Upstream stimulators for recoding

B. Larsen, J. Peden, S. Matsufuji, T. Matsufuji, K. Brady, R. Maldonado, N.M. Wills, O. Fayet, J.F. Atkins, and R.F. Gesteland

Abstract: Recent progress in elucidation of 5′ stimulatory elements for translational recoding is reviewed. A 5′ Shine–Dalgarno sequence increases both +1 and −1 frameshift efficiency in several genes; examples cited include the *E. coli prfB* gene encoding release factor 2 and the *dnaX* gene encoding the γ and τ subunits of DNA polymerase III holoenzyme. The spacing between the Shine–Dalgarno sequence and the shift site is critical in both the +1 and −1 frameshift cassettes; however, the optimal spacing is quite different in the two cases. A frameshift in a mammalian chromosomal gene, ornithine decarboxylase antizyme, has recently been reported; 5′ sequences have been shown to be vital for this frameshift event. *Escherichia coli* bacteriophage T4 gene *60* encodes a subunit of its type II DNA topoisomerase. The mature gene *60* mRNA contains an internal 50 nucleotide region that appears to be bypassed during translation. A 16 amino acid domain of the nascent peptide is necessary for this bypass to occur.

Key words: recoding, frameshifting, peptide factor, stimulatory elements.

Résumé : Les progrès récents réalisés dans l'identification des stimulateurs du recodage de la traduction, localisés en 5′, sont passés en revue. Une séquence Shine–Dalgarno augmente l'efficacité de changements de phase +1 et −1 de plusieurs gènes, dont deux de *Escherichia coli* : le gène *prfB* codant le facteur de libération 2 et le gène *dnaX* codant les sous-unités γ et τ de l'holoenzyme de l'ADN polymérase III. La distance entre la séquence Shine–Dalgarno et le site du décalage du cadre de lecture est importante pour les changements de phase +1 et −1; cependant, les distances optimales diffèrent passablement dans les deux cas. Un décalage du cadre de lecture d'un ARNm d'un gène chromosomique de mammifère, celui de l'antizyme de l'ornithine décarboxylase, a été découvert récemment; des séquences en 5′ sont essentielles pour le changement de phase. Le gène *60* du bactériophage T4 de *E. coli* code une sous-unité de l'ADN topoisomérase de type II. L'ARNm mature du gène *60* comporte une séquence interne de 50 nucléotides qui semble être sautée lors de la traduction. Un domaine de 16 acides aminés du polypeptide naissant est nécessaire pour que ce saut se produise.

Mots clés : recodage, décalage du cadre de lecture, facteur peptidique, stimulateurs.

[Traduit par la rédaction]

Introduction

Recoding is a programmed local alteration in reading of the genetic code (Gesteland et al. 1992). Instructions in a minority of sequences, probably present in all organisms, specify an alteration of linear readout (frameshifting or hopping) or the meaning of code words (e.g., stop codon readthrough). There is a site where the nonstandard decoding event occurs and often stimulatory sequences or structures in the mRNA are present which elevate the frequency of recoding at that site (Gesteland et al. 1992). Most recoding sites have a 3′ stimulator (Atkins et al. 1990; Atkins and Gesteland 1995). These stimulators are sequences of apparently linear RNA (as in Ty3 (Farabaugh et al. 1993; P.J. Farabaugh, personal communication)), stem loop structures (as in *dnaX* (Tsuchihashi and Kornberg 1990; Flower and McHenry 1990; Blinkowa and Walker 1990) and HIV (Jacks et al. 1988; Vickers and Ecker 1992; Cassan et al. 1994)), or pseudoknots (as in the Coronavirus, IBV (Brierley et al. 1989, 1991), or Murine Leukemia Virus (Wills et al. 1991; Feng et al. 1992; Wills et al. 1994)). However, 5′ stimulators have also been found and are described here.

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Abbreviations: RF, release factor; nt, nucleotide; aa, amino acid.

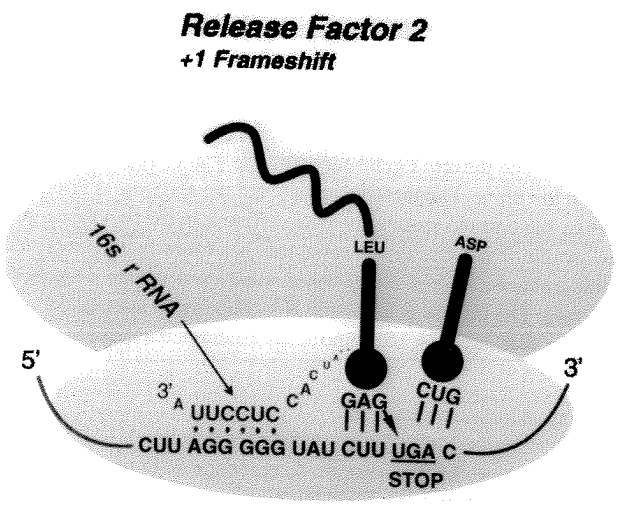
B. Larsen,[1] K. Brady, and J.F. Atkins. Department of Human Genetics, University of Utah, Salt Lake City, Utah 84112, U.S.A.
J. Peden. Department of Genetics, University of Nottingham, England.
S. Matsufuji, T. Matsufuji, R. Maldonado, N.M. Wills, and R.F. Gesteland. Department of Human Genetics and Howard Hughes Medical Institute, University of Utah, Salt Lake City, Utah 84112, U.S.A.
O. Fayet. CNRS, Toulouse, France.

[1] Author to whom all correspondence should be addressed.

An rRNA–mRNA interaction stimulates +1 frameshifting

The stimulatory effect of an mRNA–rRNA interaction on frameshifting was discovered in the expression of the *E. coli* release factor 2 (RF2) synthesis. The RF2 protein mediates polypeptide chain release at UGA and UAA codons (Capecchi 1967; Scolnick et al. 1968). Its expression is regulated by controlled variation in the efficiency of a +1 ribosomal frameshift required for its synthesis (Craigen and Caskey 1986). The zero frame of the gene for RF2, *prfB*, extends for 25 codons, terminated by a UGA codon, and the remaining 340 codons are in the +1 frame. A proportion of the initiating ribosomes shift

Fig. 1. Recoding signals for RF2. Shown are the frameshift site CUU UGA, the 0 frame stop codon, and the Shine–Dalgarno interaction, all necessary for +1 frameshifting during RF2 expression. $tRNA^{leu}$ (anticodon $^{3'}GAG^{5'}$) dissociates from the CUU leucine codon and slips +1 to re-pair with the overlapping UUU.



frame at codon 25, just before the UGA terminator, and resume triplet reading in the +1 frame to synthesize RF2 (Craigen et al. 1985). The frameshift event is autoregulatory. This has been shown both in vitro, by addition of exogenous RF2 (Craigen and Caskey 1986), and in vivo, by analysis of *prfB* mutants (Kawakami and Nakamura 1990).

The minimal sequence of *prfB* mRNA necessary for efficient +1 frameshifting has been defined by mutagenesis of a synthetic RF2 frameshift cassette cloned into a *lacZ* reporter system (Weiss et al. 1987; Curran and Yarus 1988). The frameshift site itself includes codons 25 and 26, CUU UGA. $tRNA^{Leu}$ (anticodon GAG) dissociates from the CUU leucine codon and slips +1 to re-pair with the overlapping UU U (Weiss et al. 1987) (Fig. 1). It is important that the codon 3′ of the CUU codon is a stop codon because changing it to a sense codon decreased frameshifting 10-fold (Weiss et al. 1987; Curran and Yarus 1989; Sipley and Goldman 1993). Even though the stop codon stimulates frameshifting, it is also the site of a competing reaction. RF2 binding to the UGA stop codon at the ribosomal A site competes with frameshifting leading to termination. When the concentration of RF2 is low, UUU is more frequently available for re-pairing by $tRNA^{Leu}$ and frameshifting wins out. However, when RF2 is abundant, the 3′ U is occupied in recognition by RF2 and termination wins out. The shift to the +1 frame, and thus synthesis of RF2, is therefore dependent on the existing level of RF2.

An additional factor involved in regulating RF2 expression is RF3, discovered as a third protein factor involved in stimulating termination (Milman et al. 1969; Goldstein et al. 1970; Grentzmann et al. 1994; Mikuni et al. 1994). Recently, it was shown that RF3 mainly stimulates termination at UGA stop codons, thereby indirectly affecting RF2 frameshifting (Grentzmann et al. 1995).

An additional and crucial stimulatory element for the frameshifting is a Shine–Dalgarno (SD) sequence, AGGGGG, located three bases 5′ of the CUU shift codon (Fig. 1). Though this particular SD sequence plays no role in translational initiation, like its initiation counterpart, it pairs with its complement near the 3′ end of 16S rRNA. However, here the pairing is with rRNA in elongating ribosomes (Weiss et al. 1988). The exact SD sequence and the spacing between it and the +1 shift site are critical for high-level frameshifting (Weiss et al. 1987; Curran and Yarus 1988). Single base substitutions decrease frameshifting 5-fold or more and changing the spacing by one base reduces frameshifting 15-fold. Recent results have shown that the SD interaction with elongating ribosomes may also serve to decrease the effectiveness of termination at codon 26. This is inferred from showing a positive effect of the presence of the SD sequence on in-frame readthrough (B. Larsen, J.F. Atkins, and R.F. Gesteland, in preparation). In this instance, though perhaps not in others, the stimulation of readthrough by an SD sequence is likely to be of only indirect significance. In RF2, readthrough is unproductive; the next stop codon in the zero frame is 32 codons 3′ and potentially drains ribosomes destined for frameshifting. However, increased readthrough is a reflection of decreased termination, which should also lead to increased frameshifting. It seems reasonable to infer that the SD interaction acts to stimulate frameshifting by decreasing termination (B. Larsen, J.F. Atkins, and R.F. Gesteland, in preparation). Additionally, a direct role for the SD interaction has been shown. The SD interaction stimulates frameshifting in the absence of the stop codon (Weiss et al. 1987).

SD spacing affects frameshifting direction

When SD stimulation of +1 frameshifting was discovered, equivalent effects on −1 frameshifting were sought and not found. A SD sequence placed 2, 3, or 4 nucleotides 5′ of a −1 slippery sequence inhibited frameshifting (Weiss et al. 1990*a*). However, recently, the study of several cases of programmed −1 frameshifting has shown that SD sequences can play an important stimulatory role (Larsen et al. 1994; B. Larsen, J.F. Atkins, and R.F. Gesteland, unpublished; O. Fayet, personal communication). The spacing requirement is quite different than for +1 frameshifting, and for this reason, the stimulatory effect on −1 frameshifting was missed in the early studies. The optimal spacing between the SD sequence and −1 shift site is between 9 and 14 nucleotides, though considerably more latitude is permitted than for +1 frameshifting (Larsen et al. 1994) (Fig. 2). The initial identification of a SD sequence involved in −1 frameshifting was done in *dnaX* of *E. coli* (Larsen et al. 1994) and in IS911 (O. Fayet, personal communication). Other genes that might use similar programmed frameshifting were revealed by a computer search. We looked for an internal SD sequence 9 to 14 nucleotides 5′ of A AAA AAG. This heptanucleotide sequence is particularly shifty in *E. coli* (Weiss et al. 1989). The genes found in the search are listed in Table 1. Two SD-like sequences were found 5′ of the shift site in IS1133 (Chiou and Jones 1993). The first SD is 13 nucleotides and the second SD is 5 nucleotides 5′ of the shift site. We predict that only the first SD will affect −1 frameshifting. The formation of the first SD interaction presumably prevents the formation of the second interaction (Weiss et al. 1990*a*).

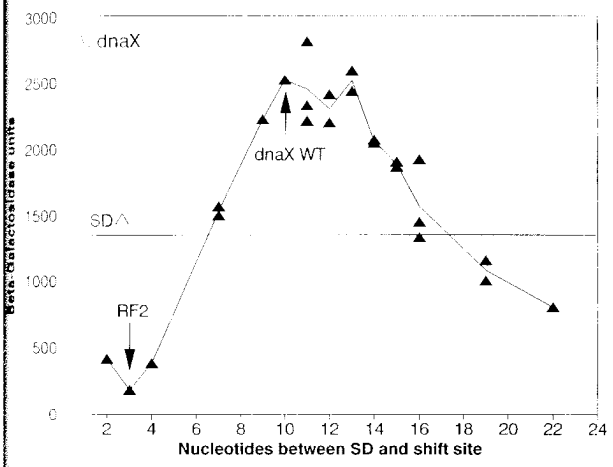
The difference in spacing requirements for stimulation of

Table 1. Sequences showing homology with the *dnaX* frameshift cassette.

Gene	Sequence	References
dnaX	AGGGAG——————— AAAAAAG	Yin et al. 1986
Bac. Di.	AGGGA——————— AAAAAAG	Harry et al. 1989
C5a	AGGAG——————— AAAAAAG	Chen and Cleary 1990
gt1	GGGAG——————— AAAAAAG	Carne et al. 1991
gt22	GGGAG——————— AAAAAAG	Lam et al. 1994
IS911	GGAG——————— AAAAAAG	Polard et al. 1991
IS861	GGAG——————— AAAAAAG	Rubens et al. 1989
IN133	GGGAGG—GGGAG——————— AAAAAAG	Chiou and Jones 1993
IS222	GGAG——————— AAAAAAG	Kropinski et al. 1994
IN1069	AGGAGG——————— AAAAAAG	Rauch et al. 1994
IS904	AGGAGG——————— AAAAAAG	Rauch et al. 1990

Note: D sequence, frameshifting site, and the spacing between the SD sequence and shift site are indicated.

Fig. 2. I ect of deletions and additions of nucleotides between he SD s uence and the shift site on the level of −1 rameshi ing. For reference, the *dnaX* WT and the RF2 WT RNA acings are indicated with arrows. SDΔ, the horizontal ine, ind tes the amount of frameshifting with the SD sequence eleted. ll constructs contain the 3′ stem–loop structure.



l and l frameshifting is perhaps indicative of a conforma- onal ange caused by movement of the ribosome with RNA d rRNA still paired in an SD interaction. The reason or the acing difference is unclear but perhaps the rRNA– RNA airing results in tension in the −1 case or compres- on in e +1 case that is relieved by mRNA slippage in the ppropte direction (Larsen et al. 1994). However, it is also ossibl hat a pause caused by the SD interaction may con- ribute or possibly even be the sole determinant of, the stim- lation frameshifting.

Distribution of SD-like motifs in coding sequences

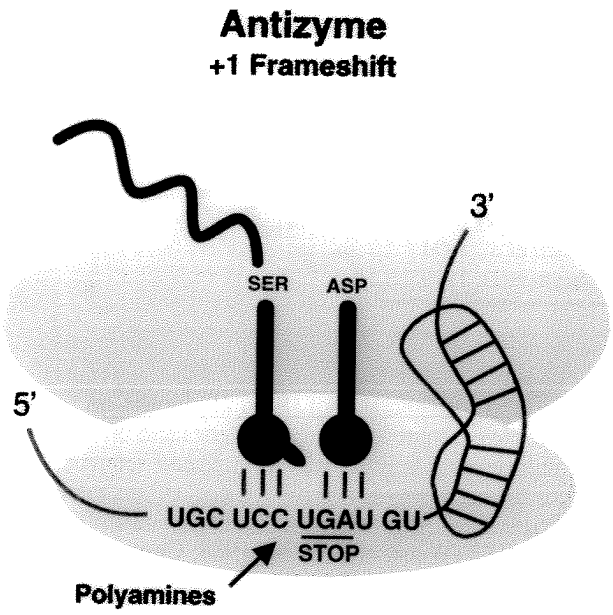
The r of SD-like sequences in programmed +1 and −1 ames fting and their ability to stimulate frameshifting in the sence of a stop codon opens up the possibility that SD-like quen s have a much wider role in recoding than previously ough (Larsen et al. 1994). We were therefore interested in examining the distribution of SD-like motifs in coding sequences. We examined 1980 *E. coli* genes and open reading frames, representing at least half of all *E. coli* sequences, for the SD consensus sequences AGGAGG, as well as three other SD-like sequences known to be involved in frameshifting, i.e., AGGGGG (RF2 (Weiss et al. 1987, 1988; Curran and Yarus 1988)), AGGGAG (*dnaX* (Larsen et al. 1994)), and GGAG (IS911 (O. Fayet, personal communication)). Relative to the expected frequencies for these sequences, estimated from codon usage and amino acid composition, these SD sequences are underrepresented in all reading frames but particularly in highly expressed genes, where codon usage is known to be biased for translational efficiency (Sharp et al. 1993). This supports the hypothesis that the 3′ end of 16S rRNA is continuously scanning the mRNA for possible SD-like interactions (Larsen et al. 1994): such interactions could result in translational pausing and would be expected to be strongly selected against in highly expressed genes.

5′ sequences also stimulate mammalian +1 frameshifting

Although frameshifting is known to be involved in decoding a substantial number of plant and animal viruses (Atkins et al. 1990; Atkins and Gesteland 1995), only recently has frameshifting in decoding a mammalian chromosomal gene been discovered. This is the antizyme gene involved in polyamine biosynthesis (Rom and Kahana 1994; Matsufuji et al. 1995). Unlike the eukaryotic viral frameshifting known to date, the obligatory frameshifting in antizyme decoding is +1. The efficiency of frameshifting is dependent on the level of polyamines. The amount of antizyme, in turn, indirectly governs the amount of polyamines synthesized to complete a regulatory circuit. The shift site, which has been identified in reticulocyte lysate cell-free translation studies, is UCC UGA U (Matsufuji et al. 1995). Re-pairing by the UCC-reading serine tRNA does not seem to be involved, rather the fourth base, the first base of the zero frame stop codon, is likely to be occluded by the serine tRNA. A 3′ pseudoknot contributes to the frameshifting (Matsufuji et al. 1995)) (Fig. 3), and recent experiments reveal an additional 5′ element.

The first hint of a 5′ stimulatory element came from analysis of 5′ deletion mutants. Deletion of antizyme sequences up

Fig. 3. The antizyme frameshift cassette. The frameshift site is UCC UGA U. UGA (underlined) is the stop codon in the 0 frame. The pseudoknot structure 3′ of the shift site enhances frameshifting.



to three codons 5′ of the zero frame stop codon reduced the frameshifting dramatically (Matsufuji et al. 1995; S. Matsufuji, T. Matsufuji, R.F. Gesteland, and J.F. Atkins, unpublished results). Any single base substitutions of the two codons upstream of the frameshift site did not significantly change the level of frameshifting, but deletions of the two codons did substantially affect the frameshift efficiency, indicating that the spacing between a 5′ element and the shift site is crucial (Matsufuji et al. 1995). Recent results have pinpointed a sequence several codons 5′ of the shift site that is important for frameshifting. The modus operandi of this 5′ stimulatory sequence is unclear. Whether it forms a structure or interacts in a linear manner with a ribosome component or is the site of binding of some nonribosomal protein remains to be investigated.

A 5′ nascent peptide is involved in recoding

The well characterized 5′ stimulatory elements described above have their effects through linear sequence elements within the mRNA that pair with ribosomal RNA. Another type of 5′ element, a nascent peptide sequence, is crucially involved in stimulating hopping–bypass in expression of bacteriophage T4 gene *60*.

The coding region of gene *60* contains an internal 50 nucleotide untranslated coding gap separating codon 46 and 47 (Huang et al. 1988). Results presented by Weiss et al. (1990*b*) suggest that *E. coli* ribosomes bypass the coding gap of gene *60* with very high efficiency by responding to recoding signals within and preceding the gap.

Five different gene *60* recoding signals have been identified for the hop–bypass (Weiss et al. 1990*b*; Gesteland et al. 1992); matched take-off and landing sites, a stop codon immediately following the take-off site, a short stem–loop structure at the take-off site, an optimal 50-nt spacing, and a cis-acting 16-aa

Table 2. Point mutations at tyrosine 17. Relative β-galactosidase activity (Miller units) in strains carrying the designated constructs compared with the wild-type construct.

Construct	Relative activity
Wild type	1.0
Asp	0.36
Ala	0.29
Phe	1.05
Gly	0.35

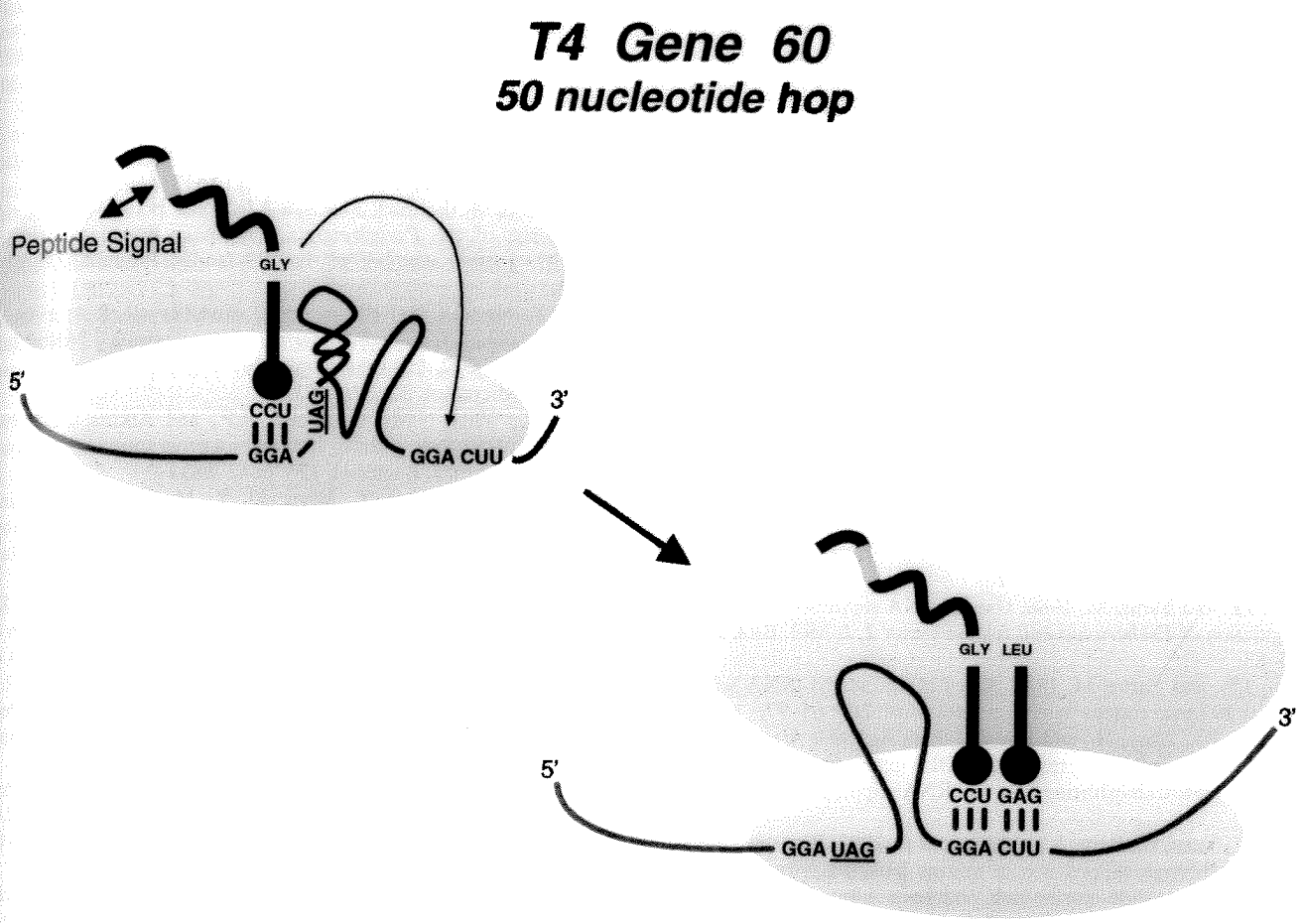
stretch of the nascent peptide translated from codons 17–32, preceding the gap (Fig. 4).

The involvement of a 5′ stimulatory element in gene *60* bypass was first indicated by 5′ deletion analysis. Deletion of gene *60* sequences located 5′ of the gap substantially reduces bypass. In addition, internal deletions narrowed the sequence element to the central region of the 5′ segment (Weiss et al. 1990*b*).

The possibility that a peptide sequence translated from this region, and not the primary mRNA sequence, is important for gap bypass was tested with a series of compensating insertions and deletions placed throughout the 5′ segment. The combination of insertions and deletions direct the ribosome to change frame, translate several codons, and then enter the zero frame again. Translating the zero frame through codons 17–32 was shown to be crucial. To clinch the importance of this amino acid sequence in the nascent chain, these codons were changed to synonymous codons that used a very different RNA sequence to encode the same peptide sequence and bypass was unabated. These mutations all indicate that it is the peptide sequence and not the primary mRNA sequence that is involved in stimulating bypass.

A ribosome protects between 30 and 40 residues of nascent polypeptide chain from proteolytic digestion (Smith et al. 1978). This indicates that residues 17 to 32 of the nascent peptide in gene *60* may be within the ribosome as the decoding site reaches the beginning of the gap sequence (Weiss et al. 1990*b*). However, a direct test of location in the ribosome of the important peptide sequence for gene *60* has not been done. It is not at all clear how the nascent peptide can act within the ribosome to stimulate hopping–bypass. Different approaches have been undertaken to obtain more information about the features of the nascent peptide. The significance of each amino acid in the nascent peptide was examined by amino acid substitutions. The 16 amino acids were changed to aspartic acid one at a time with only modest effects on hopping, except for tyrosine 17, which reduced hopping from 100% to 30% when changed to aspartic acid, glycine, or alanine (Table 2). However, phenylalanine at this position was as effective as tyrosine, implying that the aromatic nature of this residue is important (K. Brady, unpublished results). Could the nascent peptide form a secondary structure within the exit channel? The possible structure of the nascent peptide was examined by computer analysis. Different mutations, which presumably disrupt the putative structure, are being analyzed. A genetic approach has also

Fig. 4. Recoding signals for gene *60*. Shown are the take-off and landing sites, the stop codon immediately following the take-off site, the stem–loop structure, the 50 nucleotide spacer region, and the cis-acting nascent peptide. All elements are shown before and after ribosome hopping–bypass.



been taken to identify the function of the peptide in ribosomal bypass. A genetic selection is being devised for extragenic mutations that increase bypass of gene *60* constructs with a mutated nascent peptide. It is still not clear how the peptide induces bypass, but these studies may give a better understanding of the function.

Conclusions

Most stimulatory elements for recoding are 3′ of the recoding site and have been the primary focus of attention. Upstream elements are also significant. In *dnaX* and antizyme recoding, both 5′ and 3′ stimulators are operative. The known 3′ stimulators of programmed frameshifting, apart from that found in Ty3, act via secondary mRNA structure. In contrast, secondary mRNA structure is not known to be involved in the small but variable array of upstream stimulators. Determination of the mode of action of upstream stimulators is still in its infancy, but the T4 gene *60* active nascent peptide presumably interacts with the exit channel of the ribosome, while the Shine–Dalgarno sequence interacts with its 16S rRNA counterpart. The emerging antizyme 5′ stimulator remains mysterious. It remains to be seen whether all upstream stimulators cause a pause but, even if so, this effect is unlikely to be the sole direct mediator of frameshifting.

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