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Translational frameshifting in the *Escherichia coli* dnaX gene *in vitro*

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ABSTRACT

Production of the γ subunit of *Escherichia coli* DNA polymerase III holoenzyme is dependent on a very efficient translational frameshift in the dnaX gene. I used an *E.coli in vitro* translation system to analyze the mechanism of this frameshifting event. In this system, γ was produced almost to the same extent as the in-frame translation product, τ, suggesting that efficient frameshifting was reproduced *in vitro*. Coupling with transcription was not necessary for frameshifting. Addition of purified τ or γ had no effect on the frameshifting process suggesting the absence of direct feedback regulation. By use of mutant genes, a strong pausing site was identified at or very close to the frameshift site. This pausing was apparently caused by a potential stem-loop structure which was previously shown to enhance frameshifting. Thus, enhancement of frameshifting by this putative stem-loop seems to be mediated by the translation pausing at the frameshift site. Despite the apparent structural similarity of the dnaX frameshift site to that of the eukaryotic retroviral genes, dnaX mRNA synthesized *in vitro* failed to direct the production of γ in eukaryotic translation systems. This suggests that frameshifting in the dnaX gene depends on components specific to the *E.coli* translation system.

INTRODUCTION

τ (71kDa) and γ (47kDa) are two subunits of the *Escherichia coli* DNA polymerase III holoenzyme. Both are encoded by the dnaX gene (1,2). τ is translated in a single reading frame, whereas γ is generated through a very efficient -1 translational frameshift and subsequent termination at a stop codon (3,4,5) (Fig 1).

Efficient frameshifting depends on an A AAA AAG heptamer motif that provides the actual site for frameshifting, and a stable stem-loop structure that greatly enhances the frequency of frameshifting (3). A combination of a similar heptameric motif and adjacent secondary structure is also found at the frameshifting site of many eukaryotic viral genes, especially those of retroviruses (7,8,9). The heptamer motifs apparently allow tRNAs in both the A-site and the P-site of the ribosome to maintain a stable interaction with the mRNA after slipping back to -1 position (7). At least in the cases of Rous sarcoma virus and coronavirus IBV, mRNA secondary structure (either a stem-loop or psuedoknot) is clearly important for efficient frameshifting. It has been proposed that these secondary structures slow down the movement of ribosome so that it can sit on the 'slippery' part of mRNA, but there have been no published demonstrations of a pausing site around that position.

To investigate the molecular mechanism of frameshifting in the dnaX gene, it was important to develop a cell-free system in which one can detect the frameshift event efficiently. In this study I show that efficient frameshifting is observed in an *E. coli* S-30 extract *in vitro* translation system. Characterization of this event in this *in vitro* system is presented and possible mechanisms of frameshifting are discussed.

MATERIALS AND METHODS

Reagents

Sources were as follows; T7 RNA polymerase, rabbit reticulocyte lysate and *in vitro* mRNA synthesis kit were from Stratagene, wheat germ extract, *E. coli* S-30 extract and ^{35}S-methionine were from Amersham, τ and γ subunits of DNA polymerase III holoenzyme were purified as described (10). Plasmid pZT3 has a dnaX gene under the control of T7 phage gene10 promoter and T7 DNA polymerase gene Shine-Dalgarno sequence (10). Plasmid pGP1-2 contains T7 RNA polymerase gene with λP_L promoter and a gene for a temperature sensitive lcI repressor (12).

In vitro translation by a combined system

Transcription-translation combined reactions were performed using an *E. coli* S-30 kit from Amersham. The 5 μl reaction mixture containing 0.5 μg of supercoiled template DNA, 0.8 units of T7 RNA polymerase, 60 μg/ml of rifampicin and 1 μCi of ^{35}S-methionine as well as mixtures supplied from the manufacturer was incubated at 30°C for 4min. Then a methionine-chase mixture was added and the reaction was continued at the same temperature for the indicated time. After the reaction, samples were electrophoresed on a 10% SDS-polyacrylamide gel (11) and the gel was washed with 1M sodium salicylate, dried and autoradiographed at -80°C. Quantitation of the bands was done using a Molecular Dynamics scanning densitometer. Intensity of the band with the highest intensity on the gel was defined as 100 units.

Translation with *in vitro* synthesized mRNA

Capped mRNA of dnaX gene was synthesized by T7 RNA polymerase using a kit from Stratagene following the supplier's manual. PvuII-digested pZT3 was used as a template since this digestion was essential to get an mRNA capable of directing the synthesis of τ and γ in an *E. coli* S-30 system. Synthesized dnaX mRNA was purified by DNaseI treatment and phenol-chloroform extraction. 0.5 μg of purified mRNA was added to 10 μl of reaction mixture for translation in either *E. coli* S-30, wheat germ extract (Amersham) or rabbit reticulocyte lysate (Stratagene) in the presence of ^{35}S-methionine. Incubation conditions were, 30°C, 30 min for *E. coli* S-30, 25°C, 1hr for wheat germ and 30°C, 1hr for rabbit reticulocyte lysate. Products of the reaction were run on the gel and autoradiographed as described above.

Expression of τ and γ *in vivo*

E. coli cells were transformed with plasmids pZT3 and pGP1-2 (12) and grown in Luria broth supplemented with carbenicillin and kanamycin at 30°C. At OD_{595}=0.5, the temperature was raised and kept at 42°C for 1hr to induce the expression of T7 RNA polymerase from pGP1-2. Then the cells were harvested and the amounts of τ and γ were determined by western blotting using rabbit anti-γ antiserum as described (3). Blots were scanned with a Hoeffer Scientific model GS300 densitometer. Peaks of the densitogram were cut out, weighed and compared with those of known quantities of τ and γ for quantitation.

RESULTS

γ subunit is generated in a cell-free translation system

The plasmid pZT3 (10), which has a T7 RNA polymerase promoter (12) upstream of the dnaX gene, was used as a template to express the dnaX gene products *in vitro*. Addition of this DNA to an *E. coli* S-30 extract to which rifampicin and T7 RNA polymerase were added, generated polypeptides whose sizes are similar to those of τ and γ (Fig 2). Both of these polypeptides could be immunoprecipitated with anti-γ antiserum (data not shown).

In the *E. coli* cell, transcription and translation are temporally and physically coupled, while in a eukaryotic cell, there is no direct coupling between them. In *E. coli*, therefore, a DNA structure that impedes the movement of RNA polymerase might affect the movement of the translation machinery, leading to frameshifting. To address this possibility, I added purified dnaX mRNA that had been synthesized *in vitro* to an *E. coli* cell-free translation system (Fig 3A, lane 1). Both τ and γ were clearly detected, suggesting that efficient frameshifting could happen without translation-transcription coupling.

When the same mRNA was added to a rabbit reticulocyte lysate *in vitro* translation system, only τ was produced at a detectable level (Fig 3A, lane 2). A similar result was obtained with a wheat germ extract translation system (Fig 3B, lane 2). These results suggest that frameshifting in the dnaX gene is at best very inefficient when it is translated by an eukaryotic translation apparatus.

Absence of feedback regulation of frameshifting by τ and γ

To test if there is direct regulation of frameshifting efficiency by τ or γ, I added purified τ and γ to the *in vitro* translation system. Since the transcription-translation combined system supplemented with T7 RNA polymerase was much more efficient than the mRNA dependent system, the combined system was used for this analysis. As shown in Fig 4, addition of τ or γ to concentrations as high as 0.4mg/ml, which is significantly higher than their concentration in the wild type cell, did not affect the production of τ and γ. This indicates that there is no direct feedback regulation of frameshifting efficiency by the terminal products (τ and γ). Fuzzy bands of τ and γ seen with the addition of 2 μg of either protein confirm that these radioactive polypeptides are truly comigrating with purified τ and γ.

Elongation rate of translation does not affect the efficiency of frameshifting

Two kinds of experiments were performed to see how the overall speed of translation elongation would affect the frameshifting efficiency. The first experiment was to test the efficiency of γ production *in vitro* at various temperatures. The rate of translation elongation as determined from the pattern of appearance of τ and

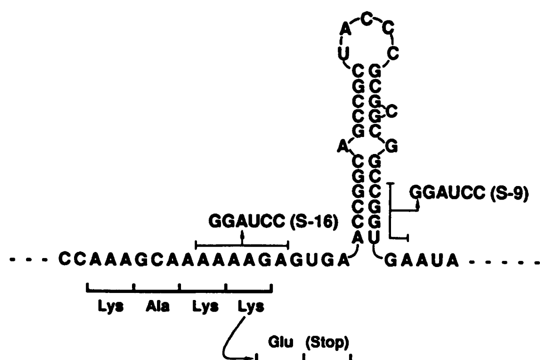

Figure 1. mRNA structure of the frameshift site of the dnaX gene. S-9 and S-16 are linker-scanning mutants with a BamHI recognition site at the position shown. Nucleotide numbering is according to Yin et al. (6).

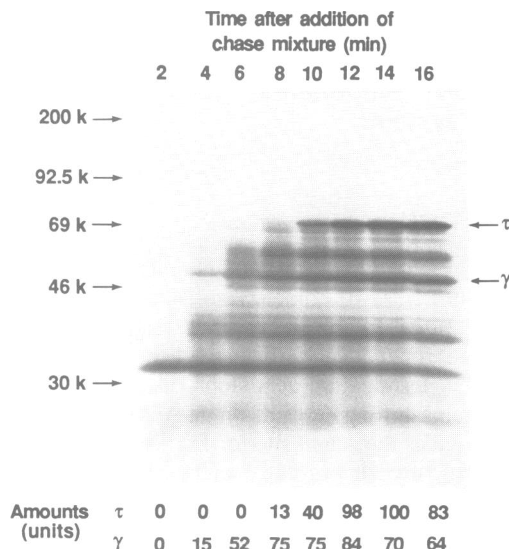

Figure 2. Production of τ and γ in a cell-free translation system. Reactions were carried out in a transcription-translation combined system with T7 RNA polymerase. After labeling with ^{35}S-methionine for 4min at 30°C, cold methionine (chase mixture) was added and the incubation was continued at 30°C for the indicated time.

γ was about three-fold higher at 37°C than at 23°C, but no significant change in the ratio of τ to γ was seen under these conditions (Fig 5).

Second, mutation of the rpsL gene, which encodes the S12 protein of the small ribosome subunit, is known to change the speed of ribosome movement (13). rpsL mutants or wild-type cells were transformed with dnaX overproducing plasmids and the amounts of τ and γ after heat induction were determined by Western blotting (Table 1). The mutant strains UK285, UK311, UK312, UK313 and UK314 all have altered rpsL genes with

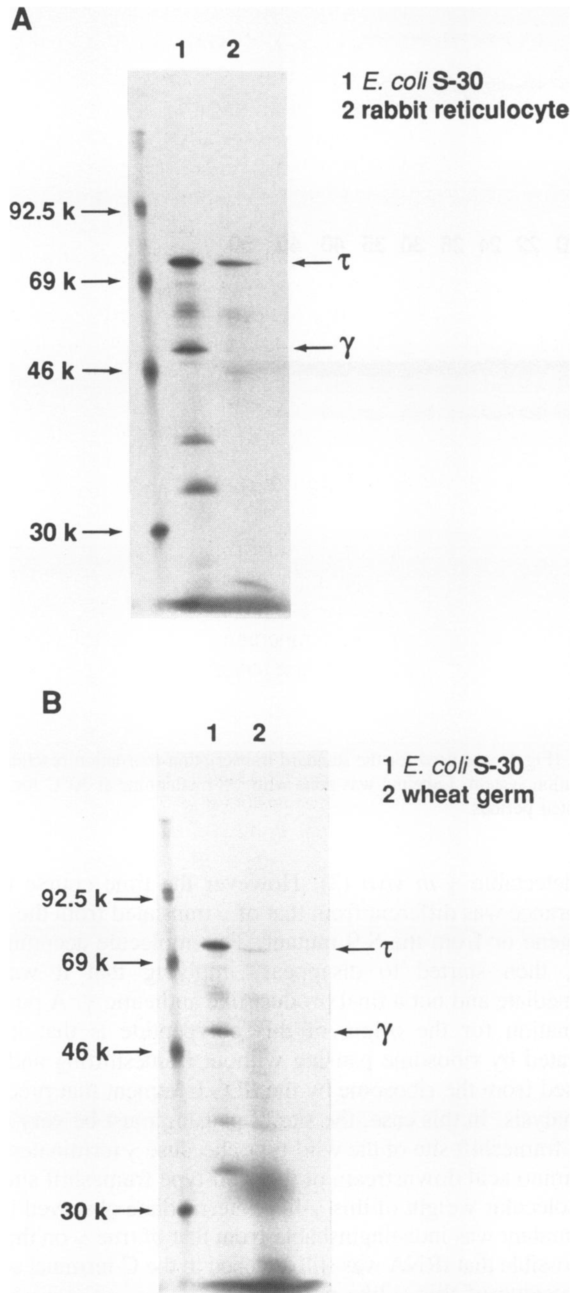

Figure 3. dnaX mRNA dependent products in various translation extracts. (A) ^{35}S-labeled products from E. coli S-30 translation system (lane 1) or rabbit reticulocyte lysate system (lane 2). (B) Same sample as the lane 1 of (A) (lane 1) or products from wheat germ extract (lane 2). All samples were run on a 10% SDS-polyacrylamide gel and labeled products were detected by fluorography.

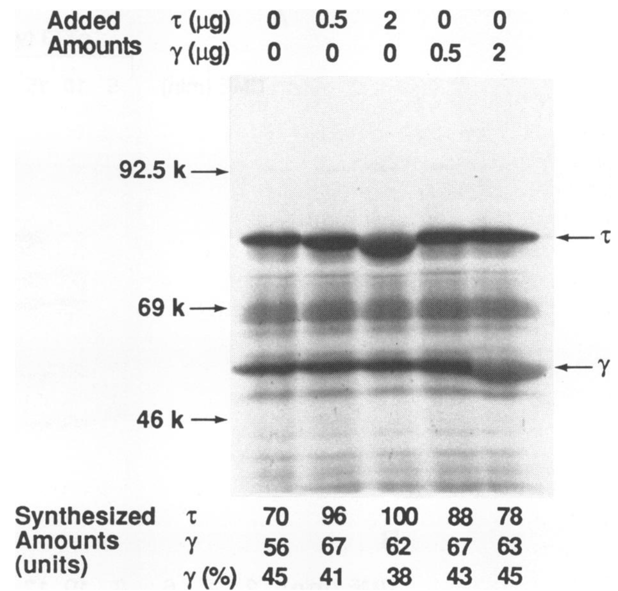

Figure 4. Addition of τ and γ to the *in vitro* translation system. A standard coupled transcription-translation reaction (5 μl) was done in the presence of purified τ or γ subunit in the indicated amount. After labeling with ^{35}S-methionine for 4 min, cold methionine was added, then incubation was continued for 30 min.

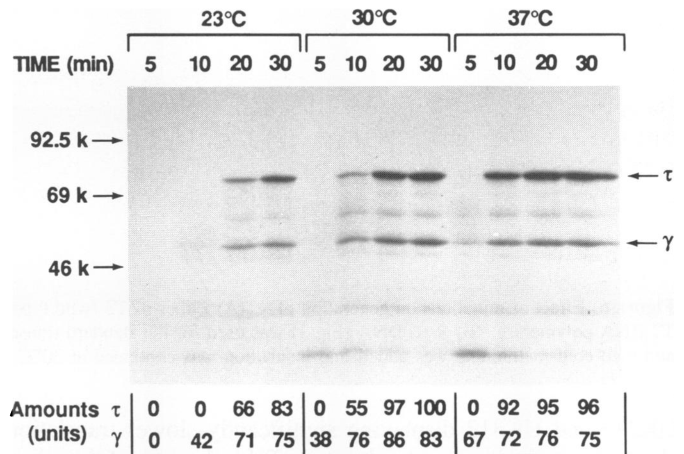

Figure 5. Effect of temperature on the production of τ and γ. Labeling was done at 30°C for 4 min with ^{35}S-methionine with the standard transcription-translation combined system. Then cold methionine was added and incubation was done at various temperatures for the indicated periods.

Table 1.

strain	genotype	γ (%)*	elongation rate (a.a./sec)**
UD132***	rpsL$^+$	60	14
UK285	rpsL282	57	7.8
UK311	rpsL221	66	14
UK312	rpsL222	56	9.3
UK313	rpsL224	62	12
UK314	rpsL226	67	14

* $\gamma/(\gamma+\tau)\times 100$ (%)
** amino acid residues elongated per minutes *in vivo* (13)
*** Δ(pro lac), Ara, gyrA, rpoB, argE. All other strains listed here share the same chromosomal markers except for rpsL gene (13).

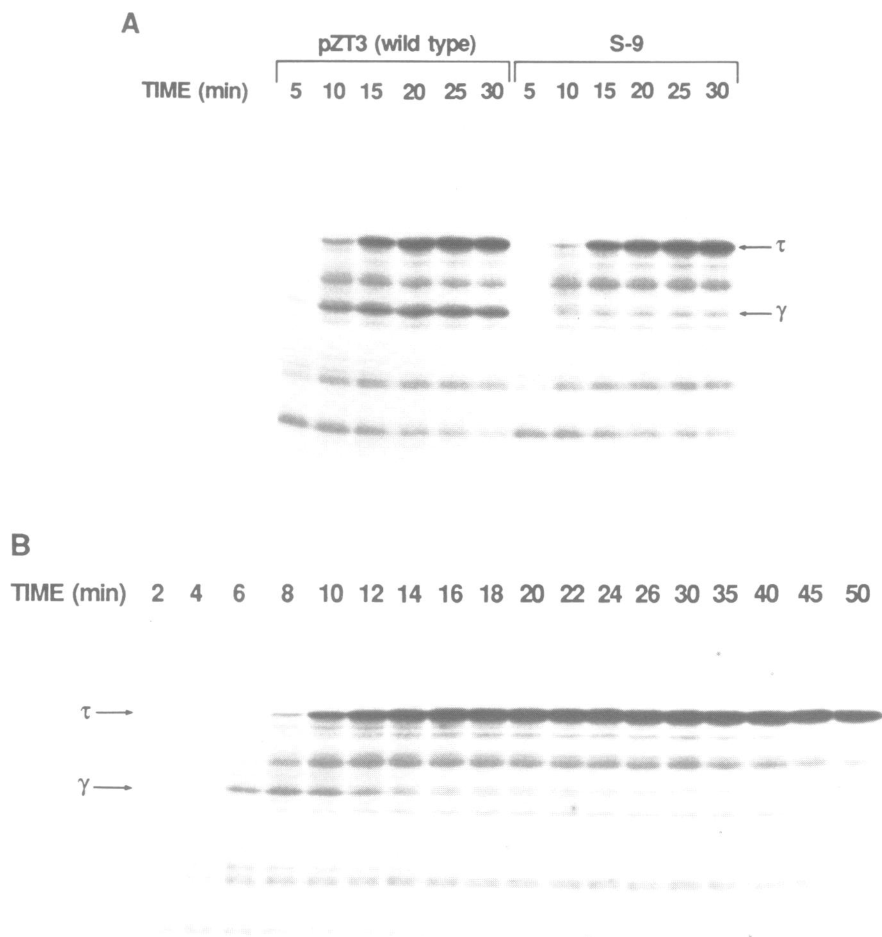

Figure 6. Effect of mutations on generation of γ. (A) Either pZT3 (wild type) or S-9 DNA (Fig 1) was used for the standard transcription-translation reaction with T7 RNA polymerase. (B) S-16 DNA (Fig 1) was used for the standard transcription-translation system. Labeling was done with ^{35}S-methionine at 30°C for 4 min and then cold methionine was added and incubation was continued at 30°C for the indicated periods.

UK285 and UK312 displaying significantly slower translation elongation rates *in vivo*. As shown in Table 1, none of them had a τ to γ ratio significantly different from that in the isogenic wild-type strain UD132.

From these experiments, I conclude that within the range tested, the overall speed of translation elongation had little or no effect on the dnaX frameshift frequency.

Effect of changes in the essential mRNA structure

To analyze the role of mRNA structure in frameshifting, I used templates with mutations in regions important for frameshifting *in vivo* (3) and compared the pattern of γ production with wild-type templates. Mutant S-9 has a BamHI linker insertion that disrupts the putative stem-loop structure (Fig 1) and shows 40% reduction in γ production *in vivo*. S-16 has a similar mutation in the heptamer motif that is the site of frameshifting (Fig 1). γ production was undetectable in this mutant *in vivo* (3). In the cell-free translation system, those mutants also showed weaker frameshifting than the wild-type gene (Fig 6).

Surprisingly, *in vitro* translation of the S-16 mutant yielded a γ-like polypeptide (Fig 6B), although this mutant did not make any detectable γ *in vivo* (3). However the time course of its appearance was different from that of γ translated from the wild-type gene or from the S-9 mutant. This molecule accumulated early, then started to disappear, implying that it was an intermediate and not a final product like authentic γ. A possible explanation for the origin of this polypeptide is that it was generated by ribosome pausing without frameshifting and was released from the ribosome by the SDS treatment that preceded gel analysis. In this case, the site of pausing must be very close to the frameshift site of the wild-type, because γ terminates only one amino acid downstream of the wild-type frameshift site and the molecular weight of this γ-like intermediate observed in the S-16 mutant was indistinguishable from that of true γ on the gel. It is possible that tRNA was still attached to the C-terminal amino acid residue of this γ-like polypeptide.

In contrast, with the S-9 mutant, the pattern of appearance of γ was similar to wild-type in a point that the amount of γ reaches a plateau at 10 min and then it remains almost constant until 30 min. But the amount of γ produced was smaller, paralleling the *in vivo* results. The ratio between the amounts of γ and τ plus γ, which reflects the frameshifting efficiency, was 0.39 for pZT3

and 0.19 for S-9 after 30 min incubation. These numbers are lower than those of in vivo expression (0.80 for pZT3 and 0.49 for S-9) (3), but the severity of inhibition by S-9 mutation was similar for *in vivo* and *in vitro*. The initial accumulation of γ and any potential γ-like products produced by pausing was even slower than in mutant S-16 (see Fig 6A,B lanes of 10 min incubation), suggesting that S-9 has a defect in the pausing step.

Since S-16 has a disruption in the frameshift site but the putative stem-loop is kept intact, while S-9 has a disruption in the stem-loop, it is likely that the putative stem-loop structure contributes to frameshifting by creating a strong pausing site. Thus, although the overall speed of translation is not critical, pausing at a specific site may be an important factor.

DISCUSSION

Role of heptameric motif

Two signals for the efficient translational frameshift in the dnaX gene are similar to signals found in frameshift sites used by many eukaryotic viruses (7); one is a heptameric motif (A AAA AAG for dnaX) and the other is a putative stable secondary structure located downstream. The heptameric motif is the place where the frameshift takes place in the case of dnaX (3) as well as in the case of retroviral genes. Jacks and colleagues proposed a 'simultaneous slippage model' in which ribosome slips back to the -1 frame on mRNA when an aminoacyl-tRNA is at the last codon of the heptamer motif (7). The sequence of this heptamer motif differs among genes, but it always allows both the A-site tRNA and the P-site tRNA to maintain basepairing at the essential first and second nucleotides of the codons after the slip. Every heptamer motif that has been identified as a frameshift site satisfies this rule and mutations that destroy this characteristic severely inhibit frameshifting (3,7).

Function of the stem-loop

The role of a downstream secondary structure is not entirely clear in the case of viral genes. In the cases of RSV and coronavirus IBV, disruption of a putative secondary structure (either stem-loop or pseudoknot) has a severe effect on the frameshift but a similar disruption in the HIV frameshift site showed no effect (14).

The stem-loop structure in dnaX is clearly important because its disruption decreased frameshifting (3). But even when this structure was completely removed, there was detectable frameshifting (about 10% of wild type) (3). Thus this structure can be considered as an 'enhancer' for frameshifting rather than a part of minimal essential region.

Jacks et al. proposed a hypothesis that these secondary structures impede the movement of the ribosome in order to prolong its interaction with the slippery heptamer motif, so that the chance of frameshifting is increased (7). Our observation that there is a strong pausing site near the frameshift site, and that the stem-loop seems to be important for pausing, provide support for this model.

In contrast, changing the overall speed of translation had no apparent effect on frameshifting. Perhaps this change did not affect the duration of the pause, or perhaps it affected pausing but even at the highest translation speed tested, the degree of pausing was already at the saturation level and its enhancement by slowed translation did not add any additionl frameshifting.

Role of tRNA structure

Frameshifting in dnaX can be classified as 'retrovirus type', since it shifts translation to the -1 frame at a slippery heptamer that is followed by a stable RNA secondary structure. It is clearly different from other examples of frameshifting such as in the *E. coli* releasing factor RF2 gene (15,16) or yeast transposable element Ty1 (17,18). In those cases translation shifts to the $+1$ frame, and the structural requirements are very different from those in the dnaX or retroviral cases. So far there are only two cases of retroviral type frameshifting known in prokaryotes: the transposase gene of insertion element IS1 (19) and dnaX.

The heptamer motif of dnaX has a sequence of A AAA AAG and although this sequence satisfies the rule proposed by Jacks et al., no heptamer motif with this sequence has yet been found in viral frameshift sites in eukaryotes. In these viruses, when the heptamer motif has a stretch of six adenine residues, it always is followed by a C residue rather than by a γ residue like dnaX. The mouse mammary tumor virus *gag/pro* frameshift site, which has an A AAA AAC motif, induces weak frameshifting in *E. coli* (2.1%), but higher frameshifting in a mammalian system (23%). Changing the heptamer from A AAA AAC to A AAA AAG sequence resulted in vigorous frameshifting (57%) in *E. coli*, indicating that the A AAA AAG motif is a more efficient signal in *E. coli* (20). In the dnaX gene, which already has the A AAA AAG motif, frameshifting was very efficient and as expected, changing the γ residue to a C residue reduced frameshifting at this site in *E. coli* (unpublished). Conversely, the natural dnaX frameshift site with the A AAA AAG heptamer motif did not give detectable frameshifting in a wheat germ or rabbit reticulocyte system (Fig. 3).

What accounts for these different sequence preferences in the different systems? One attractive model is that they are determined by the structure of the tRNAs in each species. A recent report on translational frameshifting in the yeast Ty1 element showed that one species of $tRNA^{Leu}$ that is specific to the yeast system is responsible for this frameshifting (18). Probably only certain tRNAs have a tendency to slip, and that is why only a limited set of codons is found in slippery heptamer motifs (especially at the 3′ codon of the 0 frame). In eukaryotes, only AAC, UUA and UUU codons have been found in the 3′ position of the heptamer motif (7). Interaction between these codons and cognate tRNAs ($tRNA^{Asn}$ for AAC, $tRNA^{Leu}$ for UUA and $tRNA^{Phe}$ for UUU) from eukaryotes may be particularly unstable and thus permit frameshifting. It is possible that only a subspecies of those tRNAs (such as hypomodified tRNA) are permissive for frameshifting. Infection by a retrovirus reportedly induces hypomodified tRNAs corresponding to the codons of frameshift sites, leading to the speculation that these tRNAs are involved in frameshifting (21).

Since the structures of *E. coli* tRNAs are different from eukaryotic tRNAs, the *E. coli* tRNAs susceptible to slippage may differ from those in the eukaryotes. The AAG codon is the site of the very efficient frameshifting in the dnaX gene; therefore $tRNA^{Lys}$ might be a 'slippery' tRNA in *E. coli*. If so, the efficiency of frameshifting is so high (45–80%) that most of the tRNAs (not a minor subspecies) that recognize the AAG lysine codon would be expected to have this property.

In all eukaryotic cells analyzed to date, there are two different species of $tRNA^{Lys}$ present for the non-mitochondrial translation system (22). One of the mammalian species has an anticodon of U*UU where U* is a 5-methoxycarbonylmethyl-2-thiouridine, and the other has a CUU anticodon. It is likely that the species

with CUU interacts more strongly with the AAG codon than with the AAA codon, while the one with U*UU interacts more strongly with the AAA codon. These selective interactions have in fact been demonstrated for yeast tRNAs (23). If this is the case, the A AAA AAG sequence would not promote frameshifting in eukaryotes because $tRNA^{Lys}$ bound to the AA-G codon would have a CUU anticodon, and thus interact stably with AAG, with little affinity for the AAA codon at the -1 position.

In contrast, only one species of *E. coli* $tRNA^{Lys}$ has been reported, and it has an anticodon of U**UU, where U** is a 5-methylaminomethyl-2-thiouridine (22). This tRNA must therefore decode both AAA and AAG lysine codons. But it has been proposed that interaction of the modified U residue with a γ residue is weaker than the interaction with an A residue (24,25). The interaction of this $tRNA^{Lys}$ with AAG might therefore be relatively unstable compared to its interaction with AAA. This could greatly facilitate the frameshifting to the -1 frame at an AAG codon following an A residue. To examine this hypothesis, physical understanding of tRNA structure and tRNA-mRNA interactions using natural and artificially modified tRNAs will be important. If this model is correct, one reason for the occurrence of only a single species of $tRNA^{Lys}$ in an *E. coli* cell might be to generate the γ subunit efficiently by translational frameshifting.

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