Positive and Negative Hepatic Regulation of the Human Type II Phospholipase A_2 Gene†

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ABSTRACT: To identify the elements which regulate the liver transcription of the human type II phospholipase A_2 gene and its stimulation by interleukin 6, the 5′ flanking region from −1614 to +806 and several 3′ and 5′ deleted fragments have been analyzed in CAT assays. Negative regulatory elements have been located in the regions −1614 to −326 and +20 to +806. The fragment −326 to +20 contains the main elements required for the transcription as well as for the stimulation by interleukin 6. Footprinting assays have been performed on this region and showed four protected elements, A [−35;−6], B [−125;−86], C [−209;−176], and D [−247;−211]. Deletion of element D enhanced the transcription of the reporter gene 10.5-fold compared to the [−326;+20]-CAT construct. Further deletions up to position −87 which removed both the elements B and C or the substitution of element C by a nonspecific sequence lowered the promoter activity to 23% and 70% of the control, respectively. These results indicate that element C binds positive regulatory factors and element D binds a negative regulatory factor. Furthermore, stimulation by interleukin 6 is lost when element C is substituted or deleted. As shown by the footprinting and band shift assays, the transcription factors C/EBPα and C/EBPβ can bind to elements C and D but the dissociation constant (K_d) of C/EBPα is 10 times lower for element C (0.6 nM) than for element D (5.8 nM). Band shift experiments using rat liver nuclear extracts showed that element C formed four heat stable complexes, some of which could be supershifted by anti C/EBPα antibodies. The binding of C/EBP factors to element C was confirmed by competition with previously described oligonucleotide and nucleotide substitution of element C. Band shift experiments using rat liver nuclear extracts showed that element D formed one major DNA–protein complex. This complex could be competed out by oligonucleotides containing a cAMP responsive element (CRE) but not by oligonucleotides containing the binding site of C/EBP. However, anti-CREB antibodies did not supershift this complex. Methylation interference experiments showed the involvement of a G nucleotide upstream to the sequence homologous to CRE in the binding of the hepatic nuclear factors. Further studies are required to determine the nature of the different factors which bind to element D and generated the major and minor complexes observed in band shift experiments as well as their regulatory role in the transcription of PLA_2 gene and their putative interaction with C/EBP factors bound to element C.

Phospholipases A_2 (PLA_2)[1] are a diverse family of enzymes that hydrolyze the fatty ester bond at the *sn*-2 position of glycerophospholipids, producing free fatty acids and lysophospholipids (Van Den Bosch, 1980). They can be classified as intracellular or secreted families, which exhibit very different substrate specificities and calcium requirements for the catalytic process. The intracellular PLA_2 are involved in cellular processes as diverse as phospholipid turnover (Lands & Merckl, 1963), protection of the membrane from peroxidation damage (Van Kruijf *et al.*, 1987), and receptor-mediated cell signaling (Burch, 1989). This last role is mainly due to the production of free arachidonic acid by PLA_2 and the subsequent formation of eicosanoids (Irvine, 1982) as well as of the platelet activating factor through the acetylation of 1-alkyl-*sn*-3-glycerophosphocholine (Snyder, 1985).

Secreted PLA_2 constitute a family of low molecular weight proteins (12–16.5 kDa) which are phylogenetically related (Davidson & Dennis, 1990). All of these enzymes share a common structure of the active site which involves a His/Asp diad (Verheij *et al.*, 1980) and a heptacoordinated calcium ion (Tashima *et al.*, 1989). In mammals, secreted PLA_2 are encoded by two genes. The first one is the 14-kDa type I PLA_2 gene, which is mainly expressed in the pancreas, where it controls the synthesis and the secretion of this enzyme in the pancreatic juice (Seilhamer *et al.*, 1986). The second gene encodes for the type II PLA_2, which is found in very large amounts in rheumatic arthritic synovial fluids (Seilhamer *et al.*, 1989; Kramer *et al.*, 1989). This PLA_2 is indistinguishable from that associated with sepsis and septic shock (Green *et al.*, 1991).

Type II PLA_2 are secreted by various cell types in response to a combination of cytokines. Thus interleukin 1 (IL-1) and tumor necrosis factor α (TNFα) stimulate PLA_2 synthesis and secretion in rat chondrocytes (Suffys *et al.*, 1988), rat mesangial cells (Pfeilschifter *et al.*, 1989), rat vascular smooth muscle cells (Nakano *et al.*, 1990), rat calvarial osteoblasts (Vadas *et al.*, 1991), and rat cultured astrocytes (Oka & Arita, 1991). High amounts of PLA_2 were found in synovial fluid of patients with rheumatoid arthritis (Kramer *et al.*, 1989;

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[1] Abbreviations: PLA_2, phospholipase A_2; IL-1, interleukin 1; TNFα, tumor necrosis factor α; IL-6, interleukin 6; CAT, chloramphenicol acetyl transferase; APRE, acute phase responsive element; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LPS, lipopolysaccharide; C/EBP, CAAT enhancer binding protein; PCR, polymerase chain reaction; IL-6-RE, IL-6 responsive element; CRE, cAMP responsive element; CREB, CRE binding protein; NFκB, nuclear factor for κB immunoglobulin chain; DTT, dithiothreitol; PMSF, phenylmethyl sulfonyl fluoride.

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Pruzanski *et al.*, 1985). Serum PLA_2 activity rises up to 200-fold above the normal mean value in patients with septic choc (Pruzanski & Vadas, 1991). The levels of serum PLA_2 activities correlate with the magnitude and duration of circulatory collapse (Pruzanski & Vadas, 1991). These enzymatic activities have been related to the type II PLA_2 by immunoassay in a series of patients with hematological malignancies and septic fever (Nevalainen *et al.*, 1992). In addition, these authors established a correlation between the concentrations of PLA_2 and C-reactive protein in the serum of these patients. Recently, Crowl *et al.* (1991) demonstrated that interleukin 6 (IL-6), alone or in combination with TNFα and IL-1, stimulates PLA_2 synthesis in the human hepatoma cells HepG2. This result joined with the data previously described in patients suggests that type II PLA_2 belongs to the group of acute phase responsive proteins. It is well-known that these proteins are actively regulated at the transcriptional level and factors such as the C/EBP family members are involved in the expression of these genes. The objectives of this study were to identify the regulatory DNA elements and the factors involved in the transcriptional regulation of this gene and to assess the role of C/EBP family members.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, T4 kinase, proteinase K, polyacrylamide, TEMED, ammonium persulfate, and agarose were purchased from Appligene. MSL medium was provided by Eurobio. Taq polymerase was obtained from Cetus. Materials for cell culture were purchased from Gibco BRL (Dubelcco's modified Eagle medium supplemented, fetal calf serum, HEPES, trypsin) and from Falcon Inc. (flasks and petri dishes). We used the β-galactosidase expression vector pSV40β-gal from Clontech and acetyl-CoA, poly(dI-dC), and deoxynucleotides from Pharmacia. DNase I was from Worthington and the Klenow fragment of the polymerase I from Biolabs. The radioactive products were obtained from Amersham. All other chemicals were purchased from Fluka. Purified C/EBPα protein was kindly provided by Dr. Zannis, C/EBPα and C/EBPβ expression plasmids were generously provided by Pr. Ciliberto from IRBM (Roma), and CREB expression vector was generously provided by Dr. Lee (ICRF, Clare Hall Laboratories, Herefordshire, U.K.). Anti-C/EBPα and anti-CREB antibodies were purchased from Santa Cruz Biotechnology Inc.

Synthetic Oligonucleotides and Plasmid Constructions. Oligonucleotides were synthesized by the solid-phase phosphide triester method using an automated oligonucleotide synthesizer (Applied Biosystems, Inc., Model 380-B). The oligonucleotides were purified by electrophoresis in 20% polyacrylamide 7 M urea gels. The 5′ flanking fragment of the PLA_2 gene was generated by PCR (Mullis & Faloona, 1987). Human blood was collected in the presence of EDTA and was poured on a cusion of ficoll medium (MSL, Eurobio). The red cells were pelleted by centrifugation at 3000*g*, and the refractive band halfway up the tube, which was formed by the leukocytes, was collected using a pasteur pipet. The leukocytes were rinsed twice in PBS and pelleted by centrifugation at 3000*g*. The pellet was resuspended in 10 mM Tris pH 8.0, 50 mM NaCl, 10 mM EDTA, 0.5% SDS, and the proteins were digested by 100 μg/mL proteinase K for 4 h. Finally, the human genomic DNA was extracted by phenol and chloroform and precipitated by ethanol/sodium acetate. The primers, 5′-TGATCTCTGCCTTCATCTTTGTATATG-GCCT-3′ and 5′-CAAATGCAGATGGACTGGCCTAGC-TCCTCTG-3′, were designed according to the sequence published by Kramer *et al.* (1989) and corresponded respectively to the regions [12/40] and [2403/2433] in their sequence. Thirty PCR cycles were performed on a Perkin-Elmer 480 thermal cycler in a volume of 100 μL of buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 1 μM primers, 2.5 units of Taq polymerase using 1 μg of human DNA with the following steps: denaturation of 95 °C for 1.5 min, annealing at 65 °C for 1.5 min, extension at 72 °C for 3 min. This PCR product was reamplified using the derivated primers containing the *Sal*I and *Hin*dIII restriction sites under the same conditions as indicated above and inserted into the polylinker site of the pUC-SH-CAT plasmid (Ogami *et al.*, 1990). The construct was sequenced using the Sequenase Sequencing Kit (USB) (Sanger *et al.*, 1977). The sequence was found to be identical to the data published by Kramer *et al.* (1989). This sequence is referred to as [−1614;+806] in this paper with respect to the putative transcription starting site proposed by Seilhamer *et al.* (1989). The fragments [−1614;+20], [−326;+806], and [−326;+20] were generated by PCR from pUC-[−1614;+806]-PLA_2-SH-CAT with the corresponding primers deduced from the previously published sequence and inserted at the *Sal*I and *Hin*dIII restriction sites into the pUC-SH-CAT plasmid (Ogami *et al.*, 1990). The 5′ deleted fragments [−264;+20], [−210;+20], [−159;+20], [−118;+20], [−87;+20], and [−55;+20] were amplified by PCR from the pUC-[−326;+20]-PLA_2-SH-CAT construct and subcloned at the *Xba*I/*Hin*dIII sites into the pUC-SH-CAT plasmid. The PCR reactions were performed as described above. The pUC-PLA_2-SH-CAT constructs were sequenced and found to be identical to the sequence already published (Kramer *et al.*, 1989).

Element C was substituted by the nonspecific sequence 5′-GTGAATTCGAGCTCGGTACCCGGG-3′ (Papazafiri *et al.*, 1991) in the construction pUC-[−326ΔC;+20]-PLA_2-SH-CAT. For this mutant, we performed two series of PCR. In the first step, by using pUC-[−326;+20]-PLA_2-SH-CAT as template, we amplified two overlapping fragments containing half of the substituting sequences respectively at the 3′ end of the 5′ segment and at the 5′ end for the 3′ segment. For the 5′ segment, we used the primers 5′-ATCCGTCGACT-TAGAGGCGATTGCAGGGAGGT-3′ and 5′-GGGTAC-CGAGCTCGAATTCACCGTGTCAGTAGCTGATGC-3′. For the 3′ segment, we used the primers 5′-AGCT-AAGCTTCTCAGAGGACTCCAGAGTTG-3′ and 5′-AAT-TCGAGCTCGGTACCCGGGGTCCTGCCA-GCTGATGAG-3′. We purified the PCR products by electroelution to remove the primers and the wild type template. In the second step, we amplified the mutant fragment [−326;+20], by mixing an aliquot of each primary fragment and the primers 5′-AGCTAAGCTTCTCAGAGGACTCCA-3′ and 5′-ATCCGTCGACTTAGAGGCGATTGCAGG-GAGGT-3′. We ran a first cycle with a denaturation step at 95 °C for 1.5 min, a ramping step to lower the temperature to 50 °C in 5 min, an annealing step at 50 °C for 1.5 min, and an extension step at 72 °C for 2 min. Then we performed 30 cycles under the same conditions as previously described except that the annealing temperature was 55 °C. The PCR product was purified and ligated at the *Xba*I and *Hin*dIII sites of pUC-SH-CAT plasmids (Ogami *et al.*, 1990) after digestion by the restriction enzymes. The mutated promoters were sequences before being used in the transfection experiments.

Cell Cultures, Transfections, and CAT Assays. HepG2 cells were grown in Dulbecco's modified Eagle medium

supplemented with 10% fetal calf serum. The cultures were incubated with 5% CO_2 at 37 °C. Sixteen to twenty hours prior to transfection, 75-cm^2 confluent flasks were trypsinized and HepG2 cells were plated on 60-mm dishes at a density of 10^6 cells/dish. Plasmids used in transfections were purified through two CsCl gradients in a TL100 Beckman centrifuge. HepG2 cells were transfected using the calcium phosphate DNA coprecipitation method (Graham & Van der Eb, 1973). Briefly, cells were incubated with the transfection mixture containing 12 μg of pUC-SH-CAT constructs and 2.5 μg of plasmids bearing the β-galactosidase gene (Edlund *et al.*, 1985) for 4 h and then shocked with HBS buffer (21 mM HEPES, pH 7.1, 16 mM dextrose, 0.8 mM Na_2HPO_4, 5 mM KCl, and 137 mM NaCl) containing 15% glycerol for 30 s. Eighteen hours later, the medium was changed and the cells were grown for an additional 30 h in the presence or in the absence of IL-6 at concentrations ranging from 0.01 to 10 ng/mL. The cells were harvested 48 h after the glycerol shock, resuspended in 100 μL of 0.25 M Tris-HCl pH 7.5, and lysed by three cycles of freezing/thawing (Kumar & Chambon, 1988). Cell debris was removed by low-speed centrifugation at 4 °C for 5 min in a microfuge, and the supernatants were stored at −80 °C.

The CAT assays were performed as described by Gorman *et al.* (1982). Cell lysates were incubated with 5 μL of [^{14}C]-chloramphenicol (specific activity 25 μCi/mL, 50–60 mCi/mmol) and 0.53 mM acetyl-CoA in a 150-μL total volume of 0.47 M Tris-HCl pH 7.8 for 1 h at 37 °C. The concentration of the lysates was selected to assure linear conversion of the chloramphenicol into the acetylated forms (i.e., <30% acetylation). The products were extracted with 300 μL of ethyl acetate, dried under vacuum, and resuspended into 15 μL of ethyl acetate. The monacetylated and diacetylated forms were separated by thin layer chromatography on silica and acid resistant gel using 95:5 chloroform/methanol for development. The radioactive spots, detected by autoradiography, were scraped from the thin layer plates and counted. The β-galactosidase activity of the cell lysates was determined as described (Graham & Van der Eb, 1973) and the values were used to normalize variabilities in the efficiency of the transfections.

Preparation of Nuclear Extracts from Rat Liver and from Cell Cultures. Each batch of liver extracts was prepared from six male Wistar rats as described by Gorski *et al.* (1986). The nuclear factors (40 mg in 10 mL) were dialyzed twice for 1 h against nuclear dialysis buffer (25 mM HEPES pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 μg/mL aprotinin, 0.1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL pepstatin).

Nuclear extracts from HepG2 cells were prepared following a method derived from that described by Osborn *et al.* (1989). Briefly, cells from four confluent P100 dishes were washed and scraped in PBS buffer. The cells were centrifuged at low speed, and the pellet was resuspended in 500 μL of buffer A (5 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM PMSF, leupeptin 5 μg/mL). After incubation at 4 °C for 15 min, the cells were centrifuged and the pellet was resuspended in 250 μL of buffer C (20 mM HEPES, 25% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/mL of leupeptin). The nuclei were lysed by pipetting up and down four times and incubating for 30 min at 4 °C. The lysates were centrifuged at 45 000 rpm for 30 min in a TL100 Beckman centrifuge. The supernatants were collected, divided into aliquots after measurement of protein concentration (Kalb & Bernlohr, 1977), and stored at −80 °C.

Extracts from COS-1 cells were prepared 40 h after transfection of the cells. P100 60% confluent dishes were transfected with 30 μg of C/EBP or CREB plasmids by the calcium phosphate method. Forty hours posttransfection, the cells were scraped in 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl, and pelleted by low-speed centrifugation. Cells were resuspended in 400 μL of a buffer containing 20 mM Tris-HCl, pH 7.4, 0.4 M KCl, 2 mM DTT, and 10% glycerol and were broken by freezing/thawing three times (Kumar & Chambon, 1988). Cell debris was removed by centrifugation at 4 °C for 5 min in a microfuge, and the supernatant (whole cell extracts) was divided into aliquots and stored at −80 °C.

DNase I Footprinting and Methylation Interference Assays. Synthetic DNA fragments were generated by PCR and used for footprint analysis. The 5′ primers (30 pmol) were labeled with [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) and 10 units of T4 polynucleotide kinase prior to enzymatic amplification with Taq polymerase. The PCR products were separated on 4% polyacrylamide gels. The band was cut, electroeluted, extracted by phenol/chloroform, and ethanol-precipitated. Footprint analysis was performed as described by Cardot *et al.* (1991). Briefly, 6–45 μg of rat liver nuclear protein extracts was preincubated in a 20-μL reaction volume containing 25 mM HEPES pH 7.6, 40 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 3 μg of double-stranded poly(dI-dC). When recombinant C/EBPα protein was used instead of rat liver nuclear extracts, 4–6 μg of C/EBPα preparation was preincubating in the same reaction solution except that the poly(dI-dC) mass was 0.3 μg. In order to test the heat stability of the protecting factors, nuclear extracts were heated at 85 °C for 5 min before use. After 15 min on ice, 20 000 cpm of radiolabeled PCR product was added and the incubation was continued for 90 min at 4 °C. Then, 2 μL of freshly diluted DNase I (5–75 ng) in 10 mM HEPES pH 7.6, 25 mM $CaCl_2$, was added and the reaction was allowed to proceed for 5 min at 4 °C. The reaction was stopped by the addition of 4 μL of 125 mM Tris-HCl pH 8.0, 125 mM EDTA, 3% sodium dodecyl sulfate. Forty micrograms of proteinase K and 5 μg of tRNA were added, and the reaction mixture was incubated for 30 min at 65 °C. The volume was adjusted to 100 μL with water, and the DNA was precipitated by adding 1 vol of 5 M ammonium acetate and 2.5 volumes of ethanol. After incubation at −80 °C for 1 h, centrifugation at 13 000 rpm, and evaporation under high-speed vacuum, the pellet was resuspended in 98% formamide/0.2% bromophenol blue/0.2% xylene cyanol, 5 mM EDTA, and electrophoresed on a 6% polyacrylamide 7 M urea sequencing gel (thickness 0.4 mm) at 1400 V. The gel was dried and exposed to X-ray film.

For methylation interference assays, the coding strand of a synthetic oligonucleotide corresponding to element D was labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]-ATP. Each labeled strand was annealed with the unlabeled complementary strand as described previously (Maxam & Gilbert, 1977). End-labeled double-stranded oligonucleotides (4×10^6 cpm) were partially methylated at G residues using dimethyl sulfate (Maxam & Gilbert, 1977). The methylated probes were incubated with rat liver nuclear factors, and the complexes were analyzed in a preparative mobility shift gel as described by Ladias *et al.* (1992). The bands corresponding to the bound and free probes were cut and electroeluted at 100 V for 90 min. Bound and free probes were incubated with 1 M piperidine for 30 min at 90 °C. The piperidine was evaporated under vacuum for 2 h and the pellet resuspended

Table 1: Sequences of the Oligonucleotides Corresponding to Elements B, C, and D of the PLA_2 Promoter and Oligonucleotide Competitors Used in the Band Shift Assays

Element B : Fragment [-125/-85]

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5'-GGGATCCTGAGTCCACCAACTGACCACGCCCATCCCCAGCCTTG   -3'
3'-   TAGGACTCAGGTGGTTGACTGGTGCGGGTAGGGGTCGGAACGGG-5'
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Element C : Fragment [-210/-176]

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5'-GGGACACGTAAGGTTTCCCAATCCTCAACTCTGTCCT   -3'
3'-   TGTGCATTCCAAAGGGTTAGGAGTTGAGACAGGAGGG-5'
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Element D : Fragment [-248/-210]

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5'-GGGTGCAAAACTGCCTGAAATGTGTTTTGGCATCAGCTACTG   -3'
3'-   ACGTTTTGACGGACTTTACACAAAACCGTAGTCGATGACGGG-5'
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KBE WT : NKκB binding site of the κ immunoglobin chains (Brasier *et al.*, 1990)

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5'-GGGACAGAGGGGACTTTCCGAGAGG   -3'
3'-   TGTCTCCCCTGAAAGGCTCTCCGGG-5'
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APRE WT : Rat angiotensinogen acute phase responsive element (Brasier *et al.*, 1990)

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5'-GGGACCACAGTTGGGATTTCCCAACCTGACCA   -3'
3'-   TGGTGTCAACCCTAAAGGGTTGGACTGGTGGG-5'
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C/EBP : Domain D of the mouse albumin promoter (Maire *et al.*, 1989)

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5'-GGGTGGTATGATTTTGTAATGGGGTAGGA   -3'
3'-   ACCATACTAAAACATTACCCCATCCTGGG-5'
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AP1 : SV40 enhancer (Bos *et al.*, 1988)

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5'-GGGAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCA   -3'
3'-   TCGGCGTTCACTGAGTCGCGCCCCGCACACGTGGG-5'
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CRE : CRE site of the rat somatostatin promoter (Vajello *et al.*, 1992)

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5'-GGGGATCCGGCGCCTCCTTGGCTGACGTCAGAGAGAGAGA   -3'
3'-   CTAGGCCGCGGAGGAACCGACTGCAGTCTCTCTCTCTGGG-5'
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in 98% formamide. The digested probes were electrophoresed on 20% acrylamide gel at 30 W.

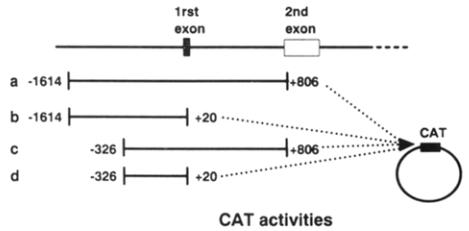
DNA Binding Gel Electrophoretic Assays. The pairs of complementary single-stranded (5 μg each) oligonucleotides corresponding respectively to the elements C and D identified in the footprint assays and to the different competitors (Table 1) were diluted in a volume of 20 μL of 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 50 mM NaCl, heated at 95 °C for 5 min and allowed to hybridize for 3 h at room temperature. Hybridization was verified on a 3% agarose gel. The solution was diluted with 80 μL of water and stored at −20 °C. The labeling of the probes was performed by incubating 100 ng (1 μL) of the double-stranded oligonucleotide with 60 μCi of [α-^{32}P]dCTP (specific activity 3000 Ci/mmol), 8 units of Klenow polymerase, and 2 μg of bovine serum albumin in a volume of 20 μL of 10 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM $MgCl_2$, for 30 min at room temperature. Free nucleotides were separated from the labeled probe on a Sephadex G50 column. The specific activity of the probe was estimated by spotting 1 μL of the labeling volume (before the G50 column) on TLC and counting the labeled probe and free nucleotide spots. The specific activities were in the range (0.8–1) × 10^8 cpm/μg.

Rat liver or HepG2 nuclear extracts or lysates from C/EBPα-, C/EBPβ-, or CREB-transfected COS-1 cells (2–8 μg of proteins) were preincubated for 15 min at 4 °C in a 20-μL reaction volume containing 25 mM HEPES pH 7.6, 8% ficoll, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 3 μg of double-stranded poly(dI-dC). To test the heat stability of the nuclear factors which interacted with elements C and D, the nuclear extracts were heated to 85 °C for 5 min prior to use. When competitor double-stranded oligonucleotides were used in the experiments, they were adeed in a 10–500-fold excess as compared to the concentration of the probe (as indicated in the figure captions) and the reaction mixture was incubated for an additional 15 min at 4 °C. The sequences of synthetic oligonucleotides used as competitors are displayed in Table 1. Labeled double-stranded oligonucleotide probes (60 000 cpm), corresponding to elements C and D, were then added, and the incubation was continued for 30 min at 4 °C. Free DNA and DNA–protein complexes were resolved in 4–6% polyacrylamide gels in buffer containing 6.7 mM Tris-HCl, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9. The gels were dried and exposed to X-ray films. In supershift experiments, rat liver nuclear extracts or lysates of HepG2 cells were preincubated overnight with various dilutions ranging from $^1/_{10}$ to $^1/_{1000}$ of anti-C/EBPα or anti-CREB antibodies. They were then use in band shift experiments as indicated above.

The dissociation constants (K_d) were obtained from binding reactions performed with a constant amount of protein extract and increasing concentrations of radiolabeled probe (5000–500 000 cpm). After gel electrophoresis and autoradiography, the radioactive bands corresponding to the bound and free oligonucleotide were excised and the radioactivity was measured. The data were computerized and fitted to the equation $1/B = K_d/P \times 1/F + 1/P$ where B and F were the bound and free probes, respectively, and P was the protein concentration. K_d values were deduced from the y values of the points where the computerized curves crossed the x axis; the coordinates of these points were $y = -1/\text{Free} = -1/K_d$ and $x = 0 = 1/\text{Bound}$ where Bound and Free were the concentrations of bound and free probes, respectively.

RESULTS

Identification of the Functional Promoter in the 5′ Flanking Region of the Human Type II PLA_2 Gene. On the basis of the transcription initiation site and the location of the first exon described by Seilhamer (Seilhamer *et al.* 1989), we obtained by PCR amplification of human genomic DNA a fragment which spans the sequence −1614 to +806 of PLA_2 gene. The first exon (20 bp), the first intron (697 bp), and a part of the untranslated region of the second exon (89 bp) are included in it. We cloned the fragment corresponding to this region in front of the CAT reporter gene of the pUC-SH-CAT plasmid (Ogami *et al.*, 1990), and we measured the promoter activity in HepG2 cells in the absence or in the presence of IL-6 (30-h incubation time with 10 ng/mL of IL-6). The importance of different regions of the cloned [−1614;+806] sequence was assessed by 3′ and 5′ deletions (Figure 1). Using as reference the CAT activity induced by the [−1614;+806] sequence, deletion of the 3′ [+20;+806] region increased transcription 8.1-fold. Deletion of the [−1614;−326] also increased transcription 7.7-fold in the absence of IL-6. Deletion of both the 3′ [+20;+806] and 5′ [−1614;−326] regions enhanced the promoter activity 65-fold in the absence of IL-6. Thus the highest promoter activity was obtained with the −326 to +20 promoter region. This region also provided the highest (2.4) stimulation by IL-6. This stimulation was found in 11 independent experiments performed in duplicate. The findings indicate that the −1614 to −326 as well as the +20 to +806 region contains negative regulatory elements which act synergistically to repress transcription of the human PLA_2 gene. This finding is in agreement with the inability of unstimulated HepG2 cells to synthesize and secrete type II PLA_2 in the absence of IL-6 (Crowl *et al.*, 1991). This analysis also localized the IL-6 responsive element in the −326 to +20 region. The response to IL-6 is time and dose dependent, and the maximal effect is achieved at 30 h for 10 ng/mL of IL-6 (Figure 2).



CAT activities

	Unstimulated	IL6 Stimulated	n_2
a [-1614; +806]	1	x1.3 ± 0.5	3
b [-1614; +20]	8.1 ± 2.3	x2.2 ± 0.3	2
c [-326; +806]	7.7 ± 1.6	x1.3 ± 0.1	2
d [-326; +20]	65 ± 15	x2.4 ± 0.06	11
n_1	3		

FIGURE 1: Effect of 5′ and 3′ deletions of the –1614 to +806 region of the 5′ region of the PLA_2 gene on the transcription activity. The PCR products were inserted at the *SalI*/*Hin*dIII site of pUC-SH-CAT and used for transient transfection in HepG2 cells as indicated in Experimental Procedures. The results for unstimulated experiments are expressed taking the activity of the pUC-[–1614;+20]-PLA_2-SH-CAT construct (a) as a reference. Results are the mean ± SEM of the independent experiments. n_1 is the number of experiments performed in duplicate which have been considered for this comparison (three experiments). The results for IL-6 (10 ng/mL, 24 h) stimulation represent the ratios between the IL-6-stimulated activities and unstimulated activities for each transfected CAT construct. n_2 is the number of independent experiments, each performed in duplicate. For the pUC-[–326;+20]-PLA_2-SH-CAT construct, three different preparations of plasmids have been used.

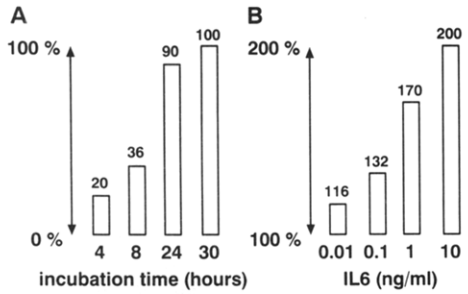


FIGURE 2: Time course (A) and dose–response curve (B) of IL-6 stimulation of the CAT activities in HepG2 cells transfected by the pUC-[–326;+20]-PLA_2-SH-CAT construct. Experimental procedure as in Figure 1. The activities are expressed as percentage of the maximal stimulated activity after a 30-h incubation time (A) or as percentage of the unstimulated activity (B). Each bar represents the mean of triplicate values.

Determination of the Regulatory Elements within the –326 to +20 Region of the Human PLA_2 Promoter. To define the regulatory elements present in the [–326;+20] region, we performed DNAse I footprint assays. Using the PCR products [–159;+20] and [–264;–119] (Figure 3) we observed four regions protected by rat liver nuclear extracts. Element A spans region [–35;+6] of the noncoding strand (Figure 3A). Element B spans region [–125;–86] of the coding strand (Figure 3B). Footprints A and B on the complementary strand were less pronounced and span from 43 to –7 and from –102 to –89 for elements A and B, respectively. Footprint B contains hypersensitive sites at positions –72 and –109 on the noncoding strand (Figure 3A). The footprints of elements C and D on both the coding and noncoding strands are shown in Figure 3C,D. The footprints of element C span region [–209;–175] of the coding strand and region [–207;–182] of the noncoding strand. The footprints of element D span the regions [–246; –211] and [–253;–221] of the coding and the noncoding strands, respectively. Elements B and C and to a lesser extent element D are protected by extracts heated to 85 °C for 5 min (Figure 3B, lanes g and h; Figure 3C,D, lanes e and f). In contrast, element A is not protected by heated extracts (Figure 3A, lanes g and h). This indicates that at least some of the factors which bind elements B, C, and D are heat stable.

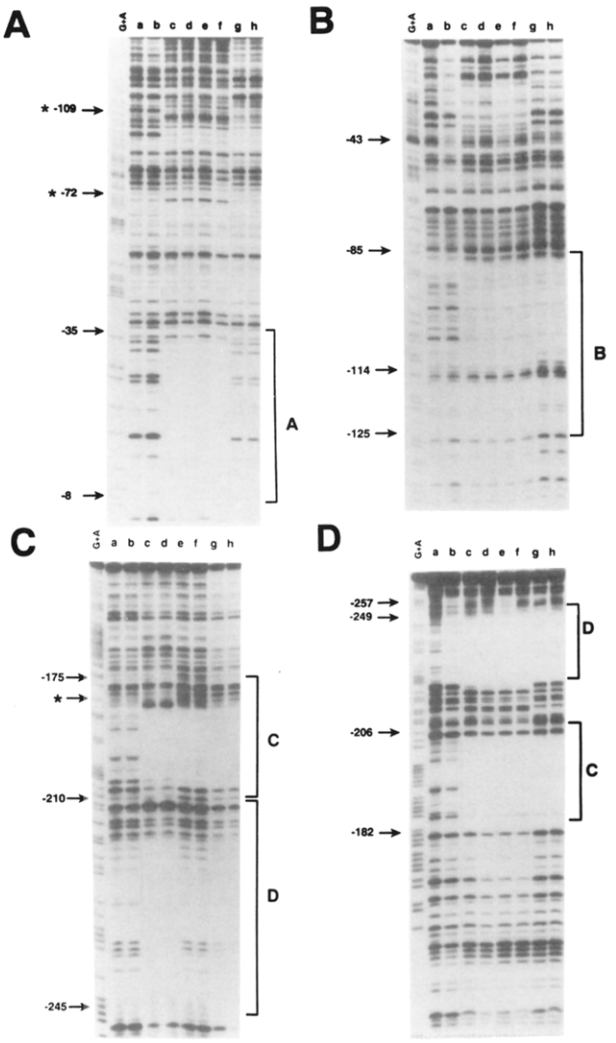


FIGURE 3: DNase I footprinting assays of the human type II PLA_2 promoter region. The fragments [–159;+20] (A, B) or [–264;–119] (C, D) were synthesized by PCR using primers which were previously labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The primers corresponded to the coding strand (B, C) or to the noncoding strand (A, D). Binding reactions and DNase I treatment were carried out as described in Experimental Procedures. (A) DNase amounts were 15 (lane a) and 25 ng (lane b) when no protein was added to the reactions and 25 (lanes c and d) and 50 ng (lanes e and f) when the reactions were performed in the presence of crude rat liver extracts. The amount of extract used was 15 μg in lanes c and e and 30 μg in lanes d and f. When rat liver extracts were heated for 5 min at 85 °C (30 μg in lane g, 40 μg in lane h), the DNase concentration was lowered to 5 ng. The stars indicate the location of hypersensitive sites at positions –109 and –72. (B) DNase amounts were 25 (lane a) and 50 ng (lane b) when no protein was added to the reactions and 50 (lanes c and d) and 100 ng (lanes e and f) when the reactions were performed in the presence of crude rat liver extracts. The amount of extract used was 15 μg in lanes c and e and 30 μg in lanes d and f. When rat liver extracts were heated for 5 min at 85 °C (30 μg in lane g, 40 μg in lane h), the DNase concentration was lowered to 15 ng. (C) DNase amounts were 15 and 25 ng for the reactions performed without proteins (lanes a and b), 100 ng for the reactions performed with 30 μg (lane c), and 40 μg (lane d) of rat liver nuclear extracts. When rat liver extracts were heated (30 μg in lane e, 40 μg in lane f) or when recombinant C/EBP wa used (4 μL in lane g, 6 μL in lane h), DNase amounts were lowered to 15 ng. The star indicates position –183, where a difference in protection by C/EBPα and rat liver extracts can be noticed. (D) The DNase and protein amounts were the same as in C except that DNase was 50 ng for lanes e and d.

Table 2: Homologies of the Regulatory Elements of the PLA_2 Promoter with Other Promoters and the Binding Sites of Previously Described Transcription Factors[a]

Element B (coding strand)	-124 TCCTGAGTCCACCAACTGACCACGCCCATCCCCAGCCTTG -85
AP1 (Wasylyk *et al.*, 1989)	G C TGA T A C A
AP2 (Williams & Tijan, 1991)	A CCC CCC N C GGG
NF-Y^m (Chodosh *et al.*, 1988)	C AG CG NNNNNN CCAA TCAN T AG TT C C A A
NF-Y^{*m} (Chodosh *et al.*, 1988)	AG NNN RCCAA TCNNN T T G G
Element C (coding strand)	-210 ACACGTAAGGTTTCCCAATCCTCAACTCTGTCCT -176
Rat angiotensinogen Acute Phase Responsive Element (Brasier et al., 1990)	ACAGTTGGGATTTCCCAACCTGACCA
C/EBP (Akira *et al.*, 1990)	T T T NNGNAA G G
Element C (non coding strand)	-176 AGGACAGAGTTGAGGATTGGGAAACCTTACGTGT -210
C/EBP (Akira *et al.*, 1990)	T T T NNGNAA G G
Element D (coding strand)	-247 GCAAAACTGCCTGAAATGTGTTTTGGCATCAGCTAC -211
T CRE (Roesler *et al.*, 1988)	TGACGTCA
C/EBP (Akira *et al.*, 1990)	G T T T T NNGNAA T NNGNAA T G G G

[a] The two residues found critical for the binding of C/EBP factors are underlined.

C/EBPα produced by expression of C/EBPα cDNA in *Escherichia coli* can protect elements C and D (Figure 3C,D, lanes g and h).

Elements A–D display partial sequence homologies to the binding sites of previously described factors (Table 2). Thus, element A contains the TATA box. Element B [-125;-86] displays homologies with the consensus binding sequences of AP1 between the positions -121 and -114 (TGAGTCCA) (Wasylyk *et al.*, 1989) and AP2 between the positions -100 and -93 (CCATCCC) (Williams & Tijan, 1991). The -122 to -102 sequence contains a CCAAT box and is homologous to the binding sites of the CCAAT box binding factors NF-Y^m and NF-Y^{*m} (Chodosh *et al.*, 1988). Element C and the rat angiotensinogen acute phase responsive element (APRE) (Brasier *et al.*, 1990) share a homologous sequence, TTTC-CCAA. Putative binding sites of C/EBP (Akira *et al.*, 1990) can also be observed on the coding strand as well as on the noncoding strand of this element. The -224 to -217 sequence of element D is homologous to the consensus sequence of cAMP responsive element (CRE) (Roesler et al., 1988). The coding strand of this element displays partial homologies with the C/EBP consensus binding site in the sequences -240 to -213 and -227 to -219. This last sequence overlaps with the sequence homologous to CRE in this element.

The results of the DNase I protection experiments allowed us to design several mutated PLA_2-SH-CAT constructs which contained deleted fragments of the [-326;+20] promoter (Figure 4). Deletion of the -326 to -210 region, which contains the regulatory element D, increases the transcription of the reporter CAT gene 10.5-fold. Further deletion of the -326 to -159 region, which contains elements C and D, increases the promoter activity 12-fold but reduces the responsiveness of the promoter to IL-6. Mutagenesis of element C, which precluded the binding of nuclear factors to it, reduced transcription to 70% of the control as well as the responsiveness of the promoter to IL-6. Deletion of the -326 to -87 regions,

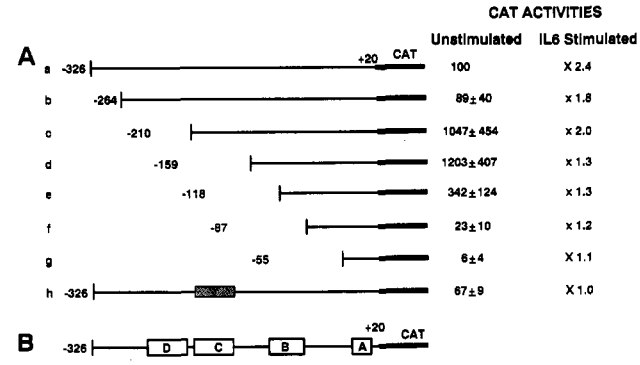


FIGURE 4: Effect of deletions and nucleotide substitutions of the fragment [-326;+20] of the PLA_2 promoter on the transcription (A) and summary of the footprint assays (B). The plasmids were designed according to the results summarized in Figure 3 and transiently transfected in HepG2 cells as indicated in Experimental Procedures. Transfected cells were either stimulated with 10 ng/mL IL-6 for 30 h or unstimulated. The results are expressed taking the basal activity of pUC-[-326;+20]-PLA_2-SH-CAT as a reference (100%). Results are the mean of three independent experiments performed in duplicate with three different batches of plasmids. The values in the column (IL-6 stimulated) represent the ratio between IL-6-stimulated and unstimulated activity for each transfected plasmid. Results are the mean of 11 independent experiments for lane a and three independent experiments for the other lanes. In lane h element C was replaced by an unspecific sequence (see Experimental Procedures).

which contain elements D, C, and B, reduced transcription to 23% of control, indicating that the factors bound to elements C and B are positive regulators of transcription. The involvement of element D in the inhibition of the transcription and element C in the IL-6 response of the promoter can also be deduced from these data. These findings prompted us to focus our attention on the regulatory elements C and D.

Identification of the Factors Which Bind Elements C and D. In order to identify the hepatic transcription factors which bind to elements C and D, we performed band shift experiments using these regulatory elements as probes.

Three closely migrating complexes and a faster migrating complex were observed with rat liver nuclear extracts using element C as a probe (Figure 5A, lane a). As expected from the DNase I footprint experiments, the factors involved in these complexes were heat stable (Figure 5A, lane b). Since element C displays strong homologies with the rat angiotensinogen APRE which binds C/EBP and NFκB related factors (Brasier *et al.*, 1990), we used the rat angiotensinogen APRE (Brasier *et al.*, 1990), element D of mouse albumin promoter (Maire *et al.*, 1989), and the NFκB binding site of the κ immunoglobulin chain promoter (Brasier *et al.*, 1990) as competitors (Table 1) in DNA binding assays. Both rat angiotensinogen APRE and element D of mouse albumin promoter competed for the binding of rat liver nuclear activities to the regulatory element C of the PLA_2 promoter (Figure 5A, lanes d, e). In contrast, the NFκB-RE did not compete (Figure 5A, lane f). In addition, neither an AP1 binding sequence (Bos *et al.*, 1988) nor element B of PLA_2 was able to compete for the binding of hepatic nuclear activities to element C (Figure 5A, lanes g, h).

We tested the ability of element C to bind C/EBP factors by using lysates of COS-1 cells transfected by C/EBPα and C/EBPβ. No formation of complexes with lysates of non-transfected COS-1 cells and element C was observed (data not shown). The band shift assay with element C and C/EBPα showed a single band which migrated at a position corresponding to the slowest migrating complex formed with the rat liver or HepG2 nuclear extracts (Figure 5B, lanes b, d, and e). The experiments performed with C/EBPβ displayed

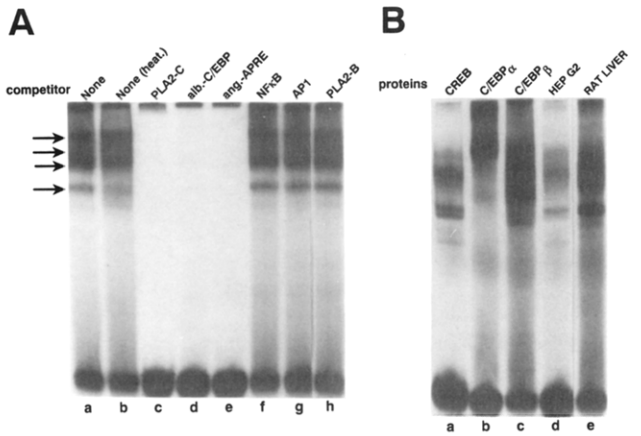


FIGURE 5: Band shift assays on the human type II PLA_2 promoter element C. A double-stranded synthetic oligonucleotide corresponding to the footprinting element C[–209;–175] was labeled with the Klenow polymerase in the presence of [α-^{32}P]dCTP as described in Experimental Procedures. (A) DNA binding assay of rat liver nuclear factors and competition experiments. Rat liver nuclear extracts (4.5 μg of proteins), heated (lane b) or not heated (lanes a, c–h), were incubated with the probe corresponding to element C (60 000 cpm) in the absence (lanes a and b) or in the presence of a 500-fold excess of unlabeled oligonucleotide competitors corresponding to element C itself (lane c), element D of mouse albumin promoter (lane d), the rat angiotensinogen APRE (lane e), the NFκB binding site of the promoter of the κ immunoglobulin chains (lane f), the SV40 enhancer which binds AP1 (lane g), and element B of human type II PLA_2 promoter (lane h). The complexes were resolved by electrophoresis on a 6% polyacrylamide gel. The arrows indicates the 4 main complexes formed between element C and rat liver extracts. (B) Formation of complexes between the probe corresponding to element C and different sources of nuclear factors. Lysate of COS-I cells overexpressing CREB (8 μg of protein, lane a), C/EBPα (2 μg of protein, lane b), C/EBPβ (8 μg of protein, lane c), lysate of HepG2 cells (18 μg of protein, lane d), or rat liver nuclear extract (4.5 μg of protein, lane e) was incubated with 60 000 cpm of the probe corresponding to element C as indicated in Experimental Procedures. The electrophoresis was performed on a 5% polyacrylamide gel.

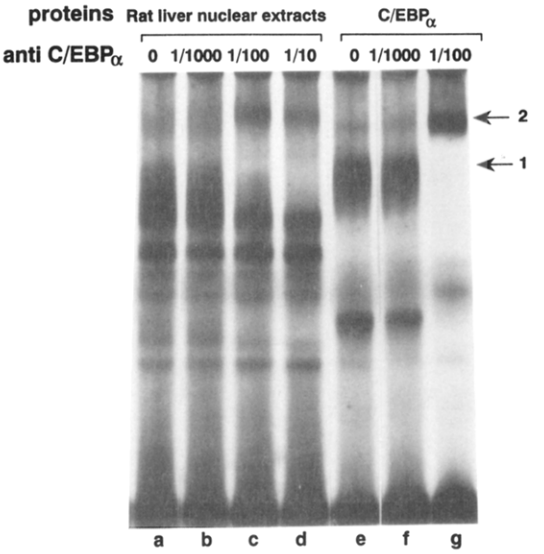


FIGURE 6: Supershift of some of the complexes formed with element C and rat liver nuclear factors preincubated with an anti-C/EBPα antibody. Rat liver nuclear extracts (4.5 μg of protein) or lysate from COS-I cells overexpressing C/EBPα (9 μg of protein) were incubated overnight with anti-C/EBPα antibodies diluted to 1/1000 (lanes b and f), 1/100 (lane c), 1/10 (lanes d and g), or with the vehicle alone (lanes a and e). Other incubation procedures as in Figure 5B except that the electrophoresis was performed on a 4% polyacrylamide gel. Arrow 1 shows the complex involving C/EBPα. Arrow 2 shows the complex shifted by the antibodies.

several complexes whose mobilities were similar to those observed with the rat liver or HepG2 nuclear extracts (Figure 5B, lanes c, d, and e). The binding of C/EBPα to the element C present in hepatic extracts was further documented by supershift experiments (Figure 6). The slowest migrating complex formed with hepatic nuclear extracts and element C was supershifted when the nuclear extracts were preincubated with anti-C/EBPα antibodies as well as the complex formed with C/EBPα obtained from COS-1 lysates (Figure 6, lanes c, d, and g).

We designed several mutated oligonucleotides to localize the binding site of the hepatic nuclear proteins which bind to element C (Figure 7). In mutants M2 and M4, the CCAAT box has been altered. Moreover, mutant 4 displays a substitution of a G by an A residue upstream from the homologous sequence TTTCCCAA shared by rat angiotensinogen APRE and PLA_2 element C. However the M2 and M4 oligonucleotides could still compete for the binding of hepatic nuclear factors to element C. Similarly, competition was observed with the mutant oligonucleotide M3. In this mutant a pair of T and G residues conserved in the rat angiotensinogen APRE, element D of mouse albumin promoter and PLA_2 element C were substituted by G and T residues, respectively. However the mutant oligonucleotide M1 did not compete for the binding of hepatic nuclear activities to element C. In mutant M1, the first two nucleotides of the triplet TTT (–199;–197) were replaced by G and C residues. This mutation alters the putative C/EBP binding site found on the noncoding strand (Table 2). This finding combined with the data of the binding assays showed in Figure 5A,B suggests that the liver nuclear factors which bind to element C belong to the C/EBP family and their site is located on the noncoding strand of the [–200;–192] sequence of the PLA_2 promoter.

Element D forms a major complex with rat liver nuclear extracts which is heat sensitive (Figure 8A, lanes a, b). Some other heat stable hepatic activities bound to element D could be observed (Figure 8A, lane b). Neither element D of murine albumin promoter, nor the rat angiotensinogen APRE, nor an NFκB binding site (Brasier *et al.*, 1990) competed for the binding of hepatic nuclear activities to element D (Figure 8, lanes d, e, and f). No competition was observed with oligonucleotides containing an AP1 binding site (Bos *et al.*, 1988) or the element B of PLA_2 (Figure 8A, lanes g and h). These data would exclude the involvement of C/EBP factors in this complex. However, element D was still able to bind C/EBPα and C/EBPβ expressed in COS-1 cells (Figure 8B, lanes b and c). No formation of complexes with lysates of nontransfected COS-1 cells and element D was observed (data not shown). The mobilities of the complexes formed by C/EBPα and C/EBPβ bound to element D are higher than those of the major complexes formed by the rat liver or HepG2 nuclear proteins bound to the same element. Oligonucleotides corresponding to element D could compete effectively for the binding of hepatic nuclear activities or C/EBPα to element C at 100- and 200-fold excesses, respectively (Figure 9, panels A and B). A 500-fold excess of oligonucleotide D was required to obtain a partial competition for the binding of C/EBPβ to element C (Figure 9C). Furthermore, a 1250-fold excess of unlabeled oligonucleotide C could not compete for the binding of the major hepatic activity to element D although a 25-fold excess of oligonucleotide C was sufficient to compete with the minor activities to element D (Figure 9D).

We performed band shift experiments with elements C and D by varying the total concentration of probe with constant amounts of rat liver extract and C/EBPα proteins and have

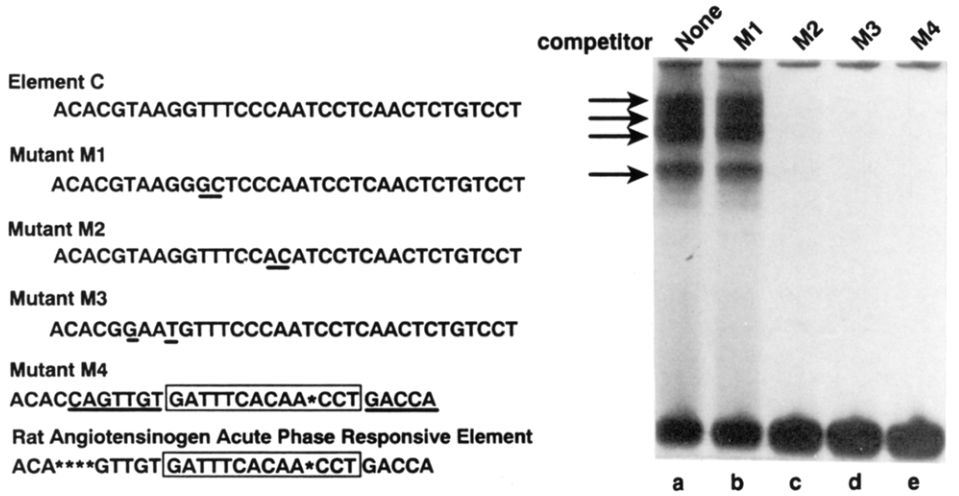


FIGURE 7: Design of the oligonucleotide mutants of element C and competition experiments for the binding of rat liver nuclear factors to element C. The experiments were performed as in Figure 5A. The oligonucleotide probe corresponding to element C (60 000 cpm) was incubated with rat liver nuclear extracts (4.5 μg of protein) in the presence of a 100-fold excess of oligonucleotide competitors as indicated in Experimental Procedures. Oligonucleotide competitors derived from element C: M1 in which the putative C/EBP binding site on the noncoding strand was altered (Table 2), lane b; M2 in which the putative C/EBP binding site on the coding strand was altered (Table 2), lane c; M3 in which two homologous residues found in rat angiotensinogen APRE were substituted, lane d. For these oligonucleotide mutants (M1 to M3), the substituted nucleotides are underlined. Oligonucleotide competitor derived from rat angiotensinogen APRE: M4 in which the homology with NFκB-RE and the CAAT box have been altered, lane e. For this mutant the sequence homologous to the PLA_2 element C and the rat angiotensinogen APRE is enclosed in a box and the nucleotides which differ from the sequence of the PLA_2 element C are underlined. The sequence of the rat angiotensinogen APRE was aligned with mutant M4. The stars represent missing nucleotides. The sequence of the mutant M4 has been published by Brasier *et al.* (1990). The arrows indicate the four complexes formed between element C and rat liver nuclear extracts.

determined the concentration of bound and free probes. The Scatchard analysis of the data showed that element D has a lower affinity for C/EBPα (K_d = 5.8 nM) than element C (K_d = 0.6 nM) (Figure 10, panels A and B). However, the affinity of the major hepatic activity which binds to element D is in the same range (K_d = 2.9 nM) as that of C/EBPα (Figure 10, panel C).

A cAMP responsive element (CRE) present in the rat somatostatin promoter (Vajello *et al.*, 1992) (Table 1) competed partially (at a ratio CRE/element D of 500/1) for the binding of hepatic nuclear activities to element D (Figure 11). This oligonucleotide could compete in a similar way with element C (Figure 11). It has been demonstrated that CRE can be used as an alternative site for C/EBP factors in the case of the human fibronectin and the phosphoenolpyruvate carboxykinase promoters (Muro *et al.*, 1992; Park *et al.*, 1990). Moreover, CREB expressed in COS-1 cells formed several complexes with elements C and D (Figures 5B and 8B, lanes a). However, anti-CREB antibodies did not supershift the major DNA–protein complex formed with element D and hepatic nuclear extracts, indicating that CREB does not bind to element D (data not shown).

We performed methylation interference experiments to define the binding site of the rat liver nuclear factor(s) in element D. This analysis showed that the C and G residues of the sequence [–224 TGGCATCA –217] homologous to CRE are not involved in the interaction with the liver proteins. Only a methylation on the G nucleotide at position –228 i.e., upstream from the CRE like sequence, influences the binding of the nuclear factor to element D (Figure 12).

DISCUSSION

Type II PLA_2 is a secreted enzyme which is mainly synthesized and secreted under pathophysiological circumstances in response to various cytokines, mainly IL-6, TNFα, and IL-1. In contrast with most other acute phase proteins, which are mainly synthesized in the liver (Schreiber, 1987), synthesis and secretion of human type II PLA_2 also occur in mesangial cells (Pfeilschifter *et al.*, 1989), smooth muscle vascular cells (Nakano *et al.*, 1990), chondrocytes (Suffys *et al.*, 1988), synoviocytes (Hulkower *et al.*, 1992), osteoblastic cells (Vadas *et al.*, 1991), placenta (Kramer *et al.*, 1989), and astrocytes (Oka & Arita, 1991). In this paper, we focused our attention on the sequence of the promoter of the human type II PLA_2 gene, which can be involved in an inflammatory response in hepatocytes. Recently, extensive information has been accumulated on the role of regulatory DNA elements which are involved in the induction of the hepatic expression of the genes of acute phase proteins. In a number of cases, the proximal promoter elements located in the first hundred base pairs upstream from the transcription start site were shown to be crucial for the stimulation of the transcription by cytokines. These elements are the binding sites of trans-acting factors, and gene expression appears to be the result of the combined effect of a set of factors which are bound to different regulatory elements.

Regulation of the Human PLA_2 Gene Is Controlled by Positive and Negative Regulatory Elements. Conventional deletion and nucleotide substitution analysis of the human PLA_2 promoter showed that it contains a set of positive and negative regulatory elements. Strong negative regulatory elements were localized in the –1614 to –210 and +20 to +806 regions. Thus CAT constructs containing the –210 to +20 promoter region stimulated transcription 780-fold as compared to the –1614 to +806 promoter region. On the other hand, the region –210 to –55 was shown to contain important positive regulatory elements. The proximal regulatory elements of the human PLA_2 promoter were localized by footprinting analysis and were designated A [–35;–6], B [–125;–86], C [–176;–209], and D [–253;–211]. Element A contains the TATA box and probably represents the binding site of TBP. The deletion and nucleotide substitution analysis showed that element D is a strong negative regulatory element and elements C and B are positive regulatory elements. The

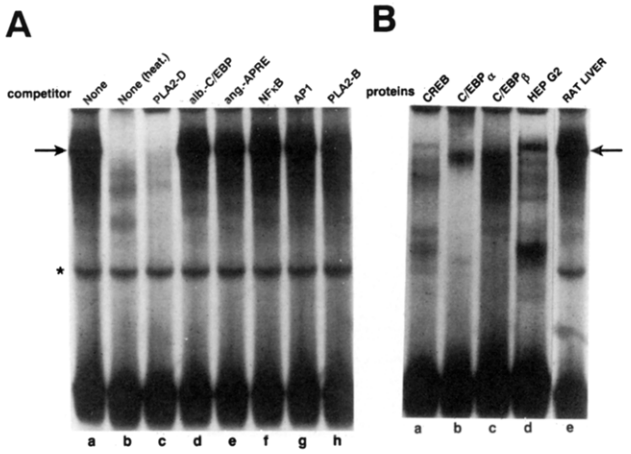


FIGURE 8: Band shift assays on the human type II PLA_2 promoter element D. A double-stranded synthetic oligonucleotide corresponding to the footprinting element D [–247;–211] was labeled with the Klenow polymerase in the presence of [α-^{32}P]dCTP as described in Experimental Procedures. (A) DNA binding assays of rat liver nuclear factors to element D and competition experiments. Rat liver nuclear extracts (9 μg), heated (lane b) or not heated (lanes a and c–i), were incubated with the probe corresponding to element D (60 000 cpm) in the absence (lanes a and b) or in the presence of a 500-fold excess of unlabeled oligonucleotide competitors corresponding to element D itself (lane c), the C/EBP binding element D of mouse albumin promoter (lane d), the rat angiotensinogen APRE (lane e), the NFκB binding site of the promoter of the κ immunoglobulin chains (lane f), the SV40 enhancer which binds AP1 (lane g), and element B of human type II PLA_2 promoter (lane h). The electrophoresis was performed on a 4% acrylamide gel. The arrow shows the single complex formed between element D and rat liver extracts. The star indicates a nonspecific band. (B) Formation of complexes between the probe corresponding to element D and different sources of nuclear factors. Lysates from COS-I cells overexpressing CREB (8 μg of proteins, lane a), C/EBPα (2 μg of proteins, lane b), C/EBPβ (8 μg of proteins, lane c), nuclear extracts from HepG2 cells (18 μg of proteins, lane d), and rat liver (9 μg of proteins, lane e) were incubated with 60 000 cpm of the probe corresponding to element D as indicated in Experimental Procedures. The complexes were resolved on a 4% polyacrylamide gel. The arrow shows the main complex formed between element D and HepG2 or rat liver nuclear extracts.

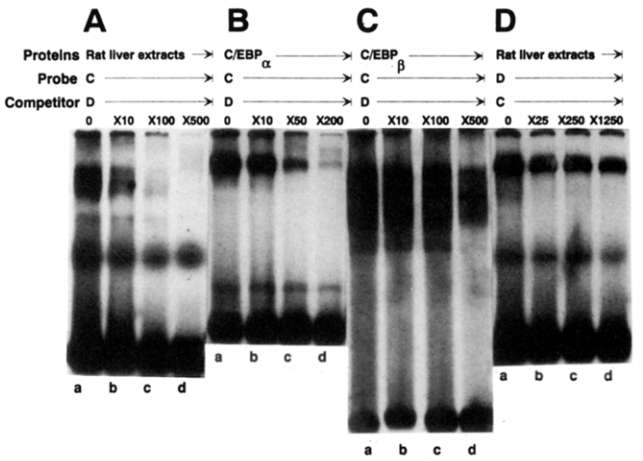


FIGURE 9: Competition between PLA_2 elements C and D for the binding of rat liver nuclear proteins and C/EBP factors. A double-stranded oligonucleotide corresponding to element C was used as probe (60 000 cpm/reaction), and a double-stranded oligonucleotide corresponding to element D was used as a competitor for the binding of rat liver nuclear factors (4.5 μg) (A), C/EBPα (2 μg) (B), and C/EBPβ (8 μg) (C). The ratios of oligonucleotide competitor/probe (D/C) are indicated at the top of the figure. A double-stranded oligonucleotide corresponding to element D was incubated (60 000 cpm/reaction) with rat liver nuclear factors (9 μg) in the presence of the oligonucleotide competitor corresponding to element C (D). The ratios of oligonucleotide competitor/probe (C/D) are indicated at the top of the figure.

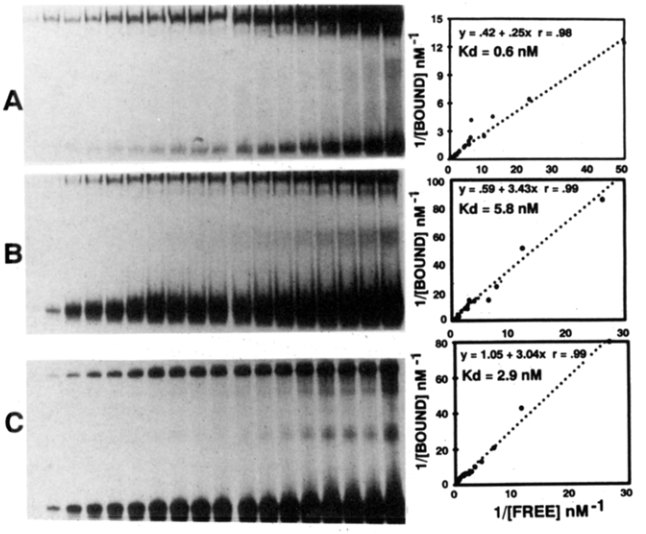


FIGURE 10: Determination of the dissociation constants (K_d) of element C for C/EBPα (A) and of element D for C/EBPα (B) and the rat liver nuclear factors (C). The band shift assays were performed by varying the concentrations of probes whose specific activities were determined. The concentrations of proteins were kept constant. The bands corresponding to the free and the bound probes were cut and counted. The K_d were calculated as indicated in Experimental Procedures.

role of element C appears quite complex. The deletion of element C did not induce a drastic decrease of the transcription activity since the fragment [–159;+20] induced CAT activities similar to those of the fragment [–210;+20]. Therefore element B would be postulated as the main positive element; but substitution of element C by a nonspecific sequence in the presence of wild type elements D and B induced a decrease of 33% of the transcription activity and a loss of IL-6 response. Elements C and D are separated by less than 15 bp, and it is tempting to assume that the main role of element C is to modulate the inhibitory effect of element D in response to IL-6. The confirmation of such interactions between elements C and D requires further studies.

Factors Bound to the Proximal Regulatory Elements D and C. DNA binding and competition assays showed that the regulatory region C forms several DNA–protein complexes with the hepatic nuclear extracts. All these complexes could be competed out only by oligonucleotides containing the binding site of C/EBP. In addition these complexes were stable to heating at 85 °C for 5 min. C/EBP represents a family of heat stable transcription factors designated α, β, γ, δ (Akira *et al.*, 1990; Williams *et al.*, 1991; Cao *et al.*, 1991; Descombes *et al.*, 1990; Chang *et al.*, 1990; Thomassin *et al.*, 1992; Poli *et al.*, 1990; Landschulz *et al.*, 1988a). They contain a leucine zipper motif that is involved in DNA binding and dimerization (Cao *et al.*, 1991; Landschulz *et al.*, 1988; Vinson *et al.*, 1989; Landschulz *et al.*, 1988b, 1989). The binding of C/EBPα and C/EBPβ to this element was further confirmed by DNA binding and footprinting of this region with C/EBPα or C/EBPβ. The mobilities of the complexes formed with C/EBPα and C/EBPβ were similar to those observed with rat liver nuclear extracts. C/EBPβ mRNA is translated in several proteins with different transactivation potentials (Descombes & Schibler, 1991). The dimerization between these different translated products can explain the formation of several complexes with the C/EBP binding element C. Similarly the heterodimerization of the different members of the C/EBP family may induce the complex band shift pattern

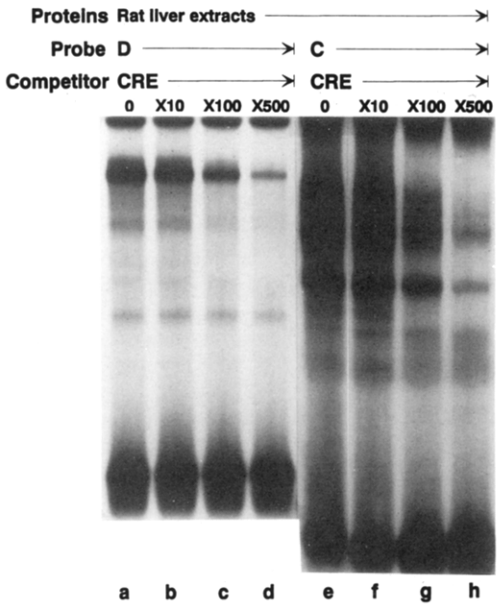


FIGURE 11: Competition between elements C and D of PLA_2 promoter and rat somatostatin CRE for the binding of rat liver extracts. Oligonucleotides corresponding to element D (lanes a–d) and element C (lanes e–h) were used as probes and labeled as indicated in Experimental Procedures. The probes (60 000 cpm/lane) were incubated with 9 μg of rat liver nuclear extracts. Rat somatostatin CRE was added in an excess of ×10 (lanes b and f), ×100 (lanes c and g), and ×500 (lanes d and h).

obtained with the probe corresponding to element C and the hepatic nuclear extracts. The alteration of a sequence homologous to the C/EBP binding site on the noncoding strand of element C made the corresponding mutant oligonucleotide unable to compete with element C. Finally a complex formed with the hepatic nuclear extracts which has the same mobility as the complex formed with C/EBPα could be supershifted by anti-C/EBPα antibodies. The C/EBP proteins bound to this region represent positive regulatory factors since mutagenesis, which precluded their binding to this region, diminishes the overall transcription and abolishes the IL-6 stimulation of transcription driven by PLA_2 promoter.

Similar DNA binding and competition assays showed that the regulatory region D forms one major heat labile DNA–protein complex which could not be competed with by oligonucleotides containing the binding site of C/EBP. Minor heat stable activities also bind to this element. These activities could be competed with by oligonucleotides corresponding to element C but not by other oligonucleotides containing the binding site of C/EBP. Unexpectedly direct binding experiments showed that C/EBPα and C/EBPβ produced by transfection in COS-1 cells could bind to element D. Some homologies can be observed between elements D and the consensus C/EBP binding sites as well as IL-6 responsive elements of human β-fibrinogen and C reactive protein promoter (Crowl *et al.*, 1991; Baumann *et al.*, 1990; Arcone *et al.*, 1988). However, the affinity of C/EBPα for element D was reduced 10-fold as compared to its affinity for element C. Element D competes poorly for the binding of C/EBPβ to element C. The overall DNA binding and competition assays suggest that the major activities which bind to the regulatory region D may be different from C/EBP family members.

Although weak competition of this hepatic activity was observed with oligonucleotides containing the binding site of CREB, anti-CREB antibodies did not supershift the heat labile major complex nor the other minor complexes, indicating that

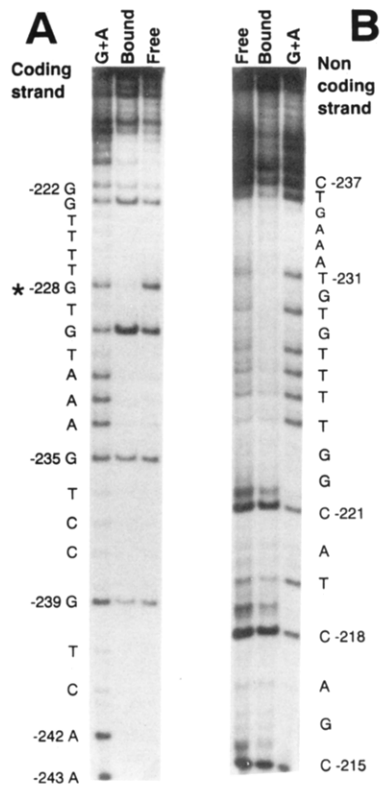


FIGURE 12: Methylation interference pattern of the main DNA–rat liver nuclear extracts complex formed with element D of type II PLA_2 promoter. (A) The oligonucleotide corresponding to the coding strand has been labeled and then annealed with the complementary strand before partial methylation on the G residues. The figure shows the electrophoresis of the bound probe (bound), the free probe (free), and a molecular weight marker (G + A) on a 20% acrylamide gel after digestion with piperidine. The star indicates the position of the G residue whose methylation impedes the interaction with the nuclear factor. The sequence of the coding strand is shown beside the molecular weight marker. (B) The oligonucleotide corresponding to the noncoding strand has been labeled and then annealed with the complementary strand before the partial methylation on the G residues. The figure shows the electrophoresis of the bound probe (bound), the free probe (free), and a molecular weight marker (G + A) on a 20% acrylamide gel after digestion with piperidine. The sequence of the coding strand (complementary strand) is shown beside the molecular weight marker.

CREB itself does not bind to element D. The competition between CRE or rat somatostatin promoter and element D may be related to a partial homology between this element and the CREB binding site (Table 2). CREB belongs to the ATF/CREB family of transcription factors. Two members of this family have been demonstrated to negatively regulate the activity of the corresponding promoters (Hoeffler *et al.*, 1988; Foulkes *et al.*, 1991). The nature of the hepatic activities bound to element D and their putative relationship with the ATF/CREB family remain to be investigated by further topographical and functional studies.

Regulatory Elements and Factors Required for the IL-6 Response of the Human PLA_2 Promoter. The deletion and substitution analysis demonstrated that the region –326 to +20 of the human type II PLA_2 promoter contains the elements required for the IL-6 response in HepG2 cells. The observed 2-fold stimulation of the basal activity for both the pUC-[–326;+20]-PLA_2-SH-CAT and the pUC-[–1614;+20]-PLA_2-SH-CAT constructs is rather low as compared with promoters of other acute phase genes. IL-6 increased the transcription driven by human β-fibrinogen or human and rat haptoglobin promoters by approximately 10-fold in transfected human hepatoma cells (Baumann *et al.*, 1990; Huber *et al.*,

1990; Oliviero & Cortese, 1989; Marinkovic & Baumann, 1990). However, this IL-6 effect was reproducible in each of the 11 independent experiments that we have performed and it was time and concentration dependent.

Acute phase reactants are classically divided into two groups. The first one, which comprises α1 glycoprotein, haptoglobin, complement component C3, and serum amyloid A protein, requires a combination of at least IL-1 and IL-6 to exhibit full stimulation, whereas the second group, including fibrinogen, α2 macroglobin thiostatin, antitrypsin, and chymotrypsin, only needs IL-6 as stimulatory cytokine (Bauman & Gauldrie, 1990). Human type II PLA_2 could barely be classified in one or the other of these two groups, since IL-1 and TNFα displayed respectively additive and synergistic effects on the endogenous gene (Crowl et al., 1991). Human type II PLA_2 may also be different from other acute phase proteins by the fact that the response to acute phase reactants may be controlled by the negative regulatory elements that are localized in the −1614 to −210 and +20 to +806 regions.

It has been suggested that C/EBPβ may participate in the regulation of IL-6 responsive gene. Cis elements which bind C/EBP factors and are responsive to IL-6 have been characterized in the promoters of many acute phase proteins, i.e., haptoglobin (Oliviero & Cortese, 1989), hemopexin (Poli & Cortese, 1989), and C-reactive protein (Majello *et al.*, 1990). In livers of LPS treated mice, C/EBPα mRNA decreased by 20–50% 4 h after injection. In contrast, C/EBPβ and C/EBPδ mRNA rose respectively to 4- and 70-fold in the same period of time (Alam *et al.*, 1992). The constitutive expression of C/EBPδ was found to be very low as compared to those of C/EBPα and C/EBPβ by these authors; but the levels of expression of C/EBPβ and C/EBPδ gene in mice were shown to be similar 4 h after LPS injection. Baumann *et al.* (1992) observed similar patterns of variations of C/EBP mRNAs in rat liver 4 h after a turpentine injection except that the amount of C/EBPβ exceeded that of C/EBPδ under control or acute phase conditions. In addition, Baumann *et al.* (1992) found 2- and 10-fold stimulations of C/EBPα and C/EBPβ, respectively, in rat hepatoma cells H35 after treatment by IL-1, IL-6, and dexamethasone. Poli *et al.* (1990) characterized C/EBPβ in the human hepatoma cell line Hep3B. Some authors (Xanthopoulos *et al.*, 1992) suggested that the level of C/EBP factors, especially C/EBPα, is lower in HepG2 cells than in normal adult hepatocytes. Spergel *et al.* (1992) demonstrated that the promoters of some gene of the adenovirus are responsive to C/EBPβ and their activities are highly stimulated in the other human hepatoma cell line HepG2. They estimated by band shift experiments that the amount of C/EBPβ in these cells is 15–20-fold higher than in the Jurkat cells, which are human T-cells devoid of IL-6 receptors. Taken together, these data suggest that C/EBP factors and noticeably C/EBPβ are present in high amounts in liver and heptoma cells even if some species- and cell-type-dependent differences can be observed. C/EBPβ mediates the effect of IL-6 through the increase of the expression of its gene (Akira *et al.*, 1990; Ranji *et al.*, 1993) or post-translational modifications (Wegner *et al.*, 1992). As discussed above, element C is the major binding site of C/EBP factors. The deletion and the nucleotide substitution mutagenesis showed that the IL-6 stimulation of the transcription driven by the PLA_2 promoter required the presence of element C. Thus it is posible that C/EBPβ bound to the regulatory element C may participate in the IL-6 stimulation. The precise mechanism of this stimulation and how the negative regulatory factor(s) bound to element D modulate this response require further studies. Such studies may for instance involve cotransfection experiments, using wild type and mutated C/EBP forms.

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