Increasing Diversity of Human Thyroperoxidase Generated by Alternative Splicing

CHARACTERIZATION BY MOLECULAR CLONING OF NEW TRANSCRIPTS WITH SINGLE- AND MULTISPLICED mRNAs*

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The human thyroperoxidase (hTPO) gene is composed of 17 exons. The longest complete cDNA sequence determined so far contains a full-length hTPO (TPO1) encoding a 933-amino acid polypeptide. Several mRNA species encoding for hTPO isoforms are present in normal thyroid tissues, including TPO2 with exon 10 deleted and TPOzanelli with exon 16 deleted. In the present study, we established the existence of two new single-spliced transcripts, TPO4 and TPO5, lacking exons 14 and 8, respectively. Upon transfecting the TPO4 cDNA into Chinese hamster ovary cells, it was observed that TPO4 is able to reach the cell surface, is enzymatically active, and is able to be recognized by a panel of 12 monoclonal antibodies directed against hTPO, whereas TPO5 does not fold correctly and is unable to reach the cell surface. In normal tissues, the expression of TPO4 mRNA was examined by performing quantitative reverse transcription PCR. The functional significance of the other newly spliced mRNA variants still remains to be elucidated, but these results might help to explain the heterogeneity of the hTPO purified from the thyroid gland.

Thyroperoxidase (TPO)† is the key enzyme in the process of thyroid hormone synthesis. The human TPO gene is about 150 kbp in size, is located on chromosome 2, locus 2p25, and consists of 17 exons and 16 introns (for a review, see Ref. 1). The complete sequence of the human TPO coding region is known (2–4). The full-length 3048-bp transcript (TPO1) codes for a protein consisting of 933 amino acids, which have a large extracellular domain, a transmembrane domain consisting of 60 residues, and a short intracytoplasmic tail consisting of 60 residues. Two other transcripts have been described, namely TPO2, in which exon 10 is spliced out, and TPOzanelli (TPO3), in which exon 16 is spliced out. TPO2 and TPO3 have been found to occur in normal thyroid tissues as well as in Graves’ tissues (2, 5, 6). These two form code for proteins consisting of 876 and 929 residues, respectively. TPO2 is rapidly degraded after its synthesis, does not reach the cell surface, and does not have any enzymatic activity (7), whereas TPO3 is able to reach the cell surface and shows enzymatic activity (8).

After being purified from the human thyroid gland, TPO is known to show up in SDS-PAGE under reducing conditions as a double band of 105 and 110 kDa. The relative intensity of these bands varies from one gland to another, and it has been established that TPO2 does not correspond to one of these bands (9, 10), certainly because it is too rapidly degraded after its synthesis (7). The difference in molecular weight between TPO1 and TPO3 (four amino acids) does not explain the existence of two bands, and glycosylation is not responsible for this heterogeneity either (11). The presence of other isoforms and/or the occurrence of endoproteolysis might explain the existence of these different species.

The aim of the present study was to search for the presence of new TPO transcripts that might help to explain this heterogeneity. Reverse transcription was carried out from the total RNAs, and PCRs were performed with various pairs of primers. Two new single spliced species (TPO4 and TPO5) were identified. After quantification of the four spliced isoforms TPO2, TPO3, TPO4, and TPO5 in normal thyroid tissues, the possible existence of multispliced isoforms was hypothesized. The existence of two isoforms with double splicing and one isoform with five spliced exons was established.

EXPERIMENTAL PROCEDURES

RNA Isolation—Frozen normal thyroid tissue was used in these experiments. Tissues were homogenized and prepared using the Promega kit (SV total RNA isolation system) according to the manufacturer’s instructions, and the preparation was then treated with DNase. The RNA concentration was determined from the spectrophotometric absorbance at 260 nm, and the RNAs were aliquoted and stored in water.

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at ~8 °C until further use. The absorption ratio (260/280) was between 1.7 and 2.0 with all the preparations.

Reverse Transcription—Depending on the experiments, reverse transcription was carried out using either 0.5 μg of oligo(dT)_{12-18}, 1 μg of random hexamers, or 2 pmol of gene-specific primer (GSP). A 40-μl reverse transcription reaction mixture containing hexamers, GSP, or oligo(dT)_{12-18}, 0.9 μg of RNA, 0.25 μM dNTP mix, 10 mM dithiothreitol, and 4 units of RNase recombinant inhibitor (Invitrogen) was incubated at 42 °C for 2 min when GSP and oligo(dT)_{12-18} were used, or at 25 °C for 10 min when random hexamers were used. Superscript II RNase H reverse transcriptase (0.2 units) (Invitrogen) was then added, and the mixture was incubated at 42 °C for 50 min. The reaction was inactivated by heating the preparation at 70 °C for 15 min. The mixture then treated with 2 units of RNase H (Escherichia coli) at 37 °C for 20 min.

PCR, Cloning, and Sequencing—Reaction mixtures (50 μl) consisted of 2 units of Fast Start Taq polymerase (Roche Molecular Biochemicals), Taq buffer, 0.9 μM oligonucleotide primers, 300 μM dNTP, 2 mM MgCl₂, and 5 μl of GC-rich solution (Roche Molecular Biochemicals). A DNA sample was added to this mixture, and PCR was performed with the following profile: 5 min at 95 °C for an initial denaturation followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at temperatures depending on the primers, and a 45-s extension at 72 °C, ending with a 5-min final extension at 72 °C and a soak at 4 °C. The products obtained were electrophoresed on agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) buffer and stained with ethidium bromide. When the 3060-bp TPO1 cDNA kindly provided by B. Rapoport was cloned into its initial proportion within the cDNA population, we performed PCR band was multiplied by a factor corresponding to this difference. To rect the difference in nucleotide length, the density of the smaller size each consecutive cycle and loaded onto 2% agarose gel. After staining of each primer, 50 ng of the pcDNA-TPO1, 200 μM dNTPs, 10% (v/v)
dimethyl sulfoxide, 2.5 units of PfuTurbo DNA polymerase (Stratagene), and streptomycin (0.1 mg/ml). Cells were transfected using LipofectAMINE (Invitrogen) with pcDNA3-TPO4 or pcDNA3-TPO5. Cells were incubated in a saturated atmosphere (5% CO₂/95% air) at 37 °C. Stable transfecants were selected in the presence of geneticin (400 μg/ml) and subcloned using limiting dilutions. Positive TPO4 and TPO5 expressing cell lines were identified by performing Western blotting or immunoprecipitation after [³⁵S][Met + Cys] labeling (EXPRE³⁵S²²S protein labeling mix, PerkinElmer Life Sciences). A significant level of TPO1, TPO4, and TPO5 expression was obtained by growing TPO1-CHO, TPO4-CHO, and TPO5-CHO cell lines as described previously (15).

Metabolic Labeling of TPO—Cells were incubated in cysteine- and methionine-free MEM supplemented with 10% FBS, 10 μM sodium butyrate, and 100 μg/ml [³⁵S][Met + Cys]. The incubation was carried out for 5, 16, or 48 h. In the pulse-chase experiments, cells were incubated for 1 h in Cys- and Met-free MEM supplemented with 10% dialyzed FBS and 10 μM sodium butyrate. Cells were then pulsed for 30 min in the presence of 100 μM [³⁵S][Met + Cys]. After the pulse, the labeling medium was removed, and the cell surface was washed three times with PBS and then replaced by Ham’s F-12 medium supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (0.1 mg/ml). Cells were labeled with LipofectAMINE (Invitrogen) with pcDNA3-TPO4 or pcDNA3-TPO5. Cells were incubated in a saturated atmosphere (5% CO₂/95% air) at 37 °C. Stable transfecants were selected in the presence of geneticin (400 μg/ml) and subcloned using limiting dilutions. Positive TPO4 and TPO5 expressing cell lines were identified by performing Western blotting or immunoprecipitation after [³⁵S][Met + Cys] labeling (EXPRE³⁵S²²S protein labeling mix, PerkinElmer Life Sciences). A significant level of TPO1, TPO4, and TPO5 expression was obtained by growing TPO1-CHO, TPO4-CHO, and TPO5-CHO cell lines as described previously (15).

Diversity of Alternatively Spliced Thyroperoxidase mRNAs

| Primer | Exon | Position | Sequence |
|--------|------|----------|----------|
| PE2F   | 2    | 53-73    | 5'-CTTCCTCCGCCCTATCCTGAA-3' |
| PE9Fa  | 9    | 1392-1413| 5'-GCTGACTCTGGTTCCTATGAA-3' |
| PE9b   | 9    | 1392-1413| 5'-AGGCTCTAGGATGATAGCT-3' |
| PE9R   | 11   | 1416-1397| 5'-GGCTGACCTCCTCCTGACGGTG-3' |
| PE11F  | 11   | 1833-1862| 5'-AGCCATCGGACGAGGGTGGCCGACAA-3' |
| PE11R  | 11   | 1974-1773| 5'-GCAGAAACCTCCCTCCTACTTGA-3' |
| PE12F  | 12   | 2111-2140| 5'-TGCCCATGGATGCCTCAGTGGCAAT-3' |
| PE15F  | 15   | 2577-2606| 5'-GCTAGGCTTCCGACGCTTCCTCCCTGACGGTG-3' |
| PE15R  | 15   | 2573-2603| 5'-TGCCAGGAGACCTGACGCTTCCTCCCTGACGGTG-3' |
| PE17Fa | 17   | 2881-2851| 5'-TTTGGCTGTGTGTTGAAAAAGTCTGTAACGG-3' |
| PE17b  | 17   | 2851-2733| 5'-TCGTGGCAAGCATGGTGTGATATC-3' |
| P-TPO4-F | 13-15 | 5'-GAGTGGATTTCGACGCTCCTCTGCAAGACTGCGGAGGCTCTATCGG-3' |
| P-TPO4-R | 15-13 | 5'-TCGAGGAGGCCCTCCCTGCTTGTCTCTTGAGGAGGCTGAAAATCCCATC-3' |
| P-TPO5-F | 7-9  | 5'-AACCCATGGTTTTTCCTAACAATTCATACCTGGTGGATTAC-3' |
| P-TPO5-R | 9-7  | 5'-GTAACCTCAGGAGTGATTTGTTAGGGAAAAACATGGTTT-3' |
experiment. 125I-labeled CHO-TPO cell lysates were immunoprecipitated for 4 h at 25 °C with 50 μg of each of the TPO-mAbs previously complexed with protein A-Sepharose 4B.

Cell Surface Biotinylation—TPO1-, TPO4-, and TPO5-CHO confluent monolayers were metabolically labeled for 18 h with 100 μCi/ml 125I (Met + Cys) in the presence of 10 μM sodium butyrate, and cell surfaces were biotinylated as described previously (15). Cells were washed twice with PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2, and exposed to a 0.5 mg/ml EZ-link sulfo NHS-SS-Biotin (Pierce) for 20 min at 4 °C. The cross-linker was removed, and the procedure was repeated once. The biotin reagent was quenched by incubating the preparation with 50 mM NH4Cl in PBS for 10 min at 4 °C. Cells were washed with PBS and harvested. To recover the immunoprecipitated antigens, the complexes were supplemented with 10 μg/ml BSA (5 mg/ml in PBS) and Na125I (106 cpm/ml), with or without 2 mM 2-mercapto-1-methylimidazole, as the control medium. The reaction was initiated by adding H2O2 to obtain a final concentration of 0.5 mM, 2-mercaptoethanol, and cell surface was biotinylated as described previously (15). Cells were

Identification of the Deletion Variants of Exon 8 and 14—A strategy was developed to search for new isoforms of hTPO. It is known that the various transcripts resulting from alternative splicing can have different lengths of the poly(A) tail (18), which exon 8 had been deleted. This variant was named TPO5 (GenBank™ accession number Y136822). Exon 14 codes for an extracellular part of the protein located in its myeloperoxidase-like domain. Juxtaposing exons 7 and 9 did not lead to any changes in the open reading frame, and the corresponding isoform corresponds exactly to its EGF-like domain (3). Juxtapos-
underlying the formation of compound I, and Glu-399, which may covalently bind to the heme prosthetic group through ester linkage (19). Two potential N-glycosylation sites (Asn-307 and Asn-342) are also spliced out. Contrary to what occurs in TPO5, the deleted exon in TPO4 mRNA codes for a whole domain (the EGF-like domain) that is not included in the main catalytic part of the molecule. It therefore seemed possible that this isoform might be active. We therefore examined the properties of the proteins corresponding to these two transcripts, focusing in particular on TPO4.

Expression of TPO4 and of TPO5 in CHO Cell Line—pcDNA3-TPO4 and pcDNA3-TPO5 were constructed from pcDNA3-TPO1 using a one stage PCR protocol compatible with the deletion of exon 14 or exon 8. CHO cells were transfected with pcDNA3-TPO4 or pcDNA3-TPO5, and several clones expressing significant levels of TPO4 or TPO5 were then isolated. After a metabolic labeling step using $[^{35}S]$(Met + Cys), immunoprecipitation was performed using the pair mAb15 + mAb47, and TPO4 and TPO5 showed up as bands with the predicted molecular weight on the SDS-PAGE analysis (Fig. 3, A and B).

Stability, Immunoreactivity, and Intracellular Trafficking of TPO4—To determine whether TPO4 has a modified three-dimensional structure in comparison with TPO1, we used a panel of 12 mAbs directed against hTPO. All of these mAbs except one, mAb47, were directed against conformational epitopes. TPO1- and TPO4-CHO cells were labeled for 16 h with $[^{35}S]$(Met + Cys), and, after the extraction step, immunoprecipitations were performed with each of the 12 mAbs (Fig. 4, A and B). TPO1 as well as TPO4 immunoreactivity was observed with all the mAbs. However, mAbs 1, 24, and 59 showed a slight decrease in immunoreactivity with TPO4 as compared with TPO1 (Fig. 4C). This seems to indicate that most of the TPO4 fold correctly in comparison with TPO1 and that none of the mAbs used were directed against the EGF-like domain. To determine whether the small differences observed affect the global half-life of the TPO4 synthesized in CHO cells, we performed a pulse-chase experiment. Cells were pulsed for 30 min with $[^{35}S]$(Met + Cys) and then chased for various times. Immunoprecipitation of TPO was performed, and samples were analyzed by SDS-PAGE. Quantification of these bands (Fig. 5) showed that TPO4 has a shorter half-life than TPO1, i.e. 5 versus 7.5 h. All of these events may affect the intracellular trafficking of TPO4 and hence its level of expression at the cell surface. To check whether TPO4 can reach the cell surface of the CHO cells, the cell surface expression of the two isoforms was determined after labeling CHO cells with $[^{35}S]$(Met + Cys) for 48 h and performing cell surface biotinylation (Fig. 6A). Quantification of the bands obtained showed that 25% of the TPO1 and only 12% of the TPO4 were present at the cell surface (Fig. 6B).

When expressed in CHO cells, TPO5 showed reactivity with mAb47 but not with mAb15, which indicates that this isoform was not able to fold correctly. In addition, this isoform is not able to reach the cell surface (data not shown).

Enzymatic Activity of TPO4—The following two methods were used to detect whether TPO4 has any enzymatic activity. In the first step, microsomes were prepared from TPO1-, TPO4-, and pcDNA3-CHO cells, and, after protein extraction, guaiacol oxidation was performed (Fig. 7A). As expected, TPO1 was enzymatically active, and three times less activity was detected with TPO4. The difference in activity was due to the fact that TPO1-CHO cells express three times more TPO than TPO4-CHO cells. Cell surface enzymatic activity was also determined. The TPO1 present at the cell surface was able to catalyze the iodination of BSA. Some activity was also detected with TPO4 (Fig. 7B). This activity was seven times lower than that obtained with TPO1; however, this difference is consistent
FIG. 5. Rate of degradation of TPO1 and TPO4. Cells were pulsed for 30 min in the presence of 100 μCi/ml of [35S](Met + Cys) in a Cys- and Met-free MEM supplemented with 10% FBS. After the pulse step, the medium was removed and replaced with Ham’s F-12 medium supplemented with 5 mM Cys and Met. At the times indicated, after the extraction step, TPO from radiolabeled cell lysate was immunoprecipitated using the pair mAb 15 + mAb 47. Immunoprecipitated TPO1 (A) and TPO4 (B) were analyzed by SDS-PAGE. Bands corresponding to TPO1 (○) and TPO4 (●) were quantified by phosphorimaging. This figure gives the results of an experiment that is representative of four identical experiments performed.

with the fact that the level of TPO4 expression is three times lower in CHO cells and only half as high as that of steady state TPO1 at the cell surface. These results show that TPO4 is enzymatically active and that it may be involved in thyroid hormone synthesis.

Quantification of TPO mRNA Variants—The level of involvement of TPO4 in thyroid hormone synthesis depends on the level of expression of this isoform in thyroid tissues. Quantitative RT-PCR was therefore performed to measure the level of TPO4 mRNA expression as compared with the other transcripts. RT was performed using random hexamers, and PCR was performed with primers PE11F and PE15R. The 493-bp and 362-bp products obtained were separated on 2% agarose gel (Fig. 8A). After quantification, a correction of 1.36 was applied because of the difference in product size. The normalized band intensities were plotted as a function of the number of cycles (Fig. 8B). The semi-logarithmic plot of the product accumulation versus the number of cycles (Fig. 8C) showed that the efficiencies of the PCRs (as given by the slopes of the lines) were the same with both of these species. Under these conditions, the relative difference between the original abundance of these two samples was taken to be 2^n, where n is the difference between the number of cycles necessary to reach a threshold value (12) (Fig. 8B). The threshold value was the value in the exponential part of the curve at which a statistically significant increase in fluorescence was detected. RNAs from 14 different thyroid tissues were used, and the results obtained showed that TPO4 mRNA amounted to 32 ± 11% of the total TPO mRNAs. In Fig. 8, the TPO1/TPO4 transcript ratio differs from that shown in Fig. 1, because the mRNAs used were obtained from different thyroid glands. The level of TPO2 mRNA expression in normal thyroid tissues has never been exactly determined, and in the case of TPO3 it is only known that in thyroid from Graves’ disease its mRNA accounts for ~50% of the total hTPO mRNA. We also quantified these two variant mRNAs by RT-PCR. Primers located in exons 9 and 11 and exons 15 and 17 were used to quantify mRNA with exons 10 (Fig. 8A) and 16 deleted (Fig. 8B), respectively. TPO2 amounted to 35 ± 12%, and TPO3 amounted to 36 ± 14%. In the case of TPO5, we did not find in this part of the molecule any pair of primers giving a similar reaction efficiency between TPO1- and TPO5-cDNAs. However the quantity of TPO5 mRNA probably accounts for ~10% (Fig. 2). The sum of the percentages of these four species (not including TPO1) was more than 100%, possibly due to the presence of multipliced mRNAs.

RT-PCR, Cloning, and Identification of a Multispliced Variant of hTPO—As it is not possible to synthesize the full-length coding region of the hTPO mRNA efficiently, we searched for the existence of multipliced mRNAs in its 3’-terminal part corresponding to the exon 10, 12, and 16 deletions. These TPO variant cDNAs can be amplified using primers located in exons 9 (PE9Fb) and 17 (PE17Rb). RT was performed using oligo(dT) 12–18, and after an amplification step, PCR products were analyzed on 1% agarose gel. Three main bands migrated with apparent sizes of 1416, 1283, and 1160 bp, and faint bands with smaller sizes were observed (Fig. 10). The expected size of TPO1 (no exon deletion) was 1413 bp, which corresponded no doubt to the widest band obtained. As we were looking for multipliced isoforms, the smaller bands were purified, and the cDNAs were subcloned and sequenced. Twenty-five clones were analyzed, and three multispliced variants were identified, namely TPO2/3 corresponding to the deletion of exons 10 and 16 (GenBank™ accession number AF533530), TPO2/4 corresponding to the deletion of exons 10 and 14 (GenBank™ accession number AF533531), and an unexpected variant, TPO6, corresponding to the deletion of exons 10, 12, 13, 14, and 16 (GenBank™ accession number AF533529). The latter clone...
certainly corresponds to one of the faint bands with a smaller size, which can be seen in Fig. 10.

It is also worth noting that when we analyzed the intensity of the bands obtained after RT using oligo(dT)12–18 and 35 cycles of PCR amplification using primers located in exons 9 and 17 (Fig. 10), the ratio between TPO1 and the various other isoforms did not correlate with the results obtained by quantitative RT-PCR, because the major mRNA species seems to be the TPO1 mRNA. One of the differences between these two experiments was that oligo(dT)12–18 was used in this experiment, and random hexamers were used in the quantitative RT-PCR. This confirms that the choice of RT procedure is of great importance when analyzing the level of expression of these various isoforms.

**DISCUSSION**

Some years ago, five variants of hTPO were characterized, *i.e.* the full-length mRNA TPO1, which consists of 17 exons (2, 3, 4), the TPO2 with exon 10 deleted (2, 5), the TPO3 with exon 16 deleted (6), TPO I consisting of exons 1–6 plus the 5′-end of intron 6, and TPO II consisting of exons 1–5 plus an unidentified DNA tract 558 bp in length (20). Studies performed by expressing the recombinant variants showed that TPO1 and TPO3 are enzymatically active and able to reach the cell surface of CHO cells (8, 21), whereas TPO2 is rapidly degraded after its synthesis and does not have any enzymatic activity (7).

After being purified from human thyroid glands by affinity chromatography using anti-TPO mAbs, hTPO shows up in SDS-PAGE as a closely migrating double band with a lower molecular weight than that of the TPO1 and TPO3 expressed in CHO cells. This difference may be due to post-transcriptional or post-translational modifications. In the present study, we investigated the possible existence of new variants of hTPO mRNAs, which might help to explain the multiple forms of hTPO obtained.

Because it is difficult to detect alternative splicing using the full-length coding region of hTPO, we synthesized smaller cDNA parts of hTPO that together cover the entire mRNA. For

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2 V. Le Fourn, M. Ferrand, and J. L. Franc, unpublished results.
Figure 9. Relative abundance of TPO mRNAs with exons 10 or 16 deleted. The following actions were taken to quantify the relative abundance of TPO mRNA. A, with exon 10 deleted, reverse transcription was performed using random hexamers, and TPO mRNAs were amplified by performing PCR as described under “Experimental Procedures” using primers PE9Fa and PE11R with an annealing temperature of 55 °C. B, with exon 16 deleted, reverse transcription was performed using random hexamers, and TPO mRNAs were amplified by performing PCR as described under “Experimental Procedures” using primers PE15F and PE17Ra with an annealing temperature of 70 °C. Aliquots of 9 μl were taken from the reaction mixture at various cycles. These aliquots were analyzed with 2% gel agarose, and the bands were detected and quantified using a Kodak Image Station 440.

Figure 10. Amplified fragments of TPO cDNAs obtained by performing RT-PCR between exons 9 and 17. Reverse transcription was performed using oligo(dT)$_{12-18}$ and 35 cycles of PCR were then performed using primers PE9Fb and PE17Rb with an annealing temperature of 61 °C. Amplification products were analyzed on 1% agarose gel. Lane 1, DNA size marker; lanes 2–5, RT-PCR products obtained using RNA from four different normal thyroid tissues.

The reverse transcription procedure, we used random hexamers, GSP or oligo(dT)$_{12-18}$. The use of random hexamers or GSP makes it possible to detect mRNA that either does not possess any tail or has only a very short poly(A) tail. Actually it is well known that various transcripts from one gene can differ in their poly(A) tail length and, in some cases, can be detected with difficulty using oligo(dT)$_{12-18}$. Using this strategy, two new monospliced variants were identified, namely TPO4 lacking exon 14, and TPO5 lacking exon 8. We estimated by quantitative RT-PCR that TPO with exon 14 deleted accounts for 32 ± 11% of the total hTPO mRNA. Using the same technique, the level of expression of the known transcript with exon 10 deleted was found to be 35 ± 12%, and that with exon 16 deleted was 36 ± 14%. The TPO5 mRNA accounts for ~10% of the total hTPO mRNAs. As the sum of these various forms, not including TPO1, was more than 100%, the existence of multispliced transcripts was hypothesized. The search for multispliced species with exons 10, 14, and 16 deleted showed the existence of two transcripts with a double splicing, namely TPO2/3, and TPO2/4. Although no variant with triple splicing TPO2/3/4 was observed, the presence of this species cannot be ruled out. We did not search for multispliced species with exon 8 deleted, but these transcripts certainly exist. Moreover, an unexpected multispliced species, TPO6, was detected with splicing of exons 10, 12, 13, 14, and 16. It is worth noting that we never detected the presence of TPO mRNA with a spliced exon 12 or exon 13 alone. We then established the existence of five new hTPO mRNAs with the other known variants; with the multiple possibilities of the multisplicing procedure, it seems quite likely that a greater number of hTPO mRNA variants may be found to exist.

It is not possible to quantify the proportions in which the different multispliced transcripts are present. However, it should be noted that although TPO5 and TPO6 mRNA are not at all abundant, the splicing of exons 10, 14, and 16 is a very common occurrence in normal thyroid tissues, and TPO1 mRNA is perhaps not the main transcript present in the thyroid gland. Northern blot data obtained in previous studies by various groups showed the presence of mRNA species of ~4.0, 3.1, 2.9, 2.1, and 1.7 kb (for a review, see Ref. 1). The 4.0-kb transcript has been thought to be an immature mRNA precursor, whereas the 3.1-kb transcript corresponds to TPO1, and the mRNA species that are 2.1 and 1.7 kb in size correspond to TPO1 and TPO II, respectively. The 2.9-kb species might correspond to TPO2, TPO3, and/or TPO4. The 3.1-kb species has been obtained by all the authors, whereas only Kimura et al. (2) have reported the existence of the 2.9-kb species. It is difficult to understand why this is so, but determining the mRNA size accurately in Northern blots is known to be rather tricky, and it is also possible that the 3.1- and 2.9-kb species may have been poorly separated because of the small difference in size. It is therefore difficult to obtain an exact idea of the proportion of wild type/splice variant transcripts from these Northern blot experiments.

As to whether the RT-PCR procedure constitutes a valid means of analyzing the expression of these various isoforms, the use of oligo(dT)$_{12-18}$ and a random hexamer or GSP clearly yielded very different results (see Figs. 8, 9, and 10). This seems to indicate that some or all of the spliced variants of hTPO have a shorter poly(A) tail than that of TPO1 as was also previously found to occur in the case of TPO I and II (20). Messenger RNAs leave the nucleus with a >200-residue poly(A) tail and are deadenylated, yielding heterogenous polymers consisting of adenosine residues. The cytoplasmic control of poly(A) length plays a key role in activating and repressing gene expression. A search for possible different lengths of poly(A) tails, depending on the isoform, is now under way.

Multiple species of MPO were also produced by performing alternative splicing. Two transcripts with deletions of 57 and 171 bp, which were generated by partially skipping exon 9 and completely skipping exon 10 (22), and two other transcripts with modifications in their 5'-ends and with insertions of 96 bp in exon 2 and 82 bp in exon 4 have been described (23). Except for the splicing of exon 10, which corresponds to TPO2, the other alternative splicing shows no similarities between the two peroxidases.

The splicing of one exon can affect the folding of the protein variably, depending on its location. The nucleotide and amino acid sequences corresponding to exons 3–11 in the hTPO gene show some significant similarities with exons 2–11 of MPO (24). The next two exons, 13 and 14, belong to the C4b and EGF gene families, respectively. Exons 15 and 16 code for the transmembrane part of the protein and for its cytoplasmic tail. These polypeptide parts do not show any similarities with other proteins. As far as TPO5 is concerned, exon 8 codes for a large (172 amino acids) and important part of the protein located in the middle of the myeloperoxidase-like domain, which includes Arg-396 and Glu-399. Arg396 corresponds to...
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The Arg-235 of MPO, which participates in the catalytic mechanism, Glu-399 corresponds to the Glu-242 in MPO and is a possible site of the covalent heme binding process (19). In addition, two potential N-glycosylation sites (Asn-307 and Asn-342) are present in this part of the protein. Therefore, as can be expected when expressed in CHO cells, TPO5 is unable to acquire a proper three-dimensional structure and reach the cell surface. The other monospliced species, TPO4, has exon 14 deleted. This exon codes for a whole domain located in the extracellular part of the molecule showing similarities with EGF and is certainly not involved in the function of the enzyme. We expressed this protein in CHO cells and investigated the structural and functional aspects of TPO4 with respect to TPO1 as was previously done with TPO2 and TPO3 (7, 8). Part of the synthesized TPO4 is able to fold correctly and reach the cell surface, but this isoform has a shorter half-life than TPO1 (5 versus 7.5 h). Cell surface biotinylation showed that only 12% of the protein is present in the steady state at the cell surface as compared with 25% in the case of TPO1. Like TPO1 and TPO3, TPO4 is enzymatically active and can therefore be expected to play a role in thyroid hormone synthesis. Some of these results are in agreement with the results of a very recent study by Guo et al. (25). To localize the immunodominant region of hTPO, these authors transfected COS-7 cells with TPO cDNA from which the EGF-like domain had been deleted. They established that this TPO (corresponding to TPO4) is able to reach the cell surface. In addition, they established that this protein can be recognized by human monoclonal autoantibodies. The results of this study confirm that the three-dimensional structure of TPO4 is very similar to that of TPO1 and definitively exclude the possibility that the juxtamembrane EGF-like domain is part of the TPO immunodominant region.

Our study also showed the presence of multispliced species. Based on the results obtained after expressing recombinant monospliced species, the effects that the absence of the same exon from multispliced species will have are predictable. For example, we have established that the lack of exon 10 in TPO2 leads to a rapid degradation of this protein (7); it can therefore be expected that all isoforms with exon 10 deleted will be rapidly degraded. By contrast, the deletion of exon 14 or 16 does not have such dramatic effects, and if TPO3/4 exists, it will therefore certainly be active.

The existence of these various transcripts certainly explains, partly, the heterogeneity of the TPO purified from human thyroid glands. The double bands obtained in SDS-PAGE obviously consisted of numerous smaller bands with very similar molecular weights.2 The existence of these various species was certainly due to the presence of various isoforms as well as to the existence of an endoproteolytic process, because we have established that hTPO, when expressed in CHO cells (26) and the rat thyroid cell line PC Cl3,3 is cleaved by proprotein convertases in its N-terminal part.

In conclusion, the present results show that the alternative splicing form of TPO mRNAs gives rise to a great number of different transcripts. Two new monospliced isoforms (TPO4 and TPO5) and three multispliced isoforms (TPO2/3, TPO2/4, and TPO6) have been shown here to exist, but the number of multispliced isoforms is certainly greater. It was established here that TPO4 is enzymatically active and able to reach the cell surface. Further studies will now be required to determine the role played by the other isoforms in the process of thyroid hormone synthesis and the true chemical structure of the thyroperoxidase present in normal human thyroid tissues.

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