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C/EBP Factor Suppression of Inhibition of Type II Secreted Phospholipase A_2 Promoter in HepG2 Cells: Possible Role of Single-Strand Binding Proteins

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We previously reported that the type II secreted phospholipase A_2 ($sPLA_2$) promoter from positions (−326 to +20) ([−326;+20] promoter) is negatively regulated by two adjacent regulatory elements, C (−210 to −176) and D (−247 to −210). This study examines in greater detail the way in which this negative regulation operates. Successive 5′ deletions of the [−326;+20] type II $sPLA_2$ promoter indicated that the region upstream of position −195 inhibits the transcription activity sixfold in HepG2 cells but not in HeLa cells. Although the whole [−326;−176] region decreased the activity of a heterologous thymidine kinase promoter, this effect was orientation and position sensitive. C/EBPβ, C/EBPα, and C/EBPδ, which bind to element C, prevented the inhibition of promoter activity. Electrophoretic mobility shift experiments identified the binding of NF1-like proteins to the [−225;−218] site, which overlaps an insulin response-like sequence, 5′-TGTTTTG-3′. This sequence bound a factor which also recognized the promoters of the apolipoproteins C-III and A-II. Substitutions preventing the binding of this factor or the NF1-like proteins did not increase the transcription activity, but substitution in the [−217;−204] sequence blocked the transcription inhibition. This sequence did not bind any double-strand binding factor, but its antisense strand is critical for the binding of single-strand binding proteins to the [−232;−191] region. We therefore suggest that these single-strand binding proteins are involved in the inhibitory mechanism.

The phospholipases A_2 constitute a large family of enzymes which hydrolyze the ester bond at the *sn*-2 position of glycerophospholipids to give the free fatty acids and lysophospholipids which are the precursors of the inflammatory lipid mediators. A secreted phospholipase A_2 ($sPLA_2$) has been purified from the synovial fluid of patients with rheumatoid arthritis (50). This enzyme is similar in molecular weight and molecular structure to the pancreatic phospholipase A_2. The same amino acids are involved in the binding of calcium and catalytic processes of both enzymes, but the enzymes are encoded by different genes (50). The $sPLA_2$ from synovial fluid is therefore named type II $sPLA_2$ to distinguish it from the pancreatic type I enzyme. High concentrations of type II $sPLA_2$ are found in the sera of patients suffering from septic shock (33) and inflammatory diseases (31). Crowl et al. (11) reported that the type II $sPLA_2$ gene in the human hepatoma cell line HepG2 is stimulated by inflammatory cytokines and suggested that this enzyme is one of the acute-phase response proteins.

Studies on the regulation of the acute-phase response genes have pointed to the role of interleukin-6 (IL-6) in these phenomena. Some members of the C/EBP family in addition to the JAK-STAT proteins (56) mediate the stimulation of transcription by IL-6. These factors bind to the promoters of many acute-phase response genes, including those of haptoglobin and C-reactive protein (44), α_1-acid glycoprotein (26, 44), and the serum amyloid A family (20, 45). The C/EBP family has three main members, C/EBPα, C/EBPβ, and C/EBPδ (8, 44). The cell contents of C/EBPδ and C/EBPβ mRNA increase markedly after incubation of the hepatocytes with IL-6 (4) or after treatment of the animals with lipopolysaccharide or turpentine (2, 4). C/EBPβ and C/EBPδ are also regulated by phosphorylations (45, 54).

We have shown that the region from positions −326 to +20 ([−326;+20] region) of the type II $sPLA_2$ gene induces the basal transcription of a chloramphenicol acetyltransferase (CAT) reporter gene in transiently transfected HepG2 cells (37). The transcription activity of this [−326;+20] promoter fragment is also stimulable by IL-6. The promoter has four regulatory elements, A (−35 to −6), B (−125 to −85), C (−176 to −210), and D (−210 to −247), and elements C and D constitute putative C/EBP binding sites. We suggested that element C is the main acute-phase-responsive element of the type II $sPLA_2$ gene. A promoter having only the proximal elements A and B has much greater transcription activity than the whole [−326;+20] region, suggesting that the distal elements C and D have negative effects on type II $sPLA_2$ gene expression. There is a DNase I-hypersensitive site, which separates the adjacent elements C and D. Negative regulatory elements have been identified in the promoters of several acute-phase genes such as the serum amyloid A1 (27) and α_1-acid glycoprotein (26) genes. Lipopolysaccharide induction of the α_1-acid glycoprotein gene correlates with a decrease in binding to the negative regulatory elements (26). These genes may therefore be induced by removal of the negative regulation by stimulatory factors such as C/EBP family members.

This study explores the negative regulation of the type II $sPLA_2$ promoter activity and its correlation with the stimulation of transcription activity by the C/EBP proteins. We identified other liver nuclear factors which bind to elements C and D but are not involved in inhibition of the promoter activity. We have also demonstrated that the junction region overlapping elements C and D is recognized by single-strand binding proteins (SSBPs). We suggest that these proteins mediate the negative regulatory effect of this region.

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MATERIALS AND METHODS

Materials. Restriction enzymes, T4 kinase, Vent polymerase, and ligase were purchased from Biolabs or Eurogentec (Seraing, Belgium). Synthetic oligonucleotides were provided by Eurogentec or Oligoexpress (Paris, France). Materials for cell culture were purchased from Gibco-BRL (Dulbecco's modified Eagle medium supplemented with fetal calf serum, HEPES, and trypsin) and from Falcon Inc. (flasks and petri dishes). The β-galactosidase expression vector CMVβ-gal was from Clontech; poly(dI-dC) and deoxynucleotides were from Pharmacia. The antibodies to rat C/EBPα, C/EBPβ, C/EBPδ, and NF1 were provided by Santa Cruz Biotechnology, Inc. The secondary peroxidase-coupled goat antibody to rabbit immunoglobulin was provided by Gibco-BRL. The radioactive products were from Amersham. The various constructs were sequenced on an ABI 373A automatic sequencer, using dye terminator FS DNA sequencing kits from Perkin-Elmer. All other chemicals were purchased from Fluka.

Plasmid constructions. The construction of the [−326;+20]-, [−264;+20]-, and [−159;+20]-sPLA$_2$-pUC-SH-CAT constructs has been described previously (37). The plasmids containing the other 5′-deleted fragments of the promoter were similarly made by enzymatic amplifications using [−326;+20]-sPLA$_2$-pUC-SH-CAT as a template and by inserting the corresponding PCR products into the *Xba*I/*Hin*dIII sites of the native pUC-SH-CAT plasmid (35). In the substitution mutant ΣC-[−326;+20]-sPLA$_2$-pUC-SH-CAT, the [−204;−181] fragment of element C was replaced by the nonspecific sequence 5′-GTGAATTCGAGCTCG GTACCCGGG-3′ (37) through two series of PCR. In the first step, pUC-[−326; +20]-sPLA$_2$-CAT was used as the template to amplify two overlapping fragments, each containing half of the substituting sequences. The primary PCR products were purified by electroelution to remove the primers and the wild-type template. Then the 347-bp-long mutated fragment was amplified from a mixture containing aliquots of both the primary fragment and the primers, which corresponded respectively to the 5′ and 3′ ends of the fragment and contained the *Xba*I and *Hin*dIII sites. The PCR product was purified and ligated to the *Xba*I and *Hin*dIII sites of pUC-SH-CAT plasmids. The same method was used for the other substitution mutant of the type II sPLA$_2$ [−326;+20] promoter. The primers which were used in the first series of PCR corresponded to the sense and antisense strands of the DM1, DM2, and JM2 oligonucleotides (see Fig. 4A and 5A). They were used to construct plasmids Σ[−228]-, Σ[−223;−218]-, and Σ[−217;−204]-sPLA$_2$-pUC-SH-CAT. The insert of plasmid Σ[−199;−197]-pUC-SH-CAT was produced by using the oligonucleotides 5′-GACACGTAAG GGCCCCCAATCCTCAACTCTGTCC-3′ and 5′-GGACAGAGTTGAGGAT TGGGGGCCCTTCGTGTC-3′ as sense and antisense primers in the first series of PCR. The constructs enclosing the [−326;−195] and [−326;−176] regions upstream of the thymidine kinase promoter were built by amplifying each fragment from the [−326;+20]-pUC-SH-CAT template and inserting the products into the *Xba*I site of plasmid pTK77CAT (19).

The C/EBPα expression vector was produced after amplification by Vent polymerase (Biolabs). The template was the rat C/EBPα cDNA (25) inserted into the PMT2 vector (22). The PCR products were digested by *Hin*dIII, blunt ended by using Klenow polymerase, redigested with *Xho*I, and then ligated into the *Xho*I and *Eco*RV sites of the PHD vector (32). The rat C/EBPδ, LIP, and C/EBPβ cDNAs in the PHD expression vector were generously provided by G. Ciliberto (IRBM, Rome, Italy). The PMT2 expression vector containing the NF1-L cDNA was a gift from P. Cardot (INSERM CJF9508, Paris, France).

Cell cultures, transfections, and CAT assays. HepG2 and HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. The cells were transfected by the calcium phosphate-DNA coprecipitation method. The cells were harvested 53 h after the beginning of transfection, resuspended in 100 μl of 0.1 M Tris-HCl (pH 7.8)–1 mM EDTA–150 mM NaCl, and collected by centrifugation at 4°C for 5 min. They were lysed by incubation in 50 μl of 100 mM Tris (pH 7.8)–0.7% Nonidet P-40 for 15 min at 4°C, and the nuclei and cell debris were pelleted by low-speed centrifugation. The supernatants were stored at −80°C.

The CAT assays were performed by the two-liquid-phase procedure of Desbois et al. (13). The reaction mixture included 1.25 mM chloramphenicol, 0.1 mM unlabeled acetyl coenzyme A, and 0.1 μCi of [^{3}H]acetyl coenzyme A (specific activity, 5 Ci/mmol) in 100 μl of 100 mM Tris-HCl (pH 7.8). Diluted cell lysate (50 μl) was added, and the reaction mixture was placed in a scintillation vial under 3 ml of hydrophobic scintillation liquid to measure the rate of chloramphenicol acetylation. The β-galactosidase activities of the cell lysates were used to normalize variations in transfection efficiency. Each transfection was repeated two to six times with two to three different preparations of plasmids as indicated in the figure legends.

Electrophoretic mobility shift assays (EMSAs). Extracts of rat liver nuclei were prepared as described by Gorski et al. (16). Nuclear extracts of HepG2 and HeLa cells were prepared as described by Osborn et al. (38). Lysates of COS-1 cells were prepared 40 h after transfection of the cells with the NF1 expression vector (37). Double-stranded probes or competitors were produced by diluting the pairs of complementary single-stranded oligonucleotides (5 μg of each) in 20 μl of 10 mM Tris HCl (pH 8)–10 mM $MgCl_2$–50 mM NaCl, heating them to 95°C for 5 min, and allowing them to hybridize for 3 h at room temperature. Hybridization was checked on a 3% agarose gel. The solution was diluted with 80 μl of water and stored at −20°C. Single-stranded or double-stranded probes were labeled by using T4 kinase and 50 μCi of [γ-^{32}P]ATP. Free nucleotides were separated from the labeled probes on a Sephadex G-50 column. The specific activities of the probes were estimated by spotting 1 μl of the labeling volume (before G-50 chromatography) on thin-layer chromatography plates and counting the labeled probes and free nucleotide spots. Extracts of rat liver, HepG2, and HeLa cell nuclei or COS-1 cell lysates were preincubated for 15 min at 4°C in 18 μl of 25 mM HEPES (pH 7.6)–8% Ficoll–40 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–3 μg of poly(dI-dC). When competitor oligonucleotides were used, they were added in 100- to 250-fold excess over the concentration of the probe (see figure legends), and the reaction mixture was incubated for a further 15 min at 4°C. Labeled probes were then added, and the incubation was continued for 30 min at 4°C. Free DNA and DNA-protein complexes were resolved in 6% 30/2 acrylamide/bisacrylamide gels (except for experiments using CWT oligonucleotide and HepG2 nuclear extracts, which were performed on 5% 30/1 acrylamide/bisacrylamide gels) in buffer containing 6.7 mM Tris-HCl, 3.3 mM sodium acetate, and 1 mM EDTA (pH 7.9). The gels were dried and exposed to X-Omat^{AR} films (Kodak).

In addition to the unlabeled oligonucleotides corresponding to the wild-type and mutant probes, the NF1 oligonucleotide (5′-ACAATTTTTTGGCAAGAA TATTAT-3′) and the C/EBP oligonucleotide (5′-TGGTATGATTTTGTAATG GGGTAGGA-3′) (elements E and D of the murine albumin promoter) (28), the HNF3 oligonucleotide (5′-GTTGACTAAGTCAATAATCAGA-3′; HNF3 binding element of the transthyretin promoter) (10), the A-IID oligonucleotide (5′-TGCTTCCTGTTGCATTCAAGTCCAAG-3′; element D of the apolipoprotein A-II promoter) (9), and the C-IIIC oligonucleotide (5′-CGCTTGCT GCATCTGGACA-3′; element C of the apolipoprotein C-III promoter) (35) were used in EMSAs. When anti-NF1 and anti-C/EBP antibodies were used in supershift experiments, 1 μl (1 μg) of each antibody was added to the nuclear extracts in a total volume of 13 μl of 25 mM HEPES (pH 7.6)–8% Ficoll–40 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol and incubated for 1.5 h at 4°C prior to addition of poly(dI-dC) and the competitor and probe oligonucleotides.

Western blot analysis. HepG2 cells (6×10^6 cells in P100 dishes) were transfected with 30 μg of the various C/EBP expression vectors or the native PHD plasmid (control cells) and 5 μg of β-galactosidase expression vector. The cells were harvested 48 h after transfection, and nuclear extracts and cytosol were prepared as described by Osborn et al. (38). β-Galactosidase activities in the cytosols were measured. The nuclear proteins were diluted in gel-loading buffer containing 60 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.025% bromophenol blue and loaded on a 1.5-mm-thick SDS–12% polyacrylamide minigel. The amounts of nuclear proteins were normalized to the β-galactosidase activities. A full set of protein molecular mass markers (Bio-Rad) was also loaded as controls to evaluate molecular masses. The minigel was run in a 25 mM Tris–192 mM glycine–0.1% SDS migration buffer at 150 V for 1 h and transferred onto a nitrocellulose filter. The filter was stained with Ponceau red, destained, and sliced into three blots. The blots were prehybridized in 10 mM Tris (pH 7.5)–100 mM NaCl–5% fat-free milk at room temperature for 1.5 h and hybridized in 10 mM Tris (pH 7.5)–100 mM NaCl–1% fat-free milk with 1/1,000 dilutions of the specific anti-C/EBP antibodies for an additional 1.5-h period. The blots were then washed three times for 15 min each and incubated for 1.5 h at room temperature in 10 mM Tris (pH 7.5)–100 mM NaCl–2% fat-free milk with 1/5,000 dilutions of the secondary peroxidase-coupled antibody to rabbit immunoglobulin. They were developed by enhanced chemiluminescence (Amersham).

RESULTS

Identification of a negative regulatory region in the type II sPLA$_2$ promoter whose effect is blocked by overexpression of C/EBP factors. The negative regulatory region in the [−326; +20] type II sPLA$_2$ promoter in HepG2 cells (37) lies upstream of the high-affinity C/EBP binding site (5′-TTGGGAA AC-3′/3′-AACCCTTTG-5′) which is in the reverse orientation between positions −200 and −191 on element C (Fig. 1A). The sequence involved in this negative effect was more precisely defined by successive 5′ deletions from elements C and D (Fig. 1A) identified in previous footprint experiments (37). The various DNA products were cloned upstream of a CAT reporter gene. We also used the previously constructed [−326; +20]-, [−264;+20]-, and [−159;+20]-sPLA$_2$-pUC-SH-CAT plasmids to transiently transfect HepG2 cells or HeLa cells. Deletions from positions −326 to −225 did not alter the CAT activities in HepG2 cells (Fig. 1B). The activity increased twofold when the [−225;−203] region was removed. A further deletion at position −195 enhanced the transcription activity sixfold over that induced by the [−326;+20] fragment (Fig. 1B). This last deletion disrupted the high-affinity C/EBP binding site (37). Deletion of the whole element C in the [−159;

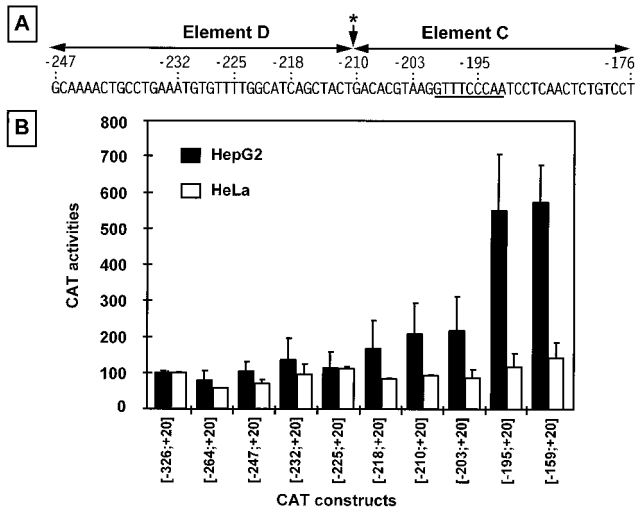

FIG. 1. Sequences of the distal footprint elements C and D of the type II $sPLA_2$ promoter (A) and transcription activities of the [−326;+20] promoter and the various 5′-deleted mutants in HepG2 and HeLa cells (B). Element C covers positions −210 to −176, and element D extends from positions −247 to −210. The [+1;+20] sequence corresponds to the whole exon 1 of the type II $sPLA_2$ gene. The asterisk indicates the DNase I-hypersensitive site (37). The high-affinity C/EBP binding site on element C is underlined. The end of each 5′-deleted construct is indicated on the element C and D sequences. P60 dishes of 60% confluent HepG2 cells were transfected by the calcium phosphate coprecipitation method, using 12 μg of the various CAT constructs and 2 μg of the CMVβ-gal expression vector, and CAT activities were measured 53 h after transfection. The activities are expressed relative to that induced by the [−326;+20] fragment in HepG2 cells and HeLa cells. The error bars indicate the standard deviations calculated from the data of four independent transfections performed in duplicate with 12 μg of plasmids prepared from two different bacterial cultures.

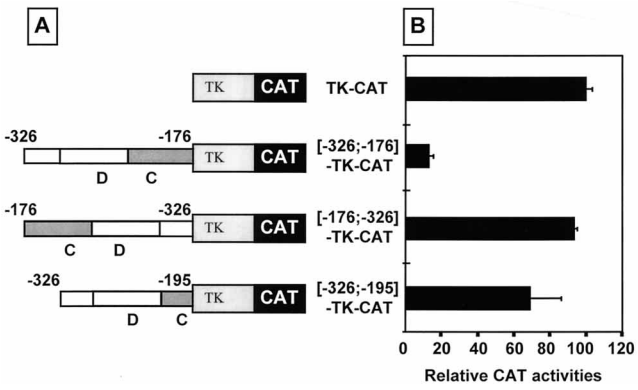

FIG. 2. Construction of the plasmid containing the HSV TK promoter (A) and effects of the [−326;−176] and [−326;−195] fragments of the type II $sPLA_2$ gene on this heterologous promoter (B). The [−326;−176] and [−326;−195] fragments were inserted upstream of the minimal 77-bp-long promoter of the HSV TK gene at the *Xba*I site of plasmid pTK77-pUC-SH-CAT (19). The orientation of the insert was determined by *Eco*RI digestion. There are two *Eco*RI restriction sites on the wild-type pTK77-pUC-SH-CAT plasmid, and one site was incorporated in the sequence of the sense primer. The HepG2 cells were transfected by the calcium phosphate coprecipitation method, using 12 μg of each pTK77-CAT construct and 2 μg of the CMVβ-gal expression vector as for Fig. 1. CAT activities were calculated relative to that of the native pTK77-pUC-SH-CAT plasmid. Means ± standard errors of the means of two independent transfections performed in duplicate are shown.

+20] construct did not change the transcription activity in HepG2 cells (Fig. 1B). The transcription activities of the various constructs in HeLa cells were similar, indicating that the [−326;−159] fragment has no influence on the transcription activity in this cell type. Furthermore, the activity of the [−159;+20] promoter fragment was very low in HeLa cells, which is consistent with the lack of type II $sPLA_2$ gene expression in this cell type.

We examined the capacity of the inhibitory region of type II $sPLA_2$ promoter to block the activity of a heterologous promoter by inserting 3′-deleted fragments of the [−326;+20] type II $sPLA_2$ promoter upstream of the herpes simplex virus (HSV) thymidine kinase (TK) minimal promoter and the CAT reporter gene (19). The [−326;−176] sequence containing the elements C and D greatly reduced the TK promoter activity (to 12% ± 2% of the control level) when inserted in the sense orientation but had no effect when inserted in the antisense orientation (Fig. 2). This result suggests that the ability of the [−326;−176] region of type II $sPLA_2$ promoter to block the transcription activity of a heterologous promoter is orientation or position dependent. The distance between this region and the minimal promoter linked to the basal transcription machinery is indeed altered when the orientation is reversed. Deletion of the [−326;−195] fragment greatly increased the transcription activity of the type II $sPLA_2$ promoter (Fig. 1), but when this sequence was inserted upstream of the TK promoter in the sense orientation, it decreased the transcription activity only to 62% ± 6% of the control level in HepG2 cells. This result may still indicate that the distance of the inhibitory region from the general transcription machinery is critical. Alternatively, the whole [−326;−176] region may be needed for full inhibition of the promoter activity.

Since we have demonstrated (37) that element C (−210 to −176) binds C/EBP family members with high affinity, we investigated the functional significance of this binding and its relationship with the inhibition. The expression vectors of the main rat C/EBP family members were cotransfected into HepG2 cells with the wild-type [−326;+20]-$sPLA_2$-pUC-SH-CAT, the 5′ deletion mutant [−159;+20]-$sPLA_2$-pUC-SH-CAT, and the substitution mutant ΣC-[−326;+20]-$sPLA_2$-pUC-SH-CAT constructs. The cDNAs of the various C/EBP family members were inserted in the same expression vector, PHD (32), to avoid discrepancies resulting from different transcription rates of the various cDNAs. Western blot analysis (Fig. 3A) showed that C/EBPα, C/EBPβ, and C/EBPδ were actually expressed at similar rates in transfected HepG2 cells, C/EBPβ being expressed slightly better than the two other C/EBP family members. The antibody to C/EBPβ recognized a band corresponding to the major 34- to 36-kDa species in nuclear proteins from HepG2 cells transfected with the C/EBPβ expression vector. It also recognized a weak band above the major one. This band has been reported elsewhere (14) and may be a posttranslationally modified form. In bandshift experiments, the double-stranded oligonucleotide corresponding to element C (CWT) formed two closely migrating complexes with the nuclear extracts from HepG2 cells transfected with the C/EBPβ PHD expression vector (Fig. 3B, lane 2). Both of these complexes were supershifted by the antibody to C/EBPβ in bandshift experiments (Fig. 3B, lane 3). C/EBPβ mRNA is translated into an N-terminal truncated inhibitory form (LIP) and two other active 297- and 276-residue forms (14). These three forms are due to the presence of three in-frame ATG codons (14). We saw no band in the 20-kDa range of LIP in Western blot analysis. The two complexes formed in bandshift experiments (Fig. 3B, lane 2) may be formed by hetero- and homodimers of the transcriptionally active species, whose molecular masses are too close for them to be resolved on the SDS-polyacrylamide gel used for Fig. 3A.

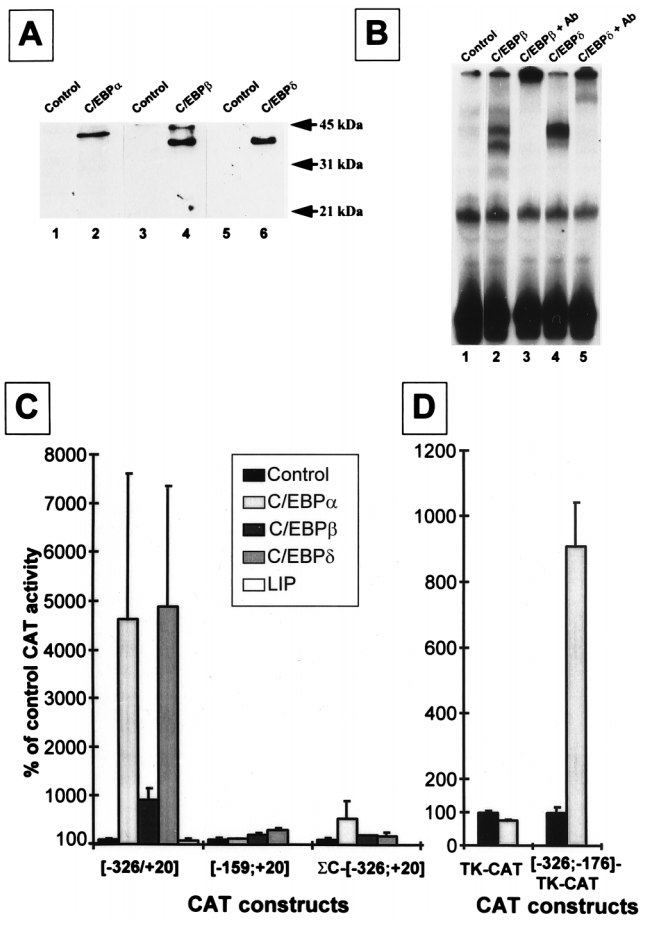

FIG. 3. Synthesis of the C/EBP family proteins in transfected HepG2 cells (A and B) and transactivations of the [−326;+20], ΣC-[−326;+20], and [−159;+20] type II sPLA$_2$ (C) and TK (D) promoters by the C/EBP family proteins in HepG2 cells. The cDNAs of the C/EBP family members were inserted in the same expression vector, PHD, downstream of a composite SV40-HSV TK promoter (32). (A) Western blot analysis of the C/EBP family proteins under the control of composite SV40-HSV TK promoter in transfected HepG2 cells. P100 dishes of 60% confluent HepG2 cells were transfected by the calcium phosphate coprecipitation method, using 30 μg of the various PHD expression vectors and 5 μg of the CMVβ-gal expression vector. Control cells were transfected with the native PHD plasmid. The amounts of nuclear proteins loaded on the 12% acrylamide gel were normalized to the β-galactosidase activities of the cytosol, i.e., 25, 30, 26, and 20 μg for the control and C/EBPα-, C/EBPβ-, C/EBPδ-expressing cells, respectively. Nuclear extracts from control cells were loaded in lanes 1, 3, and 5, and the corresponding blots were incubated with the antibodies to C/EBPα (lane 1), C/EBPβ (lane 3), and C/EBPδ (lane 5). (B) Bandshift analysis of nuclear extracts from HepG2 cells transfected with the native PHD construct (lane 1), C/EBPβ expression vector (lanes 2 and 3), and C/EBPδ expression vector (lanes 4 and 5). Nuclear extract concentrations were normalized to the β-galactosidase activities and were 2.5 μg (lane 1), 2.6 μg (lanes 2 and 3), and 2 μg (lanes 4 and 5). The probe was the oligonucleotide corresponding to element C of the type II sPLA$_2$ promoter. In lanes 3 and 5, specific antibodies (Ab) to C/EBPβ (lane 3) and C/EBPδ (lane 5) were incubated for 1.5 h at 4°C with HepG2 nuclear extracts prior to adding the poly(dI-dC) and the probe. The complexes were resolved on a 5% 30/1 acrylamide/bisacrylamide gel, which was dried and autoradiographed for 18 h. (C) The [−326;+20]-, ΣC-[−326;+20]-, and [−159;+20]-pUC-SH-CAT constructs (12 μg of each) were cotransfected with 2 μg of the wild-type expression vector PHD (control) or the various C/EBP expression vectors: C/EBPα, C/EBPβ, C/EBPδ, and the N-truncated C/EBPβ derivative LIP. The CAT activities were measured 53 h after transfection. The stimulation of each promoter by the various C/EBP family members is expressed relative to the activity of the promoter in the presence of the PHD vector. The data are means ± standard errors of the means of four independent transfections performed in duplicate. (D) Like panel A except that the TK-CAT and [−326;−176]-TK-CAT constructs were cotransfected with 2 μg of the PHD plasmid or the C/EBPα expression vector.

Extracts from HepG2 cells transfected by the C/EBPδ expression vector formed a single complex with oligonucleotide CWT, and its intensity was roughly similar to the total intensities of the two complexes formed with C/EBPβ proteins (Fig. 3B, lanes 4 and 5). No complex was obtained in assays using the extracts from cells transfected with the native PHD plasmid in these conditions (Fig. 3B, lane 1).

Overexpression of C/EBPα and C/EBPδ had similar effects on the [−326;+20] promoter and strongly enhanced its activity (Fig. 3C). C/EBPβ overexpression caused a smaller increase in promoter activity (Fig. 3C). The N-truncated derivative of C/EBPβ LIP (14) had no positive effect on the [−326;+20] promoter activity but slightly decreased it. In contrast to their effect on the activity of the wild-type [−326;+20] promoter, overexpressed C/EBPα, C/EBPβ, and C/EBPδ factors barely stimulated the activities of the [−159;+20]- and ΣC-[−326;+20]-sPLA$_2$-pUC-SH-CAT constructs (Fig. 3C). These results highlight the role of element C in mediating the stimulation of the wild-type promoter activity by C/EBP family members.

To confirm that C/EBP family members antagonize the inhibition associated with the [−326;−176] region of the type II sPLA$_2$ promoter, we tested the effect of C/EBPα overexpression on the transcription activity of the TK promoter-CAT constructs. C/EBPα exhibited no effect on the minimal TK promoter itself, since it did not alter the CAT activities of HepG2 cells transfected with the wild-type TK-CAT construct (Fig. 3D). In contrast, C/EBPα strongly reversed the inhibition of this promoter by the [−326;−176] region of type II sPLA$_2$ promoter (Fig. 3D).

No footprint element was identified on the [−326;−247] region (37), and deletion of this region modified neither the type II sPLA$_2$ promoter activity (Fig. 1) nor its activation by the C/EBP family members (not shown). The whole element C was required for full inhibition of the heterologous TK promoter activity (Fig. 2), and a double-stranded oligonucleotide corresponding to isolated element C (−210 to −176) bound C/EBP family members only at the 5′-GTTTCCCAAT-3′ site (37). We therefore examined the nature of the factors bound to the whole [−247;−176] region that could be involved in the inhibition of the type II sPLA$_2$ promoter.

Element D (−247 to −210) binds nuclear factors on overlapping insulin responsive sequence (IRS)-like and CTF/NF-1 binding sequences. Oligonucleotide DWT, corresponding to element D of the type II sPLA$_2$ promoter (Fig. 4A), formed two kinds of specific complexes with the rat liver nuclear extracts, a single major one and at least three minor ones (Fig. 4B, lanes 1 and 2). The minor complexes were eliminated by competition from a 250-fold excess of an NF1 binding oligonucleotide, which corresponds to element E of the albumin promoter, but the major complex was not (Fig. 4B, lane 9). Oligonucleotides corresponding to element C of the apolipoprotein C-III promoter and element D of the apolipoprotein A-II promoter prevented the formation of the major complex without altering the minor ones (Fig. 4B, lanes 6 and 7). All of the complexes were removed when CTF/NF1, C-IIIC, and A-IID oligonucleotides were added simultaneously as competitors (Fig. 4B, lanes 11 and 12). Despite the fact that DWT can bind C/EBPα (37), none of the complexes formed between this oligonucleotide and the rat liver nuclear extracts could be eliminated by competition from the C/EBP binding oligonucleotides corresponding to element D of the albumin promoter or element C of the type II sPLA$_2$ promoter (Fig. 4B, lanes 3 and 4).

Methylation interference experiments have shown one strong interference of binding with the G nucleotide at position −228 and several weak interferences with the immediately

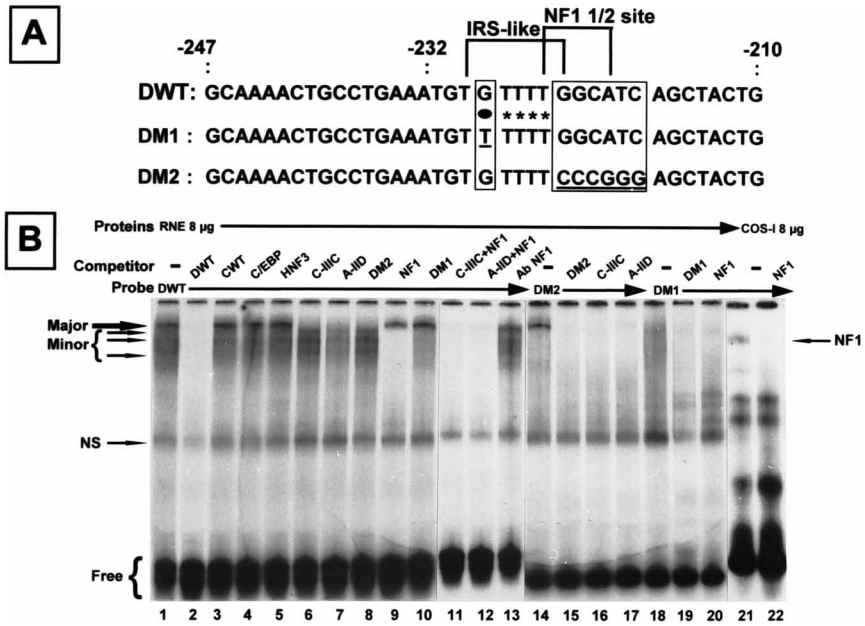

FIG. 4. Element D of the type II $sPLA_2$ promoter binds two types of activities on an IRS-like sequence and a CTF/NF1 binding site. (A) Oligonucleotides DWT, DM1, and DM2. The IRS-like and NF1 half-sites are indicated. The black circle indicates the strong interference site; asterisks indicate the weak sites identified in methylation interference experiments performed on the main complex formed with element D (37). The frames show the mutations in the DM1 and DM2 oligonucleotides. Mutant sequences are underlined. (B) The 5′-end ^{32}P-labeled probes (60,000 cpm) DWT (lanes 1 to 13), DM2 (lanes 14 to 17) and DM1 (lanes 18 to 22) were incubated for 30 min at 4°C in a mixture containing 8 μg of rat liver nuclear extracts (RNE) and 3 μg of poly(dI-dC) in 16 mM HEPES (pH 7.6)–5% Ficoll–40 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–0.1 mM EDTA. In lanes 21 and 22, the rat liver nuclear extracts were replaced by 8 μg of COS-1 cell lysate expressing NF1, and the amount of poly(dI-dC) was reduced to 1 μg. A 250-fold excess of unlabeled oligonucleotide competitor (1 μl) over the probe concentration or 1 μl of autoclaved water was added, and the mixture was incubated for 15 min prior to addition of the probe. The oligonucleotide competitors were unlabeled DWT (lane 2), DM2 (lanes 8 and 15), DM1 (lanes 10 and 19), the oligonucleotides corresponding to the type II $sPLA_2$ element C (CWT) (lane 3), the C/EBP binding element D of the murine albumin promoter (lane 4) (28), the HNF3 binding site of the transthyretin promoter (lane 5) (10), the CTF/NF1 binding element E of the murine albumin promoter (lanes 9, 11, 12, 20, and 22) (28), the apolipoprotein C-III element C (lanes 6, 11, and 16), and the apolipoprotein A-II element D (lanes 7, 12, and 17). In lane 13, rat liver nuclear extracts were incubated for 1.5 h at 4°C with the antibody (Ab) to NF1 prior to addition of the poly(dI-dC) and the probe DWT. The major complex, indicates by the arrow, and the minor complexes, indicated by the brace, were separated from the free probe on a 6% 30/2 acrylamide/bisacrylamide gel. NS indicates a nonspecific band. The gel was dried and autoradiographed for 18 h.

downstream 5′-TTTT-3′ sequence on element D (37) (Fig. 4A). Oligonucleotide DM1, which contains a T nucleotide in place of the wild-type −228 G nucleotide (Fig. 4A), did not compete with the wild-type DWT probe for the formation of the major complex (Fig. 4B, lane 10). It also did not form this complex when used as a probe (Fig. 4B, lane 18), confirming the critical role of the −228 G nucleotide in the generation of the major complex between element D and rat liver nuclear extracts. The sequence 5′-TGTTTTG-3′ mediates the negative effect of insulin on glucocorticoid-induced transcription of the genes for phosphoenolpyruvate carboxykinase and insulin-like growth factor binding protein 1 (34). HNF3 family members also bind to the IRS-like sequences on the phosphoenolpyruvate carboxykinase and α-1 antitrypsin promoters (24, 34). However, none of the complexes formed between the oligonucleotide DWT and the rat liver nuclear extracts could be disrupted by the HNF3 binding element of the transthyretin promoter (Fig. 4B, lane 5). Element D of apolipoprotein A-II encloses a 5′-TGTT-3′ motif and negatively regulates the transcription activity of the apolipoprotein A-II promoter (9), and a liver nuclear factor binds to both element D of the apolipoprotein A-II promoter and element C of the apolipoprotein C-III promoter (9, 35). This factor might be involved in the formation of the major complex between oligonucleotide DWT and rat liver nuclear extracts.

The sequence [−224;−220] 5′-TGGCA-3′, which overlaps the previously described IRS-like sequence (Fig. 4A), binds NF1-like proteins at the mouse mammary tumor virus long terminal repeat 5′ enhancer (23) and the growth factor silencer (47). This sequence corresponds to one half of the palindromic consensus site of the CTF/NF1 family members (6, 17). The mutant oligonucleotide DM1 (228 G→T) competed with the DWT probe for the formation of the minor complexes but was less efficient than the NF1 oligonucleotide (Fig. 4B, lane 10). When used as a probe, it still formed complexes which migrated like the minor complexes formed with the DWT probe and were eliminated by competition from the NF1 oligonucleotide (Fig. 4B, lanes 18 to 20). Replacement of the [−223;−218] 5′-GGCATC-3′ sequence by the nonspecific 5′-CCCG GG-3′ sequence in the DM2 oligonucleotide prevented the formation of the minor complexes when it was used as a probe (Fig. 4B, lanes 14 to 17). Also, this oligonucleotide displaced the major complex when it was used as competitor but not the minor ones formed with the DWT probe (Fig. 4B, lane 8). The DM1 oligonucleotide, which retains the 5′-TGGCA-3′ sequence (Fig. 4A), bound NF1 protein expressed in COS-1 cells and formed a single complex which migrated differently from the several minor complexes generated with the rat liver nuclear extracts (Fig. 4B; compare lanes 21 and 22 to lanes 1 and 18). Lastly, the minor complexes were not altered by prior incubation of the rat liver nuclear extract with an anti-NF1 antibody (Fig. 4B, lane 13). The minor complexes are thus generated by NF1-like proteins bound at the [−224;−220] 5′-TGGCA-3′ sequence on the DWT oligonucleotide but not by NF1 itself.

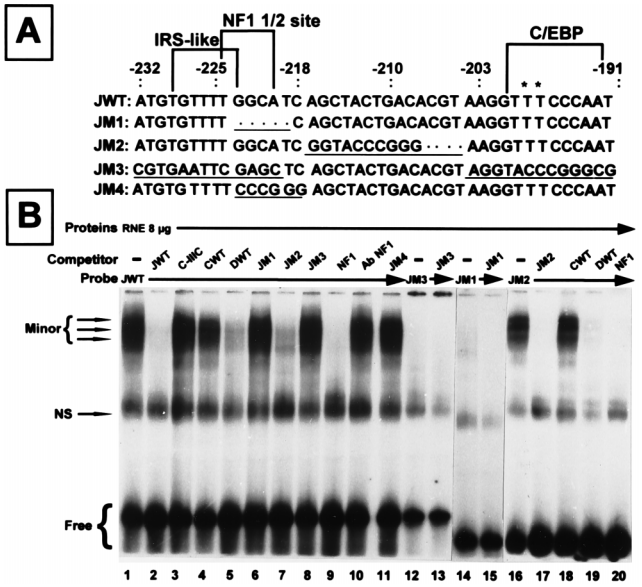

FIG. 5. Influence of element C on the binding of transcription factors to element D. (A) Sequence of oligonucleotide JWT, which spans the silencer sequence (−218 to −195) and the mutant oligonucleotides JM1, JM2, JM3, and JM4. The IRS-like sequence, the C/EBP binding site, and NF1 half-site are indicated. Mutation of the two thymidines at −199 and −198 (asterisks) inhibits the binding of C/EBP proteins (37). Mutant sequences in JM1, JM2, JM3, and JM4 are underlined. (B) Rat liver nuclear extract (RNE; 8 μg) was incubated with 3 μg of poly(dI-dC) as for Fig. 4. A 250-fold excess of the unlabeled oligonucleotide competitor C-IIIC (lane 3) (35), CWT (lanes 4 and 18), DWT (lanes 5 and 19), JWT (lane 2), JM1 (lanes 6 and 15), JM2 (lanes 7 and 17), JM3 (lanes 8 and 13), JM4 (lane 11), or NF1 (lanes 9 and 20) was then added, and incubation continued for 15 min. Finally 60,000 cpm of probe JWT (lanes 1 to 11), JM3 (lanes 12 and 13), JM1 (lanes 14 and 15), or JM2 (lanes 16 to 20) was added. In lane 10, rat liver nuclear extracts were incubated for 1.5 h at 4°C with the antibody (Ab) to NF1 prior to addition of poly(dI-dC) and the probe DWT. The reaction mixtures were electrophoresed on a 6% 30/2 acrylamide/bisacrylamide gel for 4 h. The braces and arrows indicate the probe bound to the NF1-like proteins and the free probe. NS indicates a nonspecific band.

Influence of element C on the binding of nuclear factors to element D. As the whole element C, including the high-affinity C/EBP binding site, is needed for full inhibition of TK promoter activity (Fig. 2), and as inhibition is blocked by overexpression of C/EBP family members, we examined the influence of this element on the binding of nuclear factors to element D. We designed a double-stranded oligonucleotide, JWT, spanning the [−232;−191] region, which contains the IRS-like sequence 5′-TGTTTTG-3′, the CTF/NF1 binding site 5′-TG GCA-3′ on element D, and the C/EBP binding site 5′-GTTT CCCAA-3′ on element C (Fig. 5A). The JWT oligonucleotide formed at least three intense complexes with rat liver nuclear extracts (Fig. 5B, lane 1). All of them could be disrupted with a 250-fold excess of NF1 binding oligonucleotide (Fig. 5B, lane 9) but not by the same excess of C-IIIC oligonucleotide (Fig. 5B, lane 3). Like the minor complexes formed with the DWT oligonucleotide, the NF1 protein itself was not involved in the formation of the complexes with the JWT probe, since prior incubation of the nuclear extracts with the anti-NF1 antibody did not alter their formation (Fig. 5B, lane 10). To establish the binding of NF1-like proteins to the [−224;−220] 5′-TG GCA-3′ sequence on the JWT oligonucleotide, two mutant oligonucleotides, the JM4 oligonucleotide, in which this sequence was replaced by a nonspecific sequence, and the JM1 oligonucleotide, in which the [−223;−219] sequence was deleted, were synthesized and used in bandshift experiments (Fig. 5A). Neither of them competed with the JWT probe for formation of a complex (Fig. 5B, lanes 6 and 11). The JM1 oligonucleotide formed only very faint residual complexes with rat liver nuclear extracts when used as a probe (Fig. 5B, lanes 14 and 15). Hence, NF1-like proteins bind to the critical 5′-TGGCA-3′ site on oligonucleotide JWT.

The affinity of JWT seemed to be higher than that of DWT, since the complexes formed with JWT were more intense for the same amount of probe and nuclear extract (compare Fig. 5B, lane 1, to Fig. 4B, lane 1) and since some faint complexes remained in the presence of a 250-fold excess of unlabeled DWT oligonucleotide (Fig. 5B, lane 5). This result suggests that the 5′ end of element C increases the binding of the NF1-like factors. However, alteration of the [−217;−204] sequence in the JM2 oligonucleotide (Fig. 5A) did not suppress its ability to compete with the wild-type JWT probe for the formation of complexes (Fig. 5B, lane 7), and the JM2 oligonucleotide was still able to bind NF1-like proteins (Fig. 5B, lanes 16 to 20). The complexes formed with the JM2 probe, noticeably the third one, appeared slightly less intense than those formed with JWT (Fig. 5B; compare lanes 1 and 16), which were not completely disrupted by a 250-fold excess of JM2 (Fig. 5B, lane 7). The same excess of unlabeled DWT oligonucleotide completely prevented the formation of complexes with the JM2 probe but not with JWT (Fig. 5B; compare lanes 5 and 19). Although this result suggests slight differences in the affinities of JWT and JM2 for nuclear factors, it did not show a specific binding site in the [−217;−204] sequence or the critical importance of this sequence for the binding of NF1-like proteins. To better characterize this region, we prepared the JM3 oligonucleotide, which retained only the [−219;−203] sequence with 5′ and 3′ flanking nonspecific sequences. These sequences were added to take into account the steric influence of sequences surrounding specific binding sites and to allow appropriate comparisons with the other probes. The JM3 oligonucleotide did not disrupt any complex formed with the JWT probe and did not bind rat liver proteins (Fig. 5B, lanes 8, 13, and 14). The same results were obtained with an oligonucleotide reduced to the [−219;−203] sequence (data not shown). Therefore, the higher affinity of JWT than DWT for the NF1-like proteins seems to be due to the presence of the 3′ [−210;−191] flanking sequence, including the C/EBP binding sequence 5′-GTTTCCCAA-3′. But this sequence is not specifically involved in the binding of NF1-like proteins, since a 250-fold excess of the CWT oligonucleotide did not remove the complexes formed with JWT or JM2 (Fig. 5B, lanes 4 and 18).

The JWT probe gave rise to no low-mobility complex similar to the major complex formed with the DWT probe, although the IRS-like 5′-TGTTTTG-3′ sequence is still present at the 5′ end of JWT. Similarly, JWT did not bind C/EBP family members despite the presence of the high-affinity C/EBP binding site at its 3′ end (Fig. 5B, lane 4). It is unlikely that there is competition between the NF1-like proteins and the other transcription factors (C/EBP family proteins) for binding to the JWT oligonucleotide, since the JM1 oligonucleotide, from which the NF1 half-site was deleted, formed only faint complexes (Fig. 5B, lanes 14 and 15). These complexes were not supershifted by anti-C/EBP antibodies (data not shown), which suggests that they resulted from a low-affinity binding of nonspecific factors. The binding of the nuclear factors to the IRS-like sequence or to the C/EBP binding site could require the 5′ and 3′ ends of elements D and C, respectively. If so, there could be competition between the NF1-like proteins and C/EBP or the IRS-like binding protein for binding to the whole [−247;−176] region of the sPLA$_2$ promoter. We tested this possibility by using overlapping oligonucleotides correspond-

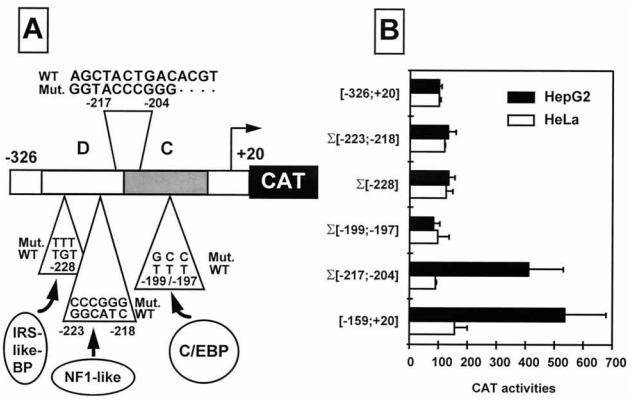

FIG. 6. Structures of the substitution mutants of the [−326;+20] promoter for the binding sites of the previously characterized double-strand binding activities (A) and activities of the corresponding pUC-SH-CAT constructs in HepG2 and HeLa cells (B). The IRS-like sequence was altered in the Σ[−228]-sPLA$_2$-pUC-SH-CAT construct; the NF1 half-site and the C/EBP binding sites were altered in the Σ[−223;−218]- and Σ[−199;−197]-sPLA$_2$-pUC-SH-CAT constructs. The Σ[−217;−204]-sPLA$_2$-pUC-SH-CAT construct contains the mutation which was made in the JM2 oligonucleotide (Fig. 5A). The positions of the mutated nucleotides, the mutated sequence (Mut.), and the corresponding wild-type sequence (WT) are indicated. The nature of the double-strand binding factor involved is indicated. CAT activities were measured 53 h after transfection of the HepG2 or HeLa cells. The activities are compared to the activity induced by the [−326;+20] wild-type promoter as the means ± standard errors of the means of four independent transfections performed in duplicate with two different preparations of reporter plasmids for HepG2 cells and two transfections for HeLa cells. BP, binding protein.

ing to the [−232;−176] and [−247;−191] fragments of the type II sPLA$_2$ promoter in EMSAs. Unfortunately, although the NF-1-like proteins seemed to be the major binding species, the probes (56 bp) were too long to provide an electrophoresis pattern with enough resolution to confirm the binding of the other factors (data not shown).

Importance of the sequence between the NF1 and C/EBP binding sites for inhibition of the [−326;+20] type II sPLA$_2$ promoter. CAT mutants based on the above-described EMSA data were constructed and used to determine the roles of the factors characterized in the EMSAs and to identify those that inhibited the activity of the [−326;+20] sPLA$_2$ promoter in HepG2 cells. Neither the G→T transversion at position −228, which abolished binding at the IRS-like sequence, nor replacement of the NF1 binding site by a nonspecific sequence in the Σ[−223;−218]-sPLA$_2$-pUC-SH-CAT construct significantly altered the promoter activity in HepG2 cells (Fig. 6). Replacement of the three adjacent thymidines (−199 to −197), which specifically prevents the binding of the C/EBP family members to element C (37) without altering the surrounding sequences, did not prevent inhibition of the transcription activity of the Σ[−199;−197]-sPLA$_2$-pUC-SH-CAT construct (Fig. 6).

An alteration in the [−217;−204] region of the Σ[−217;−204]-pUC-SH-CAT construct overcame the inhibition of transcription activity in HepG2 cells (Fig. 6), although a similar alteration in the JM2 oligonucleotide did not inhibit the binding of the NF1-like proteins (Fig. 5B, lane 17). The lack of a significant role for the NF1-like proteins was confirmed by cotransfection experiments using HepG2 cells, which showed no enhancement of the activity of the [−326;+20]-pUC-SH-CAT construct in the presence of an NF1 expression vector (data not shown). Finally, HeLa cells were transfected with the CAT constructs previously used in HepG2 cells (Fig. 6). The activities of the mutant plasmids were similar to that of the wild-type [−326;+20]-sPLA$_2$-pUC-SH-CAT construct (Fig. 6), confirming that the previously described binding sites of rat liver nuclear proteins are not functional in HeLa cells.

Recognition of the [−232;−191] sequence of the type II sPLA$_2$ promoter by SSBPs. Since inhibition of the [−326;+20] sPLA$_2$ promoter was not affected by mutation of the binding sites of the previously characterized transcription factors but was removed by altering the [−217;−204] sequence at the junction of elements C and D which was not recognized by any double-strand binding protein, we looked for the involvement of SSBPs in the negative regulation of the sPLA$_2$ promoter. SSBPs inhibit the activities of several eukaryotic promoters (7, 18, 21, 39, 51–53). The orientation and position of the DNA recognition sequence do not usually affect the regulatory activities of double-strand binding factors. However, the negative effects of SSBPs can be blocked by inverting the binding sequence in the promoter, as described by Tanuma et al. for the silencer element of the lipoprotein lipase promoter (53). The results shown in Fig. 2 are consistent with the involvement of SSBPs in the inhibition of the sPLA$_2$ promoter.

The sense (JWTs) and antisense (JWTas) strands of the JWT oligonucleotide corresponding to the [−232;−191] region were used as probes. This region encloses the [−217;−204] sequence, alteration of which enhanced the transcription activity, and its 3′ end corresponds to the limit of the inhibitory region determined in Fig. 1. The sense single-strand probe JWTs formed three main complexes with rat liver nuclear extracts, 1_{JWTs}, 2_{JWTs}, and 3_{JWTs}, (Fig. 7A), while the antisense probe JWTas formed four complexes, 1_{JWTas} to 4_{JWTas} (Fig. 7B). Complexes 3_{JWTs} and 4_{JWTas} comigrated and were only partially eliminated by competition from the corresponding unlabeled oligonucleotide (Fig. 7A and B, lanes 4). HeLa cell extracts (4 μg) produced two complexes with both the JWTs and JWTas probes (Fig. 7C, lanes 1 and 3). These complexes differed from those formed with the rat liver nuclear extracts. They were only partially disrupted by a 100-fold excess of the corresponding unlabeled oligonucleotide (Fig. 7C, lanes 2 and 4). Low-mobility complexes appeared with HeLa cell extracts in the presence of the corresponding competitors.

Since the [−217;−204] sequence was critical for the inhibition of transcription activity, we determined whether this sequence competed for the formation of the complexes with single-stranded probes JWTs and JWTas and whether it formed the same complexes. The sense strand of the JM2 oligonucleotide (JM2s), in which the [−217;−204] region was altered, competed with the JWTs probe for the formation of complexes 1_{JWTs} and 2_{JWTs} to the same extent as the unlabeled wild-type JWTs oligonucleotide (Fig. 8A, lanes 2 and 4). The sense strand of JM3 oligonucleotide (JM3s), which encloses the [−217;−204] sequence, did not compete with them (Fig. 8A, lane 5). In addition, the third complex (3_{JWTs}), which was not disrupted by all the oligonucleotide competitors, seems nonspecific (Fig. 8A, lanes 2, 4, and 5). The JM3s oligonucleotide used as a probe formed four complexes, 1_{JM3s}, 2_{JM3s}, 3_{JM3s}, and 4_{JM3s} (Fig. 8B, lane 1). These complexes were eliminated by a 100-fold excess of unlabeled JM3s oligonucleotide itself and by the JWTs oligonucleotide (Fig. 8B, lanes 2 and 5). The two low-mobility complexes (1_{JM3s} and 2_{JM3s}) were disrupted by the sense-strand DWTs (Fig. 8B, lane 4) but not by CWTs (Fig. 8B, lane 3), suggesting that they involve the [−219;−210] sequence (Fig. 5A). In contrast, the high-mobility complexes (3_{JM3s} and 4_{JM3s}) were not eliminated by a 100-fold excess of the DWTs oligonucleotide (Fig. 8B, lane 4) and were partially disrupted by the CWTs oligonucleotide (Fig. 8B, lanes 3 and 4). Furthermore, excess (100-fold) JM2s oligonucleotide eliminated complexes 3_{JM3s} and 4_{JM3s} but not complexes 1_{JM3s}

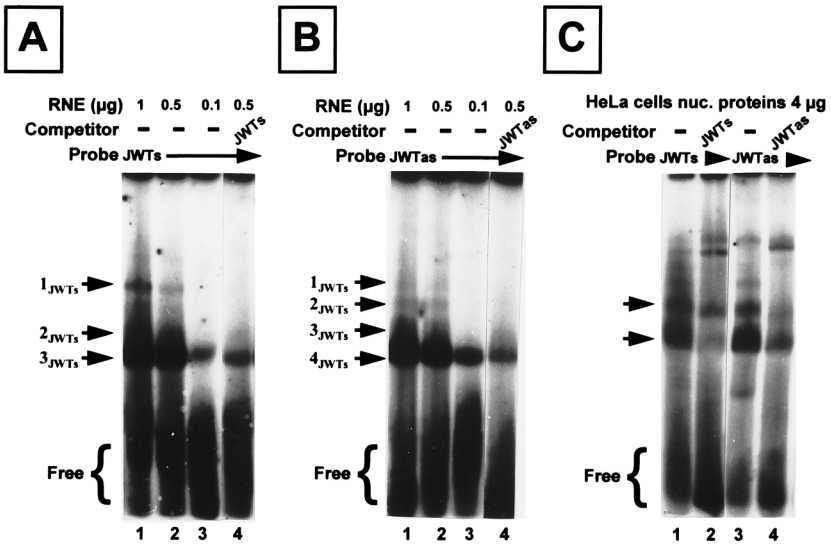

FIG. 7. The sense (A) and antisense (B) strands of the JWT oligonucleotide bind different single-strand binding activities in rat liver extracts. HeLa cells extracts form different complexes with the sense and antisense strands of the JWT oligonucleotide than rat liver nuclear extracts do (C). (A) Aliquots of 1 μg (lane 1), 0.5 μg (lanes 2 and 4), and 0.1 μg (lane 3) of rat liver nuclear extracts (RNE) were incubated for 15 min at 4°C in a mixture containing 3 μg of poly(dI-dC) in 16 mM HEPES (pH 7.6)–5% Ficoll–40 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–0.1 mM EDTA. Then 100,000 cpm of the labeled sense strand (JWTs) was added, and incubation continued for 30 min. In lane 4, a 100-fold excess of unlabeled JWTs oligonucleotide was incubated with the rat liver nuclear extract for 15 min at 4°C before addition of the probe. The reaction mixtures were loaded on 6% 30/2 acrylamide/bisacrylamide gel and electrophoresed at 4°C for 4 h. The gel was dried and autoradiographed overnight at −80°C, using intensifying screens. The three complexes 1_{JWTs}, 2_{JWTs}, and 3_{JWTs} are indicated. (B) Like panel A except that antisense strand JWTas was used as the probe and competitor. The four complexes 1_{JWTas}, 2_{JWTas}, 3_{JWTas}, and 4_{JWTas} are indicated. (C) HeLa cell nuclear (nuc.) extracts (4 μg) were incubated with the sense strand (lanes 1 and 2) or antisense strand (lanes 3 and 4) of the JWT oligonucleotide. Unlabeled oligonucleotide JWTs (lane 2) or JWTas (lane 4) was incubated with the HeLa nuclear extracts for 15 min at 4°C before addition of the probes. Other experimental conditions were as for panel A.

and 2_{JM3s}, suggesting that only complexes 1_{JM3s} and 2_{JM3s} are specific to the sense strand of the [−217;−204] sequence.

Among the four complexes formed with the antisense strand JWTas probe (Fig. 7B), a 100-fold excess of the JM3as oligonucleotide prevented the formation of complexes 1_{JWTas}, 2_{JWTas}, and 3_{JWTas} to the same extent as the unlabeled JWTas oligonucleotide (Fig. 8C, lanes 3 and 5). In contrast, the same excess of JM2as did not eliminate and even reinforced the 2_{JWTas} and 3_{JWTas} complexes (Fig. 8C, lane 4), suggesting that they are generated by proteins binding to the antisense strand of the [−217;−204] sequence. Excesses (100-fold) of CWTas and DWTas oligonucleotides completely displaced the 1_{JWTas}, 2_{JWTas}, and 3_{JWTas} complexes and partially displaced the 4_{JWTas} complex (Fig. 8C, lanes 6 and 7). Probing with the antisense strand JM3as gave four complexes (1_{JM3as} to 4_{JM3as}) similar to those formed with JWTas (compare Fig. 8D, lane 1, to Fig. 8C, lane 1). Complexes 1_{JM3as}, 2_{JM3as}, and 3_{JM3as} were eliminated by unlabeled JM3as oligonucleotide (Fig. 8D, lane 2), by the antisense CWTas, DWTas, and JWTas oligonucleotides (Fig. 8D, lanes 3 to 5), and by the oligonucleotide JRas, which strictly corresponds to the antisense strand of the [−219; −203] sequence (Fig. 8D, lane 7). They were not eliminated by the nonspecific single-stranded NSD2 oligonucleotide, which includes both the 5′ and 3′ nonspecific flanking sequence in the JM3as oligonucleotide (Fig. 5A; Fig. 8D, lane 8). Complexes 2_{JM3as} and 3_{JM3as} were not disrupted by a 100-fold excess of the antisense JM2as oligonucleotide (Fig. 8D, lane 6), suggesting that these complexes specifically involve the antisense strand of the [−217;−204] sequence. They might correspond to the complexes 2_{JWTas} and 3_{JWTas}, which were formed with the JWTas probe and were not eliminated by the JM2as competitor (compare Fig. 8D, lane 6, to Fig. 8C, lane 4). The fact that complex 1_{JM3as} was competed by the JM2as oligonucleotide, which shares only two bases with JM3as, suggests that the protein generating this complex is not strictly sequence specific. In addition, complexes 4_{JWTas} and 4_{JM3as} may involve nonspecific components since they were not completely eliminated by 100-fold excesses of the JWTas and JM3as oligonucleotides (Fig. 8C, lane 3; Fig. 8D, lane 2). Lastly, a 100-fold excess of the corresponding antisense strand added to the JWTs or JWTas probe formed double-stranded oligonucleotides, and the complexes generated by the double-strand binding NF1-like proteins were recovered (Fig. 8A, lane 3; Fig. 8C, lane 2; compare to Fig. 5B, lane 1), suggesting that the SSBPs have a low affinity for the double-stranded DNA fragments.

To summarize, the complexes formed with the sense JM3s oligonucleotide, which encloses the [−217;−204] sequence, did not exist when the larger JWTs probe was used. They were replaced by two complexes, which were eliminated by the sense JM2s oligonucleotide, which contains a mutant [−217;−204] sequence, but not by the unlabeled JM3s oligonucleotide. In contrast, two activities bind to the antisense JWTas and JM3as probes and were not competed by the antisense JM2as competitor. They were competed by the CWTas oligonucleotide, while those bound to the JM3as probe were not eliminated by the corresponding CWTs competitor. Since the alteration of the [−217;−204] sequence alleviated the inhibition of the promoter in transient transfection experiments, the proteins generating these complexes with the antisense strand could be involved in the transcription inhibition of the type II sPLA$_2$ gene.

DISCUSSION

We have shown that the [−326;−176] region of the sPLA$_2$ promoter inhibits the promoter activity. We first determined whether the [−326;−176] region itself repressed transcription or whether factors bound to regulatory elements A and B in the proximal region of the promoter were involved in the negative regulation. This is reminiscent of the situation in the

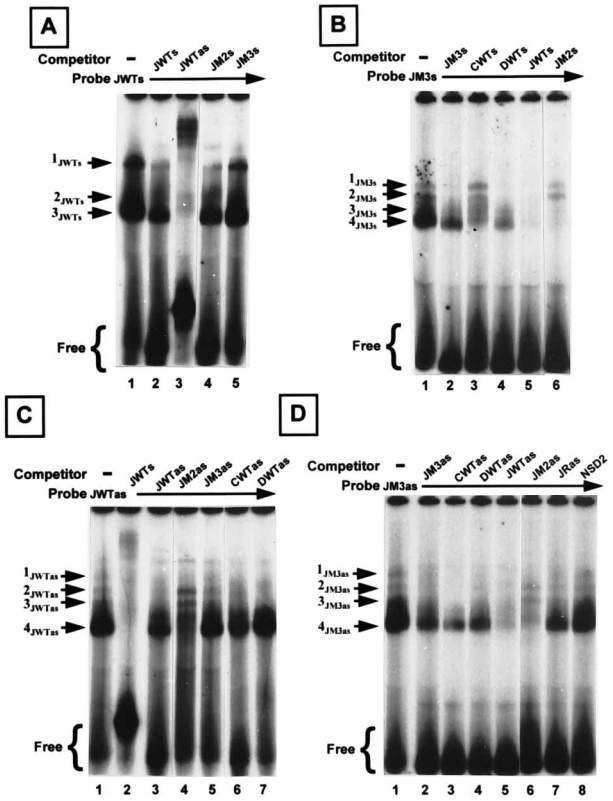

FIG. 8. Competition of the various overlapping nucleotides with the sense JWTs (A) and JM3s (B) and antisense JWTas (C) and JM3as (D) probes. (A) The sense (lane 2) and antisense (lane 3) strands of the JWT oligonucleotide and the sense strands of the JM2 (lane 4) and JM3 (lane 5) oligonucleotides (Fig. 5A) were used as competitors at a 100-fold excess and incubated for 15 min in the reaction mixture containing 1 μg of rat liver nuclear extract prior to addition of probe JWTs except in lane 1, where they were replaced by 1 μl of distilled autoclaved water. Then 100,000 cpm of the sense JWTs strand was added to the reaction mixtures, and incubation continued for 30 min. The reaction mixtures were loaded on a 6% 30/2 acrylamide/bisacrylamide gel and electrophoresed at 4°C for 4 h. The gel was dried and autoradiographed overnight at −80°C, using intensifying screens. The three complexes 1_{JWTs}, 2_{JWTs}, and 3_{JWTs} are indicated. (B) Like panel A except that the probe was the sense-strand JM3s oligonucleotide and the competitors were the sense-strand JM3 (lane 2), CWT (lane 3), DWT (lane 4), JWT (lane 5), and JM2 (lane 6) oligonucleotides. The four complexes 1_{JM3s} to 4_{JM3s} are indicated. (C) Like panel A except that the antisense-strand JWTas oligonucleotide was used as the probe (lanes 1 to 7) and competitor (lane 3). The sense-strand JWTs (lane 2) and the antisense-strand JM2as (lane 4), JM3as (lane 5), CWTas (lane 6), and DWTas (lane 7) oligonucleotides were used as competitors (100-fold excess). The four complexes 1_{JWTas}, 2_{JWTas}, 3_{JWTas}, and 4_{JWTas} are indicated. (D) Like panel A except that the probe was the antisense JM3as oligonucleotide and the competitors were the antisense JM3as (lane 2), CWTas (lane 3), DWTas (lane 4), JWTas (lane 5), JM2as (lane 6), JRas (lane 7), and NSD2 (lane 7) oligonucleotides. The four complexes 1_{JWTas}, 2_{JWTas}, 3_{JWTas}, and 4_{JWTas} are indicated. The JRas oligonucleotide corresponds to the antisense strand of the [−219;−203] sequence. The sequence of the NSD2 oligonucleotide is 5′-GGGACGCCCGGGTACCGAGCTC GAATTCACGTT-3′.

YY1 binding promoters. YY1 can act as an activator or attenuator depending on the promoter context (42). In this context, a 5′ deletion could shift the activity of the transcription factors bound to the regulatory element B of the $sPLA_2$ promoter from negative to positive. The fact that the [−326;−176] sequence itself can repress the heterologous TK promoter makes this unlikely and suggests that the promoter activity is inhibited by transcription factors bound upstream of position −176.

We have described two footprint elements, C and D, in this region. They bind three types of transcription factors: the C/EBP family members bind to element C, while an IRS-like binding protein and NF1-like proteins bind to element D. Element D contains the IRS-like sequence 5′-TGTTTTG-3′, and a factor binds to this sequence and generates a complex which is competed for by the oligonucleotides corresponding to element C the apolipoprotein C-III promoter and element D of the apolipoprotein A-II promoter. The apolipoprotein A-II element D has between positions −278 and −253 a sequence, 5′-TGTTG-3′, that is related to the $sPLA_2$ IRS-like sequence and binds four proteins. These proteins are the C/EBP family members, an Ets-related protein, a member of the HNF3 family, and a fourth factor which also recognizes the C element of the apolipoprotein C-III promoter and was originally named CIII-CI (9, 35). The −228 G nucleotide of the $sPLA_2$ IRS-like sequence is critical for the binding of the liver nuclear factor (Fig. 4). It seems to play the same role as the −270 G nucleotide of the apolipoprotein A-II promoter, which is a strong interference site with the HNF3 family member and the CIII-CI protein (9). We found that C/EBP and HNF3 family proteins are not bound. We believe that the factor which recognizes the IRS-like sequence on the type II $sPLA_2$ element D could be protein CIII-CI. However, this protein does not appear to repress the $sPLA_2$ promoter activity, since the −228 G→T mutation which prevents its binding had no effect in the transient transfection experiments.

Element D of the $sPLA_2$ promoter contains a half-site for the CTF/NF1 family members 5′-TGGCA-3′ (Fig. 4). The DNA sequence specificity of these factors is quite complex since they have been shown to bind to the dyad 5′-TGGCA NNNTGCCA-3′ as well as to the half-site 5′-TGGCA-3′ (17, 40). There are many species in this family, and their diversity is generated by the expression of several related genes as well as by alternative splicing or cleavage of larger polypeptides (15, 29, 40, 48). We failed to alter the formation of the complexes with the [−224;−220] 5′-TGGCA-3′ sequence on the $sPLA_2$ type II promoter in assays using an antibody raised against N-terminal amino acids 2 to 21 of the CTF/NF1 protein. Meisterernst et al. (29) have shown that alternative splicing can generate NF1-related proteins which lack all or part of the amino-terminal sequence. More recently, Kusk et al. (23) found that an NF1-related protein bound to the mouse mammary tumor virus long terminal repeat 5′ enhancer at NF1 half-site 5′-TTGGCAT-3′, which is homologous to the $sPLA_2$ type II promoter binding site. This protein was recognized by an anti-NF1 protein raised against a C-terminal peptide, and its binding did not depend on the presence of a 3′ half-site, unlike the prototype CTF/NF1 protein. The proteins bound to the JWT probe belong to the 5′-TGGCA-3′-recognizing group and could involve proteins related to NF1 but not the full-length NF1 protein. CTF/NF1 family members and NF1-like proteins have been implicated in the negative regulation of several eukaryotic promoters (1, 5, 39, 49). However, we find that neither the deletion nor the substitution of the NF1 binding site significantly increased the promoter activity. Therefore, NF1-like proteins do not seem to be critically involved in the negative regulation of the [−326;+20] type II $sPLA_2$ promoter, although they appear to be the major proteins bound to the [−247;−176] region of the type II $sPLA_2$ promoter in the double-stranded conformation.

The 5′ deletion of the [−203;−195] sequence which overlaps the high-affinity C/EBP binding site on element C resulted in a dramatic increase in transcription activity. C/EBP family members are known to negatively regulate the promoters of the hepatitis B and simian virus 40 (SV40) genes (43). However, the replacement of this C/EBP binding site in the ΣC-[−326;

+20]-$sPLA_2$-pUC-SH-CAT and Σ[−199;−197]-$sPLA_2$-pUC-SH-CAT constructs did not increase the basal promoter activity (Fig. 6B). By contrast, overexpression of the C/EBP family members blocked the inhibition and dramatically enhanced the $sPLA_2$ promoter activity in the presence of wild-type element C but had no effect on the ΣC-[−326;+20]-$sPLA_2$-pUC-SH-CAT construct (Fig. 3C). C/EBPα also prevented the repression of the TK promoter by the [−326;−176] region containing the whole element C (Fig. 3D). Therefore, the main function of the C/EBP family members seems to be to overcome the basal inhibition of $sPLA_2$ gene expression. C/EBPβ was less active than C/EBPα or C/EBPδ. This difference could not be due to lower expression in the transfected cell, since Western blot analyses have shown that the concentrations of the three C/EBP family members in HepG2 cells transfected by the PHD expression vectors are similar (Fig. 3A). It is also possible that the inhibitory N-truncated form LIP is produced, but it is unlikely as no 20-kDa band was observed despite the fact that the antibody to C/EBPβ was raised against an epitope covering residues 258 to 276, which are present in LIP (Fig. 3A).

Altering the [−217;−204] sequence overcomes the basal inhibition of the promoter activity (Fig. 6B) but does not affect the binding of the double-strand binding factors (Fig. 5). In addition, an oligonucleotide corresponding to the [−219;−203] sequence binds no double-strand binding activity. The bandshift assays performed with single-strand oligonucleotides show the formation of several complexes with both the sense and antisense strands. The sense strand of the [−219;−203] sequence binds some proteins generating the complexes 1_{JM3s} and 2_{JM3s}, the binding of which seems to depend on the [−219;−210] sequence overlapping the DWT oligonucleotide (Fig. 8B). When the larger sense oligonucleotide JWTs is used as a probe, other complexes are formed, and their formation is not prevented by excess of the [−219;−203] region sense strand. The relationship between the various complexes formed with the JWTs and JM3s probes remains to be determined, but these activities are not good candidates for inhibiting the promoter activity. In contrast, the antisense strands of the JWT and JM3 oligonucleotides form similar complexes (Fig. 8C and D). Two of them (2_{JWTas}/2_{JM3as} and 3_{JWTas}/3_{JM3as}) are not displaced by the antisense JM2as oligonucleotide, which has a mutated [−217;−204] region. Therefore, the proteins generating these complexes could be involved in transcription inhibition.

The transition between a double-strand and a single-strand structure might explain the occurrence of the nuclease-hypersensitive site (55), and there is a DNase I-hypersensitive site at position −210, defining the limit of elements C and D in our previous footprint experiments (37). Many cloned SSBPs have very low affinities for double-stranded DNA (7, 21, 30, 36, 51), which is consistent with our data. The heterogeneous nuclear ribonucleoprotein K (hnRNP) (30), which binds to both RNA and single-stranded DNA, and some other SSBPs (18, 21) all bind to CT- or C-rich sequences, but some other SSBPs bind to different sequences. CArG boxes in the apoVLDL II promoter (51) and GXXXXG motif in the promoter of the TSH receptor (36) bind such activities. An octamer motif is recognized by two members of the POU family on the α-internexin promoter, one an activator and the second an inhibitor (7). The [−217;−204] region of the $sPLA_2$ promoter and the flanking sequences are not C or CT rich, and we can find no obvious feature in common with published SSBP binding sites except a GXXXXG motif between positions −210 and −205 (36).

The SSBPs are believed to act by stabilizing the single-strand DNA conformation and preventing the binding of the transcription factor needed for transcription. For example, several SSBPs bind to the silencer/enhancer of the α-actin gene and replace the transcription factor TEF-1, which is essential for transcription of this gene in fibroblasts (52). But hnRNP might also interact with TATA binding protein and TATA binding protein-associated factors (30). Despite the fact that NF1-like proteins appear to be the main proteins bound in vitro to the $sPLA_2$ elements C and D, they do not seem to influence the activity of the promoter. In contrast, increased production of C/EBP factors overcomes the inhibition of transcription activity. C/EBP factors could counteract the occurrence of single-strand conformation since the C/EBP proteins covered the whole [−210;−176] region in previous footprint experiments (37) and since the antisense CWTas oligonucleotide removed the activities bound to the antisense strand of the inhibitory [−219;−203] region (Fig. 8D, lane 3). Since the binding of NF1-like proteins is recovered when an excess of the unlabeled complementary strand is added to the single-strand probe (Fig. 8A, lane 3; Fig. 8B, lane 2), such a counteracting effect of C/EBP factors would allow NF1-like proteins to bind to element D. The data obtained in cotransfection experiments suggest that the C/EBP proteins play a physiological role in regulating type II $sPLA_2$ gene expression. C/EBPδ strongly stimulates the $sPLA_2$ promoter activity and is greatly overexpressed during the acute-phase response, while C/EBPβ is predominantly regulated posttranscriptionally (2, 44). C/EBPα is associated with adipocyte differentiation (8) and seems to be required for some liver-specific physiological responses (46). Although C/EBP factors can affect the DNA conformation (3), there is still no evidence for a counteracting effect on single-strand conformation. Further studies are needed to identify the SSBPs bound to the [−217;−204] sequence, to confirm if they are involved in the blocking of the $sPLA_2$ promoter, and to define exactly how they interact with the C/EBP factors.

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