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C/EBP Binds over the TATA Box and Can Activate the M Promoter of 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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The gene A coding for 6-phosphofructo-2-kinase gives rise to three mRNA that originate from distinct promoters called F (fetal), M (muscle) and L (liver). The regulation of the M promoter is ill-understood and its TATA box region binds an unidentified factor. The aim of this work was to identify this factor and to investigate its activity. *In vitro* protein-DNA binding assays and transfection experiments showed that this factor is C/EBP and that C/EBP can stimulate the M promoter despite its potential interference with TFIID binding. The effect of C/EBP was abolished by a dominant negative variant of C/EBP. These data and other work lead to the conclusion that C/EBP may participate to the regulation of promoter switching in 6-phosphofructo-2-kinase gene A.

The expression of many genes is restricted to particular tissues in part because of a tissue-specific control of transcription. To understand the underlying mechanisms, we have been studying a gene that codes for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2). This is a bifunctional enzyme that catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, the most potent stimulator of 6-phosphofructo-1-kinase, which is a key enzyme of glycolysis (1). The PFK-2 gene of interest (gene A) gives rise to three mRNAs called F (fetal), L (liver), and M (muscle). These mRNAs originate from distinct promoters. The F promoter is active in proliferating cells and it is stimulated by the oncoproteins *ets* and E2F (2,3). The L promoter is active only in liver (4) where it is controlled by the liver-enriched transcription factors HNF-3 and HNF-6 (5,6). The M promoter is active in skeletal muscle and in adipose tissue (7,8 and our unpublished observations). Its caracterization (9) did not provide a clear understanding of its regulation, but yielded the puzzling observation that liver nuclear extracts produce a DNase I footprint over the TATA box. One possibility was that the protein(s) responsible for this footprint interfere(s) with binding of TFIID to the M promoter and prevent its transcription in liver. A similar mechanism has been described for the transcription factor GATA. This protein binds to the promoter of the rat platelet factor 4, mouse erythropoietin, and chicken β globin genes. By doing so, GATA competes with basal factors and represses transcription in nonerythropoietic cells (10). Another example was the REST (11)/NRSF (12) silencer factor, which represses in nonneuronal cells the transcription of neuron-specific genes by binding to the promoter of such genes. The second possibility was that, despite its binding over the TATA box of the M promoter, the unidentified protein is capable of transactivating it. The aim of this work was to characterize this protein and to study its activity.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The plasmid PMpGL3 was obtained by ligating the *Xho*I/*Sal*I fragment of p2KM91luc containing the sequence −97/+41 relative to the major cap site of M mRNA (9) in the *Xho*I site of the luciferase reporter gene pGL3-basic vector (Promega). The expression plasmids for C/EBPα, CHOP-10 and CHOP-10-LZ$^-$ were kindly provided by D. Ron. The sequences of the double-stranded oligonucleotides were as follows: oligo A, tcgagAATATTTCAACACTGCACACg, which corresponds to the liver footprint (−31/−12) over the TATA box of the M promoter; oligo C/EBP, GATCAATTCAATTGGGCAATCAGGAATT; oligo NF-I, ATTTTGGCTACAAGCCAATATGAT.

Band-shift assays. Rat liver or FTO-2B cell nuclear extracts were prepared as described (13,14). Two (liver) or 7 (FTO-2B) μg of nuclear

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Abbreviations used: CAT, chloramphenicol acetyltransferase; PFK-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; C/EBP, CAAT/Enhancer Binding Protein; CHOP, C/EBP-homologous protein; PLO, poly-L-ornithine.

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proteins were incubated at 4°C for 30 min in a final volume of 20 μl containing the ^{32}P-labeled probe (5000 cpm), 3 μg of poly(dI.dC), 20 mM Hepes (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 10 % (v/v) glycerol. Samples were loaded on 6% nondenaturing acrylamide gels (acrylamide/bisacrylamide ratio was 29:1) and run at 4 °C at 200 V in 25 mM Tris borate, 0.25 mM EDTA. The gels were prerun for 1 h at 200 V at 4°C. When competition experiments or immune reactions were performed, the competing oligonucleotide or the antiserum (1 μl) was preincubated for 20 min at 4°C with the nuclear proteins in the binding buffer prior to addition of the radiolabeled probe. In some experiments, liver extracts were pre-heated at the indicated temperature for 5 min.

Cell cultures and transient transfections. Rat hepatoma FTO-2B cells, grown as monolayers in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, were plated on 60-mm dishes (10^6 cells) 24 h before transfection with a calcium-phosphate-DNA coprecipitate (15) containing 5 μg of PMpGL3, different amounts of expression plasmids and 2 μg of pSV_2CAT plasmid as an internal control. The total amount of DNA was kept constant by adding pSP72 plasmid. After 16 h, the cells were washed 3 times and further incubated for 24 h before harvesting. Rat myoblast L6 cells, grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, were transfected with poly-L-ornithine (PLO) (16). Cells (2.10^5) were plated on 60-mm dishes 24 h before transfection. The PMpGL3 plasmid (4 μg) was mixed with different amounts of expression plasmids, 2 μg of pSV_2CAT and 15 μg of PLO in an Eppendorf tube, the volume being kept constant by adding water. Each dish was incubated with 2 ml of medium and the DNA/PLO mix and swirled every 90 min. After 6 h of incubation, cells were shocked for 5 min with 3 ml of medium containing 25% dimethylsulfoxide. After two washings, the cells were incubated for 36 h before harvesting. Luciferase activity was quantified on 50 μl of cell extract with the "luciferase assay system" kit (Promega) in a Lumac biocounter M2000 after cell disruption with 200 μl of lysis buffer. Cell extracts (50 μl) were also used to quantify CAT activity with the "CAT-ELISA" kit (Boehringer). Background values, obtained with extracts of cells transfected with pSP72, were about 5 U (Lumac light units) with FTO-2B cells and 20 U with L6 cells. Maximal values obtained with PMpGL3 were 55000 U in FTO-2B cells and 40000 U in L6 cells. Given that some luciferase reporter vectors such as pXP2 are artifactually stimulated by C/EBPα (17) we verified that pGL3 was insensitive to cotransfected C/EBPα in our experiments. This was the case (data not shown).

RESULTS AND DISCUSSION

To identify the factor that binds over the TATA box of the M promoter, we performed band-shift experiments with liver nuclear extracts and an oligonucleotide that corresponds to the promoter region from −31 to −12 (oligo A). Five complexes were observed, four of which (***a-d***) were specific as demonstrated by competition with excess nonlabeled probe (Fig. 1A). Both the GATA and the REST/NRSF transcription factors are zinc finger proteins. Moreover, 12 out of the 17 nucleotides located from −28 to −12 in the M promoter fit with the REST/NRSF binding consensus (12). We therefore repeated the band-shift experiments in the presence of the zinc chelator phenanthroline. This agent did not prevent the appearance of the specific complexes (Fig. 1A), excluding the involvement of zinc finger proteins. We then found that three of the specific complexes were still seen (Fig. 1A) after incubating the liver extract at

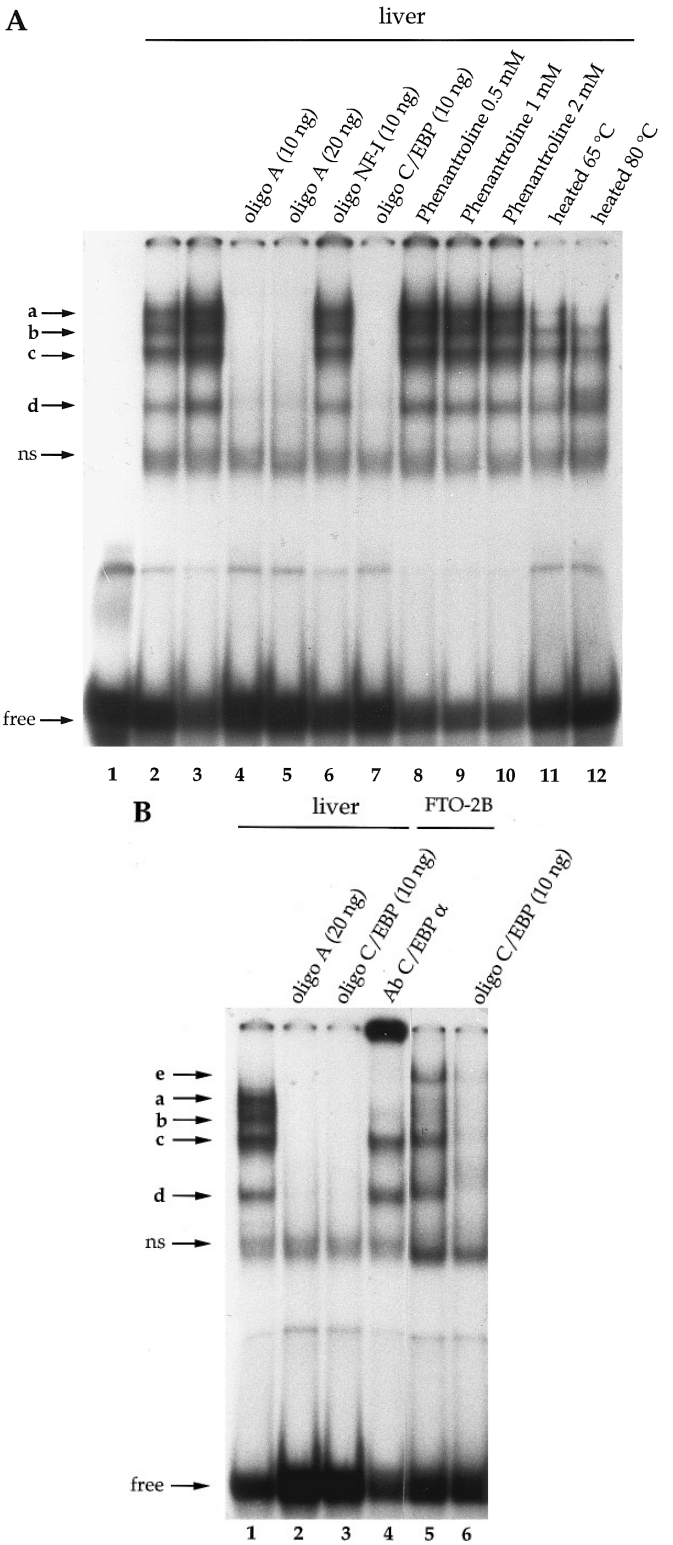

FIG. 1. Band-shift analysis of nuclear protein binding to the TATA box region of the M promoter. A radioactive probe (oligo A) corresponding to the sequence from −31 to −12 of the M promoter was incubated without (A, lane 1) or with nuclear extracts in the absence or presence of competing nonlabeled oligonucleotides, of 1,10-phenanthroline, or of an antiserum (Ab) against C/EBPα. The incubation buffer contained 5 mM $MgCl_2$, except for A, lane 2. ***a-e,*** specific complexes.

up to 80°C, suggesting that they might be due to CAAT/enhancer binding protein (C/EBP). Indeed, C/EBP is the only known liver-enriched transcription factor that is heat-stable (18). We therefore tested whether formation of the specific complexes is prevented by a competing C/EBP oligonucleotide. This was the case, as the C/EBP oligonucleotide actually inhibited all the specific complexes. A competing oligonucleotide specific for NF-I, which in the intact promoter produces a footprint immediately upstream (9), had no effect (Fig. 1A). We concluded from these experiments that the TATA box region of the M promoter binds C/EBP. The sequence from −28 to −22 indeed fits with the quite loose (19) C/EBP binding consensus.

We next investigated the possibility that C/EBP inhibits expression of the M promoter in hepatic cells. To do so, we used a dominant negative variant of C/EBP. Indeed, C/EBP belongs to the basic leucine zipper family of transcription factors and it binds DNA as a dimer. It displays several isoforms, among which CHOP-10 (GADD 53) (20). The latter is a truncated protein that cannot bind DNA but still associates through its leucine zipper with C/EBP, thereby preventing its binding to DNA. We chose FTO-2B hepatoma cells since they can transcribe transfected reporter genes driven by the L promoter (6). We reasoned that these cells could also transcribe a reporter gene linked to the M promoter if co-transfected with a CHOP-10 expression vector that would relieve the postulated inhibition exerted by C/EBP.

We first verified that the FTO-2B cells do contain endogenous C/EBP. This was the case. Band-shift experiments with FTO-2B cell nuclear extracts and oligo A showed three specific complexes (***c, d, e*** in Fig. 1B) and these were all competed by excess unlabeled oligo C/EBP. Two of these complexes (***c*** and ***d***) comigrated with complexes seen with liver nuclear extracts. Complexes ***a*** and ***b,*** detected with liver nuclear extracts, were not seen with FTO-2B cell extracts. They were attributed to C/EBPα since this isoform is absent from many hepatoma cells (21). To confirm this, we preincubated the liver extract with an antibody directed against C/EBPα and indeed complexes ***a*** and ***b*** were prevented (Fig. 1A). Complex ***a*** could correspond to binding of the long (42/40 kDA) and complex ***b*** to the short (30 kDA) forms of C/EBPα (22). We concluded that the FTO-2B cells were a convenient model to test our hypothesis.

To investigate whether C/EBP can inhibit M promoter activity, we therefore transfected FTO-2B cells with the minimal M promoter linked to the luciferase reporter gene, in the absence and in the presence of overexpressed CHOP-10. As a control, we used a leucine zipper-deletion mutant of CHOP-10 (CHOP-10-LZ$^-$) which cannot act as the natural dominant negative isoform (20). Consistent with our hypothesis, the basal transcriptional activity was increased by CHOP-10 and was not affected by CHOP-10-LZ$^-$ (Fig. 2A). However, this increase was very small and was no longer statistically significant at the outcome of three independent experiments. We reasoned that perhaps there was not enough CHOP-10 to titrate endogenous C/EBP. Another possibility was that the C/EBP isoforms present in FTO-2B cells did not mimick the situation in hepatocytes. If either one, or both, of these possibilities held true, then overexpressing C/EBPα in FTO-2B cells should inhibit transcription from the M promoter. Indeed, C/EBPα can repress transcription of the β_2-adrenergic receptor gene in rat liver (23). The result of this experiment showed that, contrary to the prediction, C/EBPα actually increased transcription of the reporter gene (Fig. 2B). Moreover, we could demonstrate that, under our experimental conditions, CHOP-10 was an adequate tool to oppose C/EBP action. To do so, we used L6 cells in which overexpressed C/EBPα stimulated the transfected M promoter more strongly than in FTO-2B cells (Fig. 3). This is consistent with the absence of endogenous C/EBP binding over the TATA box in L6 cells (9). In these cells, overexpressed CHOP-10 completely abolished stimulation of the M promoter-directed reporter gene by C/EBP (Fig. 3).

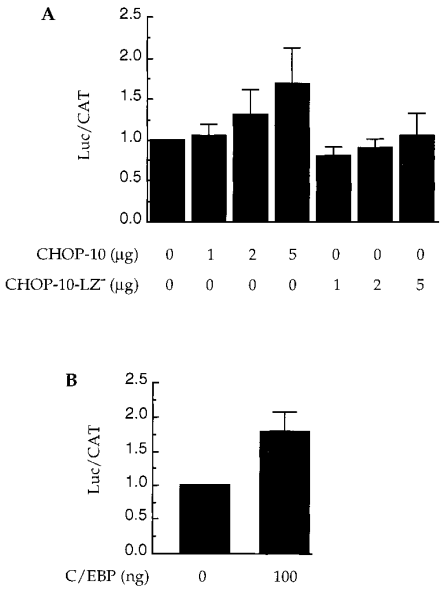

FIG. 2. Effect of overexpressing the dominant negative CHOP-10 or its inactive deletant (A) and C/EBPα (B) on M promoter activity in FTO-2B cells. Means ± S.E.M. for 3 (A) or 4 (B) independent experiments.

In conclusion, we have shown here that the transcription factor that binds over the TATA box region of the PFK-2 M promoter is C/EBP. We have also shown that this factor is capable of stimulating the M promoter. The effect is specific in that it is abolished by a dominant negative mutant of C/EBP. This raises the question of how factor binding over a region that in-

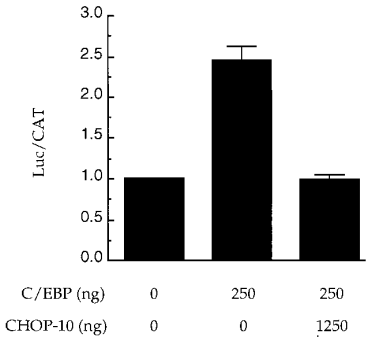

FIG. 3. Effect of overexpressing C/EBPα alone or together with CHOP-10 on M promoter activity in L6 cells. Means ± S.E.M. for 4 independent experiments.

cludes the TATA box can activate transcription. This intriguing situation has been described for a few genes, in which case it was proposed that TFIID is recruited at the promoter site by tethering with the promoter-bound transcription factor rather than via binding of TFIID to the TATA box itself (24). Such a role for C/EBP in activating the M promoter is attractive because of the poor TATA box consensus of this promoter.

Our data provide insight into the mechanism by which the M promoter is activated in white adipose tissue, as C/EBP is an important transcriptional activator of genes expressed in adipocytes (25). In this case, C/EBP could act in synergism with NF-I, which binds just upstream of C/EBP i.e. from −31 to −52 (9) in the M promoter. A cooperation between C/EBP and NF-I has indeed been postulated for the activation of the phosphenolpyruvate carboxykinase promoter (26). Another implication of our data stems from the provocative recent finding (27) that the DNA-binding and transactivation properties of C/EBP are stimulated through its direct interaction with the tumor suppressor protein Rb and that this is crucial for C/EBP-dependent terminal differentiation of fibroblasts into adipocytes. We have shown (3) that fibroblasts express PFK-2 mRNA from the F promoter and that this depends on the transcription factor E2F. The latter is inhibited by Rb, which switches off the F promoter. Thus, C/EBP could be an important component of the F-to-M promoter switch during differentiation of fibroblasts into adipocytes.

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