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Site-Specific Mutagenesis on the 32-T and 39-T of *E. coli* tRNA^{Phe} Gene

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E. coli tRNA^{Phe} 유전자의 32-T와 39-T 염기의 부위 특이적 돌연변이

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ABSTRACT : There are three pseudouridine (Ψ)bases in the *E. coli* tRNA^{Phe}. In order to study the function of the pseudouridine bases in the tRNA^{Phe}, changes of bases tRNA^{Phe}gene to other bases were undertaken by the site-specific mutagenesis. Site-specific mutagenesis of T in the *pheW* gene, a tRNA^{Phe} gene of *E. coli*,corresponding to the base at the No. 32 position to C and also T corresponding to the base at the No. 39 position to C were performed using Kunkel's uracil-containing template method.

Identification of mutants were undertaken by the DNA sequencing techniques of the mutated *pheW* genes and activities of the mutated *pheW* genes complementing to *E. coli* NP37 mutant(*pheS*$^{-ts}$) using the recombinant plasmid containing the mutated genes.

Neither NP37 harboring *pheW* gene mutated at No.32 position nor NP37 harboring *pheW* gene mutated at No. 39 position can be grown at non-permissive temperature. The result means that both mutated *pheW* genes can not complement to *E. coli* NP37, and that the pseudouridine bases are essential to the activity of the *E. coli* tRNA^{Phe} *in vivo*.

KEY WORDS □ Pseudouridine, Site-specific mutagenesis, tRNA^{Phe}, *pheW*

Transfer RNA(tRNA) interact with many different proteins including elongation factors and aminoacyl-tRNA synthetases, and ribosomal RNA. This diversity of interactions for tRNA may be one reason for its complex content of modified nucleosides. At present more than 50 different modified mucleosides have been characterized, all of which are derivatives of the normal nucleosides adenosine(A), guanosine (G), uridine(U), and cytosine(C)(Nisimura, 1979). tRNA from all orghanisms contains modified nucleosides (Björk *et. al.*, 1987). Most of them are not likely to be essential for viability. Many nucleosides in tRNA molecules are post-transcriptionally modified (Björk, 1984). The function of some of these modifications has been elucidated, but the role of the majority remains obscure. Recently, it was synthesized by *in vitro* transcription, and some of its properties in aminoacylation and protein synthesis *in vitro* were studied(Samuelson *et al.* 1988 ; Sampson and Uhlenbeck, 1988).

Pseudouridine(Ψ), 5-(β-D-ribofuranosyl)-uracil, is a modified nucleotide and abundant in cytoplasmic and organelle tRNA (Kammen *et al.*, 1988). In order to study the role of Ψ base in tRNA, Ames *et al.* (1978) reported that *hisT* mutant of *Salmonella typhimurium* which lack the enzyme that modifies uridine to pseudouridine in anticodon region of many tRNAs. In the report, the results suggested that the regulation of a large number of amino acid biosynthetic pathway was altered by the *hisT* mutation. *hisT* mutants of *E. coli* were also isolated and characterized (Balsi *et al.* 1977). However, the role of pseudouridine base in tRNA is not defined, yet.

There are three pseudouridine bases in the *E. coli* phenylalanyl tRNA(tRNA^{Phe})(Fig.1). In order to study the function of Ψ in *E. coli* tRNA^{Phe} we have subcloned the *pheW* gene, a *E. coli* tRNA^{Phe} gene, and changed the 32-T and the 39-T in the gene corresponding to the 32-Ψ and the 39-Ψ to the 32-C and the 39-C

The activities of the mutated *pheW* genes were determined by complementing to *E. coli* NP 37 mutant (*pheS*$^{-ts}$) using to recombinant plasmids containing the mutated genes.

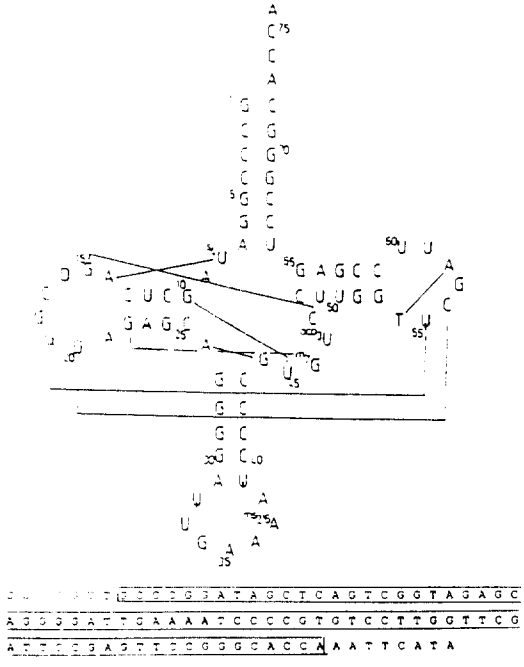

Fig. 1. *E. coli tRNA*^{Phe} *and its gene sequence*
Top, Nucleotide sequence of *E. coli* tRNA^{Phe} arranged in the classical cloverleaf structure showing the tertiary hydrogen bonds seen in the yeast tRNA ^{phe}crystal structure(Hyde and Reid, 1985). *Bottom*, sequence of the DNA fragment containing *E. coli* tRNA^{Phe} gene, *pheW* (Wilson *etal.*, 1986). The boxed region corresponds to the tRNA sequence.

MATERIALS AND METHODS

Phages, Plasmids, and Bacterial Strains

The bacterial strain for transfection after site-specific mutagenesis was *E. coli* JM103 (Messing, 1983). *E. coli* RZ1032(Kunkel *et al.*, 1983) was used for incorporation of uracil into phage DNA. *E. coli* NP37 (Schwartz *et al.*, 1984) was used for transformation of recombinant *pheW* plasmids.

M13mp9am phage was used for site-specific mutagenesis, and pHW2 plasmid which had constructed in this lab was used for gene source of *pheW*.

Isolation of Plasmid DNA

The plasmid DNA was purified according to the method described by Birnboim and Doly (1979).

Isolation of M13mp9am RF DNA

The RF DNA was prepared from infected *E. coli* JM103 as described previously(Bauer *et al.*, 1985).

Digestion and Ligation of M13 RF DNA

Cloning of *pheW* gene into M13mp9am RF DNA is schematically described in Fig. 2.

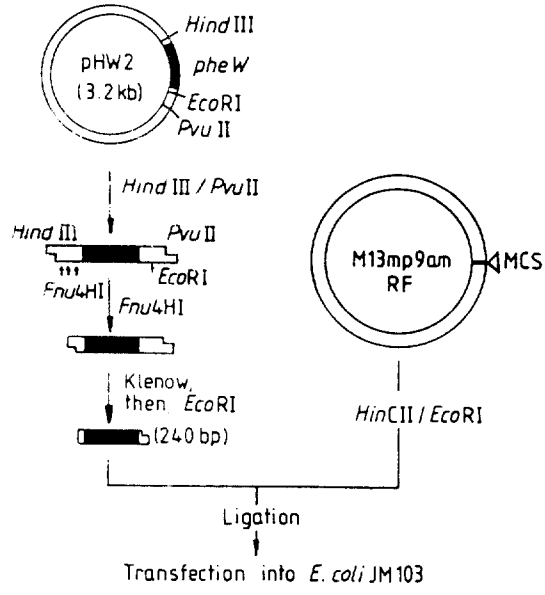

Fig. 2. *Schematic illustration of the construction of M13mp 9am-pheW*

Preparation of Uracil Containing Template DNA

M13mp9am-*pheW* template DNA containing uracil residues was prepared as described by Kunkel (1985).

Mutagenic Oligonucleotides

Mutagenic oligonucleotides, 5'-GATTTTCAGT-CCCCTGCTC- 3' containing a single-base mismatch at 32-T and 5'-GACACGGGGGTTTTCAATCC-3' containing a single base mismatch at 39-T, were obtained from KAIST.

Site-specific mutagenesis

Site-specific mutagenesis was performed according to the method described by Kunkel *et al.*(1987). And identification of mutated *pheW* genes was performed by dideoxy-sequencing method (Sanger *et al.*, 1977).

Subcloning of the Mutated *pheW* Genes into pGEMl Vector

Hind Ⅲ-*EcoR* Ⅰ fragments containing mutant *pheW* genes were eluted from M13mp9am-*pheW* after site-specific mutagenesis. The eluted DNA fragments were ligated with pGEMl vector which was also digested with *Hind* Ⅲ and *EcoR* Ⅰ at 12℃ for overnight.

Transformation of Ligated Plasmid into *E. coli* NP37

Transformation of the ligated plasmids into *E. coli* NP37 strain was performed as described (Elseviers *et al.*, 1982). Because the recipient, NP37, is temperature sensitive, LB broth(1% tryptone, 0.5% yeast extract, 1% NaCl) was inoculated with 0.005 volume of overnight culture, and cells were grown at 30℃ to and absorbance at 600nm of 0.2 to 0.6. The culture was then chilled in ice. Cells were collected by centrifugation, washed in 50mM $CaCl_2$, and kept on ice for 30min. After centrifugation, the cells were suspended in 0.1 volume of 50mM $CaCl_2$. With this procedure, 0.3ml of concentrated cells was used for a microgram of DNA. DNA and cells were mixed in prechilled tubes. The transformation mixture was incubated on ice for 40min, heat-pulsed at 42℃ for 2min, diluted 15 fold with prewarmed LB broth and allowed to grow for 2 hr at 30℃. Before plating, cells were concentrated 10-fold, and 0.1ml samples were spread on LB plates containing 50㎍ of ampicillin per ml, then incubated at 30℃ for overnight.

RESULTS AND DISCUSSION

Site-Specific Mutagenesis of *pheW* Gene

The basic scheme used to generate the 32-T to 32-C and 39-T to 39-C mutation in *pheW* gene is presented in Fig. 3.

It involves cloning of the *pheW* gene into the single stranded phage vector, M13mp9am (shown in Fig. 2), and subsquent use of the uracil-containing template DNA for *in vitro* synthesis of cccDNA. 5'-phosphorylated deoxyoligonucleotides which are 19 nucleotides long, containing a single base mismatch

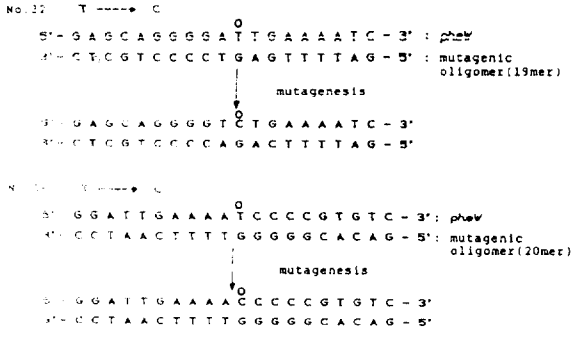

Fig. 3. *Site-specific mutagenesis of pheW gene*

at No. 32 position, and 20 nucleotides long, containing a single base mismatch at the No. 39 position were used as primer for synthesis of the cccDNA. The heteroduplex cccDNA were used for transfection *E. coli* JM103 cell.

Selection of Phages Harboring Mutated *phew* Genes

Template DNA used in this study was prepared from phage grown in *E. coli* RZ1032(*supE, ung*$^-$, *dut*$^-$). Phage DNA from the strain is biologically active in a strain incapable of removing the uracil (*ung*$^-$host). However, such a DNA can be biologically inactivated through the action of uracil glycosylase which removes uracil(Kunkel, 1985). Therefore, in this study, we supposed that almost all phages grown in *E. coli* JM103 (*supE, ung*$^-$) should contain the mutated *pheW* gene without uracil residues in their DNA. So, we randomly selected the phages grown in *E. coli* JM103 host, and isolated the single-stranded DNA from the phages.

Screening the Mutants

The ssDNA from the selected phages were screened for the presence of the mutant sequence using track sequencing. As shown in Fig. 3, we have changed the T base at the No.32 position and No.39, the muant *pheW* genes should have C base at No. 32 or No.39. Therefore, C-track sequencing was performed. C track sequencing was done exactly the same way as the dideoxy DNA suquencing, except that only C base specific reaction was performed. As the result shown in Fig. 4, while the wild type *pheW* gene did not show the band at the No.

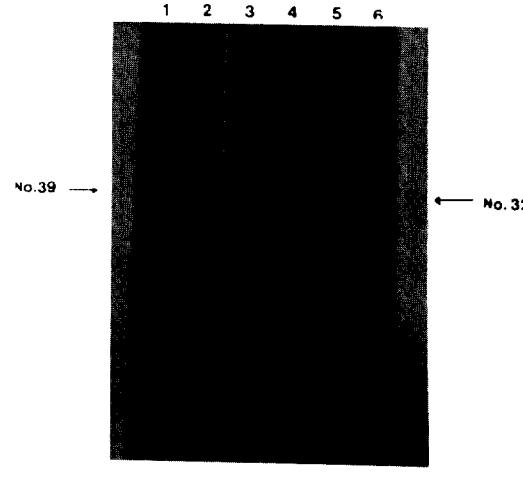

Fig. 4. *Screening of mutated pheW gene using C-track sequencing*

1 : Wild *pheW*
2 : A *pheW* mutated at the No.39 position
3 : A *pheW* mutated at the No.39 position
4 : A *pheW* mutated at the No.32 position
5 : A *pheW* mutated at the No.32 position
6 : A *pheW* mutated at the No.32 position

32 position and the No.39 position, the mutant which had been changed T to C at the No.32 position showed a C band at the expected position, and the mutant which had been changed at the No.39 position also showed a C band at the expected position. In the case of mutation at the No.32 position, two out of three candidates were the mutant, and in the case of mutation at the No.39 position, one of two candidates was the mutant.

Identification of the Mutant *pheW* Genes

When, we selected the mutants which were changed at the No.32 position or at the No.39 position in *pheW* gene, the mutants were chosen by only C track sequencing. Therefore, we determined whether other positions in the gene were changed or not by complete DNA sequencing of wild type and mutant *pheW* DNAs using the M13 dideoxy DNA sequencing techniques. As shown in Fig. 5 the mutant *pheW* DNAs have only the changed bases at the No.32, and at the No.39, and no other changes were observed except the positions which were desired. We named the mutant *pheW* gene changed at the No. 32 to *pheW*32C, and the mutant *pheW* gene changed at the No.39 to *pheW*39C.

Activities of the Mutant *pheW* Genes for Complementing to *E. coli* NP37 Mutant

An *E. coli* mutant strain, NP37, has been shown to harbor a temperature sensitive *pheS* gene, encoding phenylalanyl-tRNA synthetase. Schwartz *et al.*(1983) reported that NP37 transformed with a

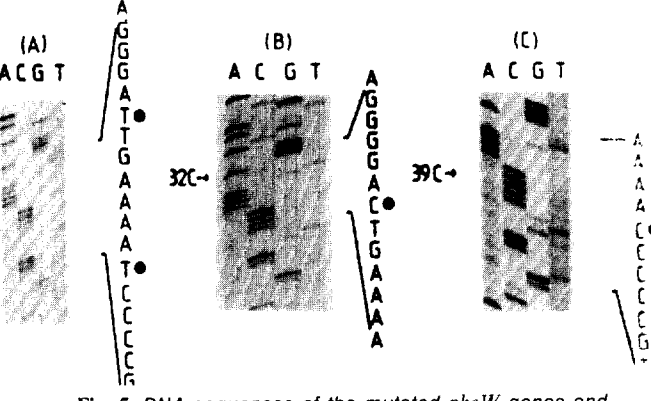

Fig. 5. *DNA sequences of the mutated pheW genes and the wild pheW gene in the region of the mutated*
(A) : Wild *pheW*
(B) : *pheW*32C
(C) : *pheW*39C

plasmid containing the coding for tRNA^{Phe} was capable of growth at the non-permissive temperature. It means that increase in active tRNA^{Phe} level complement the mutation (Goodman and Schwartz, 1988). Therefore, the mutated *pheW* genes, *pheW*32C and *pheW*39C, were subcloned into pGEM1 plasmid vector as described in the section of materials and methods. The recombinant pGEM1 plasmid which had *pheW*32C at the *Hind* III-*EcoR* I site, and the recombinant pGEM1 plasmid which had *pheW*39C at the *Hind* III-*EcoR* I site were obtained. The recombinant plasmids which were named to pHWC32 and pHWC39, respectively.

In order to determine the activities of the mutated *pheW* genes complementing to *E. coli* NP37(*pheS*$^{-ts}$), NP37 was transformed with the recombinant plasmids, pHWC32 and pHWC39. The transformed *E. coli* NP37 was incubated on LB agar plates containing 50μg/ml of ampicillin at 30℃. From the colonies grown on the plates, the plasmids were isolated and identified their insert size by *Hind*III and *Eco*RI (data not shown). The colonies identified were then incubated on LB containing ampicillin(50μg/ml) at 42℃ for overnight. The result is presented in Table 1. As the result, neither *E. coli* NP37 harboring pHWC32 nor *E. coli* NP37 harboring pHWC39 could be grown in the non-permissive temperature, 42℃, while *E. coli* NP37 harboring pHW2 which had wild *pheW* gene could be grown in the non-permissive temperature. The result shows that the mutated *pheW* genes, both *pheW*32C and *pheW*39C, can not complement to *E. coli* NP37 mutant

Table 1. *Activities of the mutated pheW genes in E. coli NP37 (pheS*$^{-ts}$*)*

	30℃	42℃
NP37/pHW2 (Wild *pheW*)	+	+
NP37/pHWC32 (*pheW*32C)	+	−
NP37/pHWC39 (*pheW*39C)	+	−

Mutated *pheW* genes cloned in pGEM1 vector, pHWC32 and pHWC39, were transformed into *E. coli* NP37 (*pheS*$^{-ts}$), and their activities were identified by their complementation at 42℃.

pheS^{-ts}). It means that Ψ bases at the No.32 position and No.39 position have very important function to the biological activities of *E.coli* tRNA^{Phe} *in vivo*.

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적 요

*E. coli*의 $tRNA^{Phe}$ 내에는 세개의 psiudouridine(Ψ) 염기들이 존재한다. 이 $tRNA^{Phe}$ 내의 pseudouridine 염기들의 기능을 연구하기 위하여 부위특이적 돌연변이를 이용하여 $tRNA^{Phe}$ 유전자의 염기를 다른 염기로 치환시켰다. *E. coli* $tRNA^{Phe}$ 유전자들중 하나인 *pheW* 유전자내에서 32번에 해당하는 T염기를 C염기로, 39번 T염기를 C염기로 Kunkel이 개발한 부위특이적 돌연변이 방법을 사용하여 각각 치환시켰다.

DNA 염기서열을 결정함으로써 돌연변이체를 확인하였으며, 이들 돌연변이 유전자를 함유한 재조합 폴라스미드를 이용하여 돌연변이된 *pheW* 유전자들의 *E. coli* NP37(*pheS*^{ts})에 대한 **complementation** 활성을 조사하였다.

32번 위치가 변이된 *pheW* 유전자 뿐만아니라 39번 위치가 변이된 *pheW* 유전자를 함유한 *E. coli* NP37들은 모두 non-permissive temperature에서 자라지 못하였다. 이 결과는 변이된 *pheW* 유전자들이 *E. coli* NP37을 complementation할 수 없으며, 또 pseudouridine 염기들이 생체내에서 *E. coli* $tRNA^{Phe}$의 활성에 필수적이라는 것을 의미한다.

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