A Potent Enhancer Made of Clustered Liver-specific Elements in the Transcription Control Sequences of Human α1-Microglobulin/Bikunin Gene*

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α1-Microglobulin (A1M) and bikunin are plasma proteins which are present both as free molecules and as complexes with either IgA heavy chains for A1M or the F1, F2, and F3 heavy chains of the inter-α-inhibitor family for bikunin. Mature A1M and bikunin originate from the cleavage of an A1M/bikunin precursor (ABP) synthesized from a single gene with liver-specific expression. Five kilobases of the 5′-flanking region of the human ABP gene were sequenced. Deletion mutants of this region subcloned upstream of a CAT reporter gene were transfected into HepG2 hepatoma cells. A segment covering the −2.7- to −2.8-kb area is required for full activity of the ABP gene. This segment contains a cluster of six elements (boxes 1–6, 5′ to 3′) which are potential binding sites for the liver-enriched trans-acting factors HNF-1, HNF-4, HNF-3, HNF-1, HNF-3, and HNF-4, respectively. This cluster enhances the activity of heterologous minimal promoters in a position- and distance-independent fashion in HepG2 cells. This enhancer activity is restricted to liver cells as the cluster is unable to activate promoters in Chinese hamster ovary (CHO) or HeLa cells. By band-shift experiments we have shown that the liver-enriched transcription factors HNF-1, or HNF-3, do bind to boxes 1 and 4, or 3, respectively. The combination of a weak promoter and a strong distant and liver-specific enhancer distinguishes the ABP gene from most other plasma protein genes expressed in hepatocytes.

α1-Microglobulin (A1M) is a plasma glycoprotein found in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X67082.

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¶ The abbreviations used are: A1M, α1-microglobulin; ABP, α1-microglobulin/bikunin precursor; CAE, cis-acting element; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; HNF, hepatocyte nuclear factor; HSV, herpes simplex virus; IL, interleukin; PCR, polymerase chain reaction; TK, thymidine kinase; kb, kilobase(s); bp, base pair(s).

a free state (M, 31,000) as well as covalently bound to some immunoglobulin A heavy chains (reviewed in Ref. 1). Although a precise function has not yet been ascribed to A1M, this protein is a member of the lipocalin superfamily which includes carrier proteins with an affinity for small hydrophobic ligands such as retinol, cholesterol, steroids, or odorant molecules (1, 2). As such, A1M has been proposed as a carrier for porphyrin (3) or retinol (4). Bikunin is another plasma glycoprotein found in a free state (M, 45,000) as well as covalently bound to the heavy chains of inter-α-inhibitor and pre-α-inhibitor. Inter-α-inhibitor and pre-α-inhibitor are two large molecules with serine protease inhibitor activity which originates from two functional tandemly arranged Kunitz-type inhibitory domains in the bikunin chain (reviewed in Refs. 5 and 6). Although A1M and bikunin have no apparent structural or functional similarity, both molecules originate from a shared precursor polypeptide and are released by cleavage of a short connecting peptide within the precursor (7). In mammals a single A1M/bikunin precursor (ABP) mRNA (1.25 kb) (7) is transcribed by a single copy gene (8, 9) with hepatocyte-restricted expression (10). The human ABP gene is comprised of 10 exons and spans about 20 kb (8). The largest intron in the gene (7 kb) separates exon 6 coding for the C-terminal end of A1M from exon 7 coding for the connecting peptide and the N-terminal end of bikunin (8). This feature has been assumed to reflect an assembly of two quite distinct ancestral genes coding for a lipocalin and a Kunitz-type protease inhibitor, respectively (8).

The primary function of A1M or bikunin is suggested by their lipocalin- or Kunitz-type-specific amino acid sequence, respectively. Furthermore, they both may be involved in a number of regulatory pathways, including immunoregulation by A1M (1) or stimulation of endothelial cell growth by bikunin (11). The ABP gene is therefore a source for two plasma proteins which could each be important for several biological events. Pathologic changes in the level of either A1M or bikunin could shed light upon their normal role(s), and therefore we have been interested in studying the overall regulation of expression of the corresponding gene under abnormal conditions. We further aim to elucidate the major mediators involved in the physiological and pathological expressions of this gene. As a first step toward this goal, we now present a structural and functional study of the 5′-flanking region of the gene. We have observed that the expression of the ABP gene is controlled by a distal cluster of liver-

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specific elements which strongly enhances transcription in a tissue-specific manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and DNA modification enzymes were from Boehringer Mannheim or Amersham. [α-32P]dATP (>600 Ci/mmol), [α-32P]dCTP (3000 Ci/mmol), [3H]Acetyl-CoA (2-4 Ci/ mmol), and x-ray films (Hyperfilm-MP) were from Amersham. Agarose was from Pharmacia. Sterile plasticware for tissue culture was obtained from Falcon. Minimal essential medium was purchased from GIBCO-BRL. Fetal calf serum and antibiotics were from Boehringer Mannheim. Culture-grade chemicals used for cell transfection were obtained from Sigma.

**Oligonucleotides**—Small linkers (5’CCCCGGGG3’) were from New England Biolabs. For sequencing, synthetic oligonucleotides were purchased from Boehringer Mannheim (pUC primers) or from Eurogentech (CAT- or ABP gene-specific primers). For PCR or band shift experiments, the following oligonucleotides were used: a (rat albumin gene) CCAAT box-containing oligonucleotide designated abCCAAT (5’GGGGTAGAAGACATGGGAAATGAATG) and its complementary strand (12), a (rat albumin gene) HindIII site designated PES6a (5’TGATGTTAATACGTATAGTTA3’) and a complementary oligonucleotide (5’TGAATACTGGTACATTAACACAC3’) (13), an (rat tyrosine aminotransferase gene) HindIII site designated HNF-3 box-containing oligonucleotide (5’GGGCTCTGCCCCCAAAACCTGCTG3’) and its complementary strand (14), a (rat L- pyruvate kinase gene) HindIII site designated HKHNF-4 box-containing oligonucleotide designated PKHNF4 (5’GGTCTCGAGCATTGCGGCCCGAGG3’) as well as its complementary oligonucleotide (5’TGATACGTTGGGC CCAAGTCCAGG3’) (15) have been described previously and were synthesized kindly provided by T. Chouard (Pasteur Institute, Paris) or T. Grange (Paris University VII) (HNF-3) and B. David- Wattier (Pasteur Institute, Paris) (HNF-4). Other oligonucleotides covering various segments of the ABP gene were synthesized on a Millipore synthesizer as follows: oligonucleotide 1, 5’CCAGGT L-CCAAT (5’GGGGTAGGAACCAATGAAATGAAAGGTTA3’) and its complementary strand (16), a (rat tyrosine aminotransferase gene) HNF-3 box-containing oligonucleotide designated PE56a (5’TCGAGT GGTCCACTTCTGAGGCGG3’) and its complementary strand (17), a (rat albumin gene) HNF-4 box-containing oligonucleotide designated HNF-4 (5’GGTTCCTGGACTCTGGCCCCAAGTG3’) and its complementary strand (18), a (rat albumin gene) HNF-1 box-containing oligonucleotide designated HNF-1 (5’TACCTCTCTGTTTGGCTC- GTGGTTAATGATCTACAGTTA3’) (19) and its complementary strand (20), a (rat albumin gene) HNF-3 box-containing oligonucleotide designated HNF-3 (5’TGATACGTTGGGTC CCAAGTCCAGG3’) (21) and its complementary strand (22), a (rat tyrosine aminotransferase gene) HNF-3 box-containing oligonucleotide designated HNF-3 (5’TACCTCTCTGTTTGGCTC- GTGGTTAATGATCTACAGTTA3’) (19) and its complementary strand (20), an (rat tyrosine aminotransferase gene) HNF-1 box-containing oligonucleotide designated HNF-1 (5’TGATACGTTGGGTC CCAAGTCCAGG3’) (21) and its complementary strand (22).

**Library Screening**—A human genomic library was made by ligating the BamHI-restricted arms of XEMBL-3 phage with partially Sau3A-digested plasmid DNA, [α-32P]dATP, labeled at 1-2×108 cpm/pg by the random oligonucleotide procedure (23) and its complementary strand to oligonucleotides 1-11, respectively.

**DNA Sequencing**—Dideoxy-sequencing reactions were carried out with [γ-32P]ATP-labeled plasmid DNA, [α-32P]dATP, and a T7 polymerase sequencing kit (Amersham or Pharmacia). Purification of inserted DNAs.

**PCR**—PCR was performed with the Taq polymerase and GeneAmp 10× PCR buffer from Perkin-Elmer Cetus in a Cetus thermocycler 480 (30 cycles: 90 s at 94°C, 90 s at 55°C, 90 s at 72°C). PCRs were performed in a 50-μl reaction volume using 0.25 μl of 10× PCR buffer, 5 μl of 10 mM MgCl2, 1 μl each of forward and reverse primers, 1 μl of chloroform-isoamyl alcohol, 2 μl of plasmid DNA, and 1 μl of [α-32P]dATP. The PCR products were purified from an agarose gel by electrophoresis, and subcloned into pUC18 vectors.

**Plasmids and Constructs**—pUC18, -19, and -BM21 were from Boehringer Mannheim. PhcH110 (Pharmacia) contains the β-galactosidase gene under the control of the SV40 early promoter.

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precipitation procedure modified from Ref. 20 with a mixture of plasmids (banded twice on CsCl gradient) made of CAT plasmid (4 μg) + pCH110 plasmid (1 μg). The cells were exposed for 4 h to the CaPO4/DNA precipitate and then shocked with 15% glycerol in culture medium for 3 min and rinsed with culture medium. Forty-eight hours later the (usually confluent) cells were washed with antiionically charged saline-buffered saline (Sigma) and harvested by gentle scraping with a rubber policeman. The cell extracts were immediately processed for β-galactosidase and CAT assays. Each set of CAT constructs to be compared was studied in at least two independent transfection experiments. Within an experiment each construct was studied in triplicate.

β-Galactosidase and CAT Assays—Our miniaturized fast procedure for simultaneous measurements of β-galactosidase and CAT activities is reported elsewhere (21). For CAT assays, the two-phase partition of [3H]acetyl-CoA and [3H]-acetylated chloramphenicol followed by scintillation counting was carried out essentially as in Ref. 22. All values of CAT activity in cell extracts were normalized to the β-galactosidase activity in the same extracts and finally expressed as: counts/min [3H]acetylchloramphenicol/β-galactosidase unit.

RNA isolation and Primer Extension Analysis—RNA from cultured hepatoma cells were obtained by in situ lysis according to Ref. 23 followed by centrifugation onto a CsCl cushion (24). Poly(A)+ RNAs were isolated onto oligo(dT) columns (Pharmacia kit). RNA integrity was monitored by visual inspection of the 28 and 18 S ribosomal bands in an agarose gel electrophoresis. A primer extension reaction was carried out onto poly(A)+ RNAs (10 μg) with reverse transcriptase and a cDNA synthesis kit (Amersham) as recommended by the manufacturer, in the presence of 1 μl of [α-32P]dCTP. The size of the extended product was determined onto a sequencing gel using a known dyeoxy sequence as a size ladder.

Preparation of Nuclear Extracts and Gel Mobility Shift Assay—Nuclei from different cell lines were prepared as described previously (25) with solutions containing 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 5 μg/ml aprotinin, pepstatin, and leupeptin. Binding reactions for band shift assays were performed in 16 μl of a reaction mixture containing 10 mM Hepes buffer, pH 7.9, 30 mM KCl, 8 mM MgCl2, 9 mM spermidine, 0.5 mM dithiothreitol, 10% glycerol, 5 μg/ml of the protease inhibitors mentioned above, 1.5 μg of poly(dI-dC), and 1 μg of sonicated salmon sperm DNA. Four μg of nuclear protein extract were preincubated in this mixture for 5 min at 4°C. Then 1 ng of kinase-labeled double-stranded oligonucleotide as a probe and competitor oligonucleotides, if any, were added and incubated for 15 min on ice. The DNA-protein complexes were loaded onto a low ionic strength (0.25 M ammonium acetate, 2 mM benzamidine, and 5 μg/ml aprotinin, pepstatin, and leupeptin) 5% acrylamide (acrylamide/bis: 29/1) gel and electrophoresed at 12 V/cm.

RESULTS

Nucleotide Sequence of the 5'-Flanking Region in the Human ABP Gene—A XEMBL-3 clone containing part of the human ABP gene was isolated by screening a genomic library with a human 179-bp cDNA probe corresponding to ABP gene exon I. By comparing a restriction map of this clone to the published map of the entire gene (8), we isolated a Sall/BamHI 5.3-kb segment. Its 3′ end sequence (not detailed) corresponded to a known ABP gene sequence (8) and indicated that this Sall/BamHI segment contained the 5′-flanking sequence of the gene. The Sall/PstI segment (−4964 to −3328) on the 5′ side of this flanking sequence was sequenced after subcloning various restriction fragments into a pUC plasmid (strategy not detailed). The PstI/BamHI 3′ side (−3327 to +335) of the flanking sequence was subcloned into pUC8BM21 and sequenced by a nested deletion strategy with exonucleases III/S1 nuclease. Some of the resulting clones were subsequently used for constructing deletion mutants in CAT plasmids.

The resulting sequence is presented in Fig. 1. It covers 4964 bp upstream of the published transcription start site numbered (+1) (8). Two shorter sequences spanning the region from −693 to exon I (8) or from −1583 (our numbering) to exon I (9) are included in the present sequence. Compared with (8) our sequence displays an extra G at −66 and a GC inversion at −25. The sequence of the entire 5′-flanking region was scanned on both strands for the presence of potential protein-binding motifs. This search was based upon published libraries of such motifs (26, 27) and a periodically updated list of consensus in our laboratory. No obvious TATA box at the usual −20/−25 position was found. Two potential CCAAT boxes are located at −162 and −316. The other, most prominent findings are indicated in Fig. 1 (see legend). Among them, an extensive series of potential IL-6α-responsive elements (28) were scattered through the entire sequence (Fig. 1). An IL-6α-responsive element, which is a weak affinity binding site for the HNF-1 transcription factor (29), was also found. Notably, numerous potential liver-specific sequences are present. They are detailed in Fig. 1 and Table I. A tight cluster of six such elements is located between −2802 and −2659 (boxed in Fig. 1). These six elements were designated boxes 1-6 (5′ to 3′) (see Fig. 1 and Table I). They contain potential binding sites for the hepatocyte-enriched nuclear factors HNF-1, HNF-4, HNF-3, HNF-1, HNF-3, and HNF-4, respectively (Fig. 1 and Table I).

Effect of Progressive 5′ Deletions on Transcriptional Activity of the ABP 5′-Flanking Region—We wished first to localize potential transcriptional control sequences in the 5′-flanking region of the ABP gene. To this end, various CAT constructs were made, all containing an identical ABP promoter 3′ end (+57) but deleted to a variable extent at their 5′ end (−2929 to −36). The promoter was positioned in a sense or antisense orientation relative to the CAT gene. These constructions were transfected into HepG2 cells. The relative CAT activities of the constructs with the promoter in a sense orientation compared with the control plasmids pFIXCAT or pTKCAT (see “Experimental Procedures”) are shown in Fig. 2. The longest three constructs, i.e. p-4964/57CAT to p-2929/57CAT, exhibited the same strong transcriptional activity, which was 50-fold higher than the Factor IX promoter (pFIXCAT) and slightly higher than the HSV TK promoter (pTKCAT). Deleting 2.5 kb at the 5′ end in the 5 kb of ABP promoter, i.e. generating p-2422/57CAT, resulted in a dramatic (50-fold) decrease in CAT activity. A further 1.7-kb deletion in ABP gene sequence (i.e. p-692/57CAT) resulted in a further 10-fold drop (activity = 0.1 relative to pFIXCAT) in CAT activity. This activity was partially recovered (4-fold above pFIXCAT activity) in p-348/57CAT; this construct corresponds to the “minimal” ABP promoter, since all constructs deleted further (p-246/57CAT to p-36/57CAT) lacked any significant CAT activity. Several control constructions made with the variably deleted ABP promoter in a reverse orientation relative to CAT gene did not exhibit significant CAT activities (data not shown). Overall, our results suggested the presence of a strong positive regulatory region located between −2929 and −2423 and a negative regulatory region between −692 and −349.

To verify the transcription start site of the ABP gene from an ABP/CAT construct, poly(A)+ RNAs isolated from HepG2 cells transfected with p-2929/57CAT were hybridized with a CAT gene-specific primer (Fig. 3, left panel) and treated with reverse transcriptase. The extended product was 136 bp in length (Fig. 3, right panel), a value which is in close agreement with the published transcription start site (+1) of the ABP gene (8).

A Strong Enhancer Containing Several Liver-Specific Elements Is Located in a Distal 5′-Flanking Region of the ABP Gene—We further analyzed the activity of the positive regulatory element described above. Various CAT constructs were prepared from a 381-bp segment (−2929 to −2549) of the ABP gene ligated in sense or antisense orientation upstream
Fig. 1. Sequence of the 5'-flanking region of the human ABP gene.
The bases are numbered positively 3' from the published (8) transcription start site (numbered +1, indicated by a broken arrow) and negatively 5' to it. Alu-repeat sequences are indicated by dotted underlining. Putative protein-binding sites in the sequence are indicated by underlined or boxed, boldface numbers. References for consensus sequences are detailed in Table I. A cluster of consensus sequences are indicated by a dotted underlining.

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of a minimal promoter or downstream to CAT gene in the plasmids pTK50 or pFIXCAT which both contain a weak minimal promoter. As shown in Fig. 4A, all such constructs transfected in HepG2 cells exhibited a strong increase in CAT activity compared with the controls, independent of the orientation or location of the 381-bp segment relative to the heterologous promoter. Specifically, the pTK50 plasmid activity was enhanced 5-fold or more whatever the orientation/location of the 5′ end. Furthermore, this sequence encompasses the set of six clusters of liver-specific factors mentioned above (Fig. 1 and Table I). To investigate whether these boxes could account for the enhancer activity, additional constructs were made with a PCR-generated 107-bp segment covering boxes 1–5 (box 6 is apparently nonfunctional as judged from band shift experiments, see below). Up to three copies of this 107-bp segment were ligated upstream of the minimal TK promoter in pTK50. As seen in Fig. 4B, this 107-bp segment increased TK promoter activity about 16-fold and therefore fully retained the enhancer activity first seen with the 381-bp segment. Additivity in enhancement was observed when two copies of this 107-bp segment were ligated upstream of the minimal TK promoter in pTK50. As seen in Fig. 4B, this 107-bp segment increased TK promoter activity about 16-fold and therefore fully retained the enhancer activity first seen with the 381-bp segment.

Liver-restricted Activity of the ABP Enhancer—To test whether the enhancer activity detected in HepG2 cells is cell-specific, the enhancer/ABP promoter-containing construct p107x3/TK50H was transfected into two nonhepatic cell lines, namely immortalized cervical carcinoma cells of human origin (HeLa) and an established Chinese hamster ovary (CHO) cell line. To allow for comparisons between lines, two internal

| Sequence in ABP gene* (location of 5′ end) | Box (derived ABP oligonucleotide pair used for band shift) | Consensus sequence (Ref.) | Cognate DNA-binding protein |
|-------------------------------------------|---------------------------------------------------|--------------------------|-----------------------------|
| GAGTATGG (--1807)*                        | TCNTACTC (37)                                     | C/EBP                     |
| TCTTACTC (--888)                          | TCNTACTC (37)                                     | C/EBP                     |
| GTTTATTTGAA (--2802)                      | 1 (1/2)                                           | HNF-1*                    |
| GCTTTATTTAAA (--2734)                     | 4 (7/8)                                           | HNF-3*                    |
| TTGGTTGCG (--2744)                        | 3 (6/6)                                           | HNF-3*                    |
| ACACAAGA (--2715)*                        | 5 (9/10)                                          | HNF-3*                    |
| GCAACAAGA (--48)*                         | TA/GATTCG(T/C) (14)                              | HNF-3p                    |
| TGGAGCGCTTGG(--2936)                      | TGGAGCGCTTGG (--2936)                             | HNF-4p                    |
| TGGACTCTGGC(--306)                       | TGGACTCTGGC (--306)                               | HNF-4p                    |
| TGGGCTCG(--342)                          | TGGGCTCG (--342)                                  | HNF-4p                    |
| GTTACCGGAGAAC(--371)                     | 2 (3/4)                                           | HNF-4p                    |

* The mismatches between ABP gene sequence and consensus are underlined.

* Paired oligos are numbered 1/2, 3/4, etc. The sequences are provided under "Materials."

* When several slightly different consensus are available, the one that best fits the ABP gene sequence is considered. Consensus ambiguities are in brackets.

* Complementary strand similar to consensus.

* HNF-1 is also designated LF-B1 or HNF-1α (31).

* HNF-3 is also designated HNF-5 (T. Grange, personal communication) and is likely identical to eH-TF (K. S. Zaret, personal communication).

* HNF-4 is also designated LF-A1 or HNF-2 (31).
FIG. 2. CAT activity of ABP-CAT constructs variably deleted at the promoter 5' end and transfected in HepG2 cells. Left column, list of CAT plasmids with the promoter segments tested. Central panel, constructs with the ABP promoter in a sense orientation relative to CAT gene. The ABP promoter is shown with a solid line, and the CAT gene is shown as an open box at the 3' end. Broken arrows indicate the transcription start site of the ABP gene. Right panel, CAT activities (fold) of ABP/CAT constructs (closed bars) are shown as mean ± S.D. (n = 3) relative to that of pFIXCAT (activity = 1, open bar) or pTKCAT (hatched bar) used as positive controls with a weak (pFIXCAT) or strong (pTKCAT) promoter activity.

FIG. 3. Determination of the transcription start site in an ABP promoter-CAT construct (p-2929/57CAT). Left panel, map of the p-2929/57CAT plasmid at the ABP/CAT junction. The 3' side of the ABP gene ends at +57 followed by CAT cDNA. The 5' end of the primer used for the extension reaction with the corresponding transcribed RNAs is 77 bp away from the ABP/CAT junction. Right panel, electrophoresis of the primer extension reaction. Lane 1, cells transfected with p-2929/57CAT; lane 2, mock-transfected HepG2 cells. A known sequence ladder (T, C, G, and A, from left to right) is used for size determination. Anode is at the bottom.

standards were used for normalization, namely pTKCAT, a plasmid with the ubiquitous HSV TK promoter, as well as pTK50, a construct which contains a minimal TK promoter (proximal SP-1 box and TATA motif). As seen in Fig. 5, p-2929/57CAT activity was 2.5-fold higher than pTKCAT activity in HepG2 cells, whereas it was 2- or 20-fold weaker than pTKCAT activity in CHO or HeLa cells, respectively. Therefore p-2929/57CAT activity was about 6- or 50-fold lower in CHO or HeLa cells, respectively, as compared with its activity in HepG2 cells. Likewise, the 6-fold enhancement of transcription provided in the construct p381/TK50H containing the 381-bp enhancer in pTK50 was observed in HepG2 but not in CHO or HeLa cells (Fig. 5). In contrast, the enhancerless construct with the minimal ABP promoter, i.e. p-348/57CAT, used as a negative control, exhibited a weak and similar activity (below pTK50 activity) in the three cell lines.

Characterization of the DNA Binding Proteins Recognizing the ABP Enhancer—To verify that the putative binding sites for liver-specific factors which we localized by homology search within the ABP enhancer (Fig. 1 and Table I) are indeed recognized in vitro, we performed band shift experiments. Nuclear extracts from the differentiated hepatoma HepG2 or H4II cell lines (human or rat origin, respectively), from the dedifferentiated rat hepatoma H5 cell line, or from the human cervical carcinoma HeLa cell line were used. The probes were double-stranded labeled oligonucleotides spanning (i) one of the ABP boxes 1–6 or (ii) established targets for the HNF-1, HNF-3, or HNF-4 transcription factors. The results are presented in Fig. 6.

The potential HNF-1 boxes 1 (oligonucleotides ABP 1/2) and 4 (oligonucleotides ABP 7/8) were both bound by the HNF-1 transcription factor (Fig. 6A). First, they both inhibited the binding of HNF-1 present in HepG2 nuclear extract to its target present in the rat albumin promoter (PE56 probe) (25) as shown in Fig. 6A, middle panels. Furthermore, they formed a complex corresponding to the electrophoretic migration of HNF-1 (Fig. 6A, right panels) which was slower than the complex formed with v-HNF-1 present in the dedifferentiated H5 hepatoma cell line (25, Fig. 6A, left panel). Finally, boxes 1 and 4 were not recognized by any protein present in HeLa extracts (data not shown). From (i) the competition of PE56, ABP 1/2, or ABP 7/8 with the complex formed by HNF-1 and PE56 and (ii) the competition of PE56 with complexes formed by HNF-1 and ABP 1/2 or ABP 7/8, it is clear that HNF-1 displays a similar affinity for PE56 and ABP 7/8 (box 4) but a weaker affinity for ABP 1/2 (box 1).

We next tested the capacity of the potential HNF-3 boxes 3 (oligonucleotides ABP 5/6) and 5 (oligonucleotides ABP 9/10) to inhibit the binding of HNF-3 to its described binding site in the human TAT promoter (TATHNF3 probe) (14). In HepG2, but not in HeLa extracts, HNF-3 bound TATHNF3 as a doublet which possibly corresponds to HNF-3α and HNF-3β (30, 31) (Fig. 6B, left panel). The oligonucleotides ABP 5/6 (box 3), but neither the oligonucleotides ABP 9/10 (box 5) nor an albCCAAT oligonucleotide, competed with this HNF-3/TATHNF3 binding. When used as a probe, the oligonucleotides ABP 5/6, but not the oligonucleotides ABP 9/10, displayed a shifted band with the HepG2 nuclear extract. This band was seen as a doublet (Fig. 6B, middle panel) whose migration was identical to the HNF-3/TATHNF3 doublet.
Finally, we failed to confirm the tentative assignment of boxes 2 and 6 as HNF-4 binding sites. The corresponding oligonucleotides ABP 3/4 (box 2) and ABP 11/12 (box 6) were both unable to displace the binding of an HepG2 nuclear protein to a functional PKHNF4 probe (15) (Fig. 6C, left panel). Furthermore, when used as radioactive probes they failed to form a complex of the HNF-4 type. The complex they formed with a nuclear protein present in HepG2, H5, and HeLa cell lines (Fig. 6C, middle and right panels) was nonspecific as it was not displaced by any of the competitors tested, including the homologous oligonucleotides.

**DISCUSSION**

Although several reports have dealt with the sequence and organization of the human ABP gene and its 5’-flanking region (8, 9), no functional analysis of the ABP gene promoter has yet been reported. Given the strong variations in bikunin mRNA in pathological states, and the potential influence of the bikunin level on inter-α-inhibitor and pre-α-inhibitor levels in plasma, we have been interested in clarifying which cis-acting elements (CAEs), trans-acting factors, and mediators drive ABP gene expression. A computer-aided search for the presence of CAEs in the 5’-flanking sequence of the ABP gene provided us with a large series of putative CAEs (Fig. 1). Investigating each potential CAE was beyond the task of this work and hence a number of 5’ deletion mutants subcloned in a CAT expression vector were used to localize the major CAEs. A basal weak promoter activity required one or more elements located between −345 and −247, possibly including a putative CCAAT box at −316 and/or a putative HNF-4 box at −306 (detailed in Table 1). The activity of the minimal ABP promoter is not tissue-specific as it reached a similar level in various cell lines when compared with pTKCAT activity (Fig. 5). A proximal negative regulatory element located in the −692 to −349 area abolishes this basal ABP promoter activity. As several constructs (p-433/57CAT to p-692/57CAT in Fig. 2) were required to progressively abolish this activity, this negative element likely covers a rather broad area, approximately from −600 to −349. Linker scanning mutations could eventually provide the location(s) of the
Fig. 6. Electrophoretic mobility shift of oligonucleotide/nuclear protein complexes. The labeled double-stranded oligonucleotides used for nuclear factor binding are indicated below the lanes and the competitor oligonucleotides are listed above the lanes. The cell lines used (H411, H5, HeLa, or HepG2, see text) are also indicated above the lanes. A, HNF-1 binding sites. PE56 is a reference probe with a known HNF-1-binding site (25). Variable amounts (0-20 ng) of competitors were used. The migration of PE56 complexed with a variant form of HNF-1, v-HNF-1 (also designated LF-B3 or HNF-1β, Ref. 31), is also indicated. B, HNF-3 binding sites. TATHNF3 is a reference probe with a known HNF-3 binding site (14). No (0) or a fixed amount (20 ng) of competitor were used. C, HNF-4 binding sites. PKHNF4 is a reference probe with a known HNF-4 binding site (15). No (0) or a fixed amount (20 ng) of competitor were used.

negative element(s), but they are beyond the scope of the present study. This proximal negative element is probably not a silencer, since the corresponding DNA segment subcloned, in either orientation, into the pTK50 plasmid did not significantly decrease the activity of a minimal TK promoter (results not shown). Finally, the basal ABP promoter activity was not significantly exceeded or even fully restored until a large construct (p-2929/57CAT) containing the 5′-flanking sequence up to −2929 was tested. This suggests that among the potential CAEs found in the area extending from −693 to −2422 (Fig. 1 and Table I), none is functionally important, at least under the conditions used in this study (heterologous reporter gene, hepatoma cell, no hormone/cytokine induction). However, given the down-regulation of the ABP gene
during the acute inflammation response, further experiments will be necessary to clarify which of the numerous potential IL-6α-responsive sites (IL-6 DNA binding protein site) found in the APB 5'-flanking sequence are indeed functional under IL-6 induction.

The strong CAT activity observed with p-2929/57CAT could be explained by a distal grouping of liver-specific elements clustered within an enhancer. This was demonstrated with various CAT constructs in which this APB enhancer drove a heterologous TK or Factor IX promoter in an orientation- and location-independent fashion. This strong CAT activity was accounted for by an increased CAT gene transcription as judged from a Northern blot experiment (not shown). When the enhancer activity was studied in the context of its own APB gene promoter, the location of the transcription start site in the ABP/CAT construct was in agreement with the location previously found (+1) by a primer extension experiment made with human liver RNAs (8). Our results did not confirm another proposal for a transcription start site at +42 (9). Finally, the APB enhancer is tissue-specific, since among the cell lines tested, it was active only in an hepatoma cell line. By a computer-aided search, we have identified in the liver-specific enhancer six potential binding sites for hepatocyte nuclear factors. Specifically, two HNF-1, two HNF-3, and two HNF-4-binding boxes were present in the order HNF-1, -4, -3, -1, -3, -4 (boxes 1-6, 5') to 3'). Band shift experiments clearly demonstrated that boxes 1 (HNF-1), 3 (HNF-3), and 4 (HNF-1) do indeed bind their cognate nuclear factor, whereas we failed to show a specific binding for boxes 2, 5, and 6. It is worth noting that for each box this binding capacity, or lack thereof, did not obviously correlate with the extent of mismatch(es) between the APB gene sequence and the reference consensus (Table 1). It is likely that box 6 is indeed nonfunctional, since the enhancer capacity of the entire cluster (boxes 1-6) did not significantly differ from the enhancer activity of a narrowed cluster made of boxes 1-5 only, as judged from the CAT activities of constructs containing either a 348 bp- or 107-bp-long ABP segment, respectively (Fig. 5). In contrast, the lack of binding capacity of boxes 2 and 5 for HNF factors in band shift experiments should not be regarded as conclusive evidence that they are nonfunctional, for two reasons. First, a weak affinity of a given box for its cognate protein may have not been detected under our experimental conditions, but it may be of functional significance in vivo. Second, our band shift experiments were performed with probes and competitors which contained only a single ABP box. Thus, we did not allow for a possible cooperativity in binding of several transcription factors, a situation which may significantly determine the overall affinity of each factor for its DNA target (19, 32). Further experiments aimed at analyzing such a possible interplay of nuclear factors are currently in progress.

Some features of the transcription control sequences in the APB gene are similar to those found in many other liver-specific genes (30), including the presence of multiple CAEs which contribute a high level of expression in hepatocytes compared with other cells and the involvement of trans-acting factors that are expressed with a broader tissue distribution than the target gene (33). Recently, however, the liver-restricted transcription of a number of mammalian genes, particularly those coding for plasma proteins, have been studied. These genes are driven by the interplay of CAEs in their 5'-flanking region and usually are under the control of a strong promoter containing tissue-specific elements driven by HNF factors (e.g. Refs. 15, 28, 34, and 35). The APB gene is distinct from such genes, as its overall activity results from the rather unusual combination of a weak promoter of potential ubiquitous expression, enhanced by a remote cluster of liver-specific elements for HNF factors, an arrangement recently described also for the human transferrin gene (36).

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REFERENCES
1. Akerstrom, B., and Legnér, L. (1990) Trends Biochem. Sci. 15, 240–243
2. Nagata, A., Suzuki, Y., Igarashi, M., Suguchi, T., Honda, U., and Hayashi, O. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4029–4024
3. Goodwin, Z., and Zimmerman, J. (1990) Trends Biochem. Sci. 15, 64–66
4. Fresco, N., and Mendez, E. (1988) Biochim. Biophys. Res. Commun. 165, 1242–1249
5. Oehmichen, W., and Hochstrasser, K. (1986) in Proteinase Inhibitors (Barrett, A. J., and Salvesen, G., eds) pp. 389–401, Elsevier Science Publishers B. V., Amsterdam
6. Salier, J. P. (1990) Trends Biochem. Sci. 15, 435–439
7. Kauanme, J. P., Polazi, J. O., and Kotick, M. P. (1986) Nucleic Acids Res. 14, 7809–7830
8. Diarra-Mehrpour, M., Bourguignon, J., Seboue, R., Salier, J. P., Leveillard, T., and Martin, J. P. (1990) Eur. J. Biochem. 191, 133–139
9. Yeatman, T. H., and Gehardi, W. (1991) Biochem. Hoppe-Seyler 371, 1185–1196
10. Salier, J. P., Diarra-Mehrpour, M., Bourguignon, J., Benarous, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 84, 8277–8276
11. McKean, W. L., Suzuki, T., Hosti, H., and McKeehan, K. A. (1986) J. Biol. Chem. 261, 5378–5383
12. Trenche, F., Roller, A., Sourdiv, D., Cereghini, S., and Yaniv, M. (1991) J. Mol. Biol. 222, 411–420
13. Chouard, T., Blumenfeld, M., Bach, I., Vandekerckhove, J., Cereghini, S., and Yaniv, M. (1990) Nucleic Acids Res. 18, 5863–5868
14. Grange, T., Roux, J., Rignaud, C., and Frénet, R. (1991) Nucleic Acids Res. 19, 131–139
15. Raymond, J., Pichard, A. L., Gregori, C., Ginot, F., and Kahn, A. (1991) Nucleic Acids Res. 19, 6146–6153
16. Ham, J., Dostati, N., Arnos, F., and Yaniv, M. (1991) EMBO J. 10, 2931–2940
17. Salier, J. P., Hiroswa, S., and Kurachi, K. (1990) J. Biol. Chem. 265, 7062–7068
18. J. Mol. Biol. 1989, 20, 3–26
19. Oehmichen, W., and Hochstrasser, K. (1990) BioTechniques, in press
20. Raymond, J., Pichard, A. L., Gregori, C., Ginot, F., and Kahn, A. (1991) Nucleic Acids Res. 19, 6146–6153
21. Ramji, D. P., Tadros, M. H., Hardon, E. M., and Cortese, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3707–3711
22. Ramji, D. P., Tadros, M. H., Hardon, E. M., and Cortese, R. (1991) Mol. Biol. Cell. 2, 957–961
23. Ramji, D. P., Tadros, M. H., Hardon, E. M., and Cortese, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3707–3711
24. Ramji, D. P., Tadros, M. H., Hardon, E. M., and Cortese, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3707–3711
25. Ramji, D. P., Tadros, M. H., Hardon, E. M., and Cortese, R. (1991) Mol. Biol. Cell. 2, 957–961