Enhanced Expression of Human Pro-urokinase cDNA in *Escherichia coli*

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Human pro-urokinase cDNA was isolated from the cDNA library constructed from human kidney mRNA using the dC/dG homopolymer tailing method and Okayama-Berg method with pBR322 as a vector. A mature polypeptide starting with Ser was produced in *Escherichia coli* under the control of the *tac* promoter and the Shine-Dalgarno sequence of the catechol 2,3-oxygenase gene derived from *Pseudomonas putida*. By replacing the sequence coding for N-terminal eight amino acids of pro-urokinase with the synthetic DNA oligomer, the bacterial pro-urokinase had a molecular weight of 47,000 daltons and accounted for 15% of the insoluble fraction of *E. coli* proteins in induced cells. Its biological activity was restored by renaturing the bacterial product. The activity of bacterial pro-urokinase was 450 IU/ml culture.

Plasminogen activators play a role in plasminogen dependent fibrinolysis in a controlling blood clot lysis system. Two types of plasminogen activators have been known; one is the urokinase type of plasminogen activator (UK), and the other is the tissue type plasminogen activator (tPA). Concerning the affinity for fibrin, tPA¹⁾ has been reported to have a higher affinity for fibrin compared with urinary high-molecular weight (HMW)-UK. Nevertheless, both plasminogen activators contain kringle domains which are considered to be crucial in fibrin affinity. Pro-UK derived from human kidney tissue (KPA), as opposed to urinary HMW-UK, has a higher fibrin affinity.²⁾ To study the role of the kringle domain of pro-UK, we tried to produce a large quantity of pro-UK in *Escherichia coli*. For the efficient expression of foreign genes in *E. coli*, transcriptional and translational signals have to be recognized by the host. In *E. coli*, the *lac*

UV5, *tac*, or phage *P_L* promoter is used as a sufficient sequence to be recognized by RNA polymerase.³⁾ As concerns the translational signal, three components are so far well-defined; (i) ATG, (ii) the distance between Shine-Dalgarno (SD) sequence and ATG, and (iii) the complementarity to the 3' end of 16S rRNA.^{4~6)} Recently, the expression of foreign genes in *E. coli* has been discussed at the level of mRNA secondary structure.^{7~9)} We also report the influence of mRNA structure on the expression of pro-UK gene since we used the catechol 2,3-oxygenase (C2,3-O) SD sequence derived from *Pseudomonas putida*.¹⁰⁾ By replacing the sequence coding for the N-terminal eight amino acids of mature pro-UK with the synthetic oligonucleotides to diminish the secondary structure of mRNA, the bacterial pro-UK production was increased 3-4-fold.

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MATERIALS AND METHODS

Strains. *E. coli* K-12 strain RR1 and DH1 were used for bacterial transformation to construct the cDNA library. *E. coli* K-12 strain JM103 was used for bacterial expression of cDNA clone for human pro-UK.

Enzymes and reagents. Restriction enzymes and other enzymes were obtained from Takara Shuzo (Kyoto, Japan), Bethesda Research Lab., Toyobo (Osaka, Japan), and New England Biolabs. AMV reverse transcriptase was purchased from Life Science. [γ - 32 P]ATP and [α - 32 P]dCTP were from Amersham. Other reagents were all the highest grade commercially available.

Oligonucleotides were synthesized by phosphotriester solid phase chemistry¹¹⁾ or with a DNA synthesizer (Nihon Zeon, Osaka, Japan.) dG tailed pBR322 vector was purchased from New England Nuclear.

Cloning of pro-UK cDNA and sequencing. Poly (A)⁺RNA was isolated from human kidney using guanidine thiocyanate/CsCl method¹²⁾ and purified by oligo dT cellulose chromatography.¹³⁾ Twenty grams of tissue yielded 6 mg total RNA and 150 μ g poly(A)⁺RNA.

The cDNA library was constructed from poly(A)⁺RNA (20 μ g) by dC/dG homopolymer tailing method¹⁴⁾ or by Okayama-Berg method.¹⁵⁾ Colony hybridization was done by the method of Hanahan *et al.*¹⁶⁾ A sixteen mixed probe used for screening consisted of the human urokinase amino acid sequence Asn¹⁶⁹ to Phe^{173/177} [5'-dAACCAAGCTTGGT(C)TGA(G)TT 3']. Similarly, as a second probe, eight mixed oligomers coding for Met²⁸³ to Pro^{287/18} [5'-dGGA(G)TCA(G)TTA(G)TACAT 3'] were used.

DNA was sequenced by the method of Maxam-Gilbert¹⁹⁾ and by the dideoxy chain termination method.²⁰⁾

Primer extension. Chemically synthesized oligonucleotide (Ser⁸⁹ to Gln⁹³, 5'-dCTGAAGAGCATCAGA 3') was 5'- 32 P-labeled using polynucleotide kinase and [γ - 32 P]ATP. Primer extension was done by the method of Agarwal *et al.*²¹⁾ Ten μ g of poly(A)⁺RNA was used as a template. Synthesized cDNA was ligated to pBR322 by the dC/dG homopolymer tailing method and then used to transform *E. coli* RR1.

Screening was done in the same way as cDNA cloning except for using nick translated 5'-upstream cDNA which corresponds to the 200 bp *Pst*I-*Bgl*II fragment of pKYU21 as a probe. Hybridization was done at 60°C.

Restriction mapping and Southern blot hybridization. Plasmid was prepared from 1.5 ml overnight culture by the method of Birnboim and Doly,²²⁾ cleaved with restriction endonucleases and Southern blotted from 1% agarose gels as described in the manual of Maniatis *et al.*³⁾

Hybridization was done as described above.

Construction of the plasmid containing full length pro-UK gene and expression plasmids. The full length gene plasmid pKYU22 was obtained by ligating the 5.7 kb *Bgl*II-*Hind*III fragment of pKYU21, the *Bgl*II-*Nco*I 66 bp fragment of pKYU21, and *Nco*I-*Hind*III 1.1 kb fragments of pPE3. To construct pKMU1 which lacks the sequence for a signal peptide, the sequence which codes for the amino terminal region of human pro-UK was replaced with the synthetic oligonucleotides as shown in Fig. 2.

The expression plasmid pMUT1L was obtained by ligating the 500 bp *Aat*II fragment of pTCM1 which contains the *P. putida* C2, 3-O SD sequence with the 6 kb dephosphorylated *Aat*II fragment of pKMU1. After digesting pMUT1L with *Dra*I, the *Hind*III linker was ligated, and then pMUT2L was constructed by ligating the 2.8 kb *Hind*III fragment of pMUT1L and the dephosphorylated *Hind*III vector fragment of pKK223-3.²³⁾ Finally self-ligation of pMUT2L digested with *Sph*I-*Tth*III and blunt-ended with T4 DNA polymerase yielded pMUT4L as shown in Fig. 2.

Expression of pro-UK gene in *E. coli*. *E. coli* K-12 strain JM103 cells containing pMUT4L were grown in 10 l of M9mE which was M9-broth supplemented with 0.1% yeast extract and 0.1% glycerol instead of casamino acids. At OD₅₅₀=0.5, 0.1 mM IPTG was added. The bacteria were collected at OD₅₅₀=2.0. The pelleted cells were resuspended in 1 l of 50 mM Tris-HCl (pH 8), 0.1 M NaCl, and 1 mM EDTA containing 0.1 mg/ml lysozyme. The suspended cells were sonicated and centrifuged. Renaturation was done by a modification of the method of Heyneker *et al.*²⁴⁾ The insoluble part was solubilized with 4 M guanidine-HCl. To solubilized material, 3 volumes of Tris-HCl buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA] containing 0.1 mM glutathione (reduced, GSH), and 0.01 mM glutathione (oxidized, GSSG) was added and left to stand overnight at room temperature.

Enzyme activity assay. Plasminogen activator activity was measured by a fibrin plate²⁵⁾ or the chromogenic substrate, S-2444 (pyroglutamyl-arginine-paranitroanilide, Kabi Diagnostica, Stockholm, Sweden).

N-terminal sequencing and amino acid composition analysis. N-terminal sequence analysis was done with a protein sequencer (Applied Biosystems 470A) and amino acid composition analysis was done by the Toray Research Center (Tokyo, Japan).

RESULTS

Pro-UK cDNA sequence and deduced amino acid sequence

The restriction map and sequencing strategy

of pro-UK which covered a 2,294 bp region including the coding sequence are shown in Fig. 1(A). The nucleotide sequence and deduced amino acid sequence of pro-UK cDNA were found by the dideoxy chain termination and Maxam–Gilbert methods [Fig. 1(B)]. The molecular weight of pro-UK estimated from the nucleotide sequence was 44,367 (411 amino acids). The deduced amino acid sequence is identical with the known amino acid sequence except for Lys¹⁵⁸. It is proposed that Lys¹⁵⁸ is removed at the time of cleavage by a serine protease when the single chain polypeptide becomes the two chains of the active form.²⁶⁾ Two other groups^{27,28)} have presented the cDNA sequence of human pro-UK independently. The differences in cDNA sequences were discussed in detail by Nagai *et al.*²⁷⁾ The cDNA obtained in our group differs by two bases from the previously reported cDNA sequence.²⁷⁾ The codons CTA for Leu²³⁹, and AAA for Lys³¹³ in our results are CTC and AAG in their report.

Construction of expression plasmid pMUT4L

We tried to express the pro-UK gene in *E. coli* by direct expression of the gene lacking the eukaryotic signal peptide but containing the methionine initiation residue at the N-terminus. We prepared two types of precursor plasmids to compare the expression level in terms of the secondary structure of 5' region of mRNA and the codon usage in *E. coli*.²⁹⁾ One plasmid (pKMU1') had the native N-terminal sequence, and the other (pKMU1) had a modified N-terminal sequence (Ser¹–Pro⁸) in which six base pairs were changed. Namely, the codons AAT for Asn², GAA for Glu³, CTT for Leu⁴, CAT for His⁵, CAA for Gln⁶, and CCA for Pro⁸ in the native sequence were AAC, GAG, CTC, CAC, CAG, and CCG in the modified sequence as shown in Fig. 2. pMUT1L was constructed by inserting a 500 bp *Aat*II fragment which contains the *tac* promoter and the *P. putida* C2, 3-O gene SD sequence into the *Aat*II site of pKMU1. The plasmid pKK223-3 was used to introduce the transcription terminator sequence, the *E. coli*

rrnB T₁, T₂ terminator, to make pMUT2L. In the construction of the final expression plasmid, pMUT4L (modified type), the pro-UK gene was placed downstream from two tandem *tac* promoters and upstream from the *rrnB* T₁, T₂ terminator. pMUT7L (native type) was constructed in the same way as pMUT4L (Fig. 2). The DNA sequence of the 5' region of the pro-UK gene contained in each pMUT4L or pMUT7L was confirmed by sequence analysis.

Expression of pro-UK gene in *E. coli*

JM103 cells containing pMUT4L were found to express pro-UK as detected by Western blot analysis. After lysis of IPTG-induced JM103 cells containing pMUT4L followed by centrifugation, pro-UK was detected in the insoluble, but not in the soluble fractions, by Coomassie Brilliant Blue staining and Western blot analysis; it may be produced as an inclusion body in *E. coli* (data not shown). No pro-UK was detected in cells without induction by IPTG. *E. coli* pro-UK was expressed as a single chain polypeptide. In the reduced conditions, the polypeptide produced in *E. coli* was represented as a 47k band in SDS-PAGE. For further analysis the *E. coli* insoluble material was solubilized in 4M guanidine-HCl, and diluted in a Tris-HCl buffer containing 0.1 mM GSH and 0.01 mM GSSG. Then the renatured solution was diluted with 50 volumes of UK dilution buffer (Tris-HCl buffer containing 0.1% gelatin). Fibrinolytic activity was measured by fibrin plate method or chromogenic substrate, S-2444, which was 450 IU/ml culture. Expression of JM103 containing pMUT7L was also investigated in the same way as JM103 containing pMUT4L. The fibrinolytic activity of pro-UK expressed in JM103 containing pMUT7L was only 110 IU/ml culture. Comparison between the amounts of expression in two cells, JM103 containing pMUT4L and JM103 containing pMUT7L, are presented in Fig. 3 and Table I. The percentage of insoluble *E. coli* protein produced by the recombinant pro-UK gene was obtained by separating the proteins by SDS-

FIG. 1(A). Restriction Map and Sequencing Strategy.

The diagram is a schematic representation of the 5' untranslated segment (open box), the signal sequence (slash box), the coding sequence (black box), and 3' untranslated sequence (open box). pKTU6 was obtained from the cDNA library prepared by the Okayama-Berg method, pKYU21 was from the cDNA library prepared by the pBR322 dC/dG homopolymer tailing method, and pPE3 was from the primer extension. Sequencing strategy is indicated by arrows, where each sequencing was done at least twice using the Maxam-Gilbert or dideoxy chain termination method.

PAGE, staining them with Coomassie Brilliant Blue and scanning the stained gel with a Helena Lab. Quick Scan II. This method showed that the pro-UK protein accounts for 15% of insoluble *E. coli* protein (2% of total bacterial protein) in the case of JM103 containing pMUT4L, but to a lesser extent in the case of JM103 containing pMUT7L. These results agree with the previous expression data.

Comparison of the partial amino acid sequence of bacterial pro-UK and urinary HMW-UK

For investigating the amino acid sequence of bacterial pro-UK, we purified the bacterial gene product. Detailed method of purification will be described elsewhere. To be brief, affinity chromatography was done, and the 47 k single band protein was identified by SDS-PAGE. The N-terminal 25 amino acids of bacterial pro-UK were identified with a protein sequencer. The N-terminal sequence of bacterial pro-UK was identical with that reported for urinary HMW-UK (data not shown).¹⁸⁾ The N-terminal amino acid of bacterial pro-UK was Ser not Met, indicating that Met was removed in *E. coli* as discussed in the expression of superoxide dismutase⁸⁾ in *E. coli*. The amino acid composition of bacterial pro-

UK was essentially identical with that of urinary HMW-UK except for the Gly content (Table II).

DISCUSSION

The cDNA sequences of human pro-UK have been reported by other groups.^{27,28)} The cDNA sequence we obtained is essentially identical with the previous results except for a few base changes. The amino acid sequence deduced from cDNA sequence is almost identical with that of urinary HMW-UK.

In the expression of the pro-UK gene in *E. coli*, we attempted to examine the effects of secondary structure of mRNA in the translation initiation region. As transcriptional and translational apparatus we used the *tac* promoter and a C2,3-O SD sequence derived from *P. putida* which had strong ribosome binding activity demonstrated in the expression of the C2,3-O gene in *E. coli* under the control of the *lac* promoter (unpublished data). We constructed two expression plasmids; one was pMUT4L which had modified DNA sequence in the N-terminal region, and the other was pMUT7L which had a native DNA sequence in N-terminal region. Possible secondary

FIG. 1(B). Nucleotide Sequence and Deduced Amino Acid Sequence of the Human Pro-urokinase Gene.

The arrow indicates the presumed signal sequence cleavage site. The two poly-A addition signals, AATAAA, are underlined. The numbers refer to nucleotide positions. Note that the codons, CTA for Leu²³⁹ and AAA for Lys³¹³, were CTC and AAG in the sequence of Nagai *et al.*²⁷⁾

FIG. 2. Construction of the Expression Plasmids.

pKMU1 contains a modified nucleotide sequence in the 5' region, and pKMU1' contains a native nucleotide sequence in the 5' region. pMUT4L and pMUT7L were used for direct expression of pro-UK gene in *E. coli*. Ap^r represents ampicillin resistance; Tc^r represents tetracycline resistance.

structures of mRNAs formed by two types of expression plasmids are shown in Fig. 4. The thermodynamic stabilities are calculated as in Tinoco *et al.*³⁰⁾ The introduced six base mutation possibly diminishes the hairpin structure, which might render the SD domain accessible to ribosomes. The effects of the mutation on gene expression were measured by both SDS-PAGE and fibrinolytic activity. The mutation increased the amount of bacterial pro-UK expressed in *E. coli* by 3.4-fold compared with that of the native type. Comparison of the expression levels of JM103/pMUT4L and JM103/pMUT7L showed that the se-

quences of the 5' flanking region containing SD and the 5' region of the pro-UK gene could influence the expression level. The expression level, however, was not increased dramatically by introducing the mutation as described by Hall *et al.*⁷⁾ This might indicate that other factors still influence the expression level, such as the sequence upstream from the SD-spacing-ATG signal or the nucleotide composition of the sequence between SD and the initial codon. Stanssens *et al.*³¹⁾ have shown that the region upstream from the SD is important in the expression of the β -interferon gene. Hui *et al.*³²⁾ have shown that the nu-

FIG. 3. Comparison of the Pro-urokinase Syntheses by the *E. coli* Cells Containing pMUT4L and the Cells Containing pMUT7L by SDS-Polyacrylamide Gel Electrophoresis under Reduced Conditions (Insoluble Fractions).

cleotide composition of the sequence between the SD and the initiation codon can dramatically alter the efficiency of initiation. The data presented here, however, suggest that the mRNA secondary structure in the initiation domain plays a certain role in the process of translational initiation concerning the expression of the pro-UK gene in *E. coli*.

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TABLE I. COMPARISON OF PRO-UKOKINASE SYNTHESIS BY THE *E. coli* CELLS CONTAINING pMUT4L AND THE CELLS CONTAINING pMUT7L

Bacteria	ΔG (kcal/mol)	Expression	
		Amount in insoluble (%)	Activity (IU/ml culture)
JM103/pMUT4L	0	15	450
JM103/pMUT7L	-10.2	5	110

TABLE II. AMINO ACID COMPOSITIONS OF PRO-UKOKINASE PRODUCED IN *Escherichia coli*

Amino acid	Calculated ^a	Found ^b
Asx	36	36.9
Thr	26	23.8
Ser	30	29.0
Glx	40	43.3
Pro	23	19.7
Gly	34	41.8
Ala	18	18.7
Cys	24	20.9
Val	19	18.2
Met	7	6.7
Ile	19	16.9
Leu	31	31.8
Tyr	18	18.1
Phe	12	12.4
Lys	27	27.1
His	18	17.1
Arg	21	22.2
Trp	8	6.4

^a Calculated from cDNA sequences shown in Fig. 2.

^b These numbers agree essentially to those which were measured with HMW-UK.^{17,18} Measured after 22 hr at 110°C hydrolysis.

support.

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FIG. 4. Possible Secondary Structures and Corresponding Thermodynamic Stabilities of Pro-urokinase mRNAs Containing Native Type Sequence (pMUT7L) and Modified Type Sequence (pMUT4L).

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