The Human Prothrombin Gene: Transcriptional Regulation in HepG2 Cells†

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Received July 15, 1992; Revised Manuscript Received October 5, 1992

ABSTRACT: The human prothrombin gene is expressed predominantly in hepatocytes. Previous work indicated that this tissue specificity is transcriptionally regulated. In order to identify the cis-acting regulatory elements in the 5′ flanking region of the human prothrombin gene which may direct the expression of prothrombin in hepatocytes, a series of hybrid plasmids were constructed linking portions of the 5′ flanking region of the human prothrombin gene to the bacterial chloramphenicol acetyltransferase gene. Expression of these hybrid plasmids was examined in calcium phosphate-mediated transient transfections of HepG2 cells, a human hepatoblastoma cell line which expresses prothrombin, and HeLa cells, an adenocarcinoma cell line which does not express detectable amounts of prothrombin. Both the prothrombin promoter and an upstream regulatory region containing sequence homologous to the hepatocyte nuclear factor 1 (HNF-1) binding site (nucleotides −919 to −790 relative to the prothrombin transcription initiation site) were required for expression in HepG2 cells. The upstream region also exhibited non-tissue-specific enhancer activity. Gel mobility shift assays confirmed cell-type-specific differences in the protein–DNA interactions between proteins in HepG2 or HeLa nuclear extracts and either the promoter region or the upstream regulatory region of the gene.

Several of the vitamin K-dependent coagulation factors (prothrombin and factors VII, IX, and X) are synthesized predominantly in the liver (Kelly & Summerfield, 1987). They are synthesized and secreted from hepatocytes as zymogens of serine proteases. Following the neonatal period and in the absence of parenchymal liver disease or ongoing coagulation, these proteins are present in human plasma at fairly stable concentrations.

Prothrombin, the most abundant of the vitamin K-dependent coagulation factors, is converted to thrombin by factor Xa in the presence of factor Va, calcium ions, and a phospholipid surface (Davie et al., 1979). Thrombin cleaves fibrinopeptides A and B from fibrinogen to form fibrin. Fibrin is then organized into a fibrin clot. Although the functions of prothrombin and the other vitamin K-dependent coagulation factors have been well characterized, little is known about the regulation of their expression or the molecular mechanisms which limit their synthesis to hepatocytes.

Tissue- or cell-specific expression of genes is often regulated at the level of transcription, mediated by the interaction of specific nuclear proteins (trans-acting factors) with cis-acting DNA sequences in or around a gene (Maniatis et al., 1987). Cis-acting regulatory elements have been identified and characterized for a number of liver-specific genes through the study in cell culture or in transgenic animals of the expression of hybrid genes containing putative regulatory sequences ligated to reporter genes, such as the bacterial gene chloramphenicol acetyltransferase (CAT)[1] (DeSimone & Cortese, 1988). We have utilized this strategy to investigate the transcriptional regulation of the human prothrombin gene and have previously identified a positive, cis-acting regulatory region between nucleotides −2969 and −797 in the 5′ flanking region of the gene (Bancroft et al., 1990). In this report we describe a set of promoter and enhancer elements in the 5′ flanking region of the human prothrombin gene which together direct expression of prothrombin–CAT hybrid plasmids in HepG2 cells, a human hepatoblastoma cell line (Knowles et al., 1980).

MATERIALS AND METHODS

The numbering of nucleotides in the human prothrombin gene and its 5′ flanking region is based on the sequence of Bancroft et al. (1990) except where noted.

Preparation of Constructs. Deletions of the 5′ flanking region of the human prothrombin gene were first obtained by restriction enzyme digestion. Fragments of 2969, 797, and 419 base pairs (bp) of flanking sequence were cloned into the promoterless CAT vector pSV0cat-Xba and have been described previously (Bancroft et al., 1990). pSV0cat-Xba is identical to pSV0cat (Gorman et al., 1982) except that the *Hin*dIII site has been changed to an *Xba*I site by use of Klenow fragment and addition of *Xba*I linkers (Bancroft et al., 1990). Constructs II(−2969/+27)cat, II(−797/+27)cat, and II(−419/+27)cat were obtained in this manner (the numbers in parentheses refer to the 5′/3′ ends of each construct relative to the sequence of the human prothrombin gene and its 5′ flanking region; Bancroft et al., 1990). The plasmid II(−1262/+27)cat was obtained by digestion of II(−2969/+27)-pUC19 with *Pst*I and *Xba*I and cloned into pSV0cat-Xba after addition of *Xba*I linkers (Bancroft et al., 1990). DNA from the 5′ flanking region in II(−1059/+27)cat, II(−989/+27)cat, II(−919/+27)cat, and II(−849/+27)cat was obtained by the polymerase chain reaction (PCR; Saiki et al.,

† This work was supported in part by Research Grant HL38232 from the National Institutes of Health and by the Pew Memorial Trust. S.J.F.D. is an Established Investigator of the American Heart Association.

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[1] Abbreviations: bp, base pairs; CAT, chloramphenicol acetyltransferase; EDTA, ethylenediaminetetraacetic acid; HGF, hepatocyte growth factor; HNF-1, hepatocyte nuclear factor 1; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; *tk*, thymidine kinase; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/92/0431-12469$03.00/0

1985) and cloned into the *Hin*dIII site of pSV0cat (Gorman et al., 1982). All oligonucleotides used for PCR reactions described in this paper included eight additional bases at the 5′ end which contain the *Hin*dIII recognition sequence except where indicated. Oligonucleotides used as 5′ primers were identical to sequence in the 5′ flanking region encompassing the following nucleotides: −1059 to −1034, −989 to −965, −919 to −895, and −849 to −825 (Bancroft et al., 1990). The oligonucleotide used as the 3′ primer was complementary to the sequence in the gene from +6 to +27 (Bancroft et al., 1990). The template used for PCR reactions was a plasmid containing the region between −6500 and +55 of the human prothrombin gene cloned into the *Xba*I site of pSV0cat-Xba (Bancroft et al., 1990). All fragments obtained by PCR were digested with *Hin*dIII and ligated into the *Hin*dIII site of pSV0cat. All inserts obtained by PCR were characterized by DNA sequence analysis and several differences were found compared to the published sequence (Bancroft et al., 1990). All four inserts had an additional G between nucleotides −646 and −645 and a C deleted at position −639. The insert in II(−1059/+27)cat contained two additional differences at nucleotides −453 and −165 where a G was substituted for an A in both cases. Finally, the insert in II(−989/+27)cat had a G substituted for an A at position −693. All of these differences are in regions that we have shown not to be involved in the liver-specific expression of the prothrombin gene and therefore should not affect the results obtained using these constructs.

PCR was used to obtain fragments from the 5′ flanking region that were then cloned upstream of the thymidine kinase (*tk*) promoter-driven CAT gene in the *Hin*dIII site of pBLcat2 to test for enhancer activity (Luckow & Schutz, 1987). The template and 5′ primers were exactly as described above. The oligonucleotide used as the 3′ primer was complementary to the prothrombin sequence from nucleotides −814 to −790. The following constructs were obtained: II(−1059/−790)-cat2, II(−989/−790)cat2, II(−919/−790)cat2, II(−790/−1059)cat2, II(−790/−989)cat2, and II(−790/−919)cat2. All inserts obtained by PCR were sequenced and were identical to the published sequence (Bancroft et al., 1990) except for the presence of an additional C at position −793 in II(−989/−790)cat2. This is in a region shown to have no liver-specific activity and therefore should have no effect on results obtained using this construct.

DNA coding for the promoter region from −73 to +27 was obtained by PCR and digested with *Hin*dIII. The template and 3′ primer were exactly as described above and the oligonucleotide used as 5′ primer was identical to sequence in the 5′ flanking region between −73 and −52 (Bancroft et al., 1990). The region from −73 to +27 was cloned into the *Hin*dIII site of pBLcat2 and pBLcat3 to test for regulatory function. pBLcat3 is identical to pBLcat2 except that it lacks the *tk* promoter (Luckow & Schutz, 1987). The resulting plasmids were labeled: II(−73/+27)cat2 and II(−73/+27)cat3. The sequence of inserts from these plasmids agreed with the published sequence (Bancroft et al., 1990).

In order to define the minimal sequences required for expression, constructs were made with the region between −1059 and −790 ligated in either orientation with respect to the region from −73 to +27 or from −108 to +27 in pSV0cat. For all constructs the region from −73 to +27 was obtained by PCR using the previously described primers except that the eight additional nucleotides on the 5′ primer included an *Xba*I recognition sequence. The region from −108 to +27 was obtained by PCR using the previously described template and 3′ primer. The 5′ primer was identical to sequence in the 5′ flanking region between −108 and −84 with eight additional nucleotides at the 5′ end including an *Xba*I recognition sequence (Bancroft et al., 1990). To obtain the constructs II[(−1059/−790)×(−73/+27)]cat and II[(−1059/−790)×(−108/+27)]cat, the region between nucleotides −1059 and −790 was generated by PCR using previously described primers except that the 3′ primer included an *Xba*I recognition sequence rather than a *Hin*dIII site. Both PCR fragments were digested with *Xba*I and *Hin*dIII followed by ligation together into the *Hin*dIII site of Bluescript SK+/−(Stratagene). The appropriate *Hin*dIII insert was isolated and ligated into the *Hin*dIII site of pSV0cat. The construct II[(−790/−1059)×(−73/+27)]-cat was obtained in exactly the same manner except that the 5′ primer for the region from nucleotides −1059 to −790 contained an *Xba*I recognition site while the 3′ primer had the *Hin*dIII sequence. The sequences of all inserts obtained by PCR were identical to the published sequence (Bancroft et al., 1990).

Transfection. HepG2 cells (Knowles et al., 1980) and HeLa cells grown at 37 °C in 5% CO_2 were transfected by calcium phosphate coprecipitation with DNA that had been purified twice on CsCl gradients (Bancroft et al., 1990). pUC19 was added to each sample to bring the total DNA per transfection to 30 μg; 6.1 pmol of each test plasmid was used per transfection. Cells were washed 24 h after transfection and cell extracts were prepared 48 h later. pD5cat was transfected in both cell lines as a positive control (Berkner & Sharp, 1985). All transfections were performed at least three times with at least two plasmid preparations.

Cotransfection of either pRSVβgal, an RSV/β-galactosidase hybrid plasmid, or pSV2A.L-AΔ5′, an adenovirus/luciferase hybrid plasmid, was done initially in an effort to control for variance in transfection efficiency (deWet et al., 1987). This failed, however, to decrease the variance in level of expression of our transfections. The assumptions underlying the use of cotransfected control plasmids include, first, that the cotransfected plasmids are expressed at a proportional and stable level and, second, that each plasmid has no effect upon the transfection efficiency or subsequent level of expression of the other plasmid. A number of investigators have demonstrated that cotransfected plasmids can interact and compete for trans-acting factors, thus altering levels of expression (Scholer & Gruss, 1984; Ciliberto et al., 1985; Colantuoni et al., 1987; Wu et al., 1987; Farr & Roman, 1992). In order to avoid competition between cotransfected plasmids, we employed a strategy used by others of reporting the mean level of expression of repeated transfections of individual plasmids in each cell line (Ott et al., 1984; Ciliberto et al., 1985; Colantuoni et al., 1987; Wu et al., 1987; Aronow et al., 1989; Leff et al., 1989; Chow et al., 1991).

Total protein in cell extracts was determined and CAT activity was assayed as previously described (Bancroft et al., 1990). ^{14}C-Chloramphenicol (Dupont NEN) had a specific activity of 195.4 cpm/pmol. Cell extracts were diluted so that the percent conversion of chloramphenicol to monoacetylated chloramphenicol was less than or equal to 40%. CAT specific activity for each sample was calculated and expressed in picomoles per minute per milligram of protein.

Isolation of RNA. Total cellular RNA was prepared from transfected HepG2 and HeLa cells 72 h after transfection by a modification of the method of Degen et al. (1985). Confluent 10-cm plates of transfected cells were washed twice with cold PBS (136 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 9.6 mM Na_2HPO_4, pH 7.3) and lysed with 2.5 mL of 1 × SET (10 mM Tris-HCl, pH 7.5, 1% SDS, and 5 mM EDTA) containing 100 μg/mL proteinase K (Sigma). Lysates were

incubated at 45 °C for 2 h followed by phenol/chloroform extraction. Total RNA was isolated by CsCl gradient centrifugation.

Primer Extension Analysis. Primer extension analysis was performed as previously described using a 33-base oligonucleotide complementary to the mRNA-like strand of the CAT gene near the 5′ end of its coding sequence (complementary to bases 257–289 of *Escherichia coli* Tn 9; Alton & Vapnek, 1979; Bancroft et al., 1990).

Preparation of Nuclear Extracts. Nuclear extracts were prepared from HepG2 and HeLa cells by a modification of the method of Zelnick et al. (1987). Confluent plates of cells were washed twice with cold PBS and then harvested in 5 mL of cold PBS with a Teflon policeman. Cells were spun at 1000*g* for 5 min. The pellet was resuspended in 5 mL/(g of cells) buffer AB [25 mM Tris-HCl, pH 7.9, 12 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol] containing 7 mM 2-mercaptoethanol, 5 μg/mL aprotinin (Calbiochem), 25 μg/mL phenylmethanesulfonyl fluoride (PMSF; Sigma) and homogenized with 10 strokes in a cold Dounce homogenizer (B pestle). Polyethylenimine (4 μL of 10% solution; Aldrich) was added to the supernatant and the sample was stirred for 30 min at 4 °C. The supernatant was spun at 13000*g* for 10 min at 4 °C, the supernatant was removed, and the pellet was resuspended in 2.5 mL/(g of cells) buffer C [50 mM Tris-HCl, pH 7.9, 25 mM KCl, 0.1 mM EDTA, 10% glycerol, and 0.2 M $(NH_2)_2SO_4$] containing 7 mM 2-mercaptoethanol, 5 μg/mL aprotinin, and 25 μg/mL PMSF. The suspension was homogenized with 10 strokes in a cold Dounce homogenizer (B pestle), stirred for 30 min at 4 °C, and spun at 11000*g* for 10 min at 4 °C. The supernatant was aliquoted and frozen at −70 °C.

Mobility Shift Assays. Gel mobility shift assays were performed initially using the Gelshift Kit (Stratagene; Hendrickson, 1985). Double-stranded deoxyoligonucleotide fragments corresponding to the human prothrombin gene sequence from nucleotide −919 to −790 and from nucleotide −108 to +27 were synthesized by PCR using the oligonucleotide primers described above. Fragments were 5′-end-labeled using T4 polynucleotide kinase (BRL) and [γ-^{32}P]ATP (3000 Ci/mmol; Dupont NEN) or were used as unlabeled competing DNA in competition experiments. HNF-1, a 28-bp double-stranded deoxyoligonucleotide containing the HNF-1 recognition site from the human fibrinogen β-chain gene (5′CAAACTGTCAAATATTAACTAAAGGGAG3′ and its complementary strand), was used as a specific competitor (Courtois et al., 1988). HGF, a 580-bp *Xho*I/*Kpn*I restriction fragment from the human hepatocyte growth factor cDNA (S.J.F.D., unpublished results), was used as a nonspecific competitor. The concentration of DNA samples was estimated by ethidium bromide dot quantitation (Seldon & Chory, 1987). Gel mobility shift experiments were performed by the addition of 5000 cpm of radiolabeled double-stranded oligonucleotide to 17 μL of buffer [47 mM NaCl, 7 mM KCl, 3.5 mM $MgCl_2$, 28.2 mM Tris-HCl, pH 7.5, 5.4 mM EDTA, 14 mM HEPES, pH 7.9, 0.05% Tween 20 (Sigma), 7% glycerol, 0.8 mM 2-mercaptoethanol, and 0.06 mg/mL poly(dI-dC)·poly(dI-dC) (Pharmacia)]. Sterile water (5 μL), HepG2 nuclear extract (2 μL), or HeLa nuclear extract (5 μL) was added to each sample and incubated for 30 min at room temperature. Specific or nonspecific competitor DNA was added to samples before addition of nuclear extract during competition experiments. Bromophenol blue (2 μL of 0.1% solution) was added to each sample before electrophoresis. Samples were electrophoresed for 2.5–3 h at 21 mA constant current on a 4% nonreducing polyacrylamide gel at 4 °C in a high ionic strength buffer (0.38 M glycine, 50 mM Tris, and 2.1 mM EDTA, pH 8.2). Gels were preelectrophoresed for 1 h at 21 mA before samples were applied, and buffer was recirculated by syringe every 45 min. Gels were dried, followed by exposure to X-Omat AR film (Kodak) at −70 °C with an intensifying screen for 12–48 h.

Table I: Expression of Prothrombin–CAT Hybrid Plasmids in HepG2 and HeLa Cells

plasmid	relative CAT activity[a] HepG2	HeLa
pSV0cat	2.5	0.2
II(−2969/+27)cat	100	0.3
II(−1262/+27)cat	79.9	0.3
II(−1059/+27)cat	22.9	0.2
II(−989/+27)cat	23.3	0.4
II(−919/+27)cat	46.5	1.2
II(−849/+27)cat	0.2	0.1
II(−797/+27)cat	1.6	0.1
II(−419/+27)cat	0.8	0.2
II[(−1059/−790)x(−73/+27)]cat	90.7	1
II[(−1059/−790)x(−108/+27)]cat	44.9	0.3
II[(−790/−1059)x(−73/+27)]cat	18.3	0.4
pD5cat	2691	841

[a] Expressed as a percentage of the expression of II(−2969/+27)cat in HepG2 cells.

RESULTS

Hybrid Plasmid Expression. We previously demonstrated that the region from −2969 to +27 relative to the transcription initiation site in the gene coding for human prothrombin directed high levels of expression of a CAT reporter gene in HepG2 cells while the regions −797 to +27 and −419 to +27 did not (Bancroft et al., 1990). None of these constructs were expressed in HeLa cells. This suggested that one or more positive cis-acting elements, which direct expression in HepG2 cells were present between nucleotides −2969 and −797 in the 5′ flanking region of the human prothrombin gene. The high content of Alu repetitive DNA between nucleotides −2969 and −1102 (five copies) suggested that the regulatory element(s) might lie between nucleotides −1101 and −798. A series of hybrid constructs containing portions of the prothrombin 5′ flanking region ligated to the CAT gene were generated with 5′ ends ranging from −1262 to −849. Equimolar amounts of plasmids were transfected into HepG2 and HeLa cells to determine the 5′ extent of sequences required to direct expression in HepG2 cells.

The CAT gene in plasmids containing 5′ flanking DNA from −2969 to +27 or −1262 to +27 was expressed at high levels in HepG2 cells (Table I). Expression of CAT in HepG2 cells transfected with plasmids containing 5′ flanking regions from −1059 to +27, −989 to +27, and −919 to +27 fell to 23–46% of the level of II(−2969/+27)cat. CAT expression fell to the level of the control plasmid, pSV0cat, for II(−849/+27)cat and shorter constructs. None of the plasmids were expressed at levels significantly above pSV0cat in HeLa cells.

In order to characterize the enhancer activity and tissue specificity of the upstream region, a series of fragments with 5′ ends ranging from −1059 to −919 and 3′ ends at −790 were generated by PCR and inserted in both orientations upstream of the *Herpes simplex tk* promoter in the plasmid pBLcat2 (Luckow & Schutz, 1987). The hybrid plasmids were transfected into HepG2 and HeLa cells. DNA coding for 5′ flanking regions −1059 to −790, −989 to −790, and −919 to −790 in either orientation with respect to the CAT gene enhanced CAT expression 2–102-fold in HepG2 cells (Table

Table II: Effect of a Prothrombin Upstream Regulatory Region on the *H. simplex tk* Promoter–CAT Plasmid pBLcat2

plasmid	relative CAT activity[a] HepG2	HeLa
II(–1059/–790)cat2	45.8	87
II(–989/–790)cat2	10.9	4.4
II(–919/–790)cat2	1.7	1.2
II(–790/–1059)cat2	5.2	3.3
II(–790/–989)cat2	8.2	3
II(–790/–919)cat2	102	31.6
pBLcat2	1	1

[a] *x*-fold increase over pBLcat2 in each cell type.

Table III: Expression of Hybrid CAT Plasmids Containing the Prothrombin Promoter Region

plasmid	relative CAT activity[a] HepG2	HeLa
pBLcat3	1	1
II(–73/+27)cat3	0.5	0.4
pBLcat2	31.8	4.7
II(–73/+27)cat2	20.5	1.4

[a] *x*-fold increase over pBLcat3 in each cell type.

II). The regions from nucleotide –1059 to –790 and –989 to –790 in either orientation and from nucleotide –919 to –790 in a reverse orientation enhanced CAT expression 3–87-fold in HeLa cells (Table II). The region from nucleotides –919 to –790 inserted in its proper orientation into pBLcat2, however, was expressed at the same level as pBLcat2 in HeLa cells (Table II). The enhanced expression of most hybrid constructs in both cell types suggests that the regulatory elements in the region from –1059 to –790 include a positive element which can act in either orientation with respect to the *tk* promoter in both cell types.

Constructs with the upstream regulatory region (–1059 to –790) ligated to either –108 to +27 or –73 to +27 in the promoterless vector pSV0cat were used to test whether the minimal regulatory elements were contained in these regions. Constructs with the upstream region ligated to either promoter region were expressed in HepG2 cells at levels comparable to the full-length plasmid, II(–2969/+27)cat (Table I). Both were expressed in HeLa cells at the level of pSV0cat. Reversal of the orientation of the upstream regulatory region as in the plasmid II[(–790/–1059)×(–108/+27)]cat resulted in expression in HepG2 cells which was decreased, but still 7.3-fold greater than pSV0cat. The same construct was expressed at control levels in HeLa cells.

A portion of the putative prothrombin promoter, nucleotides –73 to +27, was inserted upstream of the CAT gene in the promoterless plasmid, pBLcat3, and into pBLcat2 upstream of the *tk* promoter to test for possible enhancer activity. The activity of CAT in cell extracts prepared from HepG2 or HeLa cells transfected with II(–73/+27)cat3 and II(–73/+27)cat2 did not differ significantly from those transfected with the parent plasmids, pBLcat3 and pBLcat2 (Table III).

Primer Extension Analysis. Primer extension analysis was performed using RNA prepared from transfected cells and a radiolabeled oligonucleotide primer complementary to coding sequence at the 5′ end of the CAT gene in order to determine the transcription initiation site of transfected plasmids. The plasmids II(–73/+27)cat2, II(–1059/–790)cat2, II(–989/–790)cat2, II(–790/–1059)cat2, and pBLcat2 in HepG2 cells all had primer extension products corresponding to transcripts initiated at nucleotides 540–548 in the pBLcat2 sequence (Figure 1A,B). (The initiator methionine codon of the CAT gene in pBLcat2 begins at nucleotide 640; Luckow & Schutz, 1987.) Plasmid II(–2969/+27)cat in HepG2 cells had heterogeneous primer extension products with a cluster of bands corresponding to transcription initiation sites at –39, –35, and –30 with respect to the sequence of the human prothrombin gene (Figure 1C). Primer extension products for the plasmids II[(–1059/–790)×(–108/+27)]cat and II-[(–1059/–790)×(–73/+27)]cat were heterogeneous with a group of bands, similar to those observed for II(–2969/+27)-cat, corresponding to prothrombin nucleotides –37, –36, –35, and –31. Additional faint bands were noted, corresponding to longer transcripts with transcription initiation sites of –891, –793, and –790 in the prothrombin sequence for II[(–1059/–790)×(–108/+27)]cat and –926, –853, –829, –796, and –792 in the prothrombin sequence for II[(–1059/–790)×(–73/+27)]cat (Figure 1D).

Putative HNF-1 Binding Site. Since deletion of the region from –919 to –849 resulted in background levels of CAT expression in HepG2 cells, the nucleotide sequence in this region was examined for homology with sequences known to bind liver-specific transcription factors. An area homologous to the HNF-1 binding site of the human fibrinogen β-chain gene was identified (Figure 2; Courtois et al., 1988). These two sequences were identical at 8 of 12 bases.

Analysis of Protein Binding to the Upstream Regulatory Region and the Promoter Region. Gel mobility shift assays were performed to characterize the pattern of nuclear protein binding to DNA coding for nucleotides –919 to –790 and the promoter region. When radiolabeled DNA coding for nucleotides –919 to –790 was incubated with HepG2 nuclear extract, a complex pattern of bands was apparent after autoradiography (Figure 3A). Two of the bands (bands I and II) were competitively inhibited by the addition of a 10–50-fold molar excess of unlabeled DNA (–919/–790). A 20-fold molar excess of a fragment from the human HGF cDNA diminished the intensity of these bands to a lesser degree than the specific competitor. The higher molecular weight band (band I) was also competitively inhibited by a 10-fold excess of HNF-1, a double-stranded oligonucleotide containing the HNF-1 recognition sequence from the human fibrinogen β-chain gene, while the lower molecular weight band (band II) was inhibited only by a 100-fold molar excess of HNF-1 (Figure 3B). When the same radiolabeled fragment (–919/–790) was incubated with HeLa nuclear extract only a faint band which comigrated with band I was observed. Formation of this band was inhibited by both unlabeled DNA (–919/–790) and by HNF-1. This suggests that one or more nuclear proteins found in abundance in HepG2 cells binds specifically to nucleotides –919 to –790 and that one of these proteins is likely to be HNF-1.

When radiolabeled DNA coding for the promoter region of the prothrombin gene (–108/+27) was incubated with HepG2 nuclear extract, a number of retarded bands including three high molecular weight bands and a low molecular weight band were observed (Figure 4). Two of the high molecular weight bands (bands I and II) could be competitively inhibited with excess unlabeled DNA (–108/+27) but not with a 20-fold molar excess of the HGF fragment. Band II was inhibited at a 10-fold molar excess of unlabeled DNA (–108/+27) while band I was only inhibited by a 50-fold molar excess. Band III was partially inhibited by a 50-fold molar excess of unlabeled DNA (–108/+27) but not by a nonspecific competitor. This suggests that multiple nuclear proteins bind to this sequence with differing affinities.

When incubated with HeLa nuclear extract the radiolabeled promoter region produced two retarded bands, both of which were inhibited by a 10-fold excess of unlabeled specific competitor DNA (Figure 4). A 20-fold molar excess of HGF

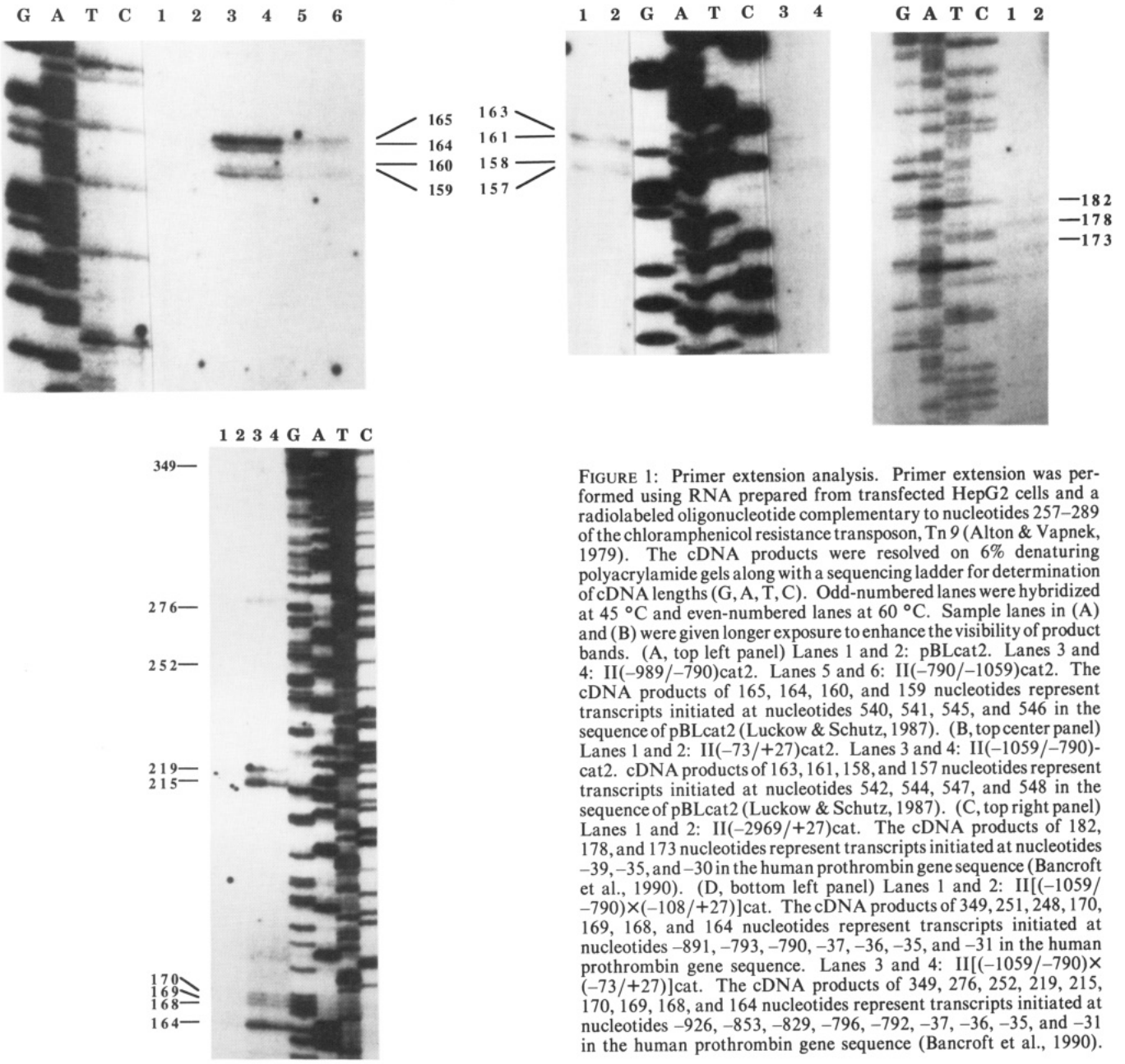

FIGURE 1: Primer extension analysis. Primer extension was performed using RNA prepared from transfected HepG2 cells and a radiolabeled oligonucleotide complementary to nucleotides 257–289 of the chloramphenicol resistance transposon, Tn *9* (Alton & Vapnek, 1979). The cDNA products were resolved on 6% denaturing polyacrylamide gels along with a sequencing ladder for determination of cDNA lengths (G, A, T, C). Odd-numbered lanes were hybridized at 45 °C and even-numbered lanes at 60 °C. Sample lanes in (A) and (B) were given longer exposure to enhance the visibility of product bands. (A, top left panel) Lanes 1 and 2: pBLcat2. Lanes 3 and 4: II(–989/–790)cat2. Lanes 5 and 6: II(–790/–1059)cat2. The cDNA products of 165, 164, 160, and 159 nucleotides represent transcripts initiated at nucleotides 540, 541, 545, and 546 in the sequence of pBLcat2 (Luckow & Schutz, 1987). (B, top center panel) Lanes 1 and 2: II(–73/+27)cat2. Lanes 3 and 4: II(–1059/–790)-cat2. cDNA products of 163, 161, 158, and 157 nucleotides represent transcripts initiated at nucleotides 542, 544, 547, and 548 in the sequence of pBLcat2 (Luckow & Schutz, 1987). (C, top right panel) Lanes 1 and 2: II(–2969/+27)cat. The cDNA products of 182, 178, and 173 nucleotides represent transcripts initiated at nucleotides –39, –35, and –30 in the human prothrombin gene sequence (Bancroft et al., 1990). (D, bottom left panel) Lanes 1 and 2: II[(–1059/–790)×(–108/+27)]cat. The cDNA products of 349, 251, 248, 170, 169, 168, and 164 nucleotides represent transcripts initiated at nucleotides –891, –793, –790, –37, –36, –35, and –31 in the human prothrombin gene sequence. Lanes 3 and 4: II[(–1059/–790)×(–73/+27)]cat. The cDNA products of 349, 276, 252, 219, 215, 170, 169, 168, and 164 nucleotides represent transcripts initiated at nucleotides –926, –853, –829, –796, –792, –37, –36, –35, and –31 in the human prothrombin gene sequence (Bancroft et al., 1990).

DNA diminished the intensity of these bands to a lesser degree than the specific competitor.

DISCUSSION

The vitamin K-dependent coagulation factors are all expressed with a high degree of tissue specificity, limited predominantly to hepatocytes (Kelly & Summerfield, 1987). The study of the regulation of expression of prothrombin, the most abundant of these evolutionarily related factors, may provide insight into the molecular mechanisms regulating tissue-specific expression of this family of genes. The liver specificity of prothrombin expression is regulated at a pretranslational level and, thus, is likely to be mediated by the interaction of nuclear proteins (trans-acting factors) with specific cis-acting DNA sequences in or around the prothrombin gene (Jamison & Degen, 1991; Maniatis et al., 1987; Mitchell & Tjian, 1989). We have identified an array of cis-acting elements in the 5′ flanking region of the human prothrombin gene which together regulate the tissue-specific expression of prothrombin in HepG2 cells.

The Prothrombin Promoter. The 5′ flanking region of the human prothrombin gene, like that of the genes encoding

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                    5'                  3'
HNF-1 consensus        GTTAATATTAAC
                       |  |||||||
Prothrombin       -876 GCAAATATTAGT   -888
                          ||||||||
β-fibrinogen      -88  TTAAATATTAAC   -77
```

FIGURE 2: HNF-1 binding sequence homology. Sequence from the noncoding strand of the 5′ flanking region of the human prothrombin gene is aligned with the homologous region from the human fibrinogen β-chain gene and the HNF-1 consensus binding sequence (Bancroft et al., 1990; Courtois et al., 1988). Numbers indicate the nucleotide position of the 5′ and 3′ ends of each sequence. Vertical lines denote positions of sequence identity.

human factor VII, factor IX, and protein C, lacks both consensus TATA and CCAAT sequences (Bancroft et al., 1990; Foster et al., 1985; O'Hara et al., 1987; Yoshitake et al., 1985). These sequences are thought to direct the accurate initiation of transcription and augment transcription by RNA polymerase II (Maniatis et al., 1987). The lack of a consensus TATA sequence may contribute to the heterogeneity observed in prothrombin transcription initiation sites (Bancroft et al., 1990; Chow et al., 1991). A number of housekeeping genes, including the mouse and human hypoxanthine phosphoribo-

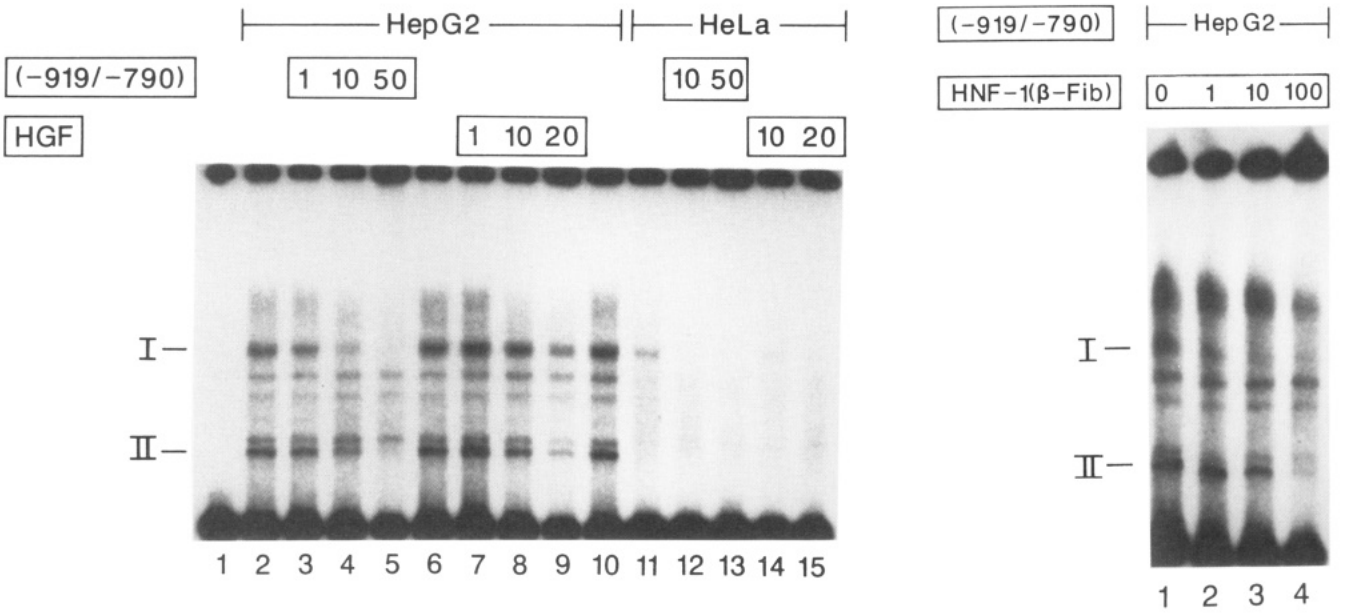

FIGURE 3: Gel-mobility shift assay with nucleotides –919 to –790 from the human prothrombin gene and nuclear extracts from HepG2 and HeLa cells. Conditions for incubation of nuclear extracts with DNA and for competition experiments are discussed in Materials and Methods. (A, left panel) Lane 1, radiolabeled (–919/–790) probe only. Lanes 2–10 and 11–15, probe incubated with HepG2 or HeLa nuclear extract, respectively. Lanes 3–5, 12, and 13 contain 1-, 10- and 50-fold molar excess of unlabeled (–919/–790) DNA as a specific competitor. Lanes 7–9, 14, and 15 contain 1-, 10-, and 20-fold molar excess of HGF cDNA as a nonspecific competitor. I and II indicate specific DNA–protein complexes. (B, right panel) Lanes 1–4, radiolabeled (–919/–790) probe incubated with HepG2 nuclear extract. Lanes 2–4 contain 1-, 10-, and 100-fold molar excess of unlabeled HNF-1 oligonucleotide (β-fib) as a competitor. I and II indicate specific DNA–protein complexes.

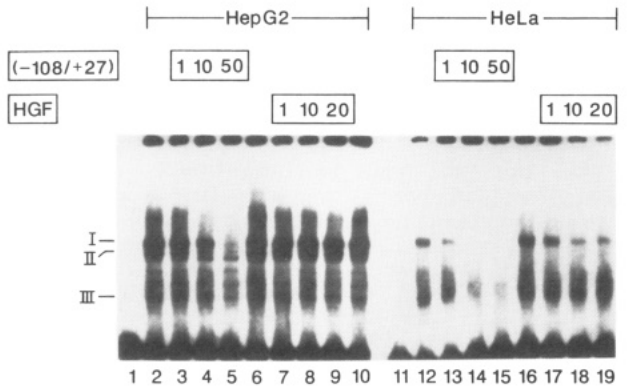

FIGURE 4: Gel mobility shift assay with nucleotides –108 to +27 from the human prothrombin gene and nuclear extracts from HepG2 and HeLa cells. Conditions for incubation of nuclear extracts with DNA and for competition experiments are discussed in Materials and Methods. Lanes 1 and 11, radiolabeled (–108/+27) probe only. Lanes 2–10 and 12–19, probe incubated with HepG2 or HeLa nuclear extract, respectively. Lanes 3–5 and 13–15 contain 1-, 10-, and 50-fold molar excess of unlabeled (–108/+27) DNA as a specific competitor. Lanes 7–9 and 17–19 contain 1-, 10-, and 20-fold molar excess of HGF cDNA as a nonspecific competitor. I, II, and III indicate specific DNA–protein complexes.

syltransferase and dihydrofolate reductase genes also lack consensus TATA sequences (Dynan, 1986). The human prothrombin gene, in addition, shares with these housekeeping genes a GC-rich region in the promoter area (Bancroft et al., 1990; Dynan, 1986). The implications of this GC-rich region and the lack of a TATA sequence for formation of the transcription initiation complex remain unclear.

The two complex constructs, II[(–1059/–790)×(–108/+27)]cat and II[(–1059/–790)×(–73/+27)]cat, had, in addition to the predominant transcription start sites at nucleotides –37 to –31 in the prothrombin sequence, minor primer extension products corresponding to start sites farther upstream in the plasmids (Figure 1D). The location of the true promoter far upstream is unlikely due to the lack of evidence for secondary structure in the prothrombin gene 5′ flanking region and the consistent results observed in primer extension and exon mapping studies of the human prothrombin gene using HepG2 cell RNA and prothrombin-specific oligonucleotides (Bancroft et al., 1990). In addition, the size of the prothrombin mRNA is consistent with the previously determined start site. Two alternative explanations which we cannot exclude are, first, that the longer products are an artifact of cloning. Removal of the upstream enhancer region from its normal position and placement close to the promoter-proximal region could result in the appearance of cryptic transcription start sites. Alternatively, the long products could be the result of readthrough transcripts initiated in the parent plasmid, pSV0cat. Such transcripts contribute to the background level of CAT expression from this promoterless plasmid (Salier & Kurachi, 1989). Most importantly, these longer products were always minor when compared to the predominant primer extension products. These predominant products correspond to the primer extension products observed for the remainder of the plasmids containing the prothrombin promoter-proximal region.

The prothrombin promoter region is transcriptionally inactive unless linked to sequences between nucleotides –919 and –849 from the 5′ flanking region. The plasmids II(–849/+27)cat, II(–797/+27)cat, and II(–419/+27)cat were not expressed above control levels in either HepG2 or HeLa cells while II(–919/+27)cat and plasmids containing additional upstream sequence [II(–989/+27)cat, II(–1059/+27)cat, II(–1262/+27)cat, and II(–2969/+27)cat] were expressed in HepG2 but not HeLa cells (Table I). Similarly, ligation of the upstream enhancer region (nucleotides –1059 to –790) directly to the promoter region (nucleotides –108 to +27 or nucleotides –73 to +27) upstream of the CAT gene resulted in high expression of CAT only in HepG2 cells (Table I). This implies that the prothrombin promoter requires this upstream region for transcriptional activity and tissue specificity. This is analogous to the human albumin promoter and its enhancer located 10 kb upstream of the promoter, which are both required for expression and liver specificity (Pinkert et al., 1987).

These observations differ from those of Chow and co-workers, who noted a low level of transcriptional activity in HepG2 cells from hybrid constructs containing the first 400 bp of the 5′ flanking region of the human prothrombin gene ligated to a human growth hormone reporter gene (Chow et al., 1991). These conflicting observations may reflect either a difference in our hybrid reporter gene constructs or a difference in sensitivity of detection methods for CAT and human growth hormone.

Upstream Enhancer Region. Our initial studies of prothrombin expression in HepG2 cells suggested that a positive cis-acting regulatory sequence between nucleotides −2969 and −797 in the 5′ flanking region of the human prothrombin gene was responsible for expression in HepG2 cells (Bancroft et al., 1990). The current investigations have demonstrated, however, that this region may be more complex, containing at least two distinct regulatory elements.

A region highly homologous to the consensus sequence for the binding site for HNF-1 was identified in the upstream enhancer region at nucleotides −888 to −876 (Figure 2; Courtois et al., 1988). Oligonucleotides containing DNA coding for this region formed specific complexes with proteins in nuclear extract from HepG2 cells as seen in gel mobility shift assays (Figure 3). The formation of these specific complexes was inhibited by excess oligonucleotide containing the HNF-1 binding sequence from the human fibrinogen β-chain gene, implying that HNF-1 protein binds this DNA sequence in HepG2 cells. Although this suggests that this is a functional HNF-1 binding site in HepG2 cells, our data do not definitively demonstrate that HNF-1 binding is necessary for tissue-specific expression of prothrombin.

HNF-1 (otherwise known as LF-B1 or APF), a DNA binding protein of 88 000 molecular weight, participates in the liver-specific expression of a number of genes including genes encoding α1-antitrypsin, albumin, α-fetoprotein, transthyretin, and the α and β chains of fibrinogen (Courtois et al., 1988; Frain et al., 1989; Monaci et al., 1988). HNF-1 is expressed predominantly in the liver, although Baumhueter and co-workers demonstrated the presence of HNF-1 mRNA and protein in kidney, intestine, spleen, and thymus (Baumhueter et al., 1990). This protein shares three functional domains with the POU family of trans-acting factors including a diverged homeodomain required for DNA binding (Baumhueter et al., 1990; Frain et al., 1989).

The HNF-1 consensus sequence identified in the 5′ flanking region of the human prothrombin gene is located farther upstream of the transcription initiation site than other previously described HNF-1 binding sites, most of which are located between −120 and +1 (Courtois et al., 1988). The significance of the distance from the transcription initiation site to the putative prothrombin HNF-1 binding sequence is unknown, since deletion of 682 bp of DNA between this site and the promoter (for example, in the plasmid II[(−1059/−790)×(−108/+27)]cat) had no effect on the tissue specificity or level of expression (Table I).

Ligation of a portion of the upstream regulatory region of the human prothrombin gene from nucleotides −1059 to −790 upstream of the *H. simplex tk* promoter in either orientation in pBLcat2 resulted [with the exception of II(−919/−790)-cat2] in plasmids expressed at a markedly higher level than the control plasmid, pBLcat2, in HepG2 and HeLa cells (Table II). The reason for baseline expression of II(−919/−790)-cat2 is unclear, as other constructs with both longer and shorter inserts of this region and the reverse orientation of this insert demonstrate non-tissue-specific enhancer activity. This non-tissue-specific effect contrasts with the observation of Monaci and co-workers that ligation of the B-box (HNF-1 binding region) of the α1-antitrypsin promoter to the *H. simplex tk* promoter converted it from ubiquitous to liver-specific expression (Monaci et al., 1988). Since HeLa cells do not express HNF-1, our findings suggest that the region from nucleotides −919 to −790 in the human prothrombin gene contains a second enhancer with non-tissue-specific activity (Frain et al., 1989). An alternative which we cannot exclude is that all of the enhancer activity expressed by the upstream region (nucleotides −919 to −790) is due to this non-tissue-specific enhancer, that the putative HNF-1 binding site is not involved in tissue-specific expression, and that the tissue specificity is mediated by sequences in the promoter-proximal region (nucleotides −73 to +27). Although the promoter-proximal region may be involved in tissue specificity, we find this alternative unlikely since linker scanning mutagenesis of the human prothrombin gene putative HNF-1 binding sequence markedly reduced expression in HepG2 cells (Chow et al., 1991).

Chow and co-workers have recently characterized an upstream enhancer element in the human prothrombin gene (Chow et al., 1991). This enhancer [between nucleotides −923 to −843 based on the sequence of Bancroft et al. (1990); nucleotides −940 to −860 using the numbering system of Chow et al. (1991)] is flanked by inverted repeat sequences ($^{5'}$CCTCCC$^{3'}$) and contains the putative HNF-1 binding site. The results of both deoxyribonuclease I footprint analysis and linker scanning mutagenesis of this enhancer element suggest that it contains multiple protein binding sites (Chow et al., 1991). The authors define the location of the human prothrombin enhancer as nucleotides −923 to −843 on the basis of the location of the inverted repeat sequences; however, neither deletion of nucleotides −948 to −903 nor linker scanning mutagenesis of nucleotides −925 to −906 had an effect upon expression of hybrid constructs. This confirms our observation that the critical enhancer elements for expression in HepG2 cells are located between nucleotides −919 to −849.

Additional Regulatory Elements. When the sequences of the 5′ flanking region of the human and bovine prothrombin genes are compared, there is an area with 69% homology located between nucleotides −717 and −581 in the human prothrombin gene and nucleotides −580 and −443 in the bovine prothrombin gene (Bancroft et al., 1990; Irwin et al., 1988). The 5′ extent of this area of homology is unknown since the sequence of the bovine prothrombin gene has only been reported to nucleotide −580 (Irwin et al., 1988). Deletion of the entire element, as in II[(−1059/−790)×(−108/+27)]cat, had no effect on tissue specificity or level of expression. Thus, although highly conserved, the function of this sequence remains unclear.

Our findings differ from those of Chow and colleagues in two respects. First, the enhancer region described by Chow and co-workers appears in our studies to have non-tissue-specific activity when linked to the heterologous *tk* promoter in addition to the tissue-specific activity which we both observed. Second, as previously noted, we detect no transcriptional activity from the prothrombin promoter in the absence of the upstream enhancer region.

The distinct difference between DNA–protein complexes formed in gel mobility shift assays using HepG2 and HeLa nuclear extracts further supports the hypothesis that both the region from −919 to −790 and the promoter contain tissue-specific regulatory elements. These tissue-specific sequences appear to bind nuclear proteins present in HepG2 but not in HeLa cells (Figures 3 and 4). Methylation interference analysis or deoxyribonuclease I footprinting analysis will further define the sequences involved in the tissue specificity

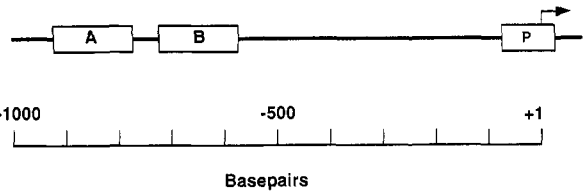

FIGURE 5: Regulatory regions of the 5'-flanking region of the human prothrombin gene. Region A includes nucleotides −919 to −790, and region B includes nucleotides −717 to −581 (see text for further discussion). P = promoter region. The arrow represents the site of initiation of transcription, which is indicated as +1 on the scale.

of expression and possibly the mechanisms of transcriptional regulation.

Our current hypothesis regarding the transcriptional regulation of the human prothrombin gene in HepG2 cells is summarized in Figure 5. Two or more enhancer sequences are present in region A: an HNF-1 binding site which is liver-specific and a second sequence with general enhancer activity. The enhancers in region A and the prothrombin promoter are the required cis-acting elements for tissue-specific expression of prothrombin in HepG2 cells. Finally, region B is a highly conserved region between the upstream enhancer and promoter regions which does not participate in regulation of the gene in HepG2 cells. Further studies will be required to define the specific sequences within these regions which direct liver-specific expression. Identification of these sequences and the trans-acting factors which bind them will facilitate evaluation of the regulatory mechanisms of the other vitamin K-dependent coagulation factors.

Generalization of these results to conclusions about the regulation of this gene in normal liver are limited by the use of tissue culture cell lines in these studies. Although HepG2 cells are well differentiated in many functions, they do not secrete normal amounts of factor IX (Crossley & Brownlee, 1990). Differences were also noted by Hammer and coworkers between the regulatory activity of three enhancers in the α-fetoprotein gene when examined in transgenic animals compared to transient expression in HepG2 cells (Godbout et al., 1986; Hammer et al., 1987). Our observations regarding the mechanism of regulation of the liver-specific expression of prothrombin must be confirmed either in primary culture of well-differentiated hepatocytes or in transgenic animals.

ACKNOWLEDGMENT

We thank Bin Wang for assistance with gel mobility shift assays and Patricia Gallagher for assistance with PCR. We also thank Debi Krauth for cloning one of the *cat* constructs, Lorie Stuart for technical assistance, and Jan Hagedorn for preparation of the manuscript.

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