We have previously proposed a molecular interaction between the liver factors that bind to the cyclic AMP response element (CRE) and CCAAT sites of the fibronectin (FN) gene based on the following evidence: (i) the close spacing of 20 base pairs between CRE and CCAAT elements is conserved in the FN genes from rats, mice, and humans; (ii) footprinting competitions showed that CRE oligonucleotides are able to detach both liver factors; (iii) CCAAT binding and transcriptional activity of liver extracts are reduced when the distance between the CRE and CCAAT elements is increased; and (iv) CCAAT-binding is stimulated by the addition of a liver extract fraction containing the CRE-binding factor ATF-2. This report provides binding and immunoochemical evidence that nuclear factor I (CTF/ NF-I) and CP1 (NF-Y or CBF) are the only liver factors that bind to the −150 CCAAT element of the FN gene, forming distinct complexes. We show that these factors bind less efficiently to the CCAAT site of a FN promoter in which the −170 CRE has been disrupted by site-directed mutagenesis and that each element contributes positively to the liver transcriptional activity assessed in vitro with a G-less cassette construct and in vivo by transfection of hepatoma cells with CAT constructs. Furthermore, using a method that combines UV cross-linking and immunoprecipitation, we show that antibodies specific to ATF-2 are able to specifically precipitate protein-protein-DNA complexes containing NF-I and CP1. This simple method preserves weak macromolecular interactions, avoiding the disruptive electrophoresis conditions of gel mobility shifts assays.

Advances in our knowledge of the physiology of gene regulation will ultimately require the precise definition of the molecular processes that are built up on a given gene’s DNA by both the well known and still unknown factors. Ideally, the model gene should be widely expressed and regulated at the level of transcription and splicing. Besides fitting these requirements (1), the fibronectin (FN) gene offers a suitable model because it has an essential role in development (2), and changes in its expression pattern are involved in pathology, particularly of the liver (3). The importance of studying FN regulation in liver is highlighted by the fact that although many tissues express FN, liver is the major source of the plasma form of FN (4), made of splicing variants different from those of the extracellular matrix forms. Moreover, liver fibrosis, a common pathological condition preceding cirrhosis, is accompanied by dramatic changes in FN expression (3), whose mechanisms are still poorly understood.

The −170 cyclic AMP response element (CRE; 5′-TGACGTCA-3′) of the FN gene appears to be involved in different regulations of the promoter. Besides mediating cAMP stimulation (5–7), this element was unexpectedly shown to be essential for responsiveness to serum (8) in an additive way and therefore independent from CAMP. Viral induction by adenovirus E1a oncoprotein has also been shown to be mediated through the FN-CRE site (9).

DNase I footprints of the proximal region (−220 to −65) of the FN gene differ considerably between liver and other cell type extracts. Although the −170 CRE is occupied by all tested extracts, strong binding to the −150 CCAAT is only observed with liver extracts. Three facts suggested an interaction between the liver factors that bind to these CRE and CCAAT sites: (i) A close spacing of 20 bp between CRE and CCAAT elements is conserved in the FN genes from rats, mice, and humans. (ii) Footprinting competitions showed that CRE oligonucleotides were able to detach both liver factors, suggesting that binding of a CRE factor to its cognate site is needed for the occupation of the neighbor CCAAT site placed two helical turns away. (iii) CCAAT binding and in vitro transcriptional activity of liver extracts were reduced when the distance between the CRE and CCAAT elements was increased in a series of spacing mutants (10).

The FN-CRE factor that cooperates with CCAAT binding in liver was shown to be an heterodimer between a 43-kDa polypeptide and the 70-kDa ATF-2 (11). 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) (12) and viral activators such as adenovirus E1a (13) and HTLV-1 Tax (14), which are then tethered to CRE-containing promoters. ATF-2 heterodimerizes with c-j UN and this heterodimer has been implicated in the viral regulation of the β-interferon gene (15) through synergistic interac-

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‡Recipient of a fellowship from the Universidad de Buenos Aires.

¶Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina. To whom correspondence should be addressed. Tel.: 54-1-784-5516; Fax: 54-1-786-8578.

1 The abbreviations used are: FN, fibronectin; CRE, cyclic AMP response element; C/EBP, CCAAT/enhancer binding protein; bp, base pair(s); CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; wt, wild type; ds, double stranded; CREB, cyclic AMP response element binding protein.

Claudio R. Alonso*, C. Gustavo Pesce‡, and Alberto R. Kornblitta†
From the Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina, Vuelta de Obligado 2490 and the Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina
tions between the ATF-2 subunit and the transcription factors HMG-I(Y) and NF-κ B. Phosphorylation of ATF-2 by the c UN NH2-terminal protein kinase has been shown to modify three ATF-2-mediated transcriptional activations: induction by serum and trans-activations by Rb and E1a (16). The central role of ATF-2 is evidenced by the severe abnormalities observed in ATF-2 knock-out mice (17).

The FN-CCAAT-binding proteins have not been identified before. At least four families of transcription factors recognize the CCAAT motif. The best characterized is that of CCAAT enhancer binding protein (C/EBP), a leucine zipper dimeric factor related to myc, fos, and CREB, with defined roles in terminal cell differentiation (18) and long term memory (19). A second family is represented by nuclear factor-κB (called CTF/NF-κB or simply NF-κB), with the dual role of transcription factor for RNA polymerase II and initiation factor for adenovirus DNA replication (20). A third group includes a ubiquitous factor known as CP2 (21), NF-Y (22), or CBF (23). It is now clear that CP1 is a heterotrimer (22). Two of its subunits, CP1A (also named NF-YB or CBF-B) and CP1 B (also named NF-YA or CBF-A) are homologous to the yeast CCAAT factors Hap3 and Hap2, respectively, which control nuclear genes required for mitochondria oxidative function (24). The fourth group is represented by CP2 (21), a heterodimer that controls the α-globin gene transcription in erythroid cells (25).

This report provides binding and immunochemical evidence that NF-1 and CP1 are the liver factors that bind to the −150 CCAAT element of the FN gene, forming distinct complexes. We show that these factors bind less efficiently to the CCAAT site of a FN promoter in which the −170 CRE has been disrupted by site-directed mutagenesis. Furthermore, using a new method that combines UV cross-linking and immunoprecipitation, we show that antibodies specific to ATF-2 are able to recognize specific protein-DNA complexes containing NF-1 and CP1.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-NF-YA (COOH-terminal peptide) and NF-YB (whole recombinant protein) sera were generously provided by R. Mantovani and D. Mathis (26). Anti-CBF/NF-1 serum was a gift from N. Tanese and R. Tjian. Anti-recombinant CP-2 IgG was obtained from M. Sheffey (25). Anti-CBF-A (CP1-B) IgG was a gift of B. De Crombrugghe (27). Anti-ATF-2 and anti-CREB IgGs were purchased from Santa Cruz Biotechnologies; anti-ATF-2 (catalog number sc-187 X) supershift reagent) is directed to an epitope corresponding to amino acids 487–505 of the carboxy terminus of human CREB and anti-CREB (catalog number sc-186 X) supershift reagent) is directed to an epitope corresponding to amino acids 295–321 mapping near the carboxy terminus of human CREB-1. Anti-human ATF-2 and anti-human CREB are also reactive to the rat and mouse counterparts.

**Protein Extracts**—Nuclei were isolated from perfused rat liver according to Gorski et al. (28). Nuclear proteins were extracted according to Dignam et al. (29) in 0.37 M NaCl, in the presence of a mixture of protease inhibitors: 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin, concentrated by ammonium sulfate precipitation, dialyzed, frozen, and stored at −70 °C.

**Experimental Design**—In multicellular organisms, the transcription of many genes is under the control of regulatory regions located at a distance from the transcription initiation site. Typically, the regulatory regions contain one or more sequence motifs that bind a specific class of transcription factors. The ability of a cell to respond to a specific signal, such as growth factors or hormones, is closely associated with changes in the transcriptional activity of these target genes. The transcription factors that control the expression of these genes are often members of the CCAAT binding protein family. One such family member is NF-1, which has been shown to play a role in the regulation of the albumin gene.

**Table I**

| Name                | Sequence*  | Ref. |
|---------------------|------------|------|
| FN-CCAT             | 5'-CCATGCCAGATGCCAGATGTTG-3' | This work |
| FN-CCAT (mut)       | 5'-CCATGCCAGATGCGCGAGG-3' | This work |
| SV40 EC             | 5'-CCATGGCTTCCACACCATCTGGG-3' | This work |
| Adenovirus NF-1     | 5'-CCATGGCTTCCACACCATCTGGG-3' | This work |
| CP1                 | 5'-CCATGGCTTCCACACCATCTGGG-3' | This work |
| C/EBP               | 5'-CCATGGCTTCCACACCATCTGGG-3' | This work |

* The sequence of only one strand is shown.

**Fig. 1. In vitro transcription of a human FN/Xenopus albumin promoter hybrid construct.** Evidence that competitions with either FN-CCAAT or FN-CRE inhibit transcriptional activity elicited in vitro by liver extracts is shown. Top, schematic drawing of the promoter and regulatory regions of the construct pwt-G-free used as template. Bottom, autoradiograph of the in vitro transcription products of pwt-G-free and of a Rous sarcoma virus G-free control construct, in which a G-free cassette is under the control of the Rous sarcoma virus LTR. Reactions were carried out using 10 μg of protein of liver nuclear extract and 0.5 μg of plasmid DNA as template. The percentage of activity was estimated from densitometric scanning quantitation of the autoradiographs.
Band Shift Assays—Nuclear extracts (up to 5 mg of protein) were preincubated for 10 min on ice in the presence of 0.5–1.0 μg poly(dI-dC), 8 mM MgCl₂/spermidine, 10 mM Hepes, pH 7.9, 30 mM KCl, 0.125 mM EDTA, 0.065 mM EGTA, 10% (v/v) glycerol, 1 mM dithiothreitol, and the "mixture" of protease inhibitors mentioned above, in a total volume of 20 μl. When indicated, antibodies or competitor oligonucleotides were added to the preincubation. Binding reactions were started by the addition of 1 ng of the labeled probe and proceeded for 10 min. Complexes were separated on a 6% polyacrylamide, 0.25 × TBE (1 × TBE is 90 mM Tris borate, 2.5 mM EDTA, pH 8.3), native gel as described by Garner and Revzin (30).

Mutagenesis—In the construct named mut CRE, we changed the TGACGTCA sequence to TGgCtTCA by site-directed mutagenesis, using a U.S. Biochemical Corp. kit. In a second construct, named iD CRE for internally deleted CRE, the CRE sequence TGACGTCA was shortened to TGCA, as described (36). The construct named mut CCAAT was obtained by site-directed mutagenesis by changing the CCAAT sequence to CCccT. Double stranded oligonucleotides carrying these mutations (FN-mutCRE, FN-iCRE, and FN-CCccT; Table I) were at least 100-fold less efficient in binding liver nuclear factors in gel shifts (not shown).

Cross-linking and Immunoprecipitation—This new method was inspired in the procedure reported by McKay (31). 20-μl binding reactions of 32P-labeled FN-CCAAT oligonucleotide to proteins in nuclear extracts were carried out in 1.5-ml Eppendorf tubes and irradiated with a 254 nm hand UV lamp (Spectrolite, model ENF 240 C/F), for 30 min. The lamp was placed on top the uncapped tubes, which were kept in an ice/water bath. After this, 1 μl of anti-ATF-2 purified IgG (see above) or other antibodies were added, and incubation proceeded at 0 °C for further 30 min. Immunocomplexes were separated by incubation for 30 min with a 10% (w/v) suspension of fixed Staphylococcus aureus (Immuno precipitin, BRL) and centrifugation at 4,000 rpm for 5 min in an Eppendorf microfuge. The amount of immunoprecipitated probe was determined by measuring Cerenkov radiation in the pellets, with or
without previously washing the pellets with phosphate-buffered saline by resuspension and centrifugation.

**DNase I Protection Assays—** For the footprinting analysis (32), 388-bp Xbal/HindIII fragments, which include FN promoter sequences between −220 and +65, were isolated from wild type or mutant constructs (a detailed description of the constructs used for obtaining the promoter fragments will be published elsewhere).2 Fragments were end-labeled with [α-32P]dATP and Klenow at their 5′-ends. Footprinting reactions were as previously reported (10), with marker G-A ladder generated as described (33).

In Vitro Transcription—In vitro transcription was performed using the template construct: wt-G-free described in Ref. 10. This construct has the proximal 220 bp of the FN promoter without its TATA box but contains the Xenopus α-amylase gene TATA box. In vitro transcription was performed as described by Sawadogo and Roeder (34) with some modifications. Reactions were carried out in a final volume of 20 μl, containing 50–800 ng of supercoiled template plasmid, 28 units of RNasin, 100 μM 3-O-methyl GTP, 600 μM ATP, 600 μM CTP, 6 μM UTP, 6 μCi of [α-32P]UTP (3,000 Ci/mmol), 6 mM MgCl2, 25 mM KCl, 15 mM Hepes, pH 7.6, and 10 μg of protein extract. After incubating for 45 min at 30 °C, transcription was stopped by the addition of 5 mM EDTA, 1% (w/v) SDS, 250 mM NaCl, and 20 mM Tris-HCl, pH 7.4 (final concentrations), and samples were treated with proteinase K, ethanediol-prepared, and analyzed in a 6% polyacrylamide urea sequencing gel.

Transfections and CAT Assays—Segments of the FN promoter (-224 to +64) carrying either wild type or mutated CRE and CCAAT sites were linked to the CAT gene. These constructs were used to transfect the human hepatoma cell line Hep3B in triplicates. Cells were co-transfected with a Rous sarcoma virus-β-galactosidase reporter plasmid as a measure of transfection efficiency. Transfection conditions and CAT assays were as described previously (46).

**Quantitation of Autoradiograms—** Bands in autoradiograms were quantitated densitometrically in a Ultrascan XL Enhanced Laser Densitometer (Pharmacia Biotech Inc.). Quantitation of protein binding equilibria in DNase I footprints and calculations of the fractional protection factor f were as described in Ref. 10.

**RESULTS**

The Liver −170 CRE- and −150 CCAAT-binding Proteins Contribute Positively to the Transcriptional Activity of the FN Gene—We have previously demonstrated that increasing the distance between the CRE and CCAAT sites of the FN gene reduced liver transcriptional activity in vitro (10). Fig. 1 shows that 50-fold molar excesses of either ds FN-CRE or FN-CCAAT oligonucleotides inhibited in vitro transcription of liver extracts under a G-less cassette template under the control of the FN promoter. At the same molar excess (50×), FN-CRE is more efficient in bringing down transcription (37% remaining activity) than FN-CCAAT (63% remaining activity), confirming the preponderant role of the CRE in the composite site. Competition with FN-CCCT, a FN-CCAAT mutant oligonucleotide unable to bind liver factors, does not affect transcription (not shown). The Rous sarcoma virus-LTR was used as reference promoter. It is worth noting that although LTR transcription is not inhibited by FN-CRE (in fact it is activated by 50%), it is negatively affected by competition with FN-CCAAT. We believe that this is due to the fact that viralLTRs, as well the adenovirus major late promoter also tested as control (not shown), contain DNA elements recognized by CCAAT-binding proteins. Anyway, the inhibition caused by FN-CCAAT on the FN-promoter is more important than that of the LTR. These results indicate that the CRE and CCAAT sites are not dispensable for transcription in liver and that the factors interacting with them are positive regulators.

The Fibronectin “NF-I Site” Not Only Binds NF-I—The liver factors that bind to the −170 CRE sequence have been characterized by Southwestern analysis and include polypeptides of 120, 70, and 43 kDa (10). The 120-kDa protein is also found in HeLa cells, and little is known about its functions (35). The 43-kDa factor is thermoresistant and resembles a member of the CREB family. The 70-kDa polypeptide was identified as ATF-2 and seems to be more interesting because it promotes the occupation of the neighboring −150 CCAAT element (11). This observation prompted us to identify the liver factors that recognize the CCAAT site. Band shifts of liver factors to the FN-CCAAT probe systematically show a pattern characterized by a sharp band on top of a smear of higher mobility (Fig. 2A, lanes 1 and 4–9). Both binding complexes are competed efficiently by homologous FN-CCAAT oligonucleotides (Fig. 2A, lanes 2 and 3) but not by as much as 100-fold excess of FN-CRE (Fig. 2A, lanes 4 and 5) or the core of the SV40 enhancer (Fig. 2A, lanes 8 and 9) used as controls.

The −150 CCAAT element of the FN gene had been described as a NF-I site (8) based on sequence comparison. However, whereas competition with cold FN-CCAAT abolished binding to both observed complexes (Fig. 2A, A, lanes 1–3, and B, lanes 1 and 2), competition with an oligonucleotide with the consensus sequence for adenovirus NF-I only eliminated the bottom smear, leaving the top band unaffected (Fig. 2B, lane 3). Accordingly, the liver complexes observed with a 30-bp NF-I-labeled probe co-migrate with the bottom smear of the 32-bp FN-CCAAT probe (Fig. 2B, lane 4). These results showed that besides NF-I, the −150 CCAAT is interacting with other CCAAT factors that form the top band complex.

The −150 FN-CCAAT also interacts with CP1 (NF-Y/CBF) but not with C/EBP nor CP2—Binding to FN-CCAAT is not competed by the consensus sequence for the C/EBP (Fig. 2A, lanes 6 and 7). We conclude that C/EBP does not interact with FN-CCAAT, which is consistent with the fact that C/EBP-binding activity is resistant to heat inactivation, whereas the liver FN-CCAAT-binding activity is inactivated by heating at

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2 C. G. Pesce, C. R. Alonso, and A. R. Kornblihtt, manuscript in preparation.
65 °C for 10 min (10). Comparison of the consensus sequences for the three CCAAT factors C/EBP, NF-I, and CP-1 with that of FN-CCAAT (Fig. 2F) confirms that the latter exhibits more sequence identity with the sites for NF-I and CP1 than for that of C/EBP. This observation led us to investigate the participation of CP1 (also named NF-Y or CBF) in the binding to FN-CCAAT, with the aid of specific antibodies. Results in Fig. 2 support the conclusion that NF-I and CP1/NF-Y are the liver factors that bind to the −150 CCAAT element of the FN gene and that C/EBP and CP2 are not involved.

Disruptive Mutations of the −170 CRE Affect the Binding to the −150 CCAAT—We have previously shown that increasing the spacing between the CRE and CCAAT elements inhibited the occupation of the CCAAT box. A similar effect was obtained with a construct in which all promoter sequences upstream of the CCAAT box, including the −170 CRE, were deleted (named ∆CRE in Ref. 10). These experiments did not exclude that the sequences upstream of the CRE cooperate with the CCAAT site. To rule out this possibility and get a more direct evidence of the role of the CRE site, we prepared two different disruptive mutations of the −170 CRE, within the context of a −220 +65 fragment of the FN promoter (see “Experimental Procedures”). DNase I footprints of the wt and ∆ CRE promoters are shown in Fig. 3. In the wild type, both the −170 CRE and −150 CCAAT are protected. In the CRE mutant, the −170 CRE remains unprotected, and this disruption affects negatively the binding to the neighbor −150 CCAAT box. Quantitation of four separate experiments is expressed as fractional occupation at the bottom of Fig. 3. Similar results were obtained with the mut CRE constructs (not shown).

Immunoprecipitation of CCAAT-binding Proteins Complexed with ATF-2—The cooperative effect of the CRE site upon the binding to the CCAAT box might involve specific protein-protein interactions between ATF-2 and CP1 or NF-I. However, we failed to demonstrate this interaction in gel shifts, probably due to disassembly of complexes during electrophoresis.

In our search for a nonelectrophoretic method that could...
detect weak but specific protein-protein interactions, we explored the hypothesis that antibodies to ATF-2 would be able to precipitate a labeled FN-CCAAT probe bound to liver nuclear proteins after the formation of a “four-member sandwich” (anti-ATF-2/ATF-2/CCAAT-binding proteins/32P-FN-CCAAT), such as the one depicted in Fig. 4A. This new approach was inspired in the “three-member sandwich” immunoprecipitation (antibody/transcription factor/labeled DNA) described by McKay (31). We found that indeed anti-ATF-2 was able to quantitatively precipitate 32P label when added to a FN-CCAAT binding reaction. Nevertheless, a series of control experiments were necessary to assure us that this was not an artifact. Fig. 4B shows that the immunoprecipitated radioactivity corresponds to a bona fide FN-CCAAT oligonucleotide and not to a degradation product. It is also shown that the amount of precipitated oligo decreases when binding is competed by unlabel FN-CCAAT but not by the mutant FN-CCcCT. The inability of anti-ATF-2 to supershift FN-CCAAT complexes and to induce additional bands in the gel shift shown in Fig. 4C clearly indicates that anti-ATF-2 does not interact directly with the CCAAT-binding proteins nor with the FN-CCAAT probe alone and that ATF-2 does not bind the FN-CCAAT probe.

Another critical point is to demonstrate that anti-ATF-2 does not precipitate
any labeled ds oligonucleotide of the same specific activity as FN-CCAAT. Fig. 5A (right) shows that the mutant FN-CCccT probe is not precipitated by anti-ATF-2, confirming the specificity of our assay. The FN-CRE is precipitated by anti-ATF-2 but not by anti-CP1 (NF-Y) and anti-NF-I (Fig. 5B), the same antisera that proved to be active in precipitating labeled FN-CCAAT (Fig. 5A, left). This might indicate that unlike anti-ATF-2, anti-CP1 (NF-Y) and anti-NF-I interfere with complex formation. Experiments shown in Fig. 6 provide further support for specificity; competitions with a 25-fold excess of unlabeled FN-CCAAT significantly inhibit the amount of labeled FN-CCAAT precipitated by anti-ATF-2 and anti-CP1 antibodies. The FN-CCccT mutant had no effect. These factors should be NF-I, CP1, or both.

To address their nature we assayed NF-I and CP1 probes in immunoprecipitation experiments. Fig. 7 (bottom) shows that anti-ATF-2 is also able to precipitate CP-1 and NF-1 labeled probes, specifically bound and cross-linked to liver nuclear proteins, as evidenced in the gel shift of Fig. 7 (top).

All these experiments and their controls suggest that immunoprecipitation of the CCAAT probe by anti-ATF-2 is reflecting a specific interaction between ATF-2 and the liver FN-CCAAT-binding factors CP-1 and NF-1. The fact that addition of the FN-CRE ds oligonucleotide to binding reactions did not stimulate immunoprecipitation of the FN-CCAAT probe by anti-ATF-2 suggests that protein complexes are formed in solution in the absence of the target DNA for ATF-2. However, this procedure does not allow us to tell if the assembly of ATF-2/CCAAT-factors complexes occurs in the absence of FN-CCAAT DNA.

Transient Expression in Human Hepatoma Cells—The cooperative effect observed in vitro with liver nuclear extracts seems to correlate with the in vivo expression of FN-CAT constructs transfected in Hep3B cells. Fig. 8 shows that mutating both the CRE and CCAAT sites decreases promoter activity by 89%. The CCAAT mutation alone inhibits transcription by 40%. If the CRE and CCAAT sites were to act in an additive way, one would expect the CRE mutation to bring down activity by about 49%. However, the CRE mutant alone causes a 70% inhibition of transcription, suggesting a synergistic function of the CRE and CCAAT sites, where altering the CRE site might affect basal promoter activity by preventing not only factor binding to CRE but also to CCAAT.

DISCUSSION

The liver-specific occupation of the CRE and CCAAT sites of the FN promoter provides a useful model for studying tissue-specific cooperation between transcription factors. The liver factors that bind to each of the two sites are positive modulators as shown by competition of in vitro transcriptions (Fig. 1). This agrees with the fact that disrupting either the CRE or the CCAAT sites inhibits transcriptional activity in transfected hepatoma cells.

Before studying the functional role of the CRE-CCAAT interaction in response to regulatory signals, it is important to characterize the liver proteins that participate in this site
Interaction. In a previous report we found that liver ATF-2 was responsible for the cooperative effect (11). We now show that NF-I and CP1 but not C/EBP or CP-2 are the liver factors that recognize the −150 FN CCAAT site. A series of complementary experiments involving competitions with sequence-specific oligonucleotides, coincidental migration of specific complexes in band shifts, supershifts, and immunodepletions with highly specific antibodies support our conclusions. It becomes clear that at least in liver, the −150 FN-CCAAT site is not simply a NF-I site (8), because after competition with a huge excess of NF-I oligonucleotide or supershifting with an anti-NF-I antibody, there still remains an important amount of bound FN-CCAAT probe. Experiments shown in Figs. 2 and 7 demonstrate that this factor is CP1. Ruling out of C/EBP is clear from experiments in Fig. 2 and from our previous observation that liver FN-CCAAT-binding factors are thermolabile (10), whereas C/EBP is typically thermostable. In fact, liver C/EBP is more likely to cross-talk with the −170 CRE, rather than with the −150 CCAAT site, as shown by Bakker and Parker (37). In any case, when antibodies specific to NF-I and CP1 were added together to a gel shift reaction, they abolished completely FN-CCAAT binding (not shown), indicating that the presence of other FN-CCAAT-binding proteins in liver extracts is highly unlikely.

Interaction between the CRE and CCAAT elements was first proposed by Muro et al. (10). Independently, Miao et al. (36) suggested a similar interaction between these two sites on the rat FN promoter. We provide here further and stronger evidence for cooperativity at the FN CRE and CCAAT sites in liver: two different mutations that disrupt the CRE site also inhibit occupation of the neighboring CCAAT box. On the other hand we found that anti-ATF-2 is able to precipitate the 32P-labeled FN-CCAAT oligonucleotide cross-linked to NF-I and CP1 in liver extracts. This precipitation is inhibited by competitions with FN-CCAAT but not with a mutant oligonucleotide (FN-CCcCT). The supershift and Western experiments in Fig. 4 respectively demonstrate that anti-ATF-2 does not react neither directly with the FN-CCAAT probe nor with NF-I or CP1. These and other experiments shown in the text not only put in evidence macromolecular assemblies between ATF-2, NF-I, and CP1 but also illustrate the use of a simple, reliable, and fast method for studying those specific protein-protein interactions that are affected by disruptive electrophoretic conditions of gel shift assays.

These in vitro studies seem to correlate well with a mild but reproducible (see triplicates of transfections in Fig. 8) synergistic function observed in vivo in Hep3B human hepatoma cells transfected with wt and mutant FN-CAT constructs.

To our knowledge, this is the first evidence of a cooperation between NF-I, CP1, and ATF-2. We have not yet used purified proteins to test it. We did try, and failed, to detect protein-protein interactions between rabbit reticulocyte lysates programmed with synthetic mRNAs for ATF-2 and CP1 (NF-YA + NF-YB, not shown). This could be due to the absence in reticulocyte lysates of co-factors present in liver nuclear extracts such as the C subunit of CP1 (23) or the lack of an appropriate protein modification. Besides, recombinant ATF-2 made in reticulocyte lysates is a unmodified homodimer, whereas natural ATF-2 requires phosphorylation for binding DNA and activating transcription (16) and acts as a heterodimer. Preliminary results indicate that the liver partner of ATF-2 is not c-jun, contrary to what was found in other systems (15).

CP1 (NF-Y or CBF) is a multimeric factor that is required for tissue-specific activation of several genes, including albumin, and that plays a negative role in the transcription of the liver-specific aldolase B promoter (38). CP1 is not itself tissue-specific but is ubiquitous, and it is thought to act through interaction with appropriate tissue-specific or regulated factors to make possible the correct pattern of expression. This is particularly well illustrated by the strict need for CP1 in the sterol-dependent transcription of farnesyl diphosphate synthase and HMG-CoA synthase genes. In both cases, CP1 is not the regulatory protein but is essential for optimal activation, playing a role similar to SP1 in sterol-dependent regulation of the LDL receptor gene (39). Both CP1 and SP1 contain glutamine-rich activation domains and would cooperate with SREBP, the sterol response element-binding protein. None of the CP1 subunits shows homology with leucine zippers, helix-loop-helix domains, or the known protein dimerization domains. CP1 subunits are bound together by unique interaction motifs (23). This implies that if ATF-2 were to contact CP1, it would not dimerize through its leucine zipper.
NF-I roles have been studied more extensively. This factor exists in several forms, one of them is particularly enriched in liver. NF-I binds simultaneously to adjacent sites within the liver-specific enhancer of the serum albumin gene, creating a composite regulatory element with properties that differ from those of either factor bound alone. NF-I modulates positively or negatively HNF3 activity depending on the context of the albumin enhancer. NF-I also cooperates with HNF1, another liver-specific transcription factor, in liver-specific transcription from the tyrosine aminotransferase proximal promoter (41).

Two domains of NF-I have attracted interest for their surprising functions. One of them bears high sequence identity with the heptapeptide repeats of carboxyl-terminal domain of RNA polymerase II, and it is usually referred to as NF-I liver. J. Jackson et al. (40) demonstrated that NF-I liver and the hepatocyte-specific transcription factor HNF3 bind simultaneously to adjacent sites within the liver-specific enhancer of the serum albumin gene, creating a composite regulatory element with properties that differ from those of either factor bound alone. NF-I modulates positively or negatively HNF3 activity depending on the context of the albumin enhancer. NF-I liver also cooperates with HNF1, another liver-specific transcription factor, in liver-specific transcription from the tyrosine aminotransferase proximal proximal promoter (41).

In conclusion, a wide variety of cooperative effects that are relevant in basal, regulated, and tissue-specific transcription have been described for CP1 and NF-I. Accumulated evidence suggests that the CRE and CCAAT elements of the F1 gene take part of a composite site. To demonstrate that it is a composite response element, such as the one described by Yamamoto and colleagues (for a review see Ref. 44), it becomes imperative to assess the role of the F1-CCAAT box, NF-I, and CP1 in modulating signals that regulate FN transcription through TGF-β (1, 3), where NF-I is an obvious candidate for signal transduction.

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