMP levels in HeLa cells (9). Test genes were introduced into HeLa cells by calcium phosphate precipitation, followed by induction with cpt-cAMP and analysis of correctly initiated RNAs at various times postinduction (Fig. 3). The VIP enhancer (attached to the RSV promoter; VIP-RSV) functioned as a cAMP-inducible enhancer in HeLa cells, as

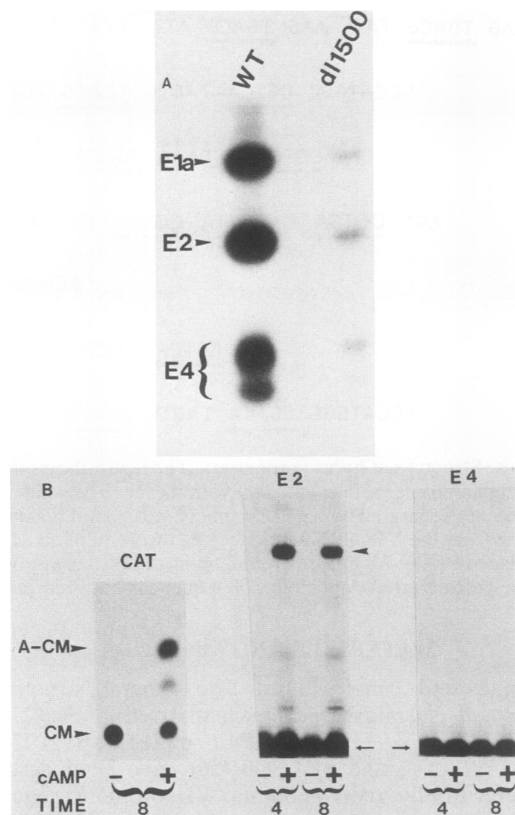

FIG. 2. (A) Transcription of early viral genes in PC12 cells in the presence (wild type [WT]) and absence (*dl*1500) of E1a. PC12 cells were infected with wild-type adenovirus (30 PFU/cell) or *dl*1500 (50 PFU/cell), followed by RNA analysis at 24 h postinfection. RNA analysis was done by primer extension as described previously (30). Correctly initiated E1a, E2, and E4 RNAs are indicated to the left of the figure. (B) cAMP induction of viral promoters in PC12 cells. PC12 cells harboring a stably integrated VIP-RSV *cat* fusion gene were infected with *dl*1500 (50 PFU/cell) as described in Materials and Methods. At 20 h postinfection, cells were induced with forskolin, and CAT or RNA analysis was performed at the times (hours) indicated below the figure. Correctly initiated E2A RNA is indicated by an arrowhead, and unextended E2A and E4 primers are indicated by the small arrows. Stimulation ratios (plus forskolin/minus forskolin) were as follows: VIP-RSV *cat* (CAT), 35-fold; E2A (RNA), >30-fold; E4 (RNA), undetectable.

indicated by induction of correctly initiated RSV *cat* RNA (3- to 20-fold induction, depending on the time postinduction). Measurement of cAMP inducibility in HeLa cells with a CAT assay indicated that induction was dependent on the VIP enhancer and was thus due to increased transcription (data not shown). The apparent difference between the activity of the highly related VIP and αCG enhancers (CREs) in HeLa cells (the results presented here and in reference 9) might reflect modulation of enhancer activity by promoter-specific elements. However, the inability of forskolin to increase cAMP levels in HeLa cells could also account for the previously reported inactivity of the αCG CRE (9). In contrast to the effects of cAMP on the VIP enhancer in HeLa cells, E4 RNA levels were not increased by cAMP, indicating a lack of response of the E4 promoter (Fig. 3). The promoter-specific effects of cAMP observed in PC12 cells are therefore mirrored in HeLa cells, further demonstrating that the ATF-binding sites in the E4 promoter do not confer an efficient transcriptional response to cAMP in this context.

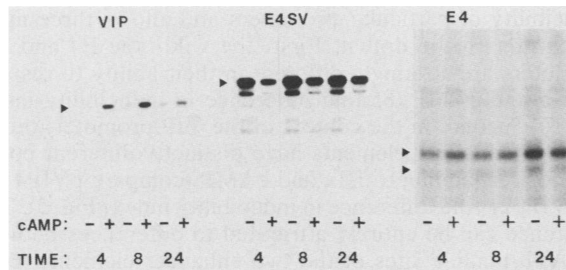

FIG. 3. cAMP induction of the VIP CRE and the E4 promoter in HeLa cells. Cells were transfected as described in Materials and Methods, followed by addition of cpt-cAMP 16 h later. Correctly initiated RNAs were analyzed by primer extension at the times indicated (hours after the addition of cpt-cAMP) below the figure and are indicated in each case by arrowheads. For E4 RNA analysis, the background bands observed that are missing in Fig. 2A, arose from variability in the primer extension assay that became apparent when very low levels of RNA were being analyzed. Plasmids used were as follows. VIP (pVIP17CAT) contains the VIP CRE fused to the RSV *cat*. pE4SV (26) contains the E4 enhancer fused to the SV40 early promoter. E4 (pE4Δ240) contains the entire E4 gene and 5'-flanking sequences to position −240.

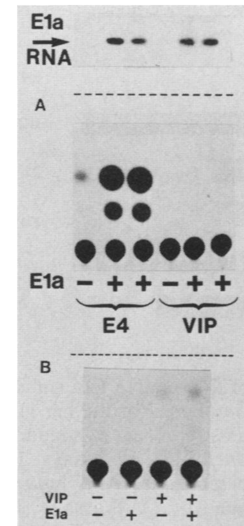

FIG. 4. E1a induction of the E4 and VIP promoters. A 5-μg amount of a plasmid containing the E4 (pE4Δ240) or VIP (pVIP4) promoter was introduced into HeLa cells in the presence of either 10 μg of pGem4 (minus E1a), 5 μg of pGem4 plus 5 μg of pH3G (plus E1a), or 10 μg of pH3G (plus E1a). CAT assays and analysis of E1a RNAs were performed at 40 h post-transfection. (A) Correctly initiated E1a RNA is indicated by an arrow at the top of the figure. Each lane corresponds to the track in the CAT assay aligned immediately below. Exposure time for autoradiography was 4 h. (B) Basal CAT activity directed by the VIP promoter under the same assay conditions. Exposure time for autoradiography was 30 h. The presence or absence of VIP (pVIP4) and E1a (pH3G) DNA is indicated below the figure.

To begin to address the reasons for the difference in cAMP responsiveness between the VIP and E4 promoters, we tested the E4 enhancer for the ability to confer cAMP responsiveness to a heterologous promoter. The E4 enhancer was fused to the simian virus 40 (SV40) early promoter (which has previously been shown to allow a cAMP response [36]) but failed to confer cAMP inducibility in this context (E4SV, Fig. 3). This result provides preliminary evidence that despite the presence of CRE consensus sequences (ATF-binding sites), the E4 enhancer does not act autonomously as a CRE.

We examined whether the difference in cAMP inducibility between the VIP and E4 promoters would be further reflected by differences in response to E1a. We tested E1a inducibility in a widely used transient expression assay (3), in which the test promoter is linked to *cat* as a reporter gene and introduced into HeLa cells in the presence and absence of E1a (Fig. 4). As reported previously, the E4 promoter responded strongly to E1a in this assay (Fig. 4A, E4, ~25-fold activation). In contrast, the VIP promoter was unresponsive (average, 1.4-fold induction) at saturating levels of E1a. Examination of E1a RNA levels showed that transfection efficiency of the E4 and VIP promoters was comparable (Fig. 4A). Longer exposure of autoradiograms demonstrated that the basal level of CAT activity directed by the VIP promoter was above background (Fig. 4B, ~7-fold above background, determined by counting radioactivity in acetylated chloramphenicol). These results demonstrate that the ATF sites of the VIP enhancer do not function as an E1a response element when in the context of the VIP promoter. In summary, although the ATF sites present in the E4 and VIP enhancers are critical for E1a (26; this study) and cAMP inducibility (15, 47), respectively, the requirements for E1a- and cAMP-inducible transcription are different. The difference is reflected by the inability of the VIP promoter to respond to E1a and the relative inability of the E4 promoter to respond to cAMP.

cAMP and E1a inducibility of E4-VIP hybrid promoters. To gain insight into the contributions of different E4 and VIP promoter elements to E1a and cAMP inducibility, we tested the activity of hybrid promoters linked to *cat* (Table 1). As reported previously (15, 47), the VIP promoter was induced ~12-fold by cAMP, and this induction was dependent on the VIP enhancer (compare pVIP4 [plus enhancer] and pVIP5 [minus enhancer]). The VIP enhancer conferred cAMP inducibility on an otherwise unresponsive truncated RSV promoter (VIP-RSV, ~12-fold induction) and to an E4 promoter lacking all sequences immediately 5' to the TATA box (pVIPE4), although the response was slightly reduced (pVIPE4, ~60% induction, compared with 100% for pVIP4). In this assay, the E4 promoter responded weakly to cAMP

TABLE 1. Inducibility by E1a and cAMP of all promoters tested[a]

Promoter or construct	Context	Assay	Inducibility[b]	
			E1a	cAMP
E4	Virus	RNA	50–100	<3
	Plasmid	RNA	ND	UD
	Plasmid	CAT	25*	3*
E2	Virus	RNA	50–100	>30
pE4SV	Plasmid	RNA	ND	UD
pVIP4	Plasmid	CAT	1.4*	12*
pVIPRSV	Plasmid	CAT	1.3*	12*
pVIP5	Plasmid	CAT	ND	1.6
pE4VIP	Plasmid	CAT	7*	3*
pE4'VIP	Plasmid	CAT	11*	4.5*
pVIPE4	Plasmid	CAT	25*	7*
pVIP'E4	Plasmid	CAT	15	ND
pE4Δ38	Plasmid	CAT	UD	UD

[a] Inducibility is expressed as the ratio of induced to uninduced levels as determined by RNA analysis or CAT assays. Data used for Fig. 5 are marked (*). All values are averages of at least three independent experiments.
[b] ND, Not determined; UD, undetectable.

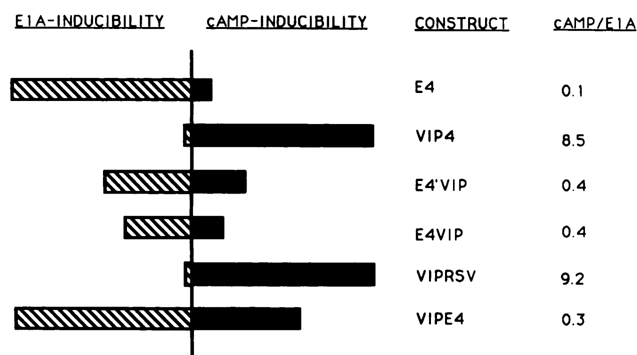

FIG. 5. Summary of the relative Ela and cAMP inducibilities of natural and hybrid promoters. For the bar graph, striped boxes to the left of the vertical axis represent Ela inducibility (ratio, induced/uninduced) as determined by CAT assays. Hatched boxes to the right of the vertical axis represent cAMP inducibility (ratio, induced/uninduced) as determined by CAT assays. The inducibility index (cAMP/E1A) for a given construct is equal to the ratio of cAMP inducibility to Ela inducibility and is indicated in the right-hand column. The indices are derived from the data marked with an asterisk in Table 1.

(maximal threefold induction, Table 1), and similarly, the E4 enhancer (sequences between −200 and −100 of the E4 promoter, containing two ATF sites [26]) weakly substituted for the VIP enhancer in the context of the VIP promoter (compare pVIP4 and pE4VIP, Table 1).

To compare the requirements for Ela and cAMP responses, we tested the series of E4-VIP hybrid promoters for Ela inducibility by the cotransfection assay (Table 1). The VIP enhancer could efficiently substitute for all E4 sequences 5′ to position −38 (pVIPE4, ~25-fold induction; compare with E4 CAT, Table 1). Deletion of a single ATF site from the VIP CRE had little effect on Ela inducibility (pVIP'E4, 15-fold induction), while deletion of the remaining ATF site (pE4Δ38) resulted in virtually undetectable expression (Table 1). Thus, the ATF sites within the VIP enhancer can efficiently substitute for all E4 promoter elements upstream of the TATA box to allow detectable Ela inducibility. This is in contrast to the inability of the VIP enhancer to participate in an Ela response in the context of the VIP promoter. These results indicate that a previously unidentified element of the E4 promoter, downstream of position −38, can significantly contribute to Ela inducibility, although we cannot rigorously rule out spacing effects at present (see Discussion). Finally, we tested the Ela inducibility of hybrid promoters in which the VIP enhancer (within the context of the VIP promoter) was replaced with E4 promoter elements containing ATF sites (Table 1). Both the E4 enhancer and the synthetic element containing two ATF sites from the E4 promoter conferred an intermediate level of Ela inducibility to the VIP promoter (pE4VIP, ~7-fold induction, and pE4'VIP, ~11-fold induction). The latter result indicates that the ATF sites within the E4 enhancer are the only elements required for Ela inducibility conferred by the enhancer.

Summary of results. The data obtained for cAMP and Ela inducibility of the VIP and E4 promoters and the E4-VIP hybrid promoters are summarized in Fig. 5. We have assigned an inducibility index (cAMP inducibility/Ela inducibility) for each of the promoters. Indices of >1 indicate that the promoter is more cAMP responsive, while indices of <1 indicate a greater degree of Ela responsiveness. Comparison of indices gives an accurate reflection of the difference in inducibility of particular promoters and allows three major conclusions to be drawn. First, the wild-type E4 and VIP promoters are strikingly different in their ability to respond to Ela and cAMP (85-fold difference in inducibility index, Fig. 5). Second, in the context of the VIP promoter, the E4 and VIP enhancer elements have distinctly different potentials for responding to Ela and cAMP (compare pVIP4 and pE4VIP, 21-fold difference in inducibility index, Fig. 5). This difference can be entirely attributed to differences between the ATF-binding sites of the two enhancer elements (compare pVIP4 and pE4'VIP, 21-fold difference in inducibility index, Fig. 5). For these two constructs, either the altered sequences immediately flanking the CGTCA motifs (Fig. 1) or the minimal spacing difference (1 base pair) between the CGTCA motifs could account for the differential effect on Ela and cAMP inducibility. A series of point and spacing mutants will be necessary to resolve this issue. Third, the activity of the VIP enhancer (and to a much smaller degree, the E4 enhancer) was strongly influenced by functional interactions with additional promoter elements (compare pVIP4 and pVIPE4, 28-fold difference in inducibility index).

Our data demonstrate that there are multiple determinants of Ela and cAMP responsiveness. In some contexts, ATF-binding sites per se have different potential for a response. In other contexts, additional promoter elements can determine the ability of particular ATF sites to participate in a response. Because for particular pairs of constructs, increases in Ela inducibility are associated with decreases in cAMP inducibility, these results unequivocally demonstrate that the promoter requirements for Ela and cAMP inducibility can be distinguished.

DISCUSSION

The E4 and VIP promoters respond differently to Ela and cAMP. Many previous studies (1, 7, 9, 11, 15, 17, 26, 37, 39, 40, 43, 47) have shown that promoter elements containing the sequence motif CGTCA (referred to here as ATF-binding sites) are critical for induction of transcription by Ela and cAMP. Consequently, it has been suggested that Ela and cAMP activate transcription through the same promoter elements. Based on the experiments described here, we find that there is not a simple correlation between the presence of ATF sites and Ela or cAMP inducibility (Fig. 5). The E4 and VIP promoters (both of which contain functional ATF sites) respond minimally to cAMP and Ela, respectively. In addition, the E4 and VIP enhancers function through the action of particular ATF sites, but have different abilities to confer Ela or cAMP responsiveness when present in certain (but not all) contexts. Our results are therefore incompatible with a simple model in which Ela and cAMP activate transcription through identical promoter elements that contain binding sites for the cellular transcription factor ATF.

Our findings might appear to be at variance with reports by Leza and Hearing (29) and Engel et al. (14) that the adenovirus E4 promoter does respond to cAMP. However, there are significant differences in the design of our experiments and those of the above authors that lead to different interpretations of the effects of cAMP on the E4 promoter. First, the use of different cell types (HepG2 [29], S49 [14], and PC12 and HeLa [this study]) might explain small differences in results. Second, our conclusion that the E4 promoter is minimally responsive to cAMP is based on a comparison with a strongly cAMP-inducible promoter (see Fig. 2B and 3; for example, in a viral context in the absence of Ela, the E4 promoter was ~30-fold less inducible than the E2A promoter

or an endogenous VIP CRE in PC12 cells). In other studies (14, 29), a weak cAMP-mediated stimulation of the E4 promoter was observed in the absence of E1a (in agreement with our transient assay in PC12 cells, Fig. 5), but this level of stimulation was not compared with a strongly cAMP-inducible promoter under the same assay conditions. Efficient cAMP inducibility of the E4 promoter has only been observed in the presence of E1a (14) and therefore under conditions that preclude a meaningful comparison with our data. In light of these considerations, our conclusion that the E4 promoter is not substantially activated by cAMP (in the absence of E1a) is significant and is not at variance with previous observations (14, 29).

ATF-binding sites differ among themselves and functionally interact with other promoter elements. The ability of the VIP enhancer to substitute for E4 ATF sites in the context of the E4 promoter but not in the context of the VIP promoter demonstrates two different effects. First, the ATF-binding sites in the E4 and VIP promoters are not functionally equivalent. Second, the ability of the ATF sites in the VIP enhancer to participate in an E1a response is strongly influenced by other promoter elements. Although our data do not entirely rule out sterospecific constraints that sometimes apply to promoter activation (4, 46), we think it unlikely that such effects play a role in specifying activation by E1a. This interpretation is based on the observation that many natural and synthetic promoters that respond to E1a contain variably located ATF sites. Instead, our results indicate that a previously unidentified element between positions −38 and +35 of the E4 promoter can cooperate with the VIP enhancer to allow an E1a response. Our preliminary data indicate that the E4 TATA box is not sufficient, suggesting that sequences around the E4 cap site (possibly in conjuction with the TATA box) contribute to the E1a inducibility of the E4 promoter.

In summary, a combination of factors determine the efficacy with which promoter elements containing ATF-binding sites confer response to E1a or cAMP. First, ATF sites differ among themselves in a manner that is probably (see below) determined by sequences immediately flanking the core motif, CGTCA. Second, ATF sites can functionally interact with other promoter elements to maximize response to E1a. Similar properties have been described for promoter elements containing the octamer motif (16, 41, 45), which functions as a lymphoid-specific promoter element for some promoters but not others. It seems likely that context effects are of general significance in modulating the activity of eucaryotic promoter elements that interact with groups of related transcription factors.

Mechanisms of E1a- and cAMP-inducible transcription. It has recently been suggested that dimerization of CREB is critical for cAMP inducibility and that functional binding of dimers is dependent on the dyad symetry (TGACGTCA) of the ATF-binding site within the somatostatin promoter (50). However, ATF-binding sites within some cAMP-inducible promoters (for example, the adenovirus E2A and E3 promoters [40; this study], CTACGTCA and TTTCGTCA, respectively) have little or no dyad symmetry. Consequently, we suggest that nucleotides immediately flanking the core motif CGTCA (that are mutated upon disruption of the dyad [50]) are important determinants of cAMP inducibility. This suggestion is consistent with many observations. First, as we have shown here, changes in sequences flanking ATF sites can have opposite effects on E1a and cAMP inducibility. Second, a G+C-rich region adjacent to the somatostatin ATF (CREB)-binding site is absolutely required for activity of the somatostatin CRE (37). Third, it has recently been directly demonstrated that flanking sequences have a marked effect on the ability of ATF sites to serve as cAMP response elements (11), even when the dyad symmetry of the ATF site is preserved. It will be of interest to systematically change sequences that flank the CGTCA motifs to define bases that are critical for E1a and cAMP inducibility and to examine the effects of such mutations on binding of the multiple factors (7, 19, 22, 36, 39) that interact with the CGTCA motif. In this regard, it is significant that certain ATF-binding sites have been shown to bind an additional factor (E4F [39]) in a manner that is dependent on sequences flanking the CGTCA motif (39).

At present, we can only speculate on mechanisms that might explain the functional complexity of ATF-binding sites. Individual ATF sites might be preferentially recognized by factors in a way that escapes detection under the conditions currently employed for DNA-binding assays. For example, in vitro assays could reflect binding of different factors (ATF-43, ATF-47 [19, 22], EivF [7], CREB [36], E4F [39]) to the core CGTCA motif with similar affinities. In vivo, this primary interaction might be modulated by sequences flanking the core, additional promoter elements, or protein factors that do not directly interact with DNA. Such factors might act by forming a terniary (or higher-order) complex with ATF and DNA that is not stable under in vitro conditions. An alternative possibility is that sequences adjacent to the core CGTCA sequence might directly alter interaction of a common activator protein with DNA and thereby differentially influence response to E1a and cAMP.

cAMP does not simply substitute for E1a in viral gene activation. Activation of at least two adenovirus early promoters by cAMP (E2A and E3 [40; our results]) suggests an involvment of cAMP in viral gene expression. The weak cAMP inducibility of the E4 promoter that is sometimes observed in the absence of E1a (14, 29) and the stronger effect that can be observed in the presence of E1a (14) are also consistent with this possibility. However, the inability of cAMP to activate the E4 promoter to a significant level in the absence of E1a (in contrast to the strong induction observed for the E2A promoter) demonstrates that cAMP cannot effectively substitute for E1a by activating all early viral genes. It is also plausible that the cAMP responsiveness of the E2A and E3 promoters resulted from adventitious acquisition of promoter elements during evolution and is not related to a specific viral function. The degenerate nature of many other eucaryotic promoter elements is consistent with this notion.

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