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Structural Characterization of the Gene for Human Histidine-Rich Glycoprotein, Reinvestigation of the 5′-Terminal Region of cDNA and a Search for the Liver Specific Promoter in the Gene[1]

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Genomic DNA libraries were screened for the human histidine-rich glycoprotein (HRG) gene and a sequence of 15,499 nucleotides was determined. The gene is composed of 7 exons and 6 introns, and all the exon-intron boundaries match the consensus GT/AG sequence for donor and acceptor splice sites. Each of cystatin-like domains I and II of HRG is encoded by three exons, exons I to III and exons IV to VI, respectively, like those of other members of the cystatin superfamily. The entire C-terminal half of the molecule is encoded by the largest exon, VII. The first 103 nucleotides of the cDNA sequence reported for human HRG [Koide, T., Foster, D., Yoshitake, S., and Davie, E.W. (1986) *Biochemistry* 25, 2220-2225] could not be found in the determined gene sequence. A homology search of this sequence against a database showed the complete matching to a part of the yeast mitochondrial DNA encoding 21S ribosomal RNA. Rapid amplification of cDNA 5′ ends (5′-RACE) analysis revealed that the cDNA has multiple 5′-ends and that a possible starting point is nucleotide 104 of the reported cDNA sequence. These results suggest that the first 103 nucleotides of the cDNA sequence reported for human HRG originated from yeast mitochondrial DNA and were incidentally incorporated into the HRG cDNA in the process of the construction of a cDNA library. Various fragments obtained on restriction endonuclease digestion of the 5′-noncoding region of the HRG gene were ligated to the chloramphenicol acetyltransferase (CAT) gene and then transfected into HepG2 and 293 cells to analyze the promoter activity. The sequence between −262 and −21 from the putative translation initiation site supported the expression of CAT in HepG2 cells but not in 293 cells, suggesting that this segment promotes the liver-specific transcription of the human HRG gene.

Key words: CAT assay, gene structure, histidine-rich glycoprotein, molecular cloning, nucleotide sequence.

Histidine-rich glycoprotein (HRG) is one of the major components of human plasma proteins. HRG is synthesized exclusively in the liver (*1*) and secreted into the plasma. Platelets and megakaryocytes also contain HRG in their intracellular granules, although it is not known whether or not HRG is synthesized in these cells (*2*). The exact physiological role of HRG has not been established yet, but it binds to a wide range of molecules. Among them, the binding of HRG to the lysine-binding site of plasminogen reduces plasminogen binding to fibrin, resulting in an antifibrinolytic effect (*3*). HRG competes for heparin binding with antithrombin, heparin cofactor II or protein C inhibitor, revealing its heparin-neutralizing ability (*4-10*). On the other hand, HRG binds to fibrinogen (*11*) and prolongs the time for the conversion of fibrinogen to fibrin by thrombin. HRG also binds to platelets by interacting with thrombospondin (*12*), although the significance of this is not well understood. Thus, HRG is thought to play regulatory roles in hemostasis and fibrinolysis. Moreover, HRG binds to several components of the complement system and T-lymphocytes, being likely to be involved in the immune system (*13-15*). The primary structure of human HRG was deduced from the nucleotide sequence of its cDNA, it being composed of 507 amino acid residues including 66 histidines and 65 prolines (*16*). HRG has two tandem repeats homologous to cystatin, a cysteine-protease inhibitor, and belongs to the cystatin superfamily (*17*). Several members with a congenital deficiency of HRG were found in two families with thrombophilia (*18, 19*). We recently analyzed the molecular and cellular bases for the deficiency in one family (HRG Tokushima) (*20*). In contrast, families with elevated levels of HRG and thrombophilia have also been reported (*21-24*). Thus, the relationship between the plasma HRG level and thrombosis is not

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Abbreviations: CAT, chloramphenicol acetyltransferase; HRG, histidine-rich glycoprotein; RACE, rapid amplification of cDNA ends.

clear at present. To analyze the molecular bases of these abnormalities, it is essential to elucidate the complete structure of the gene for HRG located on chromosome 3q28-29 (*25*).

In this paper, we describe the isolation of genomic clones for human HRG, determination of the nucleotide sequence and partial characterization of the promoter region of the human HRG gene, together with reinvestigation of the 5′-terminal region of HRG cDNA.

MATERIALS AND METHODS

Materials—A human genomic library constructed in the λ Charon 4A phage was kindly provided by Dr. T. Maniatis. Another human genomic library constructed with human leukocyte genomic DNA in the λ EMBL3 phage was purchased from Clontech (Palo Alto, CA, USA). A human liver cDNA library in λgt11 (5′-stretch library) and human liver poly(A)$^+$ RNA were also purchased from Clontech. Various restriction endonucleases were obtained from Takara Shuzo (Kyoto), Toyobo (Osaka), Boehringer Mannheim (Mannheim, Germany), or New England Biolabs (Beverly, MA, USA). The vectors for the CAT assay (pCAT Basic, pCAT Enhancer, pCAT Promoter, and pCAT Control) and pSVβG for correction of the transfection efficiency were from Promega (Madison, WI, USA). Nitrocellulose membranes (BA85S) were from Schleicher & Schuell (Dassel, Germany). All other reagents and chemicals used in this study were of the highest grade available and obtained from Wako Pure Chemicals Industries (Osaka) or Nacalai Tesque (Kyoto). Oligonucleotide primers were synthesized with an Applied Biosystems Model 394 DNA synthesizer.

Isolation of the Phage Clones Containing an Insert of the Human HRG Gene—A λ Charon 4A human genomic DNA library was screened for HRG by means of the plaque hybridization method using a radiolabeled cDNA for human HRG as the hybridization probe as described (*26*). The positive clones were plaque-purified and phage DNA was prepared by the liquid culture lysis method (*26*). The insert DNA fragments were recovered by digestion of cloned phage DNA with *Eco*RI or *Xba*I and then subcloned into the pUC19 plasmid. The screening process and partial characterization of the HRG gene were preliminarily reported previously (*27*).

Rescreening of the Human Liver cDNA Library—A human liver cDNA library in λgt11 enriched in 5′ sequences (5′-stretch cDNA library) was screened for HRG cDNA by the plaque hybridization method. A 1.9 kb insert of the human HRG cDNA clone was labeled with digoxigenin-dUTP and used as the probe for plaque hybridization according to Boehringer's protocol. After isolating the positive clones, the 5′-portion of each cDNA insert was amplified with HRG-specific primer I (Table I) and one of the λ insert amplimers placed just outside the cloning site (Clontech). The amplified fragments were digested with restriction endonucleases, *Eco*RI and *Pst*I, and then subcloned into the pUC19 plasmid for sequencing.

Rapid Amplification of cDNA 5′ Ends (5′-RACE)—5′-RACE analysis (*28*) was performed with a 5′-RACE system (GibcoBRL) according to the manufacturer's instruction. Primers I and II (Table I) were used as gene specific primers. The amplified fragments were digested with *Spe*I and *Pst*I, and then ligated to pUC19. Thirteen clones were isolated and the nucleotide sequences of their insert DNAs were determined.

Sequence Determination—The nucleotide sequence was determined by the dideoxy chain termination method with a Sequenase Ver 2.0 DNA Sequencing Kit (United States Biochemicals, Cleveland, OH, USA) and [α-^{35}S]dATP. Template DNA was alkali-denatured prior to annealing with the universal −40 or reverse sequencing primer according to USB's instructions.

Promoter Assay—The 5′-flanking region of the HRG gene was amplified by PCR using primers III and IV (Table I), and digested with *Pma*CI and *Xba*I to isolate the product. This fragment was inserted into the pCAT Basic or pCAT Enhancer plasmid, which had been pretreated with *Sal*I, blunt-ended with Klenow DNA polymerase, and then digested with *Xba*I to create blunt and cohesive ends. Then, utilizing the intrinsic *Bgl*II (−262) site of the insert and *Hind*III site of the pCAT vector, the *Hind*III/*Bgl*II fragments of these constructs were replaced by various subcloned gene fragments of 5′-noncoding region of different sizes. These plasmids (10 μg) were then transfected into human hepatoma HepG2 cells (RIKEN Cell Bank RCB0459) or human kidney 293 cells (Invitrogen) by the calcium phosphate transfection method (*26*) together with the pSVβG plasmid (5 μg) to correct possible variations in transfection efficiency. The cells were cultured under a 3% CO_2 atmosphere for 22 h, followed by under 5% CO_2 for a further 24 h in fresh medium, and then harvested. The chloramphenicol acetyl-transferase (CAT) activity of cell extracts was measured by HPLC on a mixed-functional

TABLE I. **Synthetic oligonucleotides used as primers.**

Primer No.	Sequence	(Direction)
I.	5′-CAGCACTGCAGTCAGTGGGACTCA-3′ (200 … 177)	(reverse)
II.	5′-CCAGATCTTACTGAGGGTCGAAGACTTCACA-3′ (862 … 842)	(reverse)
III.	5′-GACTCGAGATGCTGCCTAACTGGCTG-3′ (−1326 … −1309)	(forward)
IV.	5′-TCTCTAGATATGATCTGCCACTGCAGAG-3′ (−21 … −40)	(reverse)
V.	5′-CCGAATTCTAATAAAGATCAGGAAATAATTAATG-3′ (13 … 39)	(forward)
VI.	5′-GGGGATCCTTTAATTATCTAATTCACCTTCATAT-3′ (106 … 80)	(reverse)

Numbers below the underlined sequences of primers I, II, V, and VI indicate their positions in the reported cDNA sequence (*16*). Numbers below the sequences of primers III and IV indicate the positions in the gene sequence upstream from the initiation codon, ATG.

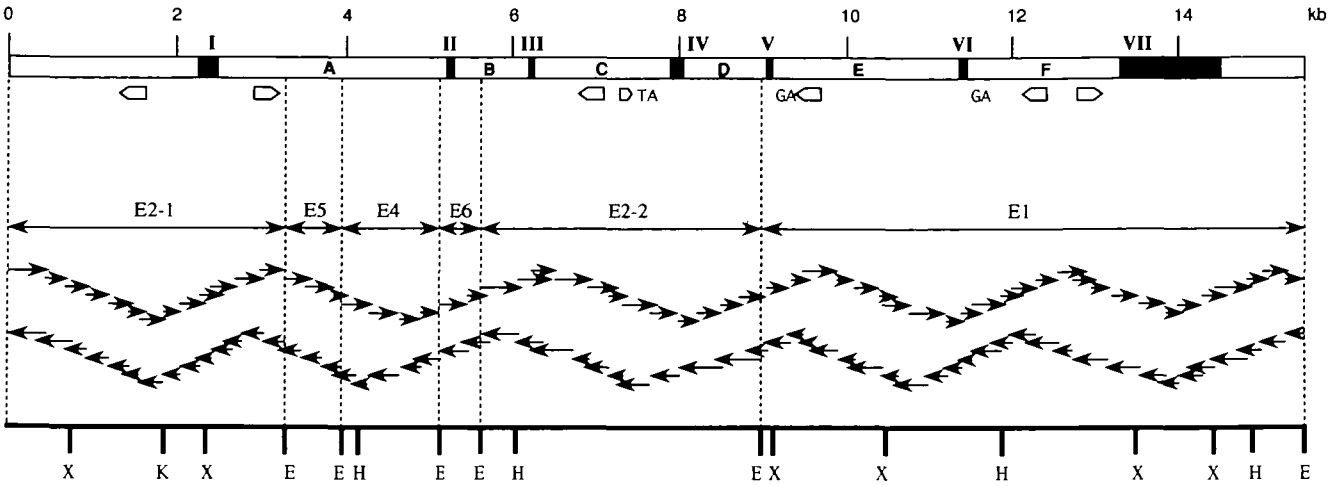

Fig. 1. **Structure of the human HRG gene.** Exons are represented as black boxes and the locations of repeat structures are indicated by "TA" or "GA." The locations and directions of the Alu repeats are shown by box-like arrows. In the middle portion, the locations of six *Eco*RI fragments are shown. The direction and extent of sequencing are indicated by arrows. A partial restriction map is shown at the bottom. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I.

reverse phase column (manuscript in preparation), and β-galactosidase activity was assayed as described (*29*).

RESULTS

Screening of the Human HRG Gene Clones and Nucleotide Sequence Determination—Several positive clones were isolated from the λ Charon 4A phage library as reported previously (*27*), but only clone 32 covered all 7 exons and thus was used for the nucleotide sequence analysis. Clone 32 had an insert of about 17 kb consisting of 7 *Eco*RI fragments, *i.e.* E1 (6.5 kb), E2-1 and E2-2 (3.3 kb each), E3 (1.5 kb), E4 (1.2 kb), E5 (0.6 kb), and E6 (0.5 kb). The nucleotide sequence of each fragment except for that of E3, which was derived from the 3′-end of the insert and located downstream of the HRG gene, was completely determined in both directions after subcloning various fragments obtained on restriction endonuclease digestion. The direction and extent of sequencing are summarized in Fig. 1, together with a partial restriction map. The nucleotide sequence of 15,499 bases was determined, as shown in Fig. 2. On comparison with the cDNA sequence, the gene for human HRG was shown to be composed of six small exons, I through VI (81 to 206 bp), and one large exon, VII (1,189 bp). The size of introns varied between 886 bp (intron B) and 2,721 bp (intron A). The nucleotide sequences around all the intron-exon boundaries are well conserved and agree with the consensus sequence of Mount (*30*). All the splice junctions are Type 0 except intron C, which is Type I (*31*).

Search for the 5′-Terminal Region of the Reported cDNA Sequence—In the course of sequencing work on the HRG gene, the first 103 nucleotide sequence of the reported HRG cDNA (*16*) was not found in the gene sequence. PCR with a pair of primers, V and VI (Table I), could not amplify this region from the λ EMBL3 human genomic library. This suggests the 103 nucleotide sequence may not be derived from the human genome. Then, we searched a database for this sequence. A part of yeast mitochondrial DNA encoding 21S ribosomal RNA (GenBank accession No. J01527) was found to match this sequence completely. Therefore, we concluded that the first 103 nucleotides of the reported cDNA sequence had been derived from the yeast mitochondrial DNA.

Determination of the 5′-End of Human HRG cDNA—To determine the real 5′-end of HRG cDNA, a new human liver cDNA library was screened and the 5′-end of the insert of each positive clone was analyzed as described under "MATERIALS AND METHODS." Connection of the unrelated sequences to the HRG cDNA were observed in 9 out of the 13 clones newly analyzed. The other four clones had less than 13 bases of 5′ non-coding sequence. A homology search of the unknown portions against databases revealed that one clone had a 214-base fragment of human mitochondrial DNA (GenBank accession No. M10546) that is attached to human HRG cDNA at C(22), and another clone had a 44-base sequence of the human homolog of mouse ribosomal protein L3 (accession No. Y00225) attached at A(−6). The numbers in parentheses indicate the positions from the initiation ATG. Other clones also had various unrelated sequences (14 to 168 bases) which were attached to the HRG cDNA at various positions (−21 to 14). Thus the real 5′-end of the cDNA for human HRG could not be determined by recloning and sequence analyses of these cDNA clones.

Then, 5′-RACE analysis was performed and the sequences of 13 clones were analyzed. Four clones had a sequence starting from A(−18), four from A(−23), three from G(−20) or C(−19), and one each from A(−28) and T(−7). The initial G(−20) could not be distinguished from C(−19) by this method since polyC was ligated to the reverse transcribed fragments in the first step. The 104th nucleotide of the reported cDNA sequence corresponds to one of the major starting points, A(−18).

Search for the Liver-Specific Promoter—The 1.5 kb fragment of the 5′-flanking region of the human HRG gene was ligated to the reporter (CAT) gene, and then transfected into human hepatoma HepG2 and human kidney 293 cells. As shown in Fig. 3, considerable CAT activity (13.6%) was detected in HepG2 cells transfected with the pCAT Enhancer-based vector which contained an SV40 enhancer.

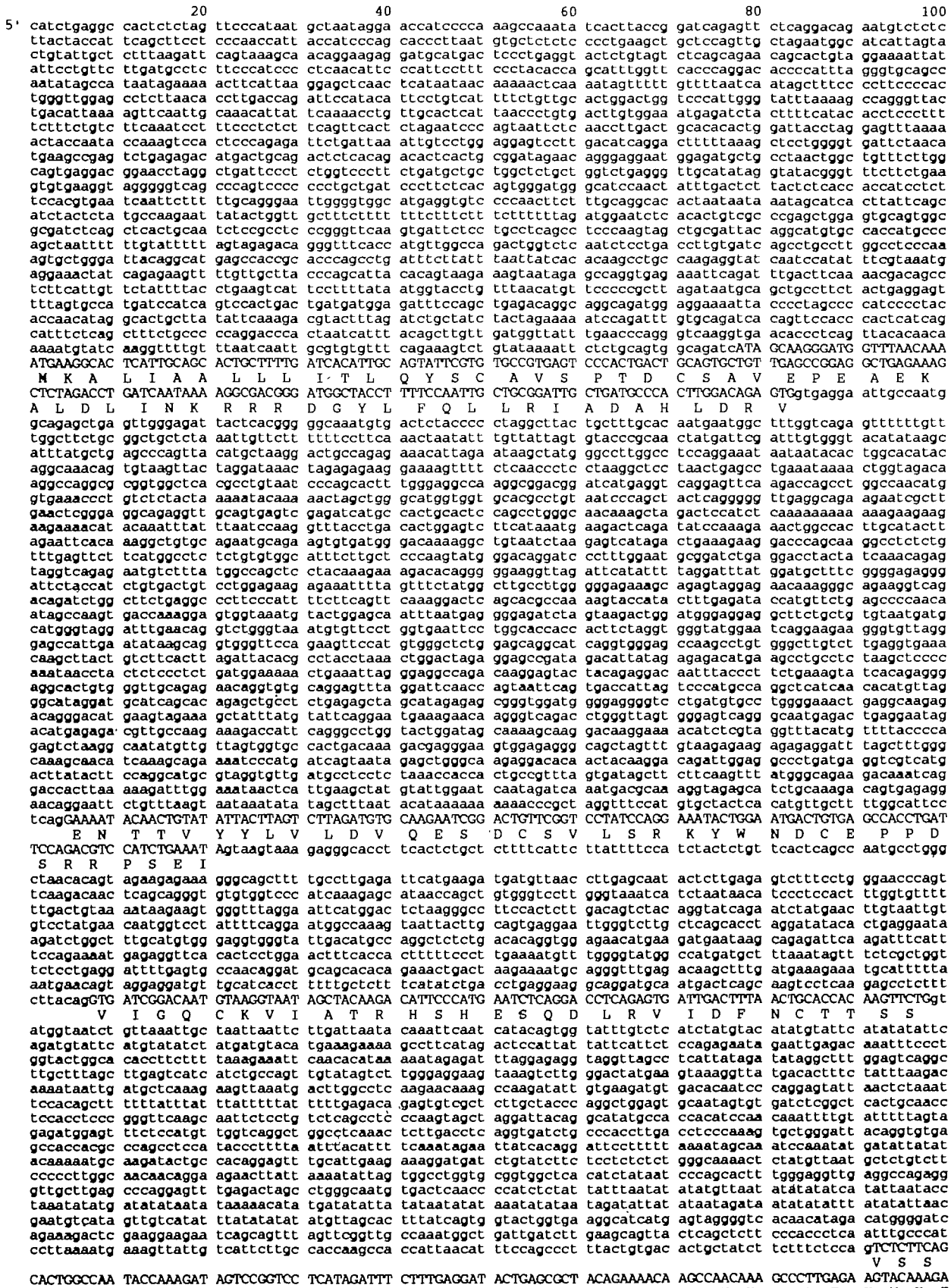

Fig. 2 (continued on next page)

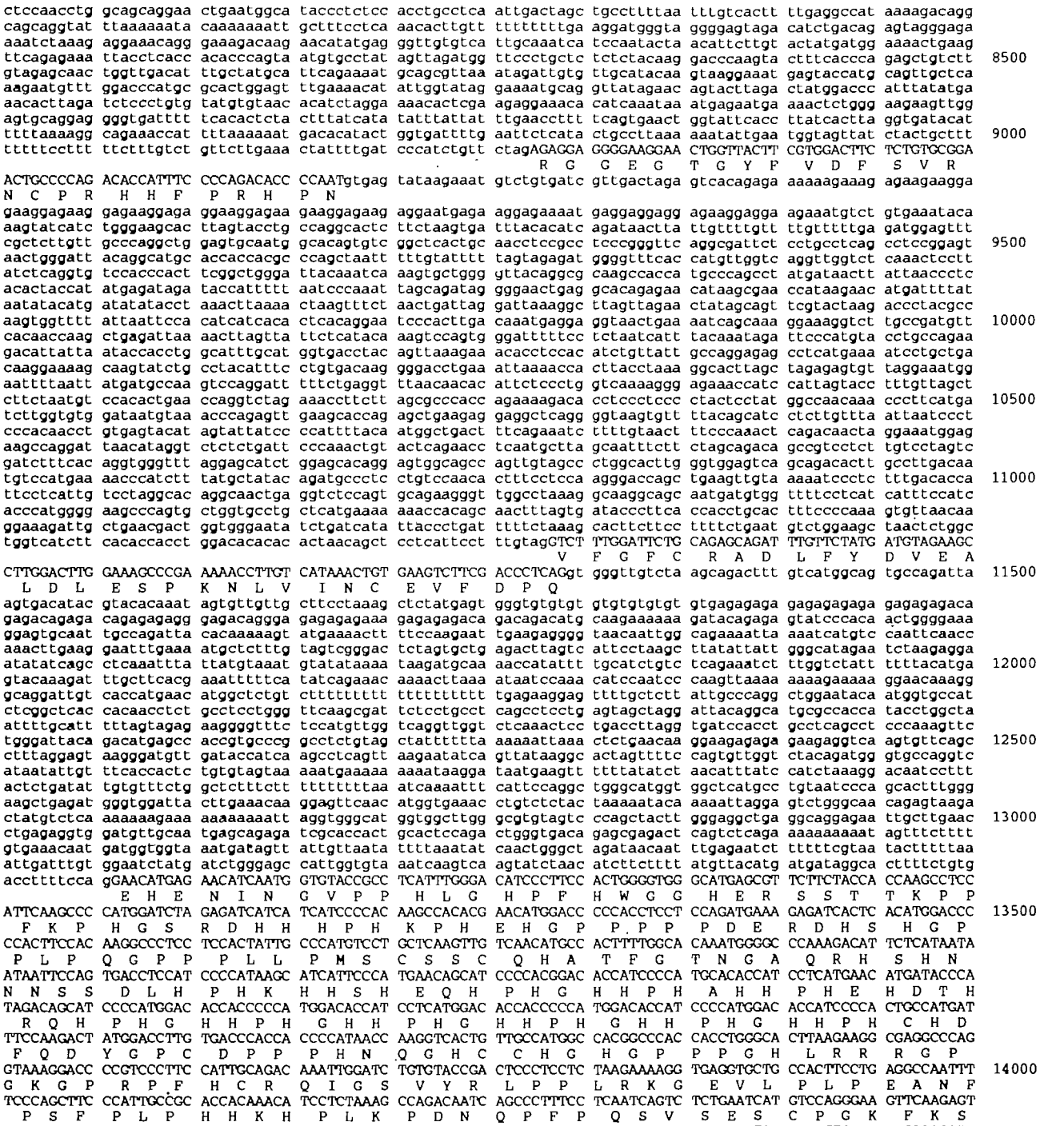

Fig. 2. **Nucleotide sequence of the human HRG gene.** Exon sequences are presented as capital letters together with the deduced amino acid sequences.

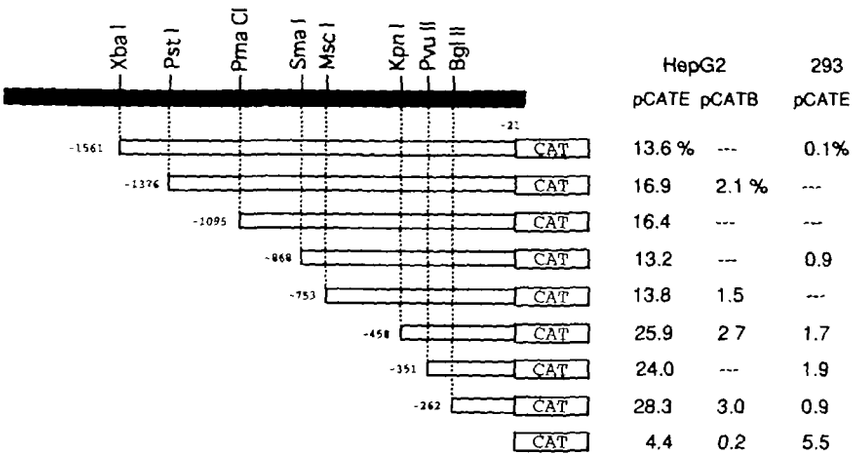

Fig. 3. **Results of CAT assays with HepG2 and 293 cells.** Various restriction endonuclease fragments were ligated to the pCAT Enhancer or pCAT Basic vector. Each CAT activity is expressed as a percentage of that obtained with the pCAT Control vector, which contains both an SV40 promoter and enhancer.

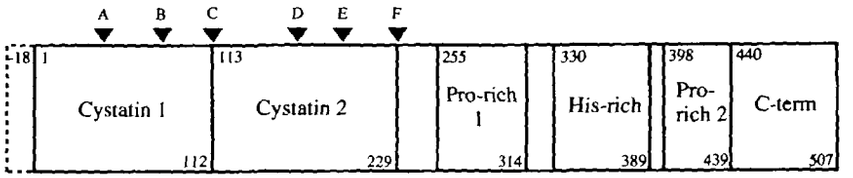

Fig. 4. **Domain structure of human HRG and locations of the introns.** The locations of introns are indicated by black triangles.

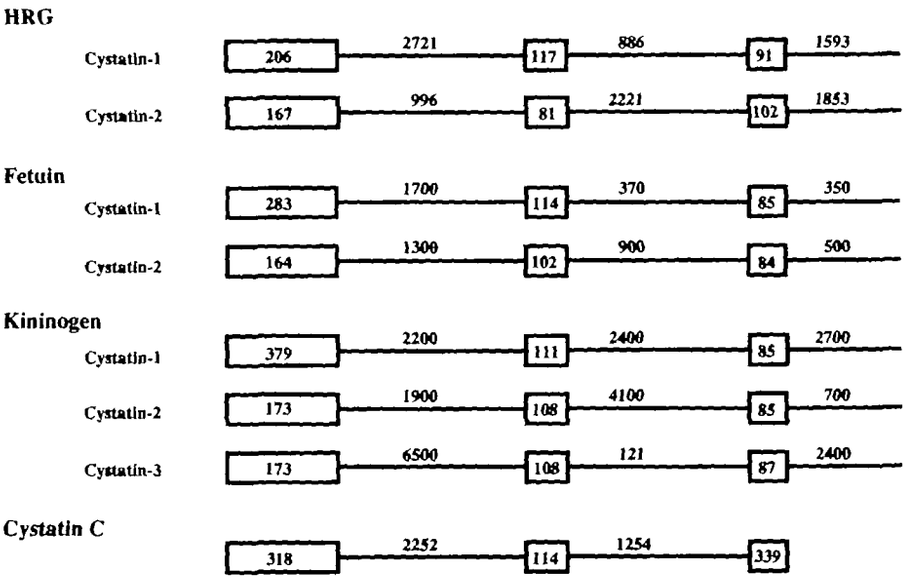

Fig. 5. **Comparison of the gene structure of each cystatin domain in human HRG, rat *fetuin* (*33*), human kininogen (*34*), and human cystatin C (*32*).** Exons are boxed. The sizes of exons and introns are expressed as the numbers of nucleotides.

Then, the insert was deleted using various restriction endonucleases and promoter activities were measured. The length of the ligated 5′-flanking region of the human HRG gene had little effect on the extent of expression, and the shortest construct that contained the fragment encompassing −262 to −21 from the translation initiation site was enough for expression of the maximum CAT activity (28.3%). The fragments in the pCAT Basic vector, which does not have an SV40 enhancer, also supported the expression of CAT in HepG2 cells, although the CAT activity was much lower than that in the case of the pCAT Enhancer vector. On the other hand, the 5′-noncoding region of the human HRG gene suppressed the expression of CAT in kidney-derived 293 cells (0.1 to 1.9%). These results suggest that there is a liver-specific promoter between −262 and −21 nucleotides from the initiation ATG codon.

DISCUSSION

In this study, we determined the total 15,499 nucleotide sequence of the gene for human HRG, as shown in Fig. 2. The gene is composed of seven exons and six introns. The domain structure of HRG and the localization of introns are

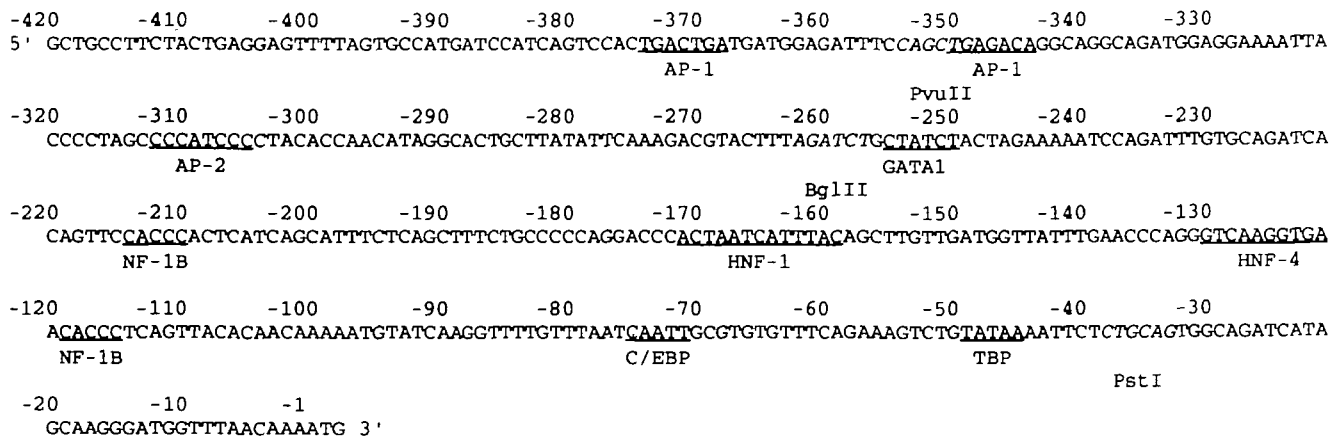

Fig. 6. **Nucleotide sequence of the 5′-noncoding region of the human HRG gene and possible recognition sites for various transcription factors.** Possible recognition sequences are underlined. Numbers indicate the positions from the starting ATG. The recognition sequences of some restriction endonucleases are shown in italics.

schematically represented in Fig. 4. Each cystatin domain is encoded by three exons and the C-terminal part by one large exon, which is quite similar to those of other members of the cystatin superfamily, cystatin C (*32*), fetuin, which is also referred to as α_2-HS glycoprotein (*33*), and kininogen (*34*). Moreover, the locations of introns in each cystatin domain are well conserved in the cystatin superfamily proteins, although the size of introns is highly variable both within and between the genes for these proteins (Fig. 5).

There are seven Alu repeats (*35*), one in the 5′-flanking region, one in intron A, two (one is half size) in C, one in E, and two in F, as shown in Fig. 1. Since the average spacing of Alu repeats was reported to be 5,000 nucleotides (*35*), the HRG gene contains this element at a higher frequency. There is one TA repeat in intron C and one GA repeat in each of introns E and F. The GA repeat in intron F was first reported by Hennis *et al.* (*25*) to be $(GT)_9(GA)_{14}(CAGA\text{-}GA)_4(GA)_2$ (GenBank Accession No. Z17218), and the dinucleotide repeat (GA) polymorphisms of this region were suggested to be related to the elevated HRG level in plasma (*36*). Similar but distinct polymorphisms in this region were also observed in the present study. Clone 32 and another clone isolated from the same Charon 4A library in this study had the sequence of $(GT)_{10}(GA)_{13}(CAGAGA)_3\text{-}(GA)_1$. This sequence was also observed in one clone isolated from the EMBL3 human genomic library, while an alternative sequence of $(GT)_9(GA)_{19}(CAGAGA)_3(GA)_1$ was found in two clones isolated from the latter library. Hennis *et al.* (*37*) also reported 5 amino acid polymorphisms detected on *in vitro* amplification in three exons of the HRG gene; Ile(ATC)/Thr(ACC)-162 in exon 4, Pro(CCC)/Ser(TCC)-186 in exon 5, and His(CAT)/Arg(CGT)-322, Arg(CGT)/Cys(TGT)-430, and Asn(AAT)/Ile(ATT)-475 in exon 7. However, the bases at all these loci in clone 32, *i.e.* bases 8039, 9106, 13589, 13912, and 14048 in Fig. 2, were T, C, A, C, and A, respectively, which were the same as those of the cDNA sequence (*16*). The presence of both Asn-475 and Ile-475 was detected by protein analysis (unpublished results). There was only one difference between the gene and cDNA sequences. C238 in the cDNA sequence was identified as T in the gene sequence (base 2417 in Fig. 2), which turned out to be due to a typing error in Ref. *16*.

The first 103 nucleotides in the previously reported cDNA sequence (*16*) were found to be derived from the yeast mitochondrial DNA. The possible cause for this might be the use of the yeast tRNA in the course of the construction of a cDNA library as a carrier in which fragments of mitochondrial DNA existed. Clontech 5′-stretch human liver cDNA also contained many artificially ligated clones, as described above. The cause of this complication is not clear at present. Minghetti *et al.* (*38*) reported that 38 nucleotides in the 5′-noncoding region of the cDNA for human albumin were not homologous to the genomic sequence but could not explain the discrepancy. This may be another example of such a happening in the course of the construction of a library.

Since the starting point of cDNA could not be determined by reinvestigation of human HRG cDNA clones, 5′-RACE analysis was carried out. Although S1 nuclease digestion or primer extension analysis should be performed to determine the real transcription initiation site, we could not perform such experiments due to the difficulty in obtaining a sufficient amount of normal human liver mRNA. It is not possible to obtain mRNA from cultured cells, because currently no human cell lines that produce HRG are available. The 5′-RACE analysis indicated that the major 5′ ends of HRG cDNA are −23, −20, and −18 from the initiation ATG codon. All cDNA clones isolated had sequences starting at nucleotide −21 or downstream, flanking the various sequences that were not found in the HRG gene sequence. Therefore, the HRG cDNA seems to start at around −23, although the real transcription starting site could not be pinpointed. In the 5′-flanking region, a TATA box-like sequence located at around −45 from the ATG codon and a CAATT sequence at around −70 are likely to conduct the transcription of the HRG gene from a defined point. However, the analyses of cDNA clones and 5′-RACE suggested that the transcription starts from multiple sites. The TATA sequence is absent in the mouse HRG gene (unpublished results).

HRG has been reported to be expressed specifically in liver parenchymal cells (*1*), and no cell lines producing HRG are currently known. So, we employed HepG2 cells to characterize the promoter region of the HRG gene and also 293 cells derived from human kidney for comparison. A

reporter assay based on the CAT activity showed that there is a critical sequence between 21 and 262 nucleotides upstream of the initiation codon, ATG, as shown in Fig. 3. This sequence supported the expression of CAT in HepG2 cells but not in kidney-derived 293 cells, indicating the liver-specific expression. The low expression level of CAT activity with pCAT Basic, a simple CAT vector without an SV40 enhancer, may be due to that HepG2 cells do not synthesize HRG (*39*). Unfortunately, no cell lines producing HRG are known yet. Nevertheless, the shortest fragment (−262 to −21) in the pCAT Basic vector still promoted the expression of CAT in HepG2 cells. The relative expression activity of each construct was similar in both the pCAT Basic and pCAT Enhancer vectors. Therefore, the promoter activities were assessed using the pCAT Enhancer vector in this study. There are many potential transcription factor recognition sites (*40*) in the 5′-flanking region of the HRG gene, as shown in Fig. 6. The CAT assay revealed that the segment between −262 and −21 from the ATG codon has liver-specific promoter activity. In this region, transcription factors such as HNF-4, HNF-1, NF-1B, C/EBP, and TBP may play critical roles in the expression of the HRG gene. Further studies will reveal the contribution of each transcription factor.

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