Expression of the human gene coding for the α-chain of C4b-binding protein, *C4BPA*, is controlled by an HNF1-dependent hepatic-specific promoter

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C4b-binding protein (C4BP) is an abundant oligomeric plasma glycoprotein which controls the activation of the complement cascade through the classical pathway. In humans, the majority form of C4BP is composed of seven α-chains and one β-chain, covalently linked by their C-termini. C4BP is mainly expressed in the liver. We have previously cloned and characterized the structure of the genes encoding the α and β chains, *C4BPA* and *C4BPB*, respectively. Here we addressed the characterization of the mechanisms controlling the hepatic restricted expression of the *C4BPA* gene. We found that the *C4BPA* promoter is contained within the first 369 bp upstream of the transcription start site. The activity of this promoter is restricted to hepatic cells in transfection experiments. The hepatic transcription factor HNF1 interacts with a region of this promoter at −38 bp. This region is absolutely required for the activity of this promoter, suggesting that HNF1 is essential for the hepatic activity of the *C4BPA* promoter. We speculate that this extreme requirement of HNF1 for the activity of the human *C4BPA* promoter is related to the fact that this promoter lacks a TATA box.

INTRODUCTION

Complement is the most important mechanism of the humoral defence and clearance systems in higher vertebrates. Activation of the complement system can proceed through two different routes known as the classical and alternative pathways. Central to both pathways of complement activation is the cleavage of the complement component C3 by a highly efficient cascade mechanism which involves multisubunit enzymes known as C3-convertases (reviewed in [1]). To avoid self damage and complement component consumption, the complement activation is tightly down-regulated. Several proteins are involved in this regulation of the complement activation. Some of these proteins, like membrane cofactor protein (MCP), decay-accelerating factor (DAF) and complement receptor 1 (CR1) are membrane-bound, while others, like H factor (H) and C4b-binding protein (C4BP) regulate the activation of the complement system in the soluble phase of the bloodstream [1]. C4BP is the most important soluble regulator of the classical pathway of complement activation [2], although CR1 may be at least as important physiologically. In humans, C4BP is a hetero-oligomer assembled by two types of polypeptide chains: seven α-chains, of 70 kDa, encoded by the *C4BPA* gene, and one β-chain, of 45 kDa, encoded by the *C4BPB* gene [3,4]. The α-chains bear the complement regulatory function of C4BP by binding and inactivating the C4b component of the classical pathway C3-convertase. The β-chain binds the anti-coagulatory serum protein S and thus may be relevant for the control of the coagulation cascade [3]. A role in localizing C4BP on the surface of injured or activated cells has been also proposed for the β-chain, which could be important for the complement regulatory function of C4BP [5]. *C4BPA* and *C4BPB* genes have been cloned and mapped to contiguous locations within the regulator of complement activation (RCA) gene cluster, on the long arm of human chromosome 1 (1q32 band) [6]. The RCA gene cluster includes the evolutionary-related genes of the proteins involved in the control of complement activation [7–14].

Recently, we have cloned and determined the exon–intron organization of the *C4BPA* gene which is split into 12 exons [15]. We have characterized the transcription start site and cloned the 5′ flanking region. Preliminary analysis of the sequence of this region showed putative sites for several transcription factors. Interestingly, no region homologous to the TATA box was observed. *C4BPA* is mainly expressed in the liver [15,16]. Here we show that the promoter is an important element for the hepatic expression of *C4BPA*. This promoter is located within the first 369 bp upstream of the cap site and showed hepatic specificity. The liver-enriched transcription factor hepatic nuclear factor 1 (HNF1) [17–21] appears to be a key element for the activity of this promoter.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides used in gel-retardation assays and PCR were synthesized in a Gene Assembler apparatus (Pharmacia). Their sequences are shown below. PE1: 5′-GGTTCTGCTGTTAATCATTCATTGGGCCC-3′, corresponds to the HNF1 site of the *C4BPA* promoter (region −57 to −31 bp of *C4BPA*). PE1M1: 5′-GGTTCTGCTGTctAgaAggCATTGGGCCC-3′, is the mutated version of PE1 (mutated nucleotides are indicated in lower-case type). PE1M1.C: 5′-ACGGGCCCAATGccTtcTagACAGCAGAA-3′, is the complementary strand of PE1M1. PE2: 5′-GGAAGGAGCTTAGGTAAACAGTGCTGCTT-3′ corresponds to the PE-2 site of the *C4BPA* promoter (region −89 to −61 bp of *C4BPA*). PE3: 5′-GGAAATGGTTGGCAGAGGAGAAAATAAACGATTGCGACAT-3′, corresponds to the PE-3 site of the *C4BPA* promoter (region −156 to −117 bp of *C4BPA*). PE4: 5′-CCAAAGAAAAAAGAATTCTGGCTTCAAATTCAAATTACCTTT-3′, corresponds to the PE-4 site of the

Abbreviations used: CAT, chloramphenicol acetyltransferase; C4BP, C4b-binding protein; CR1, complement receptor 1; DTT, dithiothreitol; βGal, β-galactosidase; HNF1, hepatic nuclear factor 1; RCA, regulator of complement activation; tk, thymidine kinase.

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C4BPA promoter (region −204 to −166 bp of *C4BPA*). Alb: 5′-TCGAGTGTGGTTAATGATCTACAGTTA-3′, corresponds to the HNF1 site of the rat albumin promoter [22]. C4BPA369: 5′-CAGTCGACGAGCTCTGGGCAAGAATATCAGTTTTC-3′, underlined nucleotides correspond to the region −369 to −340 bp of human *C4BPA*. This sequence delimits the upstream end of the *C4BPA*.PS promoter. C4BPAF20: 5′-TAAGCT-TGGTTGGCTGGTCAAGGACGGTT-3′, underlined nucleotides corresponds to the complementary strand of the region +1 to +24 of human *C4BPA*. This sequence delimits the downstream end of the *C4BPA*.PS promoter.

Cell culture and transfection procedures

HepG2, Hep3B, COS, C33 and HeLa cell lines used in these studies were cultured in Dulbecco's modified essential medium supplemented with 10% (v/v) fetal-calf serum. Transfections were carried out following the calcium phosphate co-precipitation method [23]. Briefly, 5×10^5 cells plated in 6-cm-diam. dishes were transfected with a total of 10–20 μg of plasmid DNA in a final volume of 500 μl. The DNA precipitate was maintained on the cells for 48 h. The cells were washed with PBS and collected by scraping with a 'rubber policeman'. Cell lysates were prepared in 15 mM Tris/HCl, pH 8.0, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM dithiothreitol (DTT), 0.4 mM PMSF [24]. For each cell type, the transfection efficiency variations among different experiments were less than 35%. In order to correct for these transfection efficiency differences, 2 μg of an RSV-βGal plasmid were included in all transfections. The β-galactosidase (βGal) assays were performed according to the method of Gorman [25]. For chloramphenicol acetyltransferase (CAT) assays [26], equivalent volumes of each extract, corrected for the differences in βGal activity, were used in 50 μl final volume assays. 0.2 μCi of [^{14}C]chloramphenicol were used per assay and 3 μl of 10 mM acetyl-CoA was added to the mixture every 45 min over a total of 3 h. Chloramphenicol and the different forms of acetylated chloramphenicol were resolved by TLC. Upon autoradiography, the fraction of acetylated chloramphenicol was determined by scintillation counting of the spots of the different forms of chloramphenicol, as described in [27].

Nuclear extract preparation

Nuclear extracts were prepared from different mouse organs according to [28]. Briefly, livers, lungs, testes, kidneys and spleens from three mice were excised and washed with cold PBS. They were homogenized in approximately 9 vol. of homogenization buffer (10 mM Hepes, pH 7.9, 15 mM KCl, 2 mM EDTA, 2.2 M sucrose, 0.5 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM PMSF, 2 mM benzamidine, 2.5 μg/ml of aprotinin, 2.5 μg/ml pepstatin A, 2.5 μg/ml leupeptin, and 1% non-fat milk) with a motor-driven Teflon–glass homogenizer at 0 °C. The homogenate was filtered through a cheese-cloth and nuclei were isolated by centrifugation through a cushion of the same homogenization buffer at 28000 rev./min in an SW41 rotor for 30 min at 4 °C. The pellet of nuclei was resuspended, at a concentration of 10^8 nuclei/ml, in 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 0.14 M NaCl, 25% glycerol, 0.5 mM DTT plus protease inhibitors mix (0.5 mM PMSF, 2 mM benzamidine, 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin A, 2.5 μg/ml leupeptin). Nuclei were extracted by slowly adding 1 vol. of the same buffer but with 0.7 M NaCl (0.34 M NaCl final concentration). The nuclear extracts were centrifuged at 20000 rev./min in an SW41 rotor for 30 min at 0 °C to remove chromatin and nuclear debris. Proteins were precipitated from the supernatant with $(NH_4)_2SO_4$ (0.3 g/ml final concn.) and collected by centrifugation at 25000 rev./min in an SW41 rotor for 30 min at 0 °C. The pellet was resuspended in dialysis buffer (20 mM Hepes, pH 7.9, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 20% glycerol, 1 mM DTT plus protease inhibitors) and dialysed against the same buffer for 2 h. Protein concentration of the extracts was measured by the Bio-Rad colorimetric assay.

DNase I footprinting analysis

DNA fragments containing the *C4BPA*.PS promoter were single-end labelled by filling 5′-protruding ends with [α-^{32}P]dCTP (3000 Ci/mmol), using the Klenow enzyme. Approximately 10^5 c.p.m. of end-labelled DNA fragment was incubated with 15–50 μg of protein from nuclear extracts. After 10–20 min incubation on ice, $CaCl_2$ and $MgCl_2$ were added to give final concentrations of 0.5 mM and 1 mM respectively. DNase I digestions were performed at 20 °C for 1 min with an amount of enzyme empirically calculated to leave approximately 50% of the probe undigested. Upon phenol extraction and ethanol precipitation, DNA fragments were resolved by electrophoresis in a denaturing 8% acrylamide sequencing gel.

Gel-retardation assays

Double-stranded oligonucleotides were labelled by filling the 5′-protruding ends with [α-^{32}P]dCTP (3000 Ci/mmol), using the Klenow enzyme. Gel-retardation assays were performed at 0 °C in 14 μl of gel-retardation buffer (10 mM Hepes, pH 7.9, 30 mM KCl, 5 mM $MgCl_2$, 125 μM EDTA, 62.5 μM EGTA, 0.5 mM DTT, 5 mM spermidine, 10% glycerol) with 11 fmol of labelled oligonucleotide and 0.3 to 3 μg of protein from nuclear extracts. In each assay, 1 μg of sonicated salmon sperm DNA and 1 μg of poly(dI-dC).poly(dI-dC) were included as non-specific competitors. The DNA–protein complexes were separated by electrophoresis on 6% polyacrylamide gels in 0.25 × TBE (1 × TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was performed in 0.25 × TBE at 20 V/cm for 3 h. After fixing, the gels were vacuum dried and autoradiographed.

Plasmid constructions

The constructs, including the promoter of *C4BPA* in front of the CAT reporter gene, were done in the *Hin*dIII site of the pUMS.CAT plasmid [27]. All constructs were tested by sequencing. A fragment of DNA including the *C4BPA*.PS promoter, extending for −369 bp to +24 bp of the *C4BPA* gene, was generated by PCR with specific primers (C4BPA369 and C4BPAF20) using as template the DNA of a genomic λEMBL3 clone (G562) already described [15]. The deletion mutants PSΔ1, PSΔ2, PSΔ3 and PSΔ4 were generated from the *C4BPA*.PS construct by PCR with primers C4BPF20 and PE1, PE2, PE3, and PE4 respectively. The mutant PSΔ0 was obtained from the *C4BPA*.PS construct by removing the sequences between −369 bp and −33 bp by digestion with *Sac*I and *Apa*I.

The mutant PSm1 was obtained by ligation of two fragments generated by PCR from the *C4BPA*.PS construct. One fragment was generated with primers C4BP369 and PE1M1.C. Primers PE1M1 and C4BPF20 were used for obtaining the other fragment. These two fragments were digested with *Xba*I and ligated. The resulting fragment was digested with *Hin*dIII and cloned in the *Hin*dIII-digested pUMS.CAT plasmid vector.

RESULTS

The promoter of *C4BPA* is hepatic-specific

Since promoters are essential elements for the hepatic-specific expression of many liver genes, we wanted to know whether the promoter of *C4BPA* is necessary for its liver-restricted pattern of expression. We cloned different portions of the 5′ region of *C4BPA*, upstream of the CAT reporter gene [26]. The capacity of these constructs to drive transcription was tested in transient transfection experiments in HepG2 and Hep3B hepatoma cells. For each experiment, the CAT values were corrected for transfection efficiency using the βGal activity values obtained by co-transfecting equal amounts of an RSV-βGal plasmid. We found that a region spanning from −369 to +20 bp of the *C4BPA* gene showed strong promoter activity in HepG2 (Figure 1) and Hep3B (Figure 2) cells. In these cell lines, the strength of the −369/+20 bp *C4BPA* promoter, named PS promoter, was approximately 37% that of the rat albumin promoter.

To test the activity of the PS promoter in a non-hepatic environment, we transfected the PS.CAT construct in the non-hepatic cell lines HeLa and C33. In these experiments, we also transfected, in parallel, constructs containing the CAT gene under the control of the promoters of Herpes simplex virus (HSV) thymidine kinase (tk) [29] and the rat albumin genes [30,31]. These constructs served as reference for the activity of an ubiquitous promoter, the tk promoter, and for the residual levels obtained from a proven hepatic-specific promoter (such as that of albumin) in non-hepatic cell lines. In addition, this allowed us to correct the promoter activity values in each cell line for differences in transfection efficiency and transcription capacity between cell lines. The results of these experiments are represented in Figure 2, which summarizes the combined data of at least five independent experiments performed with not less than two plasmid preparations. The PS.CAT construct showed the lowest activity values when transfected in HeLa and C33 cell lines. This promoter was more than 100-fold less efficient in promoting transcription in these cell lines than in HepG2 or Hep3B cells. This is comparable with the low relative transcriptional activity observed with the rat albumin promoter in these two non-hepatic cell lines.

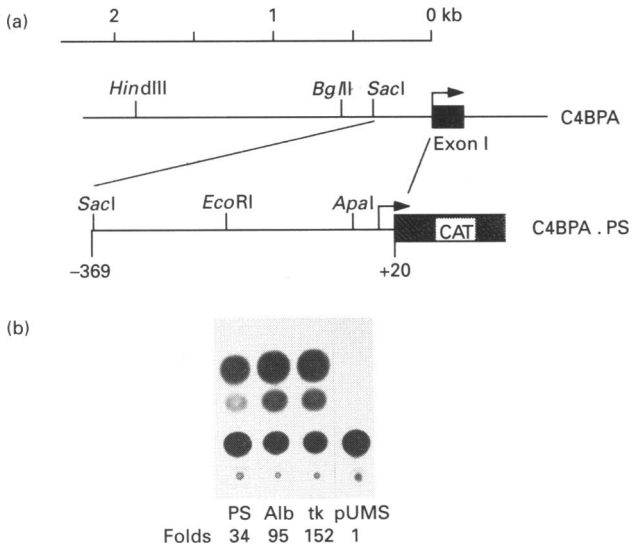

Figure 1 The promoter of *C4BPA* is contained within the first 369 bp upstream of the cap site

(**a**) Restriction map of the 5′ upstream region of the *C4BPA* gene. The PS promoter, spanning 389 bp of DNA (from +20 to −369), was cloned in front of the CAT reporter gene of the pUMS.CAT vector [27]. (**b**) Autoradiograph of CAT assays with extracts of HepG2 cells transfected with CAT reporter constructs of the PS promoter (PS), and the promoters of the HSV–tk (tk) and rat albumin (Alb) genes, included for comparisons. The two upper spots correspond to 3-acetylchloramphenicol and 1-acetylchloramphenicol respectively. The lower spot corresponds to non-acetylated chloramphenicol. The transcription activity of the PS, HSV–tk and rat albumin promoters are referred relative to the basal transcription activity of the pUMS.CAT vector alone (pUMS), arbitrarily assigned as 1. The CAT assays were corrected for transfection efficiency by including in each transfection an equal amount of an RSV-βGal expression plasmid.

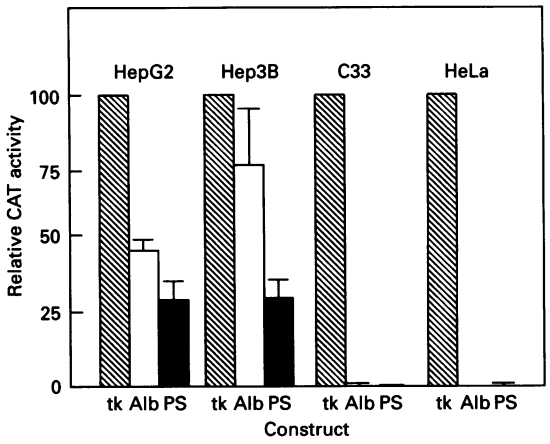

Figure 2 Relative activity of the *C4BPA*.PS promoter in several cell lines

PS, rat albumin and tk promoters were individually transfected in hepatoma cells (HepG2 and Hep3B) and in non-hepatic cell lines (C33, HeLa). The activity of each promoter is represented relative to that of the ubiquitous tk promoter, which was arbitrarily assigned as 100%. CAT activities were corrected as described in Figure 1. Bars represent the average and the S.D. of at least five independent experiments. In the conditions of the assay (see the Materials and methods section) the averaged CAT activity values, as percentages of acetylated chloramphenicol, for the tk promoter were 21.3, 7.8, 43.7, and 67.5 in HepG2, Hep3B, C33 and HeLa cells respectively.

The C4BPA.PS promoter contains at least four regions of interaction with hepatic nuclear factors

To approach the characterization of which transcription factors are responsible for the hepatic activity of the PS promoter we have performed DNaseI footprinting experiments with this region and nuclear extracts prepared from different mouse organs. As shown in Figure 3, incubation of the PS promoter with liver nuclear extracts showed at least four regions protected against DNase I digestion. These regions were named promoter element (PE)-1 (−38 to −56), PE-2 (−67 to −83), PE-3 (−119 to −156) and PE-4 (−169 to −204) (Figure 4). Regions PE-1 and PE-4 were also observed with nuclear extracts derived from kidney, but not with nuclear extracts derived from lung, testes or spleen. Regions PE-2 and PE-3 were observed exclusively with liver nuclear extracts, suggesting that the nuclear factors giving rise to these protected regions are particularly enriched in the liver.

Regions PE-2 and PE-4 showed a strong induced DNase I hypersensitive band in the coding strand (indicated with a dot in Figures 3 and 4). When the footprinting experiments were performed with the non-coding strand, the PE-3 region also showed induced DNase I hypersensitive bands. This induced hypersensitivity to DNase I suggests that the factors binding to

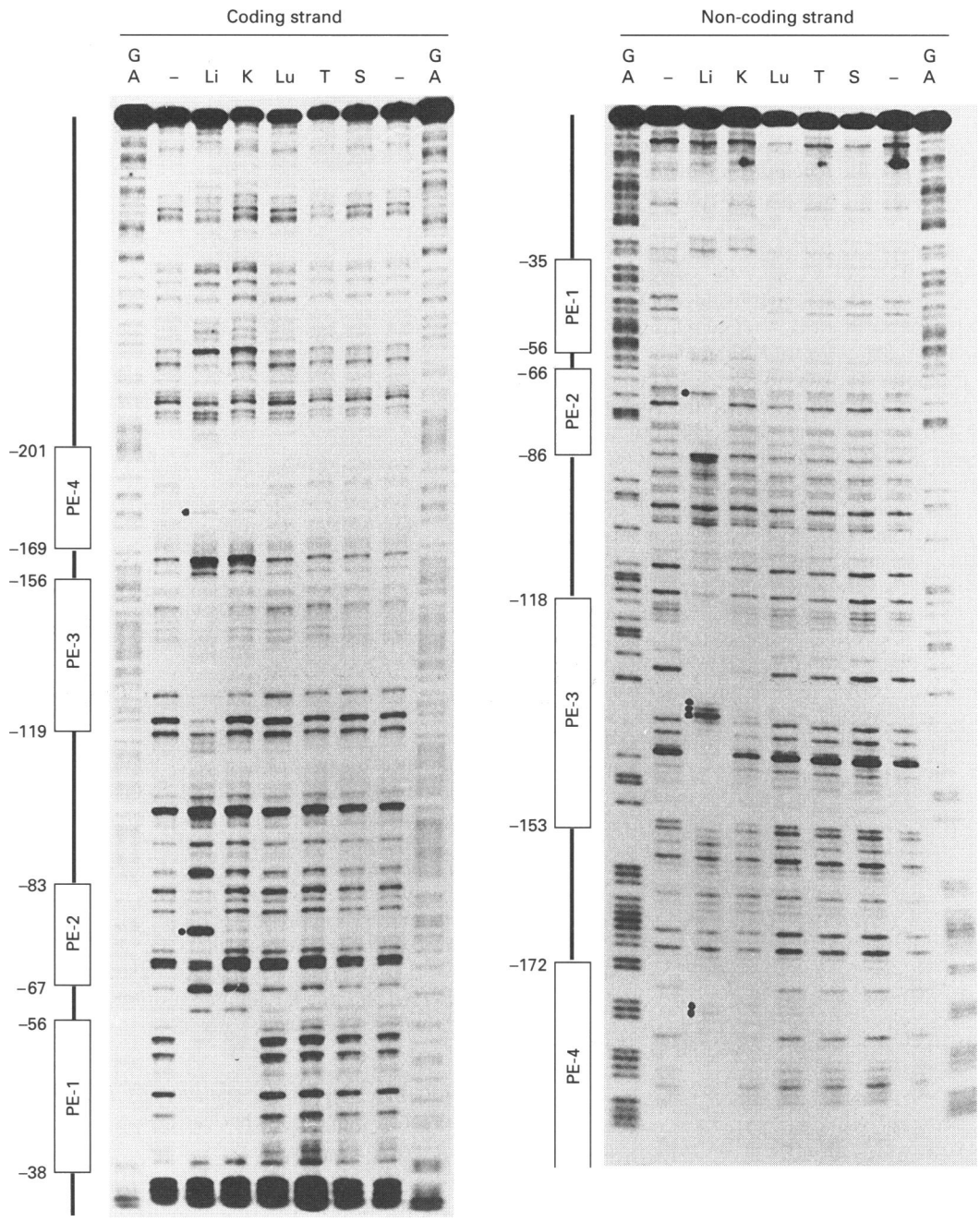

Figure 3 DNase I footprinting analysis of the *C4BPA*.PS promoter

A DNA fragment containing the PS promoter was single end-labelled with [α-^{32}P]dCTP by filling with Klenow enzyme. Approximately 10^5 c.p.m. of end-labelled DNA were incubated with 15–50 μg of nuclear extracts prepared from mouse liver (Li), kidney (K), lungs (Lu), testes (T) and spleen (S). DNase I digestions were performed as described in the Materials and methods section. The Figure shows the digestion pattern produced with the coding and non-coding strands. Dots indicate nuclear extracts-induced DNase I hypersensitive bands. GA: ladder of guanines and adenines obtained by Maxam–Gilbert chemical sequencing reactions. -: no nuclear extract added.

these regions of the *C4BPA*.PS promoter induce conformational changes on the DNA molecule, which make these regions more accessible to the DNase I action at determined nucleotide residues. The relevance of these conformational changes to the transcription-promoting activity of this region is currently unknown.

To determine the relative contribution of each PE region to the promoting activity of the PS promoter we prepared 5′-deletion mutants of the promoter, extending up to each PE region. These constructs were transfected in HepG2 cells and their transcription capacity was assessed by quantification of the expressed CAT activity. Figure 5 shows the results of these experiments. Our data indicate that each PE region has a positive effect on the global transcription activity of the PS promoter. Thus, serial deletion of each element produced a drop in the transcription-promoting capacity of each construct. The minimal construct showing promoting activity in hepatoma cells was the PSΔ1, which contains just the PE-1 element. This construct showed an

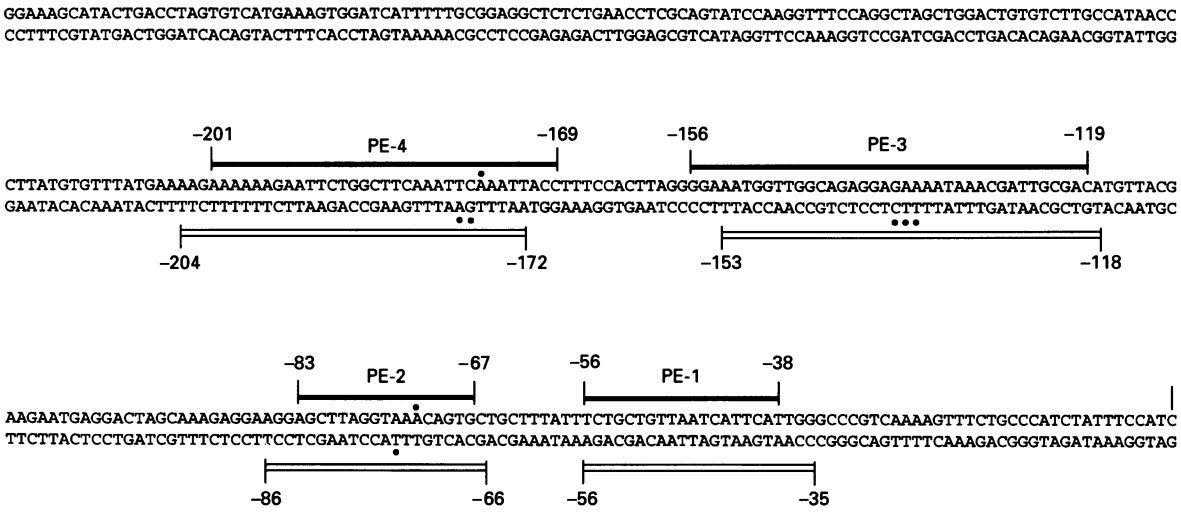

Figure 4 PE regions of the human *C4BPA*.PS promoter

Sequences protected against DNase I digestion by liver nuclear extracts are indicated by bars above (coding strand) and below (non-coding strand) the sequence of the promoter. Dots indicate nuclear extract-induced DNase I hypersensitive sites.

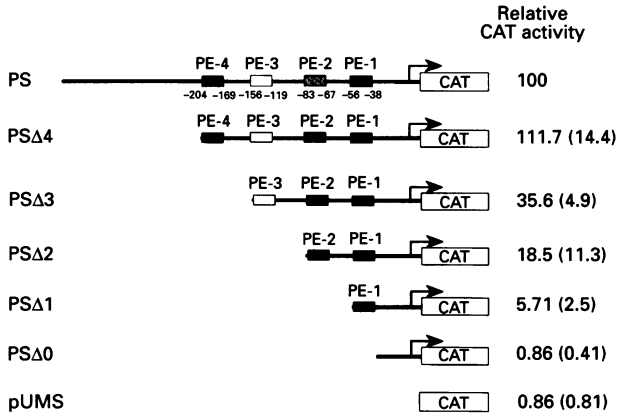

Figure 5 Transcriptional activity of the serial deletion mutants of the *C4BPA*.PS promoter

Constructs of the PS promoter extending respectively up to each PE region were tested for their transcription-promoting activity by transfection in HepG2 hepatoma cells. Figure shows the relative CAT activity of each construct with respect to the activity obtained with the whole PS promoter, considered as 100%. pUMS indicates the relative CAT activity obtained with the pUMS.CAT vector alone. Numbers in parentheses indicate the S.D. obtained in at least five independent experiments.

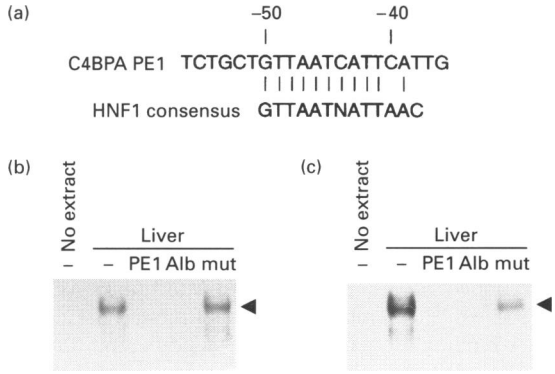

Figure 6 Gel-shift analysis of the PE-1 element of the *C4BPA*.PS promoter

(**a**) Homology between the PE-1 element of *C4BPA* and the consensus site for HNF1. (**b**) Gel-retardation assay performed with mouse liver nuclear extracts and a ^{32}P-labelled double-stranded oligonucleotide containing the PE-1 sequence. Unlabelled oligonucleotides used as competitors are indicated above each lane. They were added in a 50-fold molar excess. Abbreviations: -, no competitor added; PE1, PE-1 oligonucleotide; Alb, oligonucleotide containing the HNF1 site of the rat albumin promoter [22]; mut, PE1M1 oligonucleotide, a mutated version of PE-1 (see the Materials and methods section). (**c**) Gel-retardation assay performed as in (**b**) but with the Alb oligonucleotide as probe. Arrowheads indicate the retarded complexes.

activity of approximately 6% that of the PS full promoter. Further deletions, downstream of the PE-1 element, completely abolished the transcription-promoting activity of this region.

The hepatic activity of the human *C4BPA* promoter requires HNF1

Several transcription factors are particularly enriched in liver, and many of them have been implicated in the liver-specific transcription [32]. Among others, HNF1 was shown to be a key transcription factor essential for the hepatic specificity of the promoters of albumin and many other hepatic genes (reviewed in [32,33]). Target sequences for HNF1 are also recognized by the evolutionary-related factor vHNF1. These two factors are highly homologous in regions of the molecule important for DNA binding. Both belong to the POU-Homeodomain family of transcription factors. HNF1 and vHNF1 are apparently functionally similar, since both interact with high affinity with the same target sequences and show comparable transcription-activating capacities. However, the relative levels of expression of HNF1 and vHNF1 differ among different tissues, suggesting different regulatory roles for both factors. Examining the sequence of the *C4BPA*.PS promoter we found that the PE-1 region showed strong similarity with the HNF1 consensus site (Figure 6a). We carried out gel-retardation experiments to see

whether this region of the *C4BPA*.PS promoter was actually recognized by HNF1. As shown in Figure 6(b), a retarded band was obtained when a ^{32}P-labelled double-stranded oligonucleotide, containing the PE-1 sequence, was incubated with nuclear extracts from mouse liver. The formation of this complex was specific, as shown by competition with a 50-fold molar excess of the same unlabelled oligonucleotide. The formation of the complex was also efficiently competed with by a 50-fold molar excess of an unlabelled double-stranded oligonucleotide containing the HNF1 site of the rat albumin promoter, but not by an oligonucleotide containing a mutated HNF1 site [22]. The reciprocal experiment gave a similar result. Thus, the binding of HNF1 from nuclear extracts of mouse liver to the labelled oligonucleotide containing the HNF1 site of the rat albumin promoter, was also efficiently competed with by a 50-fold molar excess of unlabelled PE-1 oligonucleotide. Furthermore, when residues of the HNF1 consensus sequence, shown to be important for HNF1 binding, were mutated in the PE-1 sequence, the mutant oligonucleotide, PE1M1, was unable to compete with the formation of the retarded band obtained with labelled PE-1 (Figure 6c). As expected, no retarded band was observed when the PE1M1 oligonucleotide was used as a probe (not shown). These results strongly suggest that HNF1 binds to the PE-1 sequence of the *C4BPA* promoter.

To confirm that the factor interacting with the PE-1 element of the *C4BPA* promoter is, in fact, HNF1, we carried out gel-retardation experiments in the presence of antibodies specific for HNF1. When an anti-peptide serum, raised against the C-terminal part of rat HNF1 [34], was included in the gel-retardation assays with labelled PE-1 and mouse liver nuclear extracts (Figure 7), it fully displaced the retarded band to a slower migrating position. The pre-immune serum did not show any effect on the retarded band. In a similar experiment performed with a serum raised against rat vHNF1, which cross-reacts with HNF1, no retarded band was observed. Since this serum was raised against the whole rat vHNF1 molecule, this inhibitory effect of the anti-vHNF1 antibodies is probably because some of the immunoglobulins of the serum interact with the DNA-binding domain of these factors and thus inhibit binding [34]. Whereas liver expresses low levels of vHNF1, addition to the gel-retardation assays of a serum specific for the C-terminal region of rat vHNF1 [34], which does not cross-react with HNF1, did not result in any alteration of the retarded band, as did not the pre-immune sera either. When kidney or COS cell nuclear extracts, which express high levels of vHNF1 [34–36], were used in these experiments binding of vHNF1 to the PE-1 element was readily observed (results not shown). These results indicate that, indeed, the PE-1 element of the *C4BPA*.PS promoter is able to interact with the transcription factors HNF1 and vHNF1.

We assessed next the functional relevance of the binding of HNF1 to the PE-1 element of the *C4BPA*.PS promoter. We prepared a *C4BPA*.PS promoter in which the PE-1 element was substituted by the mutated PE1M1, which rendered it unable to bind HNF1. This mutant promoter was named PSm1. We also used, in these experiments, the PSΔ1 construct, the short version of the *C4BPA*.PS promoter which extends from +20 to −57, and includes the HNF1 site. The activity of these constructs was tested in transient transfection experiments of HepG2 cells. Figure 8 shows the result of these experiments. The relative activity values correspond to the averaged results of at least three independent experiments performed with not less than two preparations of plasmids. The PSΔ1 promoter was able to drive transcription of the reporter gene in HepG2 cells, but failed to drive transcription in non-hepatic cells like HeLa or C33 (results not shown). This suggests that the HNF1 site is probably sufficient to account for the hepatic specificity of the *C4BPA*.PS promoter. Interestingly, the PSm1 promoter, in which the HNF1 site has been mutated, did not show activity in any of the cell

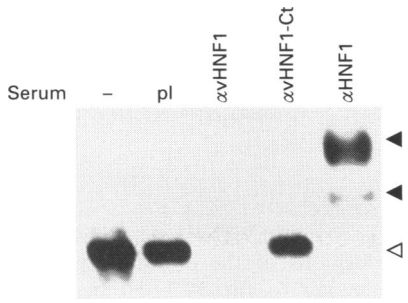

Figure 7 HNF1 interacts with the PE-1 element of the *C4BPA*.PS promoter

Gel-retardation assay performed with mouse liver nuclear extracts and the PE-1 oligonucleotide as probe, in the presence of antibodies against rat HNF1 (αHNF1), the C-terminal region of rat vHNF1 (αvHNF1-Ct), or the whole rat vHNF1 molecule (αvHNF1). Abbreviation: pI, preimmune serum. A 2 μl sample of rabbit antiserum was used in each case. - indicates that no serum was added. The open arrowhead indicates the position of the HNF1–PE-1 complex. The two closed arrowheads indicate the supershift of the HNF1–PE-1 complex obtained with the αHNF1 antiserum.

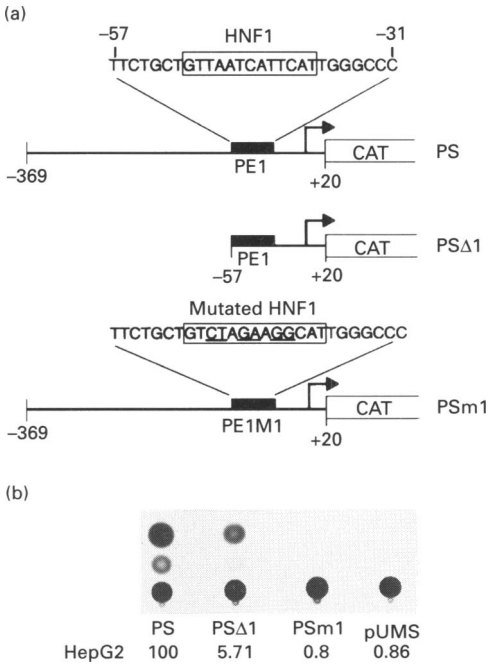

Figure 8 Effect of the HNF1 site on the transcriptional capacity of the *C4BPA*.PS promoter

(**a**) Mutant versions of the *C4BPA*.PS promoter were constructed by deleting the region upstream of the HNF1 site (mutant PSΔ1) or mutagenizing specifically the HNF1 site (mutant PSm1). The transcription activity of these constructs was tested in transfection experiments in HepG2 cells. (**b**) CAT assays performed with extracts of HepG2 hepatoma cells transfected with PSΔ1 and PSm1 constructs. PS construct and the vector pUMS.CAT alone (pUMS) were included for comparisons. Activity values are indicated below each lane, relative to the activity of the PS construct.

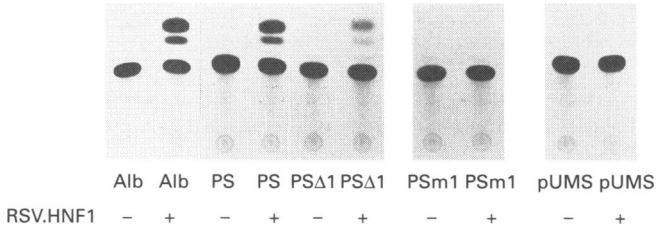

Figure 9 Activation of the *C4BPA*.PS promoter by HNF1

CAT assays were performed with extracts from C33 cells transfected with the PS, PSΔ1 and PSm1 constructs, together with an HNF1-expression vector (RSV-HNF1). 4 μg of expression vector was used in each case. When no HNF1-expression vector was used (indicated by -), 4 μg of an RSV-luciferase plasmid were added, for compensating for any effect of the RSV promoter on the transcriptional activity of the *C4BPA*.PS promoter. The rat albumin promoter (Alb) was included as a control.

lines tested. This reinforces the importance of the HNF1 site for the activity of the *C4BPA*.PS promoter, since its absence produced a dramatic effect on the capacity of this promoter to drive transcription. Moreover, this result poses HNF1 as absolutely required for the transcription of *C4BPA* from the PS promoter.

Another piece of evidence that suggests that HNF1 is necessary for driving transcription from the *C4BPA*.PS promoter, comes from co-transfection experiments of C33 cells with the PS promoter and an HNF1 expression vector. C33 cells do not express HNF1 and, consequently, the PS promoter is not active when transfected in this cell line. However, when the HNF1 expression vector RSV-HNF1 was co-transfected with the PS promoter, expression of the CAT reporter gene was observed (Figure 9). These results indicate that HNF1 is probably the only hepatic transcription factor necessary for the transcriptional activity of the PS promoter. Overexpression of HNF1 in cells which normally express it, such as HepG2 or Hep3B, further increased the activity of the PS promoter for driving transcription. Similar results were obtained with an expression vector of vHNF1 (results not shown).

Taking these data together strongly suggests that the paired HNF1-PE-1 element plays a crucial role on the hepatic-specific transcription of the *C4BPA* gene.

DISCUSSION

C4BP is an oligomeric plasma protein which plays an important role in the control of the complement activation. As with many other serum proteins, C4BP is mainly expressed in the liver. In this report we addressed the characterization of the mechanisms controlling the expression of the *C4BPA* gene, which encodes the α-chain of C4BP. We have found that the promoter, located within the first 369 bp upstream of the transcription start site, is an important element for the hepatic transcription of the *C4BPA* gene. Furthermore, the activity of the *C4BPA* promoter requires the interaction of HNF1 with a region of the promoter close to the transcription start site.

Transient transfection experiments with the first 369 bp upstream of the transcription start site of *C4BPA* showed that this region was sufficient to confer hepatic-specific expression to a CAT reporter gene. The activity of the *C4BPA* promoter was approximately 37% that of the rat albumin promoter in HepG2 and Hep3B hepatoma cells, but was virtually zero in cells with a non-hepatic origin, like HeLa and C33. In the monkey-kidney-derived COS cells, transfection of the PS promoter showed a low, although non-negligible, activity (results not shown). Interestingly, the albumin promoter also promoted transcription when transfected in this cell line, although at lower levels than in hepatoma cells. This residual activity of hepatic-specific promoters in COS cells might be explained considering the kidney origin of this cell line. It has been previously reported that kidney expresses the hepatic-enriched transcription factors HNF1 and vHNF1 [34–36]. We have found that COS cells express the latter factor at relatively high levels (J. Rey-Campos, unpublished work) that could account for the activity of the albumin and *C4BPA* promoters observed in this cell line. However, despite the presence of vHNF1, the *C4BPA* gene is not expressed in COS cells. Several possibilities could explain this discrepancy between the transfection experiments and the *in vivo* expression data. It may be possible that, in this cell line, the *C4BPA* gene bears a tight chromatin structure that hampers transcription from its promoter. On the other hand, other regions aside from the promoter may be relevant to the control of the expression of the *C4BPA* gene *in vivo*. This is the case, for example, for the albumin gene, where an enhancer region located between −8 and −10.5 kb is important for obtaining high levels of hepatic-specific expression in transgenic mice [37].

Our experiments showed that the activity of the *C4BPA* promoter is absolutely dependent on the interaction with the hepatic transcription factor HNF1. HNF1 has been shown to be an important factor for the expression of many hepatic genes [32]. Sites for this factor have been found both in promoters and enhancers. In the case of the *C4BPA* promoter, the HNF1 site appears to be essential for transcription. In fact, although the deletion mutant PSΔ1, which extends from the cap site to the HNF1 site, showed approximately 6% of the activity of the full *C4BPA* promoter, the mutant PSm1, equivalent to the whole promoter but with the HNF1 site mutated, was virtually inactive. Thus, although the rest of the factors interacting with the *C4BPA* promoter in conjunction with HNF1 raise the activity of the promoter 20-fold, they alone are unable to confer transcription-promoting activity to the mutant promoter PSm1. Hence, HNF1 seems to act as a key factor which allows all the other elements of the *C4BPA* promoter to function. This situation is rather different to the case of the rat albumin promoter and other HNF1-dependent hepatic promoters, where inactivation of the HNF1 site produces a drastic effect on the activity of these promoters, but still some transcription is observed [31].

The sensitivity of the *C4BPA* promoter to mutations on the HNF1 site may relate to the mode of action of HNF1 in activating transcription from the *C4BPA* promoter. The localization of the HNF1 sites in many hepatic promoters appears close to the TATA box. Indeed, when the HNF1 site has been located farther from a TATA box, the capacity of HNF1 to activate transcription was lowered [31], suggesting that HNF1 might directly cooperate with TFIID to efficiently promote the assembly of the initiation complex at the cap site. Interestingly, the *C4BPA* promoter lacks an obvious TATA box. This suggests one possible explanation for the deleterious effects of mutations at the HNF1 site of the *C4BPA* promoter. In the absence of a TATA box, direct binding of TFIID to the promoter is impaired. However, TFIID is an essential factor for the formation of the pre-initiation complex for all the eukaryotic promoters, regardless of whether they have a TATA box or not (reviewed in [38]). It has been proposed that in TATA-less promoters, TFIID could attach to the promoter through interaction with specific transcription factors bound to the promoter, as has been shown for the Sp1 transcription factor [39]. We would like to suggest that HNF1 might play a similar role in the *C4BPA* promoter. By binding at a location close to the transcription start site, HNF1

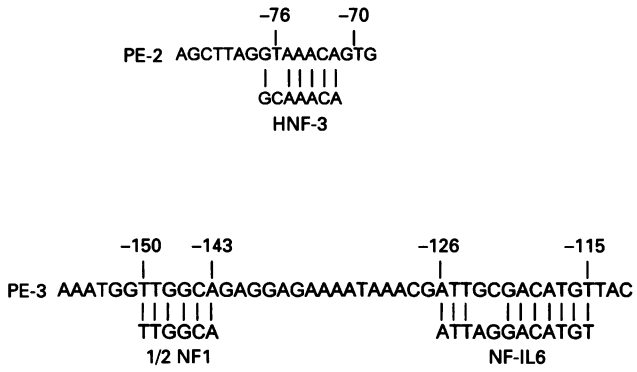

Figure 10 Homology of sequences within the PE-2 and PE-3 regions of the *C4BPA*.PS promoter with target sequences for transcription factors HNF3 [41–43], NF-IL6 [44,45], and NF1 [46]

would allow the assembling of the transcriptional complex at the cap site by tethering the TFIID through interaction with particular TATA-box-binding-protein-associated factors (TAFs). A mutated HNF1 site, unable to bind HNF1, would render the promoter incapable of interacting with TFIID and therefore inactive for promoting transcription.

An alternative explanation for the strict requirement of HNF1 for the activity of the PS promoter might be related to the particular organization of the regulatory modules that constitute this promoter. It has been proposed that transcription factors bound to regulatory regions of the genes activate transcription through interaction with adaptor molecules which ultimately promote the assembly of the initiation complex at the cap site. These adaptor molecules, in addition to contacting transcription factors could also interact one with each other, establishing a series of adaptor–adaptor interactions [40]. This series of interactions would be specific for the particular arrangement of transcription factors bound to the regulatory region. Since HNF1 binds very close to the transcription start site of the PS promoter, if binding of HNF1 is blocked then this series of adaptor–adaptor interactions would be interrupted at an early step and hence would preclude the action of the other transcription factors. If this were the case, then the transcription activity of the PS promoter would be the result of the particular organization of its regulatory modules and not only of the independent, and somehow autonomous, activity of the regulatory modules themselves.

Since the activity of the PSΔ1 promoter in transfected HepG2 cells reached just 6% that of the full promoter, factors interacting with the other regions of the *C4BPA* promoter must be important for obtaining high levels of expression. In fact, we have found that most of the regions of the PS promoter, identified by *in vitro* footprinting, had a positive effect on the activity of this promoter. Currently, we do not know the identity of the factor, or factors, interacting with the PE-2, PE-3 and PE-4 regions. However, sequence comparisons shows that the PE-2 region shares strong homology with the target sequence for the family of hepatic transcription factors HNF3 [41–43] (Figure 10). The PE-3 region contains a putative site for NF–IL-6 [44,45] and, similarly to the DEII site of the rat albumin promoter [46], a half-site for the family of transcription factors NF1 (Figure 10). PE-4 does not show clear homology with the target sequence of any of the transcription factors investigated.

Interestingly, all the regions identified by DNase I footprinting analysis in the promoter of the human *C4BPA* gene, have been conserved in other species, like rat and mouse [47,48] (Figure 11). This supports their potential relevance for the control of the expression of this gene in all these species. Furthermore, the sequences in between the PE regions have also been maintained, suggesting that they may also be relevant for the functioning of this promoter, perhaps binding factors not detected by *in vitro* footprinting or only active under certain physiological conditions. In fact, *C4BPA* is an acute-phase reactant [49]. The plasma concentration of C4BP, which normally ranges between 150 and 200 μg/ml, may increase up to 4-fold during the acute phase. It is tempting to speculate that these other conserved regions could be significant for the mechanisms modulating the plasma levels of C4BP.

The sequence identity between the human, rat and mouse *C4BPA* promoters drops drastically upstream of the position −320 (with respect to the human *C4BPA* promoter). This suggests that the *C4BPA* promoter is, in fact, fully contained in

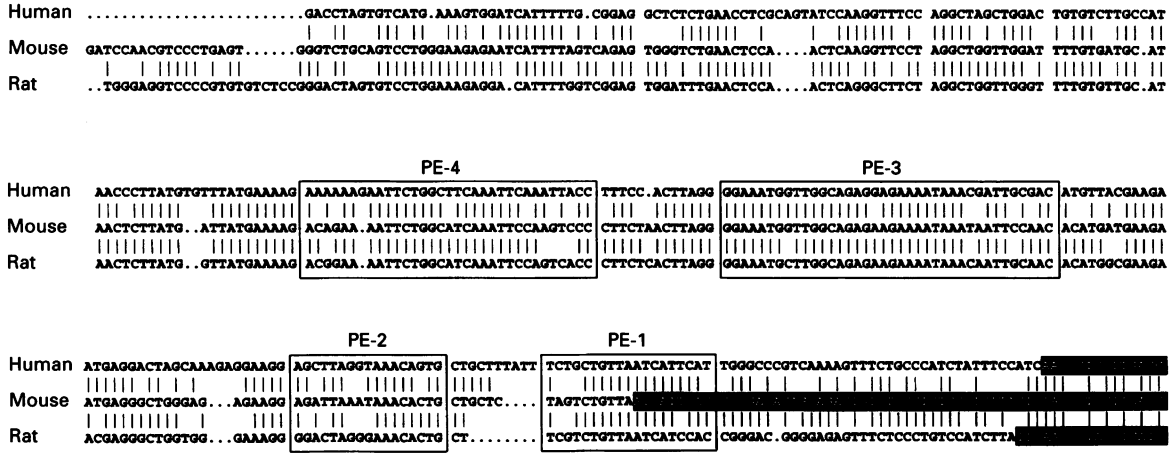

Figure 11 Comparisons of the promoter sequences of human, mouse and rat *C4BPA* genes

PE regions identified by footprinting analysis in the human *C4BPA*.PS promoter are indicated with boxes. Transcribed regions are shown in black. Gaps introduced to optimize the alignment are indicated with dashes.

the first 350 bp upstream of the cap site. Interestingly, while transcription initiates practically at equivalent positions in the rat and human *C4BPA* genes, transcription of the mouse gene starts 46 bp upstream with respect to the human gene. The transcription start site of the mouse gene lies within the PE-1 element, i.e. the HNF1 site. We do not know whether the mouse *C4BPA* promoter utilizes this site for binding to HNF1. However, if this were the case then it would impose an interesting compromise between binding of HNF1 and transcription initiation. Alternatively, mouse *C4BPA* promoter might not necessitate binding of HNF1 to this site for promoting transcription. In contrast with the case of the human *C4BPA* promoter, the mouse *C4BPA* promoter displays the sequence TTAAATAA at −30 bp, which could be used as a TATA box for correct initiation of transcription. If our hypothesis about the requirement of HNF1 binding for tethering TFIID at the correct position, and consequently all the general transcription machinery, is correct, then the presence of a TATA box at the adequate position in the mouse *C4BPA* promoter would obviate the necessity of HNF1 to correctly start transcription from this promoter. In conclusion, we postulate that the role of HNF1 in the *C4BPA* promoter would be different than in other HNF1-dependent hepatic promoters. Rather than only activating transcription, binding of HNF1 to the *C4BPA* promoter would in fact enable transcription. Experiments to test this hypothesis are in progress.

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