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Selection of Translational Frameshift Signals in *Escherichia coli*

Yuka NAKAMATSU and Senya MATSUFUJI

Department of Biochemistry (II), The Jikei University School of Medicine

ABSTRACT

We established a selection screening system using kanamycin resistance for sequences that conduct +1 frameshifting at a UGA codon in *Escherichia coli*. Screening of 5×10^5 transformants carrying randomized sequences yielded 1.1×10^3 kanamycin-resistant clones, 36% of which contained possible translational frameshift signals. The frameshift efficiencies ranged from 0.05 to 6.49 and correlated with kanamycin resistance. The frameshift efficiencies of 15 sequenced frameshift signals correlated with the ratio of the stability of peptidyl tRNA-mRNA pairing at the preframeshift and postframeshift positions. A product of one of the most efficient signals was analyzed with mass spectrometry and was identified as the +1 frameshift product. The core sequences of selected signals were found at the natural translational termination sites of genes in the *E. coli* genome. These results indicate that this system can be used to select frameshift signals and provide useful mechanistic and biologic information. (Jikeikai Med J 2004 ; 51 : 113-21)

Key words : translational frameshifting, *Escherichia coli*, selection, screening, stop codon

INTRODUCTION

Recent advances in the understanding of the structural biology of the ribosome has enabled us to clarify mechanisms of molecular movement within ribosomes during translation[1-4]. The path of the messenger RNA (mRNA) and the position of the transfer RNAs (tRNAs) in the ribosome have been determined[5]. These studies provide insight into the precise decoding mechanism[6]. The codon-anticodon base pairings are extensively monitored by bases of ribosomal RNA (rRNA) to avoid missense decoding or processing errors, such as frameshifting and tRNA drop-off[6,7].

Maintenance of the correct reading frame is a particularly accurate process[8]. The frequency of frameshift errors averages 10^{-4} per codon and is much lower than that of missense errors (5×10^{-4} per codon)[8]. However, the error rate can increase depending on the context of the mRNA sequence[9,10]. In particular cases, translational frameshifting is genetically programmed to enrich or regulate gene expression[11,12]. Programmed translational frameshifting has been found in a wide variety of genomes from viruses[13] to higher eukaryotes[14].

In *Escherichia coli* (*E. coli*), peptide release factor 2 (RF2), a translation-termination factor for UGA and UAA stop codons, is expressed by +1 frameshifting[15]. The initiating frame (ORF1) ends at the 26th codon, UGA. Some of the elongating ribosomes, however, do not terminate at UGA but translate the surrounding sequence CUU UGA (stop codon) C as leucine (CUU)-aspartate (GAC). The frameshifting is believed to occur during decoding of the UGA codon at the ribosomal aminoacyl-site (A-site)[16]. The relatively slow decoding of the termination codon by RF2

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中松　友花，松藤　千弥

Mailing address : Senya MATSUFUJI, Department of Biochemistry (II), The Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.

E-mail : senya@jikei.ac.jp

prompts slippage of peptidyl-tRNA from the cognate CUU codon to the near cognate UUU codon at the +1 position. A Shine-Dalgarno (SD)-like mRNA sequence, AGGGGG, 6 nucleotides upstream of the UGA codon stimulates the frameshifting by pairing with the 3′ end of 16S rRNA, UCCUCC[17]. When the level of RF2 protein is low, termination at the UGA codon becomes inefficient and the +1 frameshifting of RF2 mRNA increases to produce active RF2. The frameshifting thus autoregulates the RF2 level. The mechanism of RF2 frameshifting has been extensively investigated. One of these studies examined the relationship between the frameshift level and the sequence of the peptidyl (P)-site codon[18]. Each nucleotide of CUU at the RF2 shift site was substituted by other nucleotides, creating 32 alleles. The stability of codon-anticodon pairing at the postframeshift (+1) position correlated with the frameshift efficiency.

Another type of programmed frameshifting has been found in an *E. coli* cellular gene *dnaX* encoding DNA polymerase III subunits. Frameshifting to the −1 direction produces the γ subunit of the enzyme, whereas the standard decoding produces the τ subunit. The frameshift signal is the AAA AAA G sequence surrounded by an upstream SD-like sequence and a downstream stem-loop structure[19].

Some additional *E. coli* genes are translated by frameshifting with efficiency as high as 15% at the end of the annotated open reading frames[20]. In all cases the frameshift signal is located near the shift site in *E. coli*.

In the present study, we established a selection system for sequences that conduct +1 frameshifting at a UGA codon to find novel frameshift signals in *E. coli*.

Materials and Methods

Plasmid construction

Vectors for selection screening and expression were derivatives of plasmid pUC118 (Takara Shuzo, Kyoto). A kanamycin-resistance (*kan*^{r}) gene (neomycin phosphotransferase II) was amplified with the polymerase chain reaction (PCR) from pd2EGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA, USA) using the sense primer, 5′-GCGAGCTCGGTAC CGAACGGTACCGAAGGGCCCATTGAACAAGAT GGATTG-3′, and the antisense primer, 5′-GCTCTAGATTAGAAGAACTCGTCAAGAAGG-3′, and inserted at the *Apa*I and *Xba*I sites of pUC118. Oligonucleotide DNA pools with randomized sequences were chemically synthesized (library I, 5′ GCACACGCTAACGTAATNNNNNNNNNNTGAN NNNNNNNNNGTTACTAACGTATTCAA-3′, and library II, 5′-GCACACGCTAACGTAATNNNNNN NNNNTGANNVNNVNNVNGTTACTAACGTAT TCAA-3′, where N=A, G, C, or T, and V=A, G, or C), and then amplified with PCR and a sense primer containing an embedded *Kpn*I site and an antisense primer containing an embedded *Apa*I site. The PCR products were inserted into the vector at the *Kpn*I and *Apa*I restriction sites between the initiation codon and the coding region of *kan*^{r} gene so that + 1 frameshifting within the random sequence would express *kan*^{r} gene (Fig. 1A). The *Lac*Z gene was excised from pMC1871 (Amersham Biosciences, Piscataway, NJ, USA) by digestion with *Bam*HI, inserted at the *Bam*HI site of pBluescript SK(−) (Stratagene, La Jolla, CA, USA) and a clone of the correct orientation was chosen by sequencing. The *kan*^{r} gene of the screening vector was replaced by the *Apa*I and *Xba*I fragment of *Lac*Z/pBluescript SK(−) and a pair of linkers (sense 5′-CACCCGCGGTGCAACC-3′ and antisense 5′-CCGGGGTTGCACCGCGGGTGGGCC-3′) was then inserted between the *Apa*I and *Xma*I sites to adjust the reading frame. The 15-nucleotide sequence AGG GGG UAU CUU UGA (stop codon) that is necessary and sufficient for the high-level RF2 frameshifting in *E. coli*[17] was inserted in the *LacZ* expression vector and used as a control. In addition, an in-frame control was prepared by deleting the nucleotide U in the UGA codon. An expression vector with glutathione S-transferase (GST), maltose-binding protein (MBP), and His-tag (GHM57)[21] for mass spectrometry of the frameshift product was kindly provided by Drs. N.M. Wills and J.F. Atkins. The insert of a clone positive for frameshifting (clone UUU) was recloned into the *Bam*HI and *Eco*RI sites of GHM57 between the GST and MBP genes through *Bam*HI-*Kpn*I and *Apa*I-*Eco*RI

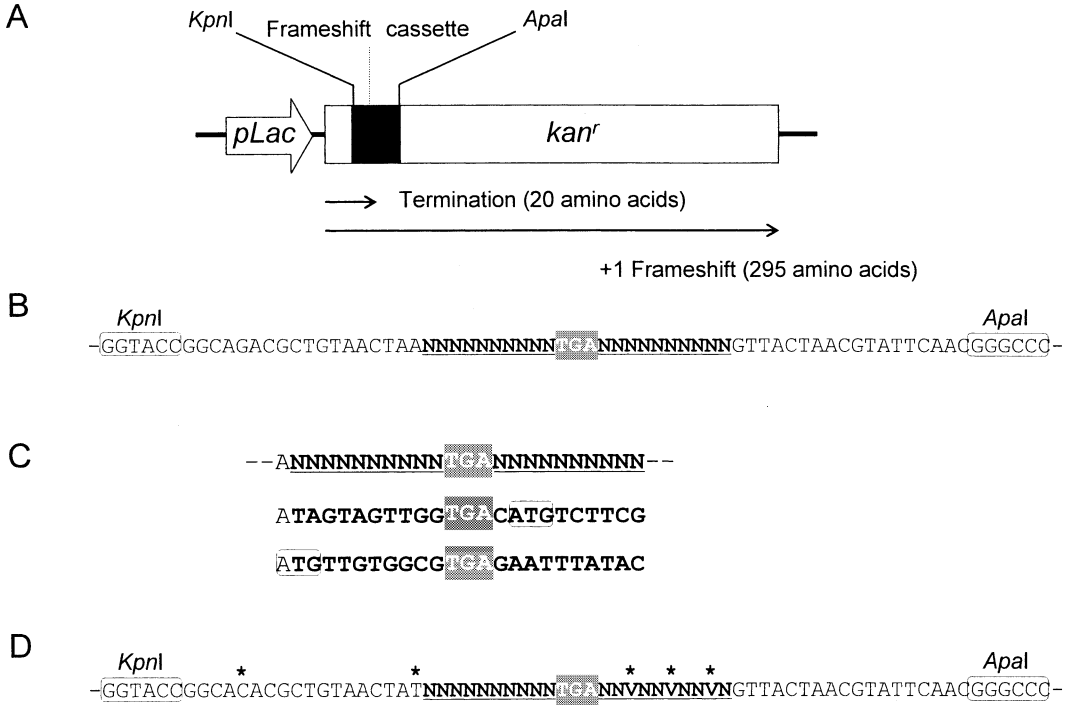

Fig. 1. Selection screening system for frameshift signals at UGA codons
A. A diagram of screening vector. A kanamycin-resistant gene (*kan*^{r}) is driven by the Lac promoter (*pLac*). Inserts containing random sequence are cloned between the *Kpn*I and *Apa*I sites. Plus 1 frameshifting within the insert will translate the full-length (295 amino acids) product.
B. Design of inserts for library I. Two completely randomized 10-nucleotide sequences flank the UGA codon. The sequence is expressed as DNA (N10-TGA-N10).
C. Two representative clones from the selection screening of library I.
D. Design of inserts for library II. The downstream random sequence is changed to a partially randomized sequence. (N10-TGA-N7V3). Two nucleotides upstream of the randomized sequences are also replaced. The alterations are marked with asterisks (*).

linkers. Detailed sequences of the PCR primers and the linkers used in this study are available from the authors upon request.

Selection screening

STBL2 competent cells [F′ *mcrA (mcrBC-hsdRMS-mrr) recA1 endA1 Δlon gyrA96 thi supE44 relA1* Δ*(lac-proAB)*] (0.2 mL) (Invitrogen, Carlsbad, CA, USA) were transformed with a plasmid library pool (0.1 pmol) and spread onto 20 LB-agar plates containing 7 μg/mL kanamycin. To examine transformation efficiency, cultures were diluted 1/10 and 1/100 and spread onto LB-agar plates containing 50 μg/mL ampicillin. The plates were incubated at 37°C for 18 hours, after which the colonies were counted.

Measurement of kanamycin resistance

To measure kanamycin resistance, the selected colonies on the kanamycin plates (7 μg/mL) were picked up and streaked on LB-agar plates containing 10, 40, or 100 μg/mL kanamycin. The plates were incubated at 37°C overnight.

Plasmid purification and sequencing

Plasmids were purified from overnight culture in LB agar containing 50 μg/mL ampicillin with a Concert System kit (Invitrogen) and sequenced with a Big Dye terminator cycle sequencing FS reaction kit (Applied Biosystems, Foster City, CA, USA) and a PRIZM377 sequencer (Applied Biosystems).

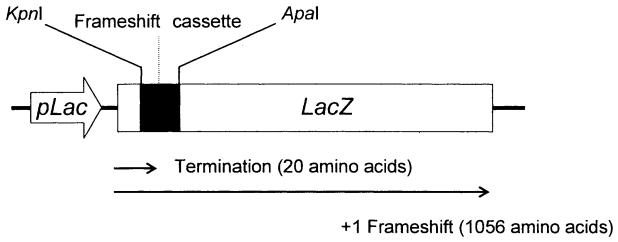

Fig. 2. Vector for β-galactosidase expression
The *kan*^{r} gene of the screening vector is replaced by the *LacZ* gene. Plus 1 frameshifting within the insert will translate the full-length (1056 amino acids) product, which can be measured with a β-galactosidase assay.

β-galactosidase assay

Frameshift efficiency was calculated by measuring of β-galactosidase activity with the *LacZ* expression vector (Fig. 2). *E. coli* SU1675 (*ara thi* Δ[*lac-pro*] *recA*/pAPIq *lacI*^{q} Km^R)[22] was transformed with the *LacZ* expression vectors. Whole-cell β-galactosidase assay was performed as previously described[23]. Overnight cultures were diluted 1/100 into M9 broth with 2% glucose and grown at 37°C with shaking for 2 hours, after which 0.5 mM of isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added. After additional growth at 37°C for 1 hour, cells were chilled on ice and diluted into 0.8 mL of ice-cold Z buffer (21.5 g/L of $Na_2HPO_4 \cdot 12\ H_2O$, 6.2 g/L of $NaH_2PO_4 \cdot 12H_2O$, 0.75 g/L of KCl and 0.246 g/L of $MgSO_4 \cdot 7H_2O$). The enzyme reaction was initiated by the addition of 0.2 mL of 4 mg/mL *O*-nitrophenyl-β-D-galactopyranoside, incubated at 30°C for 5 to 30 minutes, and terminated with 0.5 mL of 1 M Na_2CO_3.

Estimating the stability of peptidyl tRNA-mRNA pairing

Species of tRNA cognate to the P-site codon were determined with a database of tRNA sequences (http://www.staff.uni-bayreuth.de/~btc914/search/). The number of hydrogen bonds between the anticodon of the tRNA and the codon at preshift and postshift P-site positions was calculated as the sum of hydrogen bonds, that is, 3 for GC pairs, and 2 for AU, GU, and pairs with modified bases. The relative stability of peptidyl tRNA-mRNA pairing before and after frameshifting was estimated with the ratio of pairing stabilities at the preshift and postshift positions (postshift/preshift).

Mass spectrometry of a frameshift product

The expression vector GHM57 harboring the insert of clone UUU was used for tranformation of SU1675 cells. Cells cultured overnight in 10 mL LB containing 50 μg/mL ampicillin were diluted into 1 L of Terrific Broth (Fisher Biotec, West Perth, Australia). The cells were grown at 37°C for 2.5 hours and then induced with IPTG (1 mM) at 37°C for 4 hours. The cells were harvested by centrifugation at 4,500 g for 15 minutes, and resuspended in 30 mL BugBuster reagent (Novagen, EMD Biosciences, Madison, WI, USA). The fusion protein was purified with tandem chromatography on a glutathione-sepharose column (Amersham Biosciences) and a Ni-NTA Agarose column (Qiagen, Venlo, the Netherlands) according to the manufacturers' instructions. Purified protein was concentrated and washed with pure water in Centricon centrifugal filter units (Millipore, Bedford, MA, USA), digested with PreScission protease (Amersham Biosciences), and purified with a C18 Zip Tip pipette tip (Millipore). Additional clean-up of the product and mass measurements on a Quatro II mass spectrometer (Waters Micromass MS Technologies, Milford, MA, USA) were performed as described previously[21].

RESULTS

Selection of frameshift signals

A selection screening system for +1 frameshift signals at a UGA codon was established (Fig. 1A). The library prepared first (library I) contained a UGA codon flanked by 2 10-base complete random sequences (N10-UGA-N10 ; Fig. 1B). The +1 frameshifting within the random sequence was expected to express the downstream *kan*^{r} gene, and thus the *E. coli* cells carrying such a plasmid would form a colony on kanamycin plates. Preliminary screening of 10^3 transformants carrying library I yielded 72 kanamycin-resistant clones. Seventeen of these

clones were sequenced ; the sequences of 2 representative clones are shown in Fig. 1C. None of the clones had a frameshift signal. Instead, 16 of the clones were judged to express the *kan*^{r} gene from a newly introduced initiation signal. Four of the clones started upstream of random sequences with TG that created an ATG initiation codon in-frame of the *kan*^{r} gene. The AGA sequence 12 to14 nucleotides upstream may have acted as the SD sequence. In addition, a purine-rich sequence located 6 to 12 bases upstream of the initiation codon was found in a majority of the sequenced clones (72%). One of the resistant clones contained a single nucleotide deletion upstream of the UGA codon, and the translation reading frame was shifted to +1 frame at the DNA level.

To avoid initiation signals for *kan*^{r} genes newly created within the random sequence, a partially random sequence with additional modifications was used (Fig. 1D). In this library (library II), the adenine base immediately before the random cassette was substituted with a T to avoid an initiation codon at this position. The upstream SD-like AGA sequence was replaced by ACA. Three bases within the random sequence downstream of the UGA codon were changed to 3 Vs (V=A, G, or C) to prevent creation of initiation codons. The randomized bases upstream of the UGA codon were unchanged because this region is potentially crucial for a +1 frameshift signal.

Selection screening of 5×10^5 transformants carrying library II yielded 1.1×10^3 kanamycin-resistant clones. Forty-two of these clones were sequenced, of which 15 were believed to carry frameshift signals. Twenty-four of the remaining clones had initiation codons upstream of the UGA codon, and 3 of the 24 were deletion mutants. The kanamycin resistance of clones with a frameshift signal was examined on plates containing 10, 40, or 100 μg/mL of kanamycin (Table 1).

Frameshift efficiencies of positive clones

The β-galactosidase activities of the clones with the wild type and in-frame mutant of RF2 are shown in Table 2. The measured frameshift efficiency of RF2 was 20%, which agrees well with efficiencies in previous reports[17]. The efficiencies of clones positive for frameshifting selected from library II ranged from 0.05 to 6.49 by comparison with the in-frame RF2 construct and were significantly higher than the average frameshift error frequency, 5×10^{-4} per codon[8]

Table 1. Positive clone for frameshifting in library II screening

Clone	Sequence*		Kanamycin resistance (μg/mL)	β-Galactosidase activity (% in-frame)
	--AU**NNNNNNNNNN** **UGA** **NNVNNVNNVN**GU--			
UUU	**AGAUGUUUUU**	**GCACGCGUCG**	100	6.49
CGG	**GUUUCGUCGG**	**GGAUCCUAAG**	100	1.46
GCU	**GCUGCGGGCU**	**GGGCGCUGCC**	100	1.37
GGU	**GUGUUCAGGU**	**GGACCGUACG**	100	0.78
UGG	**GAGCGGGUGG**	**AUGGGGCGAA**	100	0.24
CGG	**GUUUUUUCGG**	**CUGCGCGCCU**	100	0.28
GGG	**GUUUCUAGGG**	**GCGUUGUUGU**	100	0.2
UUU^{40a}	**GUGUUUUUUU**	**CGCCGAGUCU**	40	1.3
CCU^{40}	**UUGGAGUCCU**	**UGCGCCCGCA**	40	0.55
UUU^{40b}	**GCCGGUCUUU**	**UGCGGGUGGU**	40	0.38
CUC^{40}	**AUACUUUCUC**	**UCGACGGCGU**	40	0.27
UGU^{10}	**GGAUUUAUGU**	**AUGCUCUAGG**	10	0.04
UAU^{10}	**GUUGUUGCAU**	**GGGGGGGUGU**	10	0.26
CUG^{10}	**GGAUUUUCUG**	**CUGCUGGGGG**	10	0.14
CUC^{10}	**UCAGGUGCUC**	**CGCAAGGUGG**	10	0.05

*Expressed as RNA sequence

Table 2. β-Galactosidase activity of RF2 construct

Clone	Sequence	β-Galactosidase activity	
		(units)	(% in-frame)
RF2	--AG**GGGGUAUCUU** **UGA** **CUACGACGCC**GU--	377	20.2
RF2 (in-frame)	--AG**GGGGUAUCU**−**UGA** **CUACGACGCC**GU--	1,868	100

Table 3. Possible base pairing of the P-site tRNA at the preshift and postshift positions

Clone	Anticodon of P-site tRNA (3′-5′)	Number of hydrogen bond		Ratio of stability (%) (postshift/preshift)
		Preshift	Postshift	
RF2 (CUU)	GAG	7	6	86
UUU	AAG	6	6	100
CGG	GCC	9	8	88.9
GCU	CGG, CGcmo^{5}U	8, 8	4, 4	50
GGU	CCG	8	5	62.5
UGG	ACC	8	3	37.5
CGG	GCC	9	8	88.9
GGG	CCC, CCU$^{\#}$, CCG	9, 8, 9	6, 6, 3	75
UUU^{40a}	AAG	6	6	100
CCU^{40}	GGG	8	7	87.5
UUU^{40b}	AAG	6	6	100
CUC^{40}	GAG	8	4	50
UGU^{10}	ACG	7	2	28.6
UAU^{10}	CUQ	7	2	28.6
CUG^{10}	GAU$^{\#\#}$, GAC	7, 8	2, 2	28.6
CUC^{10}	GAG	8	4	50

$^{\#}$, $^{\#\#}$ Unknown modification

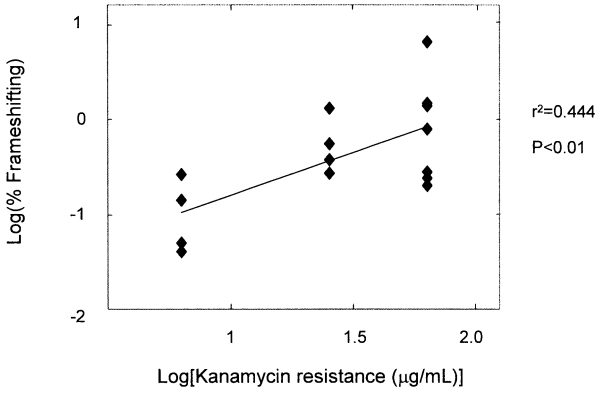

Fig. 3. Correlation between kanamycin resistance and frameshift efficiency

(Table 1). The frameshift efficiencies calculated from the β-galactosidase assay were correlated with kanamycin resistance ($p<0.01$, Fig. 3).

Relationship between frameshift efficiency and relative stability of peptidyl tRNA-mRNA pairing

The number of hydrogen bonds between codons of preshift and postshift positions and peptidyl-tRNA anticodons was estimated for each sequenced clone (Table 3). In the case of RF2 frameshifting, the peptidyl-leucyl-tRNA cognate to the CUU codon is inserted into the UUU codon at the +1 position. The number of hydrogen bonds is 7 at the preshift position but is 6 at the postshift position. For the 15 positive clones, frameshift efficiency significantly correlated with the ratio of postshift to preshift peptidyl-tRNA-mRNA stabilities ($p<0.05$, Fig. 4).

The signal of clone UUU directs +1 frameshifting

The clone UUU showed the highest frameshift

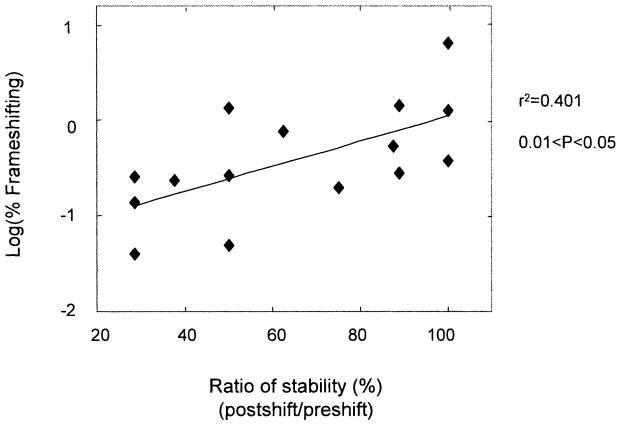

Fig. 4. Correlation between ratio of codon-anticodon stabilities and frameshift efficiency

efficiency (6.49%). To determine the type of frameshift conducted by this construct, its insert was further cloned into a GST-MBP-His-tag fusion vector (Fig. 5A). The product was purified with glutathione sepharose and Ni-NTA Agarose columns, digested with PreScission protease, and then analyzed with a Quatro II mass spectrometer. The measured mass of 44,975.8 agreed well with the mass of the +1 frameshift product at the UUU UGA G sequence (44,970.8; Fig. 5B). Although a +1 frameshift event at the 3′ adjacent codon position, GUU UUU U (GUU at P-site and UUU at A-site), would give the identified product, such an event is unlikely because the in-frame A-site sense codon should be rapidly decoded. Any other type of nonstandard decoding within the insert either cannot explain the measured mass or are very unlikely to occur.

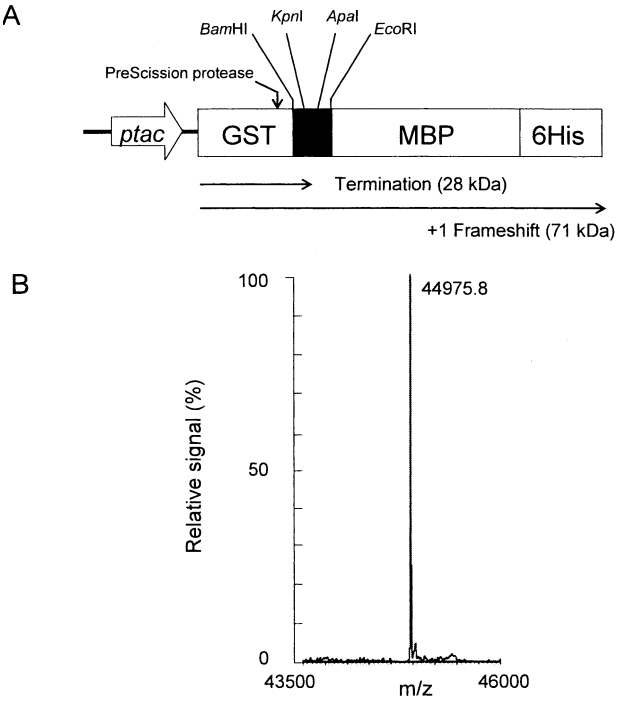

Fig. 5. Mass spectrometry analysis of the product of clone UUU
A. Diagram of GST-MBP-His-tag fusion vector.
B. Mass spectrum of the purified product. A mass range of 43,500 to 46,000 Da is shown. The major peak showed a mass value of 44,975.8 Da, which agrees well with the expected value of 44,970.8 Da.

Search of UUU UGA termination signal in the E. coli genome

Homologous sequences with the UUU codon were searched for at the end of annotated genes in *E. coli* genomes used a database, (Genobase; http://ecoli.aist-nara.ac.jp/GB5/search.jsp). A theoretical gene of unknown function, *yfjI*, matched 10 nucleotides of clone UUU, including the UGA codon and 7 nucleotides preceding to the UGA (Table 4). When +1 frameshift would occur at UUUUGA in *yfjI*, 28 extra amino acids would be added to the C terminus. In addition, *aceK* matched 8 nucleotides of clone UUU^{40a}, and *ydhL* and *friT* matched 8 nucleotides of clone UUU^{40b}.

Table 4. Search of genes matching clones UUU, UUU^{40a}, and UUU^{40b} in the *E. coli* genome

Query	Matched sequence	Gene name*	Number of codons extended
UUU	UGUUUUUUGA	*yfjI*	28
UUU^{40a}	UUUUUUGA	*aceK*	2
UUU^{40b}	UCUUUUGA	*ydhL*	34
	UCUUUUGA	*friT*	4

*The gene ID of each gene: *yfjI*, 944764; *aceK*, 944797; *ydhL*, 946168; *friT*, 946433

Discussion

We screened 5×10^5 clones of the library with partially randomized sequences surrounding a UGA codon (library II), yielding 1.1×10^3 clones (0.22%) that were resistant to 7 μg/mL kanamycin. Five percent

of these clones (42 clones) were sequenced, and 36% (15 of 42 sequenced clones) were found to contain possible frameshift signals. The occurrence of frameshift signals among random sequences around a UGA codon is thus estimated to be 0.08% (0.22%×15/42). The number of the clones we screened was only 1 to 10^6 of the entire complexity of the library (4.6×10^{11}), and further screening may identify novel frameshift signals.

The peptidyl-tRNA-mRNA pairing stability analysis of the clones positive for frameshifting revealed that the frameshift efficiency was correlated with the ratio of stability at the postshift site to that at the preshift site. The ratio of stability in 6 clones was comparable to the natural frameshift signal of RF2, and these clones showed higher frameshift efficiencies. This finding suggests that the +1 frameshifting occurs when the last sense codon is in the P-site and the UGA codon in the A-site. However, a notable exception was clone GCU, which exhibited a significant level of frameshifting (1.37%) despite a relatively low ratio of stability (50%). This finding implies involvement of another mechanism for +1 frameshifting. One possibility is that a mispairing of the aminoacyl-tRNA to the sense codon at the +1 position causes frameshifting without decoding of the first nucleotide of the UGA codon. The same mechanism has been postulated for the +1 frameshifting of yeast Ty3[24] and mammalian antizyme[14].

Of the positive clones, clone UUU showed the highest frameshift efficiency (6.49%). Although clones UUU, UUU^{40a}, and UUU^{40b} share the identical shift site (UUU UGA), their frameshift efficiencies differed. A frameshift event at a termination codon is known to be in competition with the termination. Furthermore, efficiency at a stop codon depends on the identity of the nucleotide immediately following it[25]. In *E. coli*, the termination efficiency at the UGA stop codon is greatest with UGAU, followed in descending order by UGAG and UGAC/A. The inefficient termination signals UGAC/A are often used in natural programmed translational frameshift sites. In our experiment, clone UUU^{40b} had a lower frameshift efficiency than did clones UUU and UUU^{40a}, a result that might be explained by the U base following the UGA codon. In clone UUU, the termination efficiency of the UGAG terminator is intermediate. The high frameshift level of clone UUU may be attributable to the presence of an SD-like sequence (AGA) 8 to 10 nucleotides upstream of the UGA codon. These results suggest that our screening system selects mainly the sequence upstream of the UGA codon. On the other hand, there is no common feature in the downstream sequence, although we had expected that some frameshift signals might contain a secondary RNA structure.

The frameshift signals selected in this study may be useful as clues for finding novel frameshift genes. In our database survey, 4 genes matching the partial sequences of clones UUU, UUU^{40a}, and UUU^{40b} were identified. Further analysis is needed to clarify whether frameshifting is indeed involved in the expression or regulation of these genes.

In conclusion, we have demonstrated that our system can select frameshift signals in *E. coli*. The information of the selected frameshift signals is useful for understanding the mechanism of translational frame maintenance and comprehensive features of translational frameshift events during gene expression.

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