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Transcriptional Control of the Mouse Prealbumin (Transthyretin) Gene: Both Promoter Sequences and a Distinct Enhancer Are Cell Specific

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The mouse genomic clone for the prealbumin (transthyretin) gene was cloned, and its upstream regulatory regions were analyzed. The 200 nucleotides 5′ to the cap site when placed within a recombinant plasmid were sufficient to direct transient expression in HepG2 (human hepatoma) cells, but this DNA region did not support expression in HeLa cells. The sequence of the 200-nucleotide region is highly conserved between mouse and human DNA and can be considered a cell-specific promoter. Deletions of this promoter region identified a crucial element for cell-specific expression between 151 and 110 nucleotides 5′ to the RNA start site. A region situated at about 1.6 to 2.15 kilobases upstream of the RNA start site was found to stimulate expression 10-fold in HepG2 cells but not in HeLa cells. This far upstream element was invertible and increased expression from the β-globin promoter in HepG2 cells. Unlike the simian virus 40 enhancer, the prealbumin enhancer would not stimulate β-globin synthesis in HeLa cells, and even the simian virus 40 enhancer did not stimulate the prealbumin promoter in HeLa cells. Thus, we identified in the prealbumin gene two DNA elements that respond in a cell-specific manner: a proximal promoter including a crucial sequence between −108 and −151 nucleotides and a distant enhancer element located between 1.6 and 2.15 kilobases upstream.

The molecular events that control individual vertebrate genes and coordinated groups of genes during development are being widely studied in a variety of specific cell types, e.g., liver (1, 1a, 10, 45), pancreas (18, 44, 58, 62), lens (46), lymphocytes (2, 22, 49), etc. In such studies the liver offers advantages in providing a ready source of material in which a single cell type, the hepatocyte, makes up 90% of the tissue mass (25, 26). Coordinate gene function is also prominently illustrated in the liver where, for example, the majority of the many different serum proteins are produced and secreted. From a cDNA library, bacterial plasmid-containing clones were selected that are complementary to individual mRNAs found mainly or only in liver (14, 48). The basis for the liver-specific distribution of these mRNAs was found to be transcriptional activation either exclusively in the liver or at a higher rate in liver than in other tissue (14, 48).

Sequencing of these mouse liver-specific cDNA clones has provided identification of the encoded proteins in several instances (32, 33). One of these cDNAs encodes the majority of the mRNA for prealbumin (transthyretin [16, 40, 57]). This polypeptide (14 kilodaltons) exists as a tetramer and serves as a carrier for thyroxine (13, 29, 51). It also functions indirectly to transport vitamin A by binding to retinol-binding protein (42, 47, 50). The protein has been crystallized and its structure solved at 0.18-nm resolution (5). The protein is formed not only in the liver but in the choroid plexus where it is secreted into the cerebrospinal fluid (11, 16, 30). A mutation in the prealbumin gene resulting in a single amino acid change is apparently the cause of a dominant genetic disease in humans called familial amyloidotic polyneuropathy (17, 30, 53, 59). Full or partial cDNA copies of human, rat, and rabbit mRNA have been reported (16, 40, 57), and several genomic human clones have been described (54, 61).

In this paper we describe the use of the cDNA complementary to the mouse prealbumin mRNA to select genomic clones. We determined the DNA sequence elements proximal to the cap site within these genomic clones that allow transcriptional specificity of this gene in hepatoma cells and also identified a region situated between −1.6 and −2.15 kilobases (kb) that is responsible for a high rate of cell-specific transcription. The upstream sequence can increase the expression from the prealbumin proximal promoter element in either orientation and at variable distances from the cap site and can stimulate the expression from the β-globin gene in hepatoma cells. We demonstrate that this element does not activate the β-globin promoter in HeLa cells and therefore is a cell-specific enhancer (2–5). This is only the second cell-specific enhancer located at a distance from the RNA start site to be described for a mammalian gene, the first being for immunoglobulins in lymphocytes (2, 22, 49).

MATERIALS AND METHODS

Selection of mouse prealbumin genomic clones. The prealbumin-isolated cDNA insert (100 ng) was radioactively labeled by the oligolabeling technique with random hexadeoxynucleotides (Pharmacia Fine Chemicals, Piscataway, N.J.), 100 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and *Escherichia coli* DNA polymerase I large Klenow fragment (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described by Feinberg and Vogelstein (20). This mouse prealbumin cDNA probe (5×10^8 cpm/μg) was used to select a mouse genomic clone from an *Mbo*I partial BALB/c mouse λ library (cloned in the *Bam*HI site of Charon 28 λ arms; gift from J. Ravitch, Memorial-Sloan Kettering Cancer Center) by the methods described by Maniatis et al. (36). The screening of this mouse λ bacteriophage library (10^6 plaques) yielded one prealbumin-specific genomic clone which contained the entire gene including 310 nucleotides of 5′-flanking sequence. A second library was constructed by ligating size-frac-

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tionated *Mbo*I partially digested mouse DNA (129 strain; gift from M. Wilson, Scripps Clinic and Research Foundation) into *Bam*HI- and *Eco*RI-cleaved EMBL 3 bacteriophage λ arms (Stratagene Cloning Systems). The ligated products were packaged in vitro as described by the manufacturer (Stratagene Cloning Systems) and yielded 1.5×10^6 recombinant λ phage. Here, 7×10^5 recombinant plaques from this unamplified λ library were screened with a radioactive probe obtained from the prealbumin genomic clone specific to the 5′ end of the gene (*Taq*I-*Eco*RI restriction fragment containing sequences between positions +37 and +450) and yielded several genomic clones containing between 8 and 12 kb of upstream sequences.

Sequencing of prealbumin cDNA and promoter region. The prealbumin cDNA was sequenced by the chemical cleavage of end-labeled DNA as described by Maxam and Gilbert (37). The prealbumin promoter region and exons were cloned into either gemini 1 or 2 (Promega Biotech, Madison, Wis.), and two different 15-nucleotide synthetic oligomers were made corresponding to the sequence of the Sp6 and T7 promoters on this vector. The supercoiled prealbumin subclone plasmids were denatured and hybridized with one of the synthetic oligomers and sequenced by the chain termination sequencing method described by Sanger et al. (52).

Localization of prealbumin promoter. The location of the coding regions near the 5′ end of the gene was accomplished by hybridizing 5′-specific restriction fragments from the prealbumin cDNA, labeled by extending random oligonucleotides (20), to Southern blots (56) containing various restriction digests of the genomic clone. S1 nuclease and exonuclease VII analysis of 5′-end-labeled genomic fragments (4, 63) allowed a more precise positioning of these exons. Comparison of the genomic DNA sequence with the cDNA sequence along with the splice donor and acceptor consensus sequences (41) permitted the intron-exon boundaries to be defined. Finally, the cap site was positioned by primer extension of a 5′-end-labeled synthetic oligomer hybridized to liver RNA isolated by the method described by Chirgwin et al. (9). The oligomer was 5′ end labeled (37) with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) and [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp.) and hybridized with 1 μg of liver poly(A)$^+$ RNA in 5 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–400 mM NaCl–1 mM EDTA for 3 h at either 50 or 55°C. The hybridized products were diluted to 50 μl in reverse transcription buffer (36) and extended with 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). The products were analyzed on a 15% acrylamide gel containing 8 M urea as described by Maxam and Gilbert (37).

Construction of prealbumin expression plasmids. All procedures involving manipulations of DNA restriction fragments for constructing the expression plasmids were done by methods described by Maniatis et al. (36). The prealbumin minigene was constructed by inserting the restriction fragment spanning the *Bgl*II (−329) to *Hin*dIII (+1200) site (*Hin*dIII site is located 3 nucleotides downstream from exon 2) into the *Hin*dIII-*Bam*HI sites of gemini 1. Then a 239-nucleotide *Sau*3A-*Bam*HI restriction fragment isolated from pSV2CAT (24) which contains 186 base pairs (bp) of 3′ exon and the sequences necessary for poly(A) addition from the simian virus 40 (SV40) T antigen (27) was inserted at the *Bam*HI site located in exon 2 (25 bp 5′ to the splice donor site). To construct a more universal expression plasmid, the restriction fragment spanning the *Eco*RI site (+450) in prealbumin intron 1 to the *Sph*I site in gemini 1 (2475) was extracted from the −329 prealbumin construct and inserted into the corresponding sites in gemini 2. This resulted in a plasmid which contained a polylinker 5′ to both the *Eco*RI site in intron 1 and the prealbumin minigene with its second exon fused to the SV40 poly(A) (designated as SV40 poly). The SV40 poly was utilized to make the prealbumin constructs −108 (*Hin*cII), −1400 (*Pst*I), and −3000 (*Eco*RI) by isolating the restriction fragment from the *Eco*RI site (+450) to the indicated restriction site upstream and placing it in the corresponding site in this SV40 poly vector.

The prealbumin enhancer constructions were made by removal of DNA restriction fragments, using restriction sites located within the region spanning 3 kb upstream of the cap site. The *Nco*I (−2.15 kb) deletion was constructed from the *Eco*RI (−3 kb) prealbumin expression plasmid by removing the *Eco*RI-*Nco*I restriction fragment. This was accomplished by digesting the plasmid with *Nco*I and *Sma*I (*Sma*I cleaves in the gemini polylinker) and repairing the recessed ends with Klenow fragment of DNA polymerase I followed by religation of the plasmid. The *Sst*I-*Bgl*II deletion of the *Nco*I prealbumin plasmid and the construction of the *Sst*I (−1.6 kb) enhancer deletion involved steps similar to those described above. The *Nco*I-to-*Pst*I deletion of the *Eco*RI expression plasmid was constructed by complete digestion with *Nco*I followed by a partial digestion with *Pst*I. The recessed DNA ends were repaired with the Klenow fragment and religated and screened for the proper plasmid.

The prealbumin deletion mutants were made from the −329 construct which was linearized at the *Sma*I site in the polylinker and digested with BAL 31 exonuclease (Boehringer Mannheim Biochemicals) by the methods described by Legerski et al. (34). The digested ends were repaired by the Klenow fragment of *E. coli* polymerase I. The fragment extending from the *Eco*RI (+450) site to the end of the deletion was inserted in the correct orientation by using the *Eco*RI and *Sma*I sites of SV40 poly. The endpoint nucleotide for all the prealbumin deletions was determined by DNA sequencing. The enhancer fragment *Eco*RI (−3000) to *Bgl*II (−329) was subcloned in gemini 1, and each of the different deletions containing the entire 3′ end of the minigene was inserted in the correct orientation. This was accomplished by cloning several *Xba*I to *Hin*dIII fragments, each of which contained a different prealbumin promoter deletion along with the minigene, into the corresponding sites of the prealbumin subclone (*Eco*RI-*Bgl*II). The enhancer in the reversed orientation was constructed by the addition of *Xba*I linkers to the *Eco*RI-*Bgl*II fragment and screened for the insertion of this fragment in the opposite orientation in the −329 prealbumin SV40 poly construct.

The mouse β-globin-SV40-E1B construction was a gift from J. Friedman and L. Babiss and is similar to the plasmid used to make an adenovirus vector containing the mouse β-globin promoter (21), with the addition of the SV40 enhancer at the *Hin*dIII site at −341 nucleotides 5′ to the β-globin cap site. The β-globin promoter construction (−341) was also used as a control for the level of β-globin expression lacking enhancer simulation. Addition of *Hin*dIII linkers to the prealbumin *Eco*RI-*Bgl*II (−3000 to −329 bp) enhancer fragment after repair of the sticky ends allowed the insertion of this enhancer next to the β-globin promoter. Finally, the *Hin*dIII restriction fragment containing the SV40 enhancer was placed in the corresponding site at the 3′ end of the −329 prealbumin minigene.

Transfection and T2 RNase analysis. Human hepatoma cells (HepG2 [31]) were grown in Hams F12 medium

(GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Whittacker M.A. Bioproducts). HeLa cells were grown in Dulbecco modified eagle medium (GIBCO) supplemented with 10% fetal calf serum. The plating of the cells and the formation of the calcium phosphate DNA coprecipitate were performed as described previously (8, 12).

Transient expression from the prealbumin constructs was analyzed by T2 RNase digestion of hybrids between a specific labeled RNA probe and cytoplasmic RNA from transfected cells. Cytoplasmic RNA was isolated from the transfected cells by the Nonidet P-40 lysis method (36) 36 h after the removal of the precipitate. RNA probes used in the hybridization analysis were synthesized with either bacteriophage T7 or Sp6 RNA polymerase from gemini plasmids containing either prealbumin exon 1, the adenovirus E1B 3′ end, or the β-globin exons. The prealbumin exon 1 probe was synthesized with the T7 promoter from the prealbumin gemini 1 subclone consisting of the *Eco*RI (+450) to *Hin*cII (−108) restriction fragment which was linearized at the *Hin*cII site. The β-globin first-two-exons probe was constructed by cloning the *Bam*HI (+464) to *Hin*dIII (−341) restriction fragment into gemini 1 and was synthesized with the T7 promoter after the plasmid was linearized by *Hin*dIII. The E1B probe contained a restriction fragment spanning the *Bgl*II (3328) to *Pst*I (3781) sites in the E1B region of adenovirus cloned in the *Bam*HI-*Pst*I sites of gemini 1 plasmid and linearized with *Eco*RI to synthesize the RNA from the Sp6 promoter.

Labeled RNA probe was made (39) by incubating the appropriate linearized plasmid (1 μg) in a 10-μl reaction mixture which contained 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 20 mM dithiothreitol, 100 μg of bovine serum albumin (Bethesda Research Laboratories) per ml, 500 μM CTP, ATP, and GTP, 25 μM UTP, 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; New England Nuclear Corp.), 7 U of RNasin, and 7 U of either Sp6 or T7 bacteriophage RNA polymerase (Promega Biotech). After a 60-min incubation at 40°C, 2 U of RNase-free DNase I (Promega Biotech) was added, and the reaction was incubated for an additional 15 min. The reaction was terminated by the addition of 200 μl of extraction buffer (80 mM Tris [pH 8], 150 mM NaCl, 10 mM EDTA) followed by phenol-chloroform extraction and precipitation with 2 volumes of ethanol along with 10 μg of yeast carrier RNA (Sigma Chemical Co., St. Louis, Mo.). This reaction yielded 100 ng of RNA probe at a specific activity of 5×10^8 cpm/μg of RNA. The analysis of RNA from transfected cells involved the hybridization of 25 μg of cytoplasmic RNA with 0.5 ng of labeled RNA probe in 25 μl of 80% deionized formamide containing 20 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA at 60°C for 12 to 16 h. The temperature of hybridization for the RNA probes used here was varied to optimize the hybridization conditions. The hybrids were digested by the addition of 300 μl of 50 mM sodium acetate (pH 4.6)–100 mM NaCl–2 mM EDTA containing 60 U of T2 RNase (Bethesda Research Laboratories) per ml and incubated at 30°C for 2 h. The digestion was terminated by phenol-chloroform extraction and ethanol precipitated. The T2 RNase-resistant products were analyzed on a 8% polyacrylamide gel containing 8 M urea (37) followed by autoradiography.

RESULTS

Characterization of mouse prealbumin cDNA clone. Sequence analysis of several cDNAs previously isolated from mouse hepatocytes showed that pliv5 (14) contained sequences encoding the mouse prealbumin protein, beginning with the codon for valine (at position +75) and extending 537 nucleotides to the poly(A) segment. The DNA sequence of the coding region of the mouse prealbumin gene is 82% homologous to the human gene and 90% homologous to the rat gene. The amino acid sequence homology is higher—91% homology to the human and 96% to the rat amino acid sequence. Therefore, the prealbumin protein and gene are highly conserved during evolution. (The DNA sequence of the mouse prealbumin cDNA is not presented in this paper; however, it has been submitted to Genbank.)

Selection of mouse genomic prealbumin sequences. Using the prealbumin cDNA, several genomic clones were isolated from two different mouse genomic libraries made in bacteriophage λ after partial digestion of the DNA with *Mbo*I restriction endonuclease. A restriction map and the structures of the first two exons were determined (see below). The location of the exons within the genomic λ clones was identified by using radioactive cDNA segments that were prepared after restriction enzyme digestion of the DNA. The complementarity to labeled cDNA probes of known location within the coding region allowed the 5′ direction within the genomic clone to be determined and the 5′-end region in each λ clone to be located. The exons were positioned more exactly within the genomic regions by nuclease protection experiments with liver mRNA by using end-labeled DNA (4, 63) from restriction fragments originating from the genomic clones (data not shown). The DNA sequence of the first two mouse prealbumin exons matched exactly with the corresponding cDNA sequence, and these exons in the mouse DNA had a very similar structure to those in the human gene (54, 61).

The cap site of the prealbumin mRNA was localized by primer extension of a 24-bp synthetic oligonucleotide complementary to a sequence within exon 1 of the mRNA (the sequence is indicated in Fig. 1a). This oligomer was 5′ end labeled and hybridized at two different temperatures to 1 μg of poly(A)$^+$ RNA prepared from mouse liver. A major primer-extended product (Fig. 1b) was dependent on the presence of liver RNA in the reaction (compare lanes 1, 2, and C in Fig. 1b) and was 25 nucleotides longer than the primer. This result suggests that the cap site of the prealbumin mRNA could lie 25 bp 5′ to the oligomer sequence (indicated on the DNA sequence; Fig. 1a). A partial sequence analysis of the primer-extended, terminally labeled product confirmed that the 25-nucleotide extension matched the DNA sequence upstream of the oligomer sequence. Thus, the cap site was taken to be the indicated nucleotide shown in Fig. 1a. Also shown are the 329 bp of sequence upstream of the cap site in which is marked a TATA box (6, 43) and a CCAAT box (19, 43), DNA elements known to be required for RNA initiation in many genes (shown in bold type).

Sequence homology between mouse and human prealbumin promoters. The available prealbumin upstream sequence was compared with upstream regions of three other mouse genes that are transcribed only or mainly in the liver (albumin, α-fetoprotein, α-1-antitrypsin) and with the sequences of several other rat or human genes that are expressed mainly in the liver. This homology search did not reveal any long sequences in these regions in common. However, when the mouse and human prealbumin promoters were compared, a region of strong homology was observed that began about 290 bp upstream and extended to the cap site (Fig. 2; colons represent nucleotide identities between the two se-

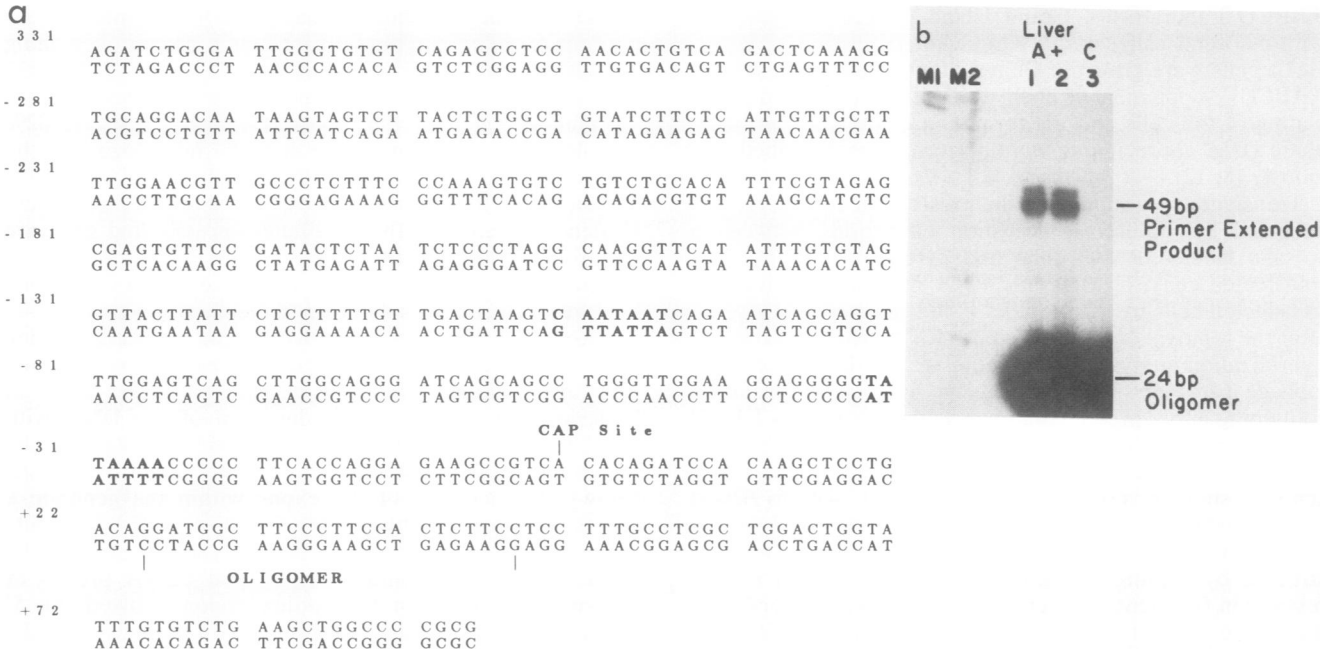

FIG. 1. DNA sequence at the 5′ end of the prealbumin gene and location of the cap site. (a) The double-stranded DNA sequence of the prealbumin promoter starting at 329 bp 5′ to the indicated cap site and extending to the end of exon 1. The oligomer that was used in the primer extension experiment described below to position the 5′ end of the mRNA is marked with vertical lines below the sequence. The consensus promoter elements beginning at −24 bp (TATA box) and at −94 bp (CCAAT box) are in bold-faced letters. (b) Primer extension of a 24-bp oligomer located in exon 1 hybridized to liver poly(A)$^+$ RNA. A primer extension was carried out with reverse transcriptase after hybridization of the kinase oligomer to liver RNA at two temperatures (0.4 M NaCl; 50°C, lane 1; 55°C, lane 2). Lane C is the primer extension experiment performed with yeast RNA as a control template. The primer-extended products (49 bp) were sized on a 15% denaturing polyacrylamide gel along with molecular weight standards (lane M1, 76 and 73 bp; lane M2, 76, 67, 34, and 26 bp).

quences). The greatest degree of homology in this region exists in the 190 bp 5′ to the cap site, where 84% identity was observed between the mouse and human prealbumin upstream regions. This homology is equal to the homology in the coding sequence of this gene. Thus, the 190 bp immediately upstream of the cap site were good candidates to function in the regulation of the prealbumin gene.

Prealbumin transient expression in HepG2 (human hepatoma) cells requires promoter-proximal sequences. To investigate the role of the upstream sequences in the expression of the prealbumin gene, we made plasmid constructions containing different amounts of upstream DNA sequence. These constructs took advantage of the structure of the first two exons of the prealbumin gene which is depicted at the top of Fig. 3a. A recombinant minigene was made by joining sequences near the end of prealbumin exon 2 to the 3′ end of the SV40 early gene containing the 3′ SV40 exon and sequences involved in polyadenylation (27). The various constructs were completed by joining different amounts of mouse DNA sequence 5′ to the prealbumin cap site with the recombinant prealbumin-SV40 segment (Fig. 3a, bottom). In addition, a plasmid was constructed which would be expressed in a variety of different cell types (1a) and used along with the prealbumin construction to control for transfection variability. As diagrammed in Fig. 3b, this control plasmid contained the β-globin promoter and the first two β-globin exons and ended with the adenovirus E1B 3′ exon and polyadenylation site. Active transcription from the β-globin promoter was ensured by including the SV40 enhancer 341 bp upstream of the β-globin mRNA cap site. This mouse β-globin-SV40-E1B construct could be used in small quantities along with test genes so that the efficiency of transfection for any particular experiment could be monitored. Transfections into human hepatoma (HepG2) cells and into HeLa cells were carried out by the calcium phosphate method (8, 12).

The expression from the prealbumin and control plasmid constructs was analyzed 36 h after transfection by isolating total cytoplasmic RNA from the transfected cells and hybridizing this RNA with labeled RNA probes prepared with either Sp6 or T7 RNA polymerase and ^{32}P-labeled UTP (39). The first probe contained a complementary sequence to the mouse prealbumin exon 1 and did not cross-hybridize under stringent hybridization conditions with any human endogenous mRNA. Therefore, the mRNA initiating from the transcription of the introduced gene could be analyzed after gel electrophoresis and autoradiography for the presence of a 93-nucleotide band corresponding to mouse exon 1. Using a second RNA probe, the expression of the β-globin-SV40-E1B control plasmid could be estimated by the presence of bands of 143 and 206 nucleotides corresponding to the two 5′ β-globin exons. (The scheme for the assays is given in Fig. 3.)

Four different constructs containing 3,000, 1,400, 300, or 110 bp of 5′-flanking prealbumin sequence were analyzed in this fashion. In the experiment (Fig. 4) three different DNA concentrations of each of the indicated constructs were cotransfected with a constant ratio of the control plasmid to obtain a DNA concentration in which the prealbumin signal was optimized. The control bands (206 and 143 bp) varied only slightly in this transfection experiment; therefore, a direct comparison of the 93-bp prealbumin exon 1 band could be used to determine the relative level of expression. As a positive control, liver mRNA was hybridized to both

FIG. 2. DNA sequence comparison between the human and mouse prealbumin promoter region and exon 1. The human prealbumin (54, 61) and mouse promoter sequence starting at 327 bp 5′ to the cap site and extending through exon 1 were compared. The colons represent nucleotide identity, and the dashes indicate where gaps were introduced in the DNA sequence to maximize the homology. The X indicates the region where the DNA sequence homology decreases. There is a 73.5% identity in the 389-nucleotide overlap.

labeled RNA probes (Fig. 4, lane liver A+). In this positive control lane (Fig. 4, lane liver A+) the 93-nucleotide band was the result of mouse prealbumin mRNA. The RNA from the whole liver also contained sufficient hematopoietic cells producing globin mRNA to protect the globin probe, yielding bands of 143 and 206 bp. The minor bands seen at 110 bp with the liver mRNA are probably due to an alternate 3′ splice site of prealbumin exon 1 and not another initiation site, for this band was not observed in the primer extension of the prealbumin mRNA (Fig. 1b).

In the experiment shown in Fig. 4, constructs containing either 1,400 or 300 bp of 5′-flanking prealbumin sequence gave identical signals when transfected in HepG2 cells (lanes marked −1400 and −300). Optimal levels of expression were observed with DNA concentrations of 40 μg/2 × 10^6 cells. In contrast, promoter deletions extending to 110 bp 5′ to the cap site resulted in a considerable decrease in expression (Fig. 4, lanes −110). This initial result affirmed the importance of the conserved prealbumin promoter region of homology (Fig. 2) that extends to 190 bp of upstream sequences. Further deletion analysis was necessary to determine more exactly the location of the necessary sequences.

In contrast to the almost identical level of expression of the plasmids with 300 or 1,400 nucleotides of upstream prealbumin sequence, expression was increased 10-fold by inclusion of 3,000 nucleotides of upstream sequence. From this result two regions of importance were preliminarily identified, a proximal region between −300 and the cap site that allowed expression in hepatoma cells and distant sequences that increased the rate of this expression (−1400 to −3000).

HeLa cell transient assays with the prealbumin promoter exhibit no detectable expression. To determine whether the expression of the prealbumin gene was cell-type specific, we performed transient assay experiments in HeLa cells at two different DNA concentrations. The three prealbumin promoter constructs (−3000, −300, and −110) that were used in HepG2 cells were cotransfected into HeLa cells together with the control plasmid (Fig. 5) as described in the previous section. Total cytoplasmic RNA (25 μg) from transfected HeLa cells was hybridized to a mixture of labeled RNA probes specific to the 3′ end of the control β-globin-SV40-E1B plasmid and to prealbumin exon 1. As a control, liver RNA was also hybridized with the two probes providing a guide to the migration of the protected 93-bp prealbumin exon 1 (Fig. 5, lane liver A+). In the HeLa cell transient assays, none of the prealbumin constructs containing different lengths of 5′-flanking sequences showed any activity (Fig. 5, lack of the 93-bp exon 1 band). In contrast, the control cotransfected β-globin-SV40-E1B plasmid was expressed at high levels in the HeLa cells (Fig. 5, protected 3′-end bands of 191 and 174 bp, lanes −110, −300, −3000), demonstrating that the transfection was successful. The results of this experiment show that neither the proximal nor the far upstream prealbumin regulatory signal leads to expression in HeLa cells. This is in contrast to the expression of these prealbumin constructs in the HepG2 experiment presented in Fig. 4. Therefore, from these data, we conclude that the proximal promoter element (spanning 300 nucleotides 5′ to the cap site) is partly responsible for the cell-type-specific expression of the prealbumin gene.

More precise location of sequences necessary for HepG2 expression. To determine more precisely which sequences define the prealbumin promoter in HepG2 cells, we made various 5′-end deletions with BAL 31 exonuclease (34). The

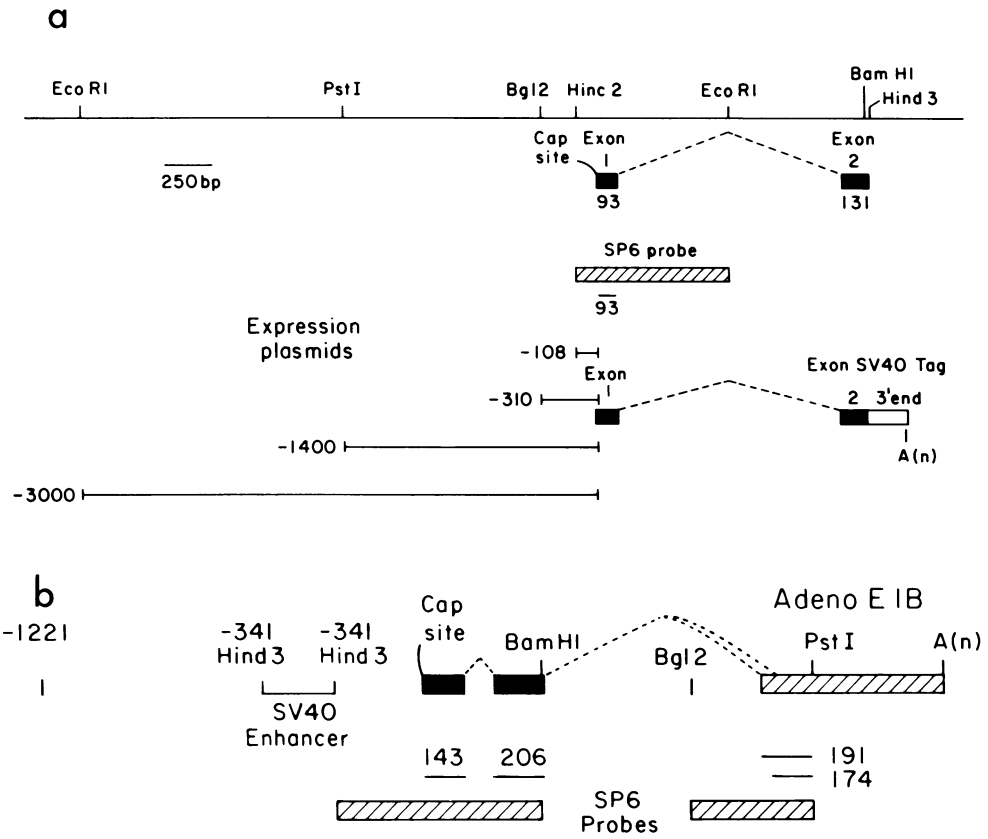

FIG. 3. Structures of the prealbumin gene, expression plasmids constructed from it, and the mouse β-globin-SV40-E1B control construct are shown. (a) At the top are the first two prealbumin exons (designated by closed boxes) and the first intron (dotted carat). Restriction endonuclease cleavage sites are shown. Below are diagrammed the prealbumin expression plasmids which contain the first two prealbumin exons at the site of union with the 3′ SV40 exon and polyadenylation site (open box). Each expression plasmid has the same 3′ segments joined to different amounts of 5′ prealbumin flanking region whose positions from the cap site are indicated by lines (−108 to −3000). The striped box represents the size and position of the T7 RNA probe used to analyze the RNA isolated from transfected cells; RNA initiated at the prealbumin cap site, that is, hybridized to this labeled probe and trimmed with T2 RNase, will protect a fragment of 93 bp. (b) Drawn to the same scale as above is the mouse β-globin-SV40-E1B plasmid utilized as a control plasmid for transfection efficiency. The construct contains the mouse β-globin gene from −1211 to +1040 bp fused to the adenovirus E1B (Adeno E1B) polyadenylation site and 3′ end (1a). The closed boxes represent the first two exons of the β-globin gene which are spliced to the E1B 3′ end where the transcript is poly(A)$^+$ (indicated by striped box). The SV40 enhancer fragment is inserted at the *Hin*dIII site, 341 bp 5′ to the β-globin cap site. Below, two Sp6 or T7 RNA probes are shown (striped boxes) which can be used to analyze the stable RNAs arising from this construct. The 5′ probe protects the first two β-globin exons giving T2 RNase-protected bands of 143 and 206 bp. The 3′-end probe yields two T2 RNase-protected bands of 191 and 174 bp.

promoter deletions that were studied started from the *Bgl*II site located 329 bp 5′ to the cap site and extended through the TATA box at position −24. These deletion plasmids (40 μg) were cotransfected into HepG2 cells together with the control construct (mouse β-globin-SV40-E1B, 5 μg) and analyzed for expression by T2 RNase analysis as described above. Indicated above each gel lane in Fig. 6 is the length of sequence upstream of the cap site that was contained in each plasmid that was analyzed. Lane C in Fig. 6 employed DNA from HepG2 cells which did not receive any transfected DNA. A sample of liver poly(A)$^+$ RNA (Fig. 6, lane liver A+) served to mark the 93-nucleotide exon 1 band and the protected β-globin control bands. Analysis of the various deletions showed that removal of sequence to −202 (i.e., 202 nucleotides 5′ to the cap site) did not affect the level of prealbumin expression (Fig. 6, enhancer −, compare lanes 312 and 202). However, removal to −151 decreased expression about 30% (Fig. 6, right side), and removal to −108 greatly decreased expression. Further deletions from the 5′ end completely eliminated specific transcription from the prealbumin promoter (Fig. 6, right side, lanes 77 and 24). These results show that at least a portion of the 190-bp upstream sequences that are conserved between the mouse and human genomes functions in initiation of transcription of this gene.

In the analysis of the most extreme deletions, −77 and −24, two aberrant bands were observed that were not present when the promoter region was left intact (bands at approximately 120 and 180 nucleotides). These bands were not observed in liver poly(A)$^+$ mRNA or in HepG2 cell RNA without transfection. It seems likely that the aberrant bands are due to transcripts that initiated within vector sequences and extended through the promoter and then were spliced correctly at the end of exon 1. These aberrant transcripts were distinguished from correct initiation by the T2 RNase analysis. If the expression had been monitored by the presence of the chloramphenicol acetyltransferase enzyme, as is often done in transfection tests of recombinant genes

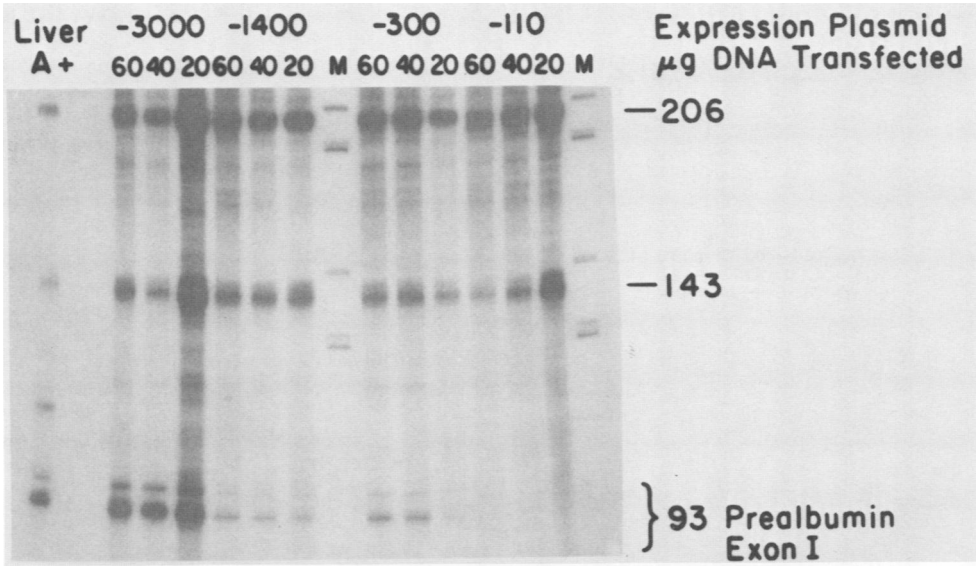

FIG. 4. Transient assays from HepG2 cells transfected with various prealbumin expression plasmids. Several DNA concentrations (60, 40, and 20 μg) of each prealbumin construct indicated were cotransfected with an equal ratio of the mouse β-globin-SV40-E1B plasmid (7.5, 5, and 2.5 μg, respectively) into a human hepatoma cell line (HepG2). Cytoplasmic RNA was harvested 36 h after the removal of the calcium phosphate DNA coprecipitate. Total cytoplasmic RNA (25 μg) was hybridized with two uniformly labeled RNA probes: the prealbumin exon 1 probe (93-bp protected fragment) and the β-globin first-two-exons probe (143- and 206-bp protected fragments). The T2 RNase-resistant fragments were sized on an 8% polyacrylamide gel containing 8 M urea followed by autoradiography. The liver A+ lane contains 0.15 μg of total liver poly(A)$^+$ RNA hybridized to both probes, and the marker lane (M) contains molecular weight markers of 220, 200, 154, and 140 bp.

(10, 24, 45), and these aberrant transcripts were translated, the level of true expression from the prealbumin promoter would have been incorrectly assayed.

Demonstration of far upstream enhancer element. Next we returned to the generation of higher levels of prealbumin expression by the far upstream sequence. The upstream sequence (−329 to −3000) was placed in the correct orientation in front of five of the deletion mutants to determine which deletions might be stimulated by its presence. The results of a transfection assay with these constructs is shown on the left side of Fig. 6. The deletion mutants from −200 and −312 were stimulated to about 10-fold-higher levels of expression by the presence of the far upstream element (Fig. 6, compare enhancer + and −). The deletion mutant −151 was also stimulated somewhat more than 10-fold to an equal level with the enhanced −202 and −312 mutants. In con-

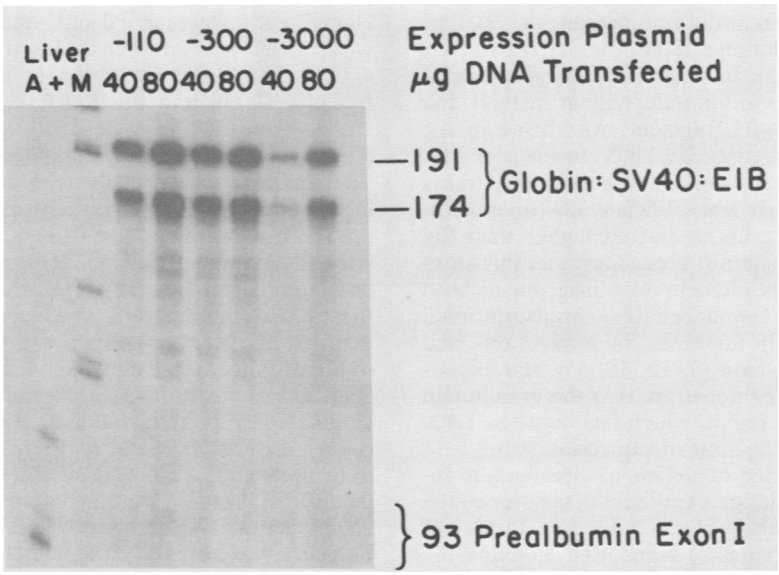

FIG. 5. Transient assay of HeLa cells transfected with prealbumin expression plasmids. HeLa cell transfections were done exactly as the HepG2 experiments were except that a 3′-end Sp6 RNA probe was used to analyze the mouse β-globin-SV40-E1B control (T2 RNase-protected fragments of 191 and 174 bp [1a, 21]). The amount of prealbumin sequences 5′ to the cap site and the concentration of DNA used for the transfection are indicated at the top of the figure.

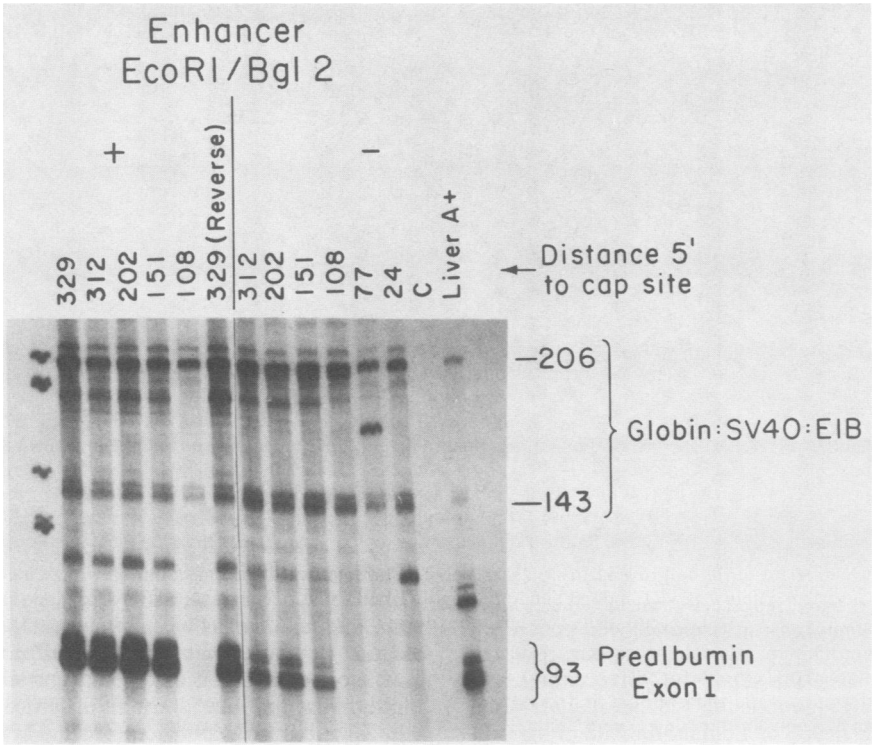

FIG. 6. Effect of the prealbumin upstream enhancer on the expression of promoter deletions during HepG2 transient assays. The number of nucleotides 5′ to the mRNA cap site contained in the prealbumin construct analyzed is indicated at the top of the gel. Each of the indicated prealbumin promoter deletions (40 μg) were cotransfected with the mouse β-globin-SV40-E1B control plasmid (5 μg) into HepG2 cells, and the transient expression was analyzed as described above (see the legend to Fig. 4 and the text). The results on the left side of the vertical line were obtained with prealbumin promoter deletions containing the upstream enhancer in the natural orientation (indicated by +). The *Eco*RI-*Bgl*II fragment (−3000 to −329) contained the enhancer element, and this fragment was placed in the opposite orientation upstream of the −329 (*Bgl*II site) deletion; the analysis of the construction with reversed orientation is shown in the lane designated 329 (reversed). The transient assay analysis shown to the right of the vertical line was performed with prealbumin promoter deletion constructs that lacked the upstream enhancer element (indicated by −). Expression from the prealbumin constructs yielded a T2 RNase-protected fragment of 93 bp from the exon 1 probe, and the control plasmid expression resulted in two protected fragments of 143 and 206 bp. DNA molecular weight markers and liver A+ control were as described in the legend to Fig. 4. Lane C, Control hybridization of the above mixed RNA probe with HepG2 RNA isolated from cells lacking exogenous added DNA.

trast, the far upstream element did not increase the level of expression of the −108 deletion mutant.

To determine whether the far upstream stimulatory element could function in a position-independent fashion, the upstream region (*Eco*RI-*Bgl*II fragment) was fused in the reverse orientation at a position 329 bp 5′ to the cap site. Equivalent expression levels were found in both constructs (Fig. 6, enhancer +, compare lanes 329 and 329 [reversed]). Again the activations were about 10-fold higher than the deletions lacking the far upstream sequences, and this stimulation occurred when this element was inserted in both orientations. Because an enhancer is a transcriptional stimulatory element which functions in a position- and orientation-independent fashion (3, 15, 35, 55), the experiments presented in Fig. 6 demonstrate that the prealbumin gene contains an enhancer region which lies between 1,400 and 3,000 bp upstream of the start of transcription.

Localization and specificity of action of prealbumin enhancer element. Further deletion experiments positioned the prealbumin enhancer region between 1.6 and 2.15 kb upstream of the cap site. Constructions that contain the prealbumin promoter (329 nucleotides of 5′-flanking sequence) were joined to various upstream DNA segments by using restriction endonuclease sites located in the enhancer region (Fig. 7). Transfection of these constructions into HepG2 cells was carried out, and assays were performed with labeled RNA probes as described in the legends to Fig. 4 through 6. In the experiment presented in Fig. 7A the *Eco*RI (−3 kb) and the *Bgl*II (−329 bp) prealbumin constructs (lanes 1 and 6, respectively; 93-bp protected fragment) were included as a comparison for enhancer function. The analysis of two enhancer deletions determined that DNA sequences located either upstream of the *Nco*I site at −2.15 kb or between the *Sst*I (−1.6 kb) and *Bgl*II (−329 bp) sites (diagrammed in Fig. 7B) were dispensible for prealbumin enhancer function (Fig. 7A, lanes 2 and 3). Both of these enhancer constructs produced an 11-fold stimulation of expression over the −329 bp expression plasmid regardless of the distance of the enhancer sequence from the promoter (Fig. 7A, compare lanes 2, 3, and 6). This stimulation was eliminated by the removal of DNA sequences either between the *Nco*I (−2.15 kb) and *Pst*I (−1.4 kb) sites (Fig. 7A, lane 4) or upstream of the *Sst*I site located at −1.6 kb (Fig. 7A, lane 5). Therefore, we concluded from these data that the 550-nucleotide fragment between the *Nco*I and *Sst*I restriction sites was necessary and sufficient to confer the high rate of expression on the DNA segment containing the prealbumin promoter region.

Experiments designed to test the cell specificity of the enhancer sequences were performed next. The control plas-

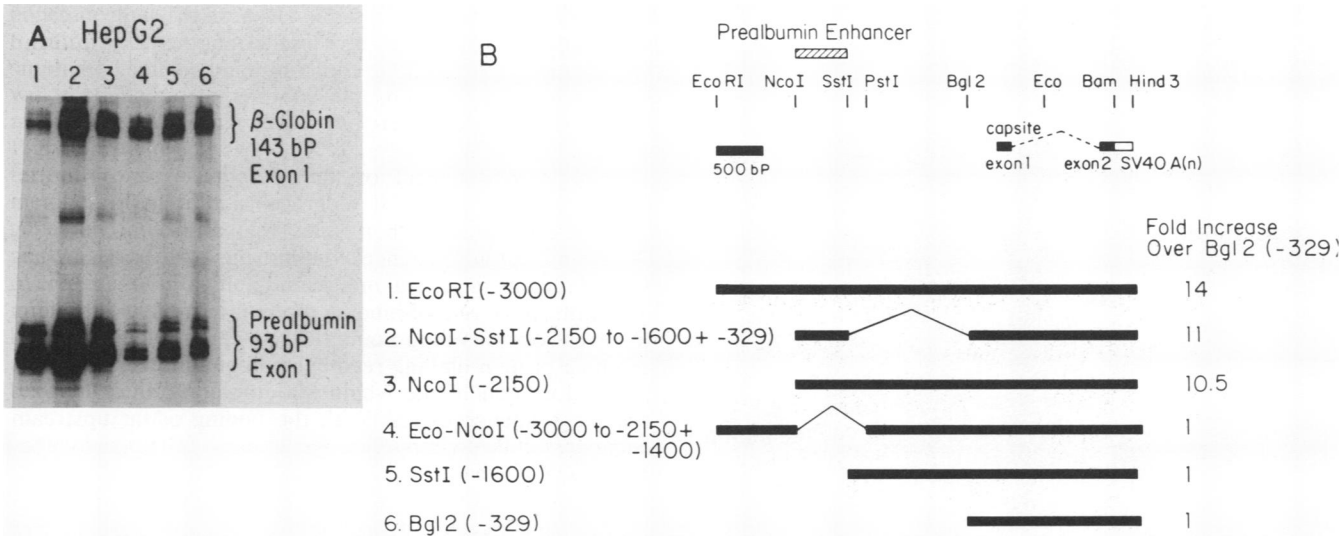

FIG. 7. Transfection of human hepatoma cells with various prealbumin enhancer constructs. (A) Transient assay of prealbumin enhancer deletions (schematically shown in Fig. 7B) in HepG2 cells to delineate the boundary of the enhancer. These deletion plasmids were constructed by using various restriction sites located in the region upstream of the cap site. Transfections and assay of cytoplasmic RNA from transfected cells were as described in the legends to Fig. 3 through 6 and in the Materials and Methods section. T2 RNase-protected bands are the 143-bp (cotransfected β-globin signal) and 93-bp (prealbumin test signal) bands. Lanes: 1, *Eco*RI (−3 kb) prealbumin wild-type enhancer construct; 2, *Nco*I-*Sst*I restriction fragment (−2.15 to −1.6 kb) joined with the *Bgl*II (−329 bp) prealbumin construct; 3, prealbumin construct containing upstream sequences ending at the *Nco*I site (−2.15 kb); 4, prealbumin *Eco*RI (−3 kb) construct lacking sequences from the *Nco*I (− 2.15 kb) to *Pst*I (−1.4 kb) sites; 5, prealbumin construct containing 5′-end sequences ending at the *Sst*I site (−1.6 kb); 6, prealbumin promoter construct containing 329 bp 5′ to the cap site. (B) Schematic representation of the enhancer constructs used in the above HepG2 transfection. At the top is drawn the prealbumin expression vector (as described in Fig. 3a) along with the location of the restriction endonuclease cleavage sites and the position of the prealbumin enhancer (designated by striped box). Below is a schematic description of the prealbumin enhancer expression plasmids in the order in which they were analyzed above. The dark bars indicate the region on the prealbumin restriction map contained in each of the constructs. The carets delineate deletions where two noncontiguous restriction fragments are brought together. On the right side is the stimulation of the enhancer constructs over the −329 prealbumin construct after normalizing for transfection efficiency with the 143-bp β-globin control signal.

mid β-globin-SV40-E1B, which earlier was used in both HepG2 cells and HeLa cells, contained the SV40 enhancer that increases transcription from the β-globin promoter. This construction obviously led to expression of β-globin in both cell types (Fig. 3 through 6). To test whether the globin gene could be stimulated in a cell-specific fashion by the prealbumin enhancer, the SV40 enhancer was removed from the β-globin-SV40-E1B construct, and the upstream region of prealbumin from −329 to −3,000 bp was inserted in its place. This plasmid (40 μg) when cotransfected with the −3 kb prealbumin construct (4 μg) into HepG2 cells led to RNA production that was within a factor of three or four equivalent to the β-globin-SV40-E1B plasmid (Fig. 8, compare 143-bp fragment in lanes 2 and 3). This stimulation is 30-fold greater than the expression from the β-globin promoter alone (Fig. 8, lanes 1 and 3, compare 143-bp band). However, in HeLa cells the prealbumin enhancer–β-globin construct expressed RNA at the same very low level as the β-globin promoter lacking an enhancer (Fig. 8, lanes 7 and 9). This is in contrast to the high level of expression from the β-globin-SV40-E1B plasmid in HeLa cells (Fig. 8, lane 8). Since the prealbumin enhancer did not increase the β-globin expression in HeLa cells and can activate this same gene in HepG2 cells, we conclude that this enhancer is cell specific.

One additional recombinant construction plus several further tests showed the importance of the cell specificity of the prealbumin promoter. When placed at the 3′ end of the −329 prealbumin construct, the SV40 enhancer could replace the prealbumin enhancer in HepG2 cells (Fig. 8, compare 93-bp band in lanes 4 and 6). The low level of cell-specific expression of the −329 prealbumin promoter region was increased by SV40 enhancer by at least 10-fold (Fig. 8, compare lanes 4 and 5). In HeLa cells, however, there was no low-level signal from the prealbumin promoter without an enhancer and neither the SV40 enhancer nor the prealbumin enhancer could increase expression sufficiently to give a signal (Fig. 8, lack of 93-bp band, lanes 10 to 12). The cells used in this experiment were capable of being transfected as indicated by strong signals from the β-globin constructs that were cotransfected together with the prealbumin expression plasmids. This result strengthens the argument for the cell specificity of the prealbumin promoter since a functional enhancer could not stimulate its expression in HeLa cells.

DISCUSSION

In the present experiments we isolated the mouse prealbumin gene and demonstrated that plasmid constructions containing the upstream regions of this gene are specifically expressed in human hepatoma and not HeLa cells. The expression of a mouse gene in a human hepatoma cell line might have been expected since there proved to be a high level of conservation between the mouse and human genes in the 190-nucleotide stretch just upstream from the cap site. Also, the rat albumin promoter incorporated into adenovirus functions in HepG2 cells (1a, 21), indicating that no general species barrier exists for liver-specific gene expression in HepG2 cells.

By deletion of various regions of upstream sequence of the prealbumin gene, a more refined picture of transcriptional

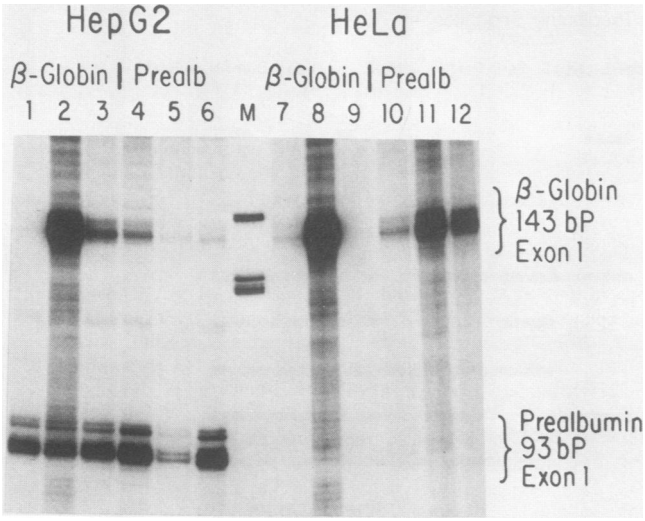

FIG. 8. HeLa and HepG2 transfections with plasmids that test the cell specificity of the prealbumin promoter and enhancer. The prealbumin enhancer was placed next to the β-globin promoter, and the SV40 enhancer was placed downstream of the prealbumin promoter and analyzed by transient assays as described in the previous figure legends and in Materials and Methods. The prealbumin −3 kb construct was used as a positive control (93-bp T2 RNase-protected fragment) in HepG2 cells for the β-globin experiments (lanes 1 to 3), and α-globin construct (data not shown) was used as a control for these constructs in HeLa cells (lanes 7 to 9). At the top of the panel is indicated the cell type, and the test plasmid is shown on top of the corresponding lanes. The following constructs were assayed after transient expression in either HepG2 (lanes 1 to 6) or HeLa (lanes 7 to 12) cells: lanes 1 and 7, β-globin construct (143-bp T2 RNase-protected fragment) containing 341 bp 5′ to the cap site; lanes 2 and 8, the SV40 enhancer driving −341 bp of the β-globin promoter; lanes 3 and 9, prealbumin enhancer (*Eco*RI-*Bgl*II restriction fragment) driving the β-globin promoter; lanes 4 and 10, prealbumin −329 promoter construct with the SV40 enhancer inserted at the 3′ end of the minigene; lanes 5 and 11, −329 prealbumin promoter plasmid; lanes 6 and 12, *Eco*RI (−3 kb) prealbumin enhancer construct. The marker lane (M) contains two bands of 154 and 140 bp.

regulatory regions emerged. The proximal upstream sequences (−329 to the cap site) were sufficient to confer specificity of transcription in HepG2 cells. Removal of 5′ sequences to −202 did not affect transcription, but deletion to −151 decreased transcription somewhat, and deletion to −108 almost completely abolished expression. Therefore, the region between −108 and −202 (particularly between −108 and −151), the region that is highly conserved between humans and mice, is critical for hepatoma-specific function. In addition to this promoter-proximal region, sequences with a great stimulatory effect on expression lie far upstream, between −1.6 and −2.15 kb. This upstream region increased expression only in HepG2 cells when present in either orientation and increased expression to about the same level in all the constructions retaining the sequences within 151 nucleotides of the cap site. However, when the only remaining proximal upstream sequence was −108 with respect to the cap site then no enhancement of expression was found in the presence of the far upstream element. This upstream element also exhibited cell specificity since it could activate β-globin RNA production in HepG2 but not HeLa cells. Therefore, the compound set of sequences required for prealbumin expression includes a proximal cell-specific promoter as well as a cell-specific upstream enhancer element(s) that raises the rate of expression. Because the same mRNAs were produced from all the constructions studied, i.e., the variations in recombinant constructions involved nontranscribed upstream regions, we assume that the effects on mRNA levels found here were all due to differences in transcription rate.

An important aspect of the present work will be to compare results obtained with the prealbumin gene with those obtained with other genes transcribed in the liver. While the initial sequence comparisons of immediate upstream regions in several mouse and human genes (albumin, α-1-antitrypsin, retinol-binding protein, and prealbumin) fail to reveal obvious long homologies, it will be necessary to examine protein-binding regions and compare their similarities before concluding whether a common proximal liver-specific sequence exists. With the finding of far upstream enhancers, it is obvious that comparisons of these sequences must also be made in the hope of further understanding coordinate cell-specific gene function. Finally, in considering coordinate gene expression it should again be noted that the prealbumin gene is expressed not only in liver cells but in the choroid plexus from which cell lines are now available (7). Thus, the basis for coordinate expression in distant tissues of possibly the same mRNA might be investigated in these cells.

The design for maximal gene expression discussed above, i.e., cell-specific promoter and distant cell-specific enhancer elements, has been found so far in only one other case in mammals, the immunoglobulins, in which the enhancer (2, 22, 45) lies in an intron. Genes in *Drosophila* sp. that are expressed in particular patterns during development may be designed in a similar way. For example, *fushi torazu* has a far upstream enhancer (28). In addition, other genes have been found to have enhancer elements whose role in differentiated expression is not yet clear. The mouse α-fetoprotein gene contains several far upstream elements that stimulate a heterologous promoter (herpes simplex virus thymidine kinase gene) in both HeLa and HepG2 cells (23). An upstream element activates chicken lysozyme (normally a differentiated product of oviduct epithelium) in a line of chicken myeloid tumor cells (60). In addition, we have recently shown that while rat albumin can be expressed from recombinant adenovirus in HepG2 cells with 441 nucleotides of upstream sequence present, the rate of transcription is very low when compared directly with that of the single endogenous human albumin gene in HepG2 cells (21). Only if an enhancer was provided (the E1A viral enhancer was used) was transcription raised to the same level as that of the endogenous gene (1a). Furthermore, an upstream element in the native rat albumin gene is suggested by the finding of a far upstream hypersensitive site at −2.8 kb in liver but not other tissues (1).

All these results plus those in this paper contrast to earlier findings suggesting that all necessary sequences for tissue-specific gene control reside close to the RNA start site. In a number of cases, e.g., chymotrypsin (62) and elastase (44, 58) in pancreatic acinar cells, insulin (18, 62) in pancreatic islet cells, and α-crystalline lens protein (46) in the eye, no distant enhancer elements were detected, but some of the necessary proximal elements behaved like enhancers. For example, sequences within about 300 nucleotides of the cap sites of insulin and chymotrypsin allow cell-specific expression when assayed in two different human cell lines (62). In the case of the insulin gene the sequence between −298 and −103 when placed in either orientation next to a heterologous promoter (thymidine kinase) can stimulate its expres-

sion in a transient assay and therefore is designated as an enhancer (18, 55). However, the distal and proximal elements of the well-characterized thymidine kinase gene itself (located within 100 nucleotides from the cap site) are also invertible (38). It is unclear whether the invertible sequences within several hundred nucleotides 5′ to the mRNA cap site perform functions similar to those of the more distant enhancers. One possibility is that the insulin or chymotrypsin gene may contain as yet undetected far upstream elements that would further stimulate transcription. The rat elastase gene that is normally transcriptionally active in pancreatic acinar cells is a dramatic case in which only proximal sequences seem to be required for maximal cell-specific expression. Here transgenic mice bearing the rat gene and only 200 nucleotides upstream produce near normal amounts of rat as well as mouse elastase (44, 58). Thus, it may be that the sequences governing cell-specific function for some genes will lie close to the initiation site for RNA transcription. However, enough genes have now been described that contain a more complex arrangement with regulatory elements located both distant and near to the cap site to make this design worth searching for in all cases.

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