Nucleic Acids Research, Vol. 19, No. 17 4601–4609

Identification and functional characterisation of the cellular activating transcription factor 43 (ATF-43) protein

Helen C.Hurst, Nicholas F.Totty[1] and Nicholas C.Jones[2]
Gene Transcription Laboratory, ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 0HS, [1]Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT and [2]Gene Regulation Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

Received July 10, 1991; Revised and Accepted August 13, 1991

ABSTRACT

The promoter motif CGTCA binds multiple cellular factors that mediate a variety of inducible events, including positive responses to raised cellular levels of cAMP and to the Adenovirus E1a protein. To date, at least ten mammalian cDNA clones have been isolated that encode distinct proteins capable of binding to this motif. However, in most cases the precise stimuli that may regulate these different factors have yet to be determined. We have previously shown that the abundant Hela protein ATF-43 forms a complex *in vivo* with the cyclic AMP response element binding protein (CREB). In this report we definitively show that ATF-43 is the product of the two published cDNA clones, ATF1 and TREB 36. We confirm that ATF1 efficiently heterodimerises with CREB and demonstrate that even though ATF1 and CREB homodimers, as well as the ATF1/CREB heterodimer efficiently bind to the CGTCA motif, the resulting DNA-protein complexes have significantly different stabilities. A region outside the DNA binding domain of ATF1 contributes to the instability of its interaction with DNA. We further show that despite ATF1's homology to CREB, it responds poorly to activation by protein kinase A. In light of our finding that in Hela cells the majority of CREB protein is heterodimerised with ATF1, we speculate on the functional significance of such heterodimers.

INTRODUCTION

The DNA sequence motif CGTCA has been identified as a functional promoter element by several groups working on a variety of agents known to stimulate gene expression. This sequence was originally identified as the cAMP responsive element (CRE) within the promoter of many cAMP-inducible genes (48; for review see 56). Subsequently, identical, or very closely related elements were identified in multiple Adenovirus early gene promoters which are transcriptionally activated by the viral E1A protein (24,34,35). In some cases the elements (termed ATF sites) have been shown to be crucial to the E1A response (33,34). Multiple copies of the motif are also found in the HTLV1 LTR and have been implicated as mediating the positive transcriptional response of this LTR to the HTLV $p40^{tax}$ protein (12,28,46). In addition, this motif overlaps the X-box sequence, a highly conserved promoter motif functionally required for constitutive expression of the murine MHC class II genes (26,38,53). All of these genes are transcribed by RNA polymerase II, but more recent studies have shown that this element may also be required for efficient expression of certain genes transcribed by RNA polymerase III (6,22).

The ATF/CRE site is recognised by a large family of transcription factors. To date at least ten different mammalian cDNA clones have been described that encode distinct members of this family (16,19,11,13,21,23,26,38,42,63). All of these sequences have a basic DNA binding domain followed by a leucine zipper dimerisation domain (collectively termed the bZIP domain; 61) analagous to similar features found in the *fos* and *jun* families of transcription factors (31). However, even within the bZIP domain the ATF family of proteins show (with one or two exceptions) surprisingly little amino acid sequence similarity to one another. Despite this, the different family members all bind the CGTCA motif as homodimers (5,9,19), and in addition certain members can dimerise with each other (19) or with members of the *jun* and *fos* families to form specific heterodimers (4,17,23,26,40,53). The overall consequences of the diversity afforded by such a large family of binding proteins are not yet fully understood. It is possible that different homo- or heterodimers recognise distinct cellular target sequences dictated either by subtle sequence differences within the consensus motif, differences in the sequences that flank the core motif or, possible differences in the nature of other factors that may bind in the vicinity of the motif. Another possibility is that the different regulatory responses that occur through the CRE/ATF sites are mediated by different homo- or heterodimer complexes. The ability to respond to a particular regulatory signal would thus depend upon the availability of a particular complex within the cell.

In order to understand regulation through the ATF/CRE binding site it is important to investigate the activity of each individual family member as well as how this activity is modulated by dimerisation with other members of the family. It is also important to define the array of different dimers that exist in any one particular cell type in order to correlate the presence of certain dimers with the regulatory responses observed

in that particular cell. One specific member, the CREB (cAMP responsive element binding protein) protein, has been investigated in detail. Its activity crucially depends upon phosphorylation at serine-133 by cAMP dependent protein kinase A (15). In contrast another member, the CRE-BP1 (ATF2,CREB2,TREB7) protein (42,39,63), is insensitive to cAMP activation despite being a substrate for kinase A, but does mediate the stimulatory activity of the E1A protein (39,10,41). In contrast to these studies, the function of the majority of these proteins is completely unknown. Moreover, a number of laboratories have described the purification of CGTCA binding proteins (2,8,18,24,27,28, 45,46,47,52,55,62). In most cases however, the nature of these proteins has yet to be reconciled with the described cDNA clones.

In this paper we show that the major Hela protein purified by binding to the CGTCA sequence (24) and formerly called ATF-43 (18,25) is encoded by a cDNA clone that has been isolated by two different laboratories (and hence given two different names: ATF1 (19) and TREB36 (63)). This protein, which we here call ATF1, is highly related to the CREB factor with which it can heterodimerise. We therefore examined the ability of ATF1 to mediate a transcriptional response to raised cAMP levels and also considered its potential role in modulating CREB activity within cells.

MATERIALS AND METHODS

Purification and sequencing of ATF-43

ATF-43 was purified from suspension cultures of Hela cells as described previously (24) with the modifications detailed in reference (25) with two rounds of DNA affinity chromatography using the ATF site at −50 in the adenovirus E4 promoter. This produced 15−20μg of highly purified ATF-43 (as estimated by gel retardation assay, see below) in 2mls of dialysis buffer (20mM HEPES [N-2-hydroxyethyl piparazine-N'-2-ethane sulfonic acid]-KOH[pH8.0], 20% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.005% LDAO; 0.5mM phenylmethylsulfonyl fluoride [PMSF]; 1mM dithiothreitol [DTT]). This sample was concentrated to 75μl using a Centricon-10 (Amicon) spin microconcentrator with a 10,000 molecular weight cut off membrane. Dilution of 1μl of this sample for binding activity estimation showed that approximately 15μg of ATF-43 activity remained. An equal volume of 2×SDS-PAGE sample buffer was added to the whole sample which was boiled and electrophoresed on a 9.5% SDS-PAGE, additionally loaded with prestained molecular weight markers (Life Technologies). Proteins were transferred to nitrocellulose in Tris-glycine buffer (3) containing 0.005% SDS for 90 minutes at 100V (0.35A) at 4°C. The nitrocellulose filter and a second backing filter were immersed in 1% acetic acid containing 0.1% Ponceau S dye for 1 minute. Excess dye was removed using 1% acetic acid thus revealing protein bands. A broad, intensly staining region of 35−40kd was detected on the filter with very much fainter species above 46kd. The backing filter showed no staining. The 35−40kd region was excised and prepared for trypsin digestion as described by Abersold et al (1). Briefly, the filter was destained in 200mM NaOH and then blocked for 30 minutes at 37°C in 0.5% PVP-40 in 100mM acetic acid. Excess PVP-40 was removed by extensive washing in water. The damp filter was then cut up into small pieces and digested with 1μg trypsin (sequencing grade; Boehringer) in 100mM Tris pH 8.2 for 16 hours at 37°C. Released peptides were loaded onto an HPLC column (1.0×100mm C18; Applied Biosystems) in buffer A (0.08% trifluoroacetic acid/H_2O/1% acetonitrile) and run at 50μl per minute at 35°C with a 0−50% gradient of buffer B (0.1% trifluoroacetic acid/H_2O/90% acetonitrile) over 45 minutes. Potential peptide-containing peaks were sequenced using a modified Applied Biosystems model 477A pulse liquid sequencer, using rapid cycle chemistry and analysis cycles (60).

ATF1 clones

The partial ATF1 cDNA clone in the pGEM3 EcoR1 site (19) was the kind gift of Dr.T.Hai. This ATF1 sequence starts with amino acid 39 Glu with respect to the full length sequence derived by Yoshimura et al (63). The 5' end of the pGEM3 clone was constructed according to this sequence using six oligonucleotides annealled to give three double stranded, sticky-ended DNA fragments with a Sal 1 site immediately prior to the first ATG and the 3' most fragment terminating in the BamH1 site at amino acid 56. The partial clone was digested with Sal 1 (in the pGEM3 polylinker) and BamH1 and ligated with the three sticky-ended fragments to generate a complete ATF1 clone in pGEM3. The construct was checked by sequencing of collapsed supercoil templates (3,43). This clone was transcribed on EcoR1 linearised template using SP6 RNA polymerase. Delta-ATF1 was made by filling in the EcoR1 ends of the partial ATF1 fragment and cloning into the vector T7βSal (51). This vector (a gift from R.Treisman) contains the bacteriophage T7 promoter upstream of the β globin 5' untranslated sequence which terminates in an Nco1 site which incorporates the first AUG. Delta-ATF1 protein produced from the T7 transcript thus starts with the sequence M K E F R E, with the methionine originating from T7βSal, the K E F R sequence from the EcoR1 linker on the partial ATF1 clone and the Glu is the first ATF1 amino acid encoded by this clone (19). DLA was made using PCR technology. The first amino acid is ATG (176) while amino acid 177 has been changed from glutamine (CAA) to glutamate (GAA) to create a 5' Nco1 site for cloning. The remaining sequence is identical to the published ATF1 sequence (19,63). The amplified piece was sequenced before subcloning into T7βSal. Protein produced from the T7 transcript represents the C-terminal portion of ATF1, but includes the entire basic domain (amino acid 215 to 240;63) and leucine zipper (amino acid 241 to 271;63).

Other clones

CREB was transcribed *in vitro* from the clone CREB1 in T7βSal described by Benbrook and Jones (4). This form of CREB does not possess the alpha-peptide sequence, therefore, the binding experiments were repeated using the rat alpha-CREB clone (15) subcloned into Bluescript plasmid and transcribed with T7 polymerase (3,43). No differences in binding or heterodimerisation patterns were found however (unpublished observations). The CREB truncation DL1 contains amino acids 236−326 and is also cloned into T7βSal (4). For the GAL4 fusion constructs, CREB or ATF1 cDNA sequences were cloned into the vector MLVGAL (1−147) deltaStu, kindly provided by S.John and R.Treisman. Briefly, this vector contains the transcriptional enhancer element of the MLV LTR, nucleotide −127 to +65 of the human β-globin gene and GAL4 cDNA sequences encoding amino acid residues 1−147. The entire CREB cDNA sequence together with 24 5' linker nucleotides, or the entire ATF1 cDNA sequences were cloned downstream of the GAL4 sequences. The inserts are followed by 3' untranslated sequences of the β-globin gene. GAL4 CREB deltaC was constructed by cloning an oligonucleotide containing stop

codons in all three frames into the Bgl II restriction site of CREB. GAL4-ATFdeltaC was similarily constructed by the cloning of an oligonucleotide into the Xho I site of ATF1. The reporter plasmid G5E4CAT was derived from G5E1BCAT (39) by replacing the E1B TATA containing fragment with an oligonucleotide containing the TATA element and flanking sequences of the Adenovirus E4 promoter. The plasmid MtC encoding the alpha isoform of the catalytic subunit of mouse protein kinase A has been previously described (44).

In vitro transcription and translation

In vitro transcription of linearized DNA templates, using T7 or SP6 RNA polymerase as appropriate (3,43), was performed using reagents supplied as a kit (Stratagene). The resulting capped transcripts were translated in nuclease treated rabbit reticulocyte lysate (Promega) including unlabelled or ^{35}S-labelled methionine. Incubations were usually for 60 minutes at 30°C, but some short labelling reactions (15 minutes) were also performed (see Fig. 1B). After translation, an equal volume of dialysis buffer was added to unlabelled samples for analysis by gel retardation assay. Yields of translated protein were such that each sample could be diluted such that any endogenous ATF/CREB binding activity present in concentrated reticulocyte extract (4) was not observed. Samples containing ^{35}S-labelled protein were analysed by SDS-PAGE.

Gel retardation assay and heterodimer formation

Two double stranded oligonucleotide probes were used in these assays representing either the ATF site at −50 within the adenovirus E4 promoter (E4ATF) or the CREB site at −45 within the rat somatostatin promoter (Som) (4). Probes were end labelled as described previously (24). Gel retardation assays using purified or *in vitro* made proteins in the presence or absense of crude antisera were performed as detailed in Hurst et al. (25). Appropriate amounts of protein were determined empirically and protein dilutions were performed in standard gel retardation buffer (25) supplemented with 20×Denhardts (43) (GRB+D). Crude Hela nuclear extract was prepared as described previously (24). Heterodimers were generated by incubating appropriate amounts of *in vitro* made proteins in GRB+D for 5 minutes at 50°C to dissociate homodimers, followed by 10 minutes at room temperature to allow reassociation. The heterodimer combination of ATF1 and CREB1 that most closely resembled crude Hela extract was determined empirically and was found to require twice as much ATF as CREB as measured by binding capacity of the individual proteins. The analyses of protein/DNA complex stability were performed largely as described by Rooney et al (57), except that the 8% gel (25) was run at 110V while samples were being loaded, and thereafter at 200V.

In vitro labelling with protein kinase A and immunoprecipitations

Crude Hela nuclear extracts were heat treated at 65°C for 10 minutes and then cleared. 5−10μg of protein was labelled with ^{32}P-gamma-ATP and protein kinase A as detailed previously (25). The labelled samples were then diluted in dialysis buffer and incubated with crude preimmune or antiserum for 2 hours at 4°C. Immunoprecipitates were collected on protein A-sepharose beads and washed as previously described (25). The beads were resuspended in SDS-PAGE sample buffer, boiled and loaded directly onto gels.

Phosphatase treatment of ^{35}S-labelled ATF1

Samples of ^{35}S-labelled ATF1 synthesised *in vitro* were incubated in 20μl reactions containing 50mM Tris-Cl,pH9.5; 50mM NaCl; 5mM $MgCl_2$ and either 3 units (low levels) or 24 units (high levels) of calf intestinal alkaline phosphatase (Boehringer) for 60 minutes at 37°C. An equal volume of 2×sample buffer was then added and the samples boiled and electrophoresed on SDS-PAGE.

Cells and transfection assays

JEG3 cells were maintained in DMEM medium supplemented with 10% foetal calf serum. Cells were transfected using the calcium phosphate precipitation method (3) and each experiment was repeated at least three times. Cell extracts were prepared and CAT activity assayed as described elsewhere (43).

RESULTS

Protein sequence analysis and identification of ATF-43

We and others have previously described the purification using DNA affinity chromatography of Hela cell nuclear factors which specifically bind to an ATF/CREB site. The material purified by this laboratory migrated as a broad band of 35−40 kd (25) and has previously been termed ATF-43 and used to raise antibodies in mice (25). Extensive treatment of the purified material with calf intestinal phosphatase resulted in a single band on Western analysis, apparently equivalent to the fastest mobility band of the native material (data not shown; but see 25). We were therefore convinced that ATF-43 represented a single polypeptide that could be multiply phosphorylated. Using the ATF-43 antibodies we have also shown that ATF-43 forms a complex with the 47kd CREB protein in a variety of cell types (25). Since this complex may modulate CREB activity it was important to determine the identity of ATF-43.

Initial attempts to sequence the intact ATF-43 protein indicated that the N-terminus was blocked, thus necessitating a peptide sequencing approach. Approximately 15μg of ATF-43 was purified from 50L of Hela cells as described previously (24,25). This material was concentrated and the entire sample electrophoresed on SDS-PAGE and proteins transferred to nitrocellulose and stained with Ponceau S (see Materials and Methods). The predominantly staining material was in a broad smear in the 35−40 kd molecular weight range. This region of the membrane was excised and prepared for trypsin digestion (see Materials and Methods) thus allowing release of peptides into the digestion buffer which was then subject to HPLC analysis (see Materials and Methods). Peak fractions from the HPLC showed low yield recovery of peptides, presumably due to the number of different phosphoforms of some peptides. However, two peaks (NT1 and NT2) contained sufficient material for peptide sequence to be determined:

NT1	A H G I L A
NT2	T T ? ? A T S L P Q T V V M T S

The NT2 sample was contaminated with another short peptide which made the assignment of the first four residues quite difficult. In both cases the amount of peptide was fairly low and it was not clear if we were sequencing to the C-terminus of the peptide. Since antibody studies had already demonstrated that ATF-43 and CREB were distinct (25), it was not surprising that neither the NT1 nor NT2 sequence was found within the predicted

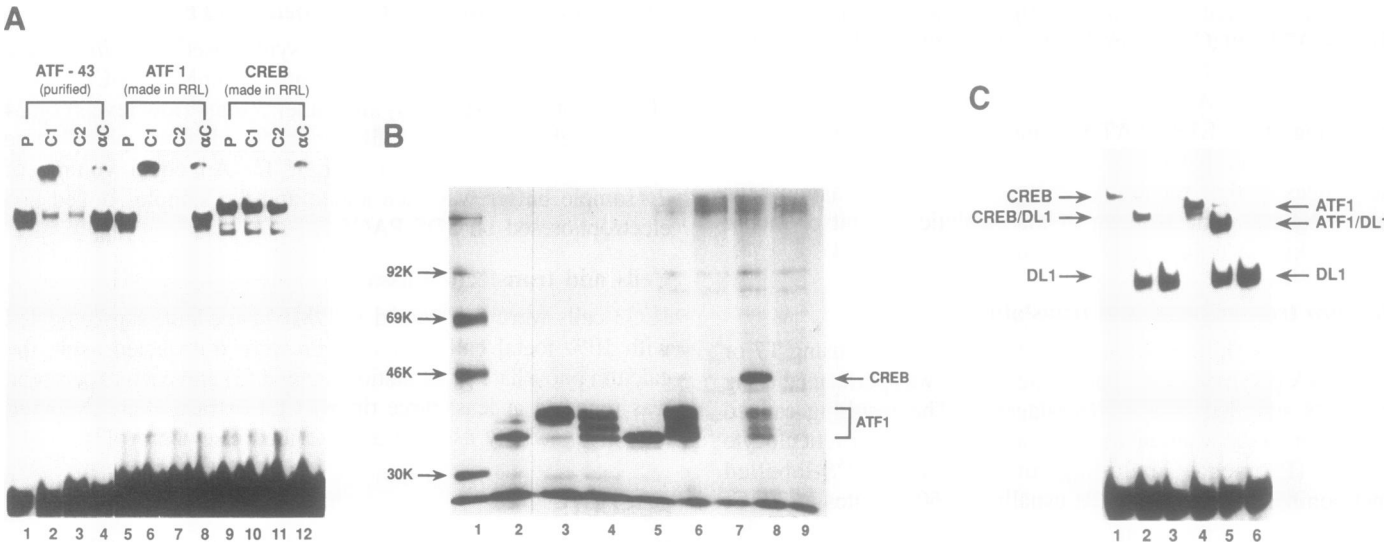

Figure 1. ATF1 has the same properties as ATF-43. **(A)** Gel retardation analysis of purified ATF-43 (lanes 1–4) and *in vitro* made ATF1 (lanes 5–8) and CREB (lanes 9–12). Each track represents a 25μl incubation containing 0.1ng of E4ATF probe with protein (see Materials and Methods for details) and including 3μl of crude antiserum as indicated: P (preimmune); C1 and C2 (anti ATF-43 sera; 25) and alphaC (CREB antipeptide antibody; 25). Lanes 1–12 were all run on one gel, but lanes 1–4 represent a shorter exposure of the gel than lanes 5–12. **(B)** SDS-PAGE of ATF1 and ATF-43. ^{35}S-labelled ATF1 (lanes 2–5) has been made *in vitro* and samples withdrawn after 15 minutes (lane 2) or 60 minutes (lane 3) of incubation. Further aliquots of the 60 minute labelling were treated with low (lane 4) or high levels (lane 5) of alkaline phosphatase. Approximately 10μg of heat-treated crude Hela nuclear extract was labelled with ^{32}P using protein kinase A catalytic subunit (25) and then divided to be immunopreciptiated with ATF-43 antiserum C2 (lane 6), CREB peptide antiserum (lane 8) or appropriate preimmune sera (lanes 7 and 9). **(C)** ATF1/CREB heterodimers. *In vitro* made CREB (lane 1), ATF1 (lane 4) and DL1 (lanes 3 and 6) are shown binding to 0.1ng of E4ATF probe. Further incubations containing both DL1 and CREB (lane 2) and DL1 with ATF1 (lane 5) show the appearance of novel complexes representing heterodimers as indicated by arrows.

sequence of CREB (16,21). NT1 and NT2 were then compared with amino acid sequences derived from other cloned bZIP transcription factors obtained by screening expression libraries with ATF/CREB binding sites. Both our peptides were found within such a cDNA clone, previously isolated by two laboratories and called ATF1 (19) and TREB36 (63). For simplicity we will hereafter refer to ATF-43 as ATF1. Within the predicted ATF1 sequence, NT1 and NT2 are preceeded by a lysine and an arginine residue respectively, showing them to be genuine tryptic peptides. NT1 is probably the complete sequence of a short peptide, whereas NT2 represents only the N-terminal sequence of a longer peptide.

As has been noted previously (19,63), although ATF1 is clearly distinct from CREB these two factors have 75% amino acid identity; the degree of homology being most marked in the basic domain and leucine zipper regions. However, both NT1 (amino acid 54–59;63) and NT2 (amino acid 543–558;63) lie within domains of ATF1 with little or no homology to CREB.

Confirmation that ATF-43 is ATF1

A full length ATF1 cDNA clone was constructed as described in Materials and Methods. The cDNA sequence was transcribed *in vitro* and the resulting RNA translated in rabbit reticulocyte lysate to generate recombinant protein which could be analysed with our specific ATF-43 antibodies (25). Purified ATF-43, together with *in vitro* synthesised ATF1 and CREB proteins were compared in a DNA binding assay using a ^{32}P-labelled oligonucleotide probe containing the E4 ATF binding site. As shown in Figure 1A, the complex generated with CREB migrated more slowly than that obtained with either ATF-43 or ATF1 and was not affected by coincubation with either preimmune serum (P) or the ATF-43 antibodies (C1 and C2). However, as has been shown previously (25), the mobility of this complex was supershifted by addition of CREB peptide antiserum (alpha-C) raised against the C-terminal ten amino acids. The complexes formed by both ATF-43 and ATF1 migrated very similarly and while both were unaffected by preimmune or CREB sera they were similarly affected by the addition of ATF-43 antisera; C1 supershifted the complexes (Figure 1A, lanes 2 and 6) while C2 reduced or abolished complex formation (Figure 1A, lanes 3 and 7). This pattern of antibody interaction with protein/DNA complexes established that ATF-43 is identical to ATF1 and distinct from the highly homologous CREB protein.

We also examined the *in vitro* synthesised ATF1 protein by SDS-PAGE. Figure 1B (lanes 2–7) shows samples of ^{35}S-labelled ATF1 synthesised in rabbit reticulocyte lysate. Protein produced within 15 minutes of the start of incubation (lane 2) ran as a single band of 35kd, but as incubation proceeded, this material was chased into a slower migrating band of 40kd (lane 3). This would be consistant with post translational modification of ATF1 taking place in the lysate and altering the mobility of the protein. To examine this possibility, samples of the 40kd material were treated with calf intestinal phosphatase to remove phosphate groups. Low levels of phosphatase produced a 35kd band plus at least two intermediate bands (lane 4), while extensive phosphatase treatment reduced the whole sample to a 35kd band (lane 5). The migration of these species was also compared to a sample of ATF-43 (lane 6) which had been immunoprecipitated from crude Hela nuclear extract labelled *in vitro* with ^{32}P by protein kinase A (see Materials and Methods). The range of mobilities and number of intermediate species of both ATF1 and ATF-43 were clearly comparable, whereas ^{32}P-labelled CREB immunoprecipitated from Hela extract (lane 8) was clearly distinct.

We have previously shown that ATF-43 forms a complex in nuclear extracts with CREB (25, Fig. 1B lane 8). Having now identified ATF-43 as the product of the ATF1 cDNA clone, it

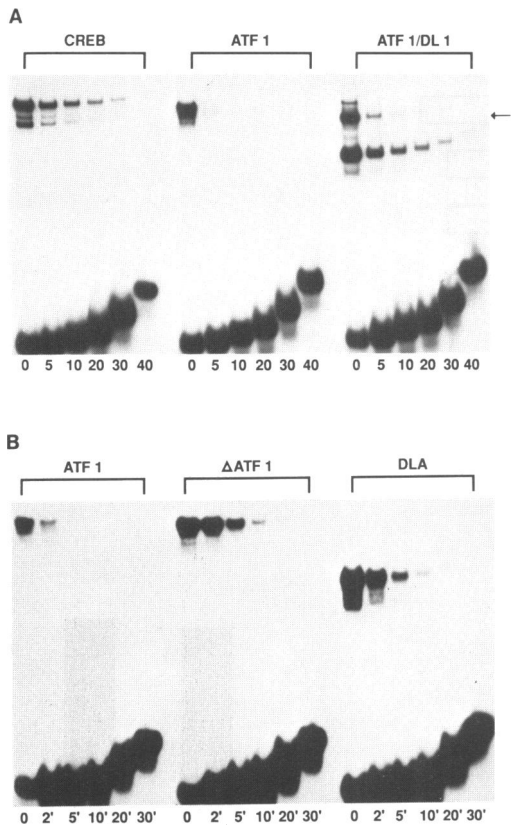

Figure 2. Stability of ATF1/DNA complexes. **(A)** Aliquots of CREB, ATF1, and ATF1/DL1 heterodimer were allowed to bind to the SOM probe to equilibrium (ATFE4 gives the same result, data not shown). An aliquot of each incubation was loaded onto a native 8% gel (lanes 0) and the gel run started at 110V. A 500 fold excess of unlabelled oligo probe was added to each incubation and samples withdrawn after 5, 10, 20, 30 and 40 minutes and loaded onto the running gel as indicated. The migration of the ATF1/DL1 complex is shown with an arrow; the faster moving complex is a homodimer of DL1 while a small amount of ATF1 homodimer can be seen above the heteromeric complex in lane 0. **(B)** Stability of complex formation between ATFE4 probe and full length ATF1 protein and two truncated forms: delta-ATF1 and DLA (see Materials and Methods). The experiment was performed essentially as in (A) above with binding incubations before (lanes 0) and 2, 5, 10, 20 and 30 minutes after addition of 500 fold excess of cold competitor, as indicated.

follows that the ATF-43/CREB complex seen *in vivo* represents a heterodimer between ATF1 and CREB. To directly show heterodimerisation between these two proteins, we used *in vitro* synthesised CREB, ATF1 and DL1, a truncated form of the CREB protein (4). As shown in the gel retardation assay in Figure 1C (lanes 3 and 6), the DL1 protein produced a faster migrating complex than either full length CREB (lane 1) or ATF1 (lane 4). If either of the full length proteins and DL1 were mixed after synthesis (see Materials and Methods) DNA complexes with intermediate electrophoretic mobility were formed (Figure 1C, lanes 2 and 5), indicating that heterodimerisation between ATF1 and CREB could indeed occur *in vitro*. The experiment was also performed using a truncated version of ATF1 with similar results (data not shown).

In summary therefore, the immunological evidence, phosphatase sensitivity and gel retardation analyses all confirm that the ATF-43 protein purified from Hela cells is the product of the ATF1 gene.

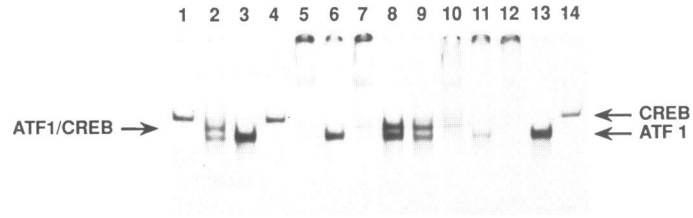

Figure 3. ATF1 and CREB complexes in crude Hela nuclear extract. Complexes formed by crude Hela nuclear extract (lanes 2 and 9–12) are compared with those formed by *in vitro* made CREB (lanes 1, 3 and 14) and ATF1 (lanes 3 and 13) and the ATF/CREB heterodimer (lanes 5–8) which are all indicated with arrows. Some incubations additionally included 1μl of crude antisera as follows: Preimmune (lanes 8 and 9); ATF-43 antiserum C1 (lanes 7 and 10); antiCREB (lanes 6 and 11); C1 plus anti CREB (lanes 5 and 12). The experiment shown here was performed using the E4ATF probe, but the SOM probe gives the same result (data not shown).

ATF1/DNA complexes have a very short half life

It has been shown repeatedly that ATF- and CREB-like proteins are able to bind very similar sites *in vitro* (8,20,24,58). However, these binding sites are not always functionally interchangeable: for example not all CGTCA motifs can mediate a transcriptional response to both E1a or $p40^{tax}$ and increased cAMP levels (29,33,49,50,57). In most studies however, the proteins are assayed with respect to their ability to recognise a particular binding site: there is no dynamic information indicating the stability of the DNA protein complex. In a situation where a number of different proteins can recognise one binding site it is important to consider the relative stability of the different protein-DNA complexes. We have previously noted that CREB and ATF1 apparently have differing abilities to interact stably with DNA (25). In order to investigate this more thoroughly, we compared the stability of ATF1 and CREB complexes formed on the CRE site within the somatostatin promoter. Figure 2A shows the results of an experiment where *in vitro* synthesised CREB, ATF1 and the heterodimer ATF1/DL1 were allowed to bind to DNA (lanes labelled O) before addition of a 500-fold excess of unlabelled DNA binding site. Aliquots of each binding reaction were subsequently loaded onto the running gel at the times indicated. The amount of labelled complex remaining at each time point allows an estimation of the half-life of each complex. As can be seen, the ATF complex was very unstable with none remaining after 5 minutes of exposure to the competitor (other experiments showed loss even after 2 minutes; see Figure 2B). The ATF1/DL1 complex also appeared to have a fairly rapid dissociation rate although it was more stable than the ATF1 homodimer. In contrast, significant CREB/DNA complex could still be detected 20 or even 30 minutes after addition of cold competitor.

ATF1 and CREB are highly homologous especially throughout the bZIP domain (19,63). The disparity in the stability of their interactions with DNA is therefore quite surprising. Apart from two or three short internal regions, the sequences of these two proteins only diverge significantly at their N-termini. The reduced stability shown by ATF1 may therefore be due to sequences within this region. We constructed two truncated clones of ATF1 (delta ATF1 and DLA; see Materials and Methods) both of which lacked the N-terminal region. Homodimers of these products were examined with respect to their protein-DNA dissociation rates. As can be seen in Figure 2B, deletion of only 38 amino acids

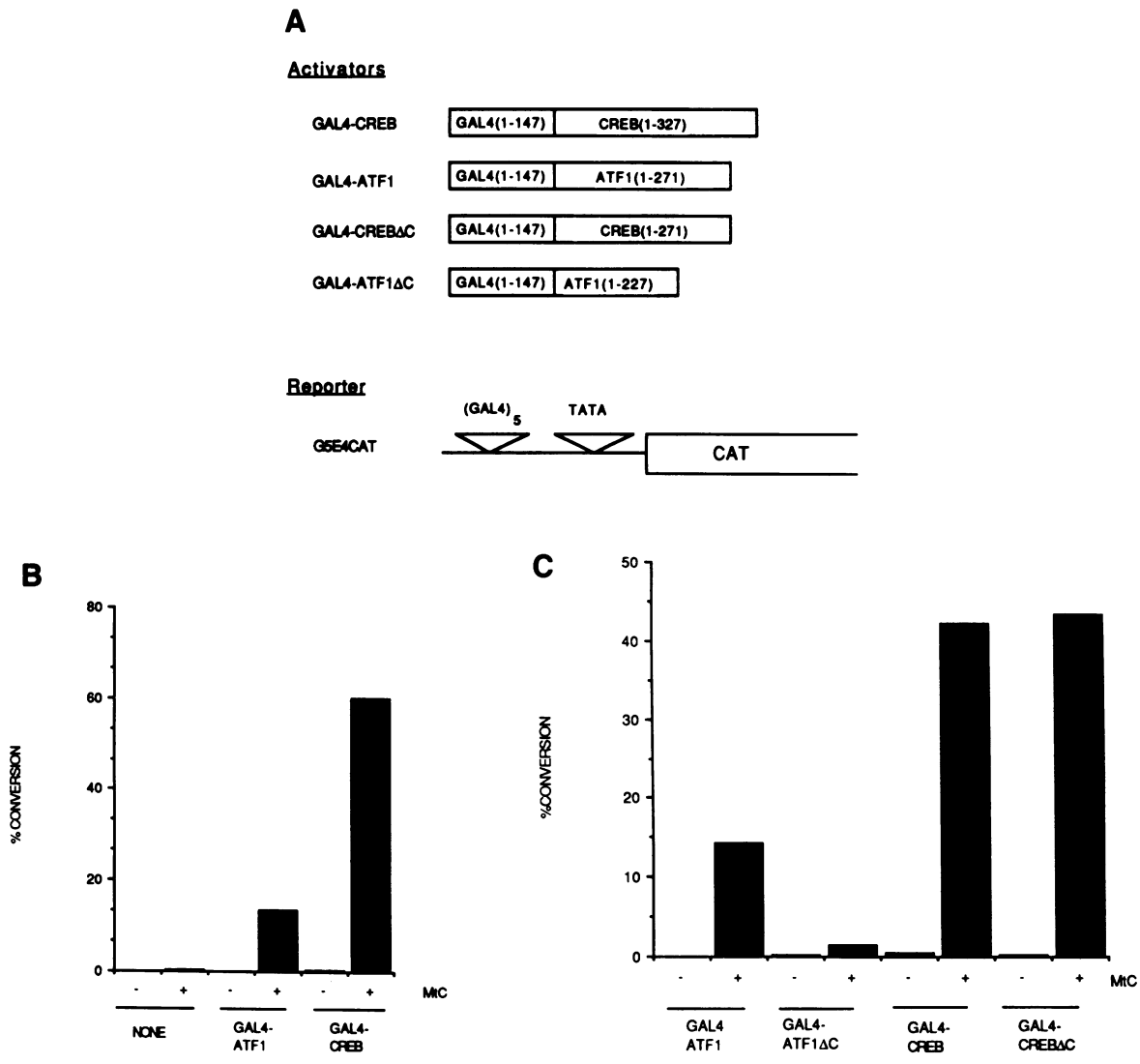

Figure 4. Activation of ATF1 by the catalytic subunit of protein kinase A. **(A)** Structure of the GAL4 derivates and CAT reporter used in this study. The activators contain full length or partial CREB and ATF1 cDNA sequences fused in frame to the N-terminal 147 amino acids of the yeast GAL4 protein. The fusion is regulated by the MLV LTR enhancer and human β-globin promoter (see Materials and Methods). **(B & C)** Human choriocarcinoma JEG-3 cells were transfected with 3μg of the indicated GAL4-derived activators, 2μg of the reporter and where indicated, 2μg of a plasmid (MtC) encoding the catalytic subunit of protein kinase A. Forty-eight hours later, cell lysates were prepared and CAT activities quantitated.

(delta ATF1) was sufficient to significantly increase the stability of the ATF1-DNA complexes. Further N-terminal deletion to amino acid 176 (DLA) had no further stabilising effect.

The majority of CREB in Hela cells is heterodimerised with ATF1

ATF-43 appears to represent the major species of ATF binding protein present within Hela cells (18,24,25,62). Indeed, our previous studies have indicated that ATF1 may be significantly more abundant than CREB and that the majority, if not all of the CREB is present in these cells as a complex with ATF1 (25). We evaluated the question of whether the CREB protein was present in extracts as a homo- or heterodimer by gel retardation analysis of Hela extracts in the presence of specific antibodies to ATF1 and CREB. In Figure 3 we show the result of one such experiment with complexes generated by crude Hela extract shown in lanes 2 and 9. *In vitro* synthesised ATF1 (lanes 3 and 13) and CREB (lanes 1, 4 and 14) were used as marker proteins and the gel was run for longer than usual to emphasise the different mobilities of these complexes more clearly. Additionally, CREB/ATF1 heterodimers (lane 8) were generated from one binding equivalent of CREB to two binding equivalents of ATF1 (see Materials and Methods) were analysed. In this case, the resulting pattern of complex formation was virtually identical to that displayed by the crude nuclear extract (lane 9). All of the specific binding activity in Hela nuclear extracts detected using ATF/CREB sites can therefore be attributed to just CREB and ATF1 proteins. The migration of the two major complexes indicated that they represented homodimeric ATF1 (lower band) and heteromeric ATF1/CREB (upper band). This is confirmed by incubation with ATF1 and CREB specific antibodies. We have previously shown (25) that the CREB antibody could recognise both CREB protein and heterodimers between CREB and ATF-43, whereas the ATF antibodies only recognise

homodimeric ATF-43. Addition of ATF antibody to the Hela extract resulted in altered mobility of the lower major complex (attributed to ATF1 homodimers; Figure 3, lane 10). Addition of CREB antibody resulted in altered mobility of the upper major complex (ATF1/CREB heterodimers; lane 11) as well as a very faint complex migrating at the position of CREB homodimers (compare lanes 9, 11 and 14). This indicates that very low levels of free CREB may be present in Hela nuclear extracts.

Regulation of ATF1 activity

The striking similarity in overall structure between ATF1 and CREB prompted us to ask whether ATF1 had similar regulatory properties. It has been shown in a number of laboratories that CREB is activated by elevated levels of cAMP. Indeed it has been implicated as being the major member of the CREB/ATF family involved in mediating the cAMP induction of gene expression (15,5,9,32,10). It has not been ruled out however, that other members of this family can also elicit such a response. The only other member that has been investigated in this regard is CRE-BP1 (ATF2, CREB2) whose activity is not stimulated by increased cAMP or kinase A levels (10).

In order to circumvent the complexity arising from multiple endogenous ATF/CRE binding proteins, the activity of ATF1 was examined in the form of a fusion with the DNA binding domain of GAL4 (Fig. 4A). Since mammalian cells are devoid of GAL4 binding activity, the regulatory activities of the fusion protein could be assayed following co-transfection of appropriate cells together with a promoter containing GAL4 binding sites linked to CAT. This approach has been successfully used to examine the regulatory activities of CREB and CRE-BP1 (5,10,39).

JEG3 cells were transfected with the GAL4-ATF1 fusion with or without a plasmid encoding the catalytic subunit of protein kinase A. As shown in Figure 4B, GAL4-ATF1 activity was significantly elevated by increased kinase A activity, although the activated level was consistantly 3−5 fold lower than that obtained with GAL4-CREB (a fusion between the GAL4 binding domain and the entire coding sequence of CREB; see Materials and Methods). This would suggest that ATF1 retains a significant capacity to elicit a cAMP response, even though it lacks the equivalent N-terminal sequences of CREB that have been implicated as being crucial for maximal CREB activity (14).

However, it is clearly possible that the GAL4-ATF1 protein may have heterodimerised with endogenous factors such as CREB. Consequently at least some of the activity seen with GAL4-ATF1 may be due to the recruitment of endogenous CREB to the promoter. A truncated version of GAL4-ATF1, no longer containing the leucine zipper domain, was constructed (see Fig. 4A) and tested. As shown in Figure 4C this truncation resulted in a 10-fold drop in the activity of GAL4-ATF1 in the presence of kinase A, whereas a similar truncation of GAL4-CREB had no effect, suggesting that although ATF1 can be activated by kinase A, its activity as a homodimer is only 3−5% of that seen with CREB. This interpretation is supported by data obtained with GAL4-CREB mutations. A number of laboratories have demonstrated the importance of serine-133 for regulation of CREB activity by cAMP (15,32). This serine is phosphorylated by kinase A. We have observed that a GAL4-CREB mutant that was no longer capable of being phosphorylated at serine-133 nevertheless responded significantly to elevated kinase A levels, albeit not as well as the wild-type fusion (K.Flint and N.Jones, unpublished results). We reasoned that the activity could be due to the recruitment of endogenous CREB protein through the leucine zipper domain with the ensuing complex being activated by kinase A, even though some members of the complex were not phosphorylated at serine-133. This possibility was supported by the construction of truncated forms of the wild-type and mutant fusions that no longer contained the leucine zipper domain. Such a deletion had no effect on the activity of GAL4-CREB, but had a drastic effect on the GAL4-CREB serine-133 mutant; removing the zipper domain resulted in almost a complete loss of activation of the mutant by kinase A.

The ATF/CRE binding site also mediates transcriptional activation by the adenovirus E1A protein (34,33,39). The CRE-BP1 (ATF2, CREB2) family member has been shown to respond to E1A activation (39,41,10), whereas CREB activity is only slightly affected (10). However, transfection studies in both Hela and CHO cells have shown that GAL4-ATF1 activity is completely unaffected by E1A (10,39).

DISCUSSION

In this paper we demonstrate that the product of the cDNA clone, ATF1, has all the properties of ATF-43, the most abundant Hela protein with specificity for binding to the CGTCA motif. The basis of concluding that ATF-43 is ATF1 is as follows: i) sequencing of peptides from affinity purified ATF-43 produced sequences present within the deduced ATF1 sequence, but absent from the highly homologous CREB sequence, ii) specific ATF-43 antisera recognise the ATF1 protein synthesised *in vitro*, iii) migration of both ATF1 and ATF-43 phosphoforms on SDS-PAGE are identical, iv) ATF1 forms heterodimers with CREB thus accounting for the previously observed CREB/ATF-43 complex found in crude cell extracts (25). ATF-43 is also a substrate for the catalytic subunit of protein kinase A. The ATF1 product clearly possesses a suitable phosphoacceptor site for kinase A (aa60−63 RRPS) and indeed ATF1 protein synthesised in bacteria is readily phosphorylated by kinase A using our standard labelling conditions (25, unpublished observations).

Due to the high degree of homology between ATF1 and CREB, it was of interest to determine whether the two proteins shared functional activities. Using GAL4 fusion proteins we have shown that ATF1 can mediate a transcriptional response to cAMP, but only very poorly (Figure 4). The level of activation detected was dependent upon the presence of the C-terminal domain of ATF1. We infer from this that full length ATF1 in the GAL4 fusion protein was able to heterodimerise with a cellular protein, probably CREB, and thereby produce a greater transcriptional response to cAMP than the truncated fusion protein. However, even in this form the response is only 20−30% of that obtained with CREB homodimers (Figure 4B). One explanation of ATF1's low activity is that it lacks the N-terminal glutamine rich domain that has been shown to be required for maximum activity of CREB (14). The exact role of this domain has not yet been established although it has been suggested that it has an activation function that is dependent upon prior phosphorylation at serine-133 (14). This phosphorylation event may induce a conformational change in the protein that renders the domain functional. If the N-terminal sequences of ATF1 are unable to functionally substitute for this CREB domain, then ATF1 activity may be very low despite being a substrate for kinase A. In addition to its poor response to cAMP, the ATF1 protein does

not respond to the stimulatory activity of the adenovirus E1A protein (39,10) or the HTLV-1 $p40^{tax}$ protein (10).

The exact role of ATF1, therefore, remains ambiguous. It retains the binding specificity of CREB together with some of the elements essential for transcriptional activation such as a kinase A phosphoacceptor site plus two important flanking domains: the immediately C-terminal acidic sequence DLSSD (14,32) (actually DLSSE in ATF1) and the immediately N-terminal serine rich domain (32). Additionally, ATF1 is at least as abundant as CREB in all the cell types we have examined (25). One possibility is that ATF1 can activate transcription but that it responds to stimuli that are different from those that have been tested to date. A second possibility is that the relationship between ATF1 and CREB is similar to that of c-*jun* and *junB*. In particular, while c-*jun* is able to activate promoters bearing a single AP1 binding site, *junB* can only activate promoters with multimeric sites as it requires cooperative interactions between adjacently bound proteins to effect a positive response (7). Conceivably therefore, ATF1 may be inactive on the classic single CRE containing promoters (such as somatostatin) but other promoters with multiple binding sites (e.g. the VIP promoter) may be efficiently activated by ATF1 or ATF1/CREB binding. Unfortunately, failure to identify a cell line which lacks ATF1 makes this difficult to test. The required protein-protein interactions implicit in this model are not necessarily reproduced using GAL4 fusion proteins and can not therefore be ruled out even though our reporter plasmid contained five tandem GAL4 binding sites. However, *in vitro* transcription assays have been reported where ATF-47 and ATF-43 purified from Hela cells, which we have shown to be the products of CREB (25) and ATF1 respectively, have been assayed for their ability to stimulate transcription from a chimeric promoter containing eight E4 ATF sites. Addition of CREB was found to stimulate transcription ten fold more than an equal binding equivalent of ATF1. Addition of heterodimeric fractions gave an intermediate level of transcription (62). These findings would therefore indicate that ATF1 is a weak activator even when bound to multimeric sites and in this instance can act to reduce levels of transcription observed with CREB alone.

Rather than behaving as an activator of transcription, it is possible that ATF1 is a repressor and more specifically, a transdominant negative regulator of CREB. One can envision that ATF1 could carry out such a regulatory role in one of two ways. First, if ATF1 were significantly more abundant than CREB, it could limit CREB's access to its cognate binding sites by direct competition. Second, ATF1 could regulate by forming heterodimers with CREB. Such heterodimers are not only less responsive to stimulation by cAMP (Figure 4), but also form less stable complexes with DNA (Figure 2A). When ATF1 is present in excess, very little CREB homodimer may be present, which indeed seems to be the case in Hela cells (Figure 3) and may offer an explanation for the relatively poor transcriptional response of these cells to elevated cAMP levels (unpublished observations). We have attempted to test this possibility by overexpressing ATF1 in JEG3 cells which respond well to cAMP, but this failed to diminish the response to cAMP shown by endogenous CREB in these cells (unpublished observations). Furthermore, the expression of an antisense ATF1 construct in Hela cells did not result in elevated cAMP response levels (unpublished data). Moreover, when we examined relative CREB and ATF1 mRNA levels in a variety of cell lines, including Hela and JEG3, we found that the 2kb ATF1 specific transcript was always at least as abundant as the 7kb CREB (16) specific transcript (unpublished data). Consequently, two cell lines with radically different responses to cAMP have very similar ratios of ATF1 to CREB and it appears unlikely therefore that ATF1 is an efficient repressor of CREB activity. These results differ somewhat from those recently reported for another small bZIP protein, CREM, which also shows high sequence similarity to the C-terminal portion of CREB, but also lacks the N-terminal glutamine rich domain (11). Expression of antisense CREM in uninduced JEG3 cells apparently raised basal levels of transcription from a suitable CAT reporter plasmid, while expression from this reporter was inhibited in cells additionally transfected with expression plasmids for kinase A and wild type CREM (11). These findings implicate CREM as being a potential repressor of CREB, but although CREM and CREB can heterodimerise *in vitro*, we found no evidence for these complexes in nuclear extracts; in particular in Hela cells where we find that CREB is complexed exclusively with ATF1 (Fig. 3;25).

ATF1 appears to be phosphorylated at multiple sites. It is a substrate for kinase A, but one or more additional kinases are required to produce the different phosphoforms found on SDS-PAGE. Moreover, our previous studies (25) examining CREB/ATF-43 complexes in a variety of cell lines showed that while ATF-43 gives a range of bands in Hela cell samples on SDS-PAGE, samples from other cell lines showed a more restricted range of apparent molecular weight. This implies that the phosphorylation state of ATF1 may be differently regulated in different cell lines and this may have important consequences for function. Clearly, further studies need to be carried out to fully elucidate the function of ATF1 and its ability to heterodimerise with CREB.

ACKNOWLEDGEMENTS

We would like to thank Dr. T.Hai for providing the partial ATF1 clone; Dr. K.Lee for providing the CREB peptide antibody; Drs. D.Bently, L.Clark, I.Cowell and S.Goodbourn for critically reading the manuscript; Z.Redley for preparing the manuscript and S.Weiss for preparing the figures.

REFERENCES

1. Abersold,R.H., Leavitt,J., Saavedra,R.A., Hood,L.E. and Kent,S.B.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84,** 6970–6974.
2. Andrisani,O.M. and Dixon,J.E. (1990) *J. Biol. Chem.*, **5,** 3212–3218.
3. Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) Current protocols in Molecular Biology (New York: John Wiley and sons).
4. Benbrook,D.M. and Jones,N.C. (1990) *Oncogene*, **5,** 295–302.
5. Berkowitz,L.A. and Gilman,M.Z. (1990) *Proc. Natl. Acad. Sci. USA*, **87,** 5258–5262.
6. Bredow,S., Sürig,D., Müller,J., Kleinert,H. and Benecke, B-J. (1990) *Nucleic Acids Res.*, **18,** 6779–6784.
7. Chiu,R., Angel,P. and Karin, M. (1989) *Cell*, **59,** 979–986.
8. Cortes,P., Buckbinder,L., Leza,M.A., Rak,N., Hearing,P., Merino,A. and Reinberg,D. (1988) *Genes Dev.*, **2,** 975–990.
9. Dwarki,V.J., Montminy,M. and Verma,I.M. (1990) *EMBO J.*, **9,** 225–232.
10. Flint,K. and Jones,N.C. (1990), Oncogene, in press.
11. Foulkes,N.S., Borrelli,E. and Sassone-Corsi,P. (1991) *Cell*, **64,** 739–749.
12. Fujisawa,J., Toita,M. and Yoshida,M. (1989) *J. Virol.*, **63,** 3234–3239.
13. Gaire,M., Chatton,B. and Kedinger,C. (1990) *Nucleic Acids Res.*, **18,** 3467–3473.
14. Gonzalez,G.A., Menzel,P., Leonard,J., Fischer,W.H. and Montminy,M.R. (1991) *Mol. Cell. Biol.*, **11,** 1306–1312.
15. Gonzalez,G.A. and Montminy,R.M. (1989) *Cell*, **59,** 675–680.

16. Gonzalez,G.A., Yamamoto,K.K., Fischer,W.H., Karr,D., Manzel,P., Biggs III,W., Vale,W.W. and Montminy,R.M. (1989) *Nature*, **337**, 749–752.
17. Hai,T. and Curran,T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3720–3724.
18. Hai,T., Liu,F., Allegretto,E.A., Karin,M. and Green,M.R. (1988) *Genes Dev.*, **2**, 1216–1226.
19. Hai,T-Y., Liu,F., Coukos,W.J. and Green,M.R. (1989) *Genes Dev.* **3**, 2083–2090.
20. Hardy,S. and Shenk,T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4171–4175.
21. Hoeffler,J.P., Meyer,T.E., Yun,Y., Jameson,J.L. and Habener,J.F. (1988) *Science*, **242**, 1430–1433.
22. Howe,J.G. and Shu,M-D. (1989) *Cell*, **57**: 825–834.
23. Hsu,J-C., Thomas,L., Mohn,K.L. and Taub,R. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3511–3515.
24. Hurst,H.C. and Jones,N.C. (1987) *Genes Dev.* **1**, 1132–1146.
25. Hurst,H.C., Masson,N., Jones,N.C. and Lee,K.A.W. (1990) *Mol. Cell. Biol.*, **10**, 6192–6203.
26. Ivashkiv,L.B., Liou,H-C., Kara,C.J., Lamph,W.W., Verma,I.M. and Glimcher,L.H. (1990) *Mol. Cell. Biol.*, **10**, 1609–1621.
27. Jalinot,P., Wintzerith,M., Gaire,M., Hauss,C., Egly,J.M. and Kedinger,C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2484–2488.
28. Jeang,K-T., Boros,I., Brady,J., Radonovich,M. and Khoury,G. *J. Virol.*, **62**, 4499–4509.
29. Kanei-Ishii,C. and Ishii,S. (1989) *Nucleic Acids Res.*, **17**, 1521–1537.
30. Kara,C.J., Liou,H-C., Ivashkiv,L.B. and Glimcher,L.H. (1990) *Mol. Cell. Biol.*, **10**, 1347–1357.
31. Kouzarides,T. and Ziff,E. (1988) *Nature*, **336**, 646–651.
32. Lee,C.Q., Yungdae,Y., Hoeffler,J.P. and Habener,J.F. (1990) *EMBO J.*, **9**, 4455–4465.
33. Lee,K.A.W., Fink,J.S., Goodman,R.H. and Green,M.R. (1989) *Mol. Cell. Biol.* **9**, 4390–4397.
34. Lee,K.A.W. and Green,M.R. (1987) *EMBO J.* **6**, 1345–1353.
35. Lee,K.A.W., Hai,T.Y., SivaRaman,L., Thimmappaya,B., Hurst,H.C., Jones,N.C. and Green,M.R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8355–8359.
36. Leza,M.A. and Hearing,P. (1988) *J. Virol.* **62**, 3003–3013.
37. Lin,Y.S. and Green,M.R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3396–3400.
38. Liou,H-C., Boothby,M.R., Finn,P.W., Davidson,R., Nabavi,N., Zeleznik-Le,N.J., Ting,J. and Glimcher,L.H. (1990) *Science* **247**, 1581–1584.
39. Liu,F. and Green,M.R. (1990) *Cell*, **61**, 1217–1224.
40. Macgregor,P.F., Abate,C. and Curran,T. (1990) *Oncogene*, **5**, 451–458.
41. Maekawa,T., Matsuda,S., Fujisawa,J., Yoshida,M. and Ishii,S. (1991) *Oncogene*, **6**, 627–632.
42. Maekawa,T., Sakura,H., Kanei-Ishii,C., Sudoi,T., Yoshimura,T., Fujisawa,J., Yoshida,M. and Ishii,S. (1989) *EMBO J.* **8**, 2023–2028.
43. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour University Press, Cold Spring Harbour.
44. Mellon,P., Clegg,C.H., Correll,L.A. and McKnight,G.S. (1989) *Proc. Natl. Acad. Aci. USA*, **86**, 4887–4891.
45. Merino,A., Buckbinder,L., Mermelstein,F.H. and Reinberg,D. (1989) *J. Biol. Chem.* **264**, 21266–21276.
46. Montagne,J., Beraud,C., Grenon,I., Lombard-Platet,G., Gazzolo,L., Sergeant,A. and Jalinot,P. (1990) *EMBO J.*, **9**, 957–964.
47. Montminy,M.R. and Bilezikjian,L.M. (1987) *Nature*, **328**, 175–178.
48. Montminy,M.R., Sevarino,K.A., Wagner,J.A., Mandel,G. and Goodman,R.H. (1986) *Proc. Natl. Acad. Sci. USA* **86**, 6682–6686.
49. Nakamura,M., Niki,M., Ohtani,K. and Sugamura,K. (1989) *Nucleic Acids Res.*, **17**, 5207–5221.
50. Nicholas,J. and Nevins,J.R. (1991) *Virology*, **182**, 156–167.
51. Norman,C., Runswick,M., Pollock,R. and Treisman,R. (1988) *Cell*, **55**, 989–1003.
52. Nyborg,J.K. and Dynan,W.S. (1990) *J. Biol. Chem.*, **265**, 8230–8236.
53. Ono,S.J., Liou,H-C., Davidson,R., Strominger,J.L. and Glimcher,L.H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4309–4312.
54. Poteat,H.T., Chen,F.Y., Kadison,P., Sodroski,J.G. and Haseltine,W.A. (1990) *J. Virol.*, **64**, 1264–1270.
55. Raychaudhuri,P., Rooney,R. and Nevins,J.R. (1987) *EMBO J.* **6**, 4073–4081.
56. Roesler,W.J., Vandenbark,G.R. and Hanson,J.R. (1988) *J. Biol. Chem.*, **263**, 9063–9066.
57. Rooney,R.J., Raychaudhuri,P. and Nevins,J.R. (1990) *Mol. Cell. Biol.*, **10**, 5138–5149.
58. Sassone-Corsi,P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7192–7196.
59. Tan,T-H., Horikoshi,M. and Roeder,R.G. (1989) *Mol. Cell. Biol.* **9**, 1733–1745.
60. Totty,N.F., Hsuan,J.J. and Waterfield,M.D. (submitted)
61. Vinson,C.R., Sigler,P.B. and McKnight,S.L. (1989) *Science*, **246**, 911–916.
62. Wada,T., Watanabe,H., Usuda,Y. and Handa,H. (1991) *J. Virol.*, **65**, 557–564.
63. Yoshimura,T., Fujisawa,J-I. and Yoshida,M. (1990) *EMBO J.*, **9**, 2537–2542.