Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 13023–13027, December 1994
Medical Sciences

Human thrombopoietin: Gene structure, cDNA sequence, expression, and chromosomal localization

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Communicated by Earl W. Davie, July 26, 1994 (received for review June 14, 1994)

ABSTRACT **Thrombopoietin (TPO), a lineage-specific cytokine affecting the proliferation and maturation of megakaryocytes from committed progenitor cells, is believed to be the major physiological regulator of circulating platelet levels. Recently we have isolated a cDNA encoding a ligand for the murine c-*mpl* protooncogene and shown it to be TPO. By employing a murine cDNA probe, we have isolated a gene encoding human TPO from a human genomic library. The TPO locus spans over 6 kb and has a structure similar to that of the erythropoietin gene (*EPO*). Southern blot analysis of human genomic DNA reveals a hybridization pattern consistent with a single gene locus. The locus was mapped by *in situ* hybridization of metaphase chromosome preparations to chromosome 3q26-27, a site where a number of chromosomal abnormalities associated with thrombocythemia in cases of acute myeloid leukemia have been mapped. A human TPO cDNA was isolated by PCR from kidney mRNA. The cDNA encodes a protein with 80% identity to previously described murine TPO and is capable of initiating a proliferative signal to murine interleukin 3-dependent BaF3 cells expressing the murine or human TPO receptor.**

The major physiological regulator of platelet levels is thought to be a humoral factor termed thrombopoietin (TPO) by Keleman *et al.* (1) in 1958. Early literature described TPO as an activity, present in plasma of thrombocytopenic animals, that regulates the number of circulating platelets by causing an increase in the number and size of the megakaryocytes in the bone marrow (2, 3). A number of laboratories have attempted the purification and characterization of TPO from thrombocytopenic plasma (4) and conditioned medium from various cell lines (5–8). Activity was assayed by several criteria including ploidization, expression of platelet-specific proteins, and platelet production *in vivo* as measured by the incorporation of radiolabeled sulfur compounds (9–12). However, the unequivocal identification of TPO was hindered by low specific activity of the starting materials and by cumbersome bioassays.

An alternative approach toward the cloning of TPO was made possible by the recent discovery of the c-*mpl* protooncogene (13–15), a member of the hematopoietin receptor family. This protooncogene was first described in a murine transducing myeloproliferative leukemia virus (MPLV) where the c-Mpl cytoplasmic signal transduction domain was expressed as a fusion with the viral envelope protein (16). Oncogenesis by the virus is believed to be due to the truncated receptor transducing a proliferative signal into infected cells independent of the presence of the receptor ligand. Several lines of evidence support the premise that c-Mpl is the receptor involved in megakaryopoiesis and that the c-Mpl ligand might be TPO. Cellular immortalization by MPLV was found to be specific to hematopoietic progenitor cells in which a large proportion of immortalized cells were megakaryocytic in nature (16). Spleen and bone cells from diseased animals were found to undergo terminal differentiation into megakaryocytic and erythrocytic colonies (16). The c-*mpl* mRNA was found to be specific to cells involved in megakaryocytopoiesis. Moreover, when antisense oligonucleotides against c-*mpl* mRNA were introduced into hematopoietic stem cells to inhibit expression of the receptor, the development of cell lineages involved in megakaryocytopoiesis was specifically inhibited (17).

We recently cloned a murine cDNA encoding a ligand for c-Mpl and have provided evidence to show that the cytokine is indeed TPO (18, 19). Recombinant murine TPO was found to increase megakaryocyte progenitor cell numbers *in vitro*, induce their differentiation, and stimulate platelet production. Intraperitoneal injection of mice with purified recombinant protein resulted in a 300% increase in circulating platelet levels. In the present report, we describe the molecular cloning and expression of a human TPO cDNA,† the structure of the human TPO gene, and the localization of the TPO locus to chromosome 3q26-27.‡

MATERIALS AND METHODS

Isolation of the Human TPO Gene. A human lung fibroblast genomic DNA library (Stratagene) was probed with a full-length mouse TPO cDNA (18) at reduced hybridization stringency. The probe was labeled by random priming using a megaprime kit (Amersham). Hybridization was carried out in 5× standard saline citrate (SSC)/0.2% SDS/1 mM EDTA at 65°C. The filters were washed in 2× SSC/0.2% SDS/1 mM EDTA at 50°C.

Isolation of Human TPO cDNA. First-strand cDNA templates for PCR were synthesized from human kidney or liver oligo(dT)-selected poly(A)$^+$ RNA (Clontech) by using SuperScript reverse transcriptase (GIBCO/BRL). First-strand cDNA templates were used to generate two TPO cDNA segments by using separate PCR reactions employing specific primers derived from human exon sequences and from conserved 5′ noncoding sequences of mouse TPO cDNA (18). The N-terminal cDNA segment containing one-third of the sequence was amplified by using the sense primer 5′-CCGGAATTCTTAGACACCTGGCCAGAATG-3′ and the antisense primer 5′-GGAAGCTGGGTACCAAGGAGGCT-3′. PCR was run for 35 cycles (1 min at 94°C, 1 min at 58°C, and 1.5 min at 72°C). The C-terminal segment containing the remaining two-thirds of the sequence was amplified by using

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Abbreviations: AML, acute myeloid leukemia; EPO, erythropoietin; TPO, thrombopoietin.

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†The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L36051 (gene) and L36052 (cDNA)].

‡The TPO locus has been assigned the symbol *THPO* in the Genome Data Base.

the sense primer 5′-AGCCTCCTTGGTACCCAGCTTCC-3′ and the antisense primer 5′-CCGGAATTCTGATGTCG-GCAGTGTCTGAGAACC-3′. PCR was run for 35 cycles (1 min at 94°C, 1 min at 65°C, and 1.5 min at 72°C). Amplification was carried out using *Taq* polymerase (Boehringer Mannheim) in the supplied buffer. After amplification, the two cDNA segments were joined via a common *Kpn* I restriction site introduced by PCR mutagenesis without alteration of the predicted amino acid sequence. The reconstructed cDNA was cloned into the mammalian expression vector pZEM229R (20) to yield the recombinant plasmid pZGTPO-124. The cloned amplified product was validated by sequencing and comparisons to the human genomic sequence.

DNA Sequencing. DNA was sequenced using AmpliTaq DyeDeoxy Terminator Cycle sequencing carried out on an Applied Biosystems model 373A sequencer (Perkin–Elmer).

Southern Blot Analysis. Genomic DNA was transferred to Hybond-N membranes (Amersham) and probed with random-primed labeled human TPO cDNA. The membranes were washed in 0.25× SSC/0.2% SDS/1 mM EDTA at 65°C and exposed for 48 hr under a phosphor plate and scanned on a Molecular Dynamics PhosphorImager.

Chromosomal Localization. Chromosomal localization of the TPO locus was carried out using a biotinylated human TPO genomic phage probe, λZGTPO-H10, and fluorescence *in situ* hybridization. Denaturation of the chromosomes, hybridization, and single-color detection were performed as described by Pinkel *et al.* (21) and Kievits *et al.* (22). Metaphase chromosome preparations were G-banded with Gurr's improved R66 Giemsa's stain (BDH), photographed, and destained in 70% absolute ethanol before being used for hybridization experiments.

Transfection. Hamster BHK 570 cells were transfected with the human TPO expression plasmid, pZGTPO-124, employing LipofectAMINE (GIBCO/BRL). Stable cell lines expressing human TPO were isolated by selection with methotrexate.

Cell Proliferation Assay. The proliferative activity of conditioned media from TPO-producing BHK cells was tested on BaF3HMPLR cells expressing the P form of the human TPO receptor (13). Cell proliferation was measured by a mitogenic assay based on the incorporation of [^{3}H]thymidine (23). The amount of activity giving rise to 50% of maximal proliferative response is defined as 10 units.

RESULTS AND DISCUSSION

Isolation of the Human TPO Gene and cDNA. A human lung fibroblast genomic DNA library was screened using the murine TPO cDNA as a hybridization probe (18). Three overlapping TPO genomic phage clones, λZGTPO-H8, λZGTPO-H10, and λZGTPO-H29, were isolated after screening $\approx 1.5 \times 10^6$ recombinant phages. Southern blot analysis of the phage inserts revealed that only the insert of λZGTPO-H10 hybridized to both 5′ and 3′ terminal murine TPO cDNA probes, suggesting that the insert may contain the entire TPO gene. A region of the genomic insert that was shown to cross-hybridize with murine TPO cDNA was sequenced. Comparison of the human genomic sequence (Fig. 1) to the murine cDNA sequence showed extensive regions of homology, including regions that correspond to the translation initiation and termination codons, indicating that all of the coding exons are present. The coding region of the TPO gene spans ≈6 kb and is organized in five coding exons, with intron/exon boundaries that precisely correspond to the intron/exon boundaries of human and murine erythropoietin genes (*EPO*) (24–26). A DNA sequence alignment of the human genomic sequence with the 5′ noncoding sequence of the murine TPO cDNA suggests that there is one additional upstream exon that codes entirely for 5′ noncoding sequence. The mouse cDNA 5′ noncoding sequence is homologous to the human genomic sequence for the first 145 nucleotides upstream of the initiation methionine codon, and then the sequences abruptly diverge, coincident with a consensus splice acceptor sequence at bp 1812–1826. Additional sequence homology can be found further upstream between the mouse 5′ noncoding sequence and human genomic sequence from bp 136 to 158, including a stretch with 22 of 24 positions identical, for 92% sequence identity. This homology terminates at a consensus splice donor sequence at positions 159–164. Finally, an *Alu*-type repeat is found at bp 1367–1647, supporting the idea that this upstream region is intronic. Taken together, these observations suggest that the 5′ noncoding region of TPO cDNA contains at least one intron. To preserve nomenclature such that corresponding TPO and *EPO* exons will have the same names, we have identified this noncoding exon as "exon 0." Since the transcription start sites have not been identified for the TPO transcript, it is not possible at this time to define the 5′ boundary of exon 0.

All of the introns in the human *EPO* and TPO gene coding regions occur in precisely the same location relative to the protein structure (Fig. 2), and in every case there is precise conservation of intron splice phase (27). These findings argue strongly that these two genes have evolved from a common ancestral sequence by gene duplication. It is notable in this respect that TPO and EPO are somewhat different in domain organization. Whereas EPO is a single-domain protein of 166 amino acids, TPO has a two-domain structure with an N-terminal domain homologous to EPO and an additional C-terminal domain rich in glycosylation sites. The biological and physiological role of the second domain of TPO is currently undefined.

Interestingly, the junction between the two TPO domains does not correspond to an intron position in the TPO gene, suggesting that the sequence encoding the TPO second domain and the *EPO* 3′ noncoding sequences might share an evolutionary origin. Our observation of nucleotide sequence similarities between these regions would support this hypothesis. While the similarity throughout the mRNAs of TPO and *EPO* are not at a level that allows unambiguous alignment, scores for alignments between the 3′ noncoding sequence of *EPO* and nucleotide sequences coding for portions of the TPO second domain as well as the TPO 3′ noncoding region are higher than alternative alignments. The observed similarity does not include the first 130 nucleotides encoding the TPO second domain, suggesting that these nucleotide sequences either represent an insertion in the TPO gene or that a similar sequence may have been deleted in *EPO* after duplication of the ancestral TPO/*EPO* gene.

To isolate a human TPO cDNA clone, we employed a PCR approach using specific primers derived from human genomic exon sequences and conserved 5′ noncoding sequences of the murine TPO cDNA (18). By using human kidney and liver cDNA templates, an amplified product corresponding to the coding region of human TPO was isolated and sequenced. The resulting human TPO cDNA encodes a polypeptide identical to that predicted from the human genomic sequence and with a similar overall size, two-domain structure, and significant sequence conservation with the murine TPO. The predicted human TPO protein is a polypeptide of 353 aa, including a 21-aa signal peptide, a 152-aa domain with homology to EPO, and a dibasic Arg-Arg pair of amino acids, followed by a 177-aa second domain. This second domain, as in murine TPO, is highly enriched in serine, threonine, and proline; contains a number of potential N-linked glycosylation sites; and bears no recognizable homology with other known protein sequences.

The signal peptidase cleavage rules of von Heijne (28) predict a cleavage site following Ser-21, leaving an N terminus for the protein that corresponds precisely to the demonstrated mature N terminus for recombinant murine TPO (18).

FIG. 1. Nucleotide sequence of the human TPO gene. Coding exon regions are translated into the amino acid sequence below. The 3′ noncoding region as well as potential 5′ noncoding regions with significant homology to the murine TPO cDNA noncoding regions are overlined. A potential splice acceptor site in the 5′ end is underlined.

The mature polypeptide has a predicted molecular mass of 35.5 kDa; however, it is anticipated that the mature protein will have a much greater molecular mass, since both the recombinant murine and human proteins have been observed to be heavily glycosylated and to be composed of as much as 50% carbohydrate (data not shown). The alignment of the human and murine proteins indicates that the mature EPO-like domain is much more highly conserved between species

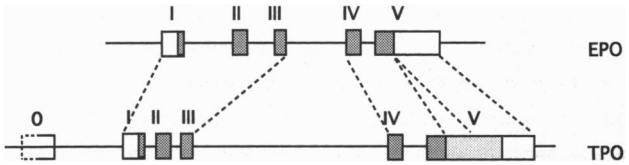

FIG. 2. Structure of the human *EPO* and TPO genes. Exon sequences are indicated by boxed regions. The exon encoding the initiation codon is numbered as exon I. Exon regions encoding the EPO-like domain are indicated with dark shading. Exon regions encoding the C-terminal glycosylation domain are indicated with light shading. Exon regions of noncoding sequence are unshaded. The proposed 5′ noncoding exon is indicated as a dashed box.

(84%; for comparison, human and murine EPO are 80% identical) than is the C-terminal glycosylated domain, which is only 60% identical. Perhaps notable is the fact that most of the sequence differences between the EPO-like domains of the human and murine sequences occur in regions surrounding predicted α-helical regions 1 and 4, which have been shown to be involved in receptor interactions in other similar cytokine structures (29). Five of the seven N-linked glycosylation sites in the murine sequence are conserved in the identical location in the human sequence, with a sixth site slightly offset from a corresponding murine site.

Chromosomal Localization of the TPO Locus. Southern blot analysis was used to determine the complexity of human genomic TPO sequences. The resulting simple hybridization pattern of *Eco*RI- and *Xba* I-digested genomic DNA, when probed with human TPO cDNA, is consistent with the presence of a single chromosomal locus (data not shown). No additional hybridizing species were evident as the washing stringency was reduced to 0.5× SSC at 50°C. Fluorescence *in situ* hybridization mapped the TPO locus to chromosome 3q26-27 (Fig. 3). Despite their common ancestry, the human *EPO* locus is located on chromosome 7 (30).

Chromosome 3q abnormalities have been demonstrated in reported cases of abnormal thrombopoiesis in acute myeloid leukemia (AML) (31, 32) and in blastic transformation of chronic myeloid leukemia (33, 34). These patients with the so-called 3q21q26 syndrome have marked thrombocythemia that is not usually associated with other types of AML. In a single case examined, elevated serum TPO activity levels were detected in the patient's serum (31). The majority of patients described above have chromosomal inversion of (3) (q21q26). The sole exception was a patient with a chromosome 3 to 9 translocation t(3;9) (q21;q34). The common chromosomal breakpoint junction of 3q21 in all patients led to the proposal that the putative TPO locus is located at 3q21 (35). The proposed 3q21 assignment, however, was weighted heavily by a single translocation event reported in one patient, and what is now known to be the correct assignment of TPO to 3q26 region was discounted.

Abnormalities at or near the 3q26 region have been implicated in a number of other leukemias. Several potential oncogenes including *EVI1* (36), a zinc finger protein whose perturbed expression may have contributed to oncogenesis, map to this region. Retroviral activation of the *EVI* locus has also been implicated in cellular transformation (36, 37). Most recently, the role of the 3q26 region in AML and in chronic myeloid leukemia in blast crisis was further indicated by translocation of chromosome 21q22 sequences to the 3q26 region (38, 39). The resulting translocation, t(3;21) (q26;q22), resulted in the production of fusion mRNAs (presumably also fusion proteins) that are initiated from the *AML1* gene located at 21q22 to several genetic loci in the 3q26 region (38–41).

Proliferative Activity of Recombinant Human TPO. The human TPO cDNA was subcloned into the mammalian expression vector pZEM229R (20) to yield the plasmid pZGTPO-124. This plasmid was stably transfected into BHK cells, and

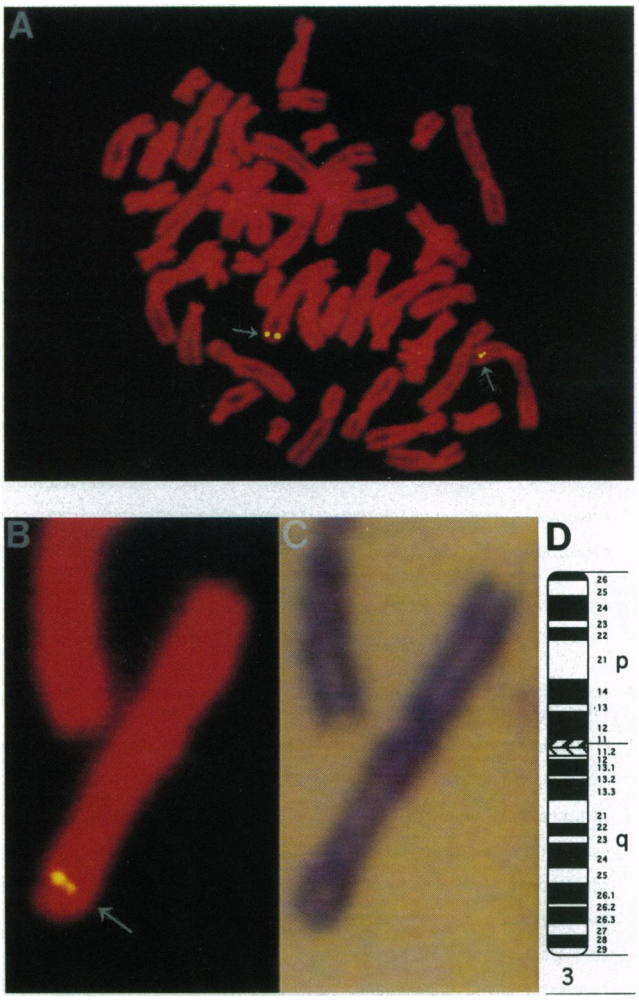

FIG. 3. Fluorescence *in situ* hybridization mapping of the TPO locus to chromosome 3q26-27. (*A*) Hybridization of a biotinylated human TPO genomic phage probe, λZGTPO1-H10, to a metaphase spread from human primary human lung cells. (*B*) Enlargement of a single labeled metaphase chromosome. (*C*) G-banding of the identical chromosome shown in *C*. (*D*) Diagram showing the G-banding pattern for a normal chromosome 3 in accordance with the International System for Human Cytogenetic Nomenclature. Arrows indicate the signal and labeling position of the TPO locus.

clonal cell lines were isolated using methotrexate selection. In previous studies we have shown that murine TPO supports the proliferation of BaF3MPLR1.1 cells, an interleukin 3-dependent murine pre-B cell line expressing a murine TPO receptor cDNA (18). To determine whether recombinant human TPO can elicit a proliferative response when bound to the human receptor, we constructed a BaF3 cell line, BaF3HMPLR, expressing the P form of the human TPO receptor cDNA (13). As shown in Fig. 4, conditioned medium from BHK cells producing human TPO supports proliferation of BaF3HMPLR cells. This result demonstrates that the recombinant protein is functional in receptor binding and signaling.

Summary. The current isolation and characterization of the human TPO gene and cDNA extend our earlier observations on murine TPO. The human sequence is highly conserved relative to the murine sequence, both structurally and functionally. Both are two-domain proteins with a structural as well as evolutionary relationship to EPO. Our earlier studies on murine TPO indicated its role as the primary physiologic regulator of platelet production, acting as both a megakaryocyte colony-stimulating factor and as a late-acting megakaryocyte maturation and platelet production factor (18, 19).

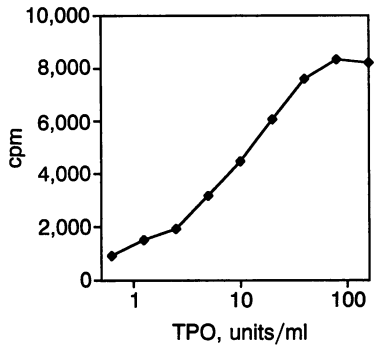

FIG. 4. Proliferative activity of human recombinant TPO. TPO activity units are arbitrarily defined; the amount giving half-maximal proliferative response is designated as 10 units/ml. The proliferation assay and incorporation of [^{3}H]thymidine is as described in *Materials and Methods*.

We have shown that murine TPO is capable of inducing >4-fold increases in circulating platelet levels in healthy mice, or ≈10-fold higher levels of induction than have been seen with stimulation by interleukins 3, 6, and 11, the c-Kit ligand, or a fusion protein called PIXY321 (42–46). The present studies with human TPO indicate a similar association with physiologic platelet regulation. Like murine TPO, the human molecule induces a strong proliferative response in cells transfected with human TPO receptor cDNA, indicating a similar coupling to the receptor. The proliferative signal transduced by the mouse receptor has clearly been shown to regulate megakaryopoiesis both *in vitro* and *in vivo* (18, 19). In addition, the mapping of the TPO locus to a chromosomal location that has been implicated in abnormal thrombopoiesis in a number of leukemias adds support to the interpretation that TPO is the primary humoral circulating regulator of platelet production in humans.

Note. After submission of this manuscript, de Sauvage *et al.* (47) reported the sequence for the human TPO cDNA including the 5′ noncoding region. Alignment of the human 5′ noncoding sequence with the present human genomic sequence supported the exact placement of the 5′ noncoding exon 0 in the human gene.

We thank M. Bernard for critical proofreading of the manuscript.

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