Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 3, 228-234 (1991)

Total Synthesis and Expression in *E. coli* of a Gene Coding for Human Interleukin-2

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인간 인터루킨-2를 코드하는 유전자의 합성과 대장균에서의 발현

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Abstract — A synthetic gene coding for human interleukin-2 (IL-2) was constructed from the oligonucleotides synthesized by an automatic DNA synthesizer. The nucleotide sequence of the synthetic gene was chosen considering the preferred codons of *E. coli* by not changing the amino acid sequence of IL-2 polypeptide. The synthetic gene was expressed in *E. coli* by placing the gene under the control of the λ P_L promoter. IL-2 was produced in the *E. coli* cytoplasm in the form of inclusion bodies. The recombinant IL-2 showed growth promoting activity on the IL-2 dependent cell line.

Interleukin-2 (IL-2), also referred to as T-cell growth factor, is a major lymphokine for T lymphocytes and delivers various signals by interacting with specific cell surface receptors (1, 2). Numerous biological activities have been ascribed to IL-2, including stimulation of T-cell clones for long-term *in vitro* growth (1, 3), induction of cytotoxic T-cell reactivity (4), augumentation of natural killer cell activity (5), generation of lymphokine activated killer cells (6).

Recombinant IL-2 has been produced using various host-vector systems (7-10). Preclinical and clinical trials using recombinant IL-2 showed optimistic results against cancer (11) and combined immunodefienciency disease (12). We have cloned and expressed the IL-2 cDNA in *E. coli* (10). We have also produced Ser^{125}-IL-2 by site directed mutagenesis, in which Cys^{125} was changed to a serine (12). In both cases, IL-2 was produced in *E. coli* in the form of insoluble inclusion bodies. Denaturation and renaturation processes which are required to purify the IL-2, might cause the reduction of the activity.

Since the cDNA is human origin, the usages of the codons are different from the preferred codons of *E. coli* (14). It has been suggested that the level of protein expression is closely correlated with the usage of codons (14). We hypothesized that by changing the codons of IL-2 cDNA into the favorable codons *E. coli*, IL-2 might be produced as a soluble protein in *E. coli* due to smooth translation of the mRNA. Furthermore the yield of IL-2 might be improved if the codons were changed to the preferred codons of *E. coli*. To change the codons in many places, chemical synthesis would be the best choice. Here we report the results of the chemical synthesis of IL-2 gene. The IL-2 gene was sucessfully expressed in *E. coli*. The recombinant IL-2 was biologically active as determined by the growth promoting activity on the IL-2 dependent

Key words: Interleukin-2, synthetic gene, expression, *E. coli*
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cell line.

Materials and Methods

E. coli strains

E. coli N4830-1; F^- su° his^- ilv^- $galK^-\Delta 8$ (ΔchlD-pgl) [$\lambda\Delta BamN^+cI857\ \Delta H1$] was purchased from Pharmacia (Sweden) and used as a host strain to express IL-2 genes. *E. coli* C600λ; F^- thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ (15) and JM109; recA1 hsdR17 gyrA96 relA1 thiΔ (lac-proAB) were also used (16).

Enzymes and chemicals

Enzymes were purchased from KOSCO Biotech (Korea), Promega Biotech (U.S.A.) or Bethesda Research Laboratories (U.S.A.). All chemicals were reagent grade either from Sigma (U.S.A.) or Merck (Germany).

Cloning procedures

All cloing procedures were mainly as those described by Sambrook *et al.* (17). Enzymatic reactions were carried out as recommened by the suppliers.

Design of the synthetic IL-2 gene

The nucleotide sequence of the synthetic IL-2 gene was designed considering the preferred codon usage of *E. coli* (14). The amino acid sequences of natural IL-2 were not changed except one position. A cysteine at position 125 was changed to a serine because, firstly, this change does not affect the activity of IL-2, and secondly, the yield of recombinant Ser^{125}-IL-2 was higher than that of Cys^{125}-IL-2 (13, 18).

Fig. 1 shows the total nucleotide sequence of the synthetic gene. Subdivision to 23 oligonucleotides are shown as arrows. The lengths of oligonucleotides were in the range of 24~41 mer. The *Xba*I site at position 174 was introduced to make the subsequent cloning convenient. *Nar*I and *Eco*RI sites were introduced to the 5′ end and 3′ end respectively.

Synthesis of oligonucleotides

Oligonucleotides were synthesized with an automatic synthesizer (Applied Biosystems, model 380A) at the Service Department of Genetic Engineering Institute, Korea Institute of Science and Technology). The 23 oligonucleotides with lengths of between 24 mer and 41 mer were synthesized. After synthesis, the oligonucleotides were separated from the support, deprotected with ammonium hydroxide. This was loaded on a denaturing polyacrylamide gel and the oligonucleotides were recovered from the gel.

Cloning of pKK511

As schematically shown in Fig. 2a, plasmid pKK 511 was prepared by cloning the first half of the IL-2 gene (171 bp) fragment into plasmid pUC18. The details are as follows. The 5′ ends of oligonucleotides ILN-2, 3, 4, 5, 12, 13, 14, and 15 were phosphorylated with T_4 polynucleotide kinase and ATP. Unphosphorylated ILN-1 and 16 were added to this and ligated together. The product was separated on a 2% agarose gel and the 173 bp fragment was eluted from the gel. This fragment possesses restriction enzyme *Nar*I and *Xba*I ends and inserted between the *Nar*I and *Xba*I sites of plasmid pUC18.

Cloning of pKKJ611

Plasmid pKKJ611, as schematically shown in Fig. 2b, was prepared by cloning the second half of the IL-2 gene, a 232 bp *Xba*I-*Eco*RI fragment, into plasmid pUC18. Details are essentially identical to the cloning procedures for pKK511.

Cloning of pKKJ711

Plasmid pKKJ711 was prepared to construct the total IL-2 gene by connecting the 171 bp and 232 bp fragments. As schematically presented in Fig. 2c, the *Nar*I-*Xba*I fragment of pKK511 was inserted into *Nar*I-*Xba*I site of pKKJ611. The resulting plasmid pKKJ711 contains the entire IL-2 gene.

Cloning of pNJ4

To express the synthetic gene in *E. coli*, an expression vector was constructed as schematically drawn in Fig. 3. Plasmid pNKM21 which expresses Ser^{125}-IL-2 from the mutated natural IL-2 gene (13; referred to 'natural' IL-2 gene in this paper) was

A

5' ILN-1

CG CCG ACT TCC TCG AGC ACC AAG AAG ACT CAG CTG CAG CTG GAA CAC
GGC TGA AGG AGC TCG TGG TTC TTC TGA GTC GAC GTC GAC CTT GTG

3' ILN-12 ILN-13

Nar I

ILN-2

CTG CTG CTG GAT CTG CAG ATG ATC CTG AAC GGT ATC AAC AAC TAC AAG
GAC GAC GAC CTA GAC GTC TAC TAG GAC TTG CCA TAG TTG TTG ATG TTC

ILN-14

ILN-3 ILN-4

AAT CCG AAA CTG ACC CGC ATG CTG ACT TTC AAG TTC TAC ATG CCG AAG
TTA GGC TTT GAC TGG GCG TAC GAC TGA AAG TTC AAG ATG TAC GGC TTC

ILN-15

ILN-5 ILN-6

AAG GCT ACT GAG CTG AAA CAC CTG CAG TGT CTA GAA GAA GAA CTG AAA
TTC CGA TGA CTC GAC TTT GTG GAC GTC ACA GAT CTT CTT CTT GAC TTT

ILN-16 ILN-17

ILN-7

CCG CTG GAA GAA GTT CTG AAC CTG GCT CAG AGC AAA AAC TTC CAC CTG
GGC GAC CTT CTT CAA GAC TTG GAC CGA GTC TCG TTT TTG AAG GTG GAC

ILN-18

ILN-8

CGC CCC AGG GAC CTG ATC TCG AAC ATC AAC GTT ATC GTT CTG GAA CTG
GCG GGG TCC CTG GAC TAG AGC TTG TAG TTG CAA TAG CAA GAC CTT GAC

ILN-19 ILN-20

ILN-9

AAA GGA TCT GAA ACC ACC TTC ATG TGT GAG TAC GCT GAT GAA ACC GCT
TTT CCT AGA CTT TGG TGG AAG TAC ACA CTC ATG CGA CTA CTT TGG CGA

ILN-21

ILN-10

ACG ATC GTT GAA TTC CTG AAC CGC TGG ATC ACC TTC TCC CAA TCG ATC
TGC TAG CAA CTT AAG GAC TTG GCG ACC TAG TGG AAG AGG GTT AGC TAG

ILN-22

EcoR I

ILN-11

3'

ATC TCT ACT CTG ACT TGA TAATTTTG
TAG AGA TGA GAC TGA ACT ATTAAAACTTAA

5'

ILN-23

B

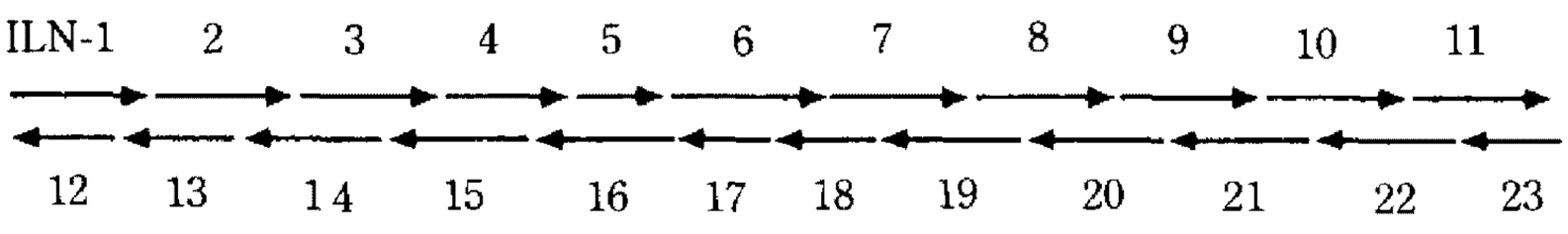

Fig. 1. (a) Nucleotide sequence of the synthetic gene coding for human IL-2. Subdivision to 23 oligonucleotides are shown as arrows.

The 5′ end is the *Nar*I cohesive end and the 3′ end is the *Eco*RI cohesive end. The *Xba*I site at position 174 was introduced to make the subsequent cloning convenient.

(b) Schematic representation or the subdivision of IL-2 gene. Oligomers are shown as arrows.

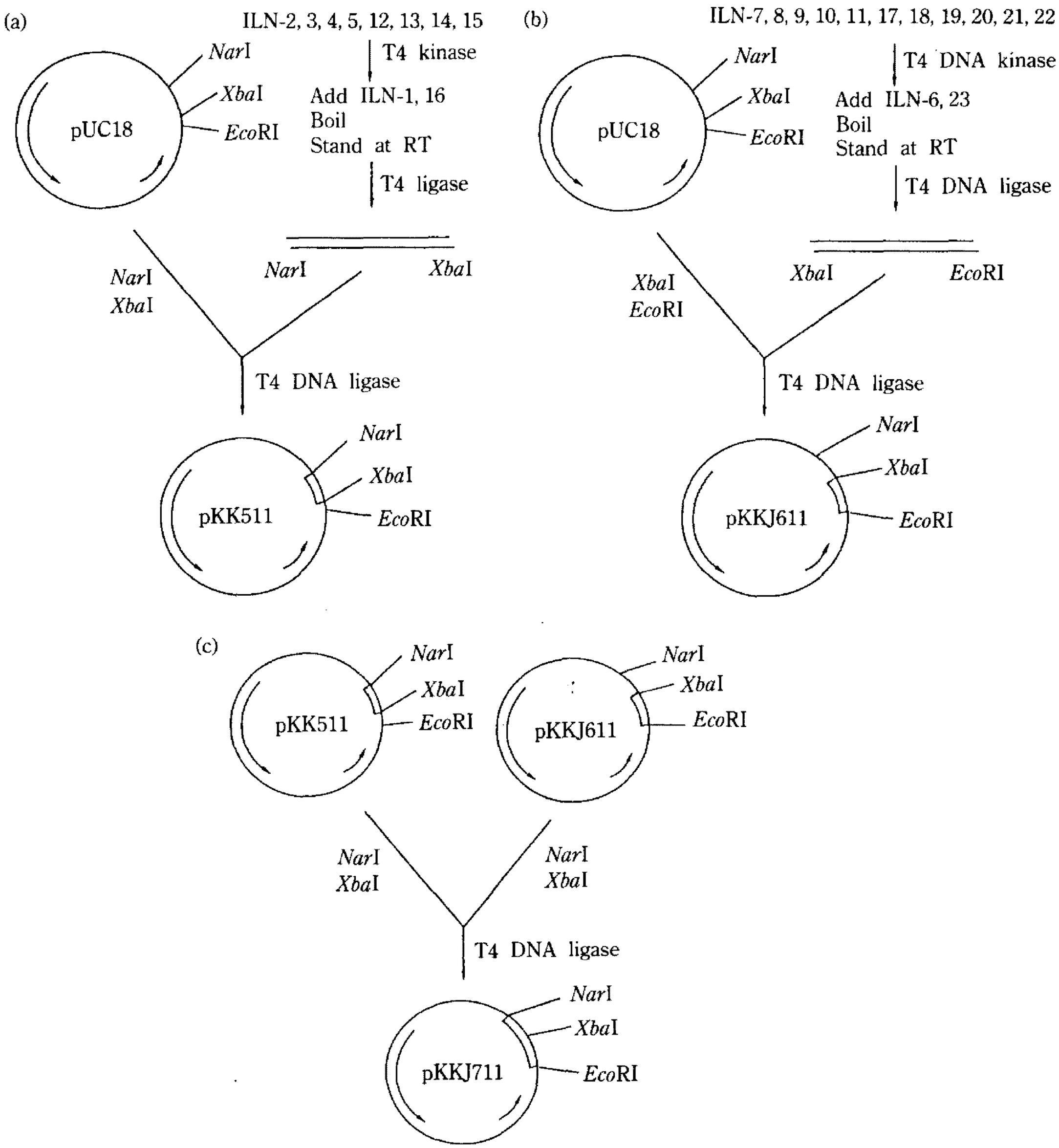

Fig. 2. Cloning scheme for the preparation of pKKJ711, a plasmid containing the synthetic IL-2 gene.
The first half of the synthetic gene is cloned into the *Nar*I-*Xba*I site of plasmid pUC18 to produce pKK511 (a). The second half of the synthetic gene is cloned into the *Xba*I-*Eco*RI site of pUC18 to produce pKKJ611 (b). Then the *Nar*I-*Xba*I fragment of pKK511 was cloned into the *Nar*I-*Xba*I site of pKKJ611 (c). The details are as described in the 'Materials and Methods' The longer arrows, shorter arrows, and the boxes on the plasmid maps indicate the ampicilline registance gene, replication origin and IL-2 gene respectively.

utilized to clone pNJ4. Plasmid pNKM21 was digested with restriction enzyme *Nar*I and *Pvu*II. This was separated on a agarose gel and the vector portion was recovered from the gel. Plasmid pKKJ711 was digested with restriction enzyme *Eco*RI and the cohesive end was filled in by filling in reaction of Klenow fragment of DNA polymerase I. This was digested with enzyme *Nar*I, separated on a gel and

the IL-2 gene fragment was recovered from the gel. This fragment was ligated to the vector portion DNA of pNKM21.

Results

Cloning of the synthetic IL-2 gene

Analysis of plasmid pKK511 using restriction enzymes showed that the 171 bp *Nar*I-*Xba*I fragment was correctly inserted as intended. The 232 bp *Xba*I-*Eco*RI fragment of pKKJ611 was also identified by agarose gel electrophoresis (data not shown). Total nucleotide sequence of the IL-2 gene in pKKJ 711 was determined by dideoxy chain termination methods using the double stranded plasmid DNA as a template (19). The results showed that the nucleotide sequences were identical to what was designed and synthesized (data not shown).

Expression of the synthetic IL-2 gene in *E. coli*

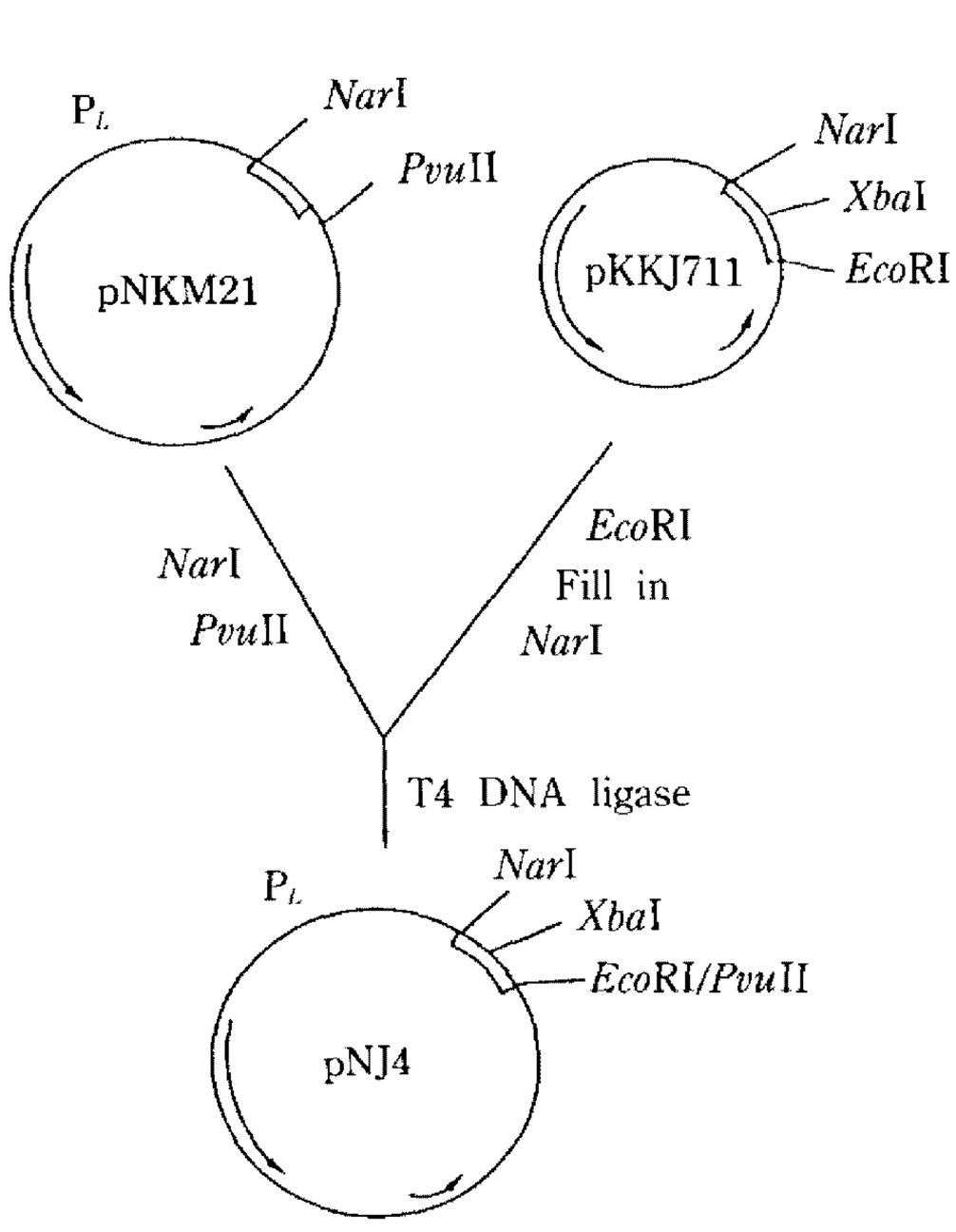

Fig. 3. Cloning scheme for the preparation of an expression vector that produces human IL-2 from the synthetic gene in *E. coli*.

The *Nar*I-*Eco*RI fragment of pKKJ711 was inserted into the *Nar*I-*Pvu*II site of pNKM21 (13). Details are as described in the 'Materials and Methods'. The arrows and boxes are as in Fig. 2.

The expression vector (pNJ4) constructed as shown in Fig. 3, contains the coding sequence under the control of the P_L promoter. *E. coli* strain N4830-1 which carries a temperature sensitive mutation of the repressor (*cI*857) was used as a host. The culture was maintained at a permissive temperature (28℃) until it reached to a proper density, and then the temperature was raised to a nonpermissive temperature (42℃) to express the IL-2 gene. Small granules were visible inside the cell within 2 hrs after the temperature shift under a phase contrast

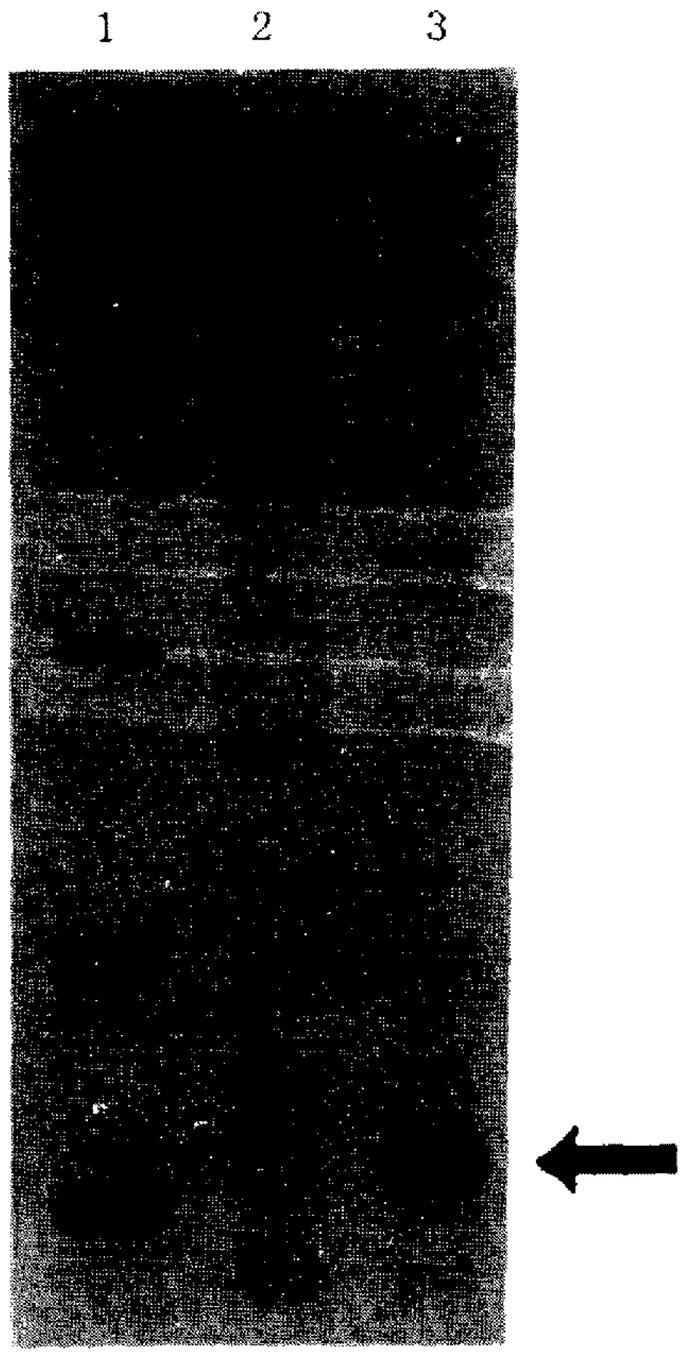

Fig. 4. Analysis of the total *E. coli* proteins by SDS-polyacrylamide gel electrophoresis.

E. coli strain harboring the recombinant plasmid was grown at 28℃ until $OD_{600}=0.6$. The temperature was raised to 42℃ and the culture was maintained at this temperature for 2 hrs. A 1.5 m*l* of the culture was harvested by centrifugation and the pellet was resuspended in a 150 μ*l* of Laemmli loading buffer (21; 0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 1.47 M β-mercaptoethanol, 0.001% bromophenol blue), boiled for 5 min and the equivalent amount of samples was loaded and separated on a 14% SDS-polyacrylamide gel. Lane 1; molecular weight standards. The sizes are 94, 67, 43, 30, 20.1, 14.4 kilodaltons from the top. Lane 2, 3; *E. coli* N4830-1/pNJ4 grown at 28℃ (2), or grown further at 42℃ (3). The arrow indicates IL-2.

microscope indicating that IL-2 was produced as inclusion bodies (10; data not shown). When the proteins in the soluble and insoluble fractions were analyzed, IL-2 was mostly present in the insoluble fraction (data not shown). Fig. 4 shows the results of the total protein alalysis by SDS-polyacrylamide gel electrophoresis. A 15 kilodalton protein was observed after, but not before, the induction of IL-2 gene expression as observed previously with the natural IL-2 cDNA (10, 13). The activity of IL-2 by the growth promoting effect on a IL-2 dependent cell line was observed from the induced culture but not from the uninduced culture (data not shown).

Discussions

Expression of heterologous gene in *E. coli* depends upon many factors, such as promoter strength, ribosome binding site (RBS), the spacing between the RBS and mRNA, the initiation codon, mRNA secondary structure, and effective termination of transcription. Biased codon usage have been observed for *E. coli* proteins which are produced at a high level. There are significant correlation between the biased codon usage and the concentration of proteins in *E. coli*. A set of preferred codons used in *E. coli* has been deduced (14). Several proteins with industrial importance have been produced from the synthetic genes in which the codons were changed to the preferred codons of *E. coli* (20). Process of polypeptide chain elongation is not smooth and the discontinuity (pausing) of translation has been observed. It was suggested that the aberrant codon usage of the mRNA is the cause of the translation discontinuity. Therefore we hypothesized that by changing the aberrant codons of natural IL-2 gene into the preferred codons of *E. coli*, IL-2 can be produced at a higher level and may be as a soluble protein. The result was rather different from our hypothesis. IL-2 produced from the synthetic gene was insoluble in *E. coli* cells (data not shown) similar to what was observed with the natural gene (10, 13).

This results suggest that aberrant translation is not the sole cause of the production of the insoluble protein. The very high rate of protein synthesis has also been suggested for the reason of the inclusion body formation. Whether IL-2 can be produced as a soluble protein *E. coli* cytoplasm by lowering the rate of synthesis is another important question and experiments are under way to answer this.

Although not throughly studied, the preliminary data showed that the level of expression from the synthetic gene was similar to that from the natural gene. Comparison of the expression levels from two IL-2 genes at various culture conditions needs a through investigation. A series of experiments, using the vectors in which different promoters are utilized, are planned and will be performed in the near future.

요 약

DNA 자동합성기를 이용하여 올리고뉴클레오타이드들을 합성하였으며 이로부터 인간 인터루킨-2를 코드하는 유전자를 제조하였다. 합성 유전자의 염기서열은 대장균에서 사용빈도가 높은 코돈을 고려하여 결정하였으며, 이때 인터루킨-2의 아미노산 서열은 변화시키지 않았다. 합성된 유전자는 람다 피엘 프로모터를 사용하여 대장균에서 발현시켰다. 인터루킨-2는 대장균내에서 불용성의 침전물로 생성되었다. 생성된 인터루킨-2는 인터루킨-2 요구 배양세포주의 성장을 촉진하였다.

Acknowledgement

This study was supported by a grant from the Ministry of Science and Technology of Korea.

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(Received May 15, 1991)