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Biochemistry

Adenoviral control regions activated by E1A and the cAMP response element bind to the same factor

(transcriptional control/DNA-binding factor/competition analysis)

STEPHEN HARDY AND THOMAS SHENK

Department of Molecular Biology, Princeton University, Princeton, NJ 08544

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ABSTRACT **Transcription of adenoviral early genes is activated by viral *E1A* gene products. Four of the five early genes contain sequence homologies to the cAMP-inducible element identified in cellular genes. Co-purification and competition assays demonstrated that the adenovirus and cAMP-inducible transcriptional control regions bind the same factor or factors. Since the binding sites map to regions necessary for transcription of the three adenoviral early genes whose control regions were analyzed, it is likely that the activities play a role in their transcription.**

The adenoviral E1A proteins are able to activate some transcription units and repress others (reviewed in refs. 1 and 2). Although it is well established that E1A-mediated activation and repression occur at the level of transcriptional initiation, the mechanisms underlying these events have remained unclear. We have found a possible link between E1A-mediated induction of viral gene expression and a basic intracellular regulatory system, cAMP-mediated transcriptional control.

The regulation of transcription by cAMP has been extensively studied in living animals and tissue culture cells. Increasing intracellular cAMP concentration by treatment with compounds such as cAMP analogs or β-adrenergic agonists induces transcription in a tissue-specific manner for many genes, including somatostatin (3), the α subunit of human chorionic gonadotropin (4), phospho*enol*pyruvate carboxykinase (5), tyrosine hydroxylase (6), and c-*fos* (7). Many cAMP-responsive genes contain a sequence homologous to the sequence TGACGTCA located on the 5′ side of their mRNA cap sites. This sequence has been termed a cAMP-responsive element (CRE) (8). Deletion mutagenesis of the phospho*enol*pyruvate carboxykinase, α subunit of human chorionic gonadotropin, and somatostatin genes has shown it to be contained within a domain necessary for cAMP-mediated induction of transcription. For somatostatin, a 31-base-pair (bp) DNA segment containing the CRE is sufficient to confer cAMP inducibility on the normally nonresponsive simian virus 40 early gene in a transient assay (3). An activity, termed CRE-binding protein (CREB), (8) has been identified that binds to this conserved sequence in the somatostatin transcriptional control region.

We noticed that four of the five early adenoviral transcriptional control regions contain one or more CRE homologies. In fact, the adenovirus *E2* and the *E4* CRE sequences are identical to those found in the c-*fos* and phospho*enol*pyruvate carboxykinase genes, respectively. These CRE homologies upstream of the mRNA caps of *E2*, *E3*, and *E4* are in regions identified (9–16) as critical for expression in transient assays. Viral transcriptional control regions that carry mutations within their CRE homologies generally exhibit reduced basal levels of transcription but remain inducible by *E1A* gene products. This suggests that CRE-like sequences are not uniquely required for E1A responsiveness. However, DNA fragments containing CRE homologies from the *E4* transcriptional control region have been shown to confer E1A inducibility to test genes (12, 16).

In this report we show that the same factor or factors bind to the CRE sequences found in cellular cAMP-responsive genes and adenoviral early genes. This suggests that the factor or factors that bind to CRE homologies are likely to be involved in the coordinate expression of early adenoviral genes. It, further, raises the possibility that these activities could play a role in E1A-mediated induction of viral gene expression.

MATERIALS AND METHODS

DNase I "Footprinting." Footprint analysis was as described by Galas and Schmitz (17). Binding reactions were identical to those described below for DNA band-shift analysis. Each reaction mixture contained 3 μl of fraction 8 from the DEAE-5PW column (see Fig. 3*B*) as the source of extract. The *E2* fragment contained adenovirus type 2 (Ad2) sequences from position −95 to position −31 relative to the mRNA cap site. The *E4* fragment contained adenovirus type 5 (Ad5) sequences from position −218 to position +256 relative to the mRNA cap site. Double-stranded oligonucleotide (1 pmol) was added to some reaction mixtures as competitor.

Chromatography. All chromatographic procedures were performed at 0 or 4°C. HeLa cell nuclear salt wash was prepared as described by Dignam *et al.* (18). Extract (10 ml) was dialyzed against 50 mM NaCl in buffer A [25 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20% (vol/vol) glycerol] and loaded onto a column (1.6 × 12 cm) containing S-Sepharose (Pharmacia). Sample was applied to the column at a flow rate of 1.5 ml/min. After loading, the column was washed with 50 mM NaCl in buffer A (15 ml), material was eluted with a linear gradient (60 ml) of 50–500 mM NaCl in buffer A, and the column was then washed with 500 mM NaCl (15 ml) and 2 M NaCl (10 ml) in buffer A. Fractions (1.5 ml) were collected, commencing with the application of the gradient. A 1-μl aliquot of each fraction was analyzed in a DNA band-shift assay. Active fractions were pooled, dialyzed against two changes of buffer B [25 mM Tris·HCl (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20% (vol/vol) glycerol], and loaded onto a DEAE-5PW column (Glass-Pak DEAE-5PW, 0.8 × 7.5 cm, LKB) at a flow rate of 0.5 ml/min on an HPLC system (LKB). After loading, the column was washed with buffer B (2.5 ml), material was eluted with a linear gradient (10 ml) of 0–400

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Abbreviations: CRE, cAMP-responsive element; CREB, CRE-binding protein; Ad2 and Ad5, adenovirus types 2 and 5, respectively.

mM NaCl in buffer B, and the column was washed with 400 mM NaCl in buffer B (2.5 ml) and a 5-ml gradient of 400–2000 mM NaCl in buffer B. Fractions (0.25 ml) were collected starting with the first gradient. Each fraction was diluted 1:3 in buffer C [25 mM Hepes (pH 7.9), 1 mM EDTA, 2 mM dithiothreitol, 50 mM NaCl, 20% (vol/vol) glycerol, and bovine serum albumin at 100 μg/ml] and analyzed in a DNA band-shift assay.

DNA-Band-Shift Assays. The protocol of Carthew *et al.* (19) with modifications was used for the analyses. Binding reactions were carried out in mixtures (10 μl) containing 1 μl of nuclear extract diluted 1:5 in buffer C or 1 μl of a column fraction, 10 fmol of 5′-end-labeled double-stranded oligonucleotide, and 1 μg of poly[d(I-C)]. The final concentrations of buffers and salts were 10 mM Hepes (pH 7.9), 75 mM NaCl, and 2 mM $MgCl_2$. Bound DNA was separated from free DNA by electrophoresis in a 4% polyacrylamide gel [1.6 mm thick, *N′,N′*-methylenebisacrylamide/acrylamide, 1:20 (wt/wt)] containing 10 mM Tris·HCl (pH 8) and 1 mM EDTA at 4°C with a voltage gradient of 15 V/cm.

DNA-band-shift assays were used to perform competition binding analysis. The affinity of CREB factor for each double-stranded oligonucleotide was determined relative to its affinity for the double-stranded oligonucleotide from the fibronectin gene to which it exhibited the highest affinity (see Table 1). To ensure an interpretable result, extracts were diluted so that the amount of bound activity was proportional to added extract. The behavior of the specific complex in response to various concentrations of NaCl and $MgCl_2$ was titrated, and optimal binding conditions were used. As a control for the length of the competing DNA, fragments of as long as 500 bp were prepared from plasmids containing *E1A*, *E2*, *E3*, and *E4* upstream regions, and they were comparable, on a molar basis, to the double-stranded oligonucleotides in their affinity for CREB factor. Relative affinity was calculated as the ratio of the molar concentration of homologous to heterologous competitor required to achieve equivalent reductions in binding of the factor to ^{32}P-labeled probe DNA.

Oligonucleotides. Synthetic DNAs were prepared on an automated DNA synthesizer (Applied Biosystems) using phosphoramadite chemistry. The yields of product were >90%, so no purification other than desalting was done. ^{32}P-labeled, double-stranded oligonucleotides were 5′-end-labeled to high specific activity as described by Maniatis *et al.* (20) with 6 μM [γ-^{32}p]ATP as the only source of ATP.

RESULTS

CRE-Like Sequences Are Present Upstream of Adenoviral Early Genes. We identified a factor that bound within the transcriptional control region of all but one of the Ad2 early genes. DNase I footprint analyses revealed that partially purified factor protected a region from position −83 to position −69 relative to the *E2* early start site (Fig. 1*A*), and this domain included an 8-bp core (TGACGTAG) homologous to the CRE sequence. Two regions were protected when *E4* DNA was used, the first extending from position −167 to position −138 relative to the *E4* start site (Fig. 1*B*), including the CRE-related TGACGTAA sequence. The second *E4* footprint was centered around position −45 and included the same 8-bp sequence. The generation of both *E4* footprints was inhibited by the addition of a double-stranded oligonucleotide containing the sequence from position −167 to position −142 relative to the *E4* cap (Fig. 1*B*, lane 5). The position −167 footprint partially protected two copies of a sequence homologous to an *E1A* 5′-flanking repeat, termed element I (21). An oligonucleotide containing element I failed to compete with the CRE-like sequences in the footprint assay (Fig. 1*B*, lane 6).

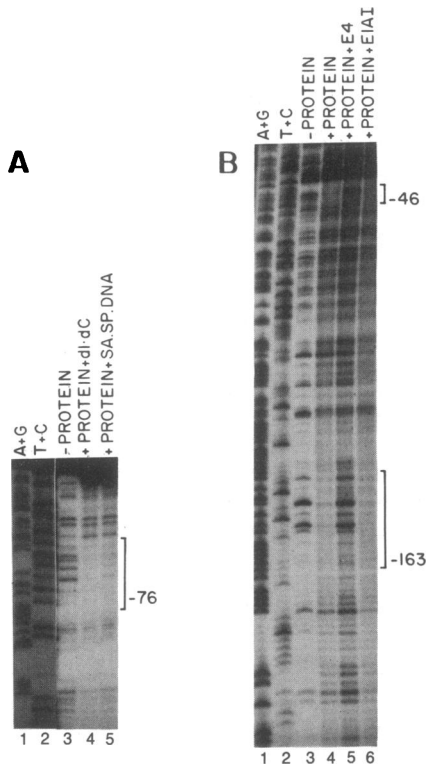

FIG. 1. DNase I footprint analysis of partially purified CREB factor on adenovirus DNA segments. (*A*) Footprint of Ad2 *E2* 5′-flanking region. Lanes 1 and 2 contain the end-labeled *E2* DNA segment subjected to chemical cleavage to produce sequence standards. Lane 3 contained DNase I-treated DNA only. Reaction products in lanes 4 or 5 received end-labeled *E2* DNA, a chromatographic fraction, and either poly[d(I-C)] or salmon sperm DNA as unlabeled nonspecific competitor DNA. (*B*) Footprint of Ad5 *E4* 5′-flanking region. Designations are as above except the reaction products in lane 5 contained a 100-fold molar excess (relative to ^{32}P-labeled *E4* DNA fragment) of the Ad5 *E4* double-stranded oligonucleotide carrying the position −163 CRE homology (Table 1). Lane 6 contained a 100-fold molar excess of an *E1A* DNA (GATC-TAAGAGGAAGTGAAATCTGGATC) containing the unrelated element I sequence.

The footprint analyses indicate that the factor under study is almost certainly the same as the activities described by Siva Raman *et al.* (22) in the context of the *E2* control region (termed E2-EF) and by Lee and Green (16) in the context of the *E4* control region (termed E4F1). Sequence homologies to the 8-bp element identified in the footprint analyses are present in the 5′-flanking regions of the Ad2 *E1A*, *E2*, *E3*, and *E4* transcription units as well as within the 103-bp inverted terminal repeats present at either end of the viral chromosome (Table 1).

A Common Factor Binds to Adenoviral Early and cAMP-Responsive Genes. A series of double-stranded oligonucleotides were prepared that ranged from 26 to 33 bp long and contained sequences including and surrounding the CRE homologies found upstream of the cap sites for early viral genes or cAMP-responsive cellular genes (Table 1). DNAs containing fibronectin, somatostatin, or viral gene CRE homologies were ^{32}P-labeled and employed as substrates in DNA-band-shift assays (Fig. 2*A*). The complexes formed with factors in HeLa cell nuclear extracts all migrated similarly in native polyacrylamide gels. Multiple bands were observed for all DNA probes. A self-competition experiment with unlabeled homologous competitor DNA demonstrated that multiple bands represent specific complexes (Fig. 2*B*). Several control DNAs were tested that lack CRE homologies, and they failed to produce complexes of similar elec-

Table 1. Relative affinities of CREB factor for oligonucleotides that contain a CRE homology

Gene	Distance to the 5′ cap, bp	Oligonucleotide sequence	Relative affinity
Fibronectin	−173	ACAGTCCCCCG**TGACGTCA**CCCGGGAGCCC	1.00
Somatostatin	−48	GCCTCCTTGGC**TGACGTCA**GAGAGAGAG	0.40
PEPCK	−90	AAAGGCCGGCCC**TTACGTCA**GAGGCGAGCCTCC	0.10
Ad5 *E4*	−163	GGAAG**TGACGTAA**CGTGGGAAAACGG	0.30
Ad2 *E4*	−163	AAATGGGAAG**TGACGTAT**CGTGGGAAAAC	0.10
Ad2 *E3*	−61	GCGGGCGGC**TTTCGTCA**CAGGGTGCGGTC	0.04
Ad2 *E2*	−76	CTGGAGA**TGACGTAG**TTTTCGCGCTTAAATTT	0.15
Ad2 *E1A*	−43	ATAGTCAGC**TGACGTGT**AGTGTATTTATACCC	0.04
Ad2,5 *E1A*	−117	ACTTTGACCGT**TTACGTGG**AGACTCGC	<0.01
Ad2 major late	−61	GGTGTAG**GCCACGTGA**CCGGGTGTTC	<0.01
Collagenase	−72	GGATGTTATAAAGC**ATGAGTCA**GACACCTCTGGCT	<0.01
Proenkephalin	−92	GGCGTAGGGCC**TGCGTCA**GCTGCAGCCCGCC	<0.01
Ad2,5 ITR	−435, 260	**TGACGTGG**	
Ad2,5 ITR	−403, 229	**TGACGTAG**	0.15

The sequence of oligonucleotides used for competition experiments and their relative affinities for CREB are tabulated. The bases indicated by bold letters in each oligonucleotide are matches to the fibronectin gene CRE homology that displayed the highest affinity for CREB. The relative affinity of each DNA for CREB versus the affinity for the fibronectin DNA was determined from competition assays displayed in part in Fig. 4. Relative affinity was calculated as the ratio of the molar concentration of homologous to heterologous competitor required to achieve equivalent reductions in binding of the factor to ^{32}P-labeled fibronectin DNA. The distance to the 5′ cap is measured from the 5′ base pair of the CRE. Somatostatin (3) and phospho*enol*pyruvate carboxykinase (PEPCK) (5) have been shown to depend on short DNA segments including the CRE for cAMP induction. Fibronectin (23) is a cAMP-inducible gene that contains a CRE homology within its 5′-flanking domain. There are two homologies in each Ad2 or Ad5 (Ad2,5) inverted terminal repeat (ITR); the first number listed in each case is the distance to the *E1A* cap site and the second is relative to the *E4* cap site. The competition assay for the inverted terminal repeat with CRE homology employed a restriction fragment containing both sites and, as a result, no oligonucleotide sequence is listed.

trophoretic mobility (Fig. 2*B*, lane 4, and data not shown). We do not yet know whether modified forms of the same factor or different factors generate the series of bands. Multiple specific bands are also produced in band-shift assays with the major late transcription factor (19). The co-migration of complexes formed on the cellular and viral DNA segments is consistent with the possibility that they all bind to the same factor or factors.

To investigate the relationship between the complexes formed with various DNA probes, the binding activities were monitored through two steps of column chromatography. In the first step, the components of a HeLa cell nuclear extract were separated on a cation-exchange matrix. Fractions were probed in band-shift assays with fibronectin, somatostatin, *E1A*, *E2*, *E3*, and *E4* oligonucleotides. Results from assays done with *E2* and somatostatin DNA as probes are shown in Fig. 3*A*. The complex pattern produced with unfractionated nuclear extract was reproduced with the column fractions, except the activities that bound to CRE homology-containing DNAs were separated into two peaks. This pattern of fractionation was reproduced with all probes. Their separation on the column indicates that the various complexes detected in a band-shift assay are not artifacts of the assay but are physically distinct entities.

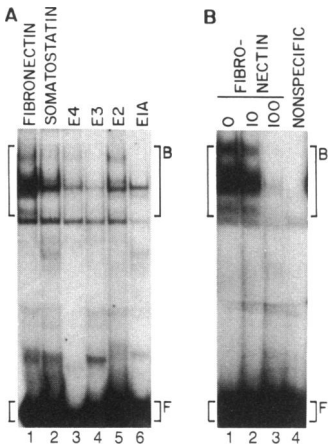

FIG. 2. (*A*) DNA-band-shift analysis of six DNA segments containing CRE homologies. The experiment employed double-stranded oligonucleotides (sequences in Table 1) and an unfractionated nuclear extract from HeLa cells. Bands labeled B and F designate the positions of the major specifically bound complexes and the free probe DNA, respectively. (*B*) DNA-band-shift analysis of a competition assay. Unlabeled fibronectin DNA was added to compete for complexes formed on the ^{32}P-labeled fibronectin DNA. Excess nuclear extract was added (1 μl of undiluted extract) so that all of the complexes could be easily seen. Lane 4 contains an unrelated ^{32}P-labeled oligonucleotide (CCATTTTCGCGGGAAA-ACTGAATAAG) as a negative control. The numbers above each lane indicate molar excess of the unlabeled DNA. Bands labeled B and F are the same as in *A*.

Fractions 4–14 were pooled and chromatographed on a high-resolution HPLC anion-exchange matrix. Once again, a complex pattern was produced. Fig. 3*B* displays the profiles obtained with the E4 and somatostatin DNA probes. Several activities that co-purified on the cation-exchange column were somewhat separated by the higher-resolution HPLC anion-exchange column. Fractions from both columns were assayed for the major late transcription factor (19) and four sequence-specific activities that bind upstream of the *E1A* cap site (unpublished results). None of these activities co-purified with the factor that bound to CRE-related sequences (data not shown). In summary, the activities measured by all six DNAs containing CRE homologies co-purified through two sequential chromatography steps. It is very probable that each DNA bound to the same factor or factors.

To confirm that the same activities bound all six DNAs, competition binding experiments were performed. In a preliminary experiment the fibronectin gene CRE sequence bound the activity most efficiently. Therefore, it was used as the ^{32}P-labeled DNA and unlabeled homologous or heterologous DNAs were employed as competitors (Fig. 4). Relative affinities of the various DNAs for the activities that bound to the fibronectin DNA are expressed as the ratio of heterologous to homologous competitor required to achieve an equivalent reduction in binding to the ^{32}P-labeled DNA (Table 1). The somatostatin DNA segment competed strongly for the factor (relative affinity, 0.40) as compared to the fibronectin sequence. Since these two DNAs share the same

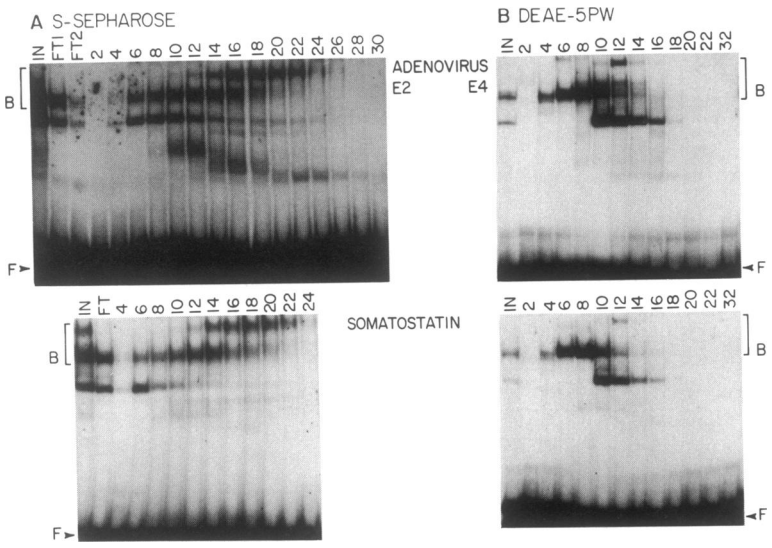

FIG. 3. DNA band-shift analysis of fractionated HeLa cell nuclear extracts. Extract was subjected to sequential chromatographic fractionations on a S-Sepharose matrix (*A*) and on a DEAE-5PW matrix (*B*). Band-shift assays employed double-stranded oligonucleotides derived from the Ad2 *E2*, Ad5 *E4*, or somatostatin gene (Table 1) as probe DNAs. Lanes labeled IN are input material loaded onto the column matrices, and lanes labeled FT are flow-through material. Bands labeled B and F designate the positions of bound and free probe DNA, respectively.

symmetrical 8-bp sequence (TGACGTCA), but their affinities for the factor differ, a larger sequence must influence binding activity. The Ad5 *E4* (position −163) segment was also a strong competitor (relative affinity, 0.30). Next in affinity were phospho*enol*pyruvate carboxykinase, Ad2 *E4* (at position −163), and Ad2 *E2* (at position −76). The sites in Ad2 *E3* (at position −59) and Ad5 *E1A* (at position −43) were relatively weak competitors. The inverted terminal repeat competed (relative affinity, 0.15) (data not shown); however, two CRE homologies were present on the fragment employed (1–106 bp from the end of the viral chromosome). Finally, there was a poor homology in the Ad5 *E1A* region (at position −117) that did not detectably compete for the factor binding, and a DNA segment containing the binding site for the major late transcription factor (19) also failed to compete for factor binding (data not shown).

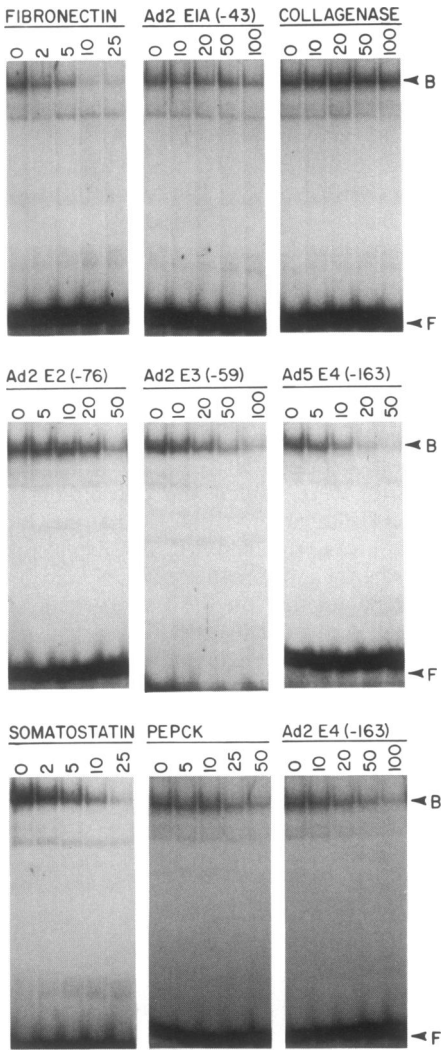

FIG. 4. Competition analyses in a DNA-band-shift assay. A double-stranded oligonucleotide derived from the fibronectin gene served as the ^{32}P-labeled substrate in all experiments, and the source of unlabeled competitor DNA is indicated (the sequences of the DNAs are in Table 1). The numbers above each lane indicate the molar excess of competitor DNA over ^{32}P-labeled DNA. Bands labeled B designate the positions of the major sequence-specific complex, and bands labeled F indicate the positions of the free DNA.

Comb *et al.* (24) have reported that a 29-bp segment from the proenkephalin gene confers cAMP inducibility on a nonresponsive gene. Within this region they noted a homology to the phospho*enol*pyruvate carboxykinase and somatostatin CRE sequences. The sequence from the proenkaphalin gene (TGCGTCAG) lacks a single base pair in comparison to the CRE sequences (TGACGTCA). The proenkephalin gene sequence is also found in the polyoma virus enhancer. This region in polyoma is thought to bind AP-1 (activator protein 1) (25), a factor known to enhance transcription of simian virus 40 early and metallothionein genes (26). To investigate the relationship between these activities and the factor under study, two additional DNAs were assayed for their ability to compete in the binding assay. The first was from the transcriptional control region of the proenkephalin gene, and the second was a collagenase gene segment containing a functional AP-1 binding site (25). Neither of these DNAs competed for binding of the CRE-specific activities (Fig. 4 and data not shown).

We conclude that there are several activities all of which bind to CRE homologies in three cAMP-inducible cellular genes, four adenovirus early genes, and the viral inverted terminal repeats.

DISCUSSION

The same activities bound to CRE homologies present in cellular and adenoviral early genes. Three lines of evidence support this assertion. First, six DNA fragments of similar size (Table 1) containing CRE homologies from cAMP-inducible cellular genes (fibronectin and somatostatin) and adenoviral early genes (*E1A*, *E2*, *E3*, and *E4*) bound activities in nuclear extracts to produce multiple complexes of similar mobilities in band-shift assays (Fig. 2). Second, the activities bound by all six DNAs co-purified through two sequential

chromatographic steps (Fig. 3). Third, all six DNAs competed for binding of the activities (Fig. 4).

Given the pattern of multiple complexes produced in a DNA-band-shift assay with DNAs containing a CRE homology and the fact that these complexes were chromatographically separable activities, it is possible that CREB is a number of proteins. Even more probable is that some of the different forms represent alternative modification states of one or more proteins. Since CREB is thought to respond to cAMP-dependent protein kinase activity, such a model is reasonable.

These activities have been termed E2-EF (22) or E4F1 (16) in the context of the adenovirus *E2* or *E4* genes, respectively, and termed CREB (8) in the context of the somatostatin gene. E2-EF, E4F1, and CREB are probably the same set of activities. Since E4F1 has been shown to function as a transcription factor in cell-free assays (16), it is very likely that E2-EF and CREB also function at the level of transcription.

The difference in affinities of CREB activities for the various oligonucleotides (Table 1) is difficult to translate into the affinities of CREB activities for different binding sites. However, two of the sites in adenoviral early genes, *E1A* and *E3*, are probably significantly weaker than the rest. The binding of CREB activity to these sites may be enhanced through interaction with factors that bind to proximal sites. Alternatively, these variations in affinity may reflect a hierarchy of binding and thus differential regulation of early gene expression through a dependence upon CREB concentration. In any case, it is clear that the *E3* CRE homology does affect expression in transient assays (11, 14).

What role do these factors play in adenovirus gene expression? CRE homologies are present upstream of the *E1A*, *E2*, *E3*, and *E4* mRNA start sites (Table 1). Mutational analyses have demonstrated that the CRE homologies are located within domains required for optimal expression of the *E2*, *E3*, and *E4* transcriptional control regions (9–16). Thus, it seems reasonable to conclude that CREB activities play a role in the expression of adenoviral early genes. What remains uncertain is whether CREB activities play a direct role in E1A-mediated induction of early viral genes. Generally, mutation of CRE homologies has led to a reduction in basal transcriptional activity without preventing an induction above the reduced level by E1A proteins (9–11, 14–16). However, when a 170-bp (12) or a 100-bp DNA fragment (16) containing *E4*-specific CRE homologies was appended to a non-E1A-responsive test gene, the resulting hybrid constructs were stimulated by E1A proteins in a short-term transfection assay. This raises the intriguing possibility that CREB activities might play a role in E1A-mediated transcriptional activation. The possibility remains, however, that another unidentified factor interacts with the *E4*-specific DNA fragments to mediate E1A inducibility. Further, the mutational studies mentioned above argue strongly that other components of the viral control regions must also be responsive or that E1A proteins somehow act on a transcription complex rather than on individual components. In fact, one E1A-induced adenoviral gene, *E1B*, does not contain a recognizable CRE homology and appears to be regulated differently than other early genes (27).

Given the possibility that CREB activities might be involved in the E1A-mediated induction of at least certain viral genes, such as *E4*, it follows that E1A proteins could interact with the cAMP-signaling system. The cAMP regulatory pathway in mammalian cells has been investigated in considerable detail (reviewed in ref. 28). Briefly, β-adrenergic receptors stimulate adenylate cyclase through guanine nucleotide-binding regulatory proteins, and cAMP-dependent protein kinases are activated in response to the increased cAMP concentration. These activated kinases then phosphorylate proteins that presumably modulate transcription. E1A proteins could directly or indirectly affect any of these steps. It is also possible that E1A-mediated activation does not functionally involve the cAMP pathway. These two transcriptional activation systems might simply share a DNA-binding factor or factors that can be activated through alternative pathways.

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