

# Human thyroid peroxidase: Complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs

( $\lambda$ gt11 library/oligonucleotide screening/Graves disease/chromosome 2)

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Communicated by Allan H. Conney, April 15, 1987 (received for review February 12, 1987)

**ABSTRACT** Two forms of human thyroid peroxidase cDNAs were isolated from a  $\lambda$ gt11 cDNA library, prepared from Graves disease thyroid tissue mRNA, by use of oligonucleotides. The longest complete cDNA, designated phTPO-1, has 3048 nucleotides and an open reading frame consisting of 933 amino acids, which would encode a protein with a molecular weight of 103,026. Five potential asparagine-linked glycosylation sites are found in the deduced amino acid sequence. The second peroxidase cDNA, designated phTPO-2, is almost identical to phTPO-1 beginning 605 base pairs downstream except that it contains 1-base-pair difference and lacks 171 base pairs in the middle of the sequence. This results in a loss of 57 amino acids corresponding to a molecular weight of 6282. Interestingly, this 171-nucleotide sequence has GT and AG at its 5' and 3' boundaries, respectively, that are in good agreement with donor and acceptor splice site consensus sequences. Using specific oligonucleotide probes for the mRNAs derived from the cDNA sequences hTPO-1 and hTPO-2, we show that both are expressed in all thyroid tissues examined and the relative level of two mRNAs is different in each sample. These results suggest that two thyroid peroxidase proteins might be generated through alternate splicing of the same gene. By using somatic cell hybrid lines, the thyroid peroxidase gene was mapped to the short arm of human chromosome 2.

Thyroid peroxidase (TPO; donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is involved in two important reactions in the biosynthesis of thyroid hormone; the iodination of tyrosine residues on thyroglobulin and the intramolecular coupling reaction of iodinated tyrosines, leading to the formation of thyroxine (T4) and 3-3'-5-triiodothyronine (T3) (1, 2). It is believed that these two reactions are carried out by the same peroxidase enzyme. Changes in the level of peroxidase have been reported in some thyroid diseases (1, 3, 4).

Most patients with autoimmune thyroid disease have circulating autoantibodies capable of reacting *in vitro* with thyroglobulin, microsomal antigen, and thyrotropin receptor (3, 5). Microsomal antigen is especially important since it is present on the surface of follicular thyroid cells and is involved in the complement-mediated cytotoxicity (6). Although the nature of this antigen has been elusive, several reports have shown (7-9) that TPO is a major component of the thyroid microsomal antigen. The significance of TPO relative to autoimmune thyroid disease, however, is not fully understood.

Although studies related to the physicochemical properties and the reaction mechanism of porcine TPO have accumulated (10-12), little information is available on the human enzyme (7, 13, 14). This is due to the difficulty in obtaining the large amounts of human thyroid tissues needed for enzyme purification. Molecular biological approaches serve as an excellent tool to circumvent this difficulty and to determine the role of TPO in autoimmune thyroid disease. To this end, we cloned and sequenced human (h) TPO cDNA and determined the chromosome location of its gene.<sup>||</sup>

## MATERIALS AND METHODS

**Materials.** Thyroid glands of patients with Graves disease were obtained from Kuma Hospital (Kobe, Japan) and normal thyroid tissue was a gift of Daniela Foti (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). hTPO was purified as described (14). The enzyme had a guaiacol oxidation activity of 290 units/mg of protein and an  $A_{413}/A_{280}$  ratio of 0.60. Oligonucleotides were synthesized using an Applied Biosystems model 380B DNA synthesizer (Foster City, CA).

**Partial Amino Acid Sequence Analysis.** Peroxidase was treated with either cyanogen bromide (15) followed by trypsin or trypsin only (16). Fragments were separated by a Hi-Pore reverse-phase column, C4 (4.6  $\times$  250 mm, Bio-Rad) and sequenced on an Applied Biosystems gas-phase sequencer (model 470A).

**Isolation and Sequencing of hTPO cDNAs.** Poly(A) RNA was isolated from thyroid tissues by the guanidine thiocyanate/cesium chloride method (17) followed by oligo(dT)-cellulose chromatography. A cDNA library was constructed in  $\lambda$ gt11 (18) by using mRNA isolated from the thyroid of a single Graves disease patient (see patient 3, Fig. 3). Plaque hybridization for screening with oligonucleotides was performed as described (19). Sequencing was carried out by the M13 shotgun cloning (20) and dideoxy (21) methods. DNA sequence alignments and other manipulations were performed with the Beckman "Microgenie" software.

**Chromosomal Mapping Using Somatic Cell Hybrids.** A human fibroblast line (GM 2658) with the karyotype 46,XX,t(2;6)(q11;q15) was used to prepare one series of human-Chinese hamster hybrids. The human and rodent parental cells, the cell fusion procedure, the isolation and

Abbreviations: nt, nucleotide(s); TPO, thyroid peroxidase; h, human.

<sup>||</sup>The sequences reported in this paper are being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J02969 and J02970).

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## Peptide

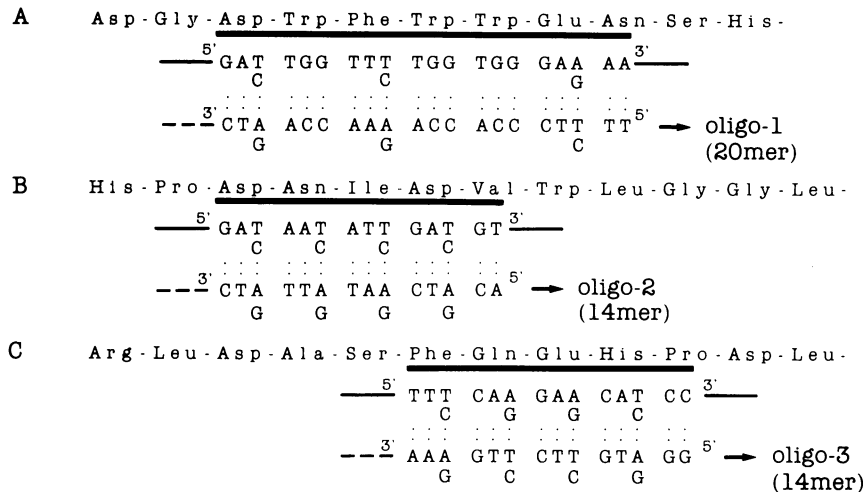


FIG. 1. Portions of the partial amino acid sequences and the deduced oligonucleotide sequences used for screening. Peptide fragments A and B were obtained from cyanogen bromide cleavage followed by trypsin digestion of hTPO, whereas fragments C and D (see Fig. 2) were from tryptic digestion only. The complete amino acid sequences of the fragments are shown in Fig. 2. The black bar indicates the amino acid sequence used for obtaining the oligonucleotide sequence. The predicted mRNA sequence and its complementary oligonucleotide sequence are shown below the bar.

characterization of hybrids, the hybrid cell lines, and the DNA analyses have been described (22–25).

## RESULTS

**Partial Amino Acid Sequences.** Four peptides derived from hTPO were isolated and sequenced, and portions of amino acid sequences were used to synthesize three different oligonucleotides (Fig. 1). Each oligonucleotide is composed of a mixture of either 20-mers (designated oligo-1) or 14-mers (designated oligos-2 and -3) representing all potential complementary sequences predicted from the hepta- or pentapeptides, respectively, excluding the third base of the last codon. These oligonucleotides were used as probes to screen a human Graves disease thyroid cDNA library.

**Isolation of phTPO cDNAs.** We originally screened  $3 \times 10^5$  individual plaques with three different synthetic oligonucleotides (Fig. 1) and isolated 28, 5, and 21 positive clones with oligo-1, -2, and -3, respectively. Fourteen plaques that hybridized to all three probes and had reasonably long inserts [ $>1.5$  kilobase pairs (kb)] were purified, and the clones with two longest inserts (2.8 kb and 2.4 kb) were sequenced (designated phTPO-2.8 and phTPO-2.4, respectively).

To determine whether the 2.8-kb clone is close to full-length or not, a primer extension experiment was carried out. The 22-mer oligonucleotide (designated oligo-5') whose sequence is complementary to that located near the 5' end of the 2.8-kb insert (Fig. 2) was synthesized and used as a primer. The primer was extended about 240 base pairs (bp), suggesting that the 2.8-kb cDNA lacked 240 bp of the 5' end of TPO mRNA (data not included). The library was, therefore, rescreened first with this 22-mer oligonucleotide and later with a 5'-most *Taq* I fragment prepared from the 2.4-kb insert [nucleotides (nt) 605–782; 177 bp, Fig. 2]. One clone (designated phTPO-5') contained an insert that extended the hTPO-2.8 sequence by 230 bp on the 5' side.

**Nucleotide Sequence of phTPO cDNAs.** The cumulative complete cDNA sequence of hTPO-2.8 and hTPO-5' is 3048 bp long and contains a putative poly(A) addition signal 18 nt upstream of the terminating poly(A) tract (Fig. 2). We believe that this cDNA sequence, designated hTPO-1, is nearly full-length based on the results obtained by the primer extension and the RNA gel blot analysis described below (Fig. 3).

Between the 2.8-kb and 2.4-kb cDNAs, the following two differences are observed. One-base change is found at nt 1570 of the complete cDNA where cytidine in the 2.8-kb clone is substituted with thymidine in the 2.4-kb clone. Furthermore, the 2.4-kb clone contains a deletion of 171 nt that corresponds

to positions 1670–1840 of hTPO-1. Interestingly, this 171-nt sequence starts with GT, ends with AG, and maintains a complete protein open reading frame.

Magnusson *et al.* (28) have reported the partial nucleotide and deduced amino acid sequences of porcine TPO. Their sequence matches with our sequence after 1861 bp. Within this limited region, hTPO nucleotide sequence showed 66% similarity to that of porcine TPO. Search of the GenBank (Beckman Microgenie software, March 1987 update) found no other known sequences with significant similarity to the hTPO sequence.

**Deduced Amino Acid Sequence of hTPO cDNA.** The first methionine codon (Met-1) in hTPO-1 is encountered at nt 73, followed by a long open reading frame 933 amino acids long (Fig. 2). The second and third in-frame methionine codons are found at 36 nt (Met-13) and 159 nt (Met-54) downstream, respectively. Both Met-1 and Met-54 are followed by a peptide similar in structure to a signal sequence (29), which is necessary for insertion of this protein into the endoplasmic reticulum. Although Met-54 also has the preceding sequence that exactly matches the consensus initiation sequence CCRCCATG (where R is a purine) derived by Kozak (30), we feel that Met-1 is more likely to be the initiation codon because of the following three reasons (30): (i) there is the invariant adenosine at the -3 position of the ATG, (ii)  $>90\%$  of eukaryotic mRNAs have the initiation site at the 5'-proximal methionine triplet, and (iii) 70% of the mRNA leader sequences are clustered in the 20- to 80-nt range. We could not, however, determine the N-terminal amino acid sequences of the hTPO protein. The reason for this is not clear, although it could be due to the difficulty of obtaining N-terminal residues from a large glycoprotein such as TPO (10). Indeed, analysis of the amino acid sequence revealed five potential asparagine-linked glycosylation sites (Asn-Xaa-Ser or Thr) (Fig. 2). The molecular weight of the unmodified protein, based on the deduced amino acid sequence, is calculated to be 103,026. One-base difference found at position 1570 in phTPO-2.4 does not change the amino acid. An interesting finding is that the 171-bp sequence that has been lost in the 2.4-kb cDNA leads to a loss of 57 amino acids (corresponding to a molecular weight of 6282) and maintenance of a complete open reading frame. Search for known amino acid sequences, including bovine thyroglobulin (31), cytochrome *c* peroxidase (32), and chloroperoxidase (33), did not reveal any significant similarity to that of hTPO except for porcine TPO (28), which has 62% amino acid similarity.

**RNA Gel Blot Analysis of hTPO mRNA: Demonstration of Expression of hTPO-1 and hTPO-2 mRNAs.** RNA gel blot

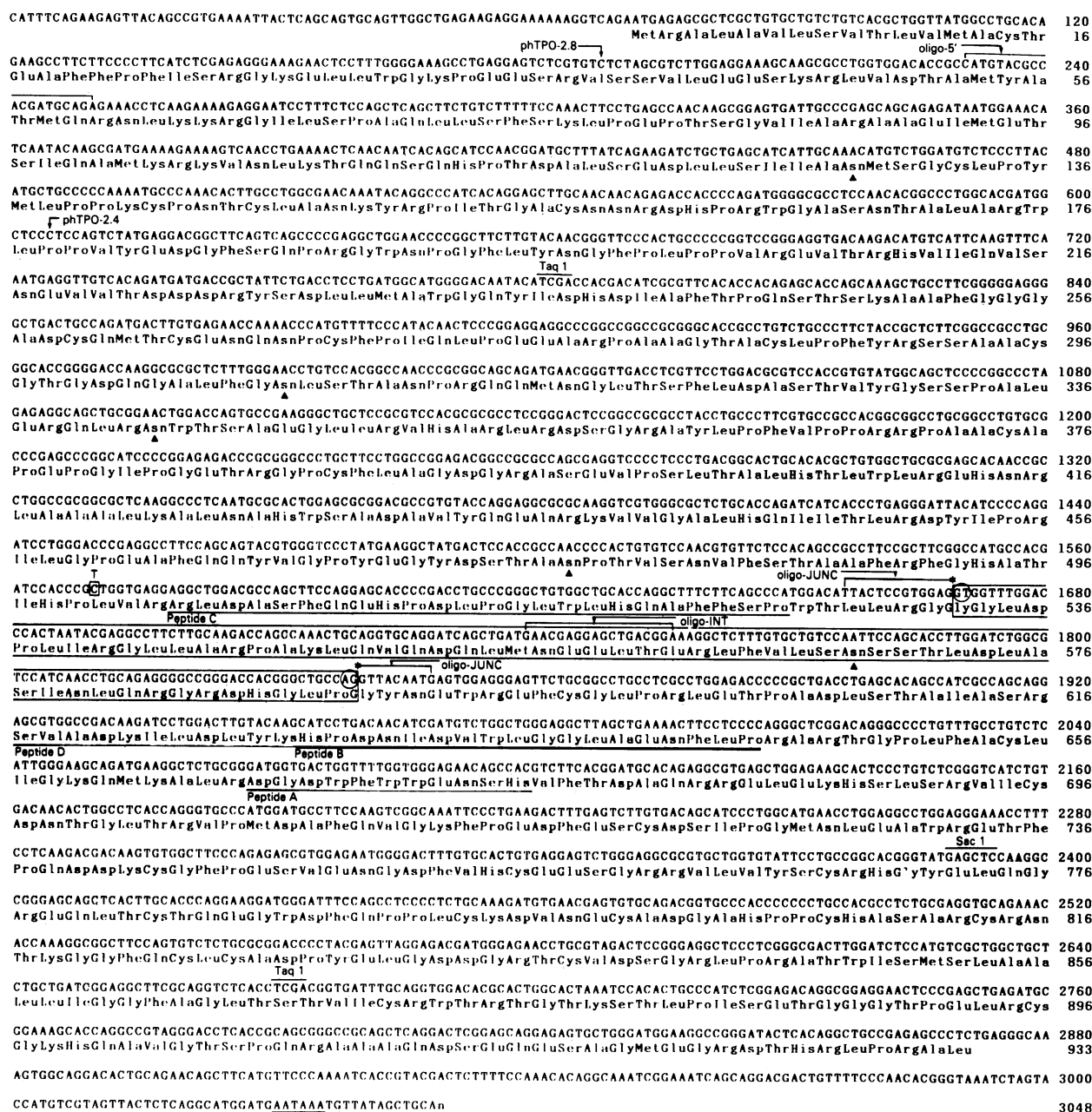


Fig. 2. Nucleotide and deduced amino acid sequence of the hTPO cDNA. The complete hTPO cDNA and protein sequences are presented. The putative poly(A) addition signal and the regions identifying partial amino acid sequences are underlined. The start positions of phTPO-2.8 and phTPO-2.4 are indicated by arrows. Asparagine residues that are potential sites for glycosylation are marked (▲). The nucleotide sequence difference found between phTPO-2.8 and phTPO-2.4 is boxed, and the difference is shown above the sequence. Restriction enzyme sites *Sac* I and *Taq* I that were used to generate probe fragments and the regions whose complementary sequences were used to synthesize oligonucleotides are overlined. Oligo-JUNC has a continuous sequence joined at \* mark. Circled residues indicate the consensus splice junction sequences. The putative intron sequence is enclosed by lines.

analysis was carried out using a 5' *Sac* I fragment of the phTPO-2.4 (nt 605–2393; 1788 bp, Fig. 2) as a probe (Fig. 3A). Human thyroid revealed an mRNA of 3 kb, although its level in three Graves-disease patient tissues was quite variable. Because of the difficulty in obtaining fresh normal human thyroid tissues, a slight background hybridization was observed that is probably due to partial degradation of the mRNA. Several distinct minor mRNA bands are also detected below the main 3-kb mRNA in normal thyroid as well as other thyroid tissues after longer exposure. A similar finding was reported for the porcine TPO mRNA (28). The nature of these RNA species, however, is unclear. Human cDNA did not show any cross-hybridization to bovine thyroid or human liver mRNAs (Fig. 3A).

To demonstrate whether hTPO-1 or hTPO-2 is actually

expressed or not, we synthesized an 18-mer and a 22-mer oligonucleotide specific to the 171-bp intron-like sequence (designated oligo-INT) and the junction sequence (designated oligo-JUNC), respectively (Fig. 2). The specificity of the oligonucleotides was verified by hybridizing a filter that contained 14 original phage DNAs to either oligo-INT or oligo-JUNC. Eleven clones out of the 14, including phTPO-2.8, hybridized to only oligo-INT probe; whereas three clones, including phTPO-2.4, hybridized to only the oligo-JUNC probe (data not shown).

RNA gel blot analysis was further carried out on a longer and, therefore, higher resolution gel using cDNA as well as the oligo-INT and oligo-JUNC as probes (Fig. 3B). When the 1.8-kb *Sac* I fragment was used as probe, two bands were detected around 3 kb in all RNA samples after longer

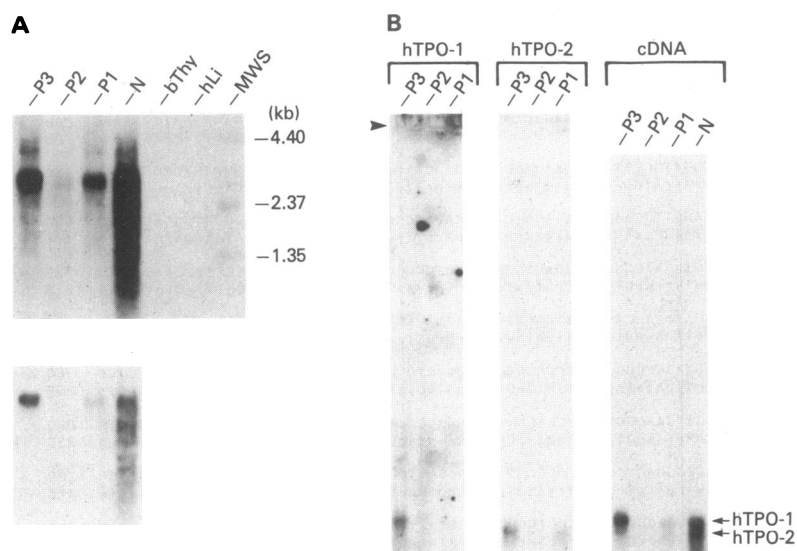


FIG. 3. RNA gel blot analysis of hTPO mRNA. Total RNA (10  $\mu$ g) isolated from thyroid tissue of normal individuals (N), patients with Graves disease (P1, P2, and P3), bovine thyroid (bThy), and human liver (hLi) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and blotted to a nylon filter (Nytran, 0.45- $\mu$ m pore size, Schleicher & Schuell). The lengths of the gels were 15 cm (A) and 30 cm (B). The electrophoresis origin of the gel in B is marked with an arrowhead. The filters were hybridized to either the 5' *Sac* I fragment of pH<sub>1</sub>TPO-2.4 (A, Upper and B, lanes cDNA), oligo-INT (B, lanes hTPO-1), or oligo-JUNC (B, lanes hTPO-2) and then subjected to autoradiography for 1 day for the cDNA fragment probe or 3 days for the oligonucleotide probes. Hybridization and washing conditions for the fragment probe were described by Church and Gilbert (26) and those for oligonucleotide by Omiecinski *et al.* (27). The filter in A was reexposed for 6 hr (A, Lower). Molecular weight standard (MWS) is a Bethesda Research Laboratories RNA ladder.

exposure, whereas oligo-INT and oligo-JUNC detected only the longer and shorter mRNA band, respectively, and the level of each approximated that detected with the cDNA probe. The relative level of these two mRNAs varied in each sample. Due to a shortage of normal human thyroid tissue, we could not obtain results with oligonucleotides.

**Chromosomal Mapping of hTPO Gene.** Mapping was performed by Southern analysis of *Eco*RI-digested DNAs isolated from a series of human-rodent hybrids segregating human chromosomes. When a 3' *Sac* I fragment of the hTPO cDNA (nt 2394 at the 3' terminus; 654 bp, Fig. 2) was used as probe, four hybridizing *Eco*RI bands (0.8, 4, 5.5, and 14 kb) were detected in parental human DNA and several hybrid cell lines (Fig. 4). Neither Chinese hamster nor mouse homologous sequences were detected under the stringent conditions of hybridization. The Southern analysis permitted unambiguous assignment of all detectable hybridizing TPO sequences to human chromosome 2 (Table 1). Analysis of hybrids containing breaks or translocations involving chromosome 2 allowed a regional assignment of the TPO gene to the short arm of this chromosome (2p). Two hybrids isolated after fusing hamster cells with human fibroblasts that contained a balanced t(2;6)(q11;q15) translocation retained one of the translocation chromosomes but lost the normal chromosome 2. One of these hybrids with the translocation chromosome containing the short arm of chromosome 2 had the TPO gene, whereas the other hybrid that retained the reciprocal translocation chromosome containing only the long arm of chromosome 2 did not have the TPO gene. This indicates that the TPO sequences are located above the translocation break point at 2q11. Other hybrids containing spontaneous breaks of chromosome 2 confirmed this interpretation.

When the blots were stripped and rehybridized with a 277-bp *Sac* I-*Taq* I fragment representing the 5' end of the original 654-bp probe (nt 2394-2671, Fig. 2), only two of the *Eco*RI bands (5.5 and 14 kb) were detected. This result strongly suggests that some of the hybridizing bands result from *Eco*RI sites within introns of a single large TPO gene, although it does not exclude the possibility of two closely linked TPO genes at this locus.

## DISCUSSION

Two human TPO cDNAs were isolated from a  $\lambda$ gt11 cDNA library constructed from the thyroid tissue of a patient with Graves disease and were sequenced. The identity of hTPO-1 was established by the following three lines of evidence: (i) sequences of four peptides isolated from a highly purified

preparation of human TPO are found in the corresponding cDNA sequence, (ii) the mass of the protein, deduced from the open reading frame of hTPO-1, is similar to the mass estimated by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis, (iii) significant amino acid similarity of 62% exists between hTPO and the partial amino acid sequence deduced from a porcine TPO cDNA (28).

The most interesting aspect in this study is that the second clone we sequenced, hTPO-2, when compared to hTPO-1, has a deletion of 171 nt in the middle of its sequence without

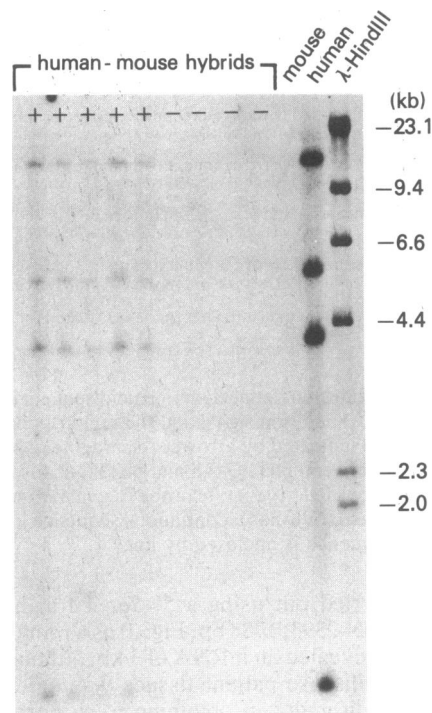


FIG. 4. Southern analysis of hybrid cell DNAs. Human-mouse hybrid cell DNA samples (10  $\mu$ g) were digested with *Eco*RI, size-fractionated by electrophoresis in 0.7% agarose gels, transferred to nylon membranes, hybridized with the 3' *Sac* I probe, and washed under high stringency (i.e., <10% divergence allowed) as described (25). A representative group of hybrid subclones is shown, and the hTPO gene is present (+) or absent (-) in lanes containing different hybrids. Parental mouse and human placental DNAs are also shown. Size standards are <sup>35</sup>S-end-labeled, *Hind*III-digested,  $\lambda$  DNA fragments.

Table 1. Segregation of TPO gene with individual human chromosomes

Human chromosome number/ % discordancy		
1/15	9/24	17/37
2/0	10/30	18/51
3/27	11/20	19/24
4/32	12/32	20/27
5/31	13/34	21/59
6/38	14/45	22/31
7/43	15/48	X/51
8/28	16/41	

Detection of four human *Eco*RI fragments hybridizing with the hTPO probe is correlated with the presence or absence of each human chromosome in 93 hybrid cell lines. Discordancy indicates presence of the gene in the absence of a specific chromosome or absence of the gene despite the presence of the chromosome, and the sum of these two numbers divided by the total number of hybrids examined  $\times 100$  represents % discordancy. The human-hamster hybrids consisted of 24 primary clones and 15 subclones (10 positives/39 total clones), and the human-mouse hybrids consisted of 14 primary clones and 40 subclones (18 positives/54 total clones).

any shift of the amino acid reading frame. Confirmation that the two mRNAs exist in Graves disease tissues was provided by RNA gel blot analysis with cDNA, and hTPO-1 and hTPO-2 mRNA-specific oligonucleotide probes. Unfortunately we have not been able to analyze the prevalence of these two mRNAs in normal tissues due to the difficulty in obtaining fresh normal thyroid tissue. Of interest is the finding that the 171-nt sequence starts with GT and ends with AG at its 5' and 3' boundaries, which are in good agreement with the donor and acceptor splice-site consensus sequences, respectively (34). These consensus sequences are believed to play an important role, yet not to be an absolute requirement in the splicing mechanism (35). Because of the absolute similarity of hTPO-1 and hTPO-2 cDNA sequences, except for a single-base difference, which could be due to a copying error by reverse transcriptase, it appears most likely that two mRNAs are generated from differential splicing of a single gene. Alternatively, although less likely, two duplicated genes may exist that generate TPO-1 and TPO-2 or two alleles of one gene are alternately transcribed; one providing TPO-1 and the other TPO-2.

Protein and enzyme analyses suggest that two forms may indeed coexist. For instance, hTPO-1 encodes a protein of 103 kDa whereas hTPO-2 would code for a protein of 97 kDa (assuming that the 5' portion of pHTP-2.4 is identical to that of pHTP-1). In an earlier study, we showed that purified human TPO has two polypeptides of  $\approx 100$  and 107 kDa as estimated on an NaDodSO<sub>4</sub>/polyacrylamide gel (14). A similar result has been obtained by Czarnocka *et al.* (7). Rawitch *et al.* (10) reported that  $\approx 10\%$  of the total weight of porcine TPO is carbohydrate. Actually, five potential asparagine-linked glycosylation sites are found in the amino acid sequence in Fig. 2. Either the 107 or 100 kDa proteins represent glycosylated and unglycosylated forms of a single protein or two different glycosylated proteins exist corresponding to hTPO-1 and hTPO-2. The difference in the molecular weight on NaDodSO<sub>4</sub>/polyacrylamide gel between the 107- and 100-kDa proteins is consistent with that calculated based on the deleted 57 amino acids of hTPO-2. These two proteins might be responsible for the two different iodination and conjugation functions of TPO (1, 2).

We thank Dr. Frank Gonzalez for his help and useful discussion throughout the course of these experiments and the preparation of

the manuscript and Dr. Harry Gelboin for support and encouragement. We are indebted to Dr. Daniela Foti and Dr. Kanji Kuma (Kuma Hospital) for providing thyroid tissues. This research was supported in part by Grants-in-Aid 61570559 and 60480274 for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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