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The CRE-binding factor ATF-2 facilitates the occupation of the CCAAT box in the fibronectin gene promoter

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The cAMP response element (CRE) and the CCAAT box of the fibronectin gene promoter are separated by only twenty base pairs. A specific factor that binds the CRE interacts cooperatively with the protein which binds to the adjacent CCAAT box, stimulating transcription [1992, J. Biol. Chem. 267, 12767–12774]. Here we show that the CRE factor is an heterodimer between a 43 kDa and the '73 kDa' CRE-binding proteins and we identify the latter as ATF-2 (also named CRE-BPI), a protein implicated in recruiting transcriptional activators to promoters, able to form heterodimers with Jun and for which a sequence-deduced MW of 55 kDa had been previously reported.

CRE; CAAT box; Fibronectin gene promoter; ATF-2

1. INTRODUCTION

The expression of fibronectin (FN), the multifunctional-multidomain adhesive glycoprotein [1,2], is regulated at the transcriptional level by cyclic AMP [3–5]. Cyclic AMP-dependent phoshorylations activate members of the CREB/ATF family of DNA binding proteins [6–8]. This family includes polypeptides encoded by at least seven distinct genes [6,9], that share the ability to bind, with different affinities, to the CRE (cyclic AMP response element, 5′-TGACGTA-3′) present in the promoter regions of genes activated by cAMP [10–13]. A wide range of CREB/ATF dimers can be formed as a consequence of the diversity of monomers. This diversity is not only generated by gene multiplicity, but also by alternative splicing [14], the degree of phosphorylation and the possibility to form heterodimers with the structurally related members of the Jun and Fos families [15,16].

In the FN gene promoter the CRE and the CCAAT box are separated by only twenty base pairs, i.e. two turns of double helix. We have recently demonstrated that there is a molecular interaction between the liver transcription factors that bind to these two elements and that this interaction stimulates transcription [17]. We have also shown that the binding to the FN-CRE of a factor having a 73 kDa CRE-binding polypeptide, but lacking CCAAT-binding activity, acts cooperatively on the occupation of the adjacent CCAAT box by its corresponding factor [17]. Here we show that the 73 kDa CRE-binding protein is ATF-2 (also named CRE-BPI), a factor implicated in recruiting transcriptional activators to promoters [18] and for which a sequence-deduced MW of 55 kDa had been reported [12].

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2. MATERIAL AND METHODS

2.1. *Protein extracts*

Nuclei were isolated from perfused liver of male Sprague–Dawley rats weighing 180–200 g, as described [19]. Nuclear proteins were extracted according to Dignam et al. [20].

2.2. *In Vitro transcription and translation*

^{35}S-labeled ATF-2 was synthesized in vitro by transcription of ATF-2 cDNA by using T7 RNA polymerase (Boehringer) and subsequent translation in rabbit reticulocyte lysate (Promega) according to the manufacturers' instructions.

2.3. *Gel retardation assays*

These were performed with nuclear extracts (up to 5 μg) or reticulocyte translation mixtures (1 μl), using the FN-CRE oligonucleotide as previously described [21,22].

2.4. *Southwestern blots*

Conditions for the transfer of proteins to nitrocellulose membranes, renaturation and binding to labeled oligonucleotides were those previously described [22].

2.5. *UV-crosslinking*

Protein/^{32}P-oligonucleotide complexes were covalently cross-linked by irradiating the retardation gels with light of 254 nm for 30 min [23]. Following autorradiography, the identified bands were excised from gels, triturated and boiled for 5 min in 50 μl 'Laemmli' buffer. Eluted complexes were separated from polyacrylamide by centrifugation over plastic columns with a porous bottom, and run in SDS–10% polyacrylamide gel electrophoresis.

3. RESULTS

The FN-CRE oligonucleotide forms complexes with two factors present in liver nuclear extracts: the low mobility complex *a*, and the high mobility complex *b* (Fig. 1A, lane 1). Both CRE-binding factors can be separated by chromatography on Mono Q. The factor forming complex *a* (fraction 15, fig. 1A, lane 2) elutes at lower ionic strength than the factor forming complex *b* (fraction 23, Fig. 1A, lane 3). As we have shown before [17], though fraction 23 has no CCAAT-binding activity per se, it acts cooperatively on the occupation of the CCAAT box of the FN gene promoter (Fig. 1B). Southwestern blots revealed that complexes *a* and *b* are associated with CRE-binding polypeptides of 43 and 73 kDa, respectively [17]. A model based on these observations is shown in Fig. 1C.

3.1. *UV crosslinking*

Protein/^{32}P-DNA crosslinking studies suggested that complex *b* contains a CRE-binding polypeptide of higher MW than the 43 kDa one present in the slow migrating complex *a*. An SDS-PAGE of the linked complexes excised from native gels is shown in Fig. 2. Complex *b* presents bands of approximately 80 and 50 kDa (lane 2), which would correspond to polypeptides of 72 and 42 kDa if an oligonucleotide mass contribution of about 8 kDa is assumed (26 nucleotides, 0.3 kDa each). On the other hand, complex *a* presents bands of lower MW, i.e. approximately 50 and 43 kDa (lane 1), which in turn would correspond to polypeptides of 42 and 35 kDa.

3.2. *SDS-PAGE of recombinant and natural ATF-2*

In our previous work [17] the 73 kDa polypeptide was thought to be a novel CRE-binding protein, since no CRE factor of such molecular weight had been previously reported [6,9,12,24]. However, since a full length cDNA clone for ATF-2 (CRE-BPI) had been isolated using as probe the CRE sequence of the FN gene promoter [12], we decided to investigate whether this factor, of deduced MW = 55 kDa, was related to the 73 kDa protein identified by UV crosslinking. Surprisingly, recombinant ATF-2 showed an apparent MW of 73 kDa in SDS-PAGE (Fig. 3, lanes 1 and 2). Consistently, an anti-hATF-2 antiserum recognizes bands that co-migrate with recombinant ATF-2 in Western blots of HeLa (lane 3) and rat liver (lane 4) nuclear extracts, as well as with the '73 kDa' band in the liver Southwestern (lane 5).

3.3. *Effect of anti-hATF-2 antiserum on complex b*

In gel retardation experiments performed with in vitro-translated hATF-2, no complexes of similar mobility to complex *b* were detected. Instead, a band corresponding to the ATF-2 homodimer, and of lower mobility than complex *a* is specifically lost in the presence of

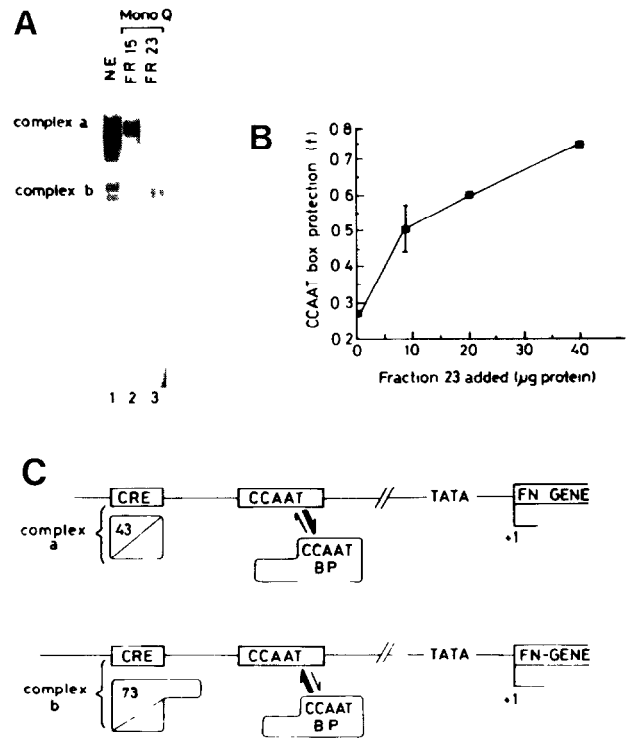

Fig. 1 (A) Gel retardation assays of binding of the FN-CRE oligonucleotide to unfractionated liver nuclear extract (lane 1), and Mono Q fractions 15 (lane 2) and 23 (lane 3). (B) Effect of the addition of increasing amounts of fraction 23 to a constant amount of fraction 15 on the fractional occupation (*f*) of the fibronectin gene CCAAT box. Factor *f* was calculated as described [17]. (C) Model for a cooperative effect of a factor containing the 73 kDa CRE binding protein on the binding to the adjacent CCAAT box in the fibronectin gene.

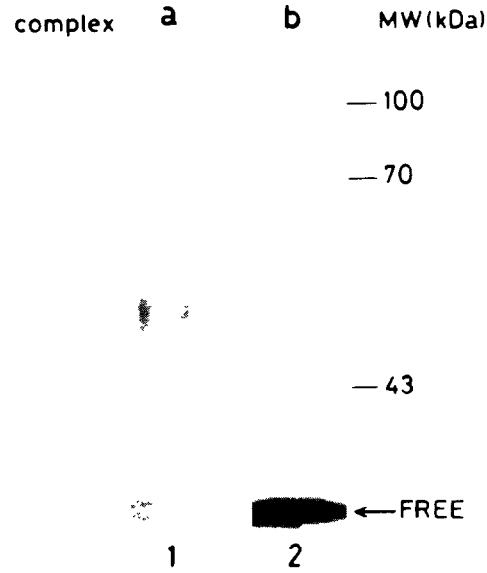

Fig. 2. UV-crosslinking of FN-CRE complexes. The ^{32}P end-labeled FN-CRE oligonucleotide was incubated with liver nuclear extract following the conditions for a typical retardation assay. Upon electrophoresis, complexes *a* (lane 1) and *b* (lane 2) were covalently crosslinked, excised, denatured and subjected to SDS-PAGE as described in section 2.

anti-ATF-2 antiserum (Fig. 4, lanes 1–4). Since the anti-hATF-2 antiserum reacts weakly with rat liver ATF-2 (compare lanes 3 and 4 in Fig. 3), we first determined which FN-CRE binding proteins were affected by incubation with this antiserum in a human nuclear extract. As rat liver, extracts of human HeLa cells generate complexes *a* and *b* in gel retardation assays. Fig. 4 (lanes 5–7) shows that HeLa complex *b* consists, at least in part, of ATF-2, as demonstrated by the loss of one of its bands in the presence of anti-hATF-2 antiserum but not with non-immune serum. Complex *a* was unaffected by either sera, indicating that ATF-2 is not part of it. Similar results were obtained when rat liver nuclear extracts were assayed for binding in the presence of the anti-ATF-2 antibody (Fig. 4, lanes 8–10). In this case, the complex *b* was 'supershifted' by the specific antiserum. These results indicate that the 73 kDa polypeptide detected in complex *b*, both by Southwestern and by UV-crosslinking, is ATF-2.

DISCUSSION

In this study, ATF-2 was identified as the '73 kDa' polypeptide present in an heterodimeric CRE binding factor that facilitates the occupation of the adjacent CCAAT sequence in the fibronectin gene promoter.

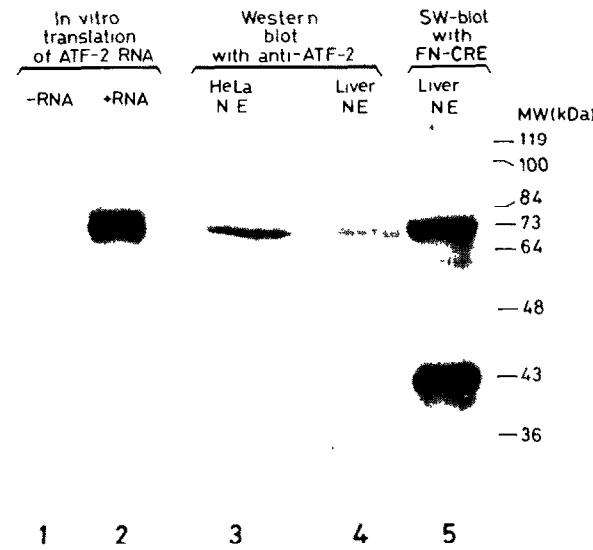

Fig. 3. SDS-polyacrylamide gel electrophoresis of recombinant and natural ATF-2. Lanes 1 and 2: [^{35}S]methionine-labeled proteins obtained in rabbit reticulocyte lysates incubated in the absence (lane 1) or in the presence (lane 2) of T7 polymerase-synthesized ATF-2 mRNA. Lanes 3 and 4: Western blot of HeLa (lane 3) and rat liver (lane 4) nuclear extracts probed with an anti-human ATF-2 antiserum. Lane 5: Southwestern blot analysis of the rat liver nuclear extract of lane 4, probed with the ^{32}P-labeled FN-CRE oligonucleotide.

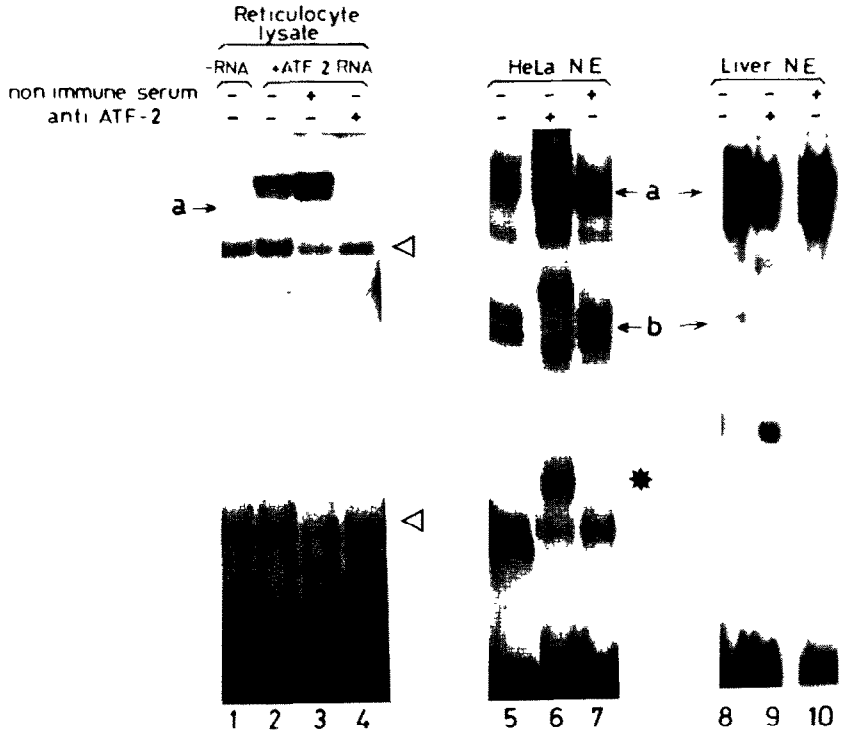

Fig. 4. Effect of the anti ATF-2 antiserum on FN-CRE complexes. Gel retardation assays were performed with unprogrammed (lane 1) or ATF-2 mRNA-programmed (lanes 2–4) rabbit reticulocyte lysates, HeLa nuclear extracts (lanes 5–7) and liver nuclear extracts (lanes 8–10). One μl of rabbit non-immune (lanes 3, 7 and 10) or anti ATF-2 (lanes 4, 6 and 9) crude antisera were added to the pre-incubation mixtures. Non-specific CRE-binding activities present in the ATF-2 antiserum and in the rabbit reticulocyte lysate produced bands which are marked with stars and open triangles, respectively.

Identification is supported by three experiments. (i) Protein/DNA crosslinking showed that the liver factor is composed of CRE binding polypeptides with SDS-PAGE MW of approximately 73 kDa and 43 kDa. (ii) Recombinant ATF-2 co-migrates in SDS-PAGE with the 73 kDa polypeptide of liver nuclear extracts detected by both a specific anti-ATF-2 antiserum and 32P-labeled FN-CRE oligonucleotide. (iii) The anti-hATF-2 antiserum specifically interferes with the formation of the gel retardation complex *b*, having the 73 kDa polypeptide.

ATF-2 (CRE-BP1) is a ubiquitously expressed CRE binding protein of cDNA with deduced MW of 55 kDa [12]. However, it migrates in SDS-PAGE with an apparent MW of 73 kDa (this report). The protein contains, at its C-terminus, leucine zipper motifs similar to those found in other enhancer binding proteins such as CREB, c/EBP and AP-1 (Jun/Fos). Recently, two shorter forms of CREBP-1 (named CRE-BP2 and 3) have been characterized [25]. The three isoforms are the result of differential splicing and transcriptional initiation. In expression studies of the T-cell receptor complex CD3 δ gene, while CRE-BP2 and 3 were potent and weak activators, respectively, CRE-BP1 was transcriptionally inert per se [25]. These findings reinforce the idea that ATF-2/CRE-BP1 acts as a specific DNA binding protein that recruits, by protein–protein interactions, other factors which provide activation domains necessary for transcriptional stimulation. In fact, Liu and Green [18] also found that ATF-2 is transcriptionally inert but becomes a potent transactivator when coexpressed with the adenovirus E1a protein, which lacks DNA binding domains. On the other hand, ATF-2 is able to form heterodimers with the 43 kDa Jun polypeptide [16,26].

The liver FN-CRE binding factor of complex *b* studied here is a heterodimer between ATF-2 and a 43 kDa-polypeptide (Figs. 2 and 4). Besides, its cooperative effect on the occupation of the CCAAT box is presumably due to protein–protein interactions with a still unidentified CCAAT-binding protein [17]. These features are consistent with the above mentioned ability of ATF-2 to heterodimerize with Jun and to interact with other transcription factors. As protein dimerization and protein–protein interactions are of relevance in transcriptional regulation, it now becomes crucial to identify the particular CCAAT binding protein that binds the CCAAT box of the fibronectin gene and determine whether it actually interacts with the ATF-2-containing complex *b*, and to investigate the putative interaction of this complex with the basic transcription machinery.

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