Sequence requirements for efficient translational frameshifting in the *Escherichia coli dnaX* gene and the role of an unstable interaction between tRNA\(^{Lys}\) and an AAG lysine codon

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Synthesis of the 7-subunit of DNA polymerase III holoenzyme depends on precise and efficient translational frameshifting to the –1 frame at a specific site in the *dnaX* gene of *Escherichia coli*. In vitro mutagenesis of this frameshift site demonstrated the importance of an A AAA AAG heptanucleotide sequence, which allows two adjacent tRNAs to retain a stable interaction with mRNA after they slip to the –1 position. The AAG lysine codon present in the 3’ half of this heptanucleotide was a key element for highly efficient frameshifting. A tRNA\(^{Lys}\) with a CUU anticodon, which has a strong affinity for AAG lysine codons, is present in eukaryotic cells but absent in *E. coli*. Expression in *E. coli* of a mutant tRNA\(^{Lys}\) with a CUU anticodon specifically inhibited the frameshifting at the AAG codon, suggesting that the absence of this tRNA in *E. coli* contributes to the efficiency of the *dnaX* frameshift.

[Key Words: *dnaX* gene; translational frameshifting; in vitro mutagenesis; tRNA\(^{Lys}\)]

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In this paper we describe an investigation of the codon–anticodon interaction at the shifty heptanucleotide motif in the dnaX gene. Our results verify the importance of favorable codon–anticodon pairing at two adjacent codons in the −1 frame, and point to a weak interaction between E. coli tRNA and an AAG lysine codon as a key determinant of efficient frameshifting in this system.

**Results**

*Importance of the A AAA AAG sequence for frameshifting*

The mRNA sequence around the frameshift site of dnaX is shown in Figure 1. The underlined A AAA AAG sequence, which satisfies the X XXX Y YZ rule for simultaneous slippage (Jacks et al. 1988b), has been shown by protein sequence analysis to be the site of a −1 frameshift. A putative downstream stem–loop structure enhances this frameshifting (Tsuchihashi and Kornberg 1990).

To investigate the role of this heptamer in dnaX, we examined a series of point mutations in the heptamer region for their effects on frameshifting. Mutated genes were expressed using an inducible T7 RNA polymerase gene. After induction by isopropyl β-thiogalactopyranoside (IPTG), amounts of γ-subunit (the in-frame product) and γ-subunit (the frameshift product) were determined by Western blotting. The frameshifting efficiency was calculated from the amounts of the two polypeptides. Figure 2A shows the results of this analysis. Any change introduced into the heptamer decreased frameshifting, whereas mutations outside the heptamer had little or no effect. The whole A AAA AAG sequence is therefore important for efficient frameshifting. Mutant ins1 (Fig. 2B), which has a 3-nucleotide insertion between the heptanucleotide sequence and the putative stem–loop, still showed efficient frameshifting, although not as efficient as wild type. Thus, there is some flexibility in the required spacing between the dnaX frameshift site and the predicted stem–loop structure, in contrast to the IBV frameshift site, where the precise spacing from the downstream pseudoknot structure is critical (Brierley et al. 1989).

Mutant 4G, which contains an A AAG AAG heptamer (Fig. 2B), retains surprisingly efficient frameshifting (46%). Simultaneous slippage on this heptanucleotide would require a codon–anticodon interaction with a G-U base pair at the first position of the triplet, which is never observed in standard codon decoding. This result, therefore, raised the possibility that slippage need not be simultaneous but can involve the upstream A AAG alone. Introduction of an additional mutation in the downstream AAG codon inhibited frameshifting (Fig. 2B, 4G6U, 4G7C, or 4G7A), however, suggesting that frameshifting still involves tRNA slippage at this codon. Perhaps the interaction between tRNA and the GAA codon in the −1 frame is stabilized to some extent by the G-U base pair at the first position of this codon. The absence of detectable frameshifting in mutant 1G4G (G AAG AAG), however, suggests that simultaneous slippage from AAG to GAA at both sites is very inefficient. Alternatively, the change of the AAA codon to an AAG codon might enhance the slippage of the P-tRNA at this position, partially compensating for the unfavorable UU/GAA pairing in the −1 frame. As we discuss later, slippage does occur more readily from an AAG codon than from an AAA codon.

The results in Figure 2, suggesting that frameshifting in dnaX occurs by a simultaneous slippage of two adjacent tRNAs, led us to examine whether other sequences that satisfy the X XY Y YZ rule can support frameshifting. Single A → G changes at position 1, 2, or 3 (first 3 nucleotides of the heptamer) reduced the frameshift frequency by at least fourfold (Fig. 2A), but when all of these positions were simultaneously changed to G, efficient frameshifting was restored (Fig. 2B, mutant 123G). Not only 3 Gs, but also 3 Cs or 3 Us in these positions supported efficient frameshifting, although none of them was as favorable as the original 3 As (Fig. 2B, mutants 123C and 123U). Thus, the identity of the first 3 nucleotides in the heptamer, which should provide the codon to which the P-tRNA slips, is of secondary importance as long as they are identical.

The sequence requirements for positions 4–7, which provide the site for the A-tRNA to slip, are different. We compared the mutant heptanucleotide sequence A AAG GGA (456G7A) with G GGA AAG (123G) and A AAG AAG (4G) (Fig. 2B,C). It is clear that 456G7A does not allow efficient frameshift but 123G and 4G do. From this result, we conclude that the sequence G GGA promotes frameshifting only when it is the site for the P-tRNA slippage, whereas A AAG can promote slippage of either A-tRNA or P-tRNA. The requirements for the slippage of the A-tRNA are thus different and, perhaps, more strict than those for the P-tRNA.

A striking implication of these results is that an AAG
The wild-type gene (pZT3) had a frameshift frequency of 81% in this experiment. (C) Expression of T and γ was determined, and frameshift frequency was calculated as the number of T plus 7 (xl00%). The regulation of T7 RNA polymerase and rifampicin produced 7 even more efficiently than the wild-type sequence (Fig. 3A). Surprisingly, when the gene was transcribed instead by E. coli RNA polymerase, very little γ was produced by this mutant, although γ was still efficiently formed from the wild-type gene under the same conditions (Fig. 3B). Thus, efficient production of γ-subunit from mutant 7A depended on transcription by T7 RNA polymerase. Moreover, mRNA transcribed from this mutant gene by T7 RNA polymerase could direct very efficient production of γ in a rabbit reticulocyte lysate translation system, although this system does not support frameshifting in the wild-type dnaX gene (Fig. 3C).

The likely explanation for this T7 RNA polymerase-dependent production of γ is that T7 RNA polymerase slips during transcription on the extended A stretch in mutant 7A, inserting an extra A. It has been shown previously that E. coli RNA polymerase can insert an extra A when it transcribes a sequence of 10 or more consecutive As. Yeast RNA polymerase II was not prone to misincorporation when the same template was used (Wagner et al. 1990). Our results suggest that T7 RNA polymerase is prone to insert an extra A at an even shorter A stretch. Because position 8 is also an A, mutant 7A has a stretch of 8 As. Because mutant 7A8G showed similar γ production in our T7 RNA polymerase-dependent expression system (Fig. 3), a stretch of 7 As seems to be sufficient. A stretch of 6 consecutive As, however, did not appear sufficient to promote this slip, because mRNA transcribed by T7 RNA polymerase from the wild-type gene, or mutants 7C or 7U, failed to direct production of γ in a reticulocyte lysate translation system (Fig. 3C).

Slippage of T7 RNA polymerase on the A stretch

Mutant 7A, which has an A AAA AAA heptanucleotide, gave fairly efficient production of γ-subunit in an in vivo assay system, which depends on T7 RNA polymerase [Fig. 2A]. In vitro transcription—translation of this mutant with an E. coli S-30 system supplied with T7 RNA polymerase and rifampicin produced γ even more efficiently than the wild-type sequence [Fig. 3A]. Surpris-
Expression of \( \tau \) and \( \gamma \) in vitro from wild type \([A AAA AAG]\) and mutants \(7A\) \([A AAA AAA]\), \(7C\) \([A AAA AAC]\), and \(7U\) \([A AAA AAU]\). (A) DNA-dependent transcription-translation coupled system with an \(E. coli\) S-30 extract supplemented with rifampicin and T7 RNA polymerase. (B) DNA-dependent transcription-translation system without rifampicin or T7 RNA polymerase. (C) Translation in rabbit reticulocyte lysate with mRNA synthesized in vitro by T7 RNA polymerase.

AAG at the second codon is essential for efficient frameshifting

Any nucleotide at position 7 can satisfy the X XXY YYZ rule, but our analysis of mutations at this position (Figs. 2 and 3A,B) showed that a G was required at position 7 for efficient translational frameshifting in \(E. coli\). A similar result was obtained by Weiss and colleagues (1989), studying the expression of wild-type and mutated mouse mammary tumor virus (MMTV) gag/pro frameshift sites in \(E. coli\). The A AAA AAC sequence at this MMTV site had to be altered to A AAA AAG to allow vigorous frameshifting in \(E. coli\), although the original heptamer promoted efficient frameshifting in a rabbit reticulocyte lysate system [Jacks et al. 1987; Moore et al. 1987; Weiss et al. 1989; Chamorro et al. 1991].

Using a rabbit reticulocyte lysate system, we detected little or no production of \(\gamma\) from wild-type (A AAA AAG), mutant 7C (A AAA AAC) or mutant 7U (A AAA AAU) mRNA (Fig. 3C). [The only exception was mutant 7A, from which \(\gamma\) production is presumed to be the consequence of a transcriptional slippage.] The failure of an A AAA AAC sequence in the context of the \(dnaX\) gene to direct efficient frameshifting in the mammalian system may be the result of the absence of a pseudoknot structure (Pleij et al. 1985) in the \(dnaX\) frameshift region. Such a structure, placed downstream of the shifty heptamer, is required for efficient frameshifting at the A AAA AAC heptamer in MMTV and in several other eukaryotic systems (Brierley et al. 1989; Chamorro et al. 1991; Dinman et al. 1991).

Absence of tRNA\(^{lys}\) with a CUU anticodon in \(E. coli\) contributes to the efficient frameshifting in the \(dnaX\) gene

Why is A AAA AAG such a good frameshift site in \(E. coli\), but not in mammalian cells (Fig. 3C; Chamorro et al. 1992) or in yeast cells (Dinman et al. 1991)? We have suggested a model involving the difference of tRNA\(^{lys}\) species in \(E. coli\) and eukaryotic cells (Tsuchihashi 1991). Figure 4 shows the anticodons of \(E. coli\) and mammalian tRNA\(^{lys}\) molecules [Spinzl et al. 1989]. In \(E. coli\), it is likely that all the tRNA genes have been identified, yet no tRNA\(^{lys}\) with a CUU anticodon has been found [Komine et al. 1990]. Thus, both the AAA and the AAG lysine codons are decoded by a single tRNA\(^{lys}\), which has a U*UU anticodon where U* is 5-methylaminomethyl-2-thiouridine. 2-Thiouridine derivatives, found at the wobble position in several tRNA species, have been shown to have a much higher affinity for A than G (Lustig et al. 1981; Yokoyama et al. 1985). The interaction of \(E. coli\) tRNA\(^{lys}\) with the AAG lysine codon may therefore be unusually weak, compared with its interaction with an AAA codon. This would favor the slippage from AAG to AAA that needs to occur at the second codon in the A AAA AAG sequence. Mammals and other eukaryotes have a tRNA\(^{lys}\) with a CUU anticodon. This tRNA is likely to decode most of the AAG lysine codons, as it has a higher affinity for this codon than does tRNA\(^{lys}\) with a U*UU anticodon [Lustig et al. 1981].
Decoding of an AAG codon by the CUU-type tRNA in eukaryotic cells might resist the slip back.

We tested whether the tRNA^{CUU}\textsuperscript{Lys} could suppress frameshifting at an AAG codon. First, we inserted a DNA fragment containing an E. coli tRNA\textsuperscript{Lys} gene downstream of the dnaX gene in plasmid pZT3 so that both tRNA\textsuperscript{Lys} and the dnaX gene were transcribed by T7 RNA polymerase. A T \rightarrow C change was then introduced in the anticodon region to generate a gene for tRNA\textsuperscript{CUU} with a CUU anticodon.

When expression of dnaX was induced along with this novel tRNA, production of \gamma was lower than when the dnaX gene was induced alone (Fig. 5A). The frameshift-suppressing effect was specific to the mutant tRNA, because overproduction of wild-type tRNA\textsuperscript{Lys} did not affect the production of \gamma (Fig. 5A). To test whether the effect of the mutant tRNA was specific for the AAG lysine codon, we constructed similar tRNA overproducers coexpressing dnaX genes with mutated frameshift sites (Fig. 5B–D). tRNA\textsuperscript{CUU} inhibited frameshifting at an A AAG AAG sequence but had no effect on \gamma production from mutant dnaX genes with either the A AAA AAA or the A AAA AAC sequence. Thus, inhibition of frameshifting by this mutant tRNA clearly depends on the presence of an AAG codon at the frameshift site. The extent of inhibition was far from complete in the experiment shown in Figure 5A (from 77–41%), probably because there was little mutant tRNA present at the early stage of dnaX induction as a result of the structure of our construct. In fact, when \textsuperscript{35}S-pulse–labeling was done 1 hr after induction of dnaX and mutant tRNA\textsuperscript{Lys} expression, inhibition of frameshifting by mutant tRNA was much more pronounced (from 84–19%, Fig. 5E). Because we do not know how much of the mutant tRNA is transcribed, correctly processed, modified, and charged by lysyl-tRNA synthetase, the observed suppression in this experiment may still be incomplete owing to inefficient production of properly charged tRNA in our system. We conclude that tRNA\textsuperscript{CUU} with a CUU anticodon can suppress frameshifting on an AAG lysine codon and that the lack of this tRNA in E. coli promotes efficient production of the \gamma-subunit.

**Heptamers related to the dnaX frameshift site in other E. coli genes**

Because A AAA AAG is such an efficient frameshifting signal in E. coli, and this sequence can cause a frameshift in the dnaX gene with 6–8% frequency even without a downstream stem-loop (Tsuchihashi and Kornberg 1990), any E. coli gene with this heptamer in the correct reading frame might be a candidate to express a frameshift product. We searched the GenBank DNA sequence data base for E. coli genes in which this motif occurred in the appropriate phase, with an open reading frame of at least 100 codons upstream, and at least 100 codons in
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either the 0 or −1 frame downstream of the heptamer. Eight sequences (the dnaX, pyrD, proU, lysR, and ereB genes, open reading frames in the ilv and glycollen gene clusters, and the Ec67 retron) were found to contain the A AAA AAG heptamer in the specified context, two genes [fumA, asnB] contained the A AAG AG heptamer, and two genes [hlyB, rhaA] had X XXA AAG heptamers with X other than A. Except for dnaX, none had a sequence immediately downstream that appeared likely to form a highly stable stem-loop structure. Nevertheless, we are currently investigating the possibility of frameshifting at these sites.

One might expect synonymous alternative sequences to be preferred to potential shifty heptamers, if these sites were prone to translational frameshifting. A search of E. coli genes in the GenBank data base for sequences synonymous to the heptamers A AAA AAG, A AAG AG, or X XXA AAG, appropriately positioned within or between open reading frames, does not reveal such a preference, when the bias in usage of individual synonymous codons is taken into account (Sharp et al. 1988) [data not shown]. Moreover, the synonymous nonshifty heptamer A AAG AAA [11 occurrences] is not found significantly more frequently than A AAG AAG (eight occurrences). In the same setting, the first nucleotide is A in 14% of N AAA AAG heptamers, versus 13% of N AAA AAA heptamers, again suggesting that there is little bias against this family of potentially shifty heptamers.

**Discussion**

Translational frameshifting in the dnaX gene can be explained by the simultaneous slippage model [Jacks et al. 1988b], which was originally proposed for retroviral frameshifting. In this study we have shown that dnaX frameshifting, as with retroviral frameshifting, requires a heptameric sequence that would allow two adjacent tRNAs to maintain a stable codon-anticodon interaction after slipping to the −1 frame.

Overall conformity to the X XXY YYZ motif was a recurring, but not universal, characteristic of the heptamer sequences that could promote frameshifting in this system. The exact identity of the codon–anticodon pairs involved was an additional important determinant of frameshifting. Our mutagenesis studies show that in the dnaX frameshift signal, the sequence requirement for the second codon, where the A-tRNA slips, is different and apparently more strict than that for the first codon, where the P-tRNA slips. For both codons, it is important that the tRNAs are able to maintain base-pairing at the first and second nucleotide positions after the slip. But for the second codon, this appears not to be sufficient—an unstable interaction between tRNA and this codon seems to be an added requirement for efficient frameshifting. In the case of the dnaX gene, it is important that this position is specifically an AAG lysine codon, presumably because of the unstable interaction of this codon and tRNA$^{15}$. One possible explanation for the different requirements at the two sites is that the interaction between P-tRNA and mRNA may be inherently weaker than that between A-tRNA and mRNA. Because the base-pairing between codon and anticodon is not strong enough to explain by itself the accuracy of translation (Hershey 1987), tRNA–mRNA pairing must involve additional interactions mediated by the ribosome or other components. These indirect interactions between P-tRNA and mRNA may be weaker than those between A-tRNA and mRNA, allowing P-tRNA to slip even in the presence of a relatively stable codon–anticodon interaction. A less likely alternative explanation is that slippage of A-tRNA can sometimes occur without slippage of P-tRNA.

The observed preference for the AAG lysine codon in E. coli frameshifting is noteworthy as it is not observed in eukaryotic frameshifting. Recent experiments confirm that the wild-type MMTV frameshift site [AAA AAA] is a much better frameshift site in eukaryotic systems than a mutant with an A AAA AAAG heptamer motif (Chamorro et al. 1991). Our results showed that the heptanucleotide sequence AAA AAG is especially slippery in E. coli because E. coli lacks a tRNA$^{15}$ with a CUU anticodon. It has been speculated that translational frameshifting is carried out by specific slippery, or shifty, tRNAs [Weiss 1984; Hatfield et al. 1990]. In the case of the frameshift in the yeast Ty1 transposon, a tRNA$^{15}$ with a UAG anticodon, which is specific to the yeast or mitochondrial system, is involved (Belcourt and Farabaugh 1990). Although, unlike retroviral or dnaX frameshifting, this particular frameshift is to the +1 frame and is caused by a slippage of a single tRNA, it is one case where frameshifting depends on the presence of a specific tRNA. Our findings with dnaX show that not only the presence of a specific tRNA, but also the absence of a specific tRNA, can be important for efficient frameshifting.

Weak codon–anticodon interactions at the second codon in the heptamer may also be important for the −1 frameshifts that have been recognized in eukaryotic systems. The codons found at this position in eukaryotic frameshift sites are enriched for As and Us, perhaps avoiding the stronger codon–anticodon interaction caused by G-C base pairing. In those sites that have an X XYY YYZ heptameric motif, only UUA, UUU, and AAC have been found as the second codon. Among the four AAX codons, AAG did not promote efficient frameshifting when it was the second codon in the frameshift site of yeast L-A virus or MMTV [Chamorro et al. 1991; Dinman et al. 1991], probably because tRNA$^{15}$ with a CUU anticodon is normally present in yeast and mammalian cells. In the L-A viral frameshift site, an AAA codon could induce frameshifting in yeast as efficiently as the original AAC codon. In the present study, efficient transcriptional slippage by T7 RNA polymerase prevented accurate measurement of translational frameshifting at the A AAA AAA heptamer, although it was clearly less efficient than at A AAA AAG [Fig. 3B].

In an MMTV gag/pro context, the A AAA AAA sequence gave little frameshifting in E. coli [∼1% of that
seen with the A AAA AAG sequence (Weiss et al. 1989). This species difference is not easily explained by differences in anticodon sequences, because in both cases, the AAA codon is expected to be decoded by a tRNA^{Lys} with an anticodon of UUU type.

Both AAC and AAU are codons for asparagine. E. coli and mammalian cells usually have only tRNA^{Asn} with the QUU anticodon, where Q is a queosine residue, although some cancer cells have tRNA^{Asn} with the GUU anticodon owing to an incomplete modification (Spinzl et al. 1989). AAC is found more frequently than AAU as the second codon in natural eukaryotic frameshift sites, and when directly compared in the MMTV gag/pro frameshift site, AAC at this position promotes frameshifting at a higher rate than AAU (Chamorro et al. 1991). It seems likely, therefore, that tRNA^{Asn}[QUU] interacts more strongly with AAU than with AAC.

For UUC and UUU phenylalanine codons, eukaryotes have only tRNA^{Phe} with a GAA (G* = 2'-O-methylguanosine) anticodon, and in E. coli the only tRNA^{Phe} has a GAA anticodon. These anticodons are expected to interact more strongly with UUC than with UUU. This might account for the observation that UUU is preferred over UUC as the second codon in eukaryotic frameshift sites, and when directly compared, promotes more frequent frameshifting (Jacks et al. 1988b). In E. coli, too, UUU supports more frameshifting than UUC, when tested in the context of the HIV frameshift site (Weiss et al. 1989). Thus, from the available data on frameshift sites, it appears that the inherent slipperiness of a tRNA can often be predicted from its anticodon sequence. More information on codon–anticodon interactions involving different tRNAs, including unnatural tRNAs modified in vitro (Bruce et al. 1986; O'Connor et al. 1989), should provide a clearer understanding of the role of specific codons in translational frameshifting.

A search of sequences in the GenBank data base identified several additional E. coli genes with an AAA AAG motif. We do not know yet whether this heptamer promotes frameshifting in any of these genes. In the case of the dnaX gene, the frameshift product is the γ-subunit of DNA polymerase III holoenzyme, the essential enzyme for DNA replication. Although the γ-subunit is clearly essential for DNA polymerase function, it remains to be determined whether the γ-subunit is required (O'Donnell 1987; Maki and Kornberg 1988). The demonstration that in vitro, the γ-subunit complements a mutant allele of dnaX more effectively than does the γ-subunit implies that the γ-subunit may have an important distinct role in DNA replication (Tsukihashi and Kornberg 1989). Perhaps this role for the γ-subunit, and the corollary requirement for efficient translational frameshifting in dnaX, is sufficient to account for the lack of tRNA^{Lys}[CUU] in E. coli and other prokaryotes. In view of this striking gap in the anticodon table and the resulting propensity for significant frameshifting from AAG codons in diverse sequence contexts, however, the phenomenon of programmed translational frameshifting from an AAG codon may not be confined to the dnaX gene.

### Materials and methods

#### DNA construction

Plasmid pZT3 contains the dnaX gene under the control of the T7 phage gene 10 promoter (Tabor and Richardson 1989; Tsukihashi and Kornberg 1989). All mutations were generated using a polymerase chain reaction (PCR)-mediated mutagenesis method (Helmley et al. 1989). To construct a plasmid expressing dnaX and tRNA^{Lys}, a DNA fragment containing the E. coli tRNA^{Lys} gene was amplified by PCR from pAp5u·β phage clone (Yoshimura et al. 1984) using a pair of PCR primers, 5'-AGC TCT GCA GCC CGC CGG CTA CGG GAT ATC A-3' and 5'-AGC TAT CGA TAA GCA CTC GGC CAG TGG-3', which contain a PstI site and a ClaI site. This fragment was cloned into the PstI and ClaI sites of pZT3 (Tsukihashi and Kornberg 1989), both of which are located downstream of dnaX gene.

#### Quantitation of frameshift efficiency

All plasmids were introduced into E. coli K12 BL21(DE3) strain (Studier et al. 1990), which contains an IPTG-inducible T7 RNA polymerase gene. Transformed cells were grown in LB medium with 70 μg/ml of carbenicillin at 37°C, and 1 mM IPTG was added when the culture reached OD_{595} = 0.5. One milliliter of the cell culture was harvested 1 hr after induction, resuspended in 47 μl of sample loading buffer (Laemmli 1970), and lysed by boiling. The cell lysates were diluted 1000-fold and 1–10 μl of the diluted sample was subjected to 10% polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose filter (Schleicher & Schuell) by electroblotting, and γ and τ were visualized using rabbit anti-τ antisem and the ECL kit (Amersham). The intensities of the bands were determined with a Molecular Dynamics densitometer and compared with those of known quantities of purified τ and γ (Tsukihashi and Kornberg 1989). For a pulse-labeling experiment, 20 μCi of [35S]methionine (Trans-label, ICN) was added to 0.2 ml of the culture and incubated for 5 min at 37°C. The cells were then harvested, and labeled proteins were analyzed by 10% polyacrylamide gel electrophoresis. Amounts of τ and γ were determined from the radioactivity in each band and corrected for methionine contents. Frameshift efficiency was calculated as [quantity of γ/quantity of τ] × 100.

#### In vitro translation

DNA-dependent transcription–translation reactions were carried out using an E. coli S-30 translation kit (Amersham), following the supplier's manual with minor modifications. Ten microliters of the reaction mixture was added to 0.5 μg of supercoiled plasmid DNA and 1.5 μCi of [35S]methionine (ICN), and was incubated at 30°C for 5 min. Cold methionine was added and incubation was continued for an additional 20 min. After the reaction, samples were run on a 10% polyacrylamide gel (Laemmli 1970) and the gel then was soaked in 1 M sodium salicylate, dried, and subjected to autoradiography. T7 RNA polymerase-dependent reactions were performed under the same conditions except for the addition of 0.4 μg of rifampicin and 2 units of T7 RNA polymerase.

The mRNAs for the rabbit reticulocyte lysate translation reactions were synthesized using T7 RNA polymerase and a capping kit (Stratagene), with PstI-digested plasmid DNA template. mRNA (0.5 μg) was added to 10 μl of rabbit reticulocyte lysate.
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[Promega] with 4 μCi of [35S]methionine and kept at 30°C for 45 min. Translation products were detected as described above.

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