JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 6842–6851
0021-9193/94/$04.00+0

Vol. 176, No. 22

rRNA-mRNA Base Pairing Stimulates a Programmed −1 Ribosomal Frameshift

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Received 11 July 1994/Accepted 16 September 1994

Base pairing between the 3′ end of 16S rRNA and mRNA is shown to be important for the programmed −1 frameshifting utilized in decoding the *Escherichia coli dnaX* gene. This pairing is the same as the Shine-Dalgarno pairing used by prokaryotic ribosomes in selection of translation initiators, but for frameshifting the interaction occurs within elongating ribosomes. For *dnaX* −1 frameshifting, the 3′ base of the Shine-Dalgarno sequence is 10 nucleotides 5′ of the shift site. Previously, Shine-Dalgarno rRNA-mRNA pairing was shown to stimulate the +1 frameshifting necessary for decoding the release factor 2 gene. However, in the release factor 2 gene, the Shine-Dalgarno sequence is located 3 nucleotides 5′ of the shift site. When the Shine-Dalgarno sequence is moved to the same position relative to the *dnaX* shift site, it is inhibitory rather than stimulatory. Shine-Dalgarno interactions by elongating ribosomes are likely to be used in stimulating −1 frameshifting in the decoding of a variety of genes.

Programmed ribosomal frameshifting is used for a variety of purposes in the decoding of a significant minority of genes in probably all organisms (1). For instance, in decoding the *Escherichia coli dnaX* gene, half of the ribosomes frameshift internally in the coding sequence and terminate to yield a short product (4, 10, 36). Accessory mRNA sequence elements, stimulators, serve to elevate the level of frameshifting at the shift site. In all known cases of −1 frameshifting, the stimulators are 3′ to the shift site. The only known 5′ stimulator is involved in +1 frameshifting. In decoding the *E. coli* polypeptide chain release factor 2 (RF2), a Shine-Dalgarno (SD)-like sequence 5′ of the shift site base pairs with its complement near the 3′ end of 16S rRNA (8, 39–41). This is the same interaction that is well known to be crucial in selection of ribosome initiation sites. It is also known that ribosome binding to SD sequences can be independent of initiation (32, 38; see also reference 14). The involvement of the internal SD sequence in RF2 frameshifting shows that elongating ribosomes must unexpectedly be able to continuously scan mRNA for potential pairing.

In this paper, we show that an upstream SD-like interaction can also mediate −1 frameshifting, as in expression of *E. coli dnaX*, which encodes DNA polymerase III subunits. *E. coli* DNA polymerase III consists of three core subunits and seven accessory subunits (20). Of the seven accessory subunits, τ and γ are both translated from the same *dnaX* transcript. τ is the 71-kDa full-length product of the *dnaX* gene translated entirely in the zero frame. γ is produced by a remarkably efficient (~50%) −1 ribosomal frameshift. After translating two-thirds of the gene, half of the ribosomes slip from the zero to the −1 frame. One codon later, the shifted ribosomes reach a UGA stop codon and produce the γ (47-kDa) subunit (4, 10, 36).

To date, two essential elements of the *dnaX* −1 frameshifting have been identified. These two *cis* elements are an A AAA AAG heptanucleotide shift sequence followed by a stem-loop stimulatory structure (10, 33, 34, 36). Two similar elements which stimulate −1 frameshifting are involved in onco- and lenti- retroviral shifting, a shift site and a 3′ RNA structure, either a stem-loop or a pseudoknot (17, 22). A common −1 shift site motif is the heptanucleotide sequence X XXY YYN. At such double slippery sites, two adjacent ribosome-bound tRNAs (at codons XXY YYN) simultaneously slip back to the −1 frame (XXX YYY) (18). The *dnaX* shifty heptanucleotide sequence matches the retroviral frameshift consensus with a sequence which is especially shifty in *E. coli* (34, 42). With the expanded use of the third element described here, a 5′ SD sequence, the sophistication of programmed ribosomal frameshift motifs (2) becomes even more apparent.

MATERIALS AND METHODS

Plasmids used for in vitro 5′ deletion experiments. The plasmids used for the in vitro 5′ deletion experiments were all constructed from plasmid pZT3 (35) kindly provided by Zenta Tsuchihashi. pZT3 contains the *dnaX* gene, which has quasi-repetitive sequences which complicate the use of PCR. Large deletions at the 5′ end of *dnaX* lead to a frameshift product too small to be seen on a standard protein gel. Therefore, each deletion had 210 codons from the chloramphenicol acetyltransferase (CAT) gene inserted in front of the *dnaX* frameshift site. Inserts in pZT3 were all constructed by a two-step PCR (Fig. 1). In the first step, pCAT control vector (Promega) was used as template. From the 5′ end, primer 1 (104 nucleotides total) had a tail consisting of an *Xba*I site, the T7 phage DNA polymerase gene SD sequence (35), and the *dnaX* sequence from nucleotide 142 to 198 (according to the numbering system in reference 43). Twenty-one nucleotides at the 3′ end of primer 1 were homologous to the CAT gene (nucleotides 29 to 49; nucleotide 1 is defined as the A in the start codon). Primer 2 contained only CAT priming sequences (nucleotides 657 to 637). For the second PCR, PCR product 1 (our designation) was gel purified and used as template. Primer 1 was also used for the second PCR along with primer 3 (Fig. 1). Different deletions were made by alteration of primer 3, varying the amount of *dnaX* sequence incorporated into the final clone. To make clone pZX1424, primer 3 (68 nucleotides

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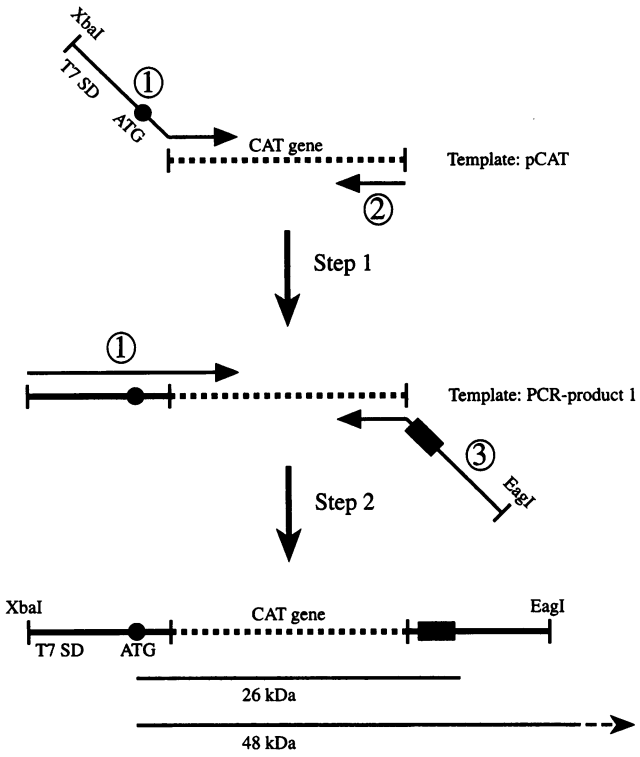

FIG. 1. Diagram of the two-step PCR procedure used to create plasmids for the 5′ deletion experiments. Primer numbers are encircled and described in detail in the text. Solid lines represent sequences from plasmid pZT3 (35). Dotted lines represent sequences from the CAT gene. Filled circle, ATG start codon; solid box, *dnaX* frameshift site; T7 SD, SD sequence of the T7 phage DNA polymerase gene. *Xba*I and *Eag*I are unique in plasmid pZT3. Translation of these plasmids in vitro generates two polypeptides: a ~26-kDa frameshift product and a ~48-kDa zero frame product.

total) had a 5′ tail consisting of an *Eag*I site and the *dnaX* sequence from nucleotide 1464 to 1424 (43), including the frameshift site. Twenty-one nucleotides at the 3′ end of the primer were homologous to the CAT gene (nucleotides 657 to 637). This PCR product was then trimmed with *Xba*I and *Eag*I and cloned into pZT3 cut with *Xba*I and *Eag*I, creating plasmid pZX1424. The *Eag*I site is located in the coding region of the *dnaX* gene. After cloning of the PCR fragment into the wild-type plasmid pZT3, the *Eag*I site is restored and the coding sequence 3′ of the frameshift site is identical to the *dnaX* wild-type gene. Translation of plasmid pZX1424 will give a 26-kDa frameshift product and a 48-kDa zero-frame product. pZX1424 has only one *dnaX* nucleotide upstream of the frameshift site.

A second plasmid, pZX1327, contained more of the *dnaX* sequences 5′ to the shift site. For the construction of pZX1327, primer 3 (91 nucleotides total) had a 5′ tail consisting of an *Asc*I site and the *dnaX* sequence from nucleotide 1382 to 1327. Twenty-one nucleotides at the 3′ end of the primer were again homologous to the CAT sequence (nucleotides 657 to 637). This PCR product was then trimmed with *Xba*I and *Asc*I and cloned into pZT3 cut with *Xba*I and *Asc*I, creating plasmid pZX1327. The *Asc*I site is located in the coding region of the *dnaX* gene but upstream of both the *Eag*I site and the frameshift site. After cloning of the PCR fragment into the wild-type plasmid pZT3, the *Asc*I site is restored and the coding sequence 3′ of the *Asc*I site is identical to the *dnaX* wild-type gene. It was now straightforward to clone synthetic oligonucleotides into pZX1327 between the *Asc*I and the *Eag*I sites; pZX1407 and pZX1398 were made in this fashion. pZX1407 has 18 *dnaX* nucleotides 5′ of the frameshift site, and pZX1398 has 26.

Plasmids used for in vivo β-galactosidase activity measurements. Site-directed mutagenesis of the *dnaX* frameshift window was accomplished by use of two complementary synthetic oligonucleotides cloned into a *lacZ*-containing plasmid, RW201 (a gift from R. Weiss) (Fig. 2). The wild-type control contains 63 bp of the *dnaX* sequence (shown in boldface type in Fig. 2), from nucleotide 1406 to nucleotide 1468 (43), with *Hin*dIII- and *Apa*I-compatible ends added. All frameshift constructs maintain the zero frame of the CAT gene at the 5′ end, and the −1 frame of *dnaX* continues into *lacZ*. To measure β-galactosidase activity for the frameshift product, the UGA stop codon (positions 1434 to 1436) in the −1 frame was modified to UGU (cysteine). The oligonucleotides were gel purified and ligated into *Apa*I- and *Hin*dIII-digested vector RW201. All plasmids were transformed into *E. coli* K-12 SU1675, a *recA56* derivative of CSH26 (21). Junctions and

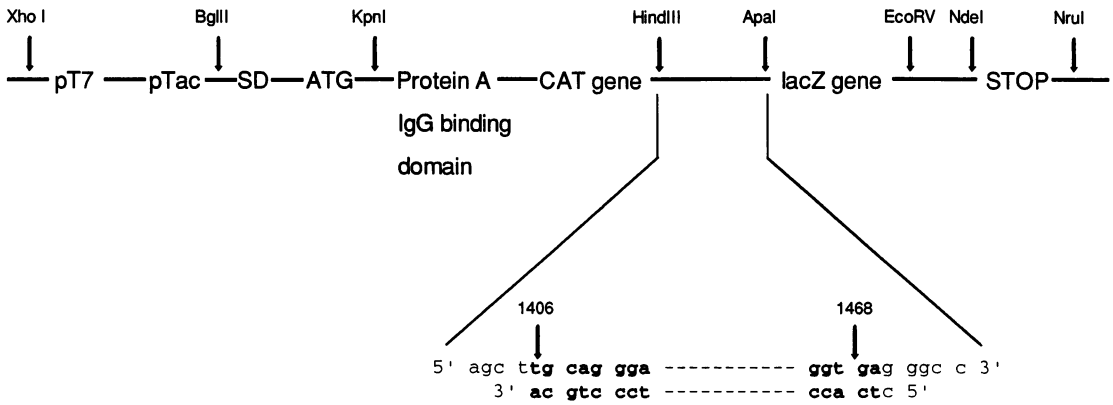

FIG. 2. Diagram of relevant portions of vector RW201. The *Hin*dIII and *Apa*I sites used for cloning of synthetic oligonucleotides are indicated. The inset shows the oligonucleotide inserts used to construct *dnaX* mutants. Nucleotides shown in boldface type are present in the *dnaX* sequence (numbering as in reference 43). The nucleotide sequence 3′ of the *dnaX* sequence is g ggc cct aat tca. tca is the fifth codon of *lacZ*. pT7, T7 promoter; pTac, Tac promoter; SD, initiating SD sequence. All restriction sites indicated are unique in the plasmid. The drawing is not to scale.

oligonucleotide inserts for all constructs were verified by sequencing.

Plasmids used for in vitro translation. RW201 was modified to maintain the unique *Hin*dIII and *Apa*I cloning sites, but with deletion of 2/3 of the *lacZ* gene sequence. RW201 was opened with *Hin*dIII and *Nde*I (Fig. 2). A PCR fragment containing the sequence between the *Hin*dIII site and the *Eco*RV site was then ligated into the digested plasmid, in effect deleting the *Eco*RV-*Nde*I fragment. The PCR primer at the *Eco*RV site had a tail which contained three UAA stop codons. The new plasmid was verified by sequencing of the junctions and the entire PCR product.

Plasmids used to test the SD/anti-SD interaction. Plasmid pASDIX-P_L, derived from pKK3535 (a pBR322 derivative) (16), was kindly provided by Marcel Brink, University of Leiden, The Netherlands. The plasmid contains a mutated *rrnB* operon; the anti-SD region at the 3′ end of the 16S rRNA gene was altered from 5′CCUCCU3′ to 5′GGAGGU3′ (15). The plasmid-borne *rrnB* operon is under λ P_L control and is temperature inducible because of the presence of the λ cI857 repressor. This repressor was provided by the low-copy-number pSC101-based plasmid pLG857, kindly provided by Michael O'Connor, Brown University, Providence, Rhode Island. To maintain compatibility between the three plasmids, the third plasmid, containing the reporter gene, was constructed from pACYC184. The *Xho*I-*Nru*I fragments from the RW201 plasmids (Fig. 2) were cloned into the *Sal*I and *Nru*I sites of pACYC184. In the plasmids IFC-SD, WT-SD, and SDM 2-SD, the SD upstream of the initiation codon of the protein A gene was changed from 5′AGGGGG3′ to 5′ACC TCC3′ to allow translation from the mutated ribosomes. This change was accomplished by use of two complementary oligonucleotides with *Bgl*II- and *Kpn*I-compatible ends (Fig. 2).

In vivo β-galactosidase assays. Whole-cell assays (100 μl in Luria-Bertani medium) were done under a modification (39) of the Miller (21) conditions. For the experiments done with the mutant 16S rRNA, the cultures were grown at 30°C to an optical density at 600 nm of 0.2 to 0.25, temperature shifted to 42°C, and grown for two cell doublings, and the β-galactosidase activity was determined.

In vitro transcription-translation assays. Coupled transcription-translation reactions were performed with an *E. coli* S-30 kit (Promega). The total reaction mixture volume was 12.5 μl. The reactions were performed according to the manufacturer's recommendations, except that 10 U of T7 RNA polymerase, 80 μg of rifampin per ml, and 7.5 μCi of [^{35}S]methionine were added to the reaction mix. Translation products were electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). Quantitation was done with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, Calif.), with correction for the number of methionine residues in each product (14 in the full-length product and 7 in the frameshift product).

Nucleotide sequence accession number. The nucleotide sequence of pACYC184 is available from GenBank under accession number X06403.

RESULTS

In vitro 5′ deletion experiments. A 3′ stem-loop structure is known to stimulate the −1 frameshifting in decoding the *dnaX* gene; sequence changes decrease the level of frameshifting (10, 36). Deletion of the 3′ end of the *dnaX* gene downstream of the stem-loop did not affect frameshifting (10, 36). If the stem-loop structure is deleted, frameshifting still occurs at 10% of the wild-type level (10, 36), fivefold higher than expected at A AAA AAG alone (42). All plasmids used in these previous 3′ deletion experiments have the full 5′ coding region of *dnaX*. Therefore, we looked for a 5′ stimulator. The preliminary experiments were done by deletion of 5′ sequences and testing of the constructs in an in vitro transcription-translation assay. The wild-type *dnaX* construct, pZT3 (Fig. 3, lane A), shows equal amounts of the frameshift product, γ, and the zero frame product, τ. Large deletions at the 5′ end of the *dnaX* gene lead to frameshift products too small to be analyzed on standard protein gels. To increase the size, part of the CAT gene was inserted upstream of the frameshift site (see Materials and Methods and Fig. 1). With a CAT construct containing 26 nucleotides 5′ of the *dnaX* shift site (pZX1398 [Fig. 3, lane D]), two polypeptides of the expected size were seen, a 26-kDa frameshift product and a 48-kDa zero frame product. The apparent frameshifting level was greater than 50%. (Note that the real efficiency might be only 50%, as continued synthesis 3′ of the shift site, to give the longer protein [the zero frame product], sometimes results in premature termination. Premature termination produces a number of minor products of intermediate sizes that, to avoid an underestimate, should be integrated into the total.) With as little as 18 nucleotides preceding the shift site, the efficiency is unchanged (pZX1407 [Fig. 3, lane C]). However, with only one nucleotide from *dnaX*, 5′ to shift site (Fig. 3, lane B), the zero frame product is dominant. This result shows that a 5′ sequence element within 18 nucleotides of the shift site is important for stimulating this −1 frameshift.

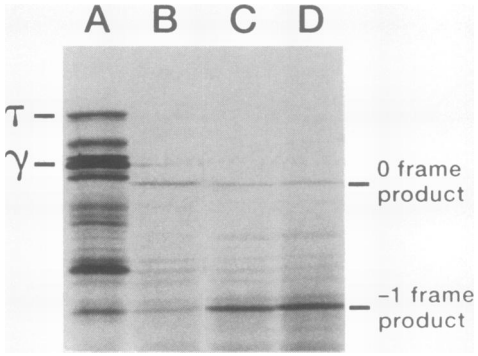

FIG. 3. In vitro 5′ deletion experiments. Autoradiograph of *E. coli* S-30 extract protein synthesis products separated by SDS–10% PAGE. Lane A, *dnaX* WT plasmid pZT3 (35); lane B, pZX1424 (one *dnaX* nucleotide 5′ of the shift site); lane C, pZX1407 (18 *dnaX* nucleotides 5′ of the shift site); lane D, pZX1398 (26 *dnaX* nucleotides 5′ of the shift site). Lane A has the WT τ and γ products indicated. The two translation products from pZX14124, pZX1407, and pZX1398 (lanes B, C, and D) are indicated as −1 frame product and 0 frame product.

This deletion analysis together with data presented earlier (10, 36) defined at most 60 nucleotides important for the *dnaX* frameshift: 18 nucleotides 5′, 7 nucleotides from the tandem shift site A AAA AAG, and 35 nucleotides from the 3′ stem loop. To analyze this region further with β-galactosidase as a reporter, synthetic oligonucleotides containing this frameshift cassette were cloned between the CAT gene in the zero frame and the *lacZ* gene in the −1 frame (Fig. 2). In these constructs, the −1 frame stop codon (UGA) was changed to UGU (Cys). The change was not expected to affect the efficiency of frameshifting (36). However, to ensure that the β-galactosidase assays would not be affected by the A-to-T change, the effect of the change was confirmed by analysis of the protein products from an in vitro transcription-translation assay (see

TABLE 1. Mutational analysis of the upstream SD-like sequence

Clone	Mutation(s)[a]	DNA sequence	β-Galactosidase	
			Whole-cell U	%
IFC[b]		gcAGGGAGcaaccaaagcA AAC AAA Gag	3,303	100
WT[c]		gcAGGGAGcaaccaaagcA AAA AAG agt	1,865	56
SDΔ[d]		aaccaaagcA AAA AAG agt	870	26
SDM 1[e]	$G_2G_3G_4$	a**CCC**ag	561	17
SDM 2	G_2–G_6	a**CCTCC**	846	26
SDM 3	G_2	a**C**ggag	2,012	61
SDM 4	G_3	ag**C**gag	1,440	44
SDM 5	G_4	agg**C**ag	1,154	35
SDM 6	A_5	aggg**T**g	1,084	33
SDM 7	A_5	aggg**G**g		53[f]
SDM 8	G_6	aggga**C**	1,797	54
SDM 9	G_3A_5	ag**T**g**T**g	1,402	43
SDM 10	G_2G_3	a**TC**gag	1,340	41
SDM 11	G_3G_4	ag**TC**ag	1,125	34
SDM 12	G_3G_4	ag**CT**ag	1,305	40
SDM 13	G_4A_5	agg**CG**g	1,430	43
SDM 14	G_4A_5	agg**CC**g	729	22
SDM 15	A_5G_6	agggg**CT**	1,132	34

[a] Our numbering begins at the 1st base in the SD sequence.
[b] Clone IFC has C (underlined) added between bases A_{19} and A_{20}, allowing translation directly into *lacZ*.
[c] The SD-like sequence is underlined in the WT construct.
[d] In SDΔ, 9 5′ nucleotides were deleted.
[e] Constructs numbered in this sequence (SDM 1 to 15) contain mutations (shown in boldface type) in the SD-like sequence.
[f] SDM 7 was tested in an experiment separate from that in which the other constructs were tested.

Fig. 6, lane B). Therefore, β-galactosidase levels directly reflect frameshifting efficiency, which was defined relative to an in-frame control (IFC, with C inserted between nucleotides A_{19} and A_{20} as well as UGA_{28} changed to UGU [subscripts are position numbers]; see Fig. 4 for numbering). As expected (4, 10, 36), the wild-type cassette showed high-level frameshifting (56% [Table 1, *dnaX* WT]).

Inspection of the 18 nucleotides 5′ of the shift site revealed an AGGGAG sequence that conforms to the SD consensus sequence typically involved in ribosome initiation (28) (Fig. 4). However, β-galactosidase expression is not due to initiation at this site (in the −1 frame relative to CAT) as there is no appropriately positioned XUG initiator codon downstream and the in vitro experiment described above (for example, see Fig. 6, lane B) shows the protein A–CAT–β-galactosidase fusion polypeptide but not β-galactosidase.

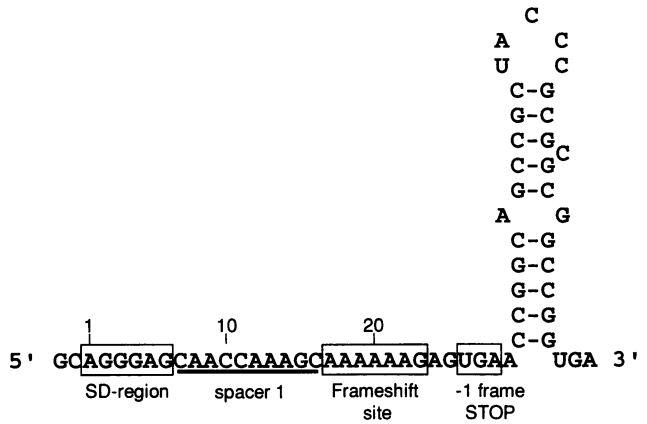

FIG. 4. The *dnaX* frameshift cassette. The boxed AAAAAAG sequence is the frameshift site. UGA (boxed) is the stop codon in the −1 frame. The stem-loop structure 3′ of the shift site enhances frameshifting. SD-region, internal SD sequence; spacer 1, nucleotides between the SD region and the frameshift site. Our numbering begins at the 1st nucleotide of the SD region. The 3′ end of the *dnaX* oligonucleotides was cloned into the *Apa*I site and thereby joined to the 5th codon of *lacZ* (see Fig. 2).

Importance of the AGGGAG sequence for frameshifting. The possible stimulatory effect of the upstream SD sequence was examined by deletion (SDΔ) and mutational analysis (SDM 1 to 15) (Table 1). All constructs in Table 1 contain the 3′ stem-loop structure. In SDΔ, nine nucleotides including the AGGGAG sequence were deleted; the frameshifting dropped from 56% to 26% (of the in-frame control). Changing $G_2G_3G_4$ to CCC (SDM 1) gave a similar drop (to 17%). Modifying the SD sequence to ACCTCC (SDM 2) also showed a significant drop (to 26%). SDM 3 to SDM 8 are single-base changes with varying results depending on which nucleotide is altered. Replacement of G_3 or G_4 with C (SDM 4 and SDM 5) reduced frameshifting levels (to 44 and 35%, respectively), whereas alteration of G_2 and G_6 (SDM 3, 61%; SDM 8, 54%) did not alter the level of frameshifting. Replacement of A_5 with T (SDM 6, 33%) showed a reduced level of frameshifting, while replacement at the same position with a G had no effect (SDM 7, 53%). A series of double mutations in the SD sequence (SDM 9 to 15) all showed reduced frameshifting compared with the *dnaX* WT clone (22 to 43%). All of these results show that there is a 5′ stimulator for the *dnaX* frameshift and that several of the nucleotides in the SD sequence are especially crucial.

Testing for interaction with 16S rRNA. The upstream sequence AGGGAG stimulates frameshifting in the *dnaX* gene. Does this sequence base pair with the anti-SD sequence in 16S rRNA? If this interaction exists, changes in crucial nucleotides in either the SD sequence or the anti-SD sequence should decrease the amount of frameshifting. Conversely, compensatory mutations in the two regions should restore frameshifting. The methodology for such experiments was developed (15, 19) and used previously in an analysis of

TABLE 2. Putative interaction between the SD-like sequence and the 16S rRNA anti-SD

ASD sequence and clone[a]	mRNA sequence		β-Galactosidase	
	Initiating SD	Frameshift SD	Whole-cell U	%
3′UUCCUCC5′[b]				
IFC	AGGGGG	AGGGAG	4,024	100
WT	AGGGGG	AGGGAG	2,309	57
SDM 2	AGGGGG	ACCTCC	839	21
IFC-SD	ACCTCC	AGGGAG	1,053	100
WT-SD	ACCTCC	AGGGAG	556	53
SDM 2-SD	ACCTCC	ACCTCC	306	29
3′UUGGAGG5′[c]				
IFC-SD	ACCTCC	AGGGAG	2,887	100
WT-SD	ACCTCC	AGGGAG	285	10
SDM 2-SD	ACCTCC	ACCTCC	852	30

[a] The first three constructs possess the wild-type initiating SD. The next six constructs have the initiating SD changed in order to base pair with the mutated ribosomes' anti-SD. The nomenclature for the constructs is defined in the text.
[b] Experiments performed at 30°C with wild-type ribosomes.
[c] Experiments performed at 42°C; mutant ribosomes are induced.

frameshifting on RF2 mRNA (41). A second plasmid provides controlled expression of a 16S rRNA gene into which alterations of choice are introduced. Upon induction, a complement of mutant ribosomes accumulates.

The original RW201 plasmid is ideally suited for these experiments. It is constructed so that the initiating SD sequence, between the *Bgl*II and *Kpn*I sites, and the frameshift window inserted between the *Hin*dIII and *Apa*I sites can be conveniently replaced with synthetic oligonucleotides. All of these sites are unique in the vector (Fig. 2). However, it is not compatible with the plasmid for expression of rRNA that is required for this experiment. To bypass this limitation, the modified *Xho*I-*Nru*I fragments from the RW201 derivatives were cloned into vector pACYC184 between the *Sal*I and *Nru*I sites (see Materials and Methods).

The mutated *rrnB* operon on plasmid pADIX-P_L is under λP_L control and is temperature inducible because of the presence of the thermolabile λ cI857 repressor on a third plasmid, pLG857. Both plasmids were introduced into the strains carrying *dnaX* constructs on pACYC184. When cells are grown at 30°C, the synthesis of mutant 16S rRNA is repressed by the cI857 repressor. By a temperature shift to 42°C, synthesis of the mutant 16S rRNA is derepressed, leading to accumulation of mutant 16S rRNA ribosomes. The level of mutated 16S rRNA reaches a maximum after 1 h of induction. Because of the wild-type *rrn* operons in the chromosome, normal ribosomes with wild-type 16S rRNA are also present in the cell (less than 50% of the total) (15, 16).

At 30°C, with no expression of the plasmid-borne rRNA gene, the ribosomes all possess a wild-type anti-SD sequence. The first three constructs in Table 2, IFC, WT, and SDM 2, all have an AGGGGG initiating SD and are translatable by wild-type ribosomes. IFC is an in-frame control made by changing the A AAA AAG frameshift site to A AAC AAA G. WT is the normal *dnaX* frameshift cassette except for a change of the −1 UGA stop to UGU. SDM 2 has the internal SD sequence mutated from AGGGAG to ACCTCC. The level of frameshifting relative to that of the in-frame control was 57% for WT and 21% for SDM 2, again showing the high level of frameshifting of the *dnaX* WT construct and the two- to threefold decrease when the internal SD is changed significantly.

In order to test the effect of the mutated ribosomes, a mutated initiating SD (AGGGGG to ACCTCC) was inserted in the same constructs, termed −SD. This sequence was chosen to complement the sequence of the mutated anti-SD in 16S RNA (15) to allow translation of these constructs by ribosomes containing mutant 16S rRNA. At 30°C, at which the synthesis of mutated rRNA is repressed, all three constructs showed reduced levels of β-galactosidase production compared with those of the wild-type initiating SD constructs (Table 2). For example, the in-frame control dropped from 4,024 (IFC) to 1,053 (IFC-SD) units. However, the relative amounts of frameshifting were unchanged (WT-SD, 53%; SDM 2-SD, 29%); only the amount of initiation was affected.

The synthesis of mutant 16S rRNA with an anti-SD sequence of 3′UGGAGG5′ complementary to both the mutant initiation SD and the mutant frameshift SD sequences was induced by temperature shifting of the cells to 42°C. Two effects are seen (Table 2). First, the total amount of initiation from the IFC-SD construct increased from 1,053 to 2,887 U, reflecting the ability of the mutant ribosomes to use the complementary mutant initiating SD sequence. Second, at 42°C there is a reversal in the efficiency of frameshifting of the two constructs with different SD sequences. Whereas at 30°C, the mutant internal SD construct has reduced frameshifting compared with WT (29% compared with 53%), at 42°C the mutant SD construct shows higher frameshifting (30%) than WT-SD (10%). In each case, the frameshift window which has the better opportunity to base pair with the predominant form of the 3′ end of 16S rRNA shows higher levels of frameshifting. These experiments directly demonstrate an interaction between the internal SD sequence from the *dnaX* message and the anti-SD sequence in 16S rRNA. (Consistent results were obtained in three separate experiments; however, the in-frame control gave some variability, possibly due to variations in the proportion of mutant ribosomes in different experiments.)

Spacer 1: the nucleotides between the SD sequence and the shift site. The stimulatory elements that have been identified in the +1 frameshifting in decoding the gene for *E. coli* RF2 are the frameshift site (CUU UGA) and an upstream SD sequence (AGGGGG) (8, 39, 41). The spacing between the SD and the shift site is critical for high-level frameshifting (39).

To see if the spacing between the SD sequence and the shift site in *dnaX* is as crucial as in the gene for RF2, several additions and deletions of nucleotides in spacer 1 were constructed, containing the 3′ stem-loop sequence. When necessary, correction of the reading frame was accomplished upstream of the SD sequence. SPA and SPD clones contain, respectively, additions and deletions in *dnaX* spacer 1 (Table 3).

Addition of 1, 2, or 3 nucleotides to spacer 1 did not affect the frameshifting level significantly (SPA 1 to 7). Additions of 4 and 5 nucleotides had little effect (SPA 8 to 11). However, addition of 6 (SPA 12 to 14), 9 (SPA 15 to 16), or 12 (SPA 17) nucleotides dramatically reduced frameshifting, and with SPA 13 to 17 the reduction was to a level comparable with that induced by the SD deletion (SDΔ). (Surprisingly, SPA 12 showed a higher frameshifting level than SPA 13 and SPA 14, even though all three have 16 nucleotides in spacer 1. Perhaps the added nucleotides result in an alternate pairing with 16S rRNA, similar to the T7 0.3 gene revertant which showed an alternate pairing with 16S rRNA nucleotides to restore expression of that gene [9].)

Deletions in spacer 1 also affected the level of frameshifting (Table 3). Deletion of 1 nucleotide had little effect (SPD 1), but deletion of 3 nucleotides reduced the frameshifting to about 1/3 of the in-frame control (SPD 2 and 3). SPD 4 to 6,

TABLE 3. Deletions and additions in spacer 1

Construct type and clone[a]	DNA sequence[b]	Spacer 1 length (nucleotides)	β-Galactosidase	
			Whole-cell U	%
Intact downstream stem-loop				
IFC	gcAGGGAGcaaccaaagcA AAC AAA G		4,593	100
WT	gcAGGGAGcaaccaaagcA AAA AAG	10	2,514	55
SDΔ	---------- FS site		1,275	28
Additions in spacer 1				
SPA 1	SD **C**---------- FS site	11	2,209	48
SPA 2	SD **T**---------- FS site	11	2,322	51
SPA 3	SD **G**---------- FS site	11	2,805	61
SPA 4	SD **GA**---------- FS site	12	2,194	48
SPA 5	SD **CG**---------- FS site	12	2,404	52
SPA 6	SD --**AAT**-------- FS site	13	2,433	53
SPA 7	SD --**CCG**-------- FS site	13	2,584	56
SPA 8	SD --**TACC**-------- FS site	14	2,063	45[c]
SPA 9	SD --**TACA**-------- FS site	14	2,041	45[c]
SPA 10	SD --**TACGG**-------- FS site	15	1,898	42[c]
SPA 11	SD --**TACCT**-------- FS site	15	1,862	41[c]
SPA 12	SD --**AATCGT**-------- FS site	16	1,913	41
SPA 13	SD --**CCTCTA**-------- FS site	16	1,445	32
SPA 14	SD --**TACTGC**-------- FS site	16	1,329	29
SPA 15	SD --**GTTTACTGC**-------- FS site	19	1,002	22
SPA 16	SD --**GTCAATCGT**-------- FS site	19	1,156	25
SPA 17	SD --**GAGGTTTACTGC**-------- FS site	22	800	18[c]
Deletions in spacer 1				
SPD 1	SD X---------- FS site	9	2,218	48
SPD 2	SD -----XXX-- FS site	7	1,561	34
SPD 3	SD XXX-------- FS site	7	1,494	33
SPD 4	SD --XXXXXX-- FS site	4	379	8
SPD 5	SD XXXXXXX--- FS site	3	182	4
SPD 6	SD XXXXXXXX-- FS site	2	413	9
Stem-loop structure deleted[d]				
SLΔ[e]	gcAGGGAGcaaccaaagcA AAA AAG	10	625	14
FSs[f]	gcA AAA AAG		85	2
Additions in spacer 1				
SLΔA 1	SD --**CCG**-------- FS site	13	643	14
SLΔA 2	SD --**TACT**-------- FS site	14	493	11[c]
SLΔA 3	SD --**TACC**-------- FS site	14	564	12[c]
SLΔA 4	SD --**TaCCG**-------- FS site	15	547	12[c]
SLΔA 5	SD --**TACAC**-------- FS site	15	562	12[c]
SLΔA 6	SD -- **TACTGC**-------- FS site	16	159	4
SLΔA 7	SD -- G**TTTACTGC**-------- FS site	19	180	4
SLΔA 8	SD --**GCTGTTTACTGC**-------- FS site	22	138	3[c]
Deletions in spacer 1				
SLΔD 1	SD XX-------- FS site	8	591	13
SLΔD 2	SD XXXX------ FS site	6	103	2
SLΔD 3	SD --XXXXXX-- FS site	4	25	0.5

[a] Clones IFC, WT, and SDΔ are defined in the text and the footnotes to Table 1.
[b] Nucleotides shown in boldface type are added. Deleted nucleotides are indicated with Xs.
[c] These constructs were tested in a separate experiment. Several other constructs were tested in the same experiment; none of the constructs showed more than 5% variation in β-galactosidase levels between the two experiments.
[d] In other respects, these are the same as the corresponding constructs listed above.
[e] SLΔ is the wild-type sequence with the stem-loop structure deleted.
[f] FSs is a deletion of 16 nucleotides upstream of the heptanucleotide sequence and also the stem-loop.

with only 4, 3, and 2 nucleotides in spacer 1, all showed drastically reduced levels of frameshifting (less than 10%), significantly lower than that of SDΔ. These limited results suggest that the SD sequence can function as a stimulator of frameshifting, given the correct spacing from the shift site, or as an inhibitor of −1 frameshifting when it is located too close to that sequence. Unexpectedly, it was not possible to convert the *dnaX* site to a +1 frameshift site by placing the SD sequence at the optimal distance (for +1 RF2 frameshift) from the shift site (data not shown).

To see if the 3′ stem loop affected the response to the SD stimulator, the SLΔA and SLΔD clones, lacking the stem-loop,

were constructed (Table 3). With the wild-type spacing, the frameshift level dropped to 14% when the stem-loop structure was deleted (SLΔ). Deletion of both the stem-loop and the 5′ stimulator (FSs) reduced the frameshifting to 2%. Addition of 3, 4, or 5 nucleotides in spacer 1 (SLΔA 1 to 5) did not affect the level of frameshifting beyond the reduction already seen in SLΔ. However, addition of 6, 9, or 12 nucleotides dramatically reduced the level to 3 or 4% (SLΔA 6 to 9), very close to the amount detected with just the heptanucleotide sequence (FSs). A 2-nucleotide deletion in the spacer had no effect (SLΔD 1), but deletion of 4 or 6 nucleotides greatly affected frameshifting (SLΔD 2 and 3). SLΔD 3 (0.5%) again shows that moving the SD too close to the shift site is inhibitory to frameshifting. Comparison of the construct containing the SD with or without the 3′ stem-loop (2,514 versus 625 U) with the construct lacking the SD with and without the stem-loop (1,275 versus 85 U) indicates that the effects of the SD and 3′ stem-loop are mostly additive but perhaps suggests some cooperativity [(1,275 − 85) + (625 − 85) = 1,730 versus (2,514 − 85) = 2,429].

The spacing results are summarized graphically in Fig. 5A and B. Figure 5C shows the results of 1- or 2-base additions and deletions in the spacer region of the gene for RF2 (data not shown in tabular form). Even single-base additions and deletions are detrimental for the +1 frameshift. This finding agrees with a previous experiment (39). A 3-nucleotide spacer is critical for frameshifting in RF2 mRNA. These results, together with those from *dnaX*, demonstrate that the spacing between the SD sequence and the shift site is more flexible in the −1 *dnaX* frameshift case than in the +1 RF2 gene case. The longer spacers with *dnaX* approach or equal the number of nucleotides 5′ of the coding site expected to be encompassed by the ribosome, on the basis of nuclease protection experiments (30, 31), sequence nonrandomness around initiation codons (27), and toe-printing P-site experiments (13).

In vitro expression of fusion proteins. In vitro frameshifting assays were done on some constructs for comparison with the in vivo results. The modified plasmid RW201 (see Materials and Methods and Fig. 2) was used as template for the expression of the fusion proteins in vitro. Translation of a *dnaX* WT construct by an *E. coli* S-30 cell-free protein-synthesizing system generated two polypeptides of the expected size (Fig. 6, lane A).

As mentioned earlier, modification of the −1 frame UGA stop codon to a UGU (Cys) codon did not show any significant effect on frameshifting in vitro (Fig. 6, lane B). The WT construct (Fig. 6, lane B) showed 72% frameshifting, and the UGA→UGU substitution showed 75% frameshifting. The size of the frameshift product was increased slightly by this modification; the next stop codon downstream in the −1 frame was used. Deletion of the stem-loop structure showed the expected effect. First, the frameshift level dropped to 38% (50% of the WT level) (Fig. 6, lane C). Second, the zero-frame peptide decreased in size because of the deletion of 10 amino acids in the coding region. Change of $G_5G_6G_7$ to CCC (Fig. 6, lane D) or deletion of 2 codons in spacer 1 (Fig. 6, lane E) produced decreases in the frameshift level to 36 and 16%, respectively. The relative in vitro frameshifting levels were comparable with the data found by the in vivo experiments, although the absolute efficiencies were somewhat higher.

DISCUSSION

In all high-level −1 frameshifting described to date, the elements which stimulate frameshifting have been 3′ of the shift site. The results presented here show the involvement of an important 5′ mRNA-rRNA interaction in addition to a 3′

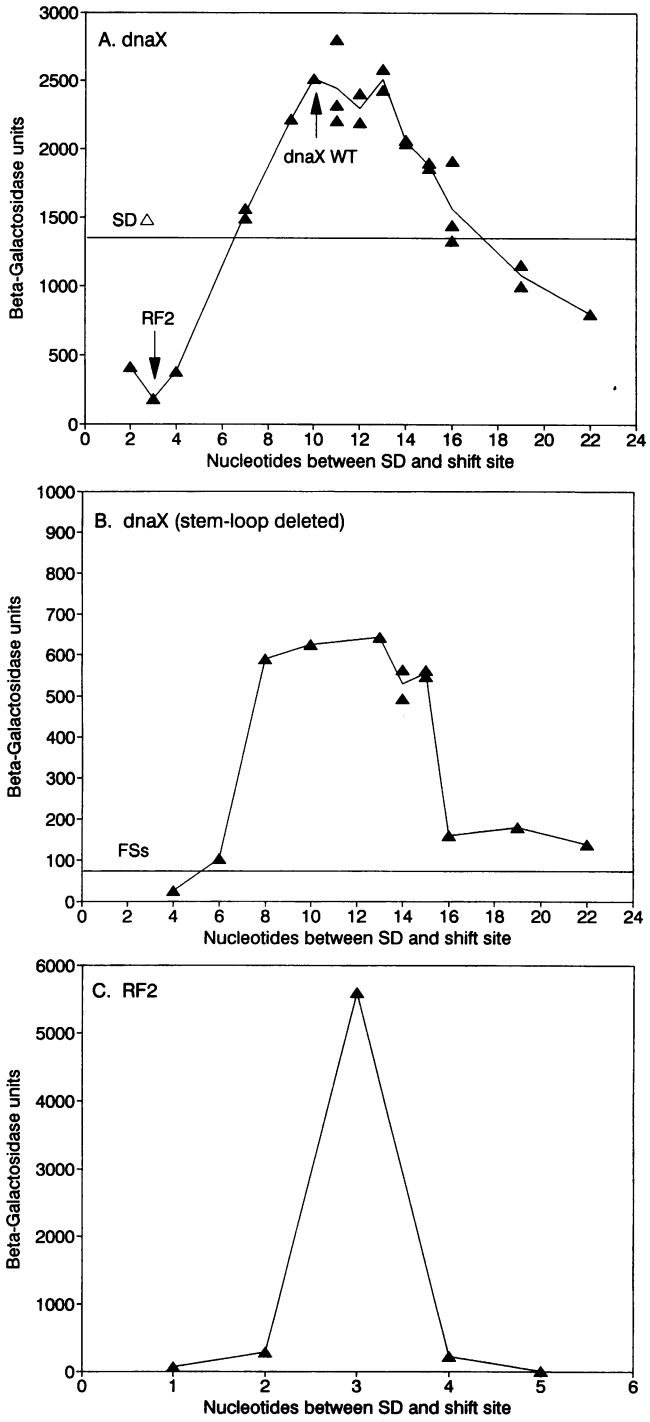

FIG. 5. Effects of deletions and additions between the SD-like sequence and the shift site on the level of frameshifting. (A) Effect of deletions and additions in spacer 1 of *dnaX*. For reference, the *dnaX* WT and the RF2 WT mRNA spacings are indicated with arrows. The horizontal line labeled SDΔ indicates the amount of frameshifting. (B) Same as panel A, except that the stem-loop structure was deleted from these constructs. (C) Effect of 1- or 2-base additions and deletions in the spacer region of RF2 mRNA.

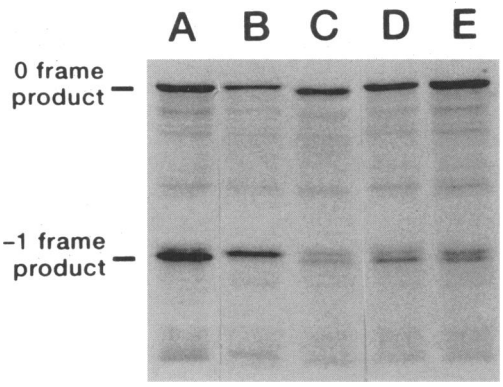

FIG. 6. In vitro expression of fusion proteins. An autoradiograph of *E. coli* S-30 extract protein synthesis products separated by SDS–10% PAGE is shown. Lane A, *dnaX* WT; lane B, replacement of the −1 frame UGA stop codon with UGU; lane C, deletion of the stem-loop structure; lane D, substitution of the SD-like sequence (AGGGAG to ACCTCC); lane E, deletion of 6 nucleotides in spacer 1. The −1 frame UGA stop codon was unchanged in the constructs used in lanes A, C, D, and E. Lanes A, C, D, and E show a doublet band around the −1 frame product (the frameshift product). The lower band is the frameshift product, and the top band is an unidentified background band. In lane B, the frameshift product increased slightly in size; the next stop codon downstream in the −1 frame was used. In lane C, the zero frame peptide decreased in size because of the deletion of the stem-loop structure (10 amino acids in the coding region).

stimulatory stem-loop structure for −1 frameshifting in the *dnaX* gene of *E. coli*. The effects of these two elements are additive. The 5′ interaction is SD pairing between 16S rRNA in elongating ribosomes and the 5′ element in the mRNA. The only other example of mRNA-rRNA pairing during elongation is for +1 frameshifting in decoding the *E. coli* RF2 (41). However, there is a substantial difference in the spacing between the SD sequence and the shift site. The distance between the SD-like sequence and the shift site in the gene for RF2 is 3 nucleotides, and increasing or decreasing it by even 1 base drastically reduces frameshifting (Fig. 5C). In *dnaX*, the distance between the SD-like sequence and the shift site is 10 nucleotides. Nevertheless, when the SD-like sequence before the *dnaX* shift site is at the same distance as its RF2 gene counterpart, it inhibits rather than stimulates −1 frameshifting (Fig. 6A and reference 40).

Inspection of the *dnaX* SD-like sequence shows only one possibility for base pairing with the 3′ end of 16S rRNA (Fig. 7A). In contrast, with RF2 mRNA there are two potential alignments (Fig. 7B and C). However, the data from a mutant SD sequence compensated by change in the anti-SD sequence of 16S rRNA (41) (Fig. 7B and C) lead to the conclusion that the pairing in Fig. 7C is correct. Thus the pairing in *dnaX* and RF2 mRNA is offset by 1 base. Replacement of the *dnaX* SD sequence with its RF2 gene counterpart promoted frameshifting equivalent to that of WT (Table 1, SDM 7). A puzzling feature of the SD-like interaction in *dnaX* is the comparison of A-U versus G-U base pairing at position 5. Replacement of the WT A-U pair with G-U in *dnaX* gave equivalent frameshifting, whereas with the RF2 gene an A-U pair at position 4 was only 1/5 as effective as a G-U pair (39). Resolution of the idiosyncrasies of mRNA-rRNA interactions of the SD type await further experiments.

How does the SD interaction promote frameshifting? One possibility is that the interaction causes a pause which gives an increased chance for dissociation and repairing at an overlapping codon. One frameshifting stimulator in yeast (37) and another in a mammalian system (29) have been shown to cause a pause which may be important for shifting. Tsuchihashi (33) found clear evidence for pausing in *dnaX* with a construct which we now know contains both the SD and downstream stem-loop stimulators. Since he was unaware of the SD sequence, he did not distinguish which, if not both, caused pausing. Experiments to distinguish between these possibilities are in progress. However, it is not clear why the space between the shift site and the SD sequence should be so divergent between *dnaX* and RF2 genes if pausing is the key element. A second effect of the SD interaction in which spacing may be crucial would be destabilization of the codon-anticodon interaction by influencing either the P-site tRNA, the mRNA, or both.

In the complex poised to frameshift, the mRNA is fixed at two points through its SD and P-site codon. Presumably, the 16S rRNA is also fixed in space by its anti-SD and the P site (since a region of rRNA near the anti-SD can be crosslinked to P-site tRNA [24]). When the SD-to-shift site (P-site codon) spacing is minimal (as in the gene for RF2), the rRNA may be compressed, forcing dissociation of the codon-anticodon pairing and relief by forward slippage (+1 frameshifting) of the mRNA. The converse argument that with maximal spacing (as in *dnaX*) rRNA may be stretched with relief provided by backward mRNA slippage (−1 frameshifting), while plausible, is weakened by the fact that in this case there is considerable latitude in the allowed spacing (9 to 14 nucleotides). Interestingly, the spacings between the SD sequences and shift codons of RF2 and *dnaX* mRNAs correspond to the minimal and maximal spacing for all the known SD-to-initiation spacings (see reference 25). Deductions about the spacing over which the SD can operate also come from a different approach. A second, upstream SD negates the effect of the SD in RF2 mRNA frameshifting only if it is closer than 15 nucleotides to the shift site (40).

Curiously, even where SD interactions are not known to be involved in initiation, as in *S. cerevisiae*, proximity of the Ty1 shift site to a translational initiator results in decreased frameshifting efficiency (3) (as if there is something special about the initiation process that affects frameshifting).

For the frameshifting stimulatory effect of SD pairing to be most effective, either the A site needs to be unoccupied by tRNA (in RF2 mRNA the codon in the A site is the stop codon UGA) or else its tRNA may need to be able to shift, as with double shifty codons.

Other genes that might use *dnaX*-like programmed frameshifting were revealed by a computer search for an internal SD sequence followed at an appropriate distance by A AAA AAG, the most shift-prone sequence in *E. coli* (42). The analysis showed the insertion sequence IS*911* (23), bacteriophage Pf1 single-stranded DNA-binding protein (5), *Streptococcus pyogenes* C5a peptidase (6), *Bacillus subtilis* cell division initiation protein (12), the insertion sequence IS*861* (26), and the insertion sequence IS*1133* (7). In one of these, IS*911*, O. Fayet and M. F. Prère identified, a few years ago, the 5′ SD sequence as a potential stimulator of −1 frameshifting. There is now strong evidence, provided by site-directed mutagenesis, that it is indeed the case (23a). A search for shift sites other than A AAA AAG is likely to show more-widespread use of internal SD-like sequences as stimulators.

The demonstration that an SD-like interaction can stimulate −1 frameshifting in addition to the previously described +1 frameshifting extends the known roles for internal SD-like sequences and makes it likely that they are more widely used than previously appreciated. Other than SD-like sequences, the known stimulatory elements for frameshifting and also for

FIG. 7. Predicted base pairing between internal SD-like sequences and the anti-SD of 16S rRNA. (A) *dnaX*; the most favorable interaction is presented. (B and C) RF2 mRNA; two likely interactions between WT RF2 mRNA and 16S rRNA are shown. Also shown are the corresponding base pairings between the altered RF2 SD sequence and the mutant 16S rRNA previously tested (41).

other types of reprogrammed decoding, or recoding, have been essentially 3′ of the recoding site (11). The findings also strengthen the conclusion that the 3′ end of 16S rRNA continuously scans the message for potential pairing during elongation. Future studies of the different features of −1 and +1 frameshifting are likely to provide new information about ribosome function.

ACKNOWLEDGMENTS

We thank Harold Swerdlow for his help with the manuscript.

This work was supported by the Howard Hughes Medical Institute and by NIH grant GM48152.

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