ATF1 and CREB Trans-Activate a Cell Cycle Regulated Histone H4 Gene at a Distal Nuclear Matrix Associated Promoter Element†

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ABSTRACT: Proteins of the ATF/CREB class of transcription factors stimulate gene expression of several cell growth-related genes through protein kinase A-related cAMP response elements. The promoter activity of cell cycle regulated histone H4 genes is regulated by at least four principal cis-acting elements which mediate G1/S phase control and/or enhancement of transcription during the cell cycle. Using protein−DNA interaction assays we show that the H4 promoter contains two ATF/CREB recognition motifs which interact with CREB, ATF1, and ATF2 but not with ATF4/CREB2. One ATF/CRE motif is located in the distal promoter at the nuclear matrix-associated Site IV, and the second motif is present in the proximal promoter at Site I. Both ATF/CRE motifs overlap binding sequences for the multifunctional YY1 transcription factor, which has previously been shown to be nuclear matrix associated. Subnuclear fractionation reveals that there are two ATF1 isoforms which appear to differ with respect to DNA binding activity and partition selectively between nuclear matrix and nonmatrix compartments, consistent with the role of the nuclear matrix in regulating gene expression. Site-directed mutational studies demonstrate that Site I and Site IV together support ATF1- and CREB-induced trans-activation of the H4 promoter. Thus, our data establish that ATF/CREB factors functionally modulate histone H4 gene transcription at distal and proximal promoter elements.

Activating transcription factors (ATF) and the cAMP response element-binding protein (CREB) are basic "leucine zippers" containing transcription factors that recognize the cAMP response element (CRE) (*1*). The ATF/CREB class of proteins forms homodimers or heterodimers and can also dimerize with fos/jun proteins of the AP1 family (*2*). CREB and ATF1 are highly related on the basis of sequence homology and similarities in phosphorylation potential (*3*−*8*). ATF/CREB factors and cAMP dependent phosphorylation pathways are components of gene regulatory signaling mechanisms that support cell proliferation and cell cycle control. It has been shown that cAMP-activated protein kinase activity associates with the cyclin-dependent kinase CDK1/cdc2, which induces cell cycle arrest at G1 phase (*9*, *10*). Inhibition of cAMP-dependent protein kinase A (PKA) in mammalian fibroblasts induces cell mitosis and nuclear envelope breakdown (*11*). Furthermore, a cAMP responsive element located in the proximal promoter of the cyclin A gene induces expression at the G1/S phase transition (*12*, *13*). These data strongly suggest that ATF/CREB transcription factors are involved in cell cycle control by directly or indirectly regulating cell cycle related genes. In this study we focus on the contribution of ATF/CREB family members to transcriptional control of cell cycle dependent histone genes.

The histone multigene family encodes five distinct nucleosomal proteins which are essential for chromatin function, and histone gene promoters represent paradigms for cell cycle control of transcription. Histone gene transcription occurs constitutively throughout the cell cycle, is upregulated upon entry into S phase, and downregulated in quiescent cells or at the onset of differentiation (*14*). These changes in histone gene promoter activity occur concomitant with modulations in histone gene transcription factors (*15*−*20*). Cell cycle dependent promoter activity is determined by the integration of signals at distinct elements by cognate transcription factors. Functional elements that are critical for maximal expression of histone genes during the cell cycle have been delineated in the promoter regions of histone H1, H2B, H3, and H4 genes (*21*−*30*). Interestingly, ATF/CRE motifs are present in several histone gene promoters (*31*−*33*). However, the functional significance of these elements and the role of the ATF/CREB class of factors in regulating histone gene transcription remain to be established.

The human H4 gene promoter has been studied extensively and contains four principal protein−DNA interacting domains, designated Sites I, II, III, and IV. There are two ATF/CRE sequence motifs in the H4 promoter at Site I (*28*) and Site IV (*34*). Mutational analysis of the ATF/CRE motif in Site I suggests that this element is important for maximal histone H4 promoter activity (*28*), but the overall functional contribution of ATF/CREB factors to H4 promoter activity has not been characterized. Interestingly, Site IV has been shown to represent a nuclear matrix attachment region (*35*), and interacts with the nuclear matrix-associated protein NMP-1/YY1 (*34*); the nuclear matrix is the filamentous, nonchromatin scaffold of the nucleus (*36*). Hence, the binding of factors to Site IV occurs within the context of histone H4 gene−nuclear matrix interactions.

In this study, we have established that ATF1, ATF2, and CREB, but not CREB2/ATF4, are capable of interacting with

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the ATF/CRE motif at Site IV. Interestingly, our data show two forms of ATF1 partition differentially between the nuclear matrix and nonmatrix compartment. Mutational analysis of the ATF/CRE motifs at Sites I and IV reveals that both elements are required for maximal induction of H4 promoter activity by ATF1 and CREB. Thus, ATF1 and CREB functionally contribute to proliferation-specific control of histone H4 gene transcription.

MATERIALS AND METHODS

Nuclear Extraction and Nuclear Matrix Preparations. Nuclear extracts (*37*) and nuclear matrix proteins (*34*, *38*) were prepared from HeLa S3 cells as described previously and stored at −80 °C. In brief, nuclear matrix proteins were subject to two consecutive detergent extractions to remove the cytoplasm. Subsequently, cells were subject to DNase I and RNase digestion followed by extraction with 0.25 M $(NH_4)_2SO_4$ (i.e. 0.5 M NH_4^+) to remove the nonmatrix chromatin fraction. Nuclear matrix proteins for EMSA and western blot analysis were obtained by dissolving nuclear matrices with 8 M urea. Upon dialysis in storage buffer (25 mM Hepes/NaOH, pH 7.5, 20% glycerol, 100 mM KCl, and 0.2 mM EDTA), intermediate filaments were reassembled and removed by centrifugation to obtain the soluble nuclear matrix fraction.

Recombinant Protein Expression and Purification. ATF1 and ATF2 were expressed in reticulocyte lysates by coupled *in vitro* transcription/translation system (Promega). The plasmids used were pGEM3-ATF1 and pGEM3-ATF2, respectively (generous gift of Michael Green, UMass Medical Center). CREB and ATF4/CREB2 cDNA were cloned into the pGEX-2T vector (Pharmacia) and expressed as GST fusion proteins in the *Escherichia coli* Bl21 strain upon induction with 1.0 mM IPTG for 4 h. GST−CREB and GST−ATF4 were affinity purified from bacterial lysates by batch-binding to glutathione-Sepharose 4B beads.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were carried out as described (*28*) using the 0.5× TBE buffer system (50 mM Tris-HCl, 50 mM borate, and 2 mM EDTA, pH 8.5). The sequences of Site IV, Site I, and ATF consensus oligonucleotides used as probes are shown in Figure 1. To optimize the CRE/ATF interaction, the final concentration of KCl was adjusted to 100 mM in the binding reactions. The antibodies (Santa Cruz Biotechnology) used in supershift assays and western blotting were directed against ATF1 antibody (C41-5.1, IgA), ATF2 (F2BR-1, IgG), CREB (24H4B, IgG), and ATF4/CREB2 (Z5, IgG). All of these antibodies (Santa Cruz Biotechnology) are non-cross-reactive with other ATF/CREB transcription factors.

Plasmid Construction and Mutagenesis. The FO108 histone H4 promoter−chloramphenicol acetyltransferase (CAT) gene fusion constructs −1039/CAT, −586/CAT, −215/CAT, and −215[Imt]/CAT have been described previously (*23*, *28*). For −1039[IVmt]/CAT in which the ATF/CRE motif in Site IV is mutated, selected base substitutions were introduced by PCR-assisted mutagenesis. The Site IV CRE sequence (5′-TGACGTCC) was mutated to create a *Bgl*II recognition sequence (5′-aGAtcTCC). The mutated Site IV sense strand was used as forward primer coupled with a reverse primer containing an *Avr*II site matching the downstream vector backbone. These primers produce the

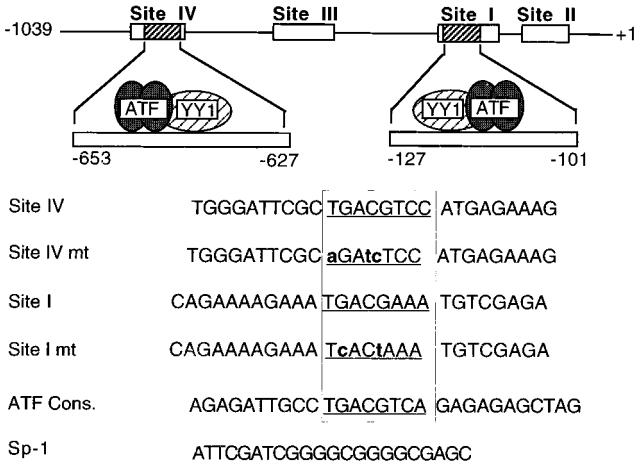


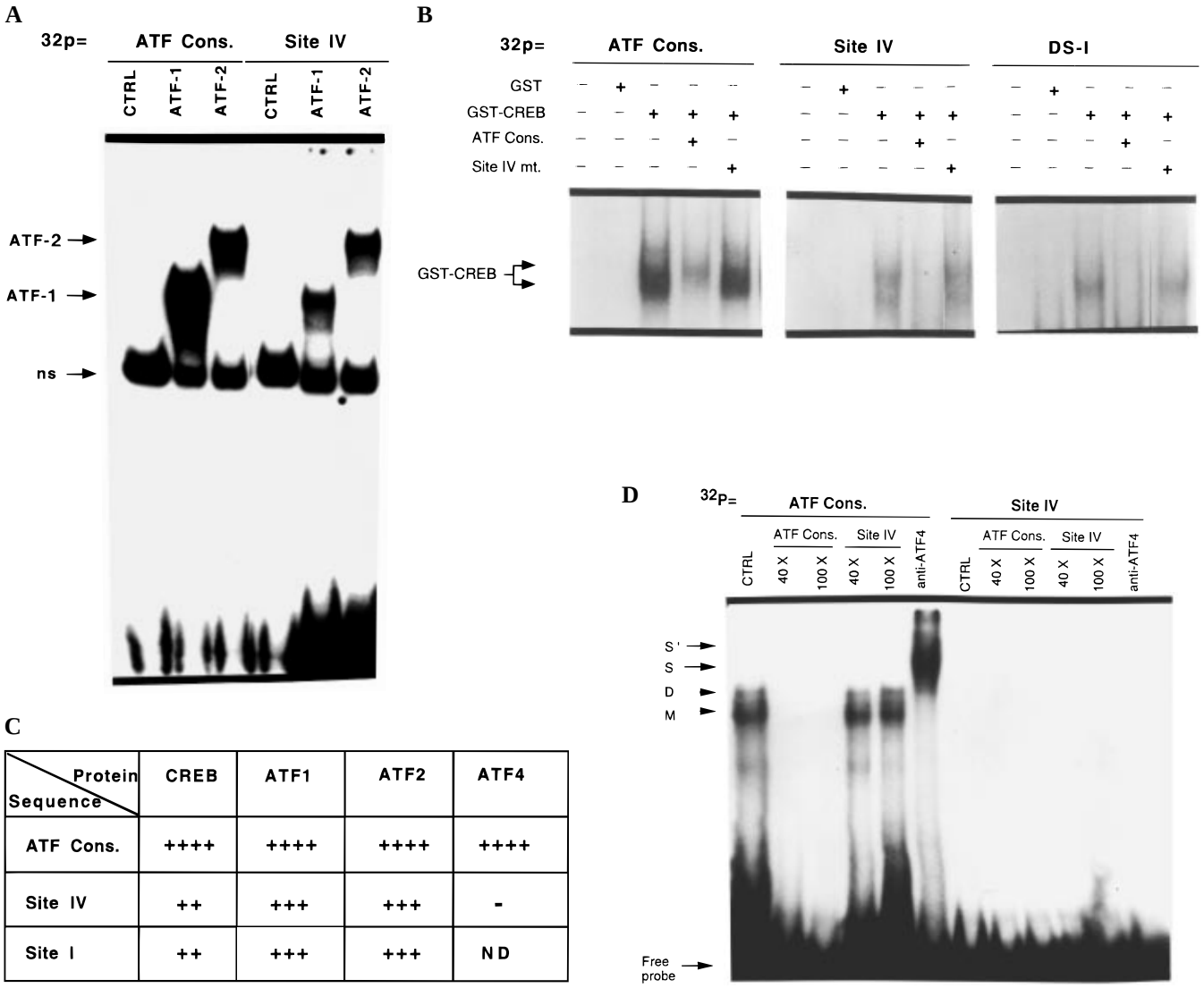
FIGURE 1: Diagram of the human histone H4 promoter (top) and sequences of oligonucleotides used in this study (bottom). The human H4 gene promoter contains four principal protein−DNA interacting domains that regulate histone H4 gene transcription (*45*): Sites I, II, III, and IV (rectangles). There are two ATF/CRE motifs in the H4 promoter, one of these motifs (5′-TGACGAAA, nt −117 to −110) is located in the proximal promoter (*28*), and the second motif (5′-TGACGTCC, nt −643 to −636) is located within Site IV in the distal promoter (*34*). The locations of these motifs in Sites I and IV are indicated by the hatched boxes. The DNA sequences of the oligonucleotides represent the sense strand. Sequences are aligned relative to the ATF/CRE motif (underlined) and nucleotide substitutions (bold face and lowercase) in Site I (Site I mt) and Site IV (Site IV mt) are indicated.

downstream promoter fragment between Site IV and the CAT gene by PCR. The mutated antisense strand was used as a reverse primer coupled with a forward primer which matches the upstream multiple-cloning site containing a *Kpn*I site. These primers produce the promoter fragment upstream of Site IV. The downstream and upstream PCR products were subject to *Avr*II/*Bgl*II and *Kpn*I/*Bgl*II digestion, respectively, and ligated to the −1039/CAT backbone digested with *Avr*II/*Kpn*I. The resulting clones were analyzed for the correct orientation and presence of the mutations by restriction enzyme digestion and sequencing.

Transient Transfection Assays. COS7, CV-1, or HeLa cells were plated in 6-well flat bottom plates (Corning) on the day prior to transfection at a density of 1.0×10^5 cells/35 mm well in 2 mL of DMEM medium. Transfections were performed using the calcium phosphate co-precipitation method as described (*39*). Cells were harvested 48 h following transfection. In each transfection experiment, the RSV-Luc plasmid was co-transfected to express luciferase as internal control. CAT activities were normalized for transfection efficiency using luciferase activity and are presented as percentage of conversion/relative light units. Each experiment was repeated at least three independent times with triplicate samples.

RESULTS

Recombinant ATF1, ATF2, and CREB but Not ATF4/CREB2 Bind to Site IV. Our recent studies on the histone H4 promoter revealed that the H4 gene regulatory domains Site I and Site IV each contain ATF/CRE motifs (Figure 1). The presence of these motifs in Sites I and IV suggests that ATF/CREB factors may interact at both distal and proximal sites in the histone H4 gene promoter. Our first objective was to assess directly whether members of the



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Protein / Sequence	CREB	ATF1	ATF2	ATF4
ATF Cons.	++++	++++	++++	++++
Site IV	++	+++	+++	-
Site I	++	+++	+++	ND

FIGURE 2: Protein−DNA interactions of recombinant CREB, ATF1, ATF2, and ATF4/CREB2 with different ATF/CREs motifs. (A) Binding of ATF1 and ATF2 to ^{32}P-labeled probes spanning the ATF consensus and Site IV was assessed by EMSAs using rabbit reticulocyte lysates programmed with constructs encoding ATF-1 or ATF-2, or lysate without *in vitro* expression plasmid (CTRL). The arrows indicate protein−DNA complexes mediated by ATF1 and ATF2; ns represents a nonspecific complex. (B) Interactions of CREB (forked arrow) with probes spanning the ATF consensus, Site IV, and distal Site I (DS-I) (as indicated above the lanes) were analyzed using EMSAs with affinity-purified GST−CREB fusion protein, or GST protein alone. Competition analysis was performed with a 100-fold excess of unlabeled ATF consensus (ATF Cons.) or mutant Site IV (Site IV mt.) oligonucleotides. Presence or absence of each protein or DNA component in the binding reactions is indicated by plus/minus signs. (C) Summary of protein−DNA interaction results of ATF/CREB factors binding to different ATF/CRE motifs as established by EMSAs. The ability (+) or inability (−) of ATF/CREB factors to bind specific ATF/CRE motifs is indicated; the number of plus signs reflects the relative strength of binding. The table also incorporates data from a previous study (*28*); ND, not determined. (D) Binding of ATF4/CREB2 to ^{32}P-radiolabeled oligonucleotides spanning the ATF consensus and Site IV (as indicated above the lanes) was monitored using purified GST−ATF4 fusion protein (CTRL). Competition analysis was performed with 40- or 100-fold molar excess of unlabeled wildtype (ATF Cons.) and mutant Site IV (Site IV mt.) oligonucleotides. The identity of the GST−ATF4 complex was confirmed by formation of supershift complexes (S and S′) in the presence of an anti-ATF4 antibody. ATF-4 complexes (M and D) are observed with the ATF consensus, but not the Site IV probe.

ATF/CREB family can bind to Site IV. We performed electrophoretic mobility shift assays (EMSAs) using oligonucleotides spanning the ATF/CRE motifs of Site IV (5′-TGACGTCC), Site I (5′-TGACGAAA), and the ATF/CRE consensus element (5′-TGACGTCA) as probes. The results show that recombinant ATF1 and ATF2 (Figure 2A) and CREB (Figure 2B) each clearly bind to Site IV, although these three proteins have higher affinity for the ATF/CRE consensus element. By contrast, ATF4/CREB2 can only bind to the palindromic ATF/CRE consensus but does not recognize the asymmetrical Site IV motif (Figure 2D). The combined results of *in vitro* protein−DNA interaction analyses (Figure 2C) indicate that selected ATF/CREB factors specifically recognize the ATF/CRE motifs in the histone H4 promoter.

Endogenous ATF/CREB Complexes from HeLa Cells Interact with Site IV. To examine whether endogenous ATF/CREB factors from HeLa cervical carcinoma cells interact with the histone promoter, we performed EMSAs using HeLa nuclear extracts. Figure 3 shows that probes spanning the ATF/CRE consensus motif or Site IV form three distinct ATF/CREB−related protein−DNA complexes with nuclear proteins from HeLa cells. To identify the components of the three complexes, we used a panel of antibodies raised

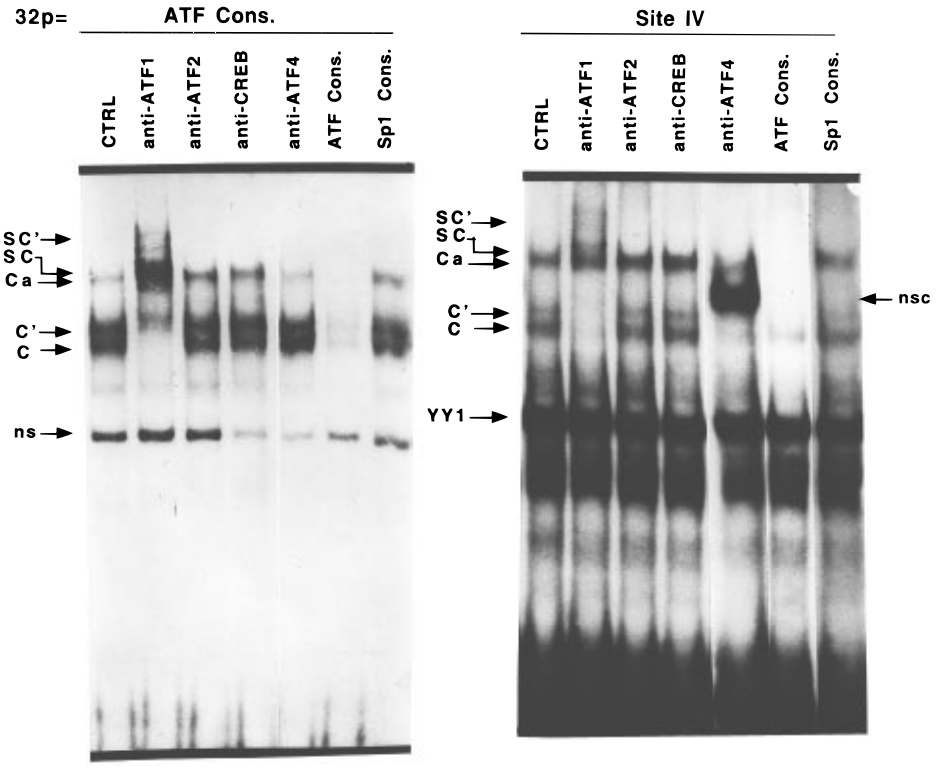

FIGURE 3: Detection of endogenous ATF/CREB proteins from HeLa S3 cells interacting with the ATF consensus and Site IV. EMSAs were performed with HeLa nuclear extracts in the presence of the ATF consensus (left) or Site IV (right) probe. Specificity of HeLa protein−DNA complexes (C, C′, and Ca) for each probe was established by competition with a 40-fold molar excess of specific (ATF Cons.) or nonspecific (Sp1 Cons.) unlabeled competitor DNAs (indicated vertically). Immunoreactivity was analyzed with a panel of specific ATF/CREB antibodies against ATF1, ATF2, CREB, and ATF4 which were pre-incubated with nuclear extract prior to the addition of probe. C and C′ are ATF1-containing complexes, and Ca refers to an unidentified ATF/CREB-related complex. SC and SC′ represent the supershifted complex formed by antibody binding to complex C and C′, respectively. The interaction of YY1 to Site IV (right panel) (*34*) is not affected by the antibodies. Addition of the ATF4 antibody to Site IV results in formation of a prominent antibody related nonspecific complex (nsc). Formation of this complex is Site IV dependent, because it is not observed with the ATF consensus probe (left panel) and competes specifically with the Site IV oligonucleotide (data not shown).

against ATF/CREB proteins by EMSA immunoanalysis. Positive controls were included to test immunoreactivity for most of these antibodies (i.e., ATF-1, ATF-2, ATF-3, and ATF-4), in addition to the quality controls by the supplier (Santa Cruz Biotechnology). These experiments involved binding reactions containing bacterially expressed ATF proteins, the cognate antibodies, and the ATF consensus oligonucleotide. We observed that each batch of antibody was active and capable of supershifting the corresponding ATF factor (Figure 2D and data not shown).

Incubation of nuclear proteins with a monoclonal ATF1 antibody (C41-5.1) results in a specific supershift of only the two lower gel shift bands (complexes C and C′). The lowest band (complex C) co-migrates with the ATF1 homodimer (data not shown). Thus, Site IV displays a protein−DNA interaction pattern (Figure 3B) that is highly similar to that observed for probes spanning Site I (*28*) and the ATF/CRE consensus element (Figure 3A): each probe mediates formation of three ATF/CREB-related complexes with similar mobility patterns, and two of these complexes contain ATF1. Interestingly, binding of CREB, ATF2, ATF3, and ATF4/CREB2 proteins from HeLa cells to the ATF/CRE consensus element, Site IV or Site I is below the level of detection under our experimental conditions [(*28*) and data not shown]. Thus, occupancy of Sites IV and I may depend on the cellular abundance of ATF/CREB factors and/or their relative affinities for ATF/CRE motifs in the histone H4 promoter.

Site IV and Site I Mediate ATF1- and CREB-Induced Trans-Activation. Mutation of the ATF/CRE motif in Site I reduces the basal transcriptional activity of the proximal histone H4 promoter (up to nt −215) in HeLa cells (*28*), suggesting that ATF/CREB family members play an important role in augmenting histone H4 gene transcription. Because endogenous ATF1 complexes from HeLa cells are capable of interacting with the ATF/CREs of the histone H4 promoter, we tested the transactivation potential of ATF1 *in vivo*. Co-transfection experiments were carried out with COS7 using pRSV-ATF1, which constitutively expresses ATF1 under control of the Rous sarcoma virus promoter, and a chimeric reporter gene construct in which the full-length histone H4 promoter (up to nt −1039) is fused to the CAT reporter gene (−1039/CAT) (Figure 4A). Over-expression of ATF1 in COS7 cells results in a 2.5-fold increase in H4 promoter activity (Figure 4A). Similar results were obtained in transfection experiments with CV1 and HeLa cells (data not shown). Because CREB and ATF1 are highly homologous transcription factors (70% amino acid identity) and share extensive similarity in the basic-zipper DNA binding domain as well as the cAMP response domain (greater than 90% identity), we also tested in parallel the potential of CREB to trans-activate the histone H4 gene promoter. Over-expression of CREB enhances the activity of the H4 promoter 3.4-fold. These results establish that ATF1 and CREB each can trans-activate the histone H4 promoter.

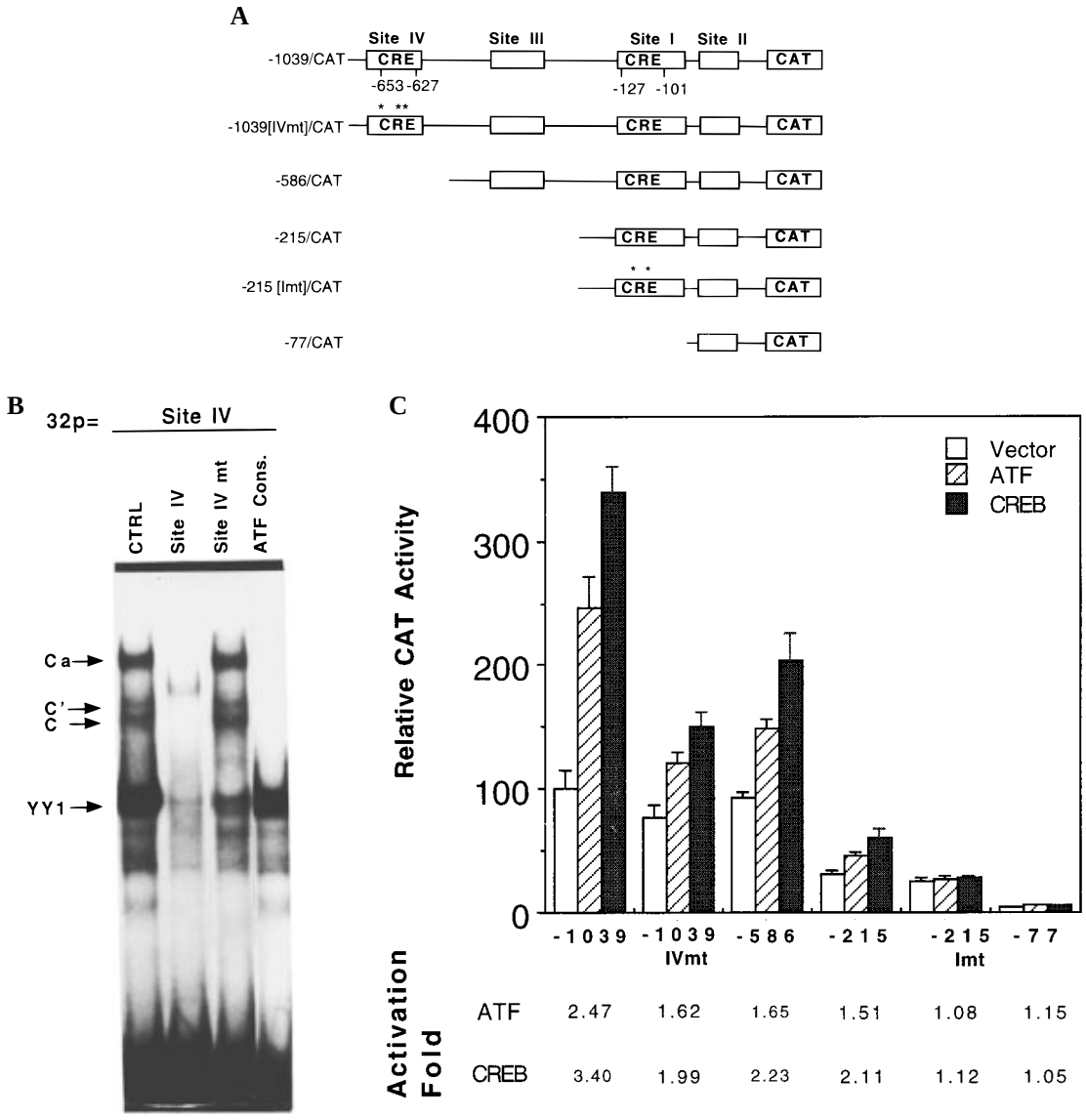

FIGURE 4: Functional contribution of ATF/CRE motifs in Sites IV and I to ATF1 and CREB induction of histone H4 promoter activity. (A) Schematic representation of histone H4 promoter/CAT reporter gene constructs. The full-length wild-type promoter spans the histone H4 promoter up to nt −1039, including Sites I, II, II, and IV (open rectangles). Also indicated are promoter deletions (−586/CAT, −215/CAT and −77/CAT) and mutant promoters with nucleotide substitutions (asterisks) in Site IV (−1039[IVmt]/CAT) or Site I (−215[Imt]/CAT). (B) Mutation of the ATF/CRE motif in Site IV selectively abrogates binding of ATF/CREB proteins but does not affect YY1 binding. EMSAs were performed by incubating HeLa nuclear extract with the Site IV probe. Competition analysis was carried out with a 40-fold molar excess of unlabeled competitor DNAs (i.e., Site IV, Site IVmt, and the ATF consensus) as indicated above the lanes. (C) Transfection results obtained with histone H4 promoter/CAT reporter gene constructs (see panel A) upon transfection in COS7 cells. The relative level of CAT activity is shown as a percentage of the activity obtained with the wild-type H4 promoter (−1039/CAT). Error bars represent the standard deviation of three independent triplicate experiments. The activation of H4 promoter activity by ATF1 or CREB is shown below.

To examine whether the ATF/CRE motif of Site IV supports ATF1 and CREB mediated trans-activation of H4 gene transcription, we introduced nucleotide substitutions into the H4 promoter (nt −1039/CAT) by PCR-assisted mutagenesis. The wild-type Site IV ATF/CRE sequence, 5′-**TGACGTCC**ATG, was changed to 5′-**aGAtcTCC**ATG (the ATF/CRE motif is shown in bold, mutations are in lowercase, and the YY1 core motif is underlined). The mutation affects critical residues of the ATF/CRE element but does not alter the YY1 binding core motif in Site IV (*34*). The effect of this Site IV mutation (Site IVmt) on ATF/CREB binding was analyzed by oligonucleotide competition assays in EMSAs. The results demonstrate that the Site IVmt oligonucleotide does not compete with wild-type Site IV for binding to ATF/CREB proteins (Figures 3B and 4B) but still competes with wild-type Site IV for binding to YY1. Hence, these findings establish that the Site IV mutation selectively abrogates the ATF/CREB interaction with Site IV.

The transcriptional consequences of the Site IV mutation were functionally analyzed in the context of the H4 promoter (−1039[IVmt]/CAT construct) by transient co-transfections with ATF1 and CREB separately into COS7 cells. Transactivation of ATF1 and CREB was decreased to 1.6-fold and 2.0-fold, respectively (Figure 4C), upon mutation of the ATF/CRE motif in Site IV (i.e., comparing −1039/CAT with −1039[IVmt]/CAT). Similarly, deletion of sequences upstream of Site I (−586/CAT), which removes distal H4 promoter sequences including the entire Site IV, results in significant reduction of the ATF1 and CREB dependent acti-

vation of H4 gene transcription (i.e., comparing the activities of −1039/CAT and −586/CAT in Figure 4c). Therefore, the ATF/CRE motif in Site IV is required for maximal responsiveness to ATF1 and CREB.

Further deletion of H4 promoter sequences including Site III (−215/CAT) reduces H4 gene transcription but does not eliminate ATF/CRE responsiveness. However, additional deletion of proximal promoter sequences encompassing Site I (−77/CAT) totally abolishes ATF1 and CREB dependent activation of H4 promoter activity (i.e., 1.1-fold for ATF1 and 1.0-fold for CREB) (Figure 4C). To address the role of the ATF/CRE motif in Site I, we mutated this sequence within the context of the histone H4 proximal promoter (−215[Imt]/CAT construct). The results show that this mutation in Site I eliminates the ATF1 and CREB responsiveness of the H4 proximal promoter (i.e., 1.1-fold induction for both ATF1 and CREB) (Figure 4C). We conclude that the two ATF/CRE motifs in Sites IV and I are critical components of gene regulatory signaling mechanisms that determine transcriptional activity of the H4 promoter in response to the cellular levels of ATF1 and CREB.

ATF1 and CREB represent nuclear transducers of the protein kinase A (PKA)-mediated and cAMP-dependent signal transduction pathway. ATF1 and CREB contain PKA phosphorylation motifs (Figure 5A), and phosphorylation of ATF1 and CREB modulates the transcriptional activities of these factors. We addressed the question whether the cAMP signaling pathway is involved in regulating histone H4 gene transcription. The −1039/CAT construct containing the full-length histone H4 promoter was co-transfected with either ATF1 or CREB into CV-1 cells which were treated with the PKA activator forskolin (10 μM) for 6 h (*40*). The results show that forskolin has only modest effects on histone H4 gene transcription under these experimental conditions (Figure 5B). Thus, regulation of histone gene transcription by ATF1 and CREB may not depend on cAMP-mediated phosphorylation events.

Different Forms of ATF1 Are Associated with the Nuclear Matrix. Histone H4 promoter Site IV is a composite promoter domain (Figure 1) which contains a binding site for the multifunctional factor YY1 overlapping the ATF/CRE motif, 5′-**TGACGTCC**ATG (the ATF/CRE motif is shown in bold, and the YY1 core motif is underlined). We have shown that Site IV represents a nuclear matrix attachment region (*35*) and that YY1 is associated with the nuclear matrix (*34*). Our studies presented here show that ATF1 is capable of binding to the ATF/CRE motif in Site IV (Figure 2). Regulation of histone gene transcription may occur by changes in the levels and/or activities of its cognate regulatory factors as well as perhaps by post-translational modifications and/or subnuclear location of histone gene promoter factors (*41*). To assess whether ATF1 is associated with the nuclear matrix, we monitored the nuclear localization of ATF1 by western blot analysis of nuclear matrix and nonmatrix (nuclear extract) fractions (Figure 6A).

As control for detection of ATF1, we analyzed recombinant ATF1 expressed in rabbit reticulocyte lysates. This recombinant ATF1 is detected as two different bands in western blot analysis. These forms are generated by lysate dependent phosphorylation of ATF1 on serine 36, 38, and/or 41, which has previously been shown to cause conformational changes resulting in a reduction of the electrophoretic mobility of ATF1 (*42*). The cAMP-dependent phosphorylation of Ser-63 by PKA, which influences transcriptional activity of ATF1, does not result in conformational changes (*42*). Western blot analysis of subnuclear fractions from HeLa cells with the same ATF1 antibody (C41-5.1) that was used in the supershift assays reveals that ATF1 is detected in both the nuclear extract and the nuclear matrix fractions. However, the ATF1 forms detected in these fractions differ in electrophoretic mobility in SDS−PAGE gels. The nonmatrix nuclear fraction (nuclear extract) contains primarily the putative hyperphosphorylated form of ATF1, whereas the putative hypophosphorylated form of ATF1 appears to be exclusively associated with the nuclear matrix.

To address whether the nonmatrix and nuclear matrix associated forms of ATF1 have different activities, we performed EMSAs using the same subnuclear fractions from HeLa cells and Site IV as probe. The results show that the level of ATF1 DNA binding activity present in the nuclear matrix is very low (Figure 6B) compared to ATF1 DNA binding activity in nuclear extracts (i.e., nonmatrix fraction). Hence, the results from our EMSA and western blot analysis together suggest that there are different forms of ATF1 which partition between distinct subnuclear compartments and that these forms may differ in competency for DNA binding.

DISCUSSION

In this study we have shown that the histone H4 promoter contains two functionally relevant ATF/CRE motifs located in Site IV (5′-TGACGTCC, nt −643 to −636 bp) and Site I (5′-TGACGAAA, nt −117 to −110). The asymmetric features of both elements provide the potential for selective recognition by different dimers formed between members of the ATF/CREB class of transcription factors. For example, homodimers and monomers of the ATF/CREB family prefer symmetric DNA sequences. Indeed, our results show that the nonpalindromic ATF/CREs in the H4 promoter, when compared with the perfect palindromic ATF/CRE consensus motif 5′-TGACGTCA, represent relatively weak binding sites for homodimers formed by recombinant ATF1, ATF2, and CREB. Notably, the ATF4 homodimer (ATF4 is synonymous with CREB2) which only recognizes the perfect ATF/CRE palindrome does not bind to the ATF/CRE motif of Site IV, and is not expected to bind to the ATF/CRE motif of Site I. Moreover, our results indicate that Site IV (this study) and Site I (*28*) are both recognized by endogenous ATF1-containing heterodimers. Thus, it appears that the asymmetric ATF/CRE sites in the H4 promoter favor binding of heterodimeric ATF/CREB proteins. Structural differences between ATF/CREB factors may directly relate to the molecular mechanisms by which ATF/CREB factors, and in particular ATF4/CREB2, exhibit selective recognition for palindromic and variant ATF/CRE motifs.

Transactivation by CREB or ATF1 is dependent on the phosphorylation status of each protein. Phosphorylation of serine 118 in CREB or serine 63 in ATF1 (*4*, *7*, *40*, *42*) modulates transcriptional activity and can be achieved by elevating intracellular Ca^{2+} and cAMP concentrations to promote the activity of Ca^{2+} and cAMP-dependent kinases. Our results indicate that forskolin, which raises intracellular cAMP levels, does not influence ATF1 or CREB dependent enhancement of histone H4 gene transcription. It is possible that histone promoter activity is regulated independently of cAMP-related phosphorylation pathways, perhaps due to, for

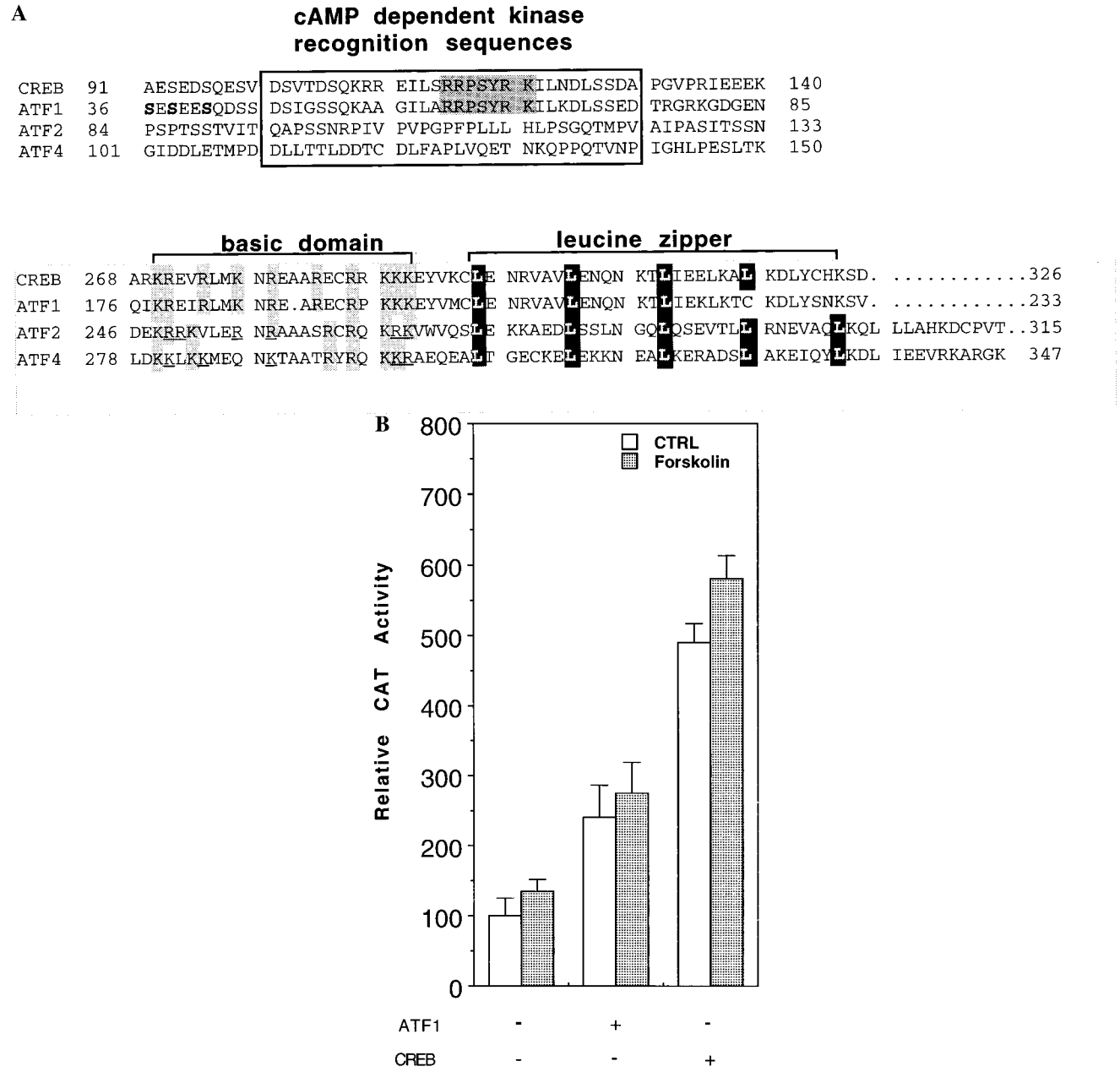

FIGURE 5: (A) Comparison of functional domains in ATF/CREB proteins. Sequences are shown in single-letter amino acid code and numbered on both sides. Motifs related to the cAMP signal response (rectangle), PKA phosphorylation (amino acids on a gray background), and serine residues (aa 36, 38, 41; bold face) that can be phosphorylated and mediate conformational changes of ATF1 (Masson et al., 1993) are indicated. The basic domain and leucine zipper regions of ATF/CREB factors are marked by brackets. Basic residues are shown on a light gray background, and basic residues differing between ATF2 and ATF4 are underlined. Leucines are depicted on a black background. Gaps are inserted for optimal alignment. This amino acid alignment of ATF/CREB factors shows that the DNA binding basic domains and leucine zipper dimerization domains between different ATF/CREB members are highly divergent. For example, ATF4/CREB2 displays the least similarity with CREB in the distribution of basic residues (Arg and Lys), and the number of leucine residues in the zipper region of ATF/CREB factors also varies dramatically (CREB has 4; ATF1 has 3; ATF2 and ATF4/CREB2 have 5). These structural differences between ATF/CREB factors may determine selective recognition for palindromic and variant ATF/CRE motifs. (B) Absence of significant effects of forskolin on histone H4 promoter activity. The H4 promoter reporter −1039/CAT reporter gene construct was co-transfected into HeLa cells with the RSV vector, RSV-ATF1, or RSV-CREB. Cells were treated for with 6 h prior to harvest with 10 μM forskolin or not treated (CTRL). The relative level of CAT activity is shown as a percentage of the activity obtained with the wild-type H4 promoter (−1039/CAT) in the absence of forskolin and expression plasmids encoding ATF1 or CREB.

example, (i) the promoter context of histone gene regulatory elements interacting with other histone-specific promoter factors, (ii) a different requirement for ATF1- and CREB-dependent cofactors, and/or (iii) inhibitory factors that antagonize the cAMP-induced transcriptional activities of ATF1 and CREB. However, we cannot dismiss the possibility that the influence of cAMP-dependent PKA activity on histone H4 promoter activity is strongly cell type or cell cycle stage specific, perhaps due to differences in pre-existing PKA activity and/or pre-activation of ATF1 and CREB by other phosphorylation dependent cell signaling mechanisms.

Site IV was initially identified as a nuclear matrix attachment region (*35*). Subsequently, the cognate nuclear matrix protein NMP-1 was identified as YY1 which partitions between nuclear matrix and nonmatrix ("bulk chromatin") compartments (*34*). Here, we show that ATF1 represents a second Site IV binding protein and that ATF1 protein is

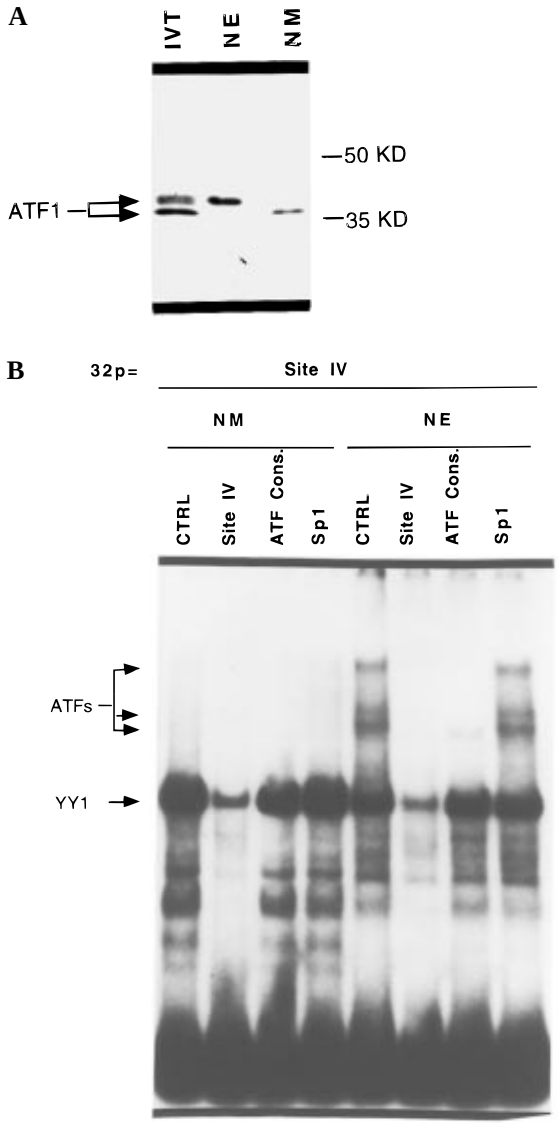

FIGURE 6: Differential partitioning of ATF/CREB proteins between nuclear extract and nuclear matrix compartments. (A) ATF1 protein levels were determined in nuclear extract (NE; 10 μg) and nuclear matrix (NM; 10 μg of protein) fractions by western blot analysis using a monoclonal ATF1 antibody (C41−5.1). For comparison of electrophoretic mobilities, we also included ATF1 protein (10 μl lysate) expressed by coupled *in vitro* transcription/translation (Promega). Because of lysate-dependent phosphorylation of ATF1, ATF1 is detected as two forms (forked arrow) which differ in the extent of phosphorylation (*42*). The position of molecular mass markers (35 and 50 kDa) is shown to the right. (B) ATF/CREB binding activities in the nuclear matrix (NM; 1 μg) and nonmatrix (NE; 1 μg) fractions were determined by EMSAs using the Site IV probe. Arrowheads indicate the locations of ATF and YY1 complexes. Specificity of binding was analyzed with binding reactions performed in the absence (CTRL) or presence of a 40-fold molar excess of unlabeled Site IV, ATF consensus, and Sp1 oligonucleotides as competitors.

present in both the nuclear matrix and the nonmatrix fractions. Western blot analysis reveals that the high-mobility (putative hypophosphorylated) ATF1 form associates with the nuclear matrix, whereas the low-mobility (putative hyperphosphorylated) ATF1 form is primarily present in the nonmatrix nuclear fraction. Results from gel shift assays indicate that the nuclear matrix-bound ATF1 form is less active with respect to DNA binding than the nonmatrix-bound ATF1 form. These data suggest that the nuclear matrix may sequester inactive ATF1 forms to control the intranuclear location and concentration of ATF1, perhaps by a phosphorylation-dependent mechanism.

The differential partitioning of the two ATF1 forms between different subnuclear compartments may be related to the presence of casein kinase II (CKII) in the nuclear matrix (*43*). Casein kinase II is capable of phosphorylating ATF1 (*42*) and may release ATF1 from the nuclear matrix upon receiving spatial and/or physiological cues. Alternatively, CKII activity in the nonmatrix chromatin compartment may phosphorylate ATF1 which would preclude association of this active ATF1 form with the nuclear matrix. These possibilities are consistent with the concept that the nuclear matrix represents a subnuclear compartment for the localized concentration and/or storage of gene regulatory factors (*35*, *37*).

It is possible that ATF1 and CREB may act additively or synergistically. The overlapping arrangements of YY1 and ATF/CRE motifs in Sites I and IV suggest that YY1 and CREB may interact in a mutually exclusive manner. We have recently provided evidence that YY1 attenuates vitamin D responsive transactivation by vitamin D receptor/retinoid X receptor heterodimers (*44*). It will be of interest to assess whether ATF/CREB responsive transcription may be similarly attenuated by YY1.

In conclusion, we have established the functional contribution of two ATF/CRE motifs present in the distal (Site IV) and proximal (Site I) domains of the histone H4 gene promoter. These results provide further evidence for the multiplicity of gene regulatory elements governing the cell cycle controlled transcription of this H4 gene. Both motifs are responsive to ATF1 and CREB in an apparent cAMP-independent manner. This finding indicates functional redundancy which may provide options for transcriptional regulation in different cell types and cell cycle stages. Furthermore, we find that different forms of ATF1 partition between nuclear matrix and nonmatrix nuclear compartments, which suggests that the subnuclear location of ATF1 may contribute to control of ATF1 mediated gene regulatory mechanisms.

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REFERENCES

1. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6682−6686.
2. Hai, T., and Curran, T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3720−3724.
3. Gonzalez, G. A., and Montminy, M. (1989) *Cell 59*, 675−680.
4. Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) *Science 252*, 1427−1430.
5. Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T.R. (1994) *J. Biol. Chem. 269*, 15520−15527.
6. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol. 14*, 6107−6116.
7. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) *Genes Dev. 8*, 2527−2539.
8. Ginty, D. D., Bonni, A., and Greenberg, M. E. (1994) *Cell 77*, 713−725.

9. Tournier, S., Raynaud, F., Gerbaud, P., Lohmann, S. M., Doree, M., and Evain-Brion, D. (1991) *J. Biol. Chem. 266*, 19018−19022.
10. Albert, D. A., Nodzenski, E., Cruz, G. H., Kuchibholtla, J., and Kowalski, J. (1991) *Exp. Cell Res. 197*, 75−81.
11. Lamb, N. J., Cavadore, J. C., Labbe, J. C., Maurer, R. A., and Fernandez, A. (1991) *EMBO J. 10*, 1523−1533.
12. Desdouets, C., Matesic, G., Molina, C. A., Foulkes, N. S., Sassone-Corsi, P., Brechot, C., and Sobczak-Thepot, J. (1995) *Mol. Cell. Biol. 15*, 3301−3309.
13. Barlat, I., Henglein, B., Plet, A., Lamb, N., Fernandez, A., McKenzie, F., Pouyssegur, J., Vie, A., and Blanchard, J. M. (1995) *Oncogene 11*, 1309−1318.
14. Stein, G.S., Stein, J. L., Van Wijnen, A. J., and Lian, J. B. (1994) *J. Cell. Biochem. 54*, 393−404.
15. Shakoori, A. R., van Wijnen, A. J., Cooper, C., Aziz, F., Birnbaum, M., Reddy, G. P., Grana, X., De Luca, A., Giordano, A., Lian, J. B., et al. (1995) *J. Cell. Biochem. 59*, 291−302.
16. van den Ent, F. M., van Wijnen, A. J., Last, T. J., Bortell, R., Stein, J. L., Lian, J. B., Stein, G. S. (1993) *Cancer Res. 53*, 2399−2409.
17. Wright, K. L., Dell'Orco, R. T., Van Wijnen, A. J., Stein, J. L., and Stein, G. S. (1992) *Biochemistry 31*, 2812−2818.
18. van Wijnen, A. J., Choi, T. K., Owen, T. A., Wright, K. L., Lian, J. B., Jaenisch, R., Stein, J. L., and Stein, G. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2573−2577.
19. van Wijnen, A. J., Wright, K. L., Lian, J. B., Stein, J. L., and Stein, G. S. (1989) *J. Biol. Chem. 264*, 15034−15042.
20. Holthuis, J., Owen, T. A., van Wijnen, A. J., Wright, K. L., Ramsey-Ewing, A., Kennedy, M. B., Carter, R., Cosenza, S. C., Soprano, K. J., Lian, J. B., and et al. (1990) *Science 247*, 1454−1457.
21. Dalton, S., and Wells, J. R. (1988) *EMBO J. 7*, 49−56.
22. La Bella, F., Gallinari, P., McKinney, J., and Heintz, N. (1989) *Genes Dev. 3*, 1982−1990.
23. Ramsey-Ewing, A., Van Wijnen, A. J., Stein, G. S., and Stein, J. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4475−4479.
24. Gallinari, P., La Bella, F., and Heintz, N. (1989) *Mol. Cell. Biol. 9*, 1566−1575.
25. Artishevsky, A., Wooden, S., Sharma, A., Resendez, E. J., and Lee, A. S. (1987) *Nature 328*, 823−827.
26. Kroeger, P., Stewart, C., Schaap, T., van Wijnen, A., Hirshman, J., Helms, S., Stein, G., and Stein, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3982−3986.
27. Birnbaum, M. J., van Wijnen, A. J., Odgren, P. R., Last, T. J., Suske, G., Stein, G. S., and Stein, J. L. (1995) *Biochemistry 34*, 16503−16508.
28. Birnbaum, M. J., Wright, K. L., van Wijnen, A. J., Ramsey-Ewing, A. L., Bourke, M. T., Last, T. J., Aziz, F., Frenkel, B., Rao, B. R., Aronin, N., Stein, G. S., and Stein, J. L. (1995) *Biochemistry 34*, 7648−7658.
29. Vaughan, P. S., Aziz, F., van Wijnen, A. J., Wu, S., Harada, H., Taniguchi, T., Soprano, K. J., Stein, J. L., and Stein, G. S. (1995) *Nature 377*, 362−365.
30. Aziz, F., van Wijnen, A. J., Vaughan, P. S., Wu, S., Shakoori, A. R., Lian, J.B., Soprano, K. J., Stein, J. L., and Stein, G. S. (1997) *Mol. Biol. Rep.* (in press).
31. Hinkley, C., and Perry, M. (1991) *Mol. Cell. Biol. 11*, 641−654.
32. Inamdar, N. M., Ehrlich, K. C., and Ehrlich, M. (1991) *Plant Mol. Biol. 17*, 111−123.
33. van Wijnen, A. J., Lian, J. B., Stein, J. L., and Stein, G. S. (1991) *J. Cell. Biochem. 47*, 337−351.
34. Guo, B., Odgren, P. R., van Wijnen, A. J., Last, T. J., Nickerson, J., Penman, S., Lian, J. B., Stein, J. L., and Stein, G. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10526−10530.
35. Dworetzky, S. I., Wright, K. L., Fey, E. G., Penman, S., Lian, J. B., Stein, J. L., and Stein, G. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4178−4182.
36. Penman, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5251−5257.
37. van Wijnen, A. J., Bidwell, J. P., Fey, E. G., Penman, S., Lian, J. B., Stein, J. L., and Stein, G. S. (1993) *Biochemistry 32*, 8397−8402.
38. Fey, E. G., and Penman, S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 121−125.
39. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J.A., and Struhl, K., Eds. (1996) *Current Protocols in Molecular Biology*, pp 9.1.7−9.1.9, John Wiley & Sons, New York, NY.
40. Liu, F., Thompson, M. A., Wagner, S., Greenberg, M. E., and Green, M. R. (1993) *J. Biol. Chem. 268*, 6714−6720.
41. Stein, G. S., Stein, J. L., Lian, J. B., van Wijnen, A. J., and Montecino, M. (1996) *J. Cell. Biochem. 62*, 198−209.
42. Masson, N., John, J., and Lee, K. A. (1993) *Nucleic Acids Res. 21*, 4166−4173.
43. Tawfic, S., Faust, R. A., Gapany, M., and Ahmed, K. (1996) *J. Cell. Biochem. 62*, 165−171.
44. Guo, B., Aslam, F., van Wijnen, A. J., Roberts, S. G. E., Frenkel, B., Green, M., DeLuca, H., Lian, J. B., Stein, G. S., and Stein, J. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 121−126.
45. Stein, G. S., Stein, J. L., van Wijnen, A. J., and Lian, J. B. (1996) *Cell Biol. Int. 20*, 41−49.

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