# Structure of the Gene Coding for the α Polypeptide Chain of the Human Complement Component C4b-binding Protein

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## Summary

The human gene coding for the 70-kD polypeptide of the complement regulatory component C4b-binding protein (C4BPα) spans over 40 kb of DNA and is composed of twelve exons. Upon transcription in liver, or in Hep-G2 cells, this gene produces a single transcript of 2,262 nucleotides, excepting the poly A tail, that presents an unusually long 5′ untranslated region (5′ UTR) of 223 nucleotides. The C4BPα gene is organized as follows: the first exon codes for the first 198 nucleotides of the 5′ UTR. It is separated by a large intron from the second exon including the remaining of the 5′ UTR and the coding region for the signal peptide. Each of the eight 60–amino acid repeats (short consensus repeats [SCRs]) that compose the C4BPα polypeptide chain is encoded by a single exon, except for the second SCR, which is split in two exons. At the 3′ end of the C4BPα gene, the twelfth exon codes for the COOH-terminal 57 amino acids of the mature protein, which have no similarities to the SCRs, and the 245 nucleotides of the 3′ UTR. Examination of the nucleotide sequence of the first exon revealed an interesting characteristic, strongly suggesting that this exon may specify a functional domain of the C4BPα transcript. It includes two in-phase ATG codons, in a different frame respect to that coding the C4BPα polypeptide, followed by an in-frame termination codon, also within the first exon. Comparison between mouse and human C4BPα transcripts indicates conservation of this structure within the 5′ UTR. C4BP is expressed in the liver and is an acute phase protein. A computer search of the genomic sequences upstream the transcription start site demonstrates the presence of potential *cis*-acting regulatory elements similar to those found in the promoters of other liver-expressed and/or acute phase genes.

Complement is a major defense and clearance system in the bloodstream which can be activated by two different routes, the classical and the alternative pathways. Activation by each pathway leads to the formation of complex enzymes, the C3-convertases, which catalyze the crucial step of complement activation, i.e., the cleavage and activation of the complement component C3 (reviewed in reference 1).

C4b-binding protein (C4BP)[1] is a regulator of complement activation. It binds to C4b, accelerates the decay of the classical pathway C3-convertae (C4b,2a), and functions as a cofactor in the factor I–mediated proteolytic inactivation of C4b (2–6). In addition, C4BP binds to the anticoagulant vitamin K–dependent protein S and renders it inactive as a cofactor to activated protein C in the inactivation of coagulation factors Va and VIIIa (7–10).

C4BP is an abundant protein. It is present in plasma at a concentration of 150 mg/liter (11) and, as many other complement components, is probably synthesized primarily by the liver. Recently, data from different laboratories (11, 12) indicate that C4BP is an acute phase protein.

C4BP shows a characteristic molecular heterogeneity. The major molecular form of C4BP is composed of eight chains, seven identical 70-kD polypeptides (α chain) and one 45-kD polypeptide (β chain), that are covalently linked by their COOH-terminal regions to give the molecule a spider-like structure (2, 13–15). Both α and β chains are evolutionarily related. They probably originated as result of a gene duplication event and then diverged to perform different functions (16). The complement regulatory functions of C4BP involve the α chain, whereas the β chain appears to be the binding site for the anticoagulant vitamin K–dependent protein S (13, 17).

The complete amino acid sequence of the human C4BPα polypeptide chain has been deduced from nucleotide sequencing of cDNA clones (18, 19). It belongs to a family

[1] *Abbreviations used in this paper:* C4BP, C4b-binding protein; DAF, decay accelerating factor; FXIII B, coagulation factor XIIIB subunit; H, factor H; MCP, membrane cofactor protein; RCA, regulator of complement activation; SCR, short consensus repeat; uORF, upstream open reading frame; UTR, untranslated region.

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of proteins with a characteristic structural organization based on a repetitive unit of 60 amino acids denominated as short consensus repeat (SCR). Because most of the proteins containing SCRs bind to C3b and/or C4b, this family is referred to as the superfamily of the C3b/C4b-binding proteins. The typical SCR framework of conserved residues includes four cysteines, two prolines, one tryptophan, and several other partially conserved glycine and hydrophobic residues (20). Starting at the $NH_2$-terminal end, the C4BP$\alpha$ polypeptide chain is composed of eight SCRs (18). The sequence ends with a COOH-terminal nonrepeat region containing two cysteines that are thought to be involved in the interchain disulfide linkage.

The human C4BP$\alpha$ gene is very closely linked to that coding for the C4BP$\beta$ polypeptide (16) and probably this is also the case in rodents (21). In humans, the C4BP$\alpha$ and C4BP$\beta$ genes are arranged in tandem with the 3′ end of the C4BP$\beta$ gene located 3.5–5 kb from the 5′ end of the C4BP$\alpha$ gene. The C4BP$\alpha$ and C4BP$\beta$ genes have been linked to the genes coding for the proteins membrane cofactor protein (MCP), CR1, CR2, decay accelerating factor (DAF), factor H (H), and coagulation factor XIII B subunit (FXIII B) (22–26). All these proteins share the same structural organization based in the presence of multiple SCR units. In addition, most of them are complement-regulatory components. This group of genes, known as the regulator of complement activation (RCA) gene cluster (22), is located on the long arm of human chromosome 1 (1q32) (27, 28).

The present report describes the structural organization of the human C4BP$\alpha$ gene and analyzes its relationship to other genes of the RCA gene cluster. It also provides the structural basis for the subsequent analysis of the regulatory elements controlling the expression of C4BP$\alpha$. In this respect, our results demonstrate a peculiar organization of the 5′ untranslated region (5′ UTR) of the C4BP$\alpha$ mRNA which may have important consequences in the control of the expression of this polypeptide.

## Materials and Methods

*Probes and Libraries.* The C4BP$\alpha$-cDNA clone used in these studies has been described previously (16). It is a 2,190-bp-long cDNA clone isolated from a human liver cDNA library (HL 1001b) purchased from Clontech Laboratories, Inc. (Palo Alto, CA). It includes a portion of the 5′ end UTR, the entire coding regions for the signal peptide and the mature secreted protein, and the 3′ UTR with a 13 nucleotide poly A tail. It is shown in this report that this cDNA clone only lacks the first 86 nucleotides of the 5′ end region of the C4BP$\alpha$ transcript. This C4BP$\alpha$-cDNA clone is apparently identical to that reported recently by Matsuguchi et al. (29). This cDNA clone, or fragments of it, was used to screen two human genomic libraries constructed in the EMBL3 vector (HL 1006d and HL 1067j; Clontech Laboratories, Inc.). Additional probes were prepared from cloned restriction fragments of the human genomic DNA isolated from the EMBL3 clones.

*Primer Extension Analysis.* A 35mer oligonucleotide (GAAGAAAACTGCCTTGATCTATGTAGTCCTGGTTC) corresponding to nucleotides 19–54 of the noncoding strand of the C4BP$\alpha$-cDNA was end labeled with T4 polynucleotide kinase to a specific activity of $\sim 10^8$ cpm/$\mu$g. 10 ng of labeled primer was annealed to 50 $\mu$g of human liver total RNA in 50 mM PIPES buffer, pH 6.5, containing 400 mM NaCl, 1 mM EDTA, and 50% formamide overnight at 40°C. Poly(A)$^+$ RNA and annealed primer were then isolated using oligo-dT cellulose and precipitated in ethanol. Reverse transcription was carried out by adding 20 $\mu$l of 50 mM TRIS-HCl buffer, pH 8.5, containing 1 mM DTT, 10 mM MgCl, 4 mM KCl, 1 mM of each dNTP, 40 U of RNasin-ribonuclease inhibitor (Promega Corp., Madison, WI), and 10 U of AMV reverse transcriptase (Promega Corp.) for 1 h at 42°C. Samples were made 0.4 M NaOH and incubated overnight at room temperature to eliminate RNA. After neutralization with acetic acid, samples were precipitated in ethanol, redissolved in 6 $\mu$l of sequencing loading buffer, and analyzed in 6% acrylamide/urea sequencing gels.

*S1 Nuclease Protection Assays.* S1 analysis of mRNA using single-stranded DNA probes were performed as described (30) with slight modifications. Briefly, single-stranded DNA probes were prepared using T7 DNA polymerase to extend 5′-end radiolabeled oligonucleotides annealed to denatured linearized-plasmid DNA templates. Labeled probes were separated from the DNA template using 6% acrylamide/urea sequencing gels, extracted from the acrylamide in 100 $\mu$l of 0.5 M Ammonium acetate, 1 mM EDTA, 1% SDS, and further purified with sephadex G-50 spun columns equilibrated with $H_2O$.

$5 \times 10^3$ cpm of labeled single-stranded probe were hybridized with 50 $\mu$g of total RNA from human liver, or with 50 $\mu$g of tRNA, in 20 $\mu$l of 50 mM PIPES buffer, pH 6.5, containing 400 mM NaCl, 1 mM EDTA, and 80% formamide overnight at 30°C. After hybridization, 300 $\mu$l of 50 mM sodium acetate buffer, pH 4.5, containing 280 mM NaCl, 4.5 mM $ZnSO_4$, 20 $\mu$g/ml salmon sperm DNA, and 120 U of S1 nuclease (Boehringer Mannheim GmbH, Mannheim, West Germany) was added to each sample, and the tubes were incubated for 1 h at 30°C. Digestions were stopped by the addition of 80 $\mu$l of 4 M ammonium acetate/20 mM EDTA and nucleic acids precipitated in ethanol. Redissolved samples were treated overnight at room temperature with 0.4 M NaOH to remove RNA, neutralized with acetic acid, and precipitated again in ethanol. Finally, samples were redissolved in 6 $\mu$l of sequencing loading buffer and analyzed in 6% acrylamide/urea sequencing gels.

*DNA Sequence Analysis.* Restriction fragments of the genomic clones hybridizing with the C4BP$\alpha$-cDNA probes were subcloned into plasmid vectors for DNA sequencing by the dideoxy chain termination technique with either Sequenase (United States Biochemical Corp., Cleveland, OH) or T7 DNA polymerase (Pharmacia LKB Biotechnology, Uppsala, Sweden) using $^{35}$S-radiolabeled dATP. Primers for DNA sequencing were mostly oligonucleotides derived from the C4BP$\alpha$-cDNA sequence. Programs included in the DNASTAR software package (DNASTAR, Inc., Madison, WI) were used for the sequence analysis and to compare our sequences with the National Biomedical Research Foundation (NBRF) protein sequence database and the Genebank nucleic acid database.

## Results

*Genomic Organization of Human C4BP$\alpha$.* We have used a 2,190-bp-long human C4BP$\alpha$-cDNA clone (16) to screen two different EMBL-3 human genomic libraries. Several overlapping clones hybridizing to this probe were identified, spanning a region of $\sim$60 kb. Five of these clones, G562, G211, G912, G105-2, and G17, were selected and characterized by restriction endonuclease digestion and Southern blot hybridization with different C4BP$\alpha$-cDNA–derived probes. The

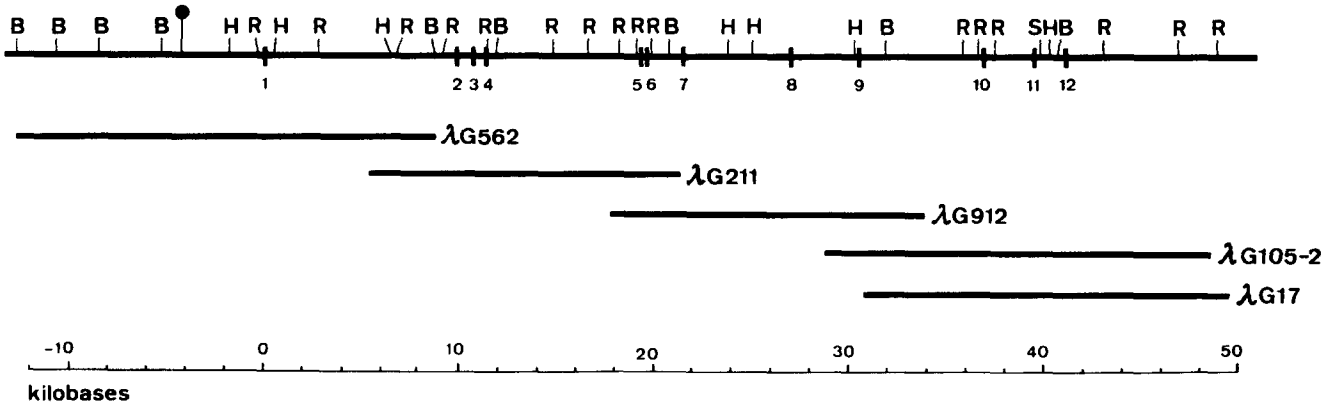



**Figure 1.** Map of the human C4BPα gene. The first line represents the complete EcoR I (*R*), BamH I (*B*), Hind III (*H*), and Sal I (*S*) restriction map of the C4BPα gene. It resulted from the alignment of the restriction maps of five selected overlapping genomic clones, G562, G211, G912, G105-2, and G17. The position of these clones is indicated below the map. The localization of the twelve exons that compose C4BPα is shown with vertical bars labeled from 1 to 12. The black solid circle (●) is to indicate the position of the 3′ end of the C4BPβ gene.

a

| EXON | (bp) | DOMAIN | INTRON | (~kb) | EXON/INTRON JUNCTIONS 3′INTRON | EXON | 5′INTRON |
|---|---|---|---|---|---|---|---|
| I | 198 | 5′ UT | 1 | 9 | | AACCGTCCT.....CTACCAAAG | gtcggt |
| II | 167 | 5′ UT/L | 2 | 1 | cagcag | AAAAACATC.....CTT G | gtgagt |
| III | 186 | SCR 1 | 3 | 0.9 | gtccag | GC AAT.....ATC T | gtaagt |
| IV | 100 | SCR 2A | 4 | 8 | tcccag | AC AAA.....GAA GG | gtgagt |
| V | 86 | SCR 2B | 5 | 0.167 | attcag | A TTT.....GAA A | gtaagt |
| VI | 192 | SCR 3 | 6 | 2 | cctcag | TT GTC.....GAA A | gtaagt |
| VII | 183 | SCR 4 | 7 | 5 | ctttag | AA ATC.....CCC A | gtaagt |
| VIII | 195 | SCR 5 | 8 | 4 | gagtag | AT AGT.....GAG G | gtgagt |
| IX | 189 | SCR 6 | 9 | 6 | tttcag | CG TTA.....GAC A | gtaaga |
| X | 171 | SCR 7 | 10 | 1.5 | ctcaag | TT TGC.....AAA G | gtaact |
| XI | 176 | SCR 8 | 11 | 1 | ttacag | CT CTG.....GAG TGG | gtaagt |
| XII | 425 | CT/3′UT | | | ttttag | GAG ACC.....TAT | |

b

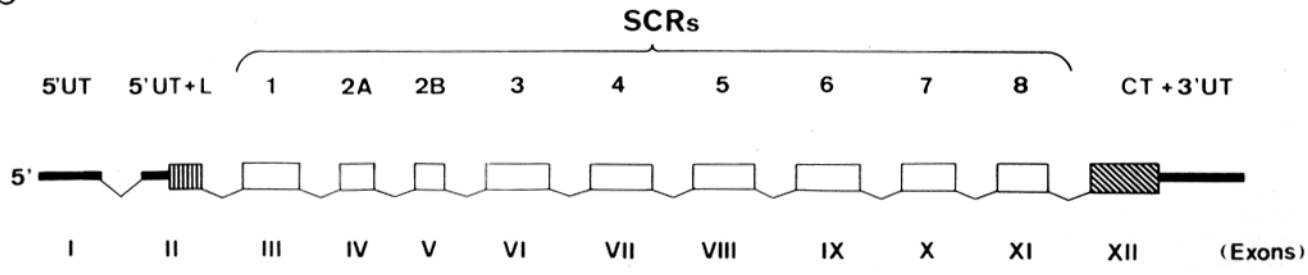



**Figure 2.** Exon/intron organization of the C4BPα gene. (*a*) The precise size of each of the C4BPα exons and the sequences corresponding to their 3′ and 5′ exon/intron boundaries are shown. Sizes of the introns, excepting intron 5 are approximate and were determined by agarose gel electrophoresis. (*b*) Diagramatic representation of the genomic organization of the C4BPα transcript. Solid bars (▬) are to indicate sequences of the mRNA corresponding to the 5′ and 3′ untranslated regions (UT). Coding regions are boxed. The signal peptide is referred to as L and the 57 amino acid COOH-terminal region as CT.

alignment of these genomic clones is presented in Fig. 1 to show the overall organization of the human C4BPα gene. C4BPα is composed of 12 exons spanning 40 kb of DNA.

The precise size of each of these 12 exons and the results of the analysis of all exon/intron junctions are described in Fig. 2. The data were obtained by comparison of the C4BPα-cDNA sequence to those sequences obtained from selected genomic subclones. Fig. 2 also presents a description of the structural domains associated with each of the exons and estimates of the length of the different introns based on agarose gel electrophoresis analyses. These results fully confirm and extend preliminary data on the organization of the human C4BPα gene (31, 32).

The 5′ UTR of the C4BPα transcript is split in two exons. The first exon includes the initial 198 nucleotides of the 5′ UTR (see below) and it is separated by a ~9-kb-long intervening sequence from the second exon (167 bp), coding for the remainder of the 5′ UTR and the predicted coding region for the signal peptide. The third exon codes for the first of the SCRs and starts the coding region for the mature secreted protein. Each of the eight SCRs that compose the C4BPα polypeptide chain is encoded by a single exon, except for the second SCR, which is split in two exons. Finally, the twelfth exon of the C4BPα gene codes for the COOH-terminal 57–amino acid region, which has no similarities to the SCRs, and the 245 nucleotides of the 3′ UTR (Fig. 2). Characterization of the precise position to which the poly A is added could not be determined since the first six A nucleotides of the poly A tail found in the cDNA clones are also present in genomic DNA.

A partial structure for the gene coding the murine homologue to human C4BPα has been reported (33). It includes six exons that are homologous to the fifth, sixth, seventh, tenth, eleventh, and twelfth exons of human C4BPα. The human and murine C4BPα transcripts, as will be shown in the Discussion, present very similar 5′ UTRs. Thus, excepting for the absence of exons 8 and 9, the organization of the murine C4BPα gene can be anticipated to be similar to that of the human C4BPα gene.

It should be mentioned that the screening of the human genomic libraries with the C4BPα-cDNA–derived probes also yield a separated set of overlapping clones with exon-like regions 80% homologous to the C4BPα exons. Pulsed field gel electrophoresis analyses of human genomic DNA, using specific probes, demonstrated that the two sets of C4BPα-specific overlapping clones map to very closely linked, but distinct, genomic regions (16). Thus, the second set of genomic clones probably identifies a "recent" duplication of the C4BPα gene, referred to as C4BPα-like gene, which will be described elsewhere (P. Sanchez-Corral, F. Pardo-Manuel, and S. Rodriguez de Cordoba, manuscript in preparation).

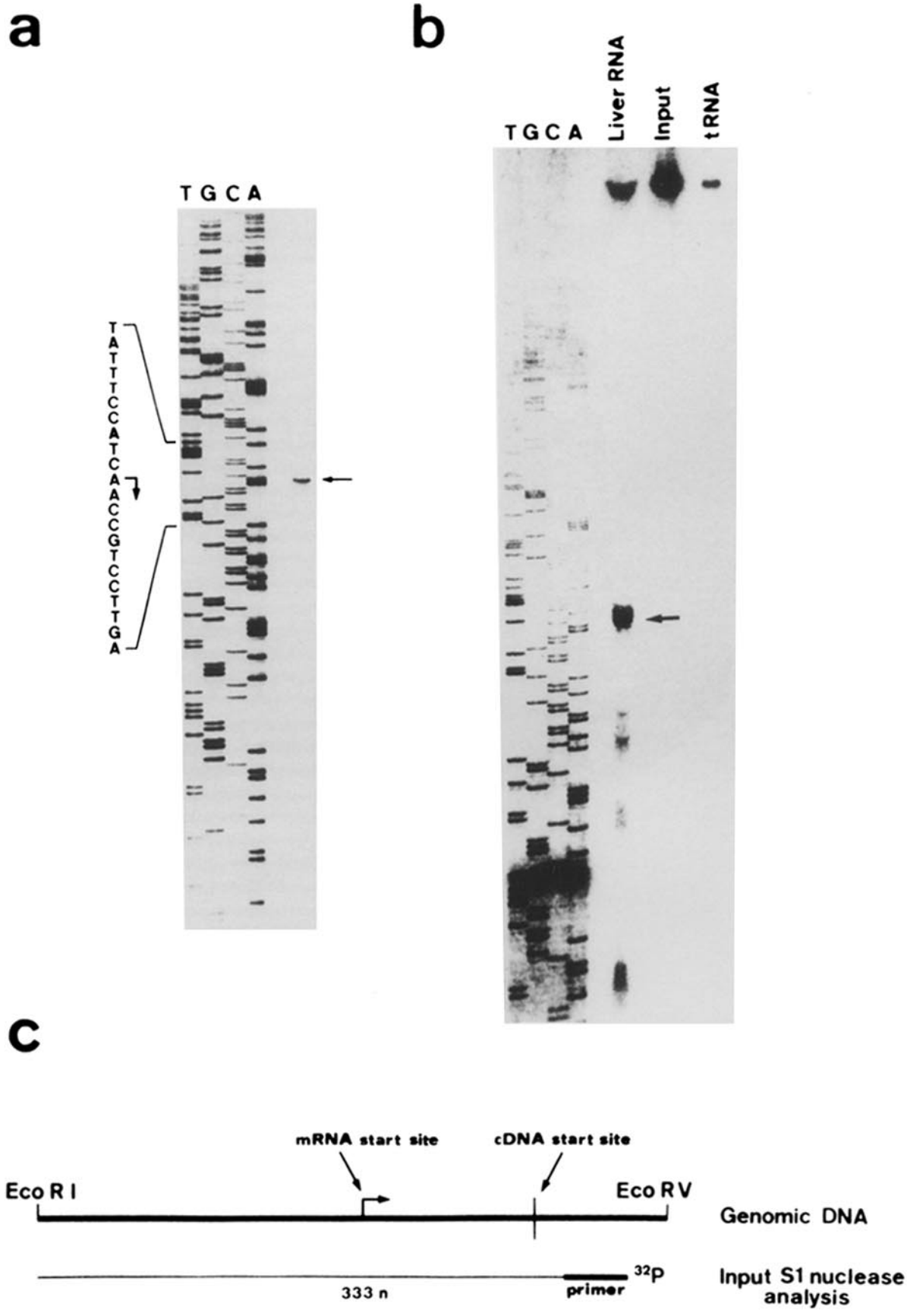
*Characterization of the Transcriptional Unit of the Human C4BPα Gene.* The 2,190-bp-long cDNA-C4BPα clone is the one among several cDNA clones that contains the longest sequence upstream the putative translational start site for the C4BPα polypeptide. Genomic sequences upstream the cDNA start site were obtained by DNA sequencing the G562RV0.3 subclone, a 352-bp-long EcoR I/EcoR V restriction fragment of the phage clone G562 including the first 98 bp of the C4BPα-cDNA, but no canonical TATA or CAAT boxes were encountered that could help to localize the cap site of the C4BPα-mRNA.

To characterize the transcription start site of the human C4BPα gene and to rule out the possibility of additional exons, we used a combination of primer extension and S1 nuclease protection assays (Fig. 3). A $^{32}$P-labeled 35mer oligonucleotide, corresponding to nucleotides 19–54 of the noncoding strand of the C4BPα-cDNA was annealed to total RNA from human liver, or from the human hepatoma cell line Hep-G2, and extended with AMV reverse transcriptase. As shown in Fig. 3 *a* the antisense 35mer oligonucleotide extended up to a single labeled fragment of 139 nucleotides, which suggests that the mRNA start site is located 104 nucleotides upstream the 3′ end of the primer. Identical results were obtained using RNA from the human hepatoma cell line Hep-G2 (data not shown).

The same 35mer oligonucleotide was also used to generate a 333-bp-long single-stranded DNA probe using the G562RV0.3 restriction fragment as the template (Fig. 3 *c*). In agreement with the results of the primer extension experiments, the S1 nuclease protection assays of this probe with total liver RNA showed a major "protected" band of nearly identical size to that found by primer extension analysis (Fig. 3 *b*). This finding further supports that the C4BPα gene transcription is initiated at a single site located 86 nucleotides upstream the 5′ most proximal nucleotide found in our longest C4BPα-cDNA clone. Taking into account these 86 nucleotides, the predicted length of the entire C4BPα transcript, excepting the poly A tail, is estimated in 2,262 nucleotides.

Close examination of the 5′ UTR sequence (198 bp) included within the first exon revealed the presence, in a different frame with respect to that encoding the C4BPα polypeptide, of two in-phase ATG codons at positions 25 and 82, which potentially could initiate translation of a small "upstream open reading frame" (uORF). This unexpected finding together with the unusual genomic organization of the 5′ UTR of the human C4BPα-cDNA prompted us to confirm

**Figure 3.** Mapping of the transcription start site of C4BPα by primer extension and S1 nuclease protection assays. (*a*) A $^{32}$P-labeled 35mer oligonucleotide, corresponding to nucleotides 19–54 of the noncoding strand of the C4BPα-cDNA, was annealed to total liver RNA to prime reverse transcription as described under Materials and Methods. The arrow shows the extended fragment of 139 nucleotides resulting from this experiment. (*b*) The 35mer oligonucleotide was also used in the generation of a 333-nucleotide-long single-stranded DNA probe for the S1 nuclease protection assays (see 3 *c*). This probe was annealed to 50 μg of total liver RNA, or 50 μg of tRNA, and digested with S1 nuclease (see Materials and Methods). The arrow shows the protected fragment of ~139 nucleotides. Minor bands below this fragment are probably consequence of S1 sensitive sites due to strong secondary structure in this region of the transcript. (*c*) Diagramatic representation of the positions of the 35mer, the cDNA start site and the transcription start site. In all the experiments the 35mer oligonucleotide was used to generate a sequence ladder from clone G562RV0.3, which was used as a size reference.


a
T G C A
TATTTCCATCAACCGTCCTTGA
b
T G C A
Liver RNA
Input
tRNA
c
mRNA start site
cDNA start site
Eco R I
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333 n
32P
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Input S1 nuclease analysis

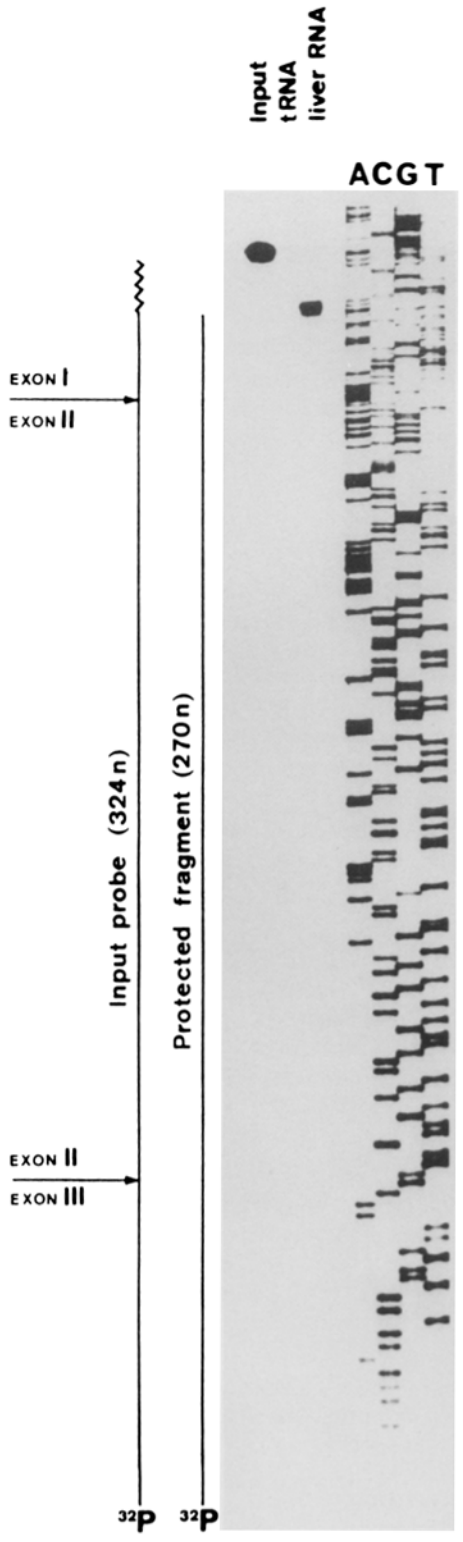


**Figure 4.** S1 nuclease analysis to identify potential alternative spliced C4BPα transcripts. S1 nuclease protection assays were performed as described under Materials and Methods, using a 324-nucleotide-long single-stranded DNA input probe spanning over sequences encoded by the first, second, and third exons. This probe, outlined in the left side of the figure, also includes an unrelated 54 nucleotide 3′ extension indicated as a zigzag line. Position of the splice junctions between exon I and II, and between exons II and III, are indicated by arrows. S1 nuclease protection using 50 μg of total liver RNA results in a single protected fragment of 270 nucleotides. The same oligonucleotide utilized in the preparation of the single stranded DNA probe was used to generate the sequence ladder that was included in the sequencing gels as a size reference for these experiments.

that the 2,262-bp-long transcript, including both the first and second exons, identifies the majority of the C4BPα-mRNA in liver.

Total RNA from human liver was used this time to protect a 324-nucleotide-long 5′-end $^{32}$P-labeled single-stranded DNA fragment including 270 nucleotides corresponding to the sequence from position 145 to position 415 of the noncoding strand of the C4BPα-mRNA; i.e., spanning the last 54 bp of exon I, exon II, and the first 49 bp of exon III. As shown in Figure 4, a "protected" band was detected that exactly corresponds to the length of the C4BPα-mRNA sequence included in the input probe. More important, no additional bands were observed (particularly in the vicinity of the boundaries between the third and second exons or between the second and the first exons), thus indicating that most, it not all, C4BPα transcripts in liver include the same 223-bp-long 5′ UTR preceding the first ATG in-frame with the coding sequence for C4BPα.

*Genomic Sequences Upstream the Transcription Initiation Site.* Over 5 kb of genomic DNA flanking the 5′ end of the C4BPα gene were sequenced using various subclones of the G562 λ genomic clone. As a first result, the analysis of this genomic sequence demonstrates that the 3′ end of the C4BPβ gene, which we have previously shown to lie adjacent to the 5′ end of the C4BPα gene (16), is exactly located 4,178 bp from the transcription start site of the C4BPα gene.

C4BP is expressed in the liver and is an acute phase protein (12, 13). Analysis of the 4,178-bp nucleotide sequence separating both genes demonstrates the presence of several sequences that share identity with the consensus sequence (TT/GNNGNAAT/G) recognized by NF-IL6, a nuclear factor implicated in the gene regulation of acute-phase genes (34). Some of these elements and their positions are shown in Fig. 5. Although no data on functional assays for these potential regulatory elements are yet available, it is suspected that some of them could be responsible for the observed enhanced secretion of C4BP by Hep-G2 cells upon stimulation by IL-6 (13). The human C4BPα gene does not present a conventional TATA promoter. However, a search of this sequence to identify potential *cis*-acting regulatory elements demonstrated the presence of the motif GTTAATCATTCAT at position −50 (Fig. 5). This 13-bp sequence matches the proposed consensus sequence for the binding site of HNF1 in the promoter region of other liver-expressed genes (35).

## Discussion

The C4BPα polypeptide belongs to a family of proteins characterized by a structural organization based on the presence of internal repeat units of 60 amino acids (SCR) that share a framework of highly conserved residues (20). Human C4BPα is composed of eight of these repeats followed by a 57–amino acid nonrepeat region (18).


**Figure 5.** Sequences flanking the transcription start site. Upper case letters indicate the nucleotide sequence included within the first exon. The sequences −118 to −126 (TGTCGCAAT), +51 to +59 (TGGAGCAAT), and +189 to +181 (TTGAGGAAG) match the consensus for the binding site of NF-IL6 (TT/GNNGNAAT/G) (34). The sequence −50 to −38 (GTTAATCATTCAT) matches the consensus for the binding site of HNF1 (GTTAATNATTNAC/T) (35). These sequences are underlined in the figure. The two upstream ATG codons and their termination codon are double underlined. The asterisk shows the 5′ most terminal nucleotide found in cDNA clones.

We present here the complete genomic organization and the structure of the transcriptional unit of the human gene coding for the α chain of the C4BP molecule. The C4BPα gene spans over 40 kb of DNA and comprises 12 exons. The organization of the C4BPα gene matches a general pattern found in other RCA genes like MCP, CR2, CR1, CR1-like, DAF, and H, for the location of most of the splice junctions (36–40). The similarities observed between C4BPα and other RCA genes can be summarized as follows: (*a*) C4BPα presents identical genomic arrangement for the boundary between the signal peptide and the mature polypeptide to that found in the CR1, CR2, and DAF genes (36, 39, 41). In these four genes the exon coding for the first, $NH_2$-terminal, SCR includes the last two base pairs of the codon for the Gly residue predicted to be the site for the cleavage of the signal peptide; (*b*) most of the SCRs that compose C4BPα are encoded by single exons and, like in other RCA genes, all the splice junctions between the SCR-coding exons occur after the first base of a codon; and (*c*) all RCA genes characterized thus far include at least one SCR split in two exons. C4BPα is not an exception to this rule and the second SCR is encoded by exons 4 and 5. In addition, the splice dividing the second SCR of C4BPα occurs at identical position as in those other RCA genes (Fig. 2).

In contrast with the similarities found between the genomic organization of the SCR-coding regions, C4BPα is clearly different from the other RCA genes in the non-SCR regions. Thus, in addition to the reported characteristic COOH-terminal region of the C4BPα polypeptide (18), the C4BPα gene presents a distinct 5′ end organization including a first exon coding exclusively for 5′ UTR sequences (see below).

The results of the comparison between the genomic organization of the C4BPα gene with other RCA genes are in agreement with the current understanding of the evolution of the RCA gene cluster. Genes encoded at this location most likely originated as a result of the duplication of an ancient gene organized by SCRs and then diverged to present distinct COOH-terminal coding regions as well as different 5′-noncoding regions. These genes maintained, however, the characteristic genetic organization of the SCR exons and it can be speculated that this fact facilitated the appearance of the successive generations of RCA genes. The peculiar uniformity of the splice junctions in this region, which permits insertion or deletion of SCR-coding exons without changing the correct reading frame, provides a particularly suitable structural organization for the evolution of these genes involving genetic mechanisms, like unequal recombination, which are normally deleterious for a gene without this feature. This model for the evolution of the RCA genes is supported by their physical localization within the RCA gene cluster. Thus, RCA genes presenting similar "non-SCR regions" map together within specific subregions of the RCA gene cluster (16).

The C4BPα gene shows a peculiar 5′ end organization. It presents an unusually long 5′ UTR that is encoded by two exons separated by a large ~9-kb intron. The first exon codes for the initial 198 nucleotides of the 5′ UTR of the C4BPα-mRNA and includes two ATG codons that lie in a sequence context suitable for being the translational start site of a small uORF. With the exception of the proto-oncogene transcriptional units, in which >65% of them include ATG codons 5′ to the authentic translation start site, uORFs are relatively rare in vertebrate mRNAs (42). The existence of uORFs in the 5′ UTR of the C4BPα gene is particularly interesting since uORFs have been shown to influence the translational efficiency of the mRNAs (43–45).

The complete amino acid sequence of the murine homologue of human C4BPα has been deduced from nucleotide sequencing of a 1,889-bp-long cDNA clone isolated from a mouse-liver–cDNA library using human C4BPα-cDNA probes (46). The predicted mouse C4BPα polypeptide chain is 51% identical at the amino acid level to its human homologue and lacks the residues corresponding to the fifth and sixth SCRs of human C4BPα. Upstream from the predicted codon starting the coding region for the mature secreted protein, the murine cDNA clone includes 370 bp with two in-phase ATG codons, positions 203 and 332, which yield putative signal peptides of 56 and 13 residues, respectively (46). Prompted by the peculiarities found in the 5′ end region of the human C4BPα transcript, we have reexamined the 5′ end region sequence of this 1,880-bp-long murine C4BPα-cDNA clone and found that further upstream of the two ATG codons in-phase with the C4BPα polypeptide there are three additional ATG codons, two of which (positions 32 and 107) could be translation start sites for two small uORFs. These uORFs lie within a region of this clone that was earlier suggested to correspond to the 3′ end of an intron sequence (46). Alignment of the murine cDNA-C4BPα sequence and the completed human C4BPα sequence, however, shows homology between both 5′ UTRs (data not shown), strongly suggesting

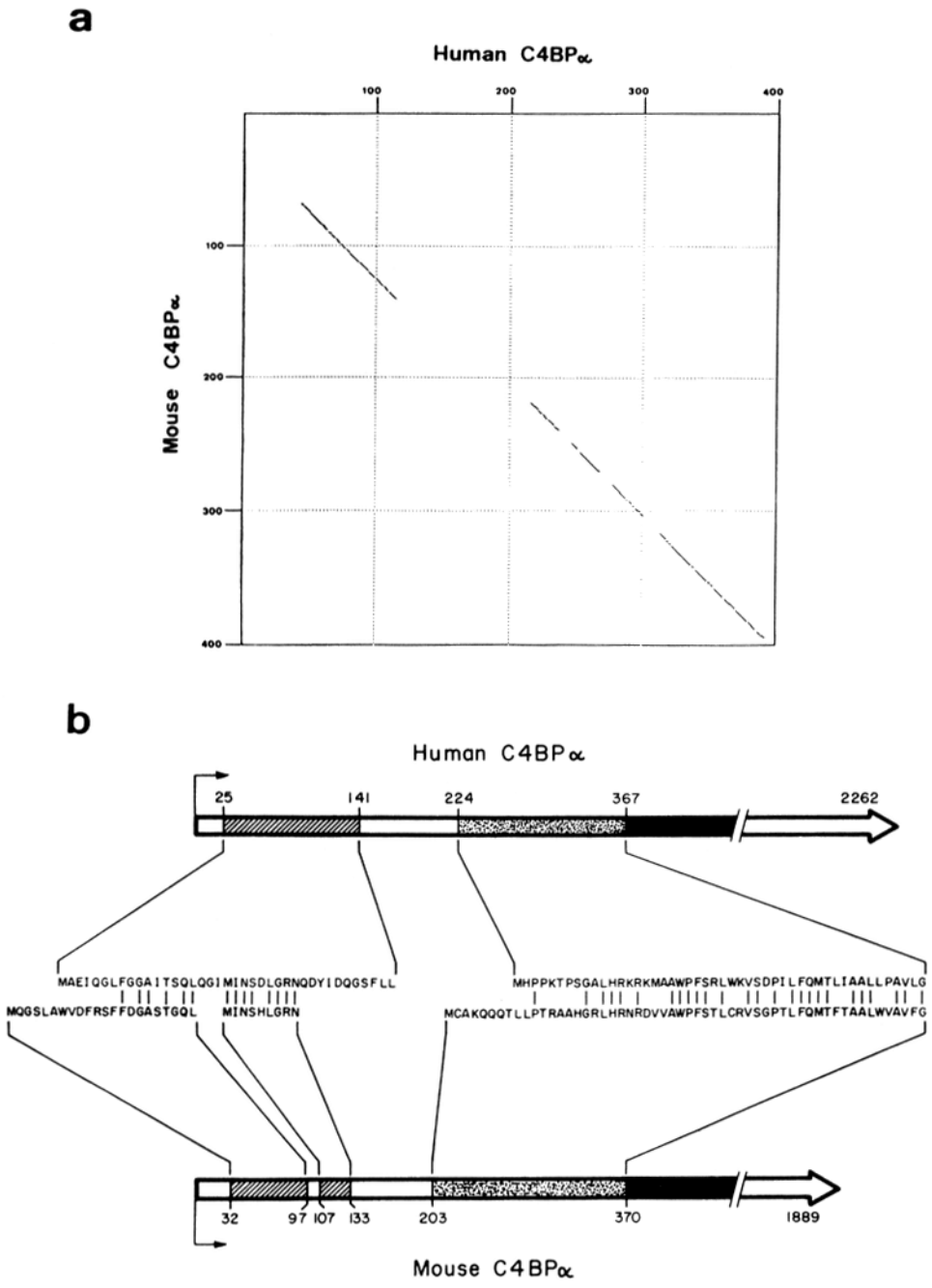


**Figure 6.** Comparison of the human and mouse C4BPα transcripts. (*a*) Dot matrix comparison of the human and mouse (46) nucleotide sequences upstream the codon for the $NH_2$-terminal amino acid residue of the mature secreted C4BPα polypeptides. Parameters were set for a window of 20 nucleotides and 80% identity. (*b*) Alignment of the human and mouse transcriptional units. Hatched boxes (▨) identify the uORFs, dotted boxes (▩) the coding regions for the signal peptide, and solid boxes (■) the regions coding for the mature secreted polypeptides.

that the sequence including the two uORFs belongs to the murine C4BPα mRNA and supporting the view that the 1,889-bp-long cDNA clone reported in reference 46 represents the entire sequence of the murine C4BPα transcript.

To identify unusually conserved sequences between human and mouse C4BPα in the region upstream from the predicted codon corresponding to the $NH_2$ terminus of the mature secreted protein, we have performed dot matrix comparisons at the nucleotide level using high stringent conditions (window = 20 n; identity = 80%). This analysis demonstrates the existence of two of such conserved regions (Fig. 6 *a*). One of these regions corresponds to that containing the uORFs and the other includes the coding sequences for the signal peptide.

Close examination of the nucleotide sequences surrounding the 5′-most proximal ATG codons starting either the upstream or the signal peptide coding frames indicates, however, that these ATG codons have not been conserved; i.e., human and mouse C4BPα present these ATG codons at distinct sequence positions. Interestingly, when we compared the deduced human and mouse amino acid sequences encoded at these locations, we found that, in spite of using different ATG codons, the reading frames have been maintained. Alignment of the deduced sequences for the signal peptide, assuming

that the translation start site is located at the most 5'-proximal in-phase ATG codon, is shown in Fig. 6 *b*. Except for nine extra residues at the beginning of the murine sequence, both signal peptides were 56% identical. In addition, they share other positions with chemically similar residues. These results strongly argue in favor of the ATG positions 203 and 224, mouse and human, respectively, as the authentic translation start sites of the C4BPα polypeptides.

The comparison of the deduced amino acid sequences of the uORFs present in the 5' UTR of the human and mouse C4BPα shows a degree of conservation between these sequences comparable to that found between those coding for the signal peptides (Fig 6 *b*) or between those coding for the mature secreted protein (46). The striking conservation of these 5' UTRs sequences between human and mouse C4BPα suggests a functional role for these uORFs. Experiments are in progress to test this hypothesis by investigating a possible role of these sequences in controlling the expression of the C4BPα polypeptide.

C4BP is unique among RCA proteins in that it is organized by two functionally distinct polypeptide chains (C4BPα and C4BPβ). They share similar COOH-terminal regions that contain the two cysteines that establish the C4BP interchain disulfide linkages (15, 18). These polypeptides are encoded by genes arranged in tandem that, as discussed above, probably originated by the duplication of a common ancestor at the C4BP-subregion of the RCA gene cluster. Because these genes code for subunits of the same molecule, it is conceivable that they also share regulatory sequences at their 5' flanking sequences which allow their coordinated expression.

As a first approach to the characterization of these regulatory sequences in the human C4BPα gene, we have sequenced >5 kb of DNA upstream the transcription start site and sought for the presence of potential *cis*-acting regulatory elements. Although the human C4BPα gene does not present a conventional TATA promoter, a number of sequences similar to known regulatory elements were identified. These include sequences that share identity with the consensus sequence for the binding sites of HNF1 or NF-IL6 (Fig. 5). The presence of these sequences is particularly suggestive because C4BP is expressed in liver and is an acute phase reactant. Whether these structural elements are functional regulatory elements involved in the control of C4BPα expression is currently under investigation in our laboratory.

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