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Frameshifting in Gene 10 of Bacteriophage T7

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Gene 10 of bacteriophage T7, which encodes the most abundant capsid protein, has two products: a major product, 10A (36 kDa), and a minor product, 10B (41 kDa). 10B is produced by frameshifting into the −1 frame near the end of the 10A coding frame and is incorporated into the capsid. The frameshift occurs at a frequency of about 10% and is conserved in bacteriophage T3. This study shows that sequences important to frameshifting include the originally proposed frameshift site, consisting of overlapping phenylalanine codons and the 3′ noncoding region that includes the transcriptional terminator over 200 bases downstream of the frameshift site. The frameshift occurs at the overlapping phenylalanine codons as determined from peptide sequencing data. Complementation studies show that there is only a very weak phenotype associated with phage infections in which there is no 10A frameshifting. Capsids from such infections are devoid of 10B and are as stable as wild-type capsids.

The accuracy by which genetic information is copied into a protein sequence is probably a trade-off between the final requirement of a certain amount of active product and the energy cost of avoiding errors during synthesis. Careful studies of the translational apparatus have revealed a background error frequency that is, as expected, very low (for an excellent review, see reference 12). However, there are now a number of examples of what appear to be mistakes that occur at frequencies far higher than expected. Perhaps the most characterized of these are the frameshifts (1). In these cases a significant proportion of ribosomes switch reading frames at a specific site in a coding region, thus entering a new frame. The result is that a single mRNA can produce two products, a protein from the normal coding frame and a transframe fusion protein. In many cases it is known that the frameshift product is essential (7). Thus it appears that, where it is advantageous to synthesize two such products, specific properties of the frame-maintaining process are exploited. Knowledge of the signals that subvert normal frame maintenance could be very informative about how the translational apparatus works and how it can be regulated.

Probably the most studied of the frameshifts are the retroviral *gag-pol* and coronaviral examples and the *Escherichia coli* release factor 2 (RF2) gene (2, 4, 7). In RF2, there is a special frameshift site bounded on the 3′ side by a stop codon and on the 5′ side by a Shine-Dalgarno-like sequence. It has been shown that the Shine-Dalgarno sequence must interact with the 3′ end of the 16S rRNA for frameshifting (21). All three signals are required for the final high-level frameshift reaction. The essential features of the retroviral and coronaviral cases are the requirement for a homopolymeric run of bases at the frameshift site and a downstream mRNA secondary structure. The function of the run of bases seems simple in that it allows the maintenance of tRNA codon-anticodon pairing before and after the frameshift. An emerging view of frameshifting is that although codon-anticodon interactions may be disrupted at certain stages during translation, correct pairing must exist before and after such events (1). However, the effect of an RNA structure on an upstream ribosome seems far from obvious. It might be acting qualitatively by binding in some way to a ribosome or quantitatively by decreasing the rate of translation at the frameshift site to allow time for the frameshift to occur. Clearly the study of other examples of frameshifting will shed light on how such mechanisms operate.

One example is gene 10 of bacteriophage T7. This gene has two products, a major product of 36 kDa, 10A, and a minor product of 41 kDa, 10B (6). Products from this gene account for about 90% of the capsid protein. From an analysis of both DNA and protein sequences, it was concluded that the minor 10B species was produced by a shift into the −1 coding frame near the end of the coding frame for the major product. Thus 10B contains most of the sequence of 10A with 52 extra amino acids from the alternate frame added onto the C terminus (6). This frameshift event appears to be conserved in bacteriophage T3, a diverged relative of T7 (3). In both cases the frameshift occurs at a frequency of about 10% of that of normal elongation, and both products are incorporated into the capsid in about this proportion. It is not known whether the frameshift is required for phage survival, because no 10B specific mutations are known. However, complementation studies show that the majority of the 10B unique coding region is dispensable for phage growth (6).

Frameshift sites have been proposed for both T7 and T3 on the basis of DNA sequence analysis and peptide labeling data in T7 (3, 6, 14). In T7 there is an overlapping phenylalanine codon (6), whereas in T3 there is an overlapping lysine codon (3). Both lie near the end of the main zero frame coding region. In both cases there is potential for the same tRNA to maintain pairing in the alternate −1 frame, and both sequences are known to facilitate frameshifting in other systems (1, 12). This frameshift site is not rare; it occurs 13 times in the coding frame for β-galactosidase, where 13 high-level frameshifts clearly would be deleterious to expression. Therefore other signals probably operate in addition to this site to promote a high-level frameshift in the gene 10 mRNA. Purified gene 10 mRNA can support frameshifting in an *E. coli* extract but not in a rabbit reticulocyte lysate cell-free translation system, indicating that the event is post-transcriptional and does not require any phage-encoded

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factors (5). This indicates that the gene 10 mRNA from the T7 RNA polymerase promoter to the gene 10 transcriptional terminator contains all *cis*-acting signals necessary for frameshifting. This system presents itself as a model for studying the function and evolution of a programmed frameshift. In this study the sequences important to frameshifting and the consequences of the reaction are examined.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. *E. coli* su1675 (21) was used for routine cloning and phage growth. For frameshift analysis, strain BL21(DE3) containing the plasmid pLys(S) (20) was used. T7 (Studier) was used as wild-type bacteriophage T7, and T7 amber 23 (17) (stop codon in gene 10) was used as the gene 10-negative phage. Both were kind gifts from F. W. Studier.

DNA manipulations. Most DNA manipulations, including plasmid purifications, restriction endonuclease digestions, T4 DNA polymerase treatment, ligations, and DNA sequencing, were performed as described previously (8). Enzymes were purchased from Bethesda Research Laboratories, Inc., except for Sequenase, which was from U.S. Biochemical Corp. Polymerase chain reaction amplifications were performed as recommended by Perkin Elmer Cetus with Taq DNA polymerase. Oligo primers were synthesized with enough complementarity to allow a melting temperature of at least 58°C and with five extra bases 5′ of the restriction site. All constructs were verified by Sanger dideoxy sequencing.

Constructs. The gene 10 clone pAR436 was a kind gift from J. Dunn (19). It was used as a template for most amplifications and to make deletions. Construct 1 is pAR436. In construct 2 there is a deletion between the *Nhe*I site at base 4 and the *Acc*I site at base 360. Both sites were blunt ended and then religated. Base numbers used here for gene 10 of both T7 and T3 are such that the translational start is base 1. Construct 3 was deleted from the *Fnu*4HI site at base 208 to the *Rsa*I site at base 969. Construct 4 was deleted from the *Esp*I site at base 1182 to the *Eco*RV site on the pBR322 vector (19). Constructs 5 to 16 were made by cloning polymerase chain reaction-amplified fragments into the unique *Hin*dIII and ApaI sites in the vector pBW1604 (21). The vector sequence that lies just 5′ to each of these deletions is 5′-A AGC TTA-3′. This vector contains a T7 RNA polymerase promoter and a reporter coding frame including the gene for protein A 5′ of the *Hin*dIII and *Apa*I cloning sites.

In vivo assays. Strain BL21(DE3) (20) contains an inducible T7 RNA polymerase gene whose basal activity is attenuated by the presence of the gene 3.5 product encoded by plasmid pLys(S). After induction of polymerase, rifampin and [^{35}S]methionine from Du Pont NEN were added to allow labeling of proteins made from plasmids with a T7 RNA polymerase promoter. All procedures were as described previously (20). Labeled proteins were resolved by 10% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Quantitative estimations of frameshifting were made by both laser densitometry of autoradiograms and phosphoimagery on a Structural Dynamics Phosphol-mager.

Protein sequencing. A fragment of gene 10, the equivalent of construct 15 except with *Eco*RI-*Bam*HI termini, was cloned 3′ of the glutathione *S*-transferase gene in the vector pGEX2 (15). Both products that are expressed from this gene were purified and subjected to thrombin cleavage as described previously (15). The entire mixture was resolved on a Pierce C-18 reversed-phase column with a gradient from 0.1% trifluoroacetic acid–20% acetonitrile to 0.1% trifluoroacetic acid–80% acetonitrile on an ABI peptide high-pressure liquid chromatography system. Peaks identified at 210 nm were collected and subjected to Edman degradation sequencing on an ABI sequenator.

Phage work. T7 was grown and handled as described previously with *E. coli* su1675 as a host (17).

RESULTS

Sequence requirements for frameshifting. Plasmid pAR436, a clone of gene 10 (19), expresses both 10A and 10B (Fig. 1, construct 1) in an *E. coli* in vivo assay system (20). Therefore this clone contains all of sequence elements necessary for frameshifting. This assay system involves the use of the strain BL21(DE3), which contains an inducible gene for T7 RNA polymerase. Therefore, after induction and in the presence of rifampin a plasmid-borne gene containing a T7 RNA polymerase promoter is expressed exclusively and so can be examined with in vivo labeling with [^{35}S]methionine and PAGE. The frequency of frameshifting with maxicells did not vary significantly under different growth conditions, in the presence of lethal amounts of kanamycin, streptomycin, or erythromycin, or from one experiment to another (data not shown). This was also true for frameshifting in maxicells with low levels of transcription by *E. coli* RNA polymerase. Therefore deletions were made in this clone to identify elements important to this reaction. Three such deletions are shown in Fig. 1 (constructs 2, 3, and 4). Constructs 2 and 3 remove most of the coding region common to both 10A and 10B but not the proposed frameshift site. Both show frameshifting (Fig. 1, lanes 2 and 3), indicating there are no unique signals necessary to frameshifting in this region. In contrast, the third deletion cannot frameshift (Fig. 1, lane 4). This deletion removes the last two codons of the 10B unique coding sequence and all of the 3′ noncoding region and would produce a frameshift product slightly larger than wild-type 10B.

To rule out the possibility that this deletion affected the amount of mRNA, it was important to directly examine transcription products from this gene. Because of the very high rate of transcription during this assay, mRNAs can be directly visualized by agarose gel electrophoresis and ethidium staining (20). Analysis of transcripts from construct 1, the intact clone of gene 10, revealed a message of the expected size for termination at the gene 10 transcriptional terminator and a much larger mRNA species that is probably the result of leaky readthrough (6) and termination of transcription 3′ of the β-lactamase gene. Only this larger species is visible with construct 4, which has the transcriptional terminator deleted (data not shown). This can be also seen as an increase in the expression of the β-lactamase gene in Fig. 1 (lane 4). This indicates that the loss of the 10B protein species in construct 4 is not due to partial degradation of the mRNA.

To investigate the extent of sequences sufficient for frameshifting, a fragment of DNA containing 91 bases of sequence upstream of the proposed frameshift site and all of the downstream sequences, including the 10B unique coding sequence and the 3′ noncoding region, was fused to the 3′ end of a reporter gene (21). This reporter sequence contains the gene for protein A with a promoter for T7 RNA polymerase. This construct (Fig. 1, construct 5) does support frameshifting, indicating that all elements necessary for

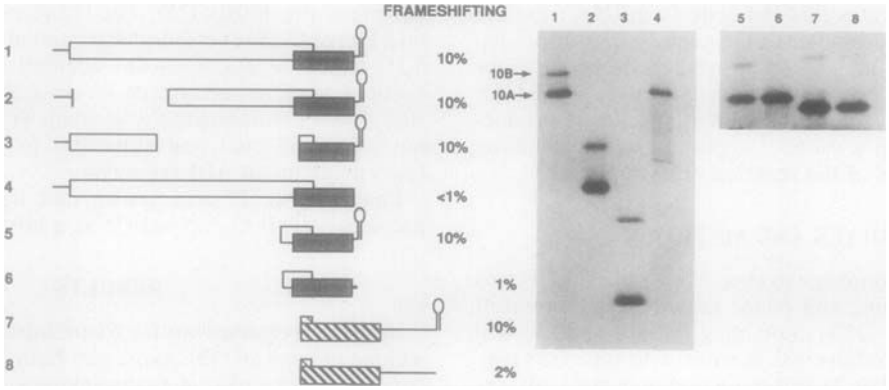

FIG. 1. Extents of a series of deletions and fragments from gene 10 used to test for sequences important to frameshifting. The schematic diagrams of gene 10 show the main 10A-10B zero coding frame and the 3′ −1 10B unique coding frame. With the translational start as base 1, the proposed frameshift site is at base 1019, the 10A stop codon is at base 1032, the 10B stop codon is at base 1187, and the transcriptional terminator is from bases 1198 to 1237. Shown are the deletions and fragments tested. Construct 1 is pAR436 and is considered to be a wild-type gene 10. The deletions were as follows: construct 2, bases 4 through 360; construct 3, bases 208 through 969; construct 4, bases 1182 through the end. Constructs 5 to 8 were cloned into the vector pBW1604, which has a protein A-based reporter gene 5′ of the fragment to be tested. The extents of the fragments were as follows: construct 5, bases 928 through 1240; construct 6, bases 928 through 1190; construct 7, bases 1016 through 1503; construct 8, bases 1016 through 1462. The latter two constructs were derived from gene 10 of bacteriophage T3. The amount of frameshifting was calculated by direct phosphoimagery of the gels. Constructs shown are overexpressed in *E. coli* cells containing T7 RNA polymerase in the presence of rifampin, labeled, and resolved by sodium dodecyl sulfate-PAGE (15% polyacrylamide gel). The band seen below 10A in lane 4 is the product of the β-lactamase gene. The increased expression of this gene is due to readthrough transcription from the terminatorless gene 10.

frameshifting are contained in this gene 10 fragment. Deleting just the 3′ noncoding region severely reduces frameshifting (Fig. 1, lane 6). Taken with the deletion data of construct 4, these data indicate that an element important to frameshifting must lie in the 3′ noncoding region, about 200 bases downstream of the proposed frameshift site.

This is also true for gene 10 of bacteriophage T3 (Fig. 1, constructs 7 and 8). Both the 10B unique coding region and the noncoding region are longer in T3 than in T7, yet deletion of the portion containing the transcriptional terminator is sufficient to reduce frameshifting in T3 (Fig. 1, lane 8). Since this terminator is the only sequence 3′ of the 10A stop codon with any homology between T7 and T3 (3), it is reasonable to assume that either this whole element or a conserved sequence within is required for frameshifting.

To determine whether any sequences 5′ of the proposed frameshift site are important to frameshifting, deletions into the 5′ end of construct 5 were made (Fig. 2). All frameshift except construct 16, in which part of the frameshift site is removed. The T3 constructs 7 and 8 (Fig. 1) contain just 9 bases 5′ of the proposed frameshift site and are thus the equivalent of the T7 construct 13. Constructs 5 to 16 were

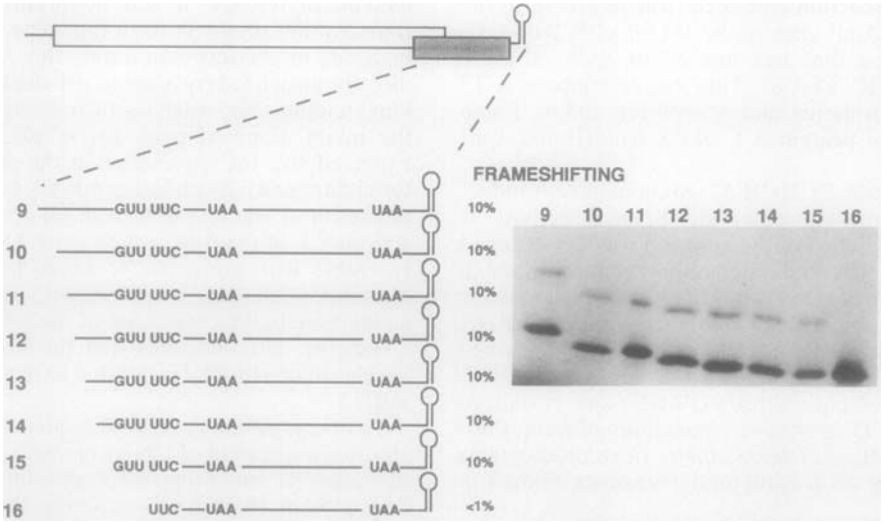

FIG. 2. Extent of deletions 5′ of construct 5 (Fig. 1) used to ascertain the 5′ boundary of sequences necessary for frameshifting. The sizes of the fragments were as follows: construct 9, bases 928 through 1240; construct 10, bases 985 through 1240; construct 11, bases 991 through 1240; construct 12, bases 1000 through 1240; construct 13, bases 1009 through 1240; construct 14, bases 1012 through 1240; construct 15, bases 1018 through 1240; construct 16, bases 1021 through 1240. The frameshift site starts at base 1019. In vivo assays of frameshifting show that all fragments can support frameshifting except construct 16, which does not contain an intact frameshift site.

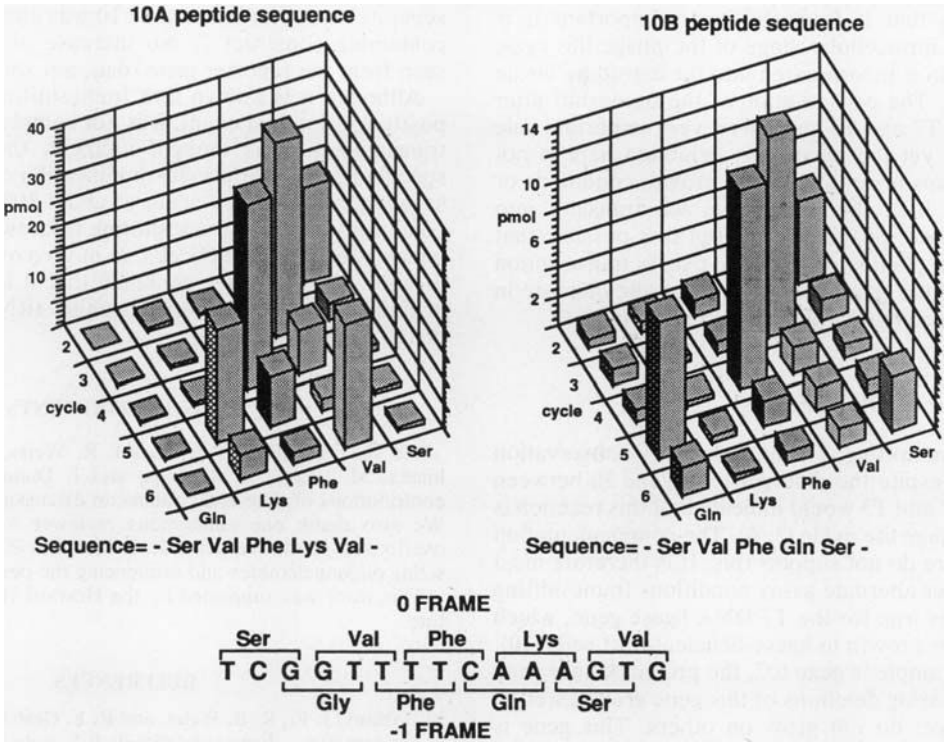

FIG. 3. Results of sequencing by Edman degradation of two peptides, one from the major product, 10A, and one from the minor product, 10B. A fragment from T7 gene 10 spanning bases 1018 to 1240, the equivalent of construct 1 (Fig. 2), was cloned 3′ of the glutathione *S*-transferase gene in the vector pGEX2. The construct produced two products; both were purified, and the C-terminal gene 10 portions were released by thrombin cleavage. Both peptides were purified by high-pressure liquid chromatography and sequenced on an ABI sequenator. Shown are the quantities of particular amino acids derived from chromatograms of cycles 2 to 6. Shown also are the DNA sequences spanning the proposed frameshift sites and the predicted zero and −1 frame-encoded amino acids. Whereas the 10A peptide sequence is compatible with normal translation of gene 10 mRNA, the sequence of the 10B peptide shows that a −1 frameshift occurs at the overlapping phenylalanine codon.

also found to show the amount of frameshifting seen in Fig. 1 and 2 when assayed in maxi cells (16) (data not shown).

Site of frameshifting. To determine the exact site of frameshifting, the amino acid sequence of peptides spanning the proposed frameshift site had to be determined from the major and minor products. Attempts to purify a tryptic peptide from the proposed frameshift site region of 10B were hampered by the extreme hydrophobicity of this peptide. Therefore a fragment of gene 10, the equivalent of construct 15 (Fig. 2), was fused to the 3′ end of the glutathione *S*-transferase gene in the vector pGEX2 (15). This vector allows the overexpression and easy purification of large amounts of fusion products. A unique recognition site of the protease thrombin is encoded at the 3′ end of the glutathione *S*-transferase gene, allowing the fused C terminus to be released by cleavage. Both fusion proteins produced from this construct were subjected to thrombin cleavage. The two resultant peptides were purified with reversed-phase HPLC, and the sequences were determined by using Edman degradation (Fig. 3). The sequence of the smaller peptide agrees with in-frame translation of the mRNA sequence, whereas that of the longer product agrees with the frameshift occurring at either the phenylalanine codon or the valine codon preceding it. Since phenylalanine codons are known to promote −1 frameshifting, it is most reasonable to assume that the frameshift occurs here, as was so insightfully proposed in an earlier study (6). Both peptides were sequenced further than shown in Fig. 3. The amino acids released agree with the zero frame (-Glu) for the 10A peptide and the −1 frame (-Gly-Val-Met-Leu-Gly-Val) for the 10B peptide.

Biology of frameshifting. Although it has been shown that clones of gene 10 in which most of the 10B-specific coding sequence was deleted could complement phage T7 mutants containing an amber stop codon in gene 10 (6), it was not clear whether these constructs were still producing some kind of necessary frameshift product. It can now be surmised that this deletion could not, in fact, frameshift because it did not contain the 3′ noncoding region. It can therefore be concluded that frameshifting, at least at high levels, is not necessary for the phage life cycle under the conditions of the assay. This was examined further by complementation of phage T7 gene 10 mutants with construct 4 (Fig. 1), which fails to frameshift. It was noticed that both the plaque size and plating efficiency were lower for complementation with this construct when compared with complementation with construct 1 (Fig. 1), which is an intact gene 10 clone. This lower plating efficiency was more pronounced at lower temperatures (about 0.68 at 24°C).

The capsids produced from infections of strains carrying construct 4 were devoid of any frameshift product. These mutant capsids showed the same decay characteristics when compared with wild-type capsids at high temperatures and in the presence of an acid, base, or detergent. No stability differences were detected between the mutant and wild-type capsids when stored at 4°C over a period of a few months.

This all indicates that if frameshifting is important it is probably at some intracellular stage of the phage life cycle and that the protein is incorporated into the capsid by virtue of its 10A moiety. The conservation of the frameshift after the divergence of T7 and T3 suggests a very important role for frameshifting, yet the phenotype exhibited here is not very strong. Perhaps under alternative growth conditions or in an alternative host the phenotype will translate into complete inhibition of phage growth. But it is possible that this weak effect on phenotype is due to excess transcription because of loss of the terminator as seen by the increase in the amount of β-lactamase expression (Fig. 1, lane 4).

DISCUSSION

Function of frameshifting. The extraordinary conservation of frameshifting despite the divergence of gene 10 between bacteriophages T7 and T3 would indicate that this reaction is essential to the phage life cycle (3, 6). The complementation data presented here do not support this. It is therefore most probable that under alternate assay conditions frameshifting is essential. This is true for the T7 DNA ligase gene, which is only required for growth in ligase-deficient host cells (10). An even better example is gene 0.7, the protein kinase gene (18). T7 strains bearing deletions of this gene grow faster on some hosts and yet do not grow on others. This gene is almost always present in strains isolated from the wild (18). Genetic studies of gene 10 indicate some alternative roles for the gene products in addition to a structural role (11, 13). Unexpected activities have been reported in a T4 capsid protein, indicating a direct enzymatic role for capsid proteins in DNA translocation and/or metabolism (9). Perhaps one way of examining the biochemical function of frameshifting would be to look for mutants of *E. coli* carrying construct 4 that fail to complement T7 gene 10 mutants. Molecular analysis of such mutants might then reveal a function for the frameshift product.

Frameshift signals. This study has shown that the minimum sequence requirement for frameshifting is the frameshift site itself and the 3′ noncoding region over 200 bases downstream. The frameshift site identified here is similar to those of other systems, indicating a common mechanism (1). The requirement for the 3′ end is unusual in its distance downstream of the frameshift site. Like other 3′ *cis*-acting signals for frameshifting studied to date, it could act via RNA structures such as stems and loops and pseudoknots (1, 2, 7). Computer analysis of the potential homologous secondary structures conserved between T7 and T3 in this region reveals only the transcriptional terminator stem and loop. It might be that the requirement for this element is only for its transcriptional termination activity and the need for a free 3′ end at this point. Alternatively, it might form a particular structure that promotes frameshifting in a way similar to that of downstream mRNA structures in retroviral and coronaviral frameshifts (1, 2, 7). Site-directed mutagenesis of the transcriptional terminator should reveal more information. This 3′ element is probably not the only signal affecting frameshifting, as can be seen in the varied amounts of frameshifting seen in constructs 4 and 6. Thus there are probably other signals between the frameshift site and the 10B stop codon that stimulate frameshifting. These would probably differ greatly between T7 and T3 because of a lack of sequence homology in this region, although structural similarities are possible. This 3′ signal only acts in *cis*. In a separate study, an intact gene 10 was cloned into the vector containing construct 7. No increase of frameshifting was seen from the reporter gene (data not shown).

Although it is known that frameshifting is in this case a posttranscriptional event, it is not completely clear that it is translational. It is thought unlikely, though, that a site-specific posttranscriptional modification of the gene 10 message would be able to occur at about 10% under the varied conditions in which frameshifting is known to occur: during phage infection, in maxicells, in in vivo overexpression, and in vitro. Therefore we conclude that it is translational and occurs by a slip of a phenylalanine tRNA catalyzed by a distal 3′ RNA structure.

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