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Expression of the EPO-like Domains of Human Thrombopoietin in *Escherichia coli*

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Abstract cDNA of human thrombopoietin (hTPO) amplified by polymerase chain reaction from a cDNA library of human fetal liver was cloned. EPO-like domains ($hTPO_{153}$ or $hTPO_{163}$) of hTPO ($hTPO_{332}$) were expressed in *Escherichia coli* using several kinds of expression systems, such as *ompA* secretion, thioredoxin fusion, and the P_L and T7 expression systems. To obtain $hTPO_{153}$ in soluble form, $hTPO_{153}$ cDNA was fused in-frame behind the gene encoding *ompA* signal sequence and thioredoxin protein. When fused with either of the genes, $hTPO_{153}$ was not expressed to the detectable level. However, a high level expression of the EPO-like domain of hTPO was obtained using the P_L and T7 expression system. $hTPO_{153}$ and $hTPO_{163}$ cDNA were subcloned into the pLex and pET-28a(+) vectors under the control of the inducible P_L and T_7 promoter, respectively. Proteins expressed using pLex vector and pET-28a(+) detected in insoluble forms with an expression level of about 14% and 9% of total cellular proteins, respectively, and the level of expression was rapidly diminished in 2 h after the maximum level of expression was reached.

Key words: Human thrombopoietin, expression, *E. coli*, EPO-like domain

Human thrombopoietin (hTPO), also referred to as the c-mpl ligand or megakaryocyte growth and development factor (MGDF), is the primary hematopoietic growth factor which stimulates the development of megakaryocyte precursors of platelets, leading to an increase in the number of circulating platelets [1-4]. The proposed clinical indication of hTPO is a therapeutic agent for treating thrombocytopenia appeared in patients undergoing high dose chemotherapy for malignant tumors and bone marrow transplantation [8].

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The human TPO cDNA has been recently cloned and sequenced by several groups [1, 2, 4]. hTPO cDNA contains an open reading frame encoding 353 amino acids including a putative signal sequence of 21 amino acids. Mature $hTPO_{332}$ has a novel two-domain structure with an amino terminal domain (amino acids 1 to 153) homologous to erythropoietin (EPO) and a carboxy-terminal domain, which contains six potential N-linked glycosylation sites [8]. The encoded full-length mature hTPO has a predicted molecular weight of 35 kDa. It is predicted that the EPO-like domain of the hTPO would adopt a four-alpha-helical structure similar to that proposed for hematopoietic cytokines [2, 3].

A recombinant form of $hTPO_{153}$ truncated at the carboxy-domain of full-length hTPO, was expressed in mammalian cells and appeared to be fully bioactive compared with full-length hTPO [3, 8]. Although expression of hTPO has been achieved in mammalian cells, there has been no report of extensive expression studies of the EPO-like domain of hTPO using *E. coli* expression vectors [12]. Since the expression level of EPO-like domain of hTPO in mammalian cells was very low, we chose *E. coli* expression systems to express the hTPO in large quantities. The large production of the EPO-like domain of hTPO allows further characterization of the structural, functional, and biochemical properties of the hTPO. In this study, we examined the usefulness of five *E. coli* expression vectors for the high level expression of the EPO-like domain of hTPO. The direct expression of $hTPO_{153}$ and $hTPO_{163}$ using pLex and pET-28a(+) represent the production of about 10~14% of total cellular proteins in *E. coli*.

MATERIALS AND METHODS

Materials

Oligonucleotide primers for cloning, sequencing, and mutagenesis were synthesized by Bioneer Corp., Korea.

All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs. Expression vectors, pLex and pTrxFus, were purchased from Invitrogen (San Diego, U.S.A.), pET-28a(+) from Novagen (Madison, U.S.A.), pBluescriptIISK(+) from Stratagene (Cambridge, U.K.), and pTED was constructed from pTE105 [14]. Anti-human TPO polyclonal antibody was purchased from R&D Systems (McKinley, U.S.A.).

Molecular Cloning and Nucleotide Sequencing

The entire coding sequence for hTPO was amplified from the human fetal liver cDNA library (Clontech, Palo Alto, U.S.A.) by the polymerase chain reaction (PCR) using oligonucleotide primers specific for the 5' (1.1S, Table 1) and 3' ends (1.1AS, Table 1) of the hTPO amino acid sequence [2]. PCR amplification for cloning and subcloning of hTPO cDNA was carried out in 30 sequential cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min. Amplified TPO cDNA was digested with *Xba*I and *Hin*dIII and subcloned into the pBluescriptIISK(+). DNA sequencing was carried out by the dideoxy chain termination method using Sequenase version 2.0 as recommended by the manufacturer (USB, Cleveland, U.S.A.). The resulting recombinant plasmid, pBlue404, contains full-length $hTPO_{353}$ cDNA containing a 21 amino acid signal polypeptide.

Construction of *E. coli* Expression Vectors and Expression of EPO-like Domains of hTPO

cDNA encoding the EPO-like domain of hTPO was amplified by PCR using pBlue404 as a template and subcloned into three kinds of *E. coli* expression vectors; pTED containing *ompA* signal sequence originating from pTE105, a secretion vector in which the expressed protein is secreted into the bacterial periplasmic space or the medium; second, pTrxFus, a thioredoxin fusion vector; Third, pLex vector in which protein is expressed under the control of P_L promoter; Fourth, pET-28a(+), a polyhistidine fusion vector which also directs intracellular protein expression. The construction of the resulting recombinant plasmids were confirmed by restriction mapping and by nucleotide sequencing.

Construction of OmpA secretion vector and expression of $hTPO_{153}$. A DNA fragment encoding $hTPO_{153}$, the EPO-like domain of hTPO, was amplified by the polymerase chain reaction (PCR) using 5'-end primer (1.2S, Table 1) and 3'-end primer (1.21AS, Table 1) containing a *Dsa*I site and a *Nsi*I site, respectively. hTPO cDNA coding mature $hTPO_{332}$ was also amplified using primers 1.2S and 1.2AS (Table 1). The amplified DNA fragments were subcloned into the *Dsa*I and *Pst*I sites of pTED. The resulting plasmids, pTE404NF and pTE404NH, contain the $hTPO_{332}$ and $hTPO_{153}$ cDNA, respectively fused in-frame behind the *ompA* signal sequence (Fig. 1). *E. coli* JM101 was transformed with the ligation mixtures and positive clones were selected. pTE404NF and pTE404NH transformants were grown in modified LB medium (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) containing tetracycline (12.5 μg/ml). When the cell density reached $OD_{595} = 0.7$, isopropyl-β-thio-D-galactoside (IPTG) was

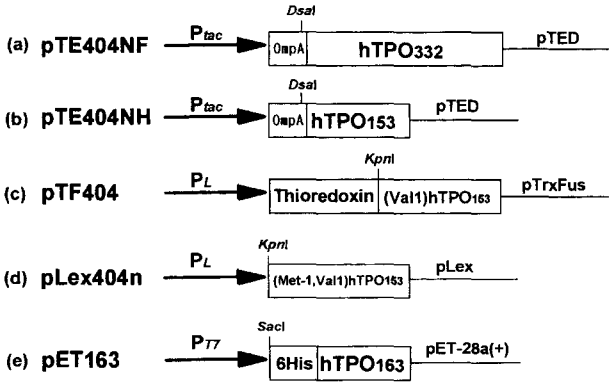

Fig. 1. Schematic representation of expression plasmids.

(a, b) Construction of OmpA secretion vectors, pTE404NF and pTE404NH. Native signal sequence of the full-length $hTPO_{332}$ gene and the EPO-like domain of the $hTPO_{153}$ gene were replaced by the *E. coli* OmpA signal sequence. White box (OmpA) indicates the OmpA signal sequence. (c) Construction of thioredoxin fusion vector, pTF404. pTF404 includes $[Val^1]hTPO_{153}$ cDNA fused with thioredoxin, the fusion partner in pTrxFus. (d, e) Construction of direct expression vectors, pLex404n and pET163. $[Met^{-1}, Val^1]hTPO_{153}$ cDNA was cloned into pLex404n under the control of P_L promoter. $hTPO_{163}$ cDNA was fused behind the gene coding six histidines and enterokinase recognition site in pET-28a(+) under the control of T_7 promoter.

Table 1. Nucleotide sequences of primers used for cloning and expression of hTPO cDNA in *E. coli* expression vectors.

Primer	Orientation	Sequence	Restriction site
1.1S	sense	5'-GGTCTAGAATGGAGCTGAATGC-3'	*Xba*I
1.1AS	antisense	5'-TTAAGCTTATCACCCTTCCTGAGACAGATT-3'	*Hin*dIII
1.2S	sense	5'-TCCGTGGCTCAAGCTAGCCCGGCTCCTCCTGCTT-3'	*Dsa*I
1.2AS	antisense	5'-CAATGCATCACCCTTCCTGAGACAGATTCT-3'	*Nsi*I
1.21AS	antisense	5'-CAATGCATCACCTGACGCAGAGGGTGGAC-3'	*Nsi*I
1.3S	sense	5'-GGTACCGGCTCCTCCTGCTTGTGACCTCC-3'	*Kpn*I
1.4S	sense	5'-ATCGAGCTCAGCCCGGCTCCTC-3'	*Sac*I
1.4AS	antisense	5'-ATCCCAAGCTTATTAGCTGGGGACAGCTGT-3'	*Hin*dIII

added to a final concentration of 1 mM. After incubation at 37°C, the induced cultures were removed by 1 ml at time-course intervals and collected by centrifugation for 1 min. The cell pellet was resuspended with 500 μl of ice-cold phosphate-buffered saline (PBS, 0.01 M NaH_2PO_4, 0.15 M NaCl, pH 7.4). After sonication, the cell suspension was frozen in a dry ice/ethanol bath and thawed at 37°C. After two more rapid sonication-freeze/thaw cycles, the samples were centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was collected and the pellet was resuspended with 100 μl of PBS.

Construction of pTF404n and expression of thioredoxin-[Val^1]hTPO$_{153}$ fusion protein. In order to express hTPO$_{153}$ as a thioredoxin-hTPO$_{153}$ fusion protein, pTrxFus (3,585 bp), a thioredoxin fusion vector, was used. To fuse hTPO$_{153}$ protein in-frame behind the thioredoxin, [Val^1]hTPO$_{153}$ cDNA was generated by amplification using 5'-end primer containing a *Kpn*I site (1.3S, Table 1) and 3' end primer containing an *Nsi*I site (1.21AS, Table 1). The PCR-generated fragment was cloned into the *Kpn*I and *Pst*I sites of pTrxFus (Fig. 1). *E. coli* GI724 was transformed with the ligation mixtures and positive clones were selected. The resulting plasmid containing the sequence for thioredoxin-[Val^1]hTPO$_{153}$ fusion protein was named pTF404 (the size of 4,020 bp). *E. coli* GI724 cells harboring pTrx vector including the thioredoxin gene only and pTF404 were grown at 30°C for overnight in RM medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 20 g casamino acids, 0.095 g $MgCl_2$ per liter) with 100 μg/ml of ampicillin. The culture was inoculated with 5% of a final concentration into an induction medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 g casamino acids, 0.095 g $MgCl_2$ per liter). When the cell density reached OD_{600} = 0.5, tryptophan was added to a final concentration of 100 μg/ml. After additional 4 h of growth, 1 ml of culture was harvested by centrifugation and resuspended with 500 μl PBS (pH 7.4). The cell pellets were sonicated and the lysates were frozen in a dry ice/ethanol bath and thawed at 37°C. After two more rapid sonication-freeze/thaw cycles, the samples were centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was collected and the pellet was resuspended with 100 μl of PBS (pH 7.2).

Construction of pLex404n and expression of [Met^{-1}, Val^1]hTPO$_{153}$. pLex was digested with *Kpn*I and *Pst*I. hTPO cDNA coding [Val^1]hTPO$_{153}$ was obtained by amplification using 5'-end primer containing a *Kpn*I site (1.3S, Table 1) and 3'-end primer containing an *Nsi*I site (1.21AS, Table 1). The *Kpn*I/*Nsi*I-digested hTPO cDNA was cloned into the *Kpn*I-*Pst*I site of pLex, and the resulting recombinant plasmid was named pLex404n. *E. coli* GI724 containing pLex404n was grown in an induction medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, 0.23 g $MgCl_2$ $6H_2O$, 5 g glucose, 2 g casamino acids per liter) to OD_{595} of 0.5 at which tryptophan was added to a final concentration of 100 μg/ml. After additional incubation at 37°C, the induced culture was removed at time-course intervals and collected by centrifugation at 12,000 rpm for 3 min. The supernatant was aspirated and the pellet was resuspended in PBS (pH 7.4) and adjusted to OD_{595} of 5.0. The cell pellet was sonicated for 20 sec at 10 cycles and centrifuged at 15,000 rpm at 4°C for 20 min. The supernatant was harvested and the pellet was resuspended with 100 μl of deionized distilled water.

Construction of pET163 and expression of (His)$_6$-hTPO$_{163}$. pET-28a(+) containing six histidine residues and thrombin recognition site at the N-terminal region of the fusion product was digested with *Sac*I and *Hin*dIII. A cDNA fragment coding mature hTPO$_{163}$ was amplified by using 5'-end primer including a *Sac*I site (1.4S, Table 1) and 3'-end primer including a *Hin*dIII site (1.4AS, Table 1). The PCR-generated hTPO cDNA fragment was cloned into the corresponding site of pET-28a(+) and the resulting recombinant plasmid was named pET163. pET163 has 38 additional amino acids in the N-terminal region of hTPO$_{163}$. *E. coli* BL21(DE3) harboring pET163 plasmid was grown in LB medium containing 30 μg/ml kanamycin to OD_{595} of 0.5 at 37°C, at which time IPTG was added to a final concentration of 1 mM. After an additional 6 h of growth, cells were harvested by centrifugation. Protein samples used for SDS-polyacrylamide gel electrophoresis and immunoblot analysis were prepared as described in an experiment performed with pLex404n vector.

Immunoblot Analysis and ELISA

The protein samples were resolved on a gradient (10~20%) or 12% SDS-PAGE (polyacrylamide gel electrophoresis) (NOVEX, San Diego, U.S.A.) according to the manufacturer's instruction. Samples run in parallel positions were either stained with Coomassie Brilliant Blue or blotted onto nitrocellulose membrane. The protein bands were scanned with a densitometer. After proteins were separated on SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose membrane. Blots were blocked for 1 h in TBS buffer (Trizma base 2.4 g/l, NaCl 29.2 g/l, pH 7.5) containing 5% skim milk and incubated with goat anti-hTPO polyclonal antibody (1:1,000 dilution) for 18 h. After washing the unbound primary antibodies with TBS buffer three times for 3 min each, the blot was treated with alkaline phosphatase-conjugated rabbit anti-goat IgG (1:10,000 dilution) and developed with BCIP/NBT substrate solution (Sigma, Saint Louis, U.S.A.). Quantitation of hTPO was performed by antigen-capture ELISA. The purified recombinant human TPO (R&D Systems, McKinley, U.S.A.) was used to generate a standard curve. The standard and protein samples were serially diluted

and added to each well of 96-well plate and then incubated at 4°C for 18 h. After blocking the plate with 5% BSA in PBS buffer, 100 μl of goat anti-human polyclonal antibody (1:1,000 dilution, R&D Systems, McKinley, U.S.A.) were added and incubated at 37°C for 2 h. Finally, after washing the plate with PBS buffer, bound IgG was detected by alkaline phosphatase-conjugated goat anti human IgG (1:10,000 dilution, Sigma, U.S.A.) and then 100 μl of *p*-nitrophenyl phosphate substrate solution (Sigma, Saint Louis, U.S.A.) was added and incubated for 30 min. The optical density was measured at 405 nm on a microplate reader.

N-Terminal Amino Acid Sequencing

Amino acid sequence determination was performed with a model 491 protein sequencer (Applied Biosystems, Foster City, U.S.A.) equipped with a miniaturized sample cartridge and phenylthiohydantoinyl amino acid analysis. After blotting of sample proteins on a polyvinylidene difluoride (PVDF) membrane, and the protein band corresponding to hTPO was excised and loaded directly onto a sample. The phenylthiohydantoinyl amino acid analysis was performed with a microliquid chromatographic system (Model 120, Applied Biosystems, Foster, U.S.A.) using dual syringe pumps and C18 narrow bore columns (2.1×250 mm) with optimized elution conditions as recommended by the manufacturer.

RESULTS AND DISCUSSION

Cloning and Expression of hTPO in OmpA Secretion Systems

cDNA coding the full length of hTPO was amplified by PCR from the human fetal liver cDNA library and cloned. The nucleotide sequence was identical with that of the previously reported hTPO cDNA [1-2] which contains an open reading frame encoding 353 amino acids including a 21 amino acid signal sequence. In order to produce hTPO in *E. coli* as a soluble form, the native signal sequence of human TPO was replaced by a signal sequence of *E. coli* outer membrane protein, OmpA. The advantage of using the *ompA* system is that an expressed protein could have its native N-terminal amino acid sequence by signal peptide cleavage [5, 12, 16, 15]. The coding sequence of the hTPO cDNA was fused with the signal sequence of OmpA protein in the secretion vector, pTED. The *ompA* secretion vector, pTE404NF, including, cDNA encoding $hTPO_{332}$, and pTE404NH including cDNA encoding $hTPO_{153}$ was constructed as diagramed in Figs. 1a and 1b.

E. coli JM101 cells harboring pTE404NF or pTE404NH were cultured and expressed as shown in Material and Methods. To determine whether the protein is soluble or insoluble, cells resuspended in the lysis buffer were broken by an ultrasonicator and fractionated into a soluble supernatant and insoluble pellet. The proteins were separated on SDS-PAGE, and subjected to immunoblot. hTPO-specific protein band was not observed by SDS-PAGE and immunoblot analysis of *E. coli* JM101 cultures containing the $hTPO_{153}$ construct, pTE404NH (Fig. 2B). However, in the case of immunoblot analysis of *E. coli* JM101 cultures harboring the $hTPO_{332}$ construct, pTE404NF, the hTPO specific band with the size of about 35 kDa in the pellet fraction only was detected (Fig. 2Ab, lanes 7-10). However, no specific hTPO band

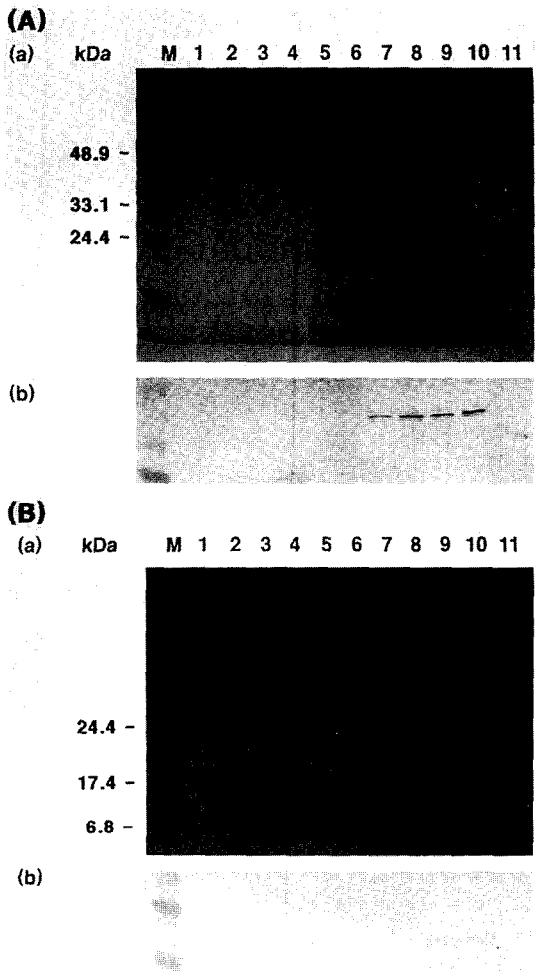

Fig. 2. SDS-PAGE (a) and immunoblot (b) analysis of *ompA*-$hTPO_{332}$ (A) and *ompA*-$hTPO_{153}$ (B).

pTE404NF plasmid including $hTPO_{332}$ gene (panel (A)) and pTE404NH including $hTPO_{153}$ gene (panel (B)) were introduced into *E. coli* JM101. They were expressed in the presence of 1 mM IPTG. The soluble (supernant) fractions and insoluble (pellet) fractions of the sonicated cells as well as culture media were analyzed as described in Materials and Methods. Lane M, prestained moleculalr weight markers (Bio-Rad); lanes 1-5, supernant fractions after 0, 2, 4, 6 and 22 h induction; lanes 6-10, pellet fractions after 0, 2, 4, 6 and 22 h induction; lane 11, culture medium after 6 h induction.

appeared in the SDS-PAGE. All of the full-length $hTPO_{332}$ was produced in insoluble forms (Fig. 2A, lanes 7-10, pellet fractions of lysates).

Expression of Thioredoxin-[Val^1]$hTPO_{153}$ in Thioredoxin Fusion System

In an attempt to achieve a high level expression of hTPO in a soluble form and to overcome a low level expression of hTPO in the *ompA* secretion system, hTPO cDNA coding [Val^1]$hTPO_{153}$ was expressed as a fusion protein with a thioredoxin fusion partner in *E. coli* GI724. The *E. coli* thioredoxin (Trx) fusion system circumvented the inclusion body formation [13] in the *E. coli* cytoplasm and dramatically increased the solubility of the fusion protein [7, 10].

pTF404 containing the thioredoxin-[Val^1]$hTPO_{153}$ gene was constructed as described in Materials and Methods (Fig. 1c). When thioredoxin-[Val^1]$hTPO_{153}$ fusion protein was expressed in *E. coli* GI724 containing pTF404, there was no obvious difference in band pattern between induced and uninduced cultures in the SDS-PAGE after staining with Coomassie Brilliant Blue (Fig. 3A). However, immunoblot analysis of the supernatant fraction in cell lysates with anti-hTPO polyclonal antibody did identify bands of 33 kDa indicating expression of thioredoxin-[Val^1]$hTPO_{153}$ by this construct, but at a very low level (Fig. 3B). The amount of thioredoxin-[Val^1]$hTPO_{153}$ fusion protein determined by ELISA was 0.08 mg/l.

Attempts to optimize expression by changing the temperature of incubation, or using protease deficient *E. coli* host strains in *ompA* secretion and thioredoxin fusion systems, failed to increase the amount of protein produced, as judged by SDS-PAGE. These results indicate that the *E. coli* secretion system using OmpA protein signal sequence and thioredoxin fusion system was not appropriate to produce the EPO-like domain to high level in a soluble form.

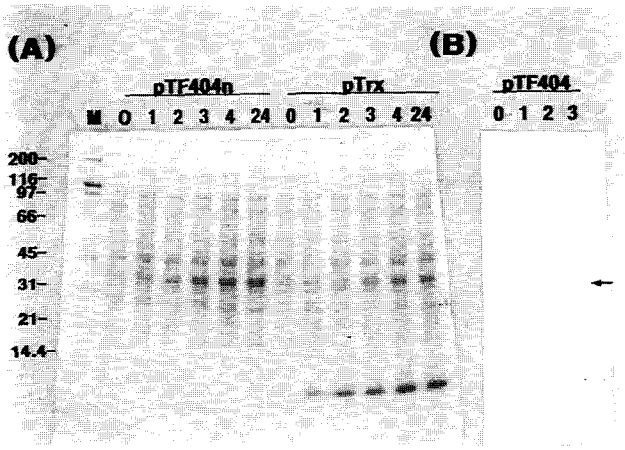

Fig. 3. SDS-PAGE and immunoblot analysis of thioredoxin-[Val^1]$hTPO_{153}$ fusion protein.

pTF404 plasmid including the thioredoxin-[Val^1]$hTPO_{153}$ fusion gene and pTrx plasmid including the thioredoxin gene only were introduced into the *E. coli* GI724. After 0, 1, 2, 3, 4, and 24 h inductions in the presence of tryptophan, 10 μl of the supernant fraction of sonicated cells were analyzed on SDS-PAGE (panel A) and immunoblot analysis (panel B). Lane M indicates prestained molecular weight markers (Bio-Rad). About 33 kDa of thioredoxin-[Val^1]$hTPO_{153}$ fusion protein (arrow) was detected in the immunoblot analysis.

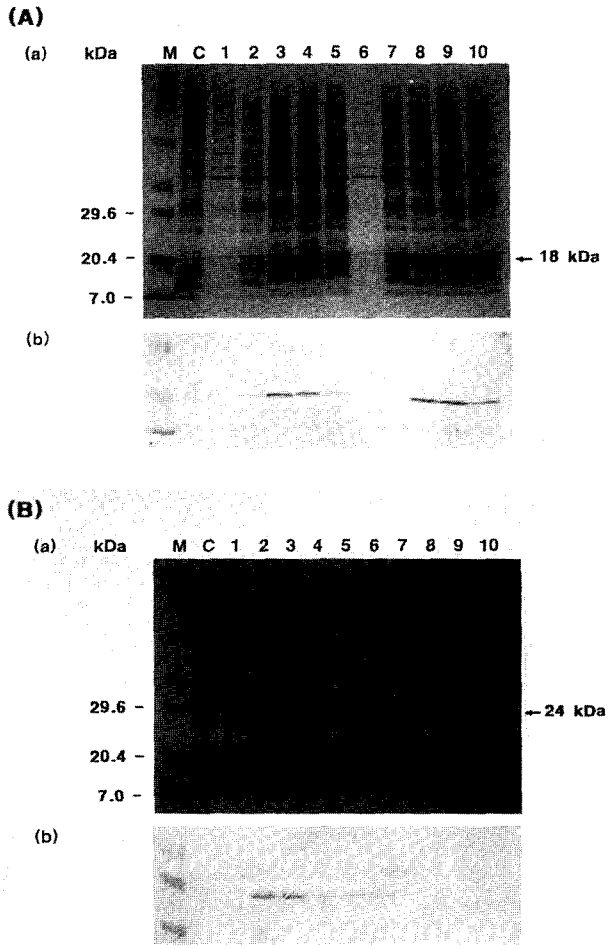

Fig. 4. SDS-PAGE (a) and immunoblot analysis (b) of [Met^{-1}, Val^1]$hTPO_{153}$ produced in *E. coli* GI724 carrying pLex404n plasmid (A), and polyhistidine-tagged $hTPO_{163}$ produced in *E. coli* BL21(DE3) carrying pET163 plasmid (B). Lane M, prestained molecular weight markers (Bio-Rad); lane C, β-galactosidase (116-kDa) expressed in control plasmid pLex*lacZ* at 6 h post-induction.

The arrow indicates the presence of β-galactosidase; (A) lanes 1-10, pellet (insoluble) fractions of sonicated *E. coli* GI724 cells harboring pLex404n. The cells were cultured in induction medium (Invitrogen) including 0.2% casamino acid (lanes 1-5) and 2% casamino acid (lanes 6-10) and harvested after 0, 2, 4, 6, and 22 h induction with tryptophan, respectively. The arrow indicates an 18-kDa $hTPO_{153}$ protein; (B) lanes 1-10, pellet (insoluble) fractions of sonicated *E. coli* BL21(DE3) cells including pET163 plasmid. The cells were cultured in LB medium and harvested after 0, 1, 2, 3, 4, 6, 8, 10, 12, and 22 h (lanes 1-10) induction in the presence of 1 mM IPTG. A 24 kDa of putative polyhistidine-tagged $hTPO_{163}$ was detected.

Expression of EPO-like Domain of hTPO Using P_L Expression System

Since the EPO-like domain of hTPO was not expressed to high level in the OmpA secretion and thioredoxin fusion systems, we tried the expression of hTPO using the direct expression vector, pLex404n, and polyhistidine fusion vector, pET163, in which the expressed protein is located in the cytoplasm.

For the direct expression of the EPO-like domain of hTPO in the cytoplasm, a 470-bp PCR product coding [Val^1]hTPO$_{153}$ cDNA was obtained and inserted into the pLex vector under the control of a strong P_L promoter. The resulting plasmid, pLex404n, encodes the [Met^{-1}, Val^1]hTPO$_{153}$. [Met^{-1},Val^1]hTPO$_{153}$ was expressed in *E. coli* GI724. Induction with tryptophan led to accumulation of the expressed protein in insoluble forms (Fig. 4A, lanes 3 and 8). On SDS-PAGE of the cell pellet fractions, [Met^{-1}, Val^1]hTPO$_{153}$ represented approximately 14% of the total protein (Table 2) indicating a molecular mass of 18 kDa, which was close to the expected size deduced from 153 amino acids. As shown in Fig. 4Ab, [Met^{-1},Val^1]hTPO$_{153}$ analyzed by anti-hTPO polyclonal antibody (R&D Systems, McKinley, U.S.A.) was detected at 2 h post-induction (lanes 2 and 7) and showed strong band intensity until 4 h post-induction and decreased at 6 h post-induction. This result reflects that the EPO-like domain of hTPO may be unstable in cytoplasm even though it was produced in insoluble forms. The N-terminal amino acid sequence of [Met^{-1},Val^1]hTPO$_{153}$ expressed in the pLex404n expression system was proved to be Met-Val-Pro-Val-Ala-Pro-Ala. This result matched the deduced N-terminal amino acid sequence from the DNA sequence and the prereported amino acid sequence of hTPO.

In order to investigate the effect of casamino acids on the expression of hTPO, 0.2% and 2% casamino acids were added to the induction medium. No significant difference was detected on the expression level of [Met^{-1}, Val^1]hTPO$_{153}$ between the treatment of 0.2% and 2% casamino acid.

Expression of Polyhistidine tag38-hTPO163 Using T7 Expression System

For the overexpression and efficient purification of hTPO$_{163}$, 513-bp of the PCR product coding hTPO$_{163}$ was obtained and inserted into the pET-28a(+), a polyhistidine vector which is under the control of the strong T_7 RNA polymerase promoter. Jiang *et al.* [6] recently reported that the full length of hTPO$_{332}$ was expressed up to 10% of total proteins in *E. coli* using a T7 expression vector, pET-11(d), in which the translation initiation region (TIR) was modified to have lower G-C content by site-directed PCR mutagenesis. Since the PCR product coding hTPO does not include the 5'-noncoding region of native hTPO cDNA, the possibility of poor expression due to high G-C content in the TIR region was excluded.

In the resulting recombinant pET163 vector, the cloned hTPO$_{163}$ cDNA is located behind the T_7 promoter, ATG initiation codon, six histidine residues, and thrombin recognition site. These pET163 vectors were introduced into *E. coli* strain BL21(DE3) and expressed in the presence of 1 mM of IPTG. After IPTG induction, the cells were harvested and examined by SDS-PAGE. As shown in Fig. 4B, polyhistidine tag-hTPO$_{163}$ was over-expressed in BL21(DE3). A major band was directly visualized at the expected molecular weights of the 24 kDa on SDS-polyacrylamide gel after Coomassie Brilliant Blue staining. For the identification of polyhistidine-tagged hTPO$_{163}$, immunoblotting with anti-hTPO polyclonal antibody was also performed. The fusion protein was expressed in insoluble form to a high amount (approximately 9% of total protein) at 1 h post-induction (Fig. 4B, a and b, lane 2) and then the fusion protein level rapidly decreased at 3 h post-induction (Fig. 4Bb, lane 4). In conclusion, we confirmed that the EPO-like domain of hTPO was not produced to large amount in soluble form

Table 2. Expression of the EPO-like domain of hTPO using *E. coli* expression vectors.

Mode of Expression	Vector	Cloned Gene Product	Promoter	Molecular Mass (kDa)	Amount of hTPO (mg/l)[a] (% of hTPO)	Type of Expressed hTPO
Secretion (*ompA*)	pTE404NF	hTPO$_{332}$	*tac*	35	Very Low Level	Insoluble
	pTE404NH	hTPO$_{153}$	*tac*	18	ND[b]	-
Fusion	pTF404	Thioredoxin$_{119}$-[Val^1] hTPO$_{153}$	P_L	33	0.08[c] (ND)	Soluble
Direct	pLex404n	[Met^{-1},Val^1]hTPO$_{153}$	P_L	18	11.7 (14.4)	Insoluble
	pET163	polyhistidine tag$_{38}$-hTPO$_{163}$	T7	24	5.0 (9.2)	Insoluble

[a]The amount of hTPO and % of hTPO were measured by using scanning densitometery based on total protein concentration. Protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard.
[b]ND, not detected
[c]The amount of Thioredoxin$_{119}$-[Val^1] hTPO$_{153}$ was determined by ELISA.

with the *ompA* secretion and thioredoxin fusion systems. The expressed protein was also unstable in *E. coli* even though a high level of expression of hTPO was achieved in P_L and T_7 direct expression systems. Although the level of hTPO expression was high, it was less than expected. We suspect that the lower level of hTPO expression may be due to the presence of a large number of rare codons in the coding sequence of hTPO. Makoff *et al.* [11] explained the low level expression of tetanus toxin fragment C in *E. coli* as the problem of 11.7% rare codons. Using their criteria of defining unfavorable codons in *E. coli*, we calculated that the coding sequence of full-length hTPO (332 amino acids) and EPO-like domain of hTPO (153 amino acids) contain 14.7% and 9.2% rare codons, respectively. Therefore, the substitution of the rare codons to the preferred codon in the hTPO gene may become a possible method for more high level expressions of hTPO in *E. coli.*

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