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Molecular cloning of cDNAs of human liver and placenta NADH-cytochrome b_5 reductase

(expression cDNA library/antibody screening/hereditary methemoglobinemia)

TOSHITSUGU YUBISUI*, YASUSHI NAITOH†, SHUHEI ZENNO‡, MINORU TAMURA*, MASAZUMI TAKESHITA*, AND YOSHIYUKI SAKAKI‡

*Department of Biochemistry, Medical College of Oita, Hazama-cho, Oita 879-56, Japan; and †Department of Internal Medicine, Faculty of Medicine, and ‡Research Laboratory for Genetic Information, Kyushu University, Fukuoka 812, Japan

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ABSTRACT **A cDNA coding for human liver NADH-cytochrome b_5 reductase (cytochrome b_5 reductase, EC 1.6.2.2) was cloned from a human liver cDNA library constructed in phage λgt11. The library was screened by using an affinity-purified rabbit antibody against NADH-cytochrome b_5 reductase of human erythrocytes. A cDNA about 1.3 kilobase pairs long was isolated. By using the cDNA as a probe, another cDNA (pb$_5$R141) of 1817 base pairs was isolated that hybridized with a synthetic oligonucleotide encoding Pro-Asp-Ile-Lys-Tyr-Pro, derived from the amino acid sequence at the amino-terminal region of the enzyme from human erythrocytes. Furthermore, by using the pb$_5$R141 as a probe, cDNA clones having more 5′ sequence were isolated from a human placenta cDNA library. The amino acid sequences deduced from the nucleotide sequences of these cDNA clones overlapped each other and consisted of a sequence that completely coincides with that of human erythrocytes and a sequence of 19 amino acid residues extended at the amino-terminal side. The latter sequence closely resembles that of the membrane-binding domain of steer liver microsomal enzyme.**

NADH-cytochrome b_5 reductase (b$_5$R; cytochrome b_5 reductase, EC 1.6.2.2) is localized mainly on the cytoplasmic side of the endoplasmic reticulum of somatic cells and functions in the desaturation and elongation of fatty acids (1, 2), cholesterol biosynthesis (3), and drug metabolism (4). The enzyme also exists in circulating erythrocytes, in which the enzyme is found in a soluble form, and its role is methemoglobin reduction (5). The deficiency of b$_5$R in human erythrocytes and somatic cells is known to occur in hereditary methemoglobinemia. Mainly two types of enzyme deficiency are known; erythrocyte type (type I), in which the enzyme is deficient only in erythrocytes with a mild cyanosis, and generalized type (type II), in which the enzyme is deficient not only in erythrocytes but also in somatic cells. Type II is a severe form accompanied with mental retardation and neurologic impairment (6, 7).

Previously we had developed a simple purification method for b$_5$R from erythrocytes (8) and determined the complete amino acid sequence of the enzyme purified from human erythrocytes (9, 10). These developments allowed us to begin the study on the genetic basis of the enzyme deficiency in this disease. As the first step of the study, we attempted to isolate a cDNA clone of membrane-bound type b$_5$R to determine the structural relationship between erythrocyte and membrane-bound type enzymes. In this paper, cloning and sequence analysis of b$_5$R cDNAs are presented. The results show that the amino acid sequence (294 residues) deduced from the nucleotide sequence of the cDNAs contains a sequence that completely coincides with that (275 residues) of b$_5$R from human erythrocytes and an extended sequence of 19 amino acid residues at the amino-terminal side. We are thus able to show that the amino acid sequence of human erythrocyte b$_5$R completely coincides with that of the catalytic domain of membrane-bound type b$_5$R of human somatic cells.

MATERIALS AND METHODS

Peroxidase-conjugated sheep antiserum against rabbit IgG was purchased from Cappel Laboratories (Cochranville, PA). Restriction endonucleases were obtained from Takara Shuzo (Kyoto, Japan) and also from Nippon Gene (Toyama, Japan). Nitrocellulose filters (0.45 μm) were a product of Schleicher & Schüll. The human liver cDNA library constructed in phage λgt11 (11) was a kind gift from Savio L. C. Woo (Howard Hughes Medical Institute, Houston, TX). The human placenta cDNA library in λgt11 (22), constructed by using the random primer method, was kindly provided by Y. Ebina (Kumamoto University). Sequencing primers of the pUC series were obtained from Pharmacia P-L Biochemicals. Specific sequencing primers of 16- or 17-mers and oligonucleotide probes deduced from the amino acid sequence of b$_5$R of human erythrocytes were synthesized by an automatic DNA synthesizer (Applied Biosystems, model 380A) and purified by reversed-phase high-performance liquid chromatography.

Human erythrocyte b$_5$R was purified as described (8–10).

Affinity-Purified Anti-b$_5$R. Rabbit antiserum against b$_5$R of human erythrocytes was obtained after subcutaneous injection of the enzyme. The antiserum was purified by fractionation with ammonium sulfate and by chromatography on a column of DEAE-cellulose/CM-cellulose as described by Palacios *et al.* (12). The partially purified antibody was purified further by immunoaffinity chromatography on a column of b$_5$R-conjugated CH-Sepharose 4B. The affinity-purified antibody could detect 1 ng of b$_5$R at a concentration of 1 μg/ml by blotting analysis (13).

Screening of cDNA Libraries. Screening of a λgt11 human liver cDNA library was performed by using the method of Young and Davis (14) using an affinity-purified antibody against b$_5$R of human erythrocytes, with the following modifications. An overnight culture of *Escherichia coli* strain Y1090 was infected with ≈2×10^5 recombinant bacteriophage λgt11 particles per plate. Induction of fusion proteins in λgt11 by an isopropyl β-D-thiogalactoside-treated filter was carried out at 37°C overnight (instead of 2–8 hr). Blocking solution used in this study consisted of TBS buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl), 3% bovine serum albumin, and 0.1% merthiolate (instead of 20% fetal calf serum). Detection of the antibody immobilized on the filter

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Abbreviation: b$_5$R, NADH-cytochrome b_5 reductase.

was performed by the reaction with peroxidase-conjugated sheep anti-rabbit IgG (instead of ^{125}I-labeled protein A). The antibody on the filter was incubated with the secondary antibody at room temperature for 2 hr, and color development by the peroxidase was detected by using the method of Hawkes *et al.* (13). Plaques corresponding to purple spots were picked up from the agar plates with a sterile toothpick. Successive rescreenings were carried out to purify the phage containing the cDNA of b_5R.

Screening of cDNA libraries using DNA probes was carried out by the plaque hybridization method (15).

Preparation of Insert DNA. Recombinant phage were grown in a large-scale liquid culture, and phage DNA was prepared as described by Maniatis *et al.* (16) with modifications. Recombinant phage DNA was digested by *Eco*RI, and the insert DNA was separated by gel electrophoresis and electroeluted into a dialysis bag (17).

Blot Hybridization with Oligonucleotide Probes. Oligonucleotides were synthesized based on the amino acid sequence of b_5R of human erythrocytes as described above and labeled at the 5′-OH end with [γ-^{32}P]ATP (2000–5000 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase (15). DNA filters were prepared as described by Maniatis *et al.* (16). Hybridization was carried out in 6× concentrated NET buffer (1× NET = 0.15 M NaCl/0.015 M Tris·HCl, pH 7.5/1 mM EDTA), 10× concentrated Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.1% sodium lauryl sulfate, 0.1% $Na_4P_2O_7$, 20 μg of yeast tRNA per ml, 2 μg of ATP per ml, and ^{32}P-labeled oligonucleotide (1 ng) at room temperature for 16–20 hr. The filter was washed twice with 0.9 M NaCl/0.09 M sodium citrate at 37°C for several minutes.

Subcloning and DNA Sequencing Analysis. The insert DNA was digested with restriction enzymes, and the DNA fragments were subcloned into pUC13 at appropriate restriction sites. The nucleotide sequence of the recombinant plasmids was determined by the modified dideoxy method using a denatured template as a template (18).

RESULTS

Isolation of cDNA Clones for Human Liver b_5R. The human liver cDNA library in λgt11 phage was screened by the polyclonal affinity-purified antibody against b_5R of human erythrocytes. The total of 28 positive pinhole-like spots was selected from ≈1.8×10^6 plaques of λgt11 library at the first screening, and among these spots only 3 clones were positive at the third successive replating. The isolated plaques that showed a strong positive signal after the third successive replating by the antibody are shown in Fig. 1. However, only one of the three clones was found to be positive by the blot hybridization analysis with an oligonucleotide probe (17-mer) encoding Glu-Ala-Trp-Asp-Tyr-Gly (Fig. 2), synthesized based on the amino acid sequence at the C-terminal region of b_5R of human erythrocytes (9, 10). The size of the insert DNA was estimated by *Eco*RI digestion and subsequent electrophoresis on agarose gel to be about 1.3 kilobase pairs (kbp) long. Sequence analysis of the clone showed that the cDNA had an open reading frame about 300 base pairs (bp) long encoding only 100 amino acid residues at the C-terminal region of human b_5R (data not shown). The size of the 3′ noncoding region was found to be about 1 kbp long. To isolate a clone carrying a longer cDNA, the λgt11 human liver cDNA library was rescreened by the plaque hybridization method (16) using the 1.3-kbp cDNA as a probe. A total of 36 positive clones was selected from 2×10^6 plaques by this rescreening, and 3 clones were positive by blot hybridization analysis with probe 1 encoding the amino acid sequence from position 13

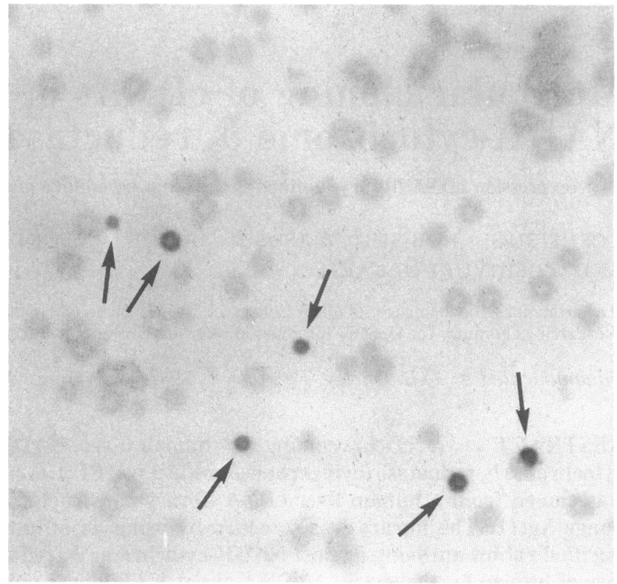

FIG. 1. Positive signals on a nitrocellulose filter of λgt11 human liver cDNA library screened with affinity-purified anti-b_5R. The arrows indicate strongly positive plaques obtained by the third successive replating.

to position 18 (Fig. 2). The size of the insert DNA was about 1.8 kbp long. Nucleotide sequence analysis of the 5′ region of one of these clones (pb_5R141) showed that the cDNA was not full size and that the amino acid sequence encoded by the first 24 bp following the *Eco*RI site was completely different from that of the N-terminal region of the erythrocyte-type b_5R (as discussed below, this may be due to an artifact of the cDNA cloning process).

Further attempts were made to obtain a cDNA clone covering more 5′ portion of b_5R mRNA. For screening, we employed a human placenta cDNA library that was constructed by using a mixture of oligonucleotides as primes. Several cDNA clones were obtained, and the one having the longest 5′ region (pb_5R-P, see Fig. 3) was further analyzed.

Restriction Map and Nucleotide Sequence of the cDNA Clones. The cDNAs (pb_5R141 and pb_5R-P) were cleaved with combinations of various restriction endonucleases, and the restriction maps were constructed (Fig. 3). The appropriate restriction fragments were subcloned into pUC13 and the nucleotide sequence was determined by the strategy shown in Fig. 3. The nucleotide sequences of these clones overlapped each other (except the first 24 bp following the *Eco*RI site of pb_5R141), and the results are summarized in Fig. 4. The deduced amino acid sequence is also shown in Fig. 4. The total size of the cDNA sequence was 1879 bp long, and it contained a coding region of 882 bp for 294 amino acid residues, termination codon, 3′ noncoding region with 985 bp, and poly(A) tail. The deduced amino acid sequence (275 amino acid residues) starting from the 66th nucleotide completely coincided with the sequence determined on the enzyme from human erythrocytes (9, 10). The cDNA also encoded a sequence of 19 amino acid residues extended at the amino-terminal side. The extended sequence is probably a portion of the membrane-binding domain, because the sequence is closely similar to that of the membrane-binding domain of steer liver b_5R (19) (Fig. 4).

DISCUSSION

In this study we screened the human liver expression cDNA library by using the affinity-purified antibody against b_5R of human erythrocytes and isolated a clone carrying b_5R cDNA of 1.3 kbp. By rescreening the same cDNA library with

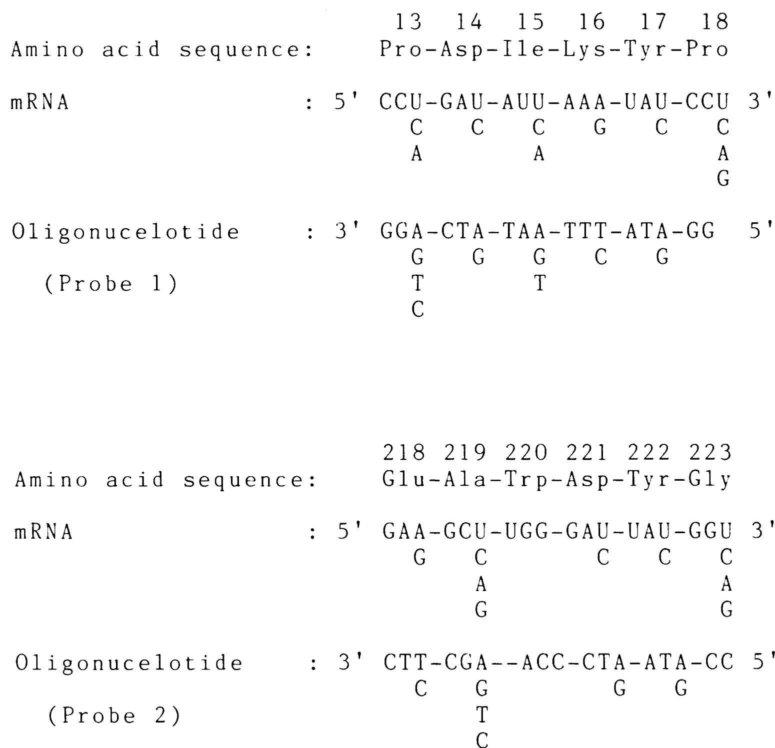

FIG. 2. Synthetic oligonucleotide probes. Probe 1 consisted of 96 different 17-mers corresponding to the amino acid sequence from position 13 to position 18, and probe 2 consisted of 32 different 17-mers corresponding to the amino acid sequence from position 218 to position 223 of b_5R of human erythrocytes (9, 10).

labeled 1.3-kbp b_5R cDNA as a probe, we isolated a cDNA clone 1.8 kbp long, designated pb_5R141. Sequence analysis revealed that pb_5R141 is definitely a cDNA clone of b_5R, although the amino acid sequence of the first eight residues deduced from the nucleotide sequence was unusual. A further attempt was made to isolate cDNA clones carrying more 5′ sequence of b_5R cDNA by screening a placenta cDNA library. Finally, we obtained cDNA clones covering 1879 bp.

The amount of b_5R in human liver would appear to be very low. We obtained one clone (including short clones) per 10^5 plaques after taking account of the orientation and fusion frame for insertion. Okada and Omura (20) found <0.01% of the enzyme, on a protein basis, in rat liver. If rat and human liver are in the same range, their value would suggest that we should find less than one clone per 10^4 colonies on the phage plaques of the cDNA library.

It is interesting and surprising that the amino acid sequence of somatic cell type b_5R deduced from the nucleotide sequence of the cDNA completely coincided with that of b_5R of human erythrocytes (9, 10). Thus, human somatic cell type b_5R has the hydrophilic catalytic domain identical to b_5R of erythrocytes, and it is conceivable that both types of enzymes are coded by the same gene at least in part. The sequence of the first 19 amino acid residues was found to be closely similar to that of the membrane-binding domain of steer liver b_5R (19). These results provide strong support for the hypothesis of Hultquist *et al.* (21), who proposed that cytochrome b_5 and b_5R bound on the membrane of the endoplasmic reticulum in erythroid cells are solubilized by

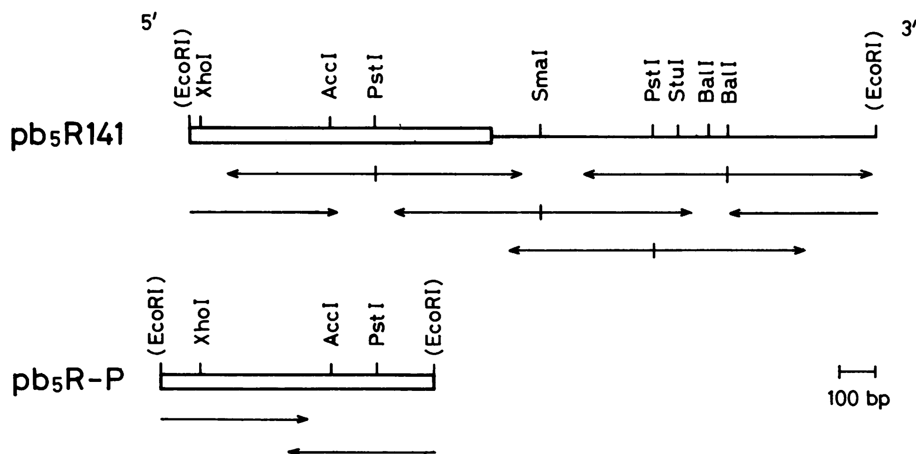

FIG. 3. Restriction map and strategy for sequence determination of cDNAs. The restriction map was constructed by digestion of the DNA with combinations of restriction enzymes as indicated. The arrows indicate the direction and extent of nucleotide sequence determination by the dideoxy method of Hattori and Sakaki (18). The boxes show the coding region.

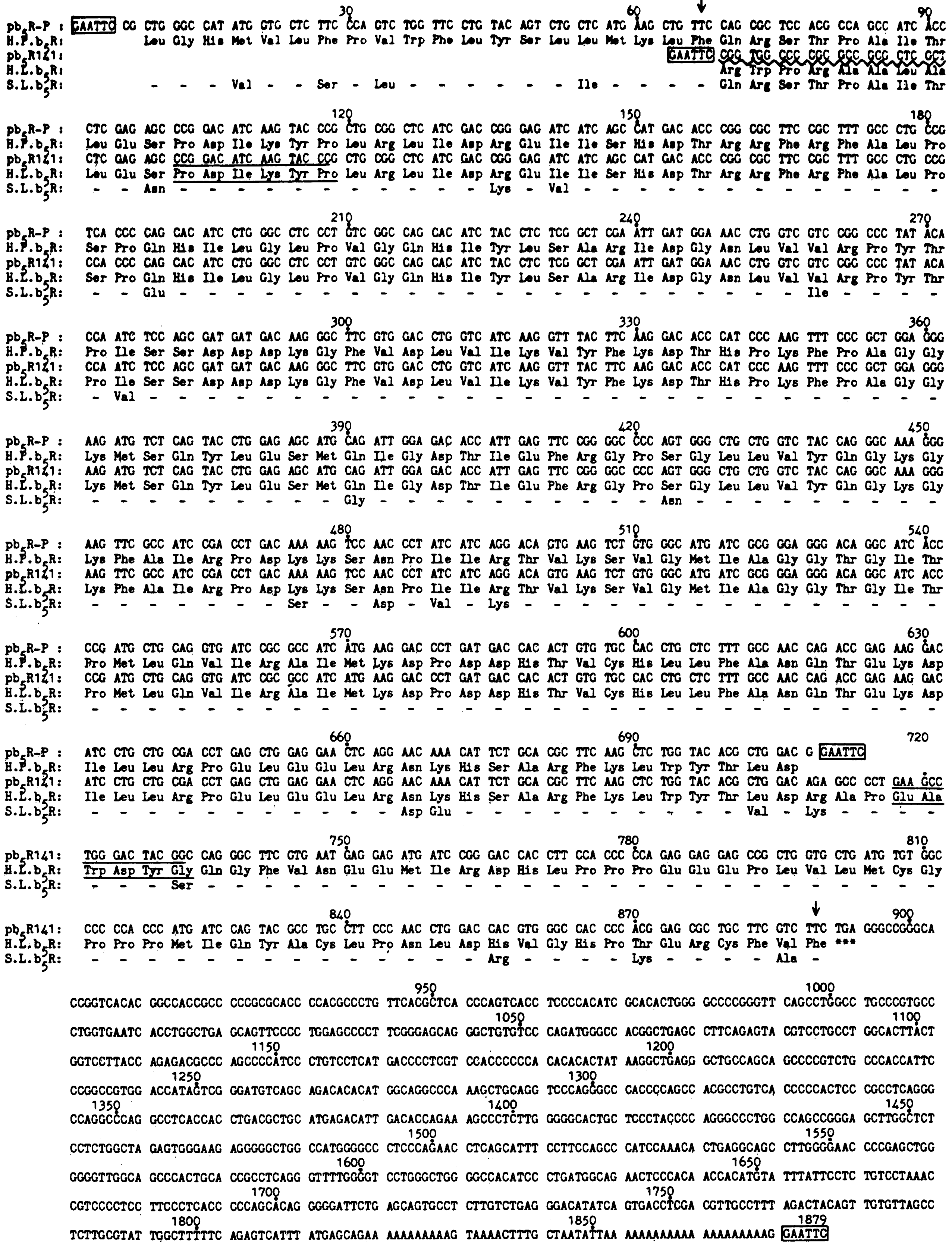

FIG. 4. Nucleotide sequence of pb_5R141 and pb_5R-P and amino acid sequence deduced from the cDNAs. *Eco*RI sites that were introduced for cDNA cloning are boxed. Regions for probes 1 and 2 are indicated by solid underlines. For comparison, the amino acid sequence of b_5R from steer liver microsomes (S.L.b_5R) (19) is shown by indicating the only residues different from those of human liver or placenta. The arrows indicate the N and C termini of human erythrocyte b_5R (9, 10) (275 residues). The wavy line shows an unusual sequence of pb_5R141 that may be introduced by an artifact of cDNA cloning (see text). H.L., human liver; H.P., human placenta.

the action of some protease, such as cathepsin D, during the maturation of erythroid cells.

In this study the full-size cDNA of b_5R was not obtained. Curiously, two independent cDNA clones obtained from the

placenta cDNA library terminated at almost identical 5′ positions (data not shown) for unknown reasons.

The genetic basis of methemoglobinemia is not well understood. Assuming that soluble b_5R of the erythrocyte type can be derived from the enzyme with the membrane-binding domain in somatic cells, the abnormality or deficiency of such processing enzyme(s) in erythroid cells may lead to the disappearance of soluble b_5R in erythroid cells and consequently cause type I methemoglobinemia. Alternatively, type I methemoglobinemia may be caused by a mutation of the b_5R gene itself, leading to production of an unstable protein. On the other hand, type II methemoglobinemia may be caused by a mutation in the b_5R gene, because enzyme activity of erythrocyte-type and somatic cell-type b_5R are both absent in this disease. The b_5R cDNA isolated in this study should lead to a means of identification of the mutation responsible for the disease and to an understanding of the process leading to the disease at the molecular level.

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