Identification and Characterization of Two Enhancers of the Human Albumin Gene*

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A 12.5-kilobase pair (kb) segment upstream of the human albumin gene was analyzed for transcription enhancing activity using transient transfection analysis, gel mobility shift assays, DNAse I footprinting, and site-specific mutagenesis. Two enhancer regions were identified, one 1.7 kb upstream of the transcription initiation site (E1.7) and the other 6 kb upstream (E6). In E1.7, a nuclear protein from HuH-7 hepatoma cells binds to an AT-rich sequence, GTTACTAAATGAC. Competition gel mobility shift assays suggested that this protein is HNF-1, which regulates the promoter of the albumin gene and several other liver-specific genes. A 60-base pair E1.7 fragment carrying the AT-rich sequence stimulates a heterologous (α-fetoprotein) promoter in a dose-dependent manner. In E6, a HuH-7 nuclear protein binds to a GT-rich sequence, TGTGGGC. A 27-base pair E6 fragment carrying this sequence is able to stimulate the SV40 promoter in an orientation-independent manner. An alteration of this sequence by site-specific mutagenesis resulted in the loss of transcriptional activity as well as binding to the HuH-7 nuclear protein. Competition gel mobility shift assays showed that homologous elements exist in the albumin promoter. These results show that the promoter and enhancer of the human albumin gene are regulated by two common transcription factors through two shared cis-acting elements, one AT-rich and the other GT-rich.

Studies of transcription of the mouse and rat albumin genes in vitro and in vivo have shown that the 170-bp1 region immediately upstream of the transcription initiation site is sufficient for tissue-specific expression of the albumin gene (Ott et al., 1984; Gorski et al., 1986; Cereghini et al., 1987; Heard et al., 1987; Babias et al., 1987). Izban and Fapaconostinou, 1989; Maire et al., 1989). At least six cis-acting elements have been identified in this region that interact with various transcription factors including HNF-1 (LF-B1, PAF, AP1), C/EBP, DBP, CTF/NF1, and NFY (Lichtsteiner et al., 1987; Johnson, 1990; Tronche et al., 1990). The corresponding region of the human albumin gene shows a 90% sequence identity and therefore is likely to be regulated by the same DNA-binding proteins.

Enhancers of the rodent albumin genes have been shown to exist far upstream (~0.5 to 8.5 kb) of the transcription initiation site (Pinkert et al., 1987; Herbst et al., 1989). In contrast, the human albumin enhancer identified so far has been mapped adjacent to the promoter (~486 to ~221 bp) (Prain et al., 1990). This difference in the position of the enhancers between the rodent and human albumin genes is at odds with the striking similarity of their promoters. In this study, we examined whether additional enhancers exist further upstream of the human albumin gene. We report here the localization of two enhancers in upstream regions (~1.7 and ~6 kb) and the identification of a nucleotide element responsible for the enhancer activity in each region. These elements are also found in the albumin promoter, indicating that two common factors participate in the regulation of the enhancer and promoter of the human albumin gene.

**Experimental Procedures**

Cell Cultures—The human hepatoma cell line HuH-7 was maintained in a chemically defined medium, IS-RPMI (Nakabayashi et al., 1984). HeLa cells were grown in IS-RPMI containing 5% fetal calf serum.

Construction of CAT Plasmids—To construct pAL1.6-CAT, a 1.6-kb HindIII fragment (~1464 to +17 kb) was isolated from pHAL-HA1-Hind-Eco3.5 (Urano et al., 1986). HindIII linkers were attached and inserted into the HindIII site of pBR-CAT. To construct pAL3.0-CAT, a HindIII-SstI fragment (~3 to ~1.1 kb) from pHAL-HA1-Hind-Eco3.5 was inserted into pAL1.6-CAT to replace the albumin 5’-flanking sequence from ~1.6 (HindIII) to ~1.1 (SstI) kb. To construct pAL12.5-CAT, the 1-kb EcoRI-SstI fragment from ~1.1 to ~2 kb in pAL1.6-CAT was replaced by SstI and partially with EcoRI to remove an 11.4-kb fragment from ~1.1 to ~12.5 kb of the albumin 5’-flanking sequence. The SstI linker was attached to the EcoRI site and religated. To construct pAL12.5-CAT, pAL12.5-CAT was digested completely with SstI and partially with EcoRI to remove a 5.7-kb fragment from ~10.8 to ~5.1 kb of the albumin 5’-flanking sequence and religated. To construct pAL12.5-CAT, pAL12.5-CAT was partially digested with SstI to remove a 9.7-kb fragment from ~10.8 to ~11.1 kb of the albumin 5’-flanking sequence and religated.

Five CAT plasmids carrying progressively shorter albumin 5’-flanking sequences from the HindIII site at ~3 kb (pAL2.6-CAT, pAL1.9-CAT, pAL1.8-CAT, pAL1.6-CAT, and pAL1.5-CAT) were prepared as follows. pAL5.0-CAT was linearized at the ClaI site, and

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1 The abbreviations used are: bp, base pairs; kb, kilobase pairs; CAT, chloramphenicol acetyltransferase; AFP, α-fetoprotein; HEPES, N-2-hydroxyethylpiperazine-N’2-ethanesulfonic acid.
the ends were filled in by the Klenow fragment of DNA polymerase I and then treated with exonuclease III. Aliquots of the reaction mixture were removed at several time points, treated with mungbean nuclease to form blunt ends, and digested with SstI. The smaller fragments released were ligated to pAL0.3-CAT prepared from pSV1'-CAT in normal and reverse orientations with Clal and SstI to remove the -3 to -1.1-kb albumin 5'-flanking DNA.

CAT plasmids containing the 168-bp AFP promoter and one or three copies of E1.7 enhancer at -1.7 kb (see below) were constructed as follows. pAF0.17[Bg]I-CAT (Nakabayashi et al., 1987) was cleaved by BglII and blunt-ended by the Klenow fragment of DNA polymerase I. One or three copies of the 60-bp fragment (3796 to 3767 bp) of E1.7 were ligated to form pAF0.17[Bg]I-[E1.7],-CAT or pAF0.17[Bg]I-[E1.7]-CAT, respectively.

pSV1'-CAT contains the SV40 TATA box and three 21-bp repeats, but only 30% of the 72-bp repeat (Watanabe et al., 1987). pBR-CAT contains the CAT-coding sequence and the SV40 polyadenylation signal, but no upstream regulatory sequences (Watanabe et al., 1987). Other CAT plasmids used in this work have been described previously (Watanabe et al., 1987; Sawadaishi et al., 1988; Nakabayashi et al., 1988).

Cell Transfection and CAT Assays—HUH-7 cells were transfected with 20 μg of plasmid DNA/75-cm² flask using the calcium phosphate precipitation method (Graham and van der Eb, 1973). Two days later, cells were harvested and lysed by five cycles of freezing and thawing. The lystate was heated at 60°C for 10 min and centrifuged at 15,000 rpm for 5 min, and the supernatant was removed to measure CAT activity as described previously (Watanabe et al., 1987).

Gei Mobility Shift Assays—Partial purification of nuclear extracts and gel mobility shift assays were conducted as described previously (Sawadaishi et al., 1988). The nuclear extract was preincubated with 5 μg of poly(dI-dC)-poly(dI-dC) for 10 min on ice and then incubated with 0.5 μg of Gei-labeled with 32P in 10 mM Tris-HCl (pH 7.5), 45 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 8% glycerol. The reaction mixture was electrophoresed on a 6% polyacrylamide gel containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. The gel was dried and autoradiographed at 70°C.

For competition experiments, a 100- or 200-fold molar excess of competitor DNA was preincubated with nuclear extracts for 5 min before the addition of end-labeled DNA.

DNase I Footprint Analysis—DNase I footprint analysis was conducted as described before (Sawadaishi et al., 1988). The reaction mixture contained HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl2, 0.12 mM EDTA, 0.3 mM dithiothreitol, 12% glycerol, 1 μg of poly(dI-dC)-poly(dI-dC), 100 or 200 μg of nuclear proteins, and 10 nmol of DNA end-labeled with 32P (50,000 cpm) in a total volume of 50 μl. This mixture was incubated on ice for 30 min and then at 25°C for 1 min with 1 μg of DNA/ml DNase I. The reaction was stopped by the addition of EDTA (final concentration, 15 mM). DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and electophoresed on an 8% polyacrylamide sequencing gel.

RESULTS

Localization of Two Enhancer Regions between -10.8 and -1.1 kb of Human Albumin Gene—Frain et al. (1990) have reported the presence of an enhancer at -486 to -221 bp of the human albumin gene that stimulates the albumin promoter ~5-fold in Hep3B cells. In our assays using Huh7 cells, however, this region exhibited little enhancer activity, possibly reflecting the difference in cell lines used. To examine whether additional enhancer activities are associated with further upstream regions, transfection assays using the CAT reporter gene were conducted in Huh7 cells. Initial analysis showed that there was no enhancer activity in the region between -1.1 and 0.5 kb. To quickly detect enhancer activity that might be present upstream of -1.1 kb, we linked the CAT gene to 12.5 kb of the 5'-flanking sequence with or without internal deletions between -10.8 and -1.1 kb (Fig. 1A). Analysis of CAT expression in Huh7 cells showed that the intact 12.5-kb fragment supported 10- to 20-fold higher CAT expression than the 1.1-kb fragment (Fig. 1B, cf. lanes 1 and 5). The deletion from -10.8 to -5 kb (pAL12[Δ1]-CAT) resulted in an ~50% decrease in transcription stimulatory activity (Fig. 1B, cf. lanes 1 and 2). The deletion from -5.1 to -1.1 kb (pAL12[Δ2]-CAT) also resulted in an ~50% decrease in CAT activity (Fig. 1B, lane 3). The deletion from -10.8 to -1.1 kb (pAL12[Δ3]-CAT) resulted in the complete loss of transcription stimulatory activity (Fig. 1B, lane 4). These results suggest that at least two enhancer regions exist between -10.8 and -1.1 kb, one between -10.8 and -5.1 kb and the other between -5.1 and -1.1 kb.

Delimitation of Enhancer Activity Present between -5.1 and -1.1 kb—To determine whether the 5'-half of this region contains the enhancer activity, we compared CAT activities supported by 3 and 5 kb of the albumin 5'-flanking DNA. No significant differences were observed, indicating that the enhancer is not present between -5 and -3 kb (Fig. 2). To localize the enhancer activity between -3 and -1.1 kb, we constructed CAT plasmids containing progressively shorter fragments from -3 kb and analyzed for their ability to support CAT expression. No significant changes in CAT activity were observed by reducing the size from 3 to 1.8 kb (Fig. 2). However, further deletion of -200 bp from -1.8 kb (1867 to -1647 bp) (pAL1.6-CAT) resulted in a 60% decrease in CAT activity. Additional reduction in size to 1.1 kb had little effect on CAT activity. These results show that an enhancer is present between -1867 and -1647 bp. This enhancer region is referred to as E1.7. The nucleotide sequence of E1.7 is shown in Fig. 3A.

Identification of Enhancer Element in E1—I—To determine a regulatory element(s) responsible for the enhancer activity of E1.7, we conducted DNase I footprint analysis. The results showed that Huh7 nuclear proteins bind to a 27-bp region from -1796 to -1770 bp in E1.7 (Fig. 4). The sequence 5'-TTGTTACTATAATGGCAA-3' contained in the protection region is similar to the HNF-1-binding site that is present in the albumin promoter (Table I) (Courtous et al., 1987, 1988; Hardon et al., 1988; Sawadaishi et al., 1988). To test whether HNF-1 in fact binds to the E1.7 element, we conducted competition gel mobility shift assays using an albumin promoter fragment (~90 to +17 bp) carrying the HNF-1 site as a competitor. The binding of protein to E1.7 was effectively prevented by this competitor (Fig. 5). A distal albumin promoter fragment (~275 to -91 bp) that lacks the HNF-1-binding site had no effect.

To test whether the HNF-1 binding sequence in E1.7 has transcription stimulatory activity, we inserted one or three copies of the 60-bp fragment carrying this element (~1796 to -1737 bp) to the 169-bp AFP promoter that is fused to the CAT gene (Fig. 6A). In Huh7 cells, one and three copies of the enhancer fragment led to 2- and 5-fold increases in CAT activity, respectively (Fig. 6B), whereas no significant CAT expression was observed in HeLa cells (data not shown). These results show that the HNF-1 binding sequence is responsible for the enhancer activity of E1.7.
Fig. 1. Transient transfection analysis of enhancer activity between -10.8 and -1.1 kb upstream of human albumin gene. A. CAT plasmids carrying a 12.5-kb albumin 5' flanking sequence with and without internal deletions. Dotted lines indicate deleted segments. B. CAT activity expressed in HUH-7 cells transfected with the plasmids described in A. Lane 1, pAL12.5-CAT; lane 2, pAL12[A1]-CAT; lane 3, pAL12[A2]-CAT; lane 4, pAL12[A3]-CAT; lane 5, pAL1.1-CAT. Cm, chloramphenicol; 1-Ac, 1-acetate chloramphenicol. Results represent the mean ± S.E. for three separate experiments.

Fig. 2. Transient transfection analysis of enhancer activity between -5 and -1.1 kb upstream of albumin gene. CAT expression activity in HUH-7 cells from each plasmid is shown as percentage of that of pAL5.0-CAT. Results represent the mean ± S.E. for three separate experiments.

Fig. 3. Nucleotide sequences of E1,7, E6, and mutant E6. A, E6. The AT-rich element (HNF-1 binding site) is underlined. Numbers indicate the positions relative to the transcription initiation site. B, E6. The GT-rich element is underlined. Numbers indicate the positions from the 5' end of the 81-bp E6 sequence. C, mutant 27-bp E6 fragment. Asterisks indicate four altered nucleotides.

fragment supported CAT expression to the same level as the 757-bp fragment (Fig. 8A, lane 5), whereas the 260-bp fragment showed little enhancer activity (lane 6). To further delimit the enhancer activity, the 497-bp fragment was digested with HindIII and HindII to yield three fragments of 174, 242, and 81 bp (Fig. 7). The enhancer activity was found to be associated with the 81-bp fragment (Fig. 8B). The 81-bp fragment was also able to stimulate the SV40 early promoter in either normal or reverse orientation in HUH-7 cells (Fig. 9, A and B). In HeLa cells, on the other hand, no enhancer activity was observed (Fig. 9C), indicating that the element contained in the 81-bp fragment is cell-specific. This enhancer region is termed E6 and its nucleotide sequence is shown in Fig. 3B.

Identification of Enhancer Element in E6—Inspection of the nucleotide sequence of E6 revealed the presence of a sequence (GCCAAAAC) that is the reverse complement of a protein-binding site (5'TGTGTTGGC3') in the albumin promoter (Cereghini et al., 1987; Godbout et al., 1988), hepatitis B virus enhancer (Shaull and Ben-Levy, 1987), and domain A of the human AFP enhancer (~3.8 kb) (Table 1). Competition gel mobility shift assays showed that the binding of HUH-7 nuclear proteins to E6 was effectively prevented by fragments of the albumin enhancer, hepatitis B virus enhancer, and AFP enhancer domain A carrying the GT-rich element (Fig. 10). This suggests that the same factor regulates all these regulatory regions.

To confirm that the GT-rich element is responsible for the enhancer activity of E6, we changed four nucleotides in the GT-rich region and analyzed for the ability to support CAT expression and to bind to HUH-7 nuclear proteins. The results show that the normal 27-bp E6 fragment was active in supporting CAT expression, whereas the same fragment carrying

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the mutated GT-rich sequence was not (Fig. 11A). Similarly, the mutant sequence failed to compete with the wild-type sequence for binding to HuH-7 nuclear proteins (Fig. 11B). These results show that the GT-rich sequence is the enhancer element in Eα.

**DISCUSSION**

In this study, we have defined two enhancers that are present 1.7 (Eα) and (Eβ) 6 kb upstream of the human albumin gene. Together with the enhancer proximal to the promoter (−486 to −221 bp) (Frain et al., 1990), the human albumin gene contains at least three enhancers in the 5′-flanking region. We have identified an AT-rich sequence (HNF-1-binding site) as the element responsible for the enhancer activity of Eα. Similar elements have been shown to exist in the proximal enhancer (−358 to −342 bp) (Frain et al., 1990) as well as in the promoter (−65 to −49 bp) of the human albumin gene (Table I). These results indicate that HNF-1 functions as a common factor regulating the promoter and two enhancers (proximal and Eα) of the human albumin gene. The AT motif is also shared by the enhancer and promoter elements in the human albumin gene. The AT motif is also shared by the enhancer and promoter elements in the human albumin gene (Table I). The AT motif is also shared by the enhancer and promoter elements in the human albumin gene (Table I).
Fig. 7. DNA fragments between −10.8 and −5.1 kb of albumin 5′-flanking sequence tested for transcription enhancing activity. Each fragment (box) was linked to the CAT gene in pAL12[Δ3]-CAT and transfected into HUH-7 cells. Numbers in the boxes indicate the size of the fragments in base pairs.

In the case of rodent albumin and AFP genes, the AT motif appears to be a characteristic feature of the human albumin promoters. However, in the case of AFP gene, there is a difference between humans and mice with respect to the distribution of GT-rich elements as competitors. Competitor DNA fragments containing TGTTTAG/G/ACT sequences are the human albumin promoter (Alb pro) (−243 to −115 bp) (lanes 3 and 4), hepatitis B virus (HBV) enhancer (943 to 1109 bp) (lanes 5 and 6), and AFP enhancer domain A (78 bp) (lanes 7 and 8). A 15-bp synthetic oligonucleotide corresponding to the AFP1 (HNF-1)-binding site in the human AFP enhancer domain B (Sawadaishi et al., 1988) was used as a competitor without the GT element (lanes 9 and 10). 100 and 200 indicate 100- and 200-fold molar excesses of competitor DNA, respectively. + and − indicate the presence and absence of HuH-7 nuclear proteins or competitors, respectively.

The E3 element (TGTTTAG) is present in the enhancer at −10 kb and binds to a mouse hepatocyte nuclear protein, eH-TF (Zaret et al., 1990). Two homologous sequences are also present in the mouse albumin promoter (Table 1). Thus, as far as GT-rich elements are concerned, the human and mouse albumin genes are similar in that these sequences are shared by the enhancers and promoters.

In the case of the AFP gene, there is a difference between humans and mice with respect to the distribution of GT-rich elements. In mice, AT-rich sequences are present in the promoter and two of the three enhancers (Table 1) (Godbout et al., 1988), whereas in humans, it is present in the enhancer, but not in the promoter.

What is the significance of the presence of AT- and GT-rich elements in the enhancer and promoter? We have shown that multimerization of the enhancer AT-rich element results in an increase in transcription stimulatory activity (Fig. 6). Similarly, Lichtsteiner and Schibler (1989) have reported that a synthetic promoter containing two B elements (albumin promoter HNF-1-binding site) is strongly activated by purified HNF-1 in vitro. It has been shown that a protein bound
to a cis-acting element often facilitates the binding of a second protein to another site and that factors bound far apart can interact, leading to transcriptional enhancement. However, in some cases, the presence and absence of HuH-7 nuclear extracts or competitors, respectively, may differ. In addition, HNF-1 has been shown to recognize DNA elements as a dimer (Courtois et al., 1990; De Simone et al., 1991; Rey-Campos et al., 1991; Mendel et al., 1991); and consequently, it can exhibit different binding or activation potential depending on the partner protein that it dimerizes (Mendel and Crabtree, 1991; Mendel et al., 1991). It is possible that HNF-1 and the partner factors change in the relative amount during liver development. This, combined with the presence of AT-rich elements in both the promoter and enhancer, would allow a wide range of expression of the albumin and AFP genes in developing liver.

At present, little is known about the protein(s) that regulates GT-rich elements. Some of the GT-rich elements so far identified exhibit a dyad symmetry, suggesting that they are also regulated by dimerization of factors. The exact mechanism of regulation by GT-rich elements and the significance of their presence in both the promoter and enhancer must await the isolation and characterization of the binding protein(s).

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