Proc. Natl. Acad. Sci. USA
Vol. 85, pp 3396–3400, May 1988
Biochemistry

Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters

(adenovirus/DNA-binding protein/DNase I "footprint"/UV crosslinking/*in vitro* transcription)

YOUNG-SUN LIN AND MICHAEL R. GREEN

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138

Communicated by Mark Ptashne, February 1, 1988 (received for review December 15, 1987)

ABSTRACT **We have previously identified a cellular transcription factor, ATF, which is involved in the expression of multiple adenovirus E1a-inducible genes. Here we show that ATF also binds to regulatory elements in cellular cAMP-inducible promoters. In these cellular promoters, ATF binds to a consensus sequence that has been previously shown to be necessary and in some instances sufficient for cAMP-inducible transcription. Furthermore, we show that binding of ATF to these promoter elements is required for the constitutive *in vitro* transcriptional activity of the cAMP-inducible somatostatin gene. Taken together, our results suggest that a common cellular transcription factor, ATF, can be regulated *in vivo* by two apparently unrelated inducing agents: the adenovirus E1a protein and cAMP.**

We have been studying the transcriptional activation of adenovirus genes by E1a, the adenovirus immediate-early protein. These studies have led to the identification of a cellular transcription factor, ATF, which is involved in expression of the E1a-inducible E4, E2, E3, and probably E1a genes (refs. 1 and 2 and refs. therein). In these viral genes, ATF binds to a common, short sequence element that in each instance has been shown to be essential for transcriptional activity (refs. 1 and 2 and refs. therein). It is likely that ATF is directly involved in the transcriptional regulation of these E1a-inducible genes since multiple ATF sites can confer (or increase) E1a-inducibility onto heterologous genes (1).

The fact that ATF is a cellular protein implies the existence of cellular genes whose expression is regulated by ATF. We have noted a strong similarity between the ATF-binding sites defined on viral promoters and the promoter regions of some cellular genes whose transcription is regulated by the intracellular cAMP concentration. This similarity suggests that a single transcription factor might mediate two very different transcriptional regulatory responses. Here we show that a common transcription factor, ATF, interacts with the regulatory elements of both E1a- and cAMP-inducible promoters.

MATERIALS AND METHODS

Constructions. The promoter regions of the various genes were cloned into the polylinker of the vector pGEM3 (Promega Biotec, Madison, WI). pE4BS contains the adenovirus type 5 E4 promoter sequences from −34 to −90 inserted between the *Bam*HI and *Sac* I sites. pSOMDB contains the rat somatostatin promoter sequences from −71 to −29 (obtained from plasmid pEJ1; ref. 3) inserted into the *Hin*cII site in the orientation in which the upstream promoter sequences are closer to the *Hin*dIII site. pSOMRB contains the rat somatostatin gene sequences from −71 to +53 inserted into the *Xba* I site in the orientation in which the upstream promoter sequences are closer to the *Hin*dIII site. pTHDM contains the rat tyrosine hydroxylase promoter sequences from −107 to −27 (obtained from plasmid 5'THCAT +27/−272; ref. 4) inserted into the *Sma* I site in the orientation in which the downstream promoter sequences are closer to the *Hin*dIII site. pαRM contains the human α-chorionic gonadotropin (α-hCG) promoter sequences from −169 to −100 (obtained from plasmid pα6/10; ref. 5) inserted into the *Hin*cII site in the orientation in which the upstream sequences are closer to the *Hin*dIII site. pMLPX contains the adenovirus type 2 major late gene sequences from −260 to +30 inserted between the *Sal* I and *Sma* I sites. pVIPRN contains the human vasoactive intestinal polypeptide (VIP) promoter sequences from −90- to −30 (obtained from pVIPΔ3'CAT1; ref. 6) inserted into the *Sma* I site in the orientation in which the upstream promoter sequences are closer to the *Hin*dIII site.

DNase I and Dimethyl Sulfate "Footprinting" and *in Vitro* Transcription. DNase I and dimethyl sulfate footprinting were carried out as described (1, 2). For DNase I footprinting, the probes were the *Hin*dIII/*Pvu* II DNA fragments from plasmids pE4BS, pSOMDB, pTHDM, pαRM, and pVIPRN 3'-end-labeled at the *Hin*dIII site. These same *Hin*dIII/*Pvu* II DNA fragments were also used as competitors. The nonspecific competitor DNA was the *Hin*dIII/*Pvu* II fragment from pGEM3.

For dimethyl sulfate footprinting, probes for the coding strand of the somatostatin, VIP, and α-hCG and for the noncoding strand of E4 and tyrosine hydroxylase promoters, were the same as those used for DNase I footprinting. For the noncoding strand of somatostatin, VIP, and α-hCG and the coding strand of E4 and tyrosine hydroxylase, the probes were the *Eco*RI/*Sph* I DNA fragments 3'-end-labeled at the *Eco*RI site. The conditions for *in vitro* transcription were as described (1) and the RNA was analyzed by primer extension (7) using the T7 promoter primer (Promega Biotec).

UV Crosslinking. Probes for UV crosslinking were prepared by the addition of 30 ng of the *Hin*dIII/*Pvu* II DNA fragments from plasmids pE4BS, pVIPRN, pSOMDB, pTHDM, or pαRM to a 100-fold molar excess of T7 promoter primer in restriction endonuclease buffer containing 50 mM NaCl, boiled for 3 min, and immediately placed on ice. To this reaction mixture was added 10 units of reverse transcriptase (Promega Biotec); dATP, dGTP, and BrdUTP to 50 μM; and [α-^{32}P]dCTP to 5 μM. The reaction mixture was incubated at 42°C for 2 hr followed by cleavage with *Eco*RI. The *Hin*dIII/*Eco*RI fragments were purified and used as probes for UV crosslinking. UV crosslinking was carried out as

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: α-hCG, human α-chorionic gonadotropin; VIP, human vasoactive intestinal polypeptide; CRE, cAMP response element.

described (8) except that UV irradiation was for 1 hr at 4°C in Sarstedt Eppendorf tubes placed 4.5 cm from the surface of a Fotodyne UV 310 transilluminator.

RESULTS

A Factor Binds to the ATF Consensus Sequence Present in Four cAMP-Inducible Promoters. Many mammalian cAMP-inducible genes contain one or more copies of a highly conserved promoter element 5′ TGACGTCA 3′ located 38–173 nucleotides upstream from the transcriptional start site (refs. 3, 4, 6, and 9–12 and refs. therein). Furthermore, a short DNA fragment that contains this sequence element, referred to as a cAMP response element (CRE), can in some instances confer cAMP inducibility when placed upstream of the "TATA box" of a heterologous gene (3, 4, 6, 9–12). We used DNase I footprinting to determine whether a factor(s) binds to the CRE in four cAMP-inducible promoters (Fig. 1*A*). In this and the other experiments presented below, extracts were prepared from HeLa cells since these cells can support both E1a-inducible (see, for examples, refs. 1 and 7) and cAMP-inducible (K. Lee and M.R.G., unpublished data) transcription. In all four promoters, the CRE is bound by a factor as evidenced by specific protection of this region from DNase I digestion. In the VIP, somatostatin, and tyrosine hydroxylase genes, a 15- to 20-base-pair protected region is located at −64 to −84, −35 to −55, and −35 to −50, respectively. In the α-hCG gene there is a 34-base-pair protected region at −111 to −145. The more extensive region of protection in the α-hCG gene is expected since this region contains two tandemly arranged copies of the CRE (5, 12). We conclude that a factor binds specifically to the CRE of cAMP-inducible promoters. This conclusion is consistent with several recent studies that have also demonstrated the binding of a factor(s) to CRE sites in some cAMP-inducible promoters (11, 13).

The Factor that Binds to the CRE Has Identical DNA-Binding Specificity to ATF. As described above, we noted that the CREs in these cAMP-inducible promoters conform to viral ATF sites. To determine whether the DNA-binding specificity of the factor(s) that interacts with the ATF sites on adenovirus promoters and the CREs on cAMP-inducible cellular promoters is identical, we performed competition experiments (Fig. 1). As expected, binding of a factor(s) to the CREs of the four cAMP-inducible promoters is competed by the homologous DNA fragments but not by nonspecific DNA (pGEM). Significantly, in each instance, binding of a factor(s) to all four cAMP-inducible genes is also competed by an adenovirus E4 promoter fragment containing a single ATF site (Fig. 1*A*). Thus, a factor(s) that binds to the CRE of cAMP-inducible promoters can also bind to the E4 promoter ATF site. This conclusion was confirmed by performing the reciprocal competition experiment, in which the E4 promoter DNA fragment, which contains a single ATF site, is used as a probe (Fig. 1*B*).

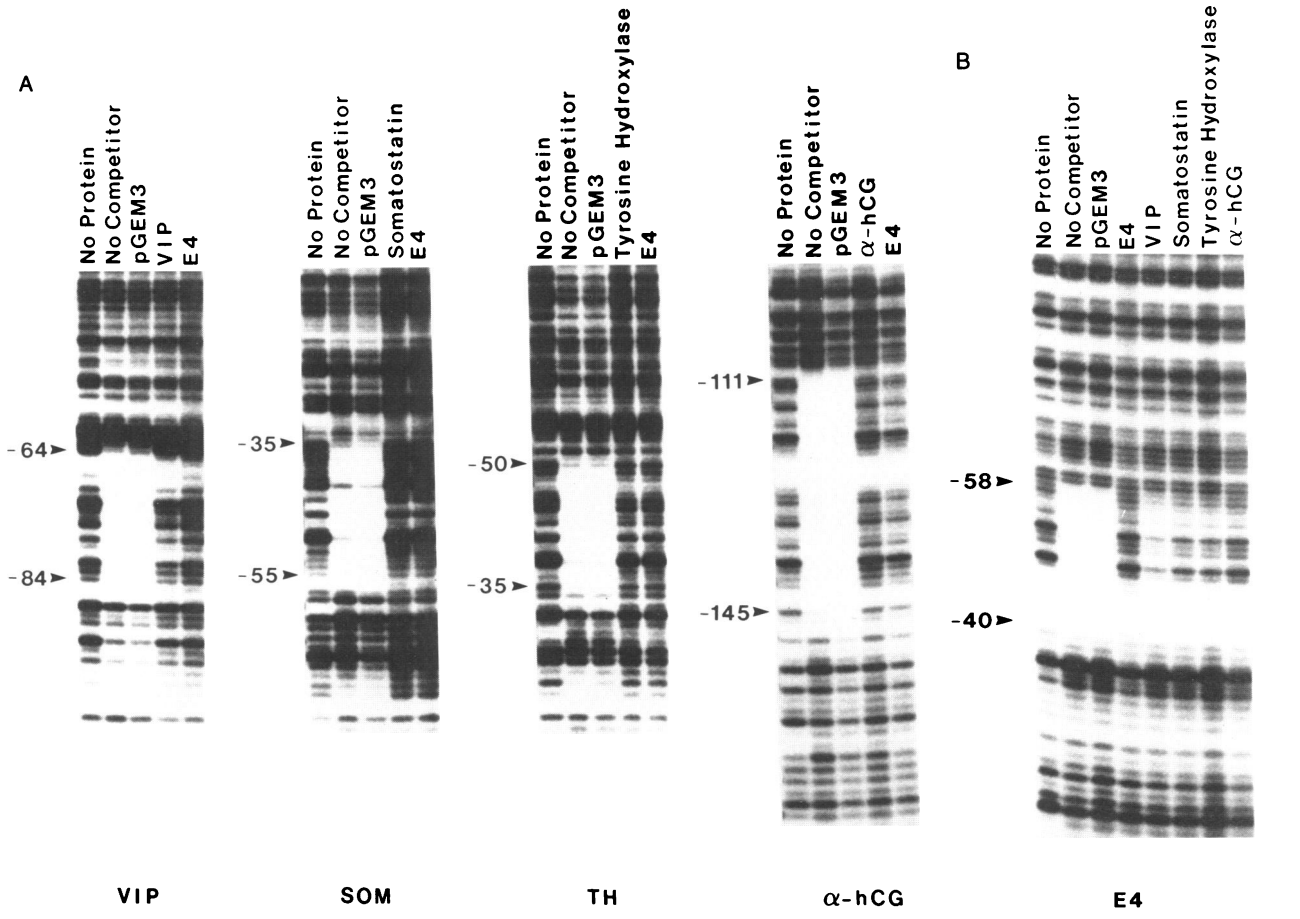

FIG. 1. A factor with related DNA-binding specificity interacts with viral ATF sites and cellular CREs. (*A*) DNase I footprint analysis of cAMP-inducible promoters. ^{32}P-end-labeled DNA probes from the VIP, somatostatin (SOM), tyrosine hydroxylase (TH), and α-hCG promoters were incubated in nuclear extract in the presence of a 100-fold molar excess of various unlabeled competitor DNAs followed by DNase I digestion. The ^{32}P-end-labeled DNA probes used are indicated below the autoradiograms. The unlabeled DNA competitor used is indicated above each lane. No protein: the ^{32}P-labeled DNA probes were incubated in buffer D instead of nuclear extract. The promoter regions protected from DNase I digestion are indicated on the left. (*B*) DNase I footprint analysis of the adenovirus E4 promoter, as in *A* except that a ^{32}P-end-labeled E4 DNA fragment was used as a probe.

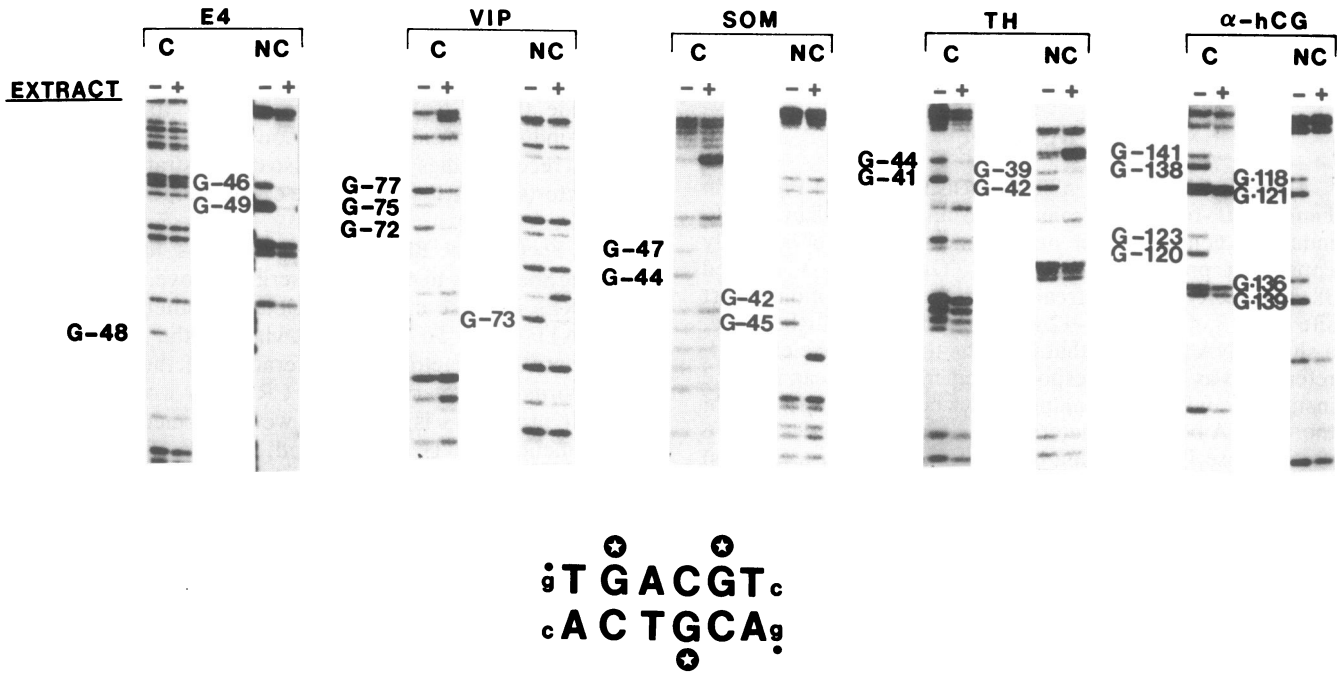

FIG. 2. Methylation protection analysis of cellular cAMP-inducible promoters. ^{32}P-end-labeled DNA probes from coding (C) or noncoding (NC) strands of the E4 (E4), α-hCG, tyrosine hydroxylase (TH), somatostatin (SOM), or VIP promoters were treated with dimethyl sulfate in the presence (+) or absence (−) of nuclear extract. The positions of the guanosine residues (relative to the transcriptional start site) protected from dimethyl sulfate methylation are indicated. The positions of the ATF sites within these promoters are listed in Table 1. Below the autoradiograms the guanosine residues within the consensus ATF site protected from methylation are marked with stars.

To define further the DNA-binding specificity of the factor(s) that interacts with the E4 and cellular cAMP-inducible promoters, we performed methylation protection experiments (Fig. 2). The ATF methylation protection pattern has been defined (2). Binding of ATF to the site 5′ TGACGT 3′ protects two guanosines on one strand and a third guanosine on the opposite strand from methylation by dimethyl sulfate (Fig. 2 *Lower*). In addition, if a guanosine is located on either strand adjacent to this core ATF sequence, it is also protected from methylation. In the CREs of the four cAMP-inducible promoters, these three guanosines in the core ATF sequence are protected (Fig. 2, see also Table 1). Furthermore, in all four cAMP-inducible promoters there is a guanosine adjacent to the core sequence that is also protected from methylation by dimethyl sulfate (Fig. 2). We conclude that the factor(s) that binds to the ATF sites in viral

Table 1. Derivation of an ATF consensus sequence

Promoter	Sequences														Positions
E4	A	A	A	T	G	A	C	G	T	A	A	C	G	G	−41 to −54
	A	A	G	T	G	A	C	G	A	T	T	T	G	A	−147 to −134
	A	A	G	T	G	A	C	G	T	A	A	C	G	T	−171 to −158
	G	G	G	T	G	A	C	G	T	A	G	G	T	T	−237 to −224
	T	T	G	T	G	A	C	G	T	G	G	C	G	C	−269 to −256
E3	C	T	G	T	G	A	C	G	A	A	A	G	C	C	−52 to −65
E2	A	G	A	T	G	A	C	G	T	A	G	T	T	T	−80 to −67
E1A	T	T	G	T	G	A	C	G	T	G	G	C	G	C	−438 to −425
	G	G	G	T	G	A	C	G	T	A	G	T	A	G	−406 to −393
	A	A	G	T	G	A	C	G	T	T	T	T	T	G	−332 to −319
α-hCG	C	C	A	T	G	A	C	G	T	C	A	A	T	T	−132 to −145
	C	C	A	T	G	A	C	G	T	C	A	A	T	T	−114 to −127
Som	C	T	C	T	G	A	C	G	T	C	A	G	C	C	−38 to −51
TH	G	G	C	T	G	A	C	G	T	C	A	A	A	G	−35 to −48
VIP	C	T	G	T	G	A	C	G	T	C	T	T	T	C	−79 to −66
Summary															
A	5	4	4	0	0	15	0	0	2	6	7	3	2	1	
G	3	4	9	0	15	0	0	15	0	2	5	3	5	4	
C	5	2	2	0	0	0	15	0	0	5	0	4	2	5	
T	2	5	0	15	0	0	0	0	13	2	3	5	6	5	
ATF consensus															
			G	T	G	A	C	G	T	(A/C)	(A/G)				

Sequences of 15 ATP-binding sites identified in this and our previous (1, 2) studies. The results for the two distal sites in the E4 promoter and the three sites in the E1a promoter are based on unpublished data from this laboratory. The sequences are aligned by the invariant motif 5′ TGACG 3′ within the ATF consensus sequence. Summary gives the frequency of the four bases at each position. Som, somatostatin; TH, tyrosine hydroxylase.

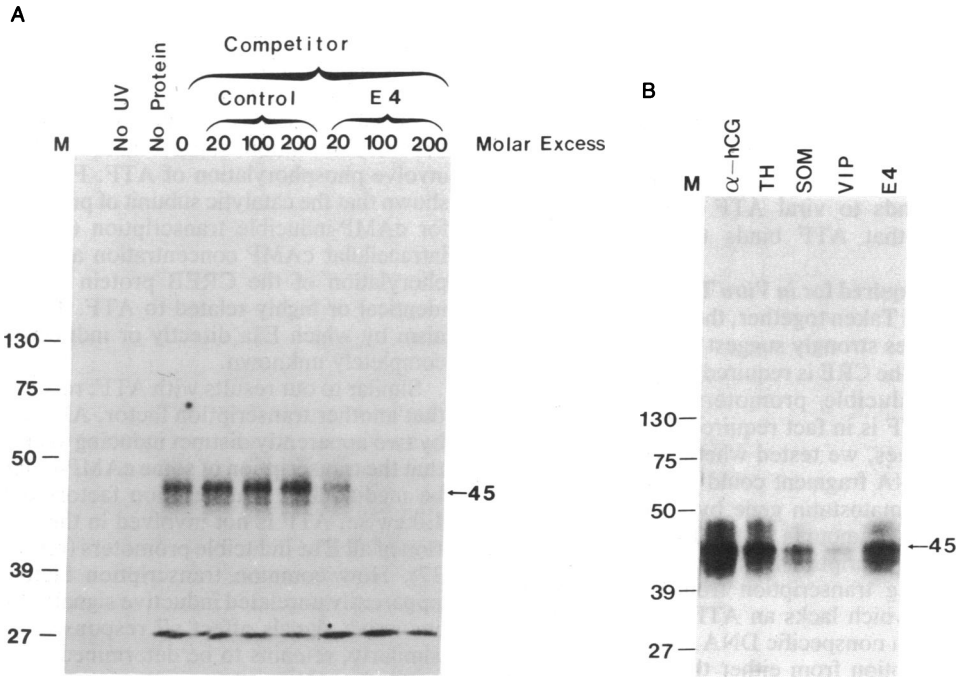

FIG. 3. Identification of the protein(s) that binds to the E4 ATF site and cellular CREs. (*A*) The E4 DNA probe was incubated in a heat-treated nuclear extract in the presence of increasing amounts of an oligonucleotide containing an ATF site (E4; E4 27-mer, see ref. 1) or an oligonucleotide lacking an ATF site (Control; βP; see ref. 2) followed by UV irradiation. The DNA competitor used and the molar excess of DNA competitor, relative to the ^{32}P-labeled DNA probe, are indicated above the lanes. No UV: no irradiation with UV light; No protein: ^{32}P-labeled DNA probe incubated in buffer D instead of nuclear extract. The positions of protein molecular mass markers are indicated on the left (in kDa). (*B*) cAMP-inducible promoters, same as in *A* except that the ^{32}P-labeled DNA probes were prepared from the E4 (E4), α-hCG, tyrosine hydroxylase (TH), somatostatin (SOM), or VIP promoters. The DNA probe used is indicated above each lane.

E1a-inducible promoters has an indistinguishable DNA-binding specificity from the factor(s) that binds to the CREs in cellular cAMP-inducible promoters.

A 45-kDa Polypeptide Binds to Viral ATF Sites and Cellular CREs. The experiments described above indicate that a protein(s) with identical DNA-binding specificity binds to viral ATF sites and cellular CREs, strongly suggesting that the same protein(s) interacts with both elements. To confirm that the same protein(s) binds to viral ATF sites and cellular CREs, we next directly identified the DNA-binding protein(s) by UV crosslinking (see, for example, ref. 8). Using the ATF site on the E4 promoter as a probe, we detected a major 45-kDa polypeptide (Fig. 3*A*). Upon longer autoradiographic exposures, several additional minor bands are revealed (Fig. 3*B*). These minor bands could be due to (*i*) different forms of ATF, (*ii*) proteolytic degradation products of ATF, (*iii*) various extents of nuclease digestion of the crosslinked [^{32}P]DNAs, or (*iv*) the existence of unrelated proteins with similar or identical DNA-binding specificity. Currently, we cannot distinguish between these possibilities. Detection of the major 45-kDa polypeptide is dependent on the addition of protein and irradiation with UV light (Fig. 3*A*). This major 45-kDa polypeptide (and the minor species) is bound specifically to the ATF site since it competes with added unlabeled oligonucleotide containing an ATF site but not by comparable amounts of an unrelated oligonucleotide (Fig. 3*A*).

Significantly, a major 45-kDa polypeptide identical in size to that observed by using the E4 promoter is also detected by [^{32}P]DNA probes containing the CREs of the four cAMP-inducible promoters (Fig. 3*B*). The variations in the intensities of the 45-kDa band among the different probes reflects the different affinities of the five probes for ATF and is consistent with the DNase I competition footprinting data (Fig. 1*B*; data not shown). Significantly, there are no differences in the pattern of crosslinked polypeptides(s) detected between the different probes, even after long autoradio-

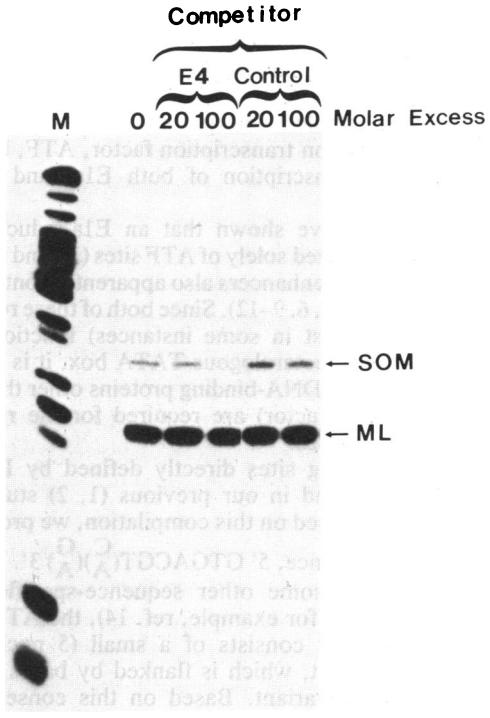

FIG. 4. ATF binding is required for *in vitro* transcription of the somatostatin promoter. pSOMRB and pMLPX (200 ng each) were cotranscribed *in vitro* under standard conditions in the presence of increasing amounts of an oligonucleotide containing an ATF site (E4) or an oligonucleotide lacking an ATF site (Control). The competitor DNAs used were the same as those in Fig. 3*A*. The competitor used and the molar excess of competitor are indicated above the lanes. Primer-extension products representing somatostatin (SOM) and adenovirus major late (ML) RNAs are indicated on the right. Lane M, ^{32}P-labeled DNA markers of *Msp* I-digested pBR322 DNA.

graphic exposures (Fig. 3*B*; data not shown). We note that a 43-kDa protein (referred to as CREB protein) has recently been purified from rat PC12 cells by sequence-specific DNA affinity chromatography and shown to interact with the CRE in the somatostatin promoter (13). It is highly likely that the CREB protein is similar or identical to ATF. Based on the indistinguishable DNA-binding specificity and size of the polypeptide(s) that binds to viral ATF sites and cellular CREs, we conclude that ATF binds to both sequence elements.

Binding of ATF Is Required for *in Vitro* Transcription of the Somatostatin Promoter. Taken together, the data presented in this and previous studies strongly suggest (but do not prove) that binding of ATF to the CRE is required for transcriptional activity of cAMP-inducible promoters. To determine whether binding of ATF is in fact required for transcription of cAMP-inducible genes, we tested whether addition of an ATF-containing E4 DNA fragment could inhibit the *in vitro* transcription of the somatostatin gene by sequestering free ATF (Fig. 4). Increasing amounts of the E4 DNA fragment significantly reduced transcription from the somatostatin promoter without affecting transcription from the adenovirus major late promoter, which lacks an ATF site. In contrast, equivalent amounts of a nonspecific DNA competitor did not affect *in vitro* transcription from either the somatostatin or adenovirus major late promoters. We conclude that binding of a factor to the ATF site is required for *in vitro* transcription of the cAMP-inducible somatostatin gene.

DISCUSSION

In this report, we demonstrate that the cellular transcription factor ATF interacts with the regulatory elements in both E1a- and cAMP-inducible promoters. Furthermore, we could not detect additional proteins that interacted with cAMP-inducible promoters but not E1a-inducible promoters, or vice versa. Thus, the differential regulation of these promoters *in vivo* is unlikely to result from selective binding of distinct transcription factors. The simplest interpretation of our combined results is that a common transcription factor, ATF, binds to and mediates the transcription of both E1a- and cAMP-inducible promoters.

Previous studies have shown that an E1a-inducible enhancer can be constructed solely of ATF sites (1), and likewise some cAMP-inducible enhancers also apparently contain only multiple ATF sites (3, 4, 6, 9–12). Since both of these regulated enhancers can (at least in some instances) function when placed upstream of a heterologous TATA box, it is unlikely that sequence-specific DNA-binding proteins other than ATF (and the TATA box factor) are required for the regulated transcriptional responses.

The 15 ATF-binding sites directly defined by DNase I footprinting in this and in our previous (1, 2) studies are aligned in Table 1. Based on this compilation, we propose an ATF consensus sequence, 5′ GTGACGT$\binom{C}{A}\binom{G}{A}$ 3′. Like the recognition sites of some other sequence-specific DNA-binding proteins (see, for example, ref. 14), the ATF recognition site apparently consists of a small (5 nucleotides) invariant core element, which is flanked by bases that are preferred but not invariant. Based on this consensus sequence, we have identified ATF sites within promoters of a variety of cellular genes whose transcriptional regulation differs significantly (unpublished observations).

Understanding the biochemical mechanism by which ATF is regulated by cAMP and E1a *in vivo* is of major importance. Several observations suggest that cAMP induction *in vivo* may involve phosphorylation of ATF. First, it has been recently shown that the catalytic subunit of protein kinase A is required for cAMP-inducible transcription (15). Second, raising the intracellular cAMP concentration appears to increase phosphorylation of the CREB protein (13), which is probably identical or highly related to ATF. The biochemical mechanism by which E1a directly or indirectly modulates ATF is completely unknown.

Similar to our results with ATF, recent studies have shown that another transcription factor, AP2, can also be regulated by two apparently distinct inducing agents (16). We also note that the transcription of some cAMP-inducible promoters can be mediated by transcription factors other than ATF (16). Likewise, ATF is not involved in the transcriptional regulation of all E1a-inducible promoters (ref. 2; for review, see ref. 17). How common transcription factors are regulated by apparently unrelated inductive signals, and whether common inductive signals affect all responsive transcription factors similarly, remains to be determined.

We gratefully acknowledge M. McCaffrey for superb technical assistance. We thank R. Goodman, D. Chikaraishi, and I. Boime for providing DNA clones. This work was supported by grants from the National Institutes of Health and The Chicago Community Trust/Searle Scholars Program to M.R.G.

1. Lee, K. A. W. & Green, M. R. (1987) *EMBO J.* **6**, 1345–1353.
2. Lee, K. A. W., Hai, T. Y., SivaRaman, L., Thimmappaya, B., Hurst, H. C., Jones, N. C. & Green, M. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8355–8359.
3. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G. & Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6682–6686.
4. Lewis, E. J., Harrington, C. A. & Chikaraishi, D. M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3550–3554.
5. Darnell, R. B. & Boime, I. (1985) *Mol. Cell. Biol.* **5**, 3157–3167.
6. Tsukada, T., Fink, J. S., Mandel, G. & Goodman, R. H. (1987) *J. Biol. Chem.* **262**, 8743–8747.
7. Lillie, J., Green, M. & Green, M. R. (1986) *Cell* **46**, 1043–1051.
8. Chodosh, L. A., Carthew, R. W. & Sharp, P. A. (1986) *Mol. Cell. Biol.* **6**, 4723–4733.
9. Short, J. M., Wynshaw-Boris, A., Short, H. P. & Hanson, R. W. (1986) *J. Biol. Chem.* **261**, 9721–9726.
10. Deutsch, P. J., Jameson, J. L. & Habener, J. F. (1987) *J. Biol. Chem.* **262**, 12169–12174.
11. Delegeane, A. M., Ferland, L. H. & Mellon, P. L. (1987) *Mol. Cell. Biol.* **7**, 3994–4002.
12. Silver, B. J., Bokar, J. A., Virgin, J. B., Vallen, E. A., Milsted, A. & Nilson, J. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2198–2202.
13. Montminy, M. R. & Bilezsikjian, L. M. (1987) *Nature (London)* **328**, 175–178.
14. Dorn, A., Bollekens, J., Staub, A., Benoist, C. & Mathis, D. (1987) *Cell* **50**, 863–872.
15. Grove, J. R., Price, D. J., Goodman, H. M. & Avruch, J. (1987) *Science* **238**, 530–533.
16. Imagawa, M., Chiu, R. & Karin, M. (1987) *Cell* **51**, 251–260.
17. Berk, A. J. (1986) *Annu. Rev. Genet.* **20**, 45–79.