FEBS Letters 353 (1994) 57–61

FEBS 14621

Molecular cloning and chromosomal localization of the human thrombopoietin gene

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Received 22 August 1994

Abstract The complete gene for human thrombopoietin (TPO) has been cloned by screening a human genomic library using human TPO cDNA as a probe. This gene is 6.2 kb in length and contains six exons and five introns. It is shown that the human genome contains a single copy of the human TPO gene according to Southern blotting analysis. The transcription initiation site was determined by S1 nuclease mapping. The human TPO gene expressed TPO activity when transfected into COS-1 cells. The human TPO gene has been mapped to chromosome 3q27 by in situ hybridization using a biotin-labeled probe.

Key words: Thrombopoietin; Genomic DNA; Chromosome mapping

1. Introduction

It has long been suggested that platelet production is regulated by a lineage-specific factor(s) which has been broadly defined as a thrombopoietin (TPO) [1]. Recently, the authors [2] and others [3,4] have individually isolated the cDNA for human TPO, the ligand for the proto-oncogene c-*mpl* product. The human TPO cDNA contains an open reading frame encoding 353 amino acids including a 21 amino acid signal peptide. The human TPO protein comprises an amino-terminal domain including 4 cysteine residues and a carboxyl-terminal domain including 6 potential *N*-glycosylation sites. The truncated TPO proteins, lacking their carboxyl-terminal regions, were purified from plasma of irradiated animals and were shown to retain their in vitro activity. Biological data showed that human TPO stimulates both megakaryocyte colony formation in vitro and platelet production in vivo [2].

Cytogenetic studies have revealed that the association of several cases of acute nonlymphocytic leukemia (ANLL) and chronic myelogenous leukemia (CML) with defects of the long arm of chromosome 3 were related to abnormal thrombopoiesis [5–7]. As TPO plays an important role in thrombopoisis, there is a possibility that the abnormal thrombopoisis is related to the TPO gene. To evaluate this, we have isolated and analyzed the human TPO gene. Here we report the primary structure, the transcription initiation site, and the chromosomal localization of the human TPO gene.

2. Materials and methods

2.1. Library screening

Standard molecular biology protocols [8] were used throughout this study unless specifically indicated. Approximately 5×10^5 plaques of a human genomic library (a generous gift from Dr. Tokuo Yamamoto of the Tohoku University Gene Research Center) [9] constructed using a λEMBL3 vector were screened using the human TPO cDNA as a probe [2]. Positive clones were checked for the presence of the entire amino acid coding region of human TPO by PCR using two sets of primers. One set of primers (L: 5′-GGCCAGCCAGACACCCCGGC-C-3′; and F: 5′-ATGGGAGTCACGAAGCAGTTT-3′) was used for the detection of the 5′ end of the amino acid coding region. Another set of primers (P: 5′-TGCGTTTCCTGATGCTTGTAG-3′; and V: 5′-AACCTTACCCTTCCTGAGACA- 3′) was used for the detection of the 3′ end of the amino acid coding region.

2.2. DNA Sequencing

Multiple restriction endonuclease digests of a positive clone were analyzed by Southern blotting using human TPO cDNA as a probe. The 11-kb *Hin*dIII fragment containing the entire coding region and the neighboring 2.4-kb *Hin*dIII fragment were subcloned into pUC13 (Pharmacia) (pHGT1 and pHGT2, respectively; Fig. 1C) and both strands of insert DNAs were sequenced using an Applied Biosystems Model 373A DNA sequencer using M13 primers or specific internal primers.

2.3. Expression of the human TPO gene in COS-1 cells

The *Eco*RI-*Hin*dIII fragment of the human TPO genomic DNA was subcloned into pEF-18S [10] (pEFHGTE1; Fig.1C). This plasmid and the human TPO cDNA expression vector (pHTF1) [2] were transfected to COS-1 cells using the DEAE-dextran method [11]. TPO activity in the culture supernatants was examined using the rat colony forming unit-megakaryocyte (CFU-MK) assay [2].

2.4. Southern blot analysis

Southern blots of 8 μg genomic DNA digested with various restriction enzymes were purchased from Clontech. The membrane was hybridized with ^{32}P-labeled human TPO cDNA under highly stringent conditions. Autoradiograms were analyzed on a BAS 2000 Bio-Imageanalyzer (Fuji Film, Tokyo, Japan).

2.5. S1 nuclease mapping

An antisense DNA probe was prepared as described by Ausubel et al. [12]. A 5′-end labeled oligonucleotide (PE3: 5′-AGGGCTCCAG-GACCCAAGTGC-3′) was used to prime a denaturing double-strand plasmid pHGT1. The primer was extended with Klenow fragment and the products were digested with *Alu*I. The probe, which was purified on a denaturing gel, covered the region −2499 to −1826 (Fig. 2) and was complementary to the mRNA. A fraction of this probe (3×10^4cpm) and 20 μg of poly(A)$^+$ RNA from normal human liver

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Abbreviatons: TPO, thrombopoietin.

The nucleotide sequence data reported in this paper have been submitted to the GSDB, DDBJ, EMBL and NCBI databases with the following accession number D32046.

SSDI 0014-5793(94)01008-0

(Clontech) were mixed, heated at 80°C for 10 min, and hybridized at 60°C for 3 h. The mixture was digested with S1 nuclease as described by Sambrook et al. [8] and analyzed on a sequencing gel.

2.6. Fluorescence in situ chromosomal hybridization

The plasmid pHGT1, carrying human TPO genomic DNA, was labeled with biotin and used as a probe. Direct R-banding fluorescence in situ hybridization (FISH), which is FISH combined with replication R-bands, has been applied to metaphase cells prepared from human peripheral lymphocytes [13,14]. For the amplification of fluorescent signals, the goat anti-biotin antibody-FITC anti-goat IgG system was used [15] with slight modifications [16,17].

3. Results and discussion

3.1. Isolation of the human TPO gene

A human genomic library was screened with the human TPO cDNA as a probe. After screening 5×10^5 phages, we obtained 13 positive clones. Five of the 13 clones were observed to contain the entire amino acid coding region of the cDNA according to PCR analysis. All these 5 clones exhibited similar restriction fragment patterns (data not shown). Therefore, only one of these clones with the longest insert (designated l HGT1; Fig. 1B) was characterized. λHGT1 was analyzed by Southern blotting using human TPO cDNA as a probe, and the 11-kb *Hin*dIII fragment was identified to contain the entire coding region. This and the neighboring *Hin*dIII fragments were subcloned into pUC13 (pHGT1 and pHGT2, respectively; Fig. 1C) and the nucleotide sequence of the insert was determined. Compared to human TPO cDNA, it was shown that the human TPO gene spans approximately 6.2 kb and contains 6 exons (Fig. 1B). The sequence of the human TPO gene is shown in Fig. 2. All exon–intron junctions conformed to the GT/AG rule [18]. The exon sequence of the human TPO gene perfectly matched its cDNA sequence [4]. Recently, the authors have isolated two types of rat TPO cDNAs containing intron sequences in their coding region. The positions where these intron sequences were inserted were precisely conserved in the human gene. These correspond to introns 2 and 5. Analysis of genomic DNA of rat and/or mouse TPO should demonstrate the evolutionary conservation of TPO gene.

3.2. Expression of the human TPO gene in COS-1 cells

COS-1 cells transfected with pEFHGTE1 clone were cultured for 3 days and TPO activity in the supernatants was examined using the rat colony-forming unit megakaryocyte (CFU-MK) assay. The results showed that the pEFHGTE1 clone expressed TPO activity (Fig. 3) suggesting that the clone may contain a functional gene.

3.3. Southern blot analysis

Southern blot analysis of human genomic DNA with the human TPO cDNA as a probe is shown in Fig. 4. One strong and one faint band were detected in an *Eco*RI digest, one strong band was seen in a *Hin*dIII digest, two strong bands were seen in a *Bam*HI digest, two strong bands were seen in a *Pst*I digest, and one strong band and one faint band were seen in a *Bgl*II digest. These bands exactly coincided with those expected from the sequence of the gene, indicating that the human TPO gene is present as a single copy gene.

3.4. Transcription initiation site

The transcription initiation site was determined by S1 nuclease protection mapping with poly(A)$^+$ RNA from normal human liver. We obtained only one signal (Fig. 5) which was assigned to the cytosine residue at the position −1949 in Fig. 2. This position was thought to be a transcription initiation site.

About 1.4-kb sequence upstream of the transcription start site were determined. In this region, no common motifs for mammalian promoters such as TATA-, GC-, and CAAT-boxes appear to be present. Further sequence analysis and promoter characterizations are needed to define the transcriptional regulation of the TPO gene.

3.5. Chromosomal localization

To localize the TPO gene, fluorescence in situ hybridization of a biotin-labeled TPO probe to normal human metaphase chromosomes was carried out. Hybridization of the TPO gene probe resulted in specific labeling only on chromosome 3, as shown in Fig. 6. A total of 100 typical R-banded metaphase spreads for human chromosomes was examined. As a result, 18% exhibited complete twin spots on both homologs and 46% had incomplete single and/or twin spots on either and both homologs. No twin spots were observed on other chromosomes. These fluorescent signals were localized to the R-positive q27 band of chromosome 3 (Fig. 6). Thus, the human TPO gene was assigned to band 3q27.

It has been reported that several cases of acute nonlympho-

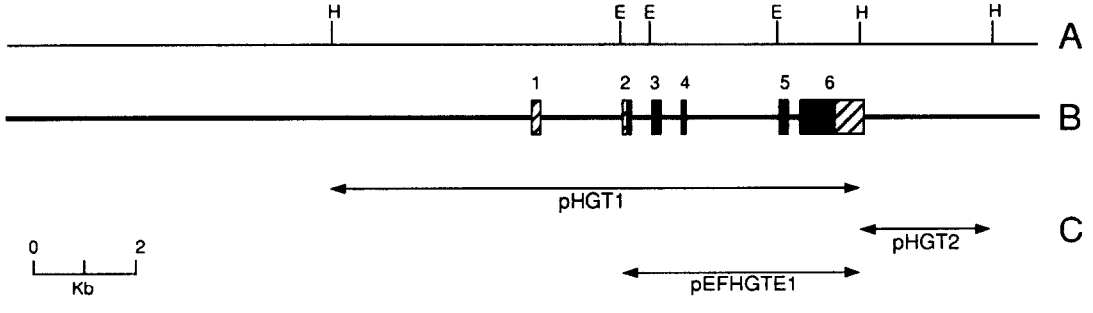

Fig. 1. Structural organization of the human TPO gene. (A) *Hin*dIII (H) and *Eco*RI (E) restriction sites are indicated. (B) The structure of l HGT1 containing all exons of the human TPO gene is shown. Exons are represented by boxes (solid for coding, hatched for 5′ and 3′ untranslated regions) and are numbered in order. (C) Subclones of the human TPO gene are indicated. The 11-kb *Hin*dIII fragment and the 2.4-kb *Hin*dIII fragment are subcloned to pUC13 and sequenced (pHGT1 and pHGT2). The 4.5-kb *Eco*RI-*Hin*dIII fragment is subcloned to pEF-18S and expressed in COS-1 cells (pEFHGTE1).

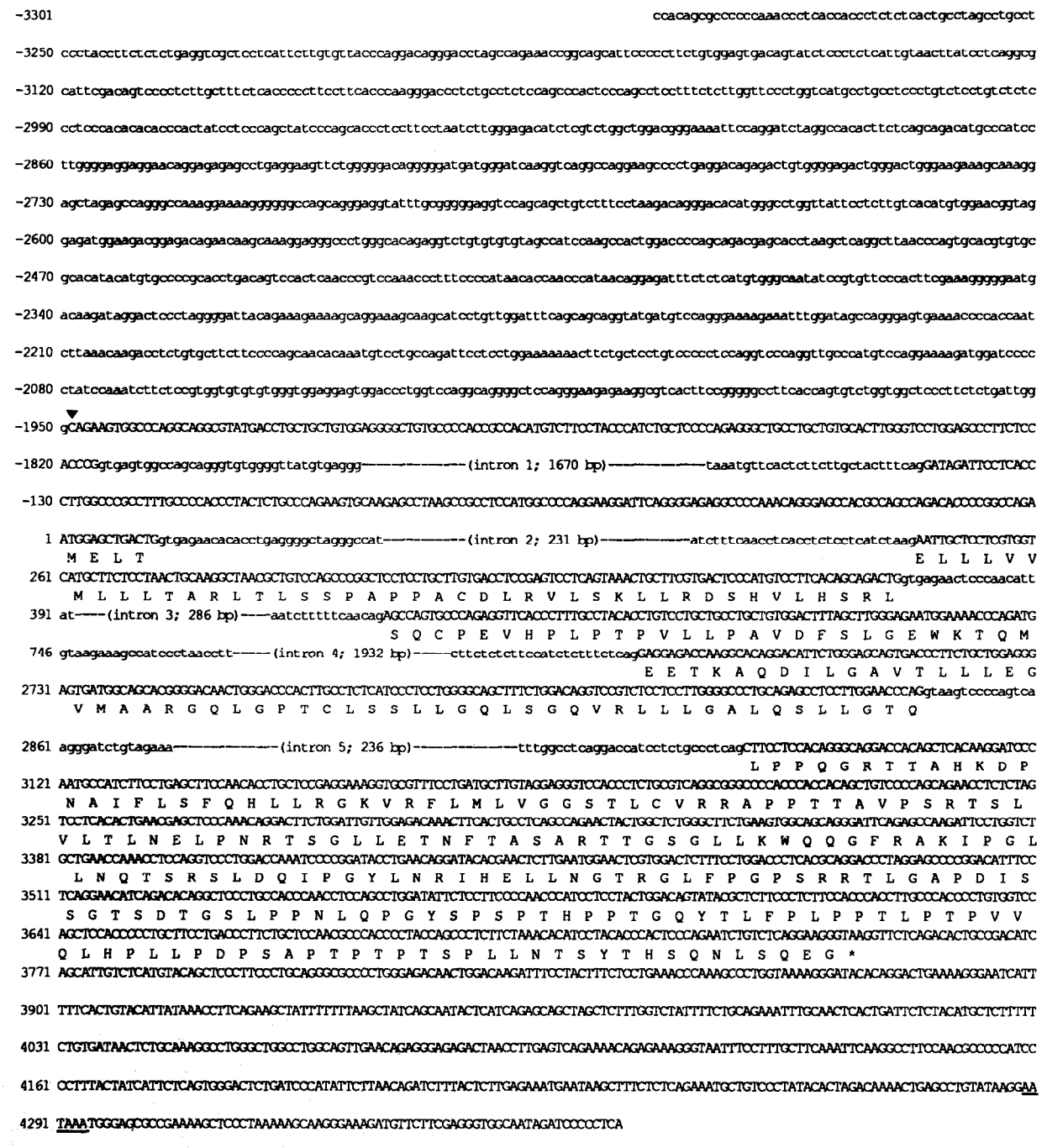

Fig. 2. Nucleotide sequences of the human TPO gene. Nucleotide 1 corresponds to A in the initiator ATG codon. Exon sequences are in uppercase letters; intron and flanking sequences are in lowercase letters. The amino acid sequence is shown under the nucleotide sequence. A stop codon is indicated by an asterisk. The putative transcription start site is indicated by an inverted triangle. The consensus polyadenylation signal is underlined. Intron sizes are indicated in parentheses.

cytic leukemia (ANLL) and chronic myelogenous leukemia (CML) with defects of the long arm of chromosome 3 were related to abnormal thrombopoiesis [5–7]. In some patients, elevated serum thrombopoietin level was associated with the inversion of chromosome 3 (q21q26) [19]. In other cases, the duplication of chromosome 3 from bands q24→q26 may be linked with megakaryocytic thrombocytopenia [20]. These data suggest that chromosomal region 3 (q21q26) contains the locus of a gene(s) regulating and/or affecting thrombopoiesis.

In the present paper, We have assigned the human TPO gene to chromosome 3q27, which is just adjacent to the chromosome breakpoint, 3q26. Our data suggest that the locus 3q26 may be related to the TPO gene in the regulation of its expression. Further analysis of these loci will clearly explain the relationship between TPO expression and abnormal thrombopoiesis.

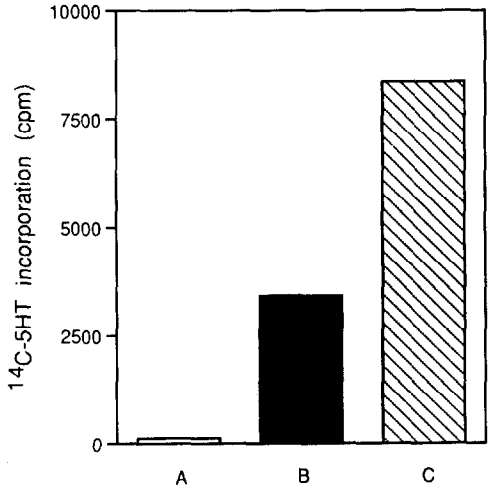

Fig. 3. TPO activity of the human TPO gene. 1:10 dilution of culture supernatants from COS-1 cells transfected with (A) Mock (pEF-18S alone), (B) pEFHGTE1 and (C) pHTF1 were assayed using the rat colony-forming unit megakaryocyte (CFU-MK) assay. The assay was carried out in duplicate and the means are shown. Each deviation was less than 5%.

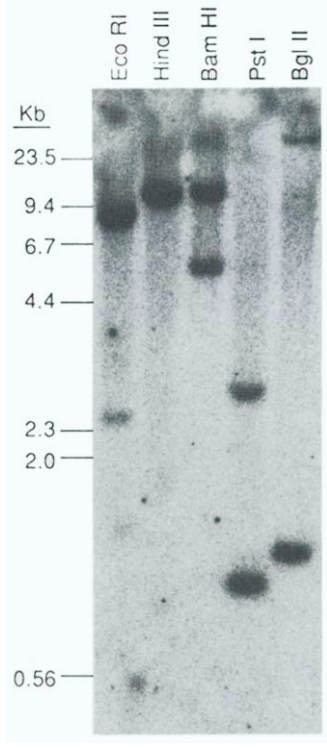

Fig. 4. Southern blot analysis of human genomic DNA. The restriction endonucleases used for digestion are indicated and the molecular sizes of DNA standards are shown to the left.

Acknowledgements: We are grateful to Dr. Tokuo Yamamoto for the gift of a human genomic library and for helpful technical advice. We also thank Drs. Akihiro Shimosaka and Tadashi Sudo for their support.

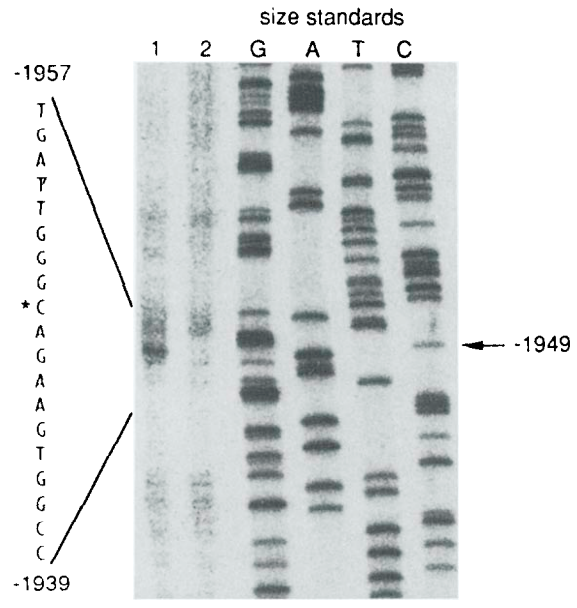

Fig. 5. Determination of the transcription initiation site of the human TPO gene by S1 nuclease mapping. The S1 probe (see section 2) was annealed with poly(A)$^+$ RNA from normal human liver (lane 1) or yeast tRNA as a negative control (lane 2). The S1 nuclease protected fragment was compared with an adjacent sequence ladder obtained with the same primer and template plasmid used to generate the probe. Numbers on the right denote the estimated position corresponding to the 5′-end of the protected fragment, according to the numbering scheme in Fig. 2. The sequence in the −1939 to −1957 region is shown on the left. An asterisk indicates the site of transcription as determined by S1 mapping.

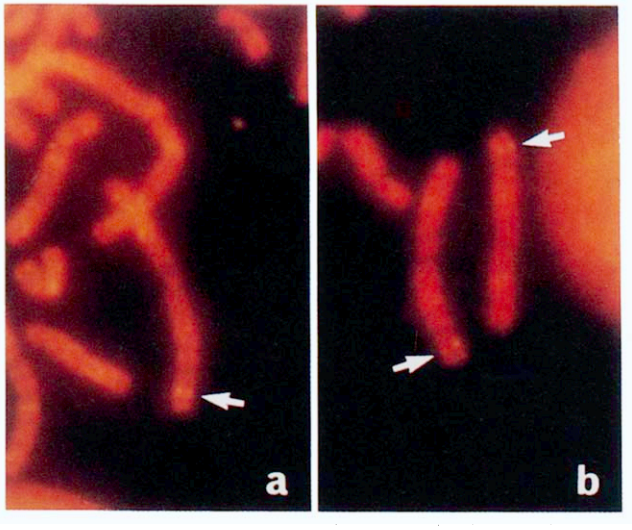

Fig. 6. Partial R-banded metaphases after fluorescence in situ hybridization with the biotinylated TPO gene. Arrows indicate twin spots of hybridization signals on single chromosome 3 (a) and on both homologs (b).

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