Interaction between the −170 CRE and the −150 CCAAT box is necessary for efficient activation of the fibronectin gene promoter by cAMP and ATF-2

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Abstract The fibronectin promoter contains an ATF/cyclic AMP (cAMP) response element (CRE) site two helical turns upstream of a CCAAT site with which it interacts. We investigated the effects of mutating these −170 CRE and −150 CCAAT elements on the promoter activity regulated by three different modulators previously known to act through CRE: ATF-2, cAMP and E1a. While the cooperation seems to play no role in E1a action, integrity of the −150 CCAAT is necessary for ATF-2 and cAMP efficient activation in a cell-specific manner. These results show that the CRE and CCAAT elements function as a 'composite element' and establish a cell-specific function for CRE-CCAAT synergy.

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Key words: Cyclic AMP response element-CCAAT cooperation; Cyclic AMP; ATF-2; E1a; Fibronectin

1. Introduction

Gene expression patterns in mammalian cells depend on the precise action of the transcription and splicing machineries. How the many DNA and RNA regulatory signals direct the timely assembly of the appropriate multiprotein complexes has been the subject of extensive research, although no general principles have emerged. The fibronectin (FN) gene offers a suitable model to study the multimolecular processes that regulate transcription and splicing [1]. It is transcribed at different levels from a single promoter in all tissues and gives rise to tissue-specific alternative splicing isoforms. FN has an essential role in development [2] and changes in its expression pattern are involved in pathology, particularly of liver [3].

The FN gene promoter contains a TATA box and several proximal regulatory sequences, including a canonical cyclic AMP (cAMP) response element (AFT/CRE or simply CRE, 5′ TGACGTCA 3′) at position −170. The factors that bind to the CRE site belong to a diversified family of leucine zipper transcriptional activators collectively known as ATF factors [4]. Some of them are preferentially activated by a particular signaling pathway, but as they all bind DNA with the same sequence-specificity, it remains unknown how they are specifically recruited to a given promoter. Interactions with other transcription factors are likely to contribute to their promoter-specificity.

The FN −170 CRE appears to be involved in different regulations of the promoter. Besides mediating cAMP stimulation in certain cell types [5,6], this element is essential for responsiveness to serum [7] and to the adenovirus E1a oncoprotein [8]. We have previously shown that the occupation of the −170 CRE site in vitro in liver extracts depends on the presence and appropriate positioning of the neighboring −150 CCAAT site, located exactly two helical turns away [9]. In this system, we showed that the FN-CRE factor that cooperates with CCAAT-binding in liver contains an ATF-2 subunit [10]. ATF-2 is a ubiquitous transcription factor containing a basic region leucine zipper motif that interacts with transcriptional activators that lack sequence-specific DNA-binding activities like the retinoblastoma protein (Rb) [11] and viral activators such as adenovirus E1a [12] and HTLV-1 Tax [13]. Phosphorylation of ATF-2 by the c-Jun NH_2-terminal protein kinase has been shown to modify three ATF-2-mediated transcriptional activations: induction by serum and *trans*-activations by Rb and E1a [14]. The central role of ATF-2 is evidenced by the severe abnormalities observed in ATF-2 knock-out mice [15].

We have previously shown that disruption of both the −170 CRE and −150 CCAAT sites was detrimental for the in vivo activity of the FN promoter and non-additive (i.e. synergistic) with respect to the single mutations [16]. Given the complexity of the signal transduction through the FN −170 CRE site, we investigate here the importance of the cooperation in regulated transcription by three different modulators previously known to act through the CRE: ATF-2, cAMP and E1a. While the cooperation seems to play no role in E1a action, integrity of the −150 CCAAT is essential for ATF-2 and cAMP activation in a cell-specific manner. In the absence of the CCAAT site, specificity is lost. These results confer the CRE and CCAAT elements the category of a 'composite element' and establish a cell-specific function for CRE-CCAAT synergy, providing a novel framework to understand the basis for the specific activity of distinct ATF/CRE sites [17,18].

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2. Materials and methods

2.1. Plasmids

2.1.1. FN promoter mutants. A USB site-directed mutagenesis kit was used to introduce disruptive mutations in p-220-FN-CAT (referred to as 'the wild-type (wt)'). This plasmid contains a −223/+44 *Bst*NI fragment of the human FN promoter cloned into the *Hin*dIII site of pSV0CAT [19]. The −170 wtCRE 5′ GTGACGTCAC 3′ site was mutated to 5′ **A**TG**G**C**T**TCAC 3′. The −150 wtCCAAT 5′ GCCAATC 3′ was mutated to 5′ GCC**C**C**T**C 3′. Three chloramphenicol acetyl transferase (CAT) expressing mutants were prepared: p-220-FN_{mutCRE}-CAT (referred to as 'the mutant CRE (mutCRE)'), p-220-$FN_{CC\underline{cc}T}$-CAT (referred to as 'the CC<u>cc</u>T mutant') and p-220-FN_{double}-CAT (referred to as 'the double mutant'). p-125-FN-CAT was obtained by deleting the 217 bp *Eag*I fragment from p-220-FN-CAT. Construction of p-500-FN-CAT was described previously [20].

2.1.2. Other expression plasmids. pMT-C-α, kindly provided by Daniel Altschuler, expresses the α-isoform of the protein kinase A catalytic subunit under the control of the human metallothionein promoter. pRSVβgal expresses bacterial β-galactosidase under the control of the Rous sarcoma virus long terminal repeat (LTR). pSV2CAT [19] produces chloramphenicol acetyl transferases from the SV40 early promoter. The 13S and 12S alternative splicing variants of the adenoviral protein E1a were expressed form plasmids pCMV13SE1a and pCMV12SE1a, respectively (kind gifts of Roberto Weinman), both controlled by the human cytomegalovirus immediate early promoter. pCEV-ATF-2, a kind gift of Silvio Gutkind, has the full length human ATF-2 cDNA under the control of the Moloney murine leukemia virus LTR. To obtain a plasmid expressing ATF-2 antisense RNA, named pCEV-2FTA, pCEV-ATF-2 was cut with *Xho*I and re-ligated, in order to allow for an inversion of the 1.5 kpb cDNA insert with respect to the promoter.

2.2. Cell cultures and transfections

The human hepatoma cell lines Hep3B and HepG2 were cultured in DMEM (low glucose) (Life Technologies, Bethesda, MD, USA) supplemented with 10% fetal bovine serum. NIH3T3 and HT1080 cells were cultured in high glucose DMEM. In all experiments, cells were transfected in 10 cm plates by the calcium phosphate method [19] and harvested and lysed 48 h after washing of DNA. Cell extracts were used to measure β-galactosidase and CAT activities as described [20]. Equal amounts of β-galactosidase activity were used in each CAT assay.

3. Results

3.1. CRE-CCAAT synergy and ATF-2

We have previously reported that the cooperation between the −170 CRE and −150 CCAAT sites detected in vitro [9,10] is also relevant for the basal activity of the FN promoter in vivo [16]. This cooperative effect correlated with the in vitro interaction between the CRE-binding factor AFT-2 and the CCAAT factors NF-I and CP1 [16]. However, we had no evidence of the in vivo role of ATF-2 on the FN promoter activity. To investigate it, we chose two strategies: (i) overexpression of human ATF-2 and (ii) expression of antisense human ATF-2 RNA. Overexpression of ATF-2 by transient

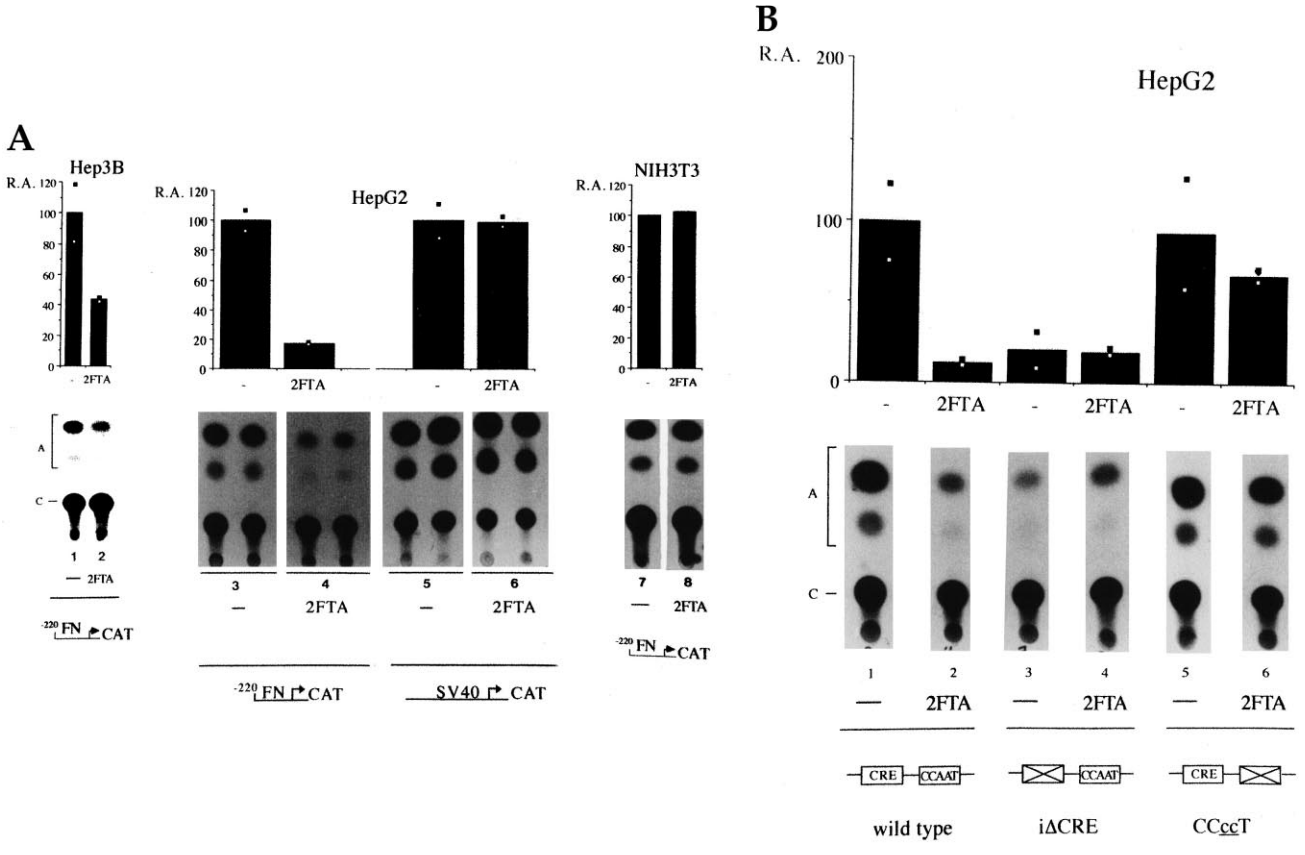

Fig. 1. Effect of the expression of ATF-2 antisense RNA on the wt and CRE/CCAAT mutant FN promoters in different cell lines. A: Hep3B (lanes 1 and 2), HepG2 (lanes 3–6) or NIH3T3 (lanes 7 and 8) cells were transfected with 10 μg of p-220-FN-CAT (lanes 1–4, 7 and 8) or SV40-CAT (lanes 5 and 6) constructs. Lanes 2, 4, 6 and 8 correspond to co-transfections with 5 μg of pCEV-2FTA, a plasmid expressing antisense ATF-2 RNA. Lane 3–6, each lane is a set of transfection duplicates. B: 10 μg of wt (lanes 1 and 2) or CRE/CCAAT mutant (lanes 3–6) was transfected in HepG2 cells. Lanes 2, 4 and 6 show co-transfections with 5 μg of pCEV-2FTA. Schematic diagrams of wt and mutant constructs are shown at the bottom of each set of duplicates of transfection. In all experiments, transfection efficiencies were determined by co-transfection with 10 μg of pRSVβgal. Relative CAT activities (R.A.) are referred to the wt. CAT assays are shown in duplicates of transfection. Bars represent the average+S.D. of transfection triplicates.

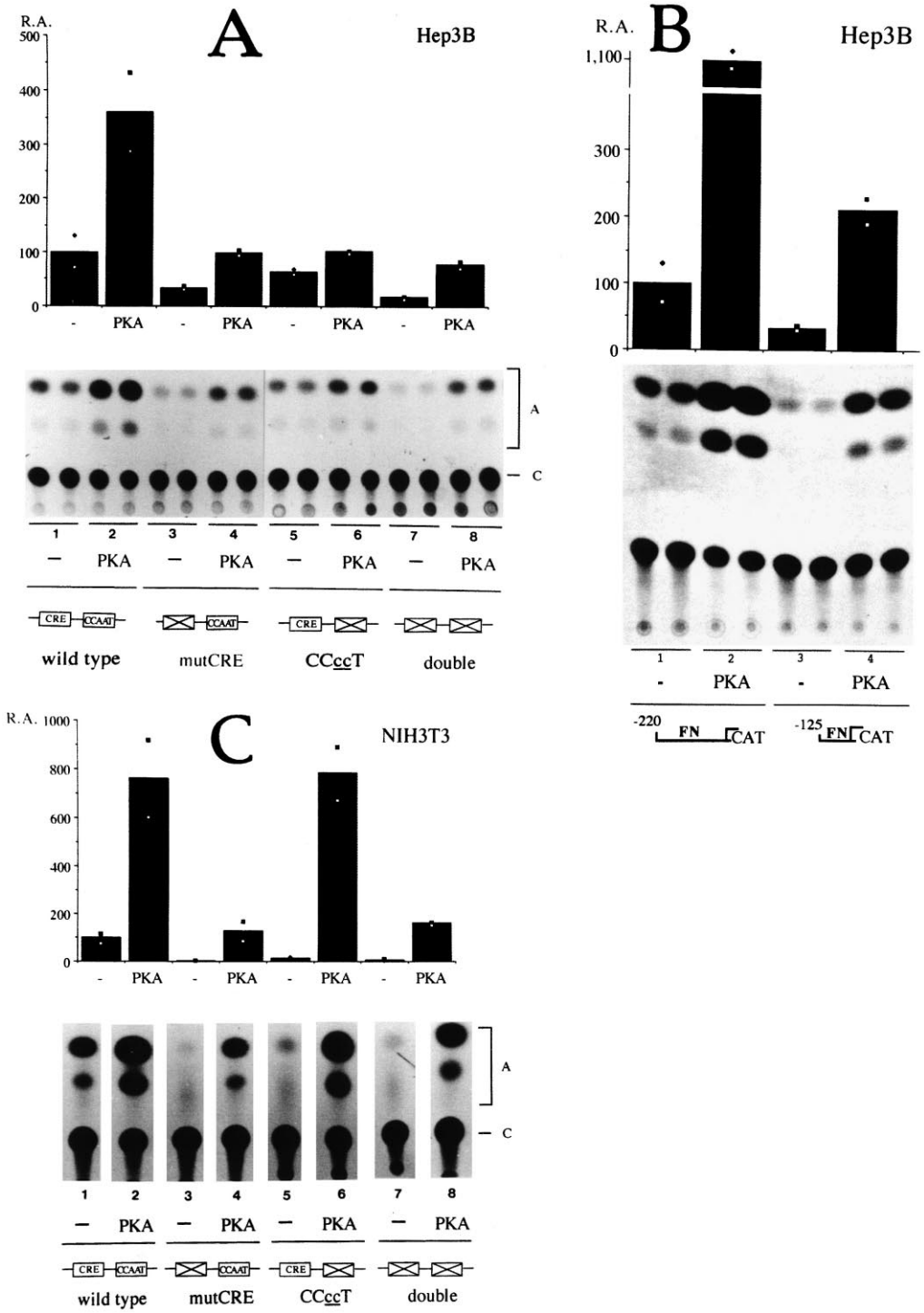

Fig. 2. Effect of cAMP-dependent protein kinase (PKA) activation of the wt and mutant promoters in Hep3B (A and B) and non-hepatic (C) cells. A: CAT assays of transfections with 10 μg of p-220-FN-CAT (lanes 1 and 2) and CRE/CCAAT mutants (lanes 3–8). Lanes 2, 4, 6 and 8 correspond to co-transfections with 5 μg of pMT-Cα, a plasmid encoding the catalytic subunit of PKA. B: Transfections with 10 μg of wt (lanes 1–4) and p-125-FN-CAT (lanes 5–9). Lanes 3, 4, 7 and 8 correspond to co-transfections with 5 μg of pMT-Cα. Each lane is a set of duplicates of transfection. C: NIH3T3 cells were transfected with 10 μg of wt (lanes 1 and 2) and CRE/CCAAT mutants (lanes 3–8). Lanes 2, 4, 6 and 8 correspond to co-transfections with 5 μg of plasmid pMT-Cα. Each lane is a single transfection but quantification bars correspond to duplicates. Heights of quantification bars correspond to the average of each duplicate value, indicated by a dot. Other conditions and diagrams are as in Fig. 1.

expression of its cDNA under the control of the Moloney murine leukemia virus LTR has no effect on the transcriptional activity of p-220-FN-CAT in hepatic cells, but stimulates by 3-fold the same promoter in non-hepatic cells (data not shown). Consistently, expression of the antisense construct, named pCEV-2FTA, inhibits transcription driven by p-220-FN-CAT in Hep3B (Fig. 1A, lanes 1 and 2) and HepG2 cells (lanes 3 and 4), but not in NIH3T3 cells (lanes

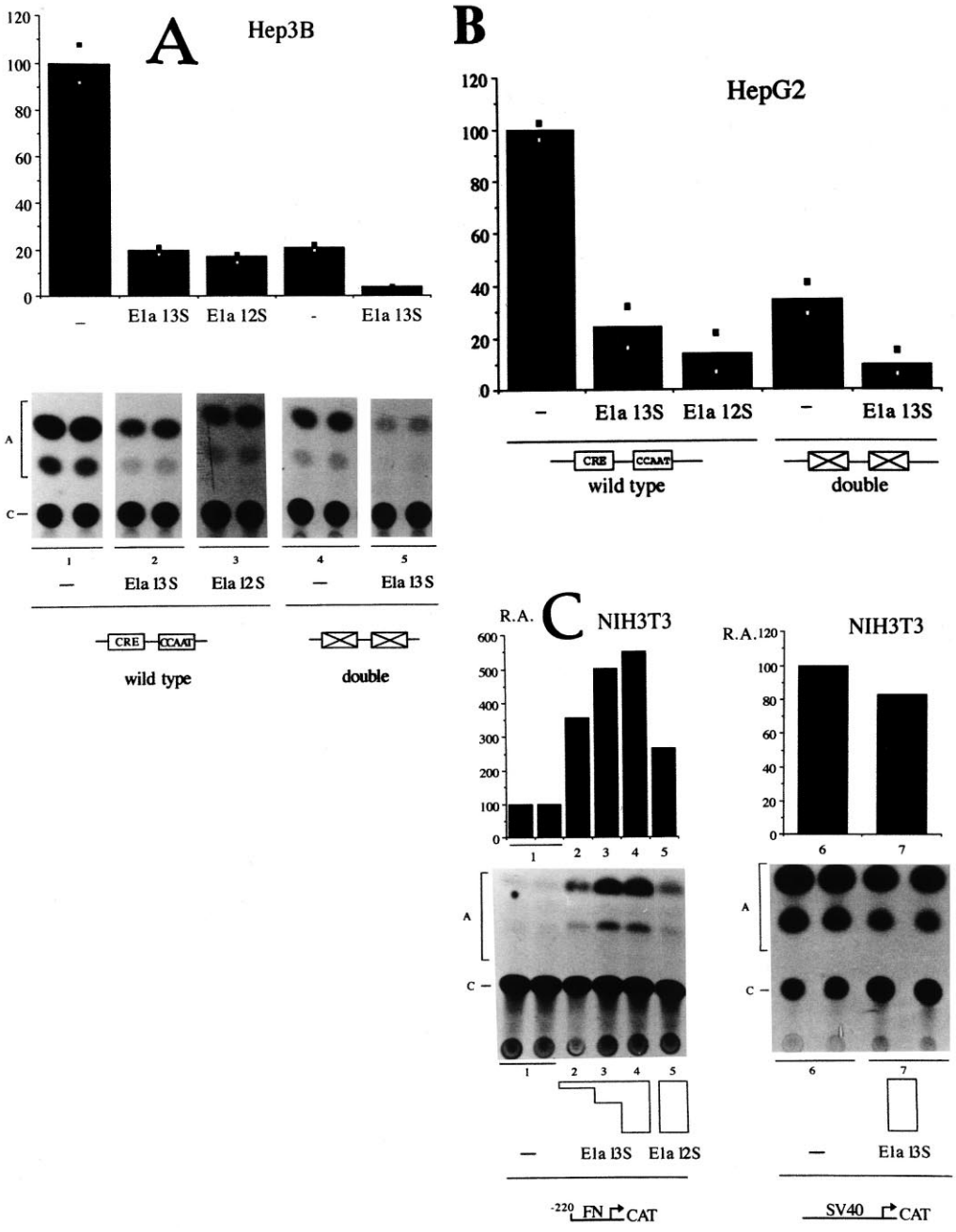

Fig. 3. Effect of the expression of two E1a alternative splicing variants on the wt and double CRE/CCAAT mutant FN promoters. A: Hep3B cells were transfected with 10 μg of wt (lanes 1–3) or double mutant (lanes 4 and 5). Co-tranfections with pCMV13SE1a (lanes 2 and 5) or pCMVE1a12S (lanes 3) are indicated. B: Experiment identical to A, but in HepG2 cells. Only quantifications are shown. C: NIH3T3 cells transfected with p-220-FN-CAT (lanes 1–5) or pSV2CAT (lanes 6 and 7) and co-transfected with pCMV13SE1a (lanes 2–4 and 7) or pCMVE1a12S (lanes 5). The amounts of E1a expressing plasmids in the transfections are: 0.2 μg (lane 2), 1.0 μg (lane 3) and 5 μg (lanes 4, 5 and 7).

7 and 8). As promoter-specificity controls, CAT activity of pSV2CAT (lanes 5 and 6) or pCMV-CAT (not shown) transfected into HepG2 cells is not inhibited by 2FTA.

Fig. 1B shows that the inhibitory effect of 2FTA over the wt FN promoter brings down transcriptional activity to a minimal level similar to the effects of CRE disruption (lanes 1–3). The inhibitory effect of the antisense is specific on the ATF/CRE element, since expression of the mutCRE construct itself is not affected by 2FTA (compare lanes 3 and 4). However, and most strikingly, activity of the CCccT mutant, that harbors an unaltered CRE, is much less inhibited by 2FTA (a 30% reduction) than the wt promoter (85%) (lanes 5 and 6).

3.2. CRE-CCAAT synergy and cAMP

To test if the −170 CRE mediated cAMP activation in hepatic cells, we studied the effect of overexpression of the catalytic subunit of protein kinase A (C-PKA) on the transcriptional activity of the p-220-FN-CAT constructs. Fig. 2A shows that in Hep3B cells, C-PKA activates the wt FN pro-

moter by about 4-fold (lane 2). Activation varies between 4- and 11-fold in different experiments. Similar levels of activation were obtained by treating the cells with 10 mM forskolin or 1 mM dibutyryl cAMP (not shown). CRE inactivation significantly reduces the activity in the presence of C-PKA (compare lanes 2 and 4). The same effect is observed when the CCAAT box is inactivated (compare lanes 2 and 6) or with the double mutant (compare lanes 2 and 8). The fact that the CCccT mutation inhibits the ability of the −170 CRE to respond to C-PKA activation strongly supports that the CRE-CCAAT interaction plays a key role in mediating cAMP induction in Hep3B cells.

In all three mutants, there is a remnant PKA-stimulated activity. This is unlikely to be caused by residual binding of transcription factors to the mutated DNA sites, as both gel shift and DNAse I footprinting experiments showed that the mutations abolish the binding of transcription factors to the sites completely ([16] and data not shown). Alternatively, it could be the consequence of cAMP activation through a site downstream of −170 CRE, presumably the AP-2 site located at position −57. To test this hypothesis, a deletion construct (p-125-FN-CAT) spanning from −125 to +44 (lacking the CRE-CCAAT region) was tested in response to C-PKA. Fig. 2B shows that although p-125-FN-CAT displays a very low basal activity, it is indeed greatly activated by C-PKA, confirming the presence of a secondary induction site.

In NIH3T3 cells, cAMP activation does not require the CCAAT box. Although CRE disruption greatly reduces PKA activation (Fig. 2C), CCAAT disruption does not affect the response (compare lanes 6 and 2), indicating that CRE-CCAAT cooperation is not essential for cAMP activation in this cell type. A similar result was observed in HepG2 cells.

3.3. Dualistic effect of E1a

A very well-characterized mechanism involving ATF-2 is transactivation by the adenovirus factor E1a. E1a exists as two alternative splicing variants: 13S and 12S. Only the 13S variant is able to interact with ATF-2 through its CR3 transactivation domain, resulting in transcriptional activation of promoters containing CRE/ATF sites [12]. To test the effect of E1a on the FN promoter in hepatic cells, we co-transfected FN-CAT constructs with 13S cDNA under the control of the human CMV promoter (pCMV13SE1a) in Hep3B cells. Fig. 3A shows that, contrary to expected, instead of activating, 13S E1a strongly inhibits the FN promoter (lane 2). The same effect is observed with the 12S variant (lane 3), indicating that this inhibition does not involve the CR3 domain. Furthermore, the inhibitory effect does not involve the CRE site (nor its interaction with the CCAAT site) because both single mutants (not shown) and the double mutant are inhibited by overexpression of 13S E1a (compare lanes 1 and 2 with 4 and 5). Similar results were obtained in HepG2 cells (Fig. 3B, only quantifications are shown).

In NIH3T3 fibroblasts, E1a activates the FN promoter mainly through its CR3 domain (Fig. 3C). In fact, the 13S variant increases the FN promoter activity by 5.5-fold (compare lanes 1 and 4), while the 12S variant is less effective (lane 5). This activation is CRE-specific, since it is impaired when the −170 CRE is disrupted (not shown) and is promoter-specific, as the SV40 promoter is unaffected by E1a 13S (lanes 6 and 7).

4. Discussion

4.1. Signal transduction through the FN gene CRE site

The −220/+44 region of the human FN promoter has all the features of a typical promoter-proximal+core promoter region [21], i.e. recognition sites for factors such as ATFs, CCAAT-binding proteins, AP-2 and Sp1, located upstream of a canonical TATA box at position −25. Several studies have demonstrated or suggested the involvement of the −170 CRE site in transcriptional activation in response to cAMP, 13S E1a and ATF-2. This variety of activators provides an ideal framework to explore the needs for signaling-specificity.

By the use of in vitro approaches, we have previously studied the properties and participants of a synergistic cooperation between the −170 CRE and −150 CCAAT sites of the FN promoter in rat liver nuclear extracts. This in vitro interaction involves protein-protein interactions between a CRE-binding factor which contains at least one ATF-2 subunit [10] and the CCAAT-binding factors NF-I and CP1 [16,9,10].

We assessed here the effect of abolishing the binding of transcription factors to either the CRE or CCAAT sites on regulatory pathways that so far were supposed to be mediated only by the CRE site: those of ATF-2, cAMP and E1a.

4.2. ATF-2

Overexpression of ATF-2 has no effect on the FN promoter in hepatic cells, but stimulates by 3-fold the same promoter in NIH3T3 fibroblasts (data not shown). Consistently, expression of the antisense ATF-2 RNA (2FTA) inhibits the FN promoter in Hep3B and HepG2 cells, but not in NIH3T3 cells (Fig. 1). A possible interpretation of this is that both Hep3B and HepG2 cells have saturating levels of endogenous active ATF-2, sufficient to maintain basal FN promoter activity, which is therefore not affected by overexpression of the factor, but can be brought down by its corresponding antisense. NIH3T3 cells seem to have limiting amounts of active ATF-2 and are able to activate the exogenous one, making the FN promoter responsive to overexpression of recombinant ATF-2.

The inhibitory effect of 2FTA on the wt FN promoter in hepatic cells brings transcriptional activity down to low levels similar to those of mutCRE. The simplest interpretation of this result is that ATF-2 is a required component of the ATF factor that binds to the CRE site and that in its absence, no other ATF factors can activate transcription through this site. This is surprising, considering that there are a variety of ATF factors able to bind to the FN-CRE site. In our gel shift experiments with extracts from liver [9,10] and the cell lines studied here (not shown), several complexes form on the FN-CRE probe and only one of them contains ATF-2. It seems that something precludes the action of the other factors in vivo. Alternatively, it might be that the antisense is having an indirect effect on the promoter by affecting other genes. We controlled for a general effect on transcription, showing that other promoters (SV40, CMV) are not inhibited. The fact that the ATF-2 antisense does not have any effect on the CRE-disrupted promoter rules out a repression of the FN promoter other than the depletion of the active CRE-binding protein itself. We conclude that ATF-2 is directly required for the activity of the −170 CRE site. The result observed with the CCccT mutation supports the idea of a selectivity for

ATF-2 that is lost when the CCAAT site is mutated. We show that expression of the ATF-2 antisense (Fig. 2B, lane 6) has only a minor effect on the activity of the CCccT mutant, suggesting that in the absence of this site, other ATF factors, besides ATF-2, must bind the CRE site and activate transcription.

4.3. cAMP

Two regions mediate stimulation of the FN promoter by the cAMP pathway (C-PKA experiments) in hepatic cells: the −170 CRE-CCAAT region and a region located downstream −125, presumably involving the −57 AP-2 site [5]. We tested the requirement for each site and found that in HepG2, Hep3B and NIH3T3, the −170 CRE site is needed for the response to cAMP. However, only in Hep3B cells, the proportional part that depends on the CRE-CCAAT region is dependent on the integrity of both elements. Disruption of either the CRE or the CCAAT site results in complete loss of signaling through that region, equivalent to simultaneous inactivation of both sites.

In previous reports, the activity of the FN −170 CRE site has been shown to depend on the promoter context. When this site was transplanted upstream of a non-responsive thymidine kinase promoter, it conferred cAMP activation to the chimera in HT1080 fibroblasts. However, the same element was shown to be dispensable for the cAMP activation of the proximal 500 bp of the human FN promoter in the same cells [22]. We have confirmed this by showing that the −220 FN promoter is completely unresponsive to cAMP in HT1080 cells (CGP and ARK, unpublished observations). This is in good agreement with our finding that context-dependent interactions are required for proper functioning of the −170 CRE site. Similar observations with respect to the need of a CCAAT box for cAMP action were reported in the rat arylalkylamine *N*-acetylatransferase gene promoter [23].

4.4. E1a

The antisense and overexpression experiments indicate that under basal conditions, ATF-2 is already in an active form in the hepatomas Hep3B and HepG2, but is inactive or less abundant in NIH3T3 fibroblasts. Consistently, 13S E1a, a viral activator of ATF-2, only activates transcription in NIH3T3 cells. ATF-2 is synthesized as an inactive protein, unable to bind DNA or activate transcription if tethered to a promoter. Interaction with E1a enables ATF-2 to become an active DNA-binding and transactivator factor. Insensitivity to 13S E1a activation in hepatic cells might be due to the fact that specifically in these cells, ATF-2 is engaged in an activating interaction with the CCAAT-binding proteins CP1 or NF-I. Instead, the oncogenic activity of CR1 and CR2 domains, present in both splicing forms of E1a, causes an important inhibition of the FN promoter, which is clearly not mediated through the CRE-CCAAT sites. A similar effect was described in the rat FN promoter: E1a repression involves GC-rich sequences downstream of −150, but cell-specificity was not investigated [24,25].

In the case of NIH3T3, a clear CRE-dependent 13S E1a activation is observed. This is likely to be mediated by ATF-2, which would be in an inactive form readily available for 13S E1a interaction.

Therefore, we are in the presence of dualistic responses of the same promoter to the same regulator in two different cell types.

4.5. Concluding remarks

Taken together, the results presented indicate that in hepatic cells, ATF-2 takes part of a composite factor, together with CCAAT-binding proteins. In the absence of binding to CCAAT, this composite factor is not able to interact with the FN promoter and other CRE-binding factors take over, explaining the differential sensitivity of the wt and CCccT-mutated promoters to 2FTA co-transfection.

We have performed gel mobility shift experiments looking for cell-specific complexes that might correlate with the observed transcriptional responses. Either with FN-CRE or FN-CCAAT probes, the same gel shift patterns were observed with Hep3B, HepG2 and NIH3T3 nuclear extracts (data not shown), which are in turn similar to those observed with rat liver nuclear extracts [16,9,10]. This is not surprising because binding to short probes does not reflect the complexity and selectivity of protein-protein and protein-DNA interactions occurring in long promoter strings. For example, hepatic-specific occupation of the CRE-CCAAT region is readily seen in DNAse I footprintings of a 220 bp promoter fragment, but not in gel shifts with short oligonucleotides [9].

ATF-2 is neither a substrate nor a mediator of PKA activation [26] and we know that the liver CCAAT-interacting factor is different from the ATF-2 homodimer ([10] and unpublished results). We therefore envision that, within the composite factor, ATF-2 exists as an heterodimer with a PKA-responsive ATF factor, like ATF-1 or CREB. The fact that in Hep3B cells, mutCRE causes the same reduction in cAMP activation as the CCccT mutant or the double mutant supports the concept that the CRE-CCAAT region is a composite element, similar to the one described by Yamamoto and coworkers [17] for the AP-1 and glucocorticoid response element sites, which confer glucocorticoid responsiveness to the proliferin gene promoter. Composite elements like the FN-CRE-CCAAT do not only regulate transcription, but also participate in the modulation of alternative splicing, as we have shown in a recent report where disruption of both sites greatly altered the pattern of exon selection in the downstream transcript [27].

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