In vitro phosphorylation studies of a conserved region of the transcription factor ATF1

Norma Masson, Joseph John and Kevin A.W.Lee*
Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, UK

Received July 8, 1993; Revised and Accepted August 10, 1993

ABSTRACT

A large family of mammalian transcription factors including multiple variants of CREB, CREM and ATF1 have been implicated in signal transduction by cAMP and other cellular pathways. Although the roles of some members of the family have been characterised the function of ATF1 is poorly understood. We have identified one or more key serine residues that are required for a phosphorylation-induced conformational change in ATF1. The critical serines map to a putative transcriptional activation domain of ATF1 and affect the stability of ATF1 DNA-binding. Intriguingly phosphorylation is modulated by ATF1 homodimerisation and by ATF1 binding to DNA. One of the key serine residues required for ATF1 phosphorylation is not conserved in CREB and CREM suggesting that it is likely to determine some specialised function of ATF1.

INTRODUCTION

Phosphorylation is one of the major mechanisms known to govern transcription often providing the key link for relay of intracellular signals to the transcription machinery (1, 2, 3). The activity of several transcription factors is directly influenced by phosphorylation, in a manner that affects their nuclear translocation, DNA-binding or transactivation functions. The basic/leucine zipper (bZIP) class of dimeric transcription factors have been shown to be the nuclear targets for many intracellular signalling pathways including those involving phosphorylation by protein kinase A (4, 5, 6, 7, 8), the calmodulin/Ca^{2+}-dependent protein kinase (9, 10), protein kinase C (11) and MAP kinases (12). Regulation of the cAMP-response-element-binding protein (CREB) by protein kinase A (PKA) is one of the best characterised pathways (13, 14, 15, 16, 17).

Regions of CREB required for transcriptional activation include a glutamine-rich N-terminal transactivation domain (18), the alpha peptide (19) and a complex region termed the kinase-inducible-domain (KID (18)) or the phosphorylation box (P box (6)). The P box includes the protein kinase A (PKA) phosphoacceptor site and two other regions termed PDE1 and PDE2 which flank the PKA site (6, 18). Activation of CREB is thought to involve an increase in both transcriptional (6, 7) and DNA-binding activity (5). Although functional characterisation of CREB has led to significant advances in understanding cAMP-signalling, the existence of a large number of CREB relatives (20, 21, 22) has raised many new questions. At least two other genes (CREM and ATF1) encode close relatives of CREB and the total number of distinct proteins is more complex due to heterodimerisation and a high degree of differential splicing of the CREB (23, 24, 25) and CREM (21) genes. Characterisation of CREB has provided clues to the properties of other family members but to date their functions are not well characterised.

ATF-1 exhibits 70% overall homology to CREB with an essentially identical bZIP domain and strong conservation of the P box. As expected from these structural similarities there is evidence that ATF1 can act as a cAMP-inducible transcription factor (8, 26). However there are a number of features which suggest that ATF1 will exhibit some novel functions and regulation. First, when fused to the DNA binding domain of the yeast activator gal4, transcriptional activation by gal4/ATF1 is strongly stimulated (26). Second the N-terminal region of ATF1 (~27 amino acids) is not highly related to CREB and determines the stability ATF1 DNA-binding (26). Third, during differentiation of F9 embryonal carcinoma cells expression of ATF1 is down-regulated while CREB remains constant (27). Finally, ATF-1 exists in multiple cell type-specific forms that arise, at least in part, due to differential phosphorylation (22, 26). To date the phosphorylation state of ATF-1 and its functional significance have not been addressed. Here we describe in vitro assays for ATF1 phosphorylation. Phosphorylation of a single serine residue in ATF1 that is not conserved in CREB and CREM is correlated with a dramatic conformational change in ATF1. Our results therefore suggest that phosphorylation will determine some specialised function of ATF1.

MATERIALS AND METHODS

Cell culture and transfections

Human choriocarcinoma JEG3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% foetal calf serum. Cells were transfected using the calcium phosphate coprecipitation method CAT assays performed as previously described (28).
Plasmids and peptides

pANTR encodes wild type ATF1 lacking the amino-terminal 27 amino acids. pANTR was derived from pGFLATF1 (a full length ATF1 cDNA cloned in the polylinker of pGEM3 (26)) by replacing ATF1 sequences upstream of the BamHI site (amino acid 56) with an oligonucleotide encoding amino acids 28-56 in ATF1. pmNTR was derived from pGFLATF1 by replacing the amino-terminal sequences of ATF1 with an oligonucleotide encoding alanine in the place of serine or threonine as indicated in figure 3. The pSM series of mutant plasmids were derived from pGFLATF1 by site-directed mutagenesis using PCR. All mutations were confirmed by sequencing. pGATF1 and pFGSM456 were also derived from pGFLATF1 as follows. A unique NdeI site was created by a single point mutation at position 628 in the ATF1 sequence. The NdeI-EcoRI fragment containing the ATF1 bZIP domain was then replaced with a hybrid bZIP domain consisting of the basic domain of c-fos and the leucine zipper GCN4. pSP6F6G2 (29) was used to generate the PCR insert and the resulting hybrid protein consists of ATF-1 (amino acids 1-210) linked to the F5G2 peptide at amino acid 135. pATFT1T and pSMS456T contain full length ATF1 (in which the bZIP domain is replaced with the bZIP domain from CREB) with a 7 amino acid T-antigen epitope for the monoclonal antibody KT3 (30) fused to the carboxy terminus and cloned into pSG424 (31). The bacterial expression vectors pET15bATF1 and pET15bSM4A were obtained by inserting the complete ATF1 cDNA sequence into pET-15b (Novagen). pGEX-ATF1 is described elsewhere (27). Synthetic peptides corresponding to the leucine zipper regions of CREB, c-Jun and c-Fos are also described elsewhere (27).

Western blotting and immunoprecipitations

Preparation of nuclear extracts and antibodies to CREB and ATF1 have been previously described (22). Anti-peptide antibodies were raised against synthetic peptides corresponding to the carboxy terminal 10 amino acids of CREB or carboxy terminal 13 amino acids of ATF1. Polyclonal antibody was raised against ATF1 amino acids 1-210) linked to the GCN4. pSP6F6GCN4 (29) was used to generate the PCR insert and the resulting hybrid protein consists of ATF-1 (amino acids 1-210) linked to the F5G2 peptide at amino acid 135. pATFT1T and pSMS456T contain full length ATF1 (in which the bZIP domain is replaced with the bZIP domain from CREB) with a 7 amino acid T-antigen epitope for the monoclonal antibody KT3 (30) fused to the carboxy terminus and cloned into pSG424 (31). The bacterial expression vectors pET15bATF1 and pET15bSM4A were obtained by inserting the complete ATF1 cDNA sequence into pET-15b (Novagen). pGEX-ATF1 is described elsewhere (27). Synthetic peptides corresponding to the leucine zipper regions of CREB, c-Jun and c-Fos are also described elsewhere (27).

In vitro transcription and translation

35S-labelled ATF-1, ATF-1 mutants and CREB proteins were synthesised in vitro by transcription of their corresponding cDNAs (26) as previously described (20) using SP6 or T7 RNA polymerase (Boehringer) and subsequent translation in rabbit reticulocyte lysate (Amersham). For phosphatase treatment of in vitro translation products, rabbit reticulocyte lysate (2μl) was treated with 5U of calf intestinal phosphatase for 2 hours at 37°C in 12μl reactions containing 20 mM HEPES pH8.0, 20 mM MgCl2, 40 mM KCl and 0.6 mM PMSF. Phosphatase inhibitors were used at the following concentrations. 60 mM NaF, 20 mM EDTA, 2 μM L-cys and 2 μM okadaic acid.

Gel retardation experiments

Gel retardation assays were performed as previously described (22). Oligonucleotides containing consensus wild-type or mutated ATF-binding sites were as follows. The ATF-binding site core motif TGACGTCA or the corresponding mutated sequence is underlined. The wild type competitor oligonucleotide contains the sequence GGATCCATGACGTCAATGGATC and the mutant oligonucleotide sequence is GGATCCATGATCATGATC.

Bacterial expression of ATF

Plasmids pGEX-ATF1 and pET15bATF1 were used to express glutathione-S-transferase fusion protein (GST-ATF1) and histidine tagged fusion protein (H6-ATF1) respectively. Purification of GST-ATF1 was as previously described (27). Purification of H6-ATF1 was performed in a similar manner and protein was eluted from the Ni2+-agarose beads using 250mM imidazole and quick frozen.

Phosphorylation by casein kinase 2 and phosphopeptide analysis

Bacterially expressed H6-ATF1 protein was 32P-labelled with purified casein kinase 2 (CK2) by incubation at 37°C for 1h in buffer containing 20 mM tris (pH 7.5), 75 mM NaCl, 12 mM MgCl2 and 50 μM ATP. CK2 was obtained from J.Woodgett and was purified as described elsewhere (32). Labelled protein was passed through a G-50 spin column (Pharmacia) to remove unincorporated label and prepared for sequencing as follows. Lyophilised protein was dissolved in 25 mM NH4HCO3 containing 1M urea and digested with trypsin (5% W/W) for 5 hours at 37°C. The digest was acidified with 2μl TFA and injected directly onto an RP300 C8 reverse-phase column equilibrated with 0.05% v/v aqueous TFA. Peptides were eluted with a 0-80% gradient of acetonitrile and the major radio labelled peak dried onto an alylamine-substituted PVDF membrane and covalently attached using ethyl dimethylamino propyl carbodiimide (33). The peptide was then sequenced by Edman degradation using a MilliGen 6600 sequenator (34) and fractions representing different cycles collected for counting.

RESULTS

Assays for phosphorylation of ATF1

To gain insight into ATF1 phosphorylation we have utilised various in vitro assays (figure 1). Phosphorylations that decrease the mobility of ATF1 on SDS gels occur during in vitro translation of ATF1 mRNA in rabbit reticulocyte lysate (RRL) (26, 27). A complex pattern of ATF1 polypeptides are produced in RRL and this is due to a high degree of differential phosphorylation as described below (see also figure 4). Throughout the results we will not distinguish between the multiple phosphovariants produced because we are not monitoring phosphorylation directly. We will simply refer to decreases in mobility of ATF1 due to phosphorylation as 'phosphorylation' without implying that all effects observed are due to the same phosphorylation events. Complete phosphorylation can result in a single ATF1 species with the lowest mobility (see figure 4B) and conversely, phosphatase treatment produces a single band with the highest mobility (figure 1A and 4A). Interestingly the mobility of the highly homologous CREB protein is not affected under these conditions (figure 1A). Because the change in mobility of ATF1 due to phosphorylation is so large (an ~ 7 kDa shift) we interpret this to indicate that phosphorylation(s) induces a change in conformation of ATF1.

To develop an assay for ATF1 kinases independently of RRL we have utilised bacterially expressed ATF1 protein as a substrate. As expected bacterial ATF1 migrates on SDS gels with a mobility that indicates it is unphosphorylated. Compare 35S-
methionine-labelled bacterial ATF-1 (figure 1B, lanes 1 and 5) with phosphorylated ATF1 made by translation in RRL (lane 4). Incubation with RRL for 10 minutes results in efficient phosphorylation of bacterial ATF1 (figure 1B, lane 6) and longer incubations are no more efficient (lane 7) suggesting that the kinases present in RRL rapidly lose activity (see also figure 9). We also tested extracts from HeLa cells for ATF1 kinases. Incubation of bacterial ATF1 with HeLa cell nuclear extract resulted in efficient phosphorylation (figure 1B, lane 2) dependent on exogenous Mg ++ and ATP (compare lanes 2 and 3). In summary a variety of in vitro assays can be used to study phosphorylations of ATF1 that alter mobility on SDS gels.

Figure 1. In vitro assays for ATF-1 phosphorylation. (A) Translation of ATF1 in rabbit reticulocyte lysate (RRL). 35S-labelled ATF-1 and CREB were produced by translation in RRL, mixed and treated with calf intestinal phosphatase in the presence or absence of phosphatase inhibitors, resolved by SDS-PAGE and autoradiographed. Molecular weight standards (Biodrad prestained low size range) are shown to the right. (B) Phosphorylation of bacterially expressed ATF1. Purified 35S in vivo-labelled ATF1 (~ 10 ng) was incubated with 5 μl of reticulocyte lysate (lanes 5—7) or HeLa cell nuclear extract (lane 1—3) for the times indicated below the figure in the presence of 1mM Mg ++ and 0.5 mM ATP as indicated. Lane 4 contains ATF1 produced by in vitro translation in RRL.

Figure 2. Mutational analysis of the N-terminal region (NTR) of ATF1 in RRL. The sequence of the NTR and homologous regions of ATF1 and CREB are shown at the top. For a detailed description see figure 3. ΔNTR lacks the N-terminal 27 amino acids of ATF-1 and mNTR is full-length with serine and threonine residues (as indicated by the filled circles) changed to alanine. 35S-labelled ΔNTR, mNTR and wild type ATF1 proteins were produced by in vitro translation in RRL and analysed as described in figure 1A. Molecular weight standards (Biodrad prestained low size range) are shown to the left.

Figure 3. Functional regions of ATF1, CREB and CREM. The known functional regions of CREB and homologous regions of ATF1 are shown. The basic (BASIC) and leucine zipper (ZIP) (regions comprise the carboxy terminal bZIP domain that is necessary and sufficient for dimerisation and DNA-binding (20, 47). Several distinct elements comprise the phosphorylation box (6) (otherwise called the kinase-inducible-domain (18)) defined by discrete deletions (6, 7). These are referred to as PDE1, PKA and PDE2 in the figure and correspond to the boxed elements shown for sequence homologies within the P box. PKA represents a single protein kinase A phosphoacceptor site (serine 133) in CREB, PDE1 is amino acids 106—122 in CREB and PDE2 is amino acids 135—144 in CREB. CREB contains the α-peptide (α) and a glutamine rich activation domain (Q), both of which contribute to transcriptional activity (18, 19). ATF1 and CREB have overall homology of ~ 70% and 95% in the bZIP region (20) but diverge at their amino-termini. ATF1 has a small region at the amino-terminus (the NTR, see figure 2) that is not obviously related to CREB. The NTR influences ATF1 DNA-binding activity (26). % homology (including conservative changes) within the P box for CREB, ATF1 and CREM is indicated at the bottom of the figure.

Requirements for ATF1 phosphorylation in vitro

Despite the high degree of homology between ATF1 and CREB, CREB does not exhibit any alteration in mobility upon treatment with phosphatase ((27) and figure 1A). This suggested that at least some of the phosphorylations occurring in RRL might be specific for ATF1. ATF1 diverges from CREB at the amino-terminus (see figures 2 and 3) and this region of ATF1 (the NTR) is highly serine/threonine-rich. We therefore examined the involvement of the NTR in phosphorylation of ATF1. An ATF1 mutant RNA lacking the NTR (ΔNTR) was translated in RRL (figure 2). Phosphatase treatment has only a small effect on the mobility of ΔNTR protein indicating that the NTR is important for the phosphorylation-induced change in ATF1. To gain further insight into the role of the NTR we tested a full length ATF1 protein (mNTR) in which all the serines and threonines in the NTR were mutated to alanine. mNTR behaved identically to wild type ATF1 in the RRL assay (figure 2). We conclude therefore that the NTR is important for a phosphorylation-induced conformational change but that the phosphorylation sites lie outside of the NTR. Because it has been suggested that activation of CREB is achieved by a PKA-dependent conformational change (6, 7) we next asked whether the PKA phosphoacceptor site of ATF1 (serine 12, see figure 4) is required for the phosphorylation-induced conformational change of ATF1 (figure 4A). Mutation of the PKA phosphoacceptor site to alanine (SM12) has no effect on ATF1 mobility on SDS gels indicating that phosphorylation of this site is not required for the conformational change in ATF1 observed in RRL.

Homology between ATF1, CREB and CREM is shown in figure 3. Immediately downstream of the NTR, ATF1 is again highly serine-rich and is homologous to the phosphorylation box or P-BOX (6, 18) in CREB. This region contains the PDE1 region of CREB that is important for transcriptional activity (6,
Figure 5. Phosphorylation of ATF1 in vivo. ATF1T or SM456T proteins are tagged at their C-termini with a 7 amino acid T-antigen epitope recognised by the monoclonal antibody KT3 (30). JEG3 cells were transfected with vectors expressing T-tagged proteins in the presence or absence of an expression plasmid for the catalytic subunit of protein kinase A (48). Nuclear extracts were prepared 36h after transfection and analysed by Western blotting using the KT3 monoclonal antibody. Molecular weight standards (Biorad prestained low size range) are indicated to the left.

Figure 6. Phosphorylation of ATF1 by CK2. Bacterially expressed ATF1 was incubated with HeLa cell nuclear extract (NE) or CK2, resolved by SDS/PAGE and detected by Western blotting using anti-ATF1 antibody (26). γ-32P-ATP was included in the CK2 incubation, the dried blot subjected to autoradiography and the autoradiogram (marched 32p) aligned with the Western blot (WESTERN). Purified bacterial expressed SM4A protein has serine 4 changed to aspartic acid. Molecular weight standards (Biorad prestained low size range) are indicated to the left.

Figure 4. Identification of serine residues important for phosphorylation of ATF1 in RRL. (A) Mutation of the PKA site. 32S-labelled SM12 protein containing a mutation of the PKA phosphoacceptor site (see nomenclature below) was produced by in vitro translation in RRL and compared with wild-type ATF1 as described in figure 2A. (B, C and D) Mutation of serines within the PDE1 homology. The sequence of ATF1 including the region of the PDE1 phosphoacceptor site and the PDE1 homology is shown and the serine residues present assigned a number from 1—12. Nomenclature is as follows. SMI is a mutant in which serine 1 has been changed to alanine. All mutations are serine to alanine except for SM4A which contains a serine to aspartic acid change at serine 4. Parts B, C and D represent different experiments each including a co-translated wild type ATF1 protein for comparison. Molecular weight standards (Biorad prestained low size range) are indicated to the left for each part of the figure.

18) and appears to be highly phosphorylated (6). We therefore tested ATF1 mutants in this region for the ability to undergo the phosphorylation-induced conformational change in RRL (figure 4B). Serine residues in the region were assigned a number from 1—11 and mutants named according to the serines that are changed to alanine. Thus SM1—4 has serines 1—4 all mutated to alanine. Mutation of two groups of serines (SM1—4 and SM5—11) has a significant effect on ATF1 mobility suggesting that at least two serines are involved in ATF1 phosphorylation (figure 4B). Further mutational analysis points to the serines responsible. Most mutations have little effect but dramatic effects are observed for mutants in which serines 4, 5 and 6 are changed to alanine (figure 4C and 4D). Significantly mutation of serine 4 alone has a dramatic effect on mobility. However the double mutants SM4/5 and SM4/6 both give a greater effect compared with SM4 alone, suggesting that serines 5 and 6 are also important. In summary, we conclude that the region of ATF1 containing serines 4, 5 and 6 is most important for the phosphorylation-induced conformational change of ATF1 in RRL. Because serines 4, 5 and 6 are adjacent serines and because mutation of these serines mimics the effect of phosphatase treatment, these results strongly suggest that phosphorylation of serines 4, 5 and 6 accounts for the induced conformational change of ATF1.

Requirements for ATF1 phosphorylation in vivo
The in vivo significance of ATF1 phosphorylation by as yet unidentified protein kinase(s) present in RRL is not clear. To examine this we expressed normal and mutant ATF1 proteins in JEG3 cells and analysed the conformation of the expressed
Serines 4, 5 and 6 map to a region of homology with CREB that is important for CREB transcriptional activity (6, 18). We therefore sought to test the effect of mutating these serines on ATF1 transcriptional activity. Since ATF1 cannot be tested directly in vivo (because of interference from endogenous ATF proteins) we employed an established assay in which ATF1 is fused to the DNA-binding domain of yeast activator gal4 (26) and transcription monitored using a reporter driven by gal4 binding sites. A panel of gal4/ATF fusion constructs were prepared to cover all the possible combinations of mutations which could arise from serines 4, 5 and 6. These constructs were transfected into JEG3 cells, and PKA-inducible transcriptional activity of the mutant proteins compared with that of wild-type gal4/ATF1 (figure 7A). For all of the mutants analysed there is a strong induction by PKA and the absolute activity is comparable with wild type ATF1. Thus in this assay mutation of serines 4, 5 and 6 has no effect on ATF1 transcriptional activity.

The phosphorylation state of ATF1 does not appear to affect the ability to recognise DNA (22). However, in light of the observations that the NTR affects the stability of ATF1 DNA-binding (26) and is required for the phosphorylation-induced conformational change, we tested the effect of mutating serines 4, 5 and 6 on the stability of ATF1 binding (figure 7B). The wild type ATF1 and SM456 proteins were produced in RRL and allowed to bind to a 32P-labelled DNA probe containing an ATF binding site from the adenovirus E4 promoter (37). The off-rate was examined by addition of specific competitor DNA for various times after equilibrium binding was established (figure 7B). The
mobility of DNA/protein complexes formed by ATF1 and SM456 proteins is different (figure 7B, compare left hand panel (ATF1) with centre panel (SM456)). Thus the phosphorylation-induced conformational change in ATF1 is apparent under native conditions and is not peculiar to the denatured protein as visualised on SDS gels. ATF1 dissociates more or less completely from DNA following 40 minutes of competition (the faint complexes remaining are present in RRL only) whereas most of the SM456 protein remains bound over this time period. Specificity of DNA-binding and the ability of the competitor DNA to compete for binding of the SM456 protein is indicated by competition with an excess of wild-type competitor DNA added at zero time (figure 7B, right hand panel). These results demonstrate that the integrity of serines 4, 5, and 6 is important for unstable binding of ATF1 to DNA and that phosphorylation of one or more of these serines probably accounts for unstable binding.

Figure 8. Effect of dimerisation on phosphorylation of ATF1 in RRL. \(^{32}\)P-
labelled ATF1 was produced by in vitro translation in RRL and analysed on SDS
gels. Reactions contained increasing amounts of a CREB leucine zipper peptide
(CREB LZ) or JUN and FOS leucine zipper peptides as indicated below the figure.
Translations of \(^{35}\)S-labelled SM456, FGATF1, and FGSM456 proteins were
performed in the absence of competition peptides. FGATF1 and FGSM456 are
hybrid proteins in which the basic and leucine zipper regions of ATF1 are replaced
with the basic region of c-fos and the leucine zipper of GCN4. Molecular weight
standards (Biorad pre-stained low size range) are indicated to the left.

Homodimerisation alters phosphorylation of ATF1
Because ATF family members function as homodimers or selectively form heterodimers (20, 22, 38) it was pertinent to examine whether homodimerisation had any effect on ATF1 phosphorylation. For this purpose we exploited the ability of a synthetic leucine zipper (LZ) to compete for homodimerisation and thereby produce essentially monomeric forms of ATF1 (27). We have previously shown that a CREB LZ peptide can dimerise with CREB and ATF1 proteins (27). Dimerisation is highly specific since LZ peptides representing leucine zippers of proteins that do not dimerise with ATF1 (for example JUN and FOS) do not compete for dimerisation (27).

Addition of increasing amounts of CREB LZ peptide to a RRL translation reaction causes an increase in mobility of ATF1 on SDS gels whereas addition of JUN and FOS LZ peptides has no effect (figure 8). This result indicates that certain ATF1 phosphorylations are inhibited by the CREB LZ peptide. The CREB LZ peptide does not directly inhibit protein kinases in RRL because inhibition is dependent on the presence of the peptide during translation (data not shown). Inhibition of phosphorylation may not be complete either because monomers are phosphorylated to some extent or because the synthetic LZ peptide is not efficient at preventing dimer formation. In any event it is clear that homodimerisation of ATF1 reduces certain phosphorylations in RRL. We also asked whether phosphorylation of ATF1 was influenced by a specific bZIP domain. Wild type (FGATF1) and serine mutant (FGSM456) hybrid proteins in which the ATF1 bZIP domain is precisely replaced by the basic region of Fos and the GCN4 leucine zipper were produced in RRL and compared with wild-type ATF1 (figure 8). The differential migration of the unmutated and mutated hybrid proteins on SDS gels is very similar to the corresponding forms of ATF1. Thus although homodimerisation modulates ATF1 phosphorylation a specific bZIP domain is not required.

Phosphorylation of ATF1 is inhibited by DNA-binding
It has recently been shown that phosphorylation of functionally important sites in the bZIP protein c-Jun is dependent upon the intracellular concentration of binding sites (39). This prompted us to examine the effect of DNA-binding on ATF1 phosphorylation. ATF1 was produced in RRL in the absence of DNA (figure 9, lane 1), the presence of an excess of oligonucleotide containing a wild-type ATF binding site (WT. OLIGO, lane 2) or an oligonucleotide containing a mutated ATF binding site (MUT. OLIGO, lane 3). An aliquot of the translation shown in lane 1 was then treated with RNase A to prevent further translation and either analysed directly on the gel (lane 4) or incubated with fresh RRL in the presence of WT. OLIGO (lane 5) or MUT. OLIGO (lane 6) for 30 minutes prior to gel analysis.

Figure 9. Effect of DNA-binding on phosphorylation of ATF1 in RRL. \(^{35}\)S-
labelled ATF1 was produced by in vitro translation in RRL in absence of DNA
(lane 1), 100ng of an oligonucleotide containing a consensus ATF-binding site
(WT. OLIGO, lane 2) or 100ng of an oligonucleotide containing a mutated ATF
binding site (MUT. OLIGO, lane 3). An aliquot of the translation shown in lane
1 was then treated with RNase A to prevent further translation and either analysed
directly on the gel (lane 4) or incubated with fresh RRL in the presence of WT.
OLIGO (lane 5) or MUT. OLIGO (lane 6) for 30 minutes prior to gel analysis.
oligonucleotide (lane 5). Addition of fresh lysate increases the overall state of phosphorylation in the presence of mutant oligonucleotide (compare lanes 4 and 5) and as expected this increase is prevented by wild type oligonucleotide (compare lanes 5 and 6). Importantly the presence of the wild type oligonucleotide does not result in dephosphorylation of ATF1 (compare lane 4 with 6). We conclude therefore that binding of ATF1 to its cognate DNA sequence markedly reduces phosphorylation by kinases present in RRL.

**DISCUSSION**

We have identified many requirements for ATF1 phosphorylation in vitro by protein kinases present in RRL. Because several serine point mutations have similar effects on the conformation of ATF1 in vitro and in vivo it is likely that the important residues identified using the in vitro assay are important for ATF1 conformation in vivo. It is clear however that the pattern of ATF1 phosphorylation is highly complex and our experiments do not address the important question concerning the protein kinases involved in vivo. Besides the effect of serine 4 phosphorylation, additional phosphorylation sites may have a related effect on the conformation of ATF1. We have provided indirect evidence that phosphorylation of serines 5 and 6 can alter the conformation of ATF1 and it is of interest that serine 6 is a potential site for the DNA-dependent protein kinase (40) which can phosphorylate ATF1 in vitro (K.L and S.J unpublished data). However our data do not rule out the possibility that the effects of mutating serines 5 and 6 are due to adventitious structural changes in this critical region of ATF1. In summary, we conclude that the region of ATF1 containing serines 4, 5 and 6 is most important for the phosphorylation-induced conformational change of ATF1 in RRL. Because serines 4, 5 and 6 are adjacent serines and because mutation of these serines mimics the effect of phosphatase treatment, we interpret our results to indicate that phosphorylation of serines 4, 5 and 6 almost certainly accounts for the induced conformational change of ATF1.

CK2 can phosphorylate serine 4 in vitro and this phosphorylation is correlated with the mobility shift of ATF1 on SDS gels. Thus phosphorylation of serine 4 appears to have a dramatic effect on ATF1 conformation, indicating that this site is likely to be important for ATF1 activity. The observation that CK2 can phosphorylate ATF1 in vitro raises the question of the in vivo role of CK2. CK2 is a ubiquitous, predominantly nuclear kinase (41) with a wide spectrum of proposed physiological substrates (1, 42, 43) including several transcription factors (44, 45, 46). However, CK2 has not been shown to be stringently regulated in different cell types and it would therefore be surprising if regulation of CK2 activity per se contributed to the cell-type specific phosphorylation of ATF1 observed in vivo (22). If CK2 does phosphorylate ATF1 in vivo, cell type differences could reflect differences in phosphatase activity or result from interactions of ATF1 with DNA or other bZIP proteins as discussed below.

Since serines 4, 5 and 6 map to the region with homology to CREB that is highly phosphorylated and required for full transcriptional activity (6, 18) it seems likely that these serines will be important for ATF1 transcriptional activity. However we were unable to detect any effect of mutating serines 4, 5 and 6 on activity of a gal4/ATF1 fusion protein in vivo. It is possible that the effect of mutations is masked by the ability of gal4/ATF1 to recruit endogenous bZIP proteins (notably CREB or ATF1) to the promoter. In this regard it is significant that when fused to gal4 the activity of ATF1 is decreased 10-fold by deletion of the leucine zipper whereas the activity of CREB is independent of the leucine zipper (26). Thus it would appear that ATF1 must be dimerised either to itself or to another bZIP protein to efficiently activate transcription. In addition, because we have shown that homodimerisation is required for ATF1 phosphorylation in vitro it may therefore not be possible to test the effects of mutations in vivo in the absence of the ATF1 leucine zipper.

Several observations suggest that potential functional effects of ATF1 phosphorylation within the PDE1 homology may not be related to similar phosphorylations occurring in CREB. First, phosphorylation of the PDE1 region in CREB is proposed to be dependent upon prior phosphorylation at the PKA site (6) while phosphorylation of ATF1 in RRL is not. Second, CREB can be activated by Glycogen Synthase Kinase-3 (GSK-3) via a phosphorylation within PDE1 (C.Fiol and O.Andrisani, unpublished results) and the GSK-3 phosphoacceptor site (serine 129 in CREB) is not conserved in ATF1. Third, serine 4 (phosphorylation of which is correlated with the conformational change of ATF1) is not conserved in CREB. Thus despite their close homology within the P-box, some of the phosphorylation events that regulate ATF1 and CREB are likely to be distinct. The degree of conservation within the P-box for ATF1, CREB and CREM is consistent with this idea (see figure 3). The C-terminal region of the P-box (including the PKA site and PDE2) is highly conserved whereas the N-terminal region is much less so. We therefore suggest that while the rudimentary function of the P-box is likely to be the same for ATF1 and CREB, distinct phosphorylation events may confer unique properties to either protein. To address this issue it is clearly of prime importance to develop assays for ATF1 transcriptional activity in vivo. In this connection our finding that the bZIP domain of ATF1 can be substituted by a heterologous bZIP domain without affecting phosphorylation, should enable the design of novel hybrid proteins that circumvent the problem of interference from relatives of ATF1.

Despite their apparently identical DNA-binding specificity CREB and ATF1 bind to DNA with markedly different stability. Binding of ATF1 is very unstable and this characteristic is dependent on long range interactions between the bZIP domain and the NTR (26). Our results show that unstable binding of ATF1 is dependent on the integrity of serines 4, 5 and 6 and is therefore likely to result from the phosphorylation-induced conformational change. This interpretation is supported by the observation that the NTR is required for unstable binding of ATF1 (26) and for the phosphorylation-induced conformational change. Interestingly there is a dynamic interplay between phosphorylation and DNA-binding in vitro since ATF1 binding is inhibitory to phosphorylation by protein kinases present in RRL. A likely interpretation of this is that the kinase(s) present in RRL do not recognise the DNA-bound conformation of ATF1. Since our data indicates that underphosphorylated ATF1 (in the form of SM456) binds more stably to DNA than does phosphorylated ATF1, equilibrium will favour accumulation of unphosphorylated ATF1 bound to DNA. Such an effect has recently been shown to occur in vivo for c-jun. Phosphorylation of three sites adjacent to the DNA-binding domain of c-jun impairs DNA-binding (11, 45) and phosphorylation is decreased as a function of the intracellular concentration of specific DNA target sites (39). We can only speculate on the consequences of
the effects of DNA binding on ATF1 phosphorylation contingent on whether ATF1 is activated or inactivated by phosphorylation. Reversal of phosphorylation upon DNA-binding might provide a mechanism for attenuation of signals transmitted via ATF-1 phosphorylation. Alternatively dephosphorylation may activate ATF1 thus coupling transcriptional activation and DNA binding.

ATF1 phosphorylation is altered by homodimerisation, indicating that accessibility of the phosphorylation sites is altered due to homodimerisation. This raises the intriguing possibility that phosphorylation of ATF1 may be influenced by other dimerisation partners. ATF1 is known to form heterodimers with CREB in vivo (22) but different ATF1 phospho variants appear to associate equally with CREB (22). ATF1 almost certainly dimerises with other bZIP proteins and the number of potential partners is quite large and includes multiple forms of CREB and CREM. In summary our data indicate that the phosphorylation state of ATF1 is likely to be influenced by a variety of factors including cell type, the level of occupancy of target promoters and the availability of particular dimerisation partners. Moreover, the phosphorylations that we have described appear to be unique to ATF1 and are therefore likely to determine some specialised function(s) of this member of the ATF family.

ACKNOWLEDGEMENTS

We are grateful to Jim Woodgett for supplying purified CK2, Daryl Pappin for peptide sequencing and Helen Hurst for ATF1 antibody. We also thank John Diffley, Noel Lowndes and Rick Wood for useful comments on the manuscript and Helen Hurst and Nic Jones for discussions during the course of this work.

REFERENCES