An analysis of sequences stimulating frameshifting in the decoding of gene 10 of bacteriophage T7

Barry G.Condron[+], Raymond F.Gesteland* and John F.Atkins

Department of Human Genetics and Howard Hughes Medical Institute, 6160 Eccles Genetics Building, University of Utah, Salt Lake City, UT 84112, USA

ABSTRACT

The signals necessary for the translational frameshift in the gene 10 message of bacteriophage T7 include the previously identified frameshift site and the 3' non-coding region, over 200 bases downstream. The functional components of the frameshift site are identified in this study and show that the site most probably operates by the retroviral type two site mechanism. However, the base pairing requirements for the first tRNA are much more relaxed after the slip than is seen in other examples. The element at the 3' end of the gene, also necessary for frameshifting, is examined but only the extreme 5' side of the transcriptional terminator stem-loop structure in the 3' non-coding region seems to be required. No simple secondary structural model can explain the involvement of this sequence. The T7 frameshift site can be replaced with either a T3 site or a E.coli dnaX site. Both show higher levels of frameshifting than with the T7 site.

INTRODUCTION

It is generally accepted that there are three standard kinds of signals in translation: start signals, stop signals and 61 codons that signal elongation. It has now become clear that there exists another set of signals that can promote alternative events such as frameshifting and hopping (1) during the elongation phase of translation. Although these latter signals were discovered because of their roles in stimulating what appeared to be specific non-standard decoding in translation, there could nevertheless be an important role for such signals during conventional translation. Typically these signals involve a site of action which must conform to particular primary sequence requirements and a stimulator that involves either a specific sequence or a complex RNA secondary structure.

In frameshifting, the site of action usually involves a homopolymeric run of bases such that correct triplet codon-anticodon base pairing can occur in an alternative reading frame

(1). This can be true for just one codon or for two adjacent codons. For instance, the mouse mammary tumor virus (MMTV) frameshift site is $5'$A AAA AAC$^{3'}$. A variant of this sequence, $5'$A AAA AAG$^{3'}$, frameshifts more efficiently in E.coli than the wild type sequence (2). The variant site is also used in the dnaX gene frameshift of E.coli (18). The codon at which the frameshift occurs of the variant, or the second codon, $5'$AAG$^{3'}$, is in the initial frame. A −1 frameshift at the $5'$AAG$^{3'}$ codon occurs by the slippage of a lysine tRNA. The lysine tRNA reads $5'$AAG$^{3'}$, but then slips back one base and now reads $5'$AAA$^{3'}$. The lysine tRNA can maintain correct base pairing with both codons (24). This rule of maintaining correct base pairing in the alternative frame also applies to the first codon. It has been observed that although the −1 frameshift event occurs at the second codon, the first codon must also have the potential to maintain pairing in the new frame (2,4). Variations of this theme also exist. For example, the coronaviral frameshift site consists of the sequence $5'$U UUA AAC$^{3'}$ (3). Here again, both tRNAs can slip back one base and still maintain sufficient pairing. The model proposes that frameshifting occurs by the simultaneous slippage of tRNAs in both the A and P sites (2,4). Such two site slips seem to be much more efficient than single site slips when frameshifting is observed in the absence of stimulatory sequences (5).

There are stimulatory sequences that can act with the frameshift site to increase the efficiency of frameshifting. While the importance of frameshift stimulators is certain, their mechanism of action is very unclear. The high level frameshift in the decoding of the release factor two gene of E.coli (6), is stimulated by both a stop codon on the 3' side and a Shine-Dalgarno-like sequence on the 5' side (7). It has been shown that the Shine-Dalgarno-like sequence must interact with the 3' end of the 16S rRNA during elongation for frameshifting to occur at high efficiency (7). In the cases of retroviruses (4), retroviral-like elements (22), the bacterial transposon IS1 (23), the dnaX (18) gene of E.coli and coronaviruses (3) there is a requirement for a downstream RNA secondary structure for frameshifting. While the nature of the active structure can be defined, there is no obvious mechanism. For instance, the elegant studies of the sequences

* To whom correspondence should be addressed

[+] Present address: Division of Biology 216-76, California Institute of Technology, Pasadena, CA 91125, USA
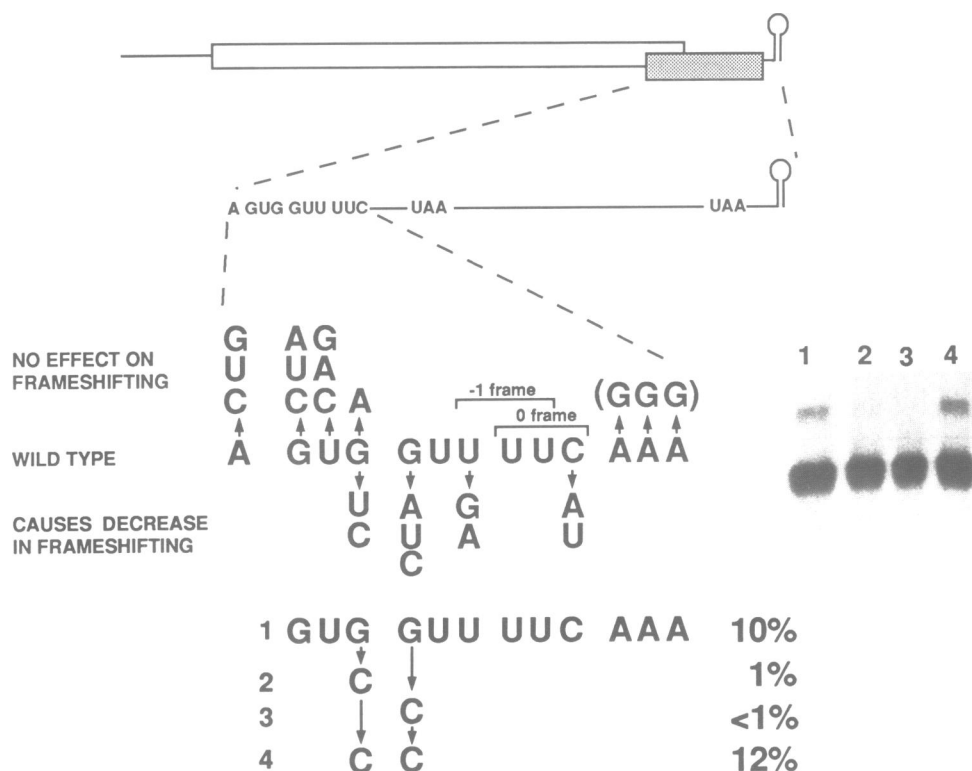
Fig. 1. This shows the effect of changes at or around the frameshift site on the frameshift reaction. Shown at top is a schematic diagram of gene 10 showing the 5' non-coding region, the 10A/10B zero frame (open bar), the −1 10B unique coding frame (shaded bar) and the 3' non-coding region including the gene 10 transcriptional terminator. Below this is shown an expanded view of the gene 10 fragment necessary for frameshifting including the frameshift site, the 10A stop codon and the 10B stop codon. This fragment is cloned into the unique HindIII/ApaI site 3' and in frame with the protein A coding region of the vector pBW1604. Gene products overexpressed from these constructs are examined by *in vivo* labeling followed by 10% SDS-PAGE. Frameshifting is calculated from the ratio of counts, measured by direct phosphoimagery of the gel incorporated into each product. All changes are single point mutations except for the $^{5'}AAA^{3'}$ to $^{5'}GGG^{3'}$, 3' of the frameshift site and the double G to C changes, construct 4.

necessary for the coronaviral frameshift have demonstrated that a pseudoknot structure is required (3,19). The exact bases in the stems seem not to be important, but the overall structure must be maintained. 3' RNA structures are also important for the specific encoding of selenocysteine (8) and the readthrough of a stop-codon (20). Clearly more examples of high level frameshifts need to be characterized for a better understanding of this type of gene expression. One such example is in the decoding of gene 10 of bacteriophage T7.

Gene 10, the major capsid gene of bacteriophage T7, produces two products, 10A and 10B (9). The 10B product is synthesized by an efficient −1 translational frameshift near the end of the main 10A coding frame (11,21). This reaction is conserved in bacteriophage T3 (10), a diverged relative of T7. A role for either the product or the reaction has not been discovered (11). Frameshifting in gene 10 mRNA has been assayed directly by quantitation of products produced *in vivo* (12). This was used to identify both the site of frameshifting and the 5' and 3' boundaries of the major signals necessary for the reaction (11). The frameshift site consists of the sequence $^{5'}GUU\ UUC^{3'}$ with the $^{5'}UUC^{3'}$ phenylalanine codon being in the original frame. From peptide sequencing data, it was concluded that the frameshift occurs at the $^{5'}UUC^{3'}$ phenylalanine codon with a phenylalanine tRNA slipping −1 from $^{5'}UUC^{3'}$ to $^{5'}UUU^{3'}$ (11). Deletion of the $^{5'}GUU^{3'}$ codon, which creates the sequence $^{5'}GUG\ UUC^{3'}$, abolishes frameshifting. The primary structural

requirements of this sequence for frameshifting are examined in this study using site directed mutagenesis.

The 3' stimulator sequence was shown to include the 3' non-coding region which contains the transcriptional terminator (11). It is over 200 bases downstream of the frameshift site which makes this an unusual case. A distal 3' stimulator has also been shown to be important for gene 10 of bacteriophage T3 (11). Although there is very little homology between T7 and T3 in this region of gene 10, deleting the portion containing only the transcriptional terminator in both cases greatly reduces frameshifting. The functional components of this and other sequences important to the T7 3' stimulator are examined in this study.

METHODS

DNA manipulations

All DNA manipulations including plasmid purifications, restriction digestions, ligations and transformations were performed as described (13). Enzymes were purchased from USB. All constructs were made by PCR amplification with mismatched oligonucleotides from the gene 10 plasmid clone, pAR436 (14). PCR was performed according to Perkin Elmer Cetus. The fragments were cloned into the unique HindIII/ApaI sites of the vector pBW1604 (7). All were confirmed by dideoxy DNA sequencing using Sequenase according to the manufacturer, USB.

In vivo frameshift assays

The *in vivo* assay used in this study was performed as described (12). Overexpression was accomplished by induction of T7 RNA polymerase in the strain BL21(DE3). ^{35}S methionine labeled proteins were resolved by 10% PAGE and the amount of incorporated label quantitated by direct phosphoimagery using a Structural Dynamics Phosphoimager. The termination product has 11 methionines and the frameshift product has 12. This slight difference was calculated into the final ratio. In the translational initiation studies, kasugamycin was added up to a final concentration of 10mg/ml at which point there is about 90% inhibition of total translation.

In vitro transcriptional termination

For the *in vitro* transcriptional termination assays, plasmid constructs 5,6,10 and 11 were cut at the unique EcoRV site within the *lacZ* gene, 3' of the unique ApaI site (7). *In vitro* transcriptions were performed using T7 RNA polymerase according to USB. The products were resolved by electrophoresis in 1.2% agarose. Construct 5 produced about a 4 to 1 ratio of termination to extension product while the other three constructs produced only the extension product.

RESULTS

Mutagenesis of the frameshift site

It has been shown that a fragment of gene 10 from the frameshift site to the 3' end of the transcriptional terminator is sufficient for the normal efficiency of frameshifting of 10% (11). The frameshift site has been identified by both deletion analysis and peptide sequencing. In order to examine the nature of the frameshift site further, point mutations were introduced around this region and their effects on frameshifting assayed, fig 1. The gene 10 fragment to be assayed was fused to the 3' end of the protein A coding region of the vector pBW1604 (7). This can be transcribed *in vivo* by endogenous T7 RNA polymerase in the *E. coli* strain BL21(DE3) (12). The gene is overexpressed by induction of T7 RNA polymerase while genomic expression is suppressed with rifampicin. After metabolic labeling with ^{35}S methionine, the products are resolved by SDS-PAGE. The amount of frameshifting was measured by quantitation of phosphoimages of both the 37kd frameshift product and the normal 34kd termination product. The values for frameshift efficiency measured for all constructs are shown in table 1. From previous peptide sequencing, it can be inferred that the phenylalanine tRNA reading the in-frame $^5'$UUC$^{3'}$ codon slips back one base to read $^5'$UUU$^{3'}$. As can be seen, fig 1, mutations that are predicted to affect this kind of slip are very deleterious to frameshifting. Changing the U that lies just 5' of the $^5'$UUC$^{3'}$ phenylalanine codon to either A or G abolishes frameshifting. Changing the C wobble base to A alters this codon from phenylalanine to a $^5'$UUA$^{3'}$ leucine codon and abolishes frameshifting. This might be due to the inability of the leucine tRNA to make the −1 slip. A uridine at this position introduces a stop codon, thus abolishing the possible appearance of a frameshift product.

An unusual effect is seen in the adjacent codon 5' of the phenylalanine codon where the T7 frameshift occurs. This codon is $^5'$GUU$^{3'}$. The first base of this codon can only be G while the last base of the codon before it can only be A or G. The T3 frameshift site is $^5'$C CCA AAG$^{3'}$. Therefore both of these bases in this homologous site in T3 are Cs. It seems then that

CONSTRUCT	FIG	N	Mean (%)
T7	–	9	10.7±1.8
1	1	28	10.3±1.3
2	1	2	0.6±0.3
3	1	1	«1
4	1	1	12
5	2	28	10.3±1.3
6	2	2	11.9±2.5
7	2	4	10.9±1.3
8	2	4	10.0±1.9
9	2	4	10.5±3.1
10	2	2	10.3±3.5
11	2	1	1
12	2	2	«1
13	3	28	10.3±1.3
14	3	3	10.0±0.9
15	3	3	10.7±0.6
16	3	3	11.8±0.8
17	3	3	0.9±0.3
18	4	6	17.3±2.6
19	4	1	1
20	4	2	17.0±1.0
21	4	2	7.0±1.3

Table 1. This table summarizes the quantitation of frameshifting with gene 10 constructs described in figures 1 to 6. The construct number and figure number are indicated in the first two columns. The number of independent gel assays are indicated by N while the last column shows the mean and standard deviation. These numbers were derived by quantitation of both the frameshift and termination products after gel electrophoresis. This was done with either laser densitometry of autoradiograms or by direct phosphoimagery. The ratio of the counts in the 10B band divided by the sum of the counts in the 10A and the 10B band was taken as the frameshift percentage. The variation in the values is probably most related to sample preparation as all samples in one experiment tended to be either all higher or lower than their respective means. The above normal values measured for constructs 4 and 6 probably represent sampling errors. The T7 row represents measurements of frameshifting in wild type phage infections.

both of these bases must be similar. To test if the specific identity of the bases in these positions is unimportant but that they be just similar, a double mutant of both deleterious G to C changes was examined, fig 1, constructs 1 to 4. As can be seen, while the single mutants cannot support frameshifting, the double mutant can. Since a different tRNA is involved in reading the double mutant, the specific identity of this tRNA seems not to be important. All of this data can be best explained in terms of the double site model, where both P and A site tRNAs slip simultaneously (2,4).

3' non-coding region

In a previous study, it was shown that the 3' non-coding region was required for frameshifting in gene 10 (11). In T7, this region contains essentially only the gene 10 transcriptional terminator. In T3, on the other hand, this region is much larger and therefore contains more non-coding sequences than the transcriptional terminator alone (10). The transcriptional terminator is the only sequence, 3' of the 10A stop codon and 5' of gene 11, that is conserved at the primary structural level between gene 10 of T7 and T3 (10). Deletion of just this sequence in both T7 and T3 is sufficient to greatly reduce frameshifting (11). To examine this further, a series of deletions into the terminator in T7 was made and frameshifting assayed, fig 2. As can be seen, all of the stem-loop sequence, except for the extreme 5' end, is dispensable for the frameshift reaction. The only sequence conserved in this
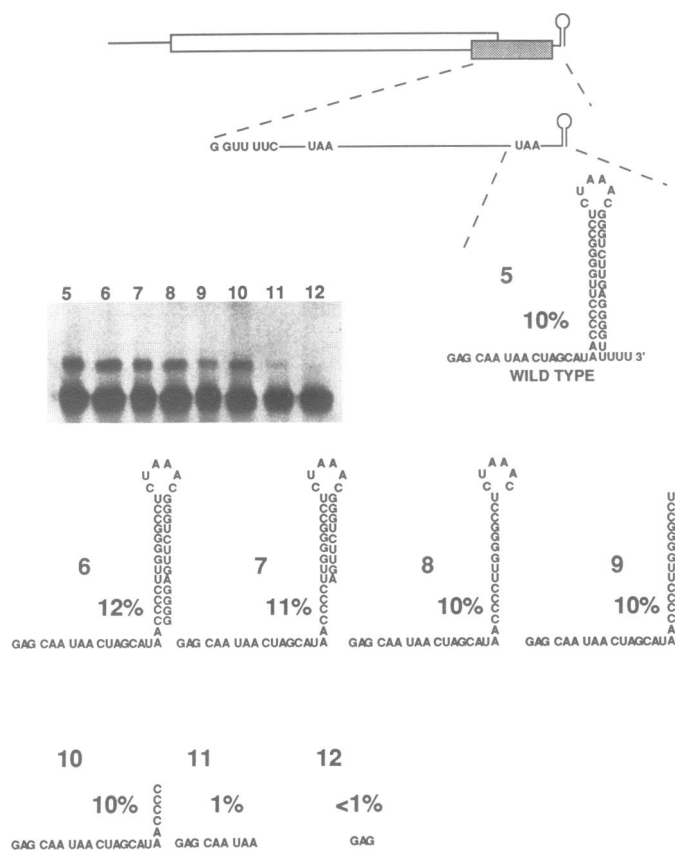
Fig. 2. This shows a series of deletions into the 3' non-coding region of a gene 10 fragment and their effects on frameshifting. The 3' end is shown in the stem-loop configuration of the gene 10 transcriptional terminator below a schematic diagram of gene 10 described in fig 1. As can be seen, only sequences 5' of those shown in construct 10 are important to the reaction. Therefore, most of the transcriptional terminator stem-loop structure is dispensable for the frameshift reaction except for the extreme 5' end. Only construct 5 shows *in vitro* transcriptional termination activity.

Fig. 3. This shows the effect of various changes in the transcriptional terminator stem-loop structure on frameshifting. Constructs 15 and 17 are second site compensatory changes for the changes in constructs 14 and 16 respectively. Only the changes in construct 17 have an effect on frameshifting. Together with the effects seen by deletion analysis in fig 2, the sequence $^{5'}$CCCC$^{3'}$ is an essential part of the 3' frameshift signal.

region between T7 and T3 is $^{5'}$CCCC$^{3'}$. *In vitro* transcriptional termination assays with constructs 5,6,10 and 11 show that only construct 5 can terminate transcription (data not shown). It is possible, that vector sequences introduced by the deletions, could fortuitously repair the stem-loop structure, though this is not obvious from visual inspection. Two changes were made in the stem, both with second site compensatory double changes, fig 3. If the stem-loop structure was important to frameshifting, it would be expected that the single mutations would lower the rate of frameshifting and the double changes would restore the rate. However, the only change to affect frameshifting is the construct that includes the $^{5'}$CCCC$^{3'}$ to $^{5'}$GGGG$^{3'}$ change. This means that the 3' non-coding region signal is not the transcriptional terminator structure. While this does not prove that the sequence $^{5'}$CCCC$^{3'}$ is the signal, the effect of deleting the transcriptional terminator on frameshift efficiency can be explained entirely in terms of the loss of this sequence. There are no obvious RNA structures that involve this sequence in gene 10 of bacteriophage T7 that are also conserved in T3.

Experiments with heterologous frameshift sites

To ask if the T7 3' stimulator stimulator is specific to its own frameshift site, constructs where the T7 frameshift site was replaced with either a T3 site or a variant of the mouse mammary
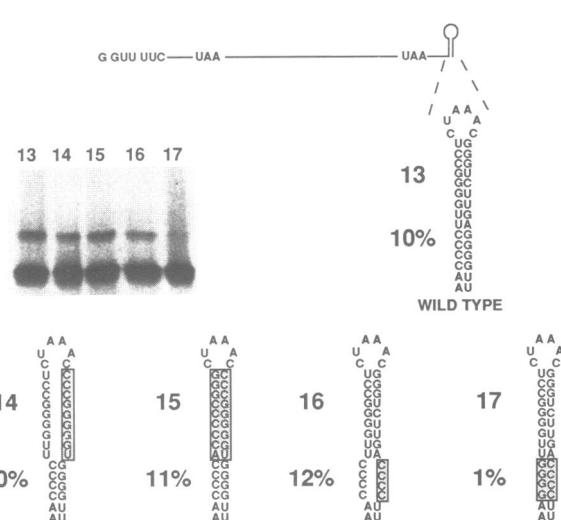
tumor virus (MMTV) site were examined, fig 4. The normal MMTV site is $^{5'}$A AAA AAC$^{3'}$. The variant sequence at the second position is $^{5'}$AAG$^{3'}$ instead of the normal $^{5'}$AAC$^{3'}$ and frameshifts at a much higher level in *E. coli* than the wild type sequence (2). Additionally, this variant is stimulated in *E. coli* by downstream RNA secondary structures and this site is used in the expression of the *dnaX* gene of *E. coli* (2,18). The unstimulated level of frameshifting in *E. coli* at the T3 site is about 1% (11) and at the variant MMTV site 6.7% (2). Both sites show comparable levels of frameshifting with incomplete 3' non-coding regions, but are stimulated to about 17% with the complete stimulator, fig 4. The MMTV site can be stimulated to frameshift to over 50% with other 3' stimulators indicating that the T7 stimulator is not very strong. 17% frameshifting is seen for both the T3 and MMTV sites. This probably represents an upper limit to the amount of frameshifting that the T7 3' stimulator can promote.

DISCUSSION
Frameshift site

The data presented here indicate that the T7 frameshift site operates according to the retroviral two site model. Such sites are known to function in *E. coli* (2). The second tRNA requires exact pairing before and after the slip, while the first tRNA can accommodate mis-pairing at the middle base. This contrasts with other examples of two site frameshifting (1). The differences in the base pairing requirements might reflect the differing environments of the two tRNAs on the ribosome. The second tRNA slips from $^{5'}$UUC$^{3'}$ to $^{5'}$UUU$^{3'}$. Both of these codons are read by phenylalanine tRNA which has the anticodon $^{5'}$GAA$^{3'}$. Therefore there is no mismatching in the codon-anticodon interaction after this shift. The first tRNA on the other hand, shifts back onto a mismatched codon-anticodon interaction. It shifts from $^{5'}$GUU$^{3'}$ to $^{5'}$GGU$^{3'}$. The tRNA that slips is valine tRNA which has the anticodon $^{5'}$VAC$^{3'}$ (where V is the

18 C CCA AAG——UAA———————————UAA┘ 17%

19 C CCA AAG——UAA———————————UAA 1%

20 A AAA AAG——UAA———————————UAA┘ 17%
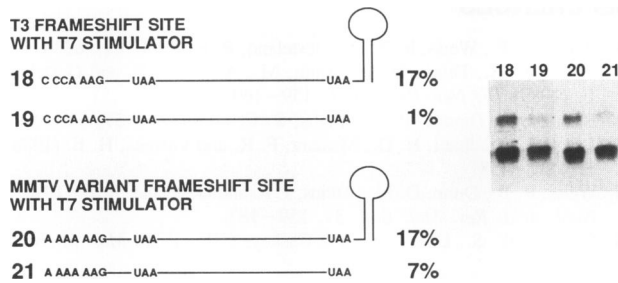
21 A AAA AAG——UAA———————————UAA 7%

Fig. 4. This shows the effects of replacing the T7 frameshift site with either the bacteriophage T3 site, constructs 18 and 19 or the mouse mammary tumor virus (MMTV) variant frameshift site, constructs 20 and 21. The wild type MMTV frameshift site is $^{5'}$A AAA AAC$^{3'}$. The variant used here is $^{5'}$A AAA AAG$^{3'}$, which frameshifts more efficiently in *E.coli*. This variant site is also used in the *dnaX* gene in *E.coli*. Constructs 18 and 20 contain all sequences necessary for full stimulation of the T7 frameshift site and constructs 19 and 21 are missing necessary sequences in the 3' non-coding region of gene 10. The amount of unstimulated frameshifting estimated for the T3 frameshift site is about 1% and for the MMTV site 6.7%. These values are seen in constructs 19 and 21 respectively. Thus, both of these constructs are acting as if there is no frameshift stimulator present. As can be seen, both are stimulated to 17% by the intact T7 gene 10 frameshift stimulator, constructs 18 and 20.
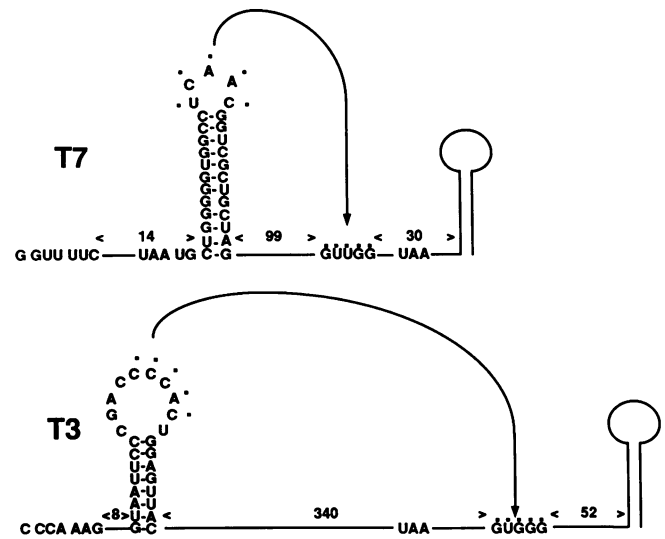

Fig. 5. This shows a hypothetical pseudoknot structure 3' of the frameshift site in gene 10 of both T7 and T3. The T3 10A stop codon is contained in the 5' portion of the T3 stem 1. No other secondary structures were found in the same region by computer analysis that are conserved in both phages.

modified base uridine 5-oxyacetic acid). This normally reads only $^{5'}$GUA$^{3'}$, $^{5'}$GUG$^{3'}$ or $^{5'}$GUU$^{3'}$. Therefore there is an A:G mismatch in the middle position after the slip.

It can be seen from the heterologous frameshift site data that the T7 3' stimulator is not very efficient (17% as opposed to a possible 50% frameshifting). While it is clear that the effects of a frameshift site and a frameshift stimulator are not a simple addition of the two separate contributions, it is probable that one of the parts might limit the overall reaction through some inherent inefficiency. The T7 3' stimulator might therefore be limiting the rate of frameshifting. If this is so, then the frameshift site might not be optimized for maximum frameshifting. The T3 frameshift site, which allows pairing at the middle base after the frameshift, frameshifts more efficiently than T7. Two site frameshifts appear more efficient than single site frameshifts (5). The T7 frameshift site might represent an intermediate between two site and single site frameshifting sequences. The T3 frameshift site (shown in fig 4) allows better pairing for the first tRNA in the −1 frame than its equivalent in T7. In T3 a proline tRNA shifts from $^{5'}$CCA$^{3'}$ to $^{5'}$CCC$^{3'}$. The valine tRNA in the T7 site must mismatch after the slip at the middle codon base while in T3 there is a mismatch at the wobble position. Presumably, mismatching at the wobble position is more favorable for codon-anticodon interactions then mismatching at the middle base. This could explain the increase in frameshifting seen in construct 18, fig 4, over wild type T7 frameshifting. Likewise, the MMTV site allows better pairing at the first codon position after the slip than the T3 site does and it can frameshift more efficiently (i.e. construct 21 and construct 19 fig 4). But this kind of comparison cannot be carried too far. Although the stimulated T3 site can frameshift more than the stimulated T7 site, both sites frameshift at the same level, 1%, when missing the non-coding region (i.e. construct 11, fig 2 and construct 19, fig 4).

T7 3' stimulator

The mode of action of the extreme 3' end of the gene 10 mRNA frameshifting is elusive. There is no obvious mRNA secondary

structure to link this with other known 3' frameshift stimulators. It could therefore operate in novel manner. However, as discussed by other workers (26) RNA structures such as pseudoknots can be very complex. The 3' non-coding region of gene 10 could be just subtly effecting a more conventional stem type frameshift stimulator. As already discussed concerning the frameshift site mutagenesis, the 3' stimulator might be the weak link of the two parts of this frameshift apparatus. Thus it might be especially sensitive to changes within and surrounding a conventional stimulating sequence such as a stem-loop or pseudoknot. It would be very interesting, if it were possible, to see how changes in the 3' non-coding region would affect a frameshift site that was considerably weaker than the T7 site. In this scenario, the frameshift site would become the weak link in the frameshift site − frameshift stimulator collaboration and so many changes in the stimulator might not be noticed.

It was originally shown that deletion of the 3' non-coding region of gene 10, including the transcriptional terminator sequence, abolished efficient frameshifting (11). This can now be equated with the loss of a specific sequence at the 5' end of the transcriptional terminator, which includes the sequence $^{5'}$CCCC$^{3'}$. Other signals operate to stimulate frameshifting at the T7 frameshift site in gene 10 in addition to the signal in the transcriptional terminator. For instance there is a difference in frameshifting between constructs 11 and 12 in fig 2. Deletion of the $^{5'}$CCCC$^{3'}$ sequence at the 3' end of gene 10 drops frameshifting from 10% to 1%, fig 2. However deleting more sequence drops frameshifting even further to where it is immeasurably low. This shows that other signals exist. No explanation in terms of secondary structure can be found to explain this effect. A computer analysis 3' of the 10A stop codon in T7 and T3 reveals a possible pseudoknot structure, fig 5. Although the size of the T3 loop 2 might seem extreme in this case, it is nevertheless similar in size to other proposed second loops (15).

Preliminary data supports the existence of at least part of this structure in T7 (25). A limited number of changes were

introduced into this hypothetical structure to test the importance to frameshifting of stem 1 and stem 2 (stem 1 includes the most 5' sequences and is closest to the frameshift site and stem 2 is formed in part by the loop of stem 1). Evidence was found for the existence of stem 2 but not stem 1, although sequences within stem 1 are definitely important. Changes in the base composition of stem 2 that maintain base pairing reduced frameshifting by 60% indicating the importance of specific structure in this region. More mutants will be required to completely describe the functional components of the mRNA 3' of the 10A stop codon.

The existence of secondary structure important to frameshifting is nevertheless strongly indicated by data presented here. Such structures would presumably be transiently destroyed by passage of a frameshifted ribosome. The overall effect of this on the upstream frameshift reaction would be related to the number of frameshifted ribosomes and hence the rate of translational initiation. Therefore one might expect in certain circumstances the rate of frameshifting to be inversely proportional to the rate of translational initiation. The rate of translational initiation can be controlled in E.coli by either the 5' non-coding sequences that promote ribosome binding or with certain antibiotics (27). The signals for translational initiation in gene 10 are possibly the strongest known in E.coli (17). This mRNA, which may have near the highest concentration of ribosomes possible on a message in E.coli supports 10% frameshifting. This level of frameshifting is also seen with constructs used in this study even though they are probably less efficient at translational initiation than wild type gene 10. Additionally, even when kasugamycin, an antibiotic that inhibits translational initiation (27), was added until 90% inhibition of overall translation, it did not affect the efficiency of the frameshift reaction in these constructs (data not shown). Given that no effect on frameshifting can be seen over such a broad range of translational initiation rates, it is reasonable to assume that if a frameshifted ribosome can affect the frameshift reaction, the rate of translational initiation cannot get high enough in E.coli to allow this. The value of 17% frameshifting seen with constructs 18 and 20 (T7 3' stimulator with T3 site and MMTV variant site) might represent an upper limit imposed by the negative effect of a frameshifted ribosome. However when frameshifting with construct 20 was examined in the presence of kasugamycin it showed no increase in frameshifting. Perhaps if the gene 10 mRNA could frameshift at a higher level, then an effect might be seen.

While it is known that specific structures can promote certain translational events, it is not at all clear how they do it. It has been postulated that they function by inducing a site specific pause in translation which can then amplify an otherwise low level translational event (4). This pause is postulated to occur by direct stalling of ribosomes trying to melt secondary structure. The elements of the gene 10 frameshift stimulator identified in this study are very different from those previously characterized. This indicates that such secondary structures might operate in a more complicated manner than just providing a difficult structure to translocate through.

ACKNOWLEDGEMENTS

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