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Identification of Five New Isoforms of Murine Thrombopoietin mRNA

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Thrombopoietin (Tpo) is the major physiologic regulator of platelet production. Its gene is expressed in many organs and appears constitutive in liver and kidney. However, inducible gene expression in the bone marrow and spleen have been reported as well as the presence of a number of isoforms, presumably arising from alternative splicing. We have identified five new murine Tpo isoforms, designated Tpo 5 to Tpo 9. Alternative splicing of these isoforms, in addition to the already-reported four isoforms, occurs around exon 7, the last exon, with insertion of some intron sequences or deletion of exon sequences. Studies of tissue distribution indicate that Tpo 4 is the major isoform in lymph nodes and bone marrow. The roles of these isoforms in hematopoietic regulation are unknown, but the presence of inducible Tpo mRNA in the marrow microenvironment may contribute to platelet or stem cell homeostasis.

Thrombopoietin (Tpo), the primary regulator of platelet production, stimulates both the proliferation and differentiation of megakaryocytes and promotes the growth in culture of megakaryocytic progenitor cells (1–4). Tpo also sustains very early progenitor cells *in vitro* and, following myelosuppression, promotes the recovery *in vivo* of multiple hematopoietic lineages (5, 6).

The regulation of Tpo gene expression is complex, and at least two mechanisms have been identified. First, Tpo is produced constitutively in liver and kidney, and Tpo mRNA levels in those organs are unaffected by changes in circulating platelet number. Plasma Tpo levels, however, vary inversely with the platelet count and megakaryocyte mass, apparently due to Tpo receptor-mediated binding and uptake on the surface of these cells (7–9). Alternatively, in bone marrow and spleen, Tpo gene expression has been reported to be inversely related to the platelet count (10, 11). Thus, with induced thrombocytopenia, Tpo mRNA levels increase. The importance of this latter mechanism of Tpo regulation in hematopoiesis is unclear, but local regulation of megakaryopoiesis or early progenitor cell maintenance could be an important role for Tpo in the marrow microenvironment.

The human Tpo gene is comprised of seven exons and six introns spanning eight kilobases (12), and this structure is largely conserved in the mouse (13). Tpo gene transcription is initiated at multiple sites (13–15), and preliminary results suggest that the different transcription initiation sites are responsible for tissue-specific expression (16). In addition to the complexity of transcription initiation, multiple alternatively spliced human and murine Tpo mRNA forms have been described. Four Tpo isoforms in the mouse have been isolated. Except for the predominant isoform, Tpo 1, the other three isoforms exhibited similar mRNA expression levels *in vitro*, but were poorly secreted (12, 14, 17). Alternative splicing occurs in the last exon. Interestingly, Tpo 4, identified in a murine stromal cell line, has a 197 base pair deletion at the beginning of the last exon, exon 7. This results in a frame shift and 113 different amino acids at the carboxy terminus. This isoform, although poorly secreted, showed bioactivity comparable to that of Tpo 1 when tested for its growth-promoting activity on a Tpo-dependent cell line (18).

To investigate the possible contribution of alternative splicing to tissue-specific Tpo gene expression, we amplified the murine Tpo gene by PCR using primers spanning the last intron. As a result, we have identified a total of nine Tpo isoforms, including five not previously reported. Our results also show that Tpo 4 is the major isoform in bone marrow and lymphoid tissue.

MATERIALS AND METHODS

Total RNA isolation. Freshly harvested C57Bl mouse tissue (0.1 gm) was disrupted in 1 ml Trazol solution (GIBCO BRL, Gaithersburg, MD) using a tissue homogenizer. Total RNA was extracted following the manufacturer's protocol, and RNA purity and integrity were determined by spectrophotometry using a Du-70 Spectropho-

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TABLE 1

Mouse Tpo Primers Used in This Study

Name	Direction	Location	Sequence
Tpo173	forward	Exon 3	AAT GGA GCT GAC TGA TTT GCT CCT G
Tpo383	forward	Exon 5	AGA ATG GAA AAC CCA GAC GGA ACA G
Tpo510	reverse	Exon 6	GGA GGC GAA CCT GCC CAG AAA GCT G
Tpo806	reverse	Exon 7	AAG TCC AGG GCC AGC AGT TCT G
Tpo854	reverse	Exon 7	GGA GCA AGG CTT GGA GAA GGA GGA A
Tpo885	reverse	Exon 7	GAG ATT TGG ACT GGG GAC CTG GA
Tpo1217	reverse	Exon 7	TGT TTC CTG AGA CAA ATT CCT GGG A

tometer (Beckman Dickinson, San Jose, CA) and formaldehyde denaturing electrophoresis.

Primer selection strategy. Based on the published sequence of the murine Tpo gene (3), a set of primers was designed with Oligo 5 (Pharmacia, Peapack, NJ). To clarify the origin of the amplified Tpo fragments, forward and reverse primers located in different exons were designed (Table 1).

Reverse transcription-polymerase chain reaction (RT-PCR). One microgram of total RNA from various tissues was subjected to RT primed with 0.5 ug oligo$(dT)_{15}$ using Superscript II RT Reverse Transcriptase (GIBCO) and 1/10 of the RT product was used as PCR template. To minimize the likelihood of generating non-specific bands, highly stringent PCR conditions were used: denaturation was carried out at 95°C for 5 min; 35 cycles at 95°C for 30 s, then at 68°C for 40 s, extend at 72°C for 10 min. The predicted sizes of the amplified cDNA fragments depend on the different primer combinations.

Sequencing and cloning of the murine Tpo cDNA fragments. PCR products were recovered using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and inserted into a pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA), and transduced into *E. coli.* Positive clones were picked and grown up, and plasmid DNA was isolated using the RMP kit (Bio 101, Vista, CA). After the correct length inserts were confirmed with EcoR I digestion, plasmids were sequenced on an Applied Biosystems (Foster City, CA) 371A automated sequencer.

Expression of Tpo isoforms. Primer combinations Tpo173 and Tpo1217 or Tpo173 and Tpo854 were used, respectively, to create Tpo 1 and Tpo 4 open reading frames (ORFs) lacking a stop codon. DNA fragments of the expected sizes were inserted into a pCR2.1 TA cloning vector, and confirmed by sequencing. pEGFP N3 vector (Clontech, Palo Alto, CA) was digested with EcoR I, filled with Klenow fragment, and digested with BamH I. The Tpo 1 or Tpo 4 ORFs were cut out from the recombinant pCR2.1 plasmid with EcoR V and BamH I and inserted into digested pGFP N3, resulting in fusion expression of green fluorescence protein (GFP) and Tpo.

Cos7 and CHO cells (American Type Culture Collection) were maintained in Ham's F12 medium and DMEM with 10% fetal bovine

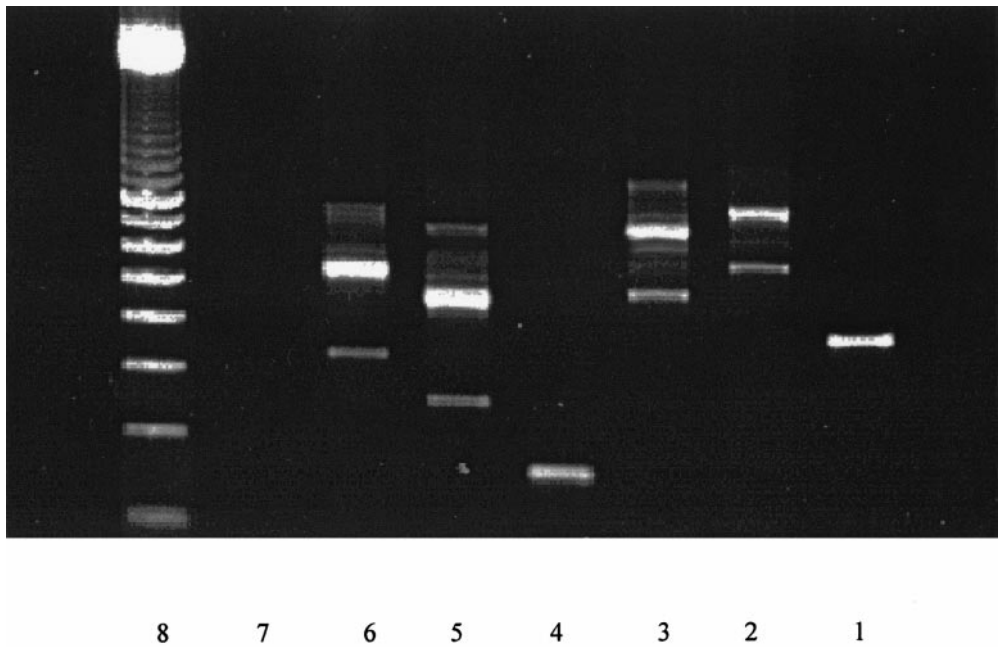

FIG. 1. PCR-amplified Tpo mRNA from murine liver. Lane 1, primers 174–510. Because the primers do not span exon 6, only a single PCR product is seen. Lane 2, primers 174–806. In this lane and in lanes 3, 5, and 6, the more prominent (upper) band represents Tpo 1; the less prominent (lower) band represents Tpo 4. Lane 3, primers 174–885. Lane 4, primers 383–510. Lane 5, primers 383–806. Lane 6, primers 383–885. All nine Tpo mRNA isoforms were identified from PCR products amplified with these primers. Lane 7, control. Lane 8, size markers.

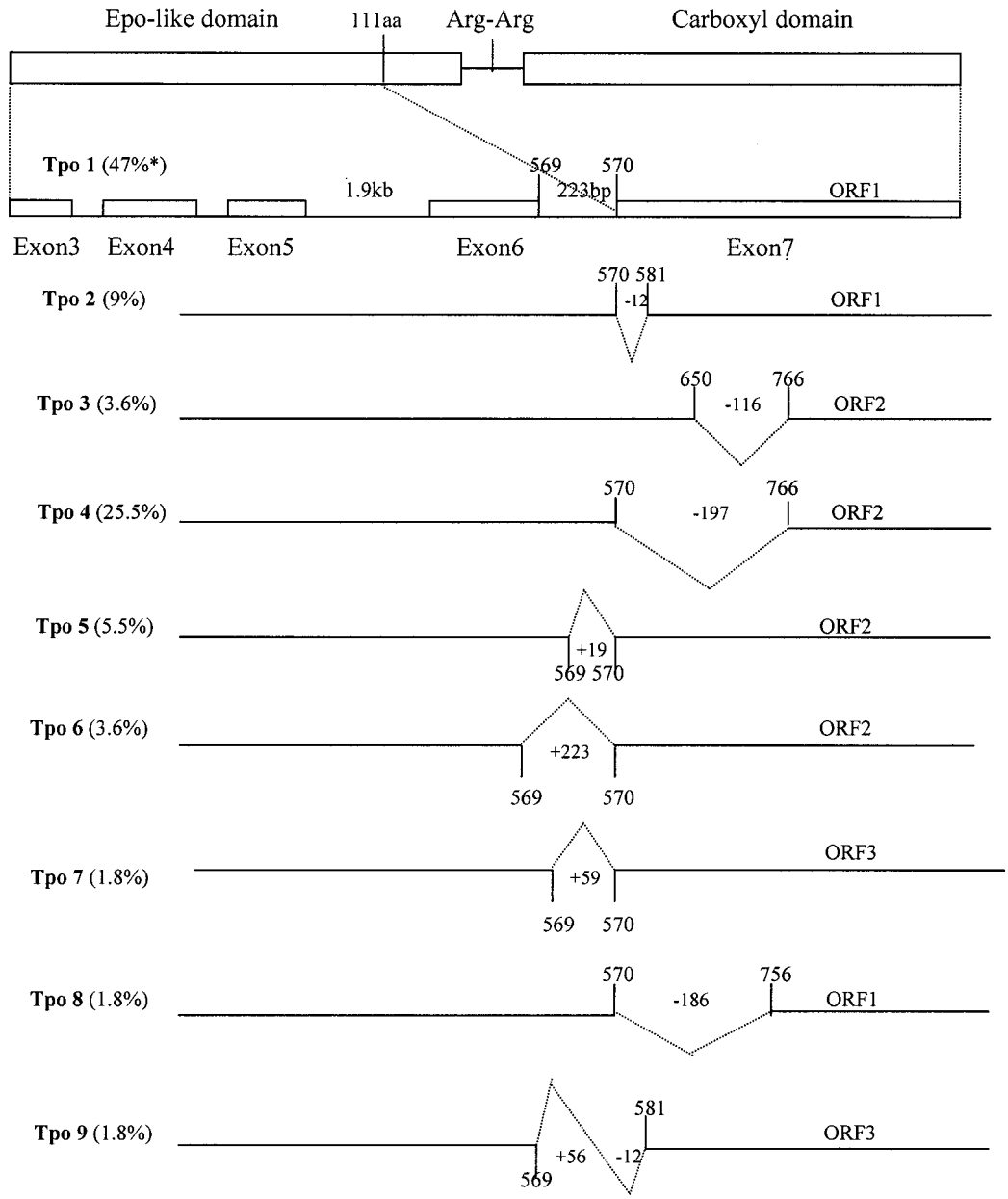

FIG. 2. Alternative RNA splicing patterns identified for murine Tpo. *, frequency of each isoform.

serum (all from GIBCO). After confirmation of inserts by sequencing, recombinant plasmid DNAs were transfected into both cell lines with lipofectAMINE (GIBCO) according to the manufacturer's instructions. Three days later, subcellular localization was observed using confocal laser scanning fluorescence microscopy (Biorad, Hercules, CA).

RESULTS

Identification of Tpo isoforms. When RT-PCR analysis was performed using total RNA from murine liver, only a single band was observed if the primer combination did not span intron 6 (Fig. 1). When the primers spanned intron 6, multiple bands appeared. In addition to the predominant Tpo 1 and the three previously reported truncated Tpo isoforms, we also observed bands consistently appearing with different primer combinations. Similar band patterns were observed using a variety of stringent PCR conditions designed to minimize non-specific PCR product formation (data not shown).

PCR products from the Tpo383-Tpo885 primer combination were recovered and inserted into a pCR2.1 TA cloning vector; fifty-five plasmids with inserts were sequenced. All contained Tpo fragments with insertions or deletions of some sequences at the exon 6-exon

A

Exon Intron Exon

C a u u u u u u u u u u u u c G

5'---or AG gu or agu--------A--------or or or or or or or or or or or or N or ag or

A a c c c c c c c c c c c c u A

B

Tpo 1 ACC **CAG** gta agt gtc ccc aaa ----------ctc tgc cct cag **A**AG AGG

Tpo 2 ACC **CAG** gta agt gtc ccc aaa------------ctt cct cta cag **G**GC AGG

Tpo 3 GGA **AAG** gtg cgc ttc ctg ctt---------aag ttc cca aac ag **G**AC TTC

Tpo 4 ACC **CAG** gta agt gtc ccc aaa--------aag ttc cca aac ag **G**AC TTC

Tpo 5 ACC **CAG** gta agt gtc ccc aaa-----------cac tgg cct cag **G**CC CAT

Tpo 6 ACC **CAG** GTA AGT GTC----------TGC CCT CAG **A**AG AGG

Tpo 7 ACC **CAG** gta agt gtc ccc aaa-------------ctc ctt att cag **A**AG AGG

Tpo 8 AAC **CAG** gta agt gtc ccc aaa-----------aca cta aac aag **T**TC CCA

Tpo 9 ACC **CAG** gta agt gtc ccc aaa-------------ctt cct cta cag **G**GC AGG

FIG. 3. The intron-exon structure of the murine Tpo gene. (A) The consensus sequences for RNA splicing in higher eukaryotes. (B) The intron-exon junction sequences of the nine Tpo isoforms. The highlighted bases are highly conserved in RNA splicing. The bolded bases shown are less conserved. The capitalized bases are exon sequences. The bases shown in lower case are intronic sequences.

7 junction area. Overall, 9 inserts, designated Tpo 1 through Tpo 9, were identified (Fig. 2). Of the 55 plasmids, 47% were Tpo 1, 25% were Tpo 4, 9% were Tpo 2, 5.5% were Tpo 5, 3.6% each were Tpo 3 or Tpo 6, and 1.8% each were Tpo 7, Tpo 8, and Tpo 9. Tpo 1 and three isoforms, Tpo 2, Tpo 3, and Tpo 4, which have, respectively, 12, 116, and 197 bp deletions in exon 7, have been reported previously (12, 14, 17). Tpo 5, Tpo 6, and Tpo 7 have 19, 223, and 56 bp inserts at the junction of exons 6 and 7. Since the genomic sequence of murine Tpo has not been reported, we employed a set of primers spanning exons 6 and 7 to amplify intron 6 from mouse liver genomic DNA. Sequence analysis indicated that Tpo 5 and Tpo 7 came partially, and Tpo 6 completely, from intron 6. Tpo 8 has the first 186 bp of exon 7 spliced out. Tpo 9 was formed with the last 56 bp of intron 6 replacing the first 12 bp of exon 7—equivalent to the combination of Tpo 7 and Tpo 2. Consensus sequences for RNA splicing are conservative in all isoforms (Fig. 3).

Tissue distribution of Tpo isoforms. Using the primer set Tpo383-Tpo885, we determined the distribution of the Tpo isoforms in different tissues (Fig. 4). Liver, kidney, heart, thymus, and testis showed similar expression patterns; the presence of all isoforms was identified in those tissues. Tpo 3, Tpo 4, and Tpo 8 were not found in lung, spleen or blood. The predominant bands found in bone marrow and lymph nodes, two major hematopoietic tissues, were confirmed to be Tpo 4.

Subcellular localization of Tpo 4. Several hematopoietic growth factors, such as macrophage colony-stimulating factor and c-kit ligand, have both soluble and membrane-bound forms as a result of using different splice acceptors. These forms play important and different roles in the regulation of hematopoiesis. Since Tpo 4 is the major Tpo mRNA isoform in lymph nodes and bone marrow, and because there is preliminary evidence that locally produced Tpo may be important as a regulator of hematopoiesis (10, 11), we wished to determine if Tpo 4 was a membrane-bound form of Tpo.

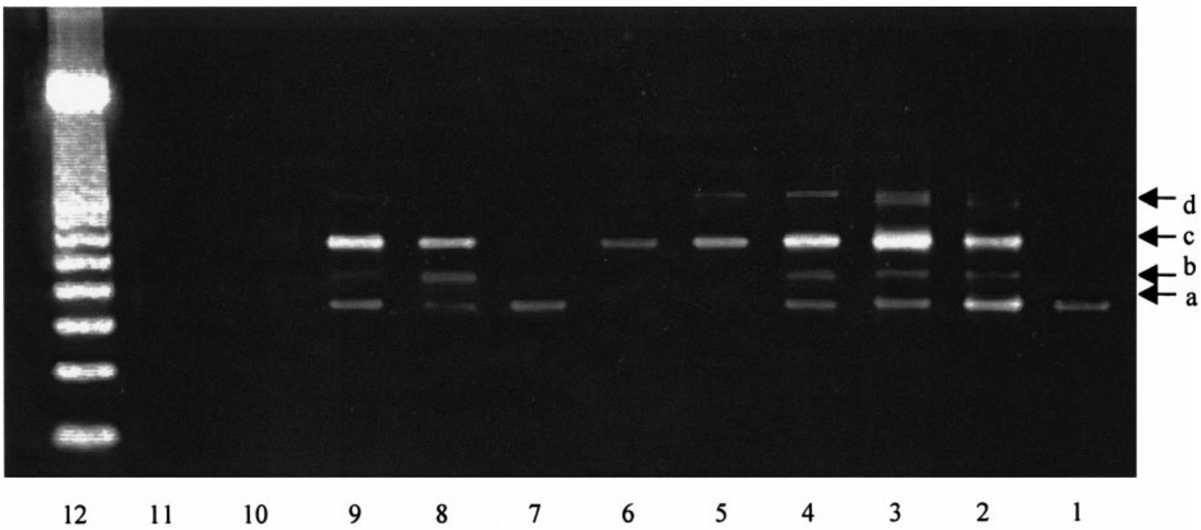

FIG. 4. Tissue distribution of Tpo isoforms. a, Tpo 4 and Tpo 8; b, Tpo 3; c, Tpo 1, 2, 5, 7, and 9; d, Tpo 6. Lanes: 1, bone marrow; 2, liver; 3, kidney; 4, heart; 5, spleen; 6, lung; 7, lymph node; 8, thymus; 9, testis; 10, blood; 11, control; and 12, size markers.

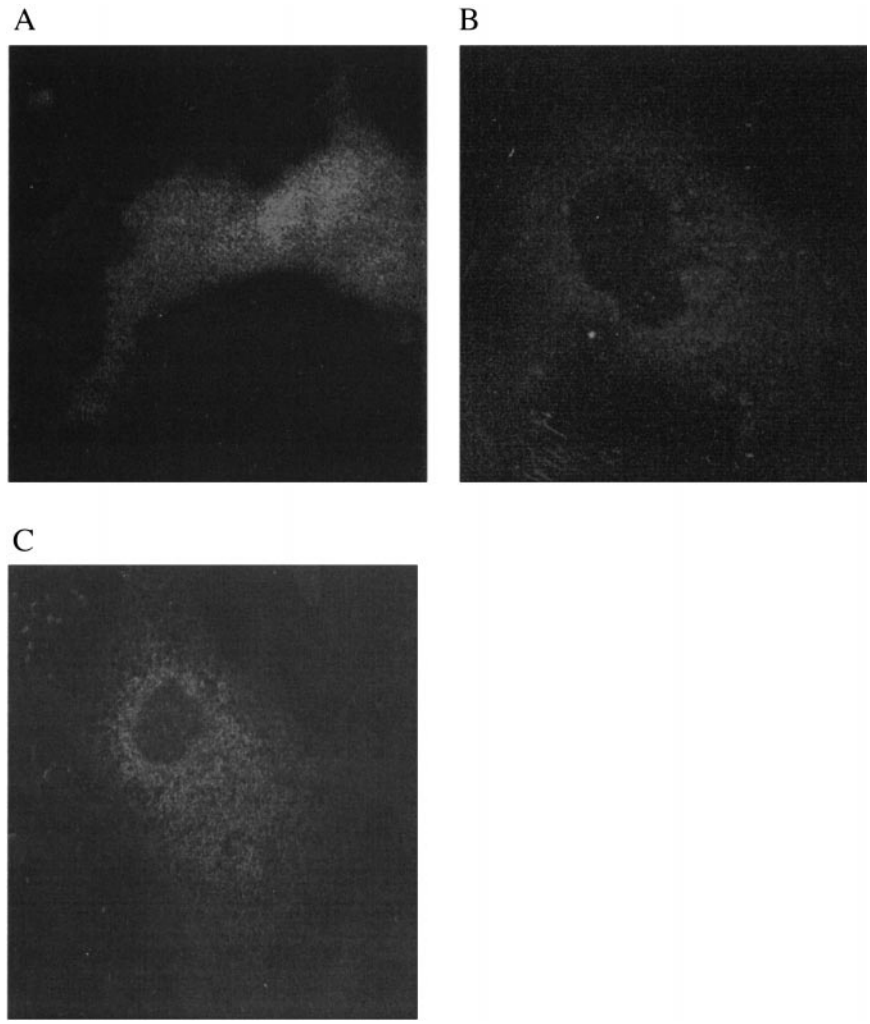

FIG. 5. Subcellular localization of Tpo isoforms. (A) Cos-7 cell transfected with GFP. (B) Cos-7 transfected with Tpo1-GFP. (C) Cos-7 cell transfected with Tpo 4-GFP.

Computer-based analysis of the Tpo 4 sequence indicated no membrane-spanning region produced by the frame shift mutation, but there is a potential N-myristylation site near the carboxy terminus which could act to anchor protein to the membrane. Therefore, we expressed Tpo 1 and Tpo 4 as fusion proteins with a GFP tag, and observed their cellular location with confocal fluorescence microscopy. As shown in Fig. 5, GFP was found uniformly distributed throughout the cell. With Tpo 1-GFP, the fluorescence was located mainly in the cytoplasm. As a secretory cytokine, Tpo 1 should be rapidly and continually secreted from the cell via the constitutive secretory pathway. The faint fluorescence seen may reflect the fact that most of the Tpo 1-GFP is efficiently secreted. The expression pattern of the Tpo 4-GFP fusion protein is similar to that of Tpo 1-GFP, with no obvious plasma membrane-related fluorescence. The fluorescence of Tpo 4-GFP in the cytoplasm was much stronger than that of Tpo 1-GFP, suggesting either a slower process of secretion, or that Tpo 4-GFP was retained in the cytoplasm after synthesis. In either case, the results do not support the likelihood that Tpo 4 is membrane-bound.

DISCUSSION

With different primer sets and temperature combinations, we consistently amplified multiple isoforms of Tpo mRNA from murine tissues. The inserted or deleted sequences of the isoforms resulted predominantly from changes around intron 6. All isoforms retain a conserved consensus sequence for RNA splicing at the junction of introns and exons, so it is very likely that these newly-identified Tpo isoforms result from alternative splicing, not from non-specific PCR products or immature pre-mRNA.

Mature Tpo consists of two domains: the first 153 amino acids of the amino terminus form the erythropoietin (Epo)-like domain which has approximately 50% similarity with Epo. The remaining 179 amino

acids form the carboxy terminal domain. This portion of the Tpo molecule has no similarity to other known growth factors or cytokines. The Epo-like domain confers the full biological activity seen with full-length Tpo *in vitro.* Recent reports (21, 22) indicate that the carboxy terminal portion of the molecule plays an important role in protein secretion.

The Tpo gene is made up of seven exons and six introns spanning eight kilobases. The last exon, exon 7, encodes the last 40 amino acids of the Epo-like domain, including the fourth cysteine which may be essential for Tpo conformation and bioactivity (4, 19). All isoforms result from alternative splicing at the junction of exon 6 and exon 7. In mouse liver, the major Tpo isoform present is Tpo 1 (47%); the other isoforms together make up the remaining 53%. Three ORFs would be required if all isoforms were translated into protein. Tpo 1, Tpo 2, and Tpo 8 would arise in ORF 1; Tpo 3, Tpo 4, Tpo 5, and Tpo 6 in ORF 2; Tpo 7 and Tpo 9 in ORF 3, the last of which (ORF 3) terminates prematurely at a stop codon in intron 6.

The physiological role(s) of alternatively spliced Tpo is unclear. Stoffel *et al.* (7) reported that the expression profile of mRNA for Tpo 1, Tpo 2, Tpo 3, and Tpo 4 remained unchanged in the liver and kidney of thrombocytopenic mice. However, the probe that they used for the RNase protection assay could not distinguish the newly identified isoforms reported here. As a result, we cannot discount the possibility that murine Tpo isoforms generated by alternative splicing may play a role in the regulation of Tpo activity.

Tpo 2, Tpo 3, and Tpo 4 have been reported previously to be efficiently expressed intracellularly *in vitro;* however, little, if any, bioactivity was detected in the medium. Hoshi *et al.* (17) showed that on a mole basis, Tpo 4 fusion protein had bioactivity comparable to Tpo 1. Recently, Ahn *et al.* (20) expressed human Tpo variants, truncated in the carboxy terminal region, in insect cells. Most of the proteins of recombinant human (h)Tpo 163 (1–163 aa), hTpo 198 (1–198 aa) and hTpo 245 (1–245 aa) remained within the cell. Our data also showed that Tpo 4-GFP gave stronger fluorescence in the cytoplasm than did Tpo 1-GFP. The Tpo 4-GFP possibly was retained in the endoplasmic reticulum and the Golgi (Fig. 4). These findings raise the possibility that the truncated Tpo variants are stored in the cytoplasm after their synthesis and, conceivably, are released upon cellular activation or stimulation. Since Tpo 4 is the major isoform in bone marrow and lymph nodes, and if this mechanism exists, it suggests a role for this Tpo isoform in the local regulation of hematopoiesis, in general, and megakaryopoiesis in particular.

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