The Interplay of mRNA Stimulatory Signals Required for AUU-Mediated Initiation and Programmed –1 Ribosomal Frameshifting in Decoding of Transposable Element IS911^{▽†}

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The IS911 bacterial transposable element uses –1 programmed translational frameshifting to generate the protein required for its mobility: translation initiated in one gene (*orfA*) shifts to the –1 frame and continues in a second overlapping gene (*orfB*), thus generating the OrfAB transposase. The A-AAA-AAG frameshift site of IS911 is flanked by two stimulatory elements, an upstream Shine-Dalgarno sequence and a downstream stem-loop. We show here that, while they can act independently, these stimulators have a synergistic effect when combined. Mutagenic analyses revealed features of the complex stem-loop that make it a low-efficiency stimulator. They also revealed the dual role of the upstream Shine-Dalgarno sequence as (i) a stimulator of frameshifting, by itself more potent than the stem-loop, and (ii) a mandatory determinant of initiation of OrfB protein synthesis on an AUU codon directly preceding the A6G motif. Both roles rely on transient base pairing of the Shine-Dalgarno sequence with the 3' end of 16S rRNA. Because of its effect on frameshifting, the Shine-Dalgarno sequence is an important determinant of the level of transposase in IS911-containing cells, and hence of the frequency of transposition.

In the decoding of a minority of genes in probably all organisms, ribosomes shift reading frames at specific places for gene expression purposes (1, 2, 31). While the sites utilized are intrinsically shift prone, the proportion of ribosomes that shift frames is most of the time greatly enhanced by recoding signals embedded in the mRNA; the frameshifting is programmed. Programmed ribosomal frameshifting (PRF) commonly leads to the synthesis of a product encoded by two partially overlapping open reading frames (ORFs). In some cases, especially with +1 shifts, the function is regulatory. However, in most cases, including the product of the programmed ribosomal –1 frameshifting (PRF-1) analyzed in this work, special features of the novel transframe-encoded product are what is important.

The richest known source of programmed –1 frameshifting sequences is the bacterial transposable insertion element (IS), especially that of the IS3 family (10, 16, 28; see the IS Finder database [http://www-is.biotoul.fr]). Most members of the IS3 group contain only two consecutive and partially overlapping genes (*orfA* and *orfB*) (Fig. 1). With IS3 elements, frameshifting is utilized to synthesize a transframe product (the OrfAB protein) possessing the transposase activity required for mobility (28, 36). An interesting example is provided by IS911. 5'

adjacent to its frameshift site, IS911 has an initiation codon that permits synthesis of some OrfB products on its own (Fig. 2A). This initiation codon is AUU, which is only known to be used for this purpose elsewhere for bacterial initiation factor 3 (IF3) and *pcnB* synthesis (6, 42) and a tiny number of eukaryotic genes (20). The 5' stimulatory upstream Shine-Dalgarno (SD)-like sequence (SD_B) for the IS911 AUU initiator also serves as one of the stimulatory recoding elements for frameshifting by ribosomes that had initiated far upstream at the *orfA* initiator. The SD_B is 11 bases 5' from the shift site, a very similar distance to that between the internal SD sequence and the shift site for *Escherichia coli dnaX* frameshifting (Fig. 2B) (23), which is utilized to synthesize an additional DNA polymerase subunit. Earlier data provided evidence that, at this spacing, an rRNA-mRNA interaction stimulates –1 frameshifting (1, 23), and this model is more thoroughly investigated in the present work.

The second interesting feature of IS911 frameshifting is the nature of the second recoding signal which occurs at the 3' end of the shift site. It is a branched stem-loop, as shown by probing (chemical and enzymatic) and mutagenesis studies (39), while other 3' frameshifting stimulators are nearby stem-loops (as in *dnaX* [24]) or pseudoknots (9, 18, 44), sometimes involving far distant sequences (3, 30).

The apparent efficiency of IS911 frameshifting was found to be 12 to 14% (36). This value is lower than that found for *dnaX* frameshifting (7, 17, 51), but the difference is not a reflection of the shift site, A-AAA-AAG, which is identical to its *dnaX* counterpart. The available evidence strongly suggests that at this site there is –1 frame realignment of tandem tRNAs, from AAA-AAG to AAA-AAA, just as has been shown to occur with different heptanucleotide sequences for the retroviral –1

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FIG. 1. Localization, sequence and features of the IS911 frameshift region. The A-AAA-AAG frameshift motif is underlined, the stimulatory upstream SD-like sequence (SD_B) and the AUU initiation codon of *orfB* (in_B) are boxed and the basal stem of the downstream stimulatory structure is marked by two arrows. The bottom part of the figure shows a pulse-labeling analysis of the translation products generated by frameshifting and initiation when the IS911 frameshift region is cloned into the pOFX302 reporter plasmid. Position of gene 10 (G10), AUU-initiated (IN), and frameshift (FS) products are indicated. The constructs tested are vector pOFX302 [noninduced (ni) and induced (i) in lanes 1 and 2, respectively]; construct *m199*, in which AUU is changed to AUG (cloned into pAN127, i.e., gene 10 is absent) and which serves as a 100% reference for the AUU-initiated product (marked as 100_{Ref} under lane 3); construct *m200*, where gene 10 and *lacZ* are in the same frame and which serves as a 100% reference for the frameshift product (marked as 100_{Ref} under lane 4); and construct *m201*, containing the IS911 wild-type frameshift region (lane 5).

frameshifting required for synthesis of the Gag-Pol polyprotein (21).

The present work focuses on the distinctive features of IS911 frameshifting and also includes an in-depth analysis of some more general features of bacterial -1 frameshifting.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and transposition assay. The *E. coli* K-12 strain JS238 [MC1061, *araD* Δ (*ara leu*) *galU galK hsdS rpsL* Δ (*lacIOPZYA*)X74 *malP::lacI^r srlC::Tn10 recA1*] was used for all experiments. Bacteria were routinely grown in LB medium (43). MOPS (morpholineethanesulfonic acid) medium (32) supplemented with glucose (0.5%), thiamine (2 mg/liter), and all amino acids at 50 mg/liter each (except methionine, tryptophan, and tyrosine) was used for pulse-labeling with [35 S]methionine; 0.5% Casamino Acids (CSA; Difco) replaced the amino acid cocktail in some experiments (MOPS-CSA). For the β -galactosidase assay of strains carrying "specialized" ribosomes, overnight cultures in LB medium were diluted 100-fold and incubated for 4 h at 37°C in MOPS medium containing 0.5% Casamino Acids, 0.9% glycerol, 0.2% arabinose, and 2 mM IPTG. Rambach agar plates (Merck) served to identify clones expressing β -galactosidase. Ampicillin (40 mg/liter) plus oxacillin (200 mg/liter) and kanamycin (25 mg/liter) were added when necessary.

Transposition frequencies of wild-type IS911 and its SD_B -mutated derivative were determined by a mating-out assay as described previously (39).

DNA techniques and quantitation of radioactive macromolecules. Plasmid DNA was prepared using the QIAprep or Qiagen-tip 100 purification systems as recommended by the supplier (Qiagen). Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England BioLabs. AmpliTaq DNA polymerase and the AmpliCycle sequencing kit were from PE-Applied Biosystems. Cloning, transformation, agarose gel electrophoresis, and sequencing gel experiments were carried out according to standard procedures (43). Radioactive products ([γ - 33 P]ATP and [35 S]methionine) were obtained from Amersham. The Fujix BAS-1000 PhosphorImager and the PCBAS software (Raytest GmbH) were used for the quantitative analysis of denaturing acrylamide gels in which radioactively labeled proteins were separated.

Plasmid constructions. The pOFX302 reporter plasmid (39) was used, unless otherwise specified, for the analysis of the frameshifting regions. The wild-type IS911 frameshift cassette (Fig. 1) was also cloned into the pAN127 vector (in this plasmid, the AAA and AAG codons of the frameshift motif are, respectively, the 12th and 13th codons after the AUG initiator in frame 0) (53). The various frameshift regions (see Table S1 in the supplemental material) were synthesized as overlapping oligonucleotides and inserted between the HindIII and ApaI sites of both vectors, i.e., between T7 gene 10 and *lacZ* for pOFX302 and in front of *lacZ* for pAN127.

A plasmid containing a p15A replication origin, possessing a kanamycin resistance gene and the arabinose-inducible P_{BAD} promoter, was used for the generation of specialized ribosomes (47). The *rmB* operon of strain W3110 was cloned, and a restriction site was added after the end of the 16S rRNA gene (pOFX503). This allowed substitution, via cloning of oligonucleotides, of the anti-SD sequence of 16S rRNA (CCUCCUUA-3') by an entirely different one (GGAGGTTA-3'), thus resulting in plasmid pOFX504 (47). A special vector, pOFX305 (see Table S1 in the supplemental material), had to be designed for insertion of the frameshift regions to be tested in conjunction with specialized ribosomes: the *lacZ* translation initiation region of pAN127 was modified to ctcgagTAACCTCCATAAATATATTAAAAACGTAATTTAagctt (the modified SD sequence and ATT initiation codon are underlined) via insertion of oligonucleotides into its XbaI and HindIII sites (in lowercase). Translation of *lacZ* in pOFX305 relies on a new SD sequence, UAACCUCC, complementary to the anti-SD sequence of the specialized ribosomes and, for reasons described in Results, on a properly positioned AUU initiator codon.

The neutral mutations in the SD_B region of construct *m235* were introduced into the IS911 copy carried by plasmid pOFT139 by PCR mutagenesis as previously described for stem-loop neutral mutations (39).

Measurement of frameshifting frequency by β -galactosidase assay. Assays were carried out according to the methods of Licznar et al. (27), except that exponentially growing non-IPTG (isopropyl- β -D-thiogalactopyranoside)-induced cultures (obtained by a 1/250 dilution of stationary phase cultures in LB medium, followed by a 270-min incubation at 37°C) were used instead of stationary-phase cultures. Exponentially growing cultures in MOPS-CSA medium, plus arabinose and glycerol, were assayed according to the method of Bertrand et al. (4) for the specialized ribosomes experiment. An in-frame control, construct *m200* (or *m297* for the specialized ribosomes), was used as 100% reference; it contains the 2 frameshift stimulators but not anymore the A-AAA-AAG motif nor the AUU initiator codon.

Measurement of frameshifting frequency by *in vivo* pulse-labeling of proteins with [35 S]methionine. Labeling of each strain was carried out with three independent clones, following the protocol previously described (39). To calculate the frequency of frameshifting (or of AUU initiation), the fraction of the total radioactivity present in the frameshift (or initiation) product band (FS or IN in

FIG. 2. Comparative analysis of the frameshift regions of IS911 (A) and *dnaX* (B). The features of each region are indicated at the top part of each panel, while the bottom part shows the incidence on frameshifting frequency of the mutation of one or both stimulatory elements. Frameshifting (and AUU initiation for IS911) was assessed by protein pulse-labeling with [³⁵S]methionine in IPTG-induced cultures. These two regions and their variants were inserted into the HindIII and ApaI sites of plasmid pOFX302 (see Table S1 in the supplemental material for the sequence of each construct). To allow continuation of synthesis in *lacZ*, the UGA stop codon in the -1 frame present in the natural *dnaX* region has been changed to a UGU sense codon (underlined with *** in panel B). The IS911 no-FS/IN construct (mutated motif and AUU changed to the noninitiator ACU codon) and the *dnaX* no-FS construct (mutated motif) were used for background corrections. SD+/SD° and SL+/SL°, respectively, indicate presence or absence of the upstream stimulatory Shine-Dalgarno sequence or of the downstream stem-loop. FS stim indicates the frameshift stimulation factors (i.e., observed frameshift frequency for a given construct divided by the frameshift frequency obtained for the relevant SD°SL° construct).

Fig. 1) was divided by the corresponding value obtained for the FS band in the in-frame control (construct *m200*) or the IN band from construct *m199* (AUU replaced by AUG). The migration front, which contains small proteins and peptides, was kept within the gels to ensure accurate assessment of all protein-incorporated [³⁵S]methionine. The standard deviation for frameshifting (or AUU initiation) frequency was ±7% on average.

Sequence analyses. In order to determine the position of initiating SD sequences among *E. coli* K-12 ORFs, a script for the Perl language (v.5.10.0 from ActiveState) was devised to extract from the genome sequence (GenBank accession number U00096) 30 nucleotides 5' of the start codon of each ORF. A second script was used to look for SD sequences (the search was restricted to the following SD sequences: NGAGG, GGAGH, HAGGAHN, NGGGG, GGGGH, NGTGG, and GGTGH where the rightmost base correspond to the last base of the GGAGG consensus core SD sequence) and to calculate the corrected spacing of each (see Fig. 6A and Results) (41). A similar analysis was carried out on 198 elements of the IS3 family possessing a potential frameshift stimulatory SD sequence upstream of a frameshift motif (Fig. 6B).

For codon analysis, a segment of 39 nucleotides (nt) from the frameshift region (30 nt upstream of the ZZN codon of the motif and 6 nt downstream) was retained for each of the 355 selected IS. Sequences were formatted with a Perl script to separate codons and were then entered into a Microsoft Excel table to allow sorting and calculation of the codon frequency at each position.

Potential RNA secondary structures were assessed with the RNAstructure v.5.1 software (40).

RESULTS

Cassette for frameshifting and internal initiation. The region necessary for maximum frameshifting is present between nucleotides 302 and 402 of IS911 (36, 39). The frameshifting analyses were performed with this 101-nt cassette fused to the 3' end of phage T7 gene 10 and the 5' end of *lacZ* in vector pOFX302 (Fig. 1). It gave an apparent frameshifting frequency of about 10% ± 2% when measured by pulse-labeling. The gene 10 translation frame defines the 0 phase and ends at the stop codon of the *orfA* gene of IS911. The *lacZ* translation frame continues the IS911 *orfB* gene and is therefore in the -1 phase. Standard translation of gene 10 gives a G10-OrfA' product (G10 protein, 29 kDa). Expression of *lacZ* comes either from a shift in frame, from the 0 to the -1 phase, generating a G10-[OrfA-OrfB]'-LacZ product (FS protein, 143 kDa), or from internal initiation at the AUU start codon of *orfB*, giving an OrfB'-LacZ product (IN protein, 114 kDa). *In vivo* [³⁵S]methionine pulse-labeling of IPTG-induced cultures, followed by SDS-PAGE of the total cellular extract, allows detection and quantification of all three products (Fig. 1). In some cases, assessment of frameshifting was carried out by

measuring the β -galactosidase activity in noninduced cultures. To ensure that the enzymatic activity is a direct measure of the amount of FS protein, the AUU initiation codon of IS911 was mutated to ACU as in construct *m211* (see Table S1 in the supplemental material) (36). We observed that pulse-labeling gave higher apparent frameshifting frequencies than the enzymatic assay (a detailed graph is presented in Fig. S1 in the supplemental material). The divergence probably originates from the very high level of transcription of the induced P_{tac} promoter. This likely precludes full translation of all mRNAs, with a deficit increasing with PRF-1 frequency and reaching a maximum for the in-frame construct used as 100% reference.

In this study, we have nevertheless mostly used pulse-labeling to measure expression because it allows discrimination between the frameshift and AUU-initiated products characteristic of the IS911 system.

Comparison of IS911 and *dnaX* frameshifting efficiencies.

The *dnaX* -1 frameshifting region has been extensively studied and is a paradigm of bacterial frameshifting signals. To carry out a comparative analysis of the *dnaX* and IS911 cassettes, four mutants were generated for each (Fig. 2A and B): an in-frame construct (100% reference) (Fig. 2A and B, lane 6), a Shine-Dalgarno minus mutant (GGAG changed to CCUC) (Fig. 2A and B, lane 3), a stem-loop deletion (Fig. 2A and B, lane 4), and a combination of the last two changes (Fig. 2A and B, lane 5). Frameshifting frequencies were then assessed by protein pulse-labeling. This experiment corroborates the expected greater efficiency of the *dnaX* signal and provides information on the nature of the concerted action of the two stimulators. Removing both stimulators leads to a low level of recoding (1.3% and 0.6%) (Fig. 2A and B, lane 5). Mutating the SD sequence only has a more severe effect on IS911 frameshifting and not on the deletion of the branched stem-loop region (Fig. 2A, lane 3 versus lane 4). The opposite effect is found with the *dnaX* region (Fig. 2B, lane 3 versus lane 4). Whereas the two SD sequences have similar effects, the *dnaX* stem-loop structure, though having a lower calculated $\Delta G_{37^\circ\text{C}}$ (standard Gibbs free energy of formation at 37°C) than its IS911 counterpart (-16.2 versus -20.2 kcal/mol), is a 3.4-times-more-potent stimulator of frameshifting. In IS911, association of the SD (4.2-fold stimulation by itself) and the stem-loop (2.5-fold by itself) leads to a 20-fold stimulation. This is about two times more than would be expected if they were acting according to a simple multiplicative model in which the joint effect is equal to the product of the two individual factors. In *dnaX*, the concerted action of the SD (4.3-fold stimulation by itself) and the stem-loop (8.5-fold stimulation by itself) results in a 45-fold stimulation of frameshifting (i.e., 1.23 times more than 4.3 multiplied by 8.5). In conclusion, with both frameshift signals, the joint effect of the two stimulators clearly departs from a simple multiplicative model. There is either moderate (1.23-fold for *dnaX*) or significant (2-fold for IS911) synergy.

With the IS911 cassette, AUU initiation disappears when the SD sequence is mutated (Fig. 2A, lanes 3 and 5) and, surprisingly, increases 3-fold when the branched stem-loop is removed (Fig. 2A, lane 4). This unusual initiation event therefore relies on the same mRNA-16S rRNA interaction as standard initiation, but it is negatively affected by the downstream sequence.

Dual role of the branched stem-loop: a moderate stimulator of frameshifting and inhibitor of AUU initiation. Mutants of the IS911 branched stem-loop were analyzed to gain a better understanding of which features are responsible for its low stimulatory capacity. When the Y-shaped structure is reinforced by suppressing all mismatches in the 3 stems (Fig. 3, *m219*), there is a 3-fold increase in frameshifting. However, it remains slightly less efficient than the *dnaX* structure (Fig. 2B), while requiring pairing over a longer RNA segment. In a second set, loop 2 of IS911 (AUAAU) (Fig. 2A) was positioned above a continuous stem of various lengths, from 6 to 15 bp (Fig. 3, *m223* to *m226*). The shorter of these (*m223*; 5 G-C pairs) is already two times more efficient than the original structure. Bringing it to 9 and 12 pairs (*m224* and *m225*; mostly G-C pairs) increases its efficiency progressively, but adding 3 more pairs does not (*m226*). With 12 pairs, frameshifting reaches a maximum level of nearly 80%, which is higher than the 58% produced by the *dnaX* signal. In a third set, either stem 2 or stem 3 was deleted, resulting in a single extended stem, with one unpaired base on each side, ending with loop 1 or loop 2 (*m217* and *m218*). Both are better stimulators than the original design and are equivalent to the *dnaX* region. Finally, stem 1 was kept constant but capped by loops of various lengths (5, 14, or 32 nucleotides chosen so as not to form any significant structure by Watson-Crick pairing). The construct with the shorter loop, *m220*, is about 5 times more active than the wild type (51% versus 10.5%). Thus, stem 1, though having an unpaired U, is a rather strong stimulator when associated with a short loop. Increasing the loop to 14 nt reduces frameshifting moderately (*m221*; 38%). Extending the loop to 32 nt (i.e., close to the length of the two branches in the wild-type structure) results in a major drop in frameshifting, down to a value 3 times lower than that with the original structure (*m222*; 3.7%).

A characteristic feature of nearly all 3'-frameshifting stimulators is their spacing 3' from shift sites: a spacing of 5 to 8 nt has been found for most of them (14, 22, 49). Moving them closer or further downstream reduces their effectiveness (8, 22, 23), sometimes with implications for frameshift directionality (29). A similar analysis carried out on the IS911 signal shows that it follows the common rule (Fig. 4A). The original spacing of 6 nt is clearly better for -1 frameshifting than either 0, 12, or 18 nt. AUU initiation also is sensitive to spacing, but in a different way. The farther the structure is, the better initiation fares; there is a 6.5-fold difference between the extremes (1.7% versus 11.1%). These data, as well as those obtained with deletions extending into the stem-loop region (Fig. 4B), confirm that the stem-loop interferes with initiation on the AUU codon.

Dual role of SD_B, a stimulator of both frameshifting and AUU initiation. Mutants of SD_B were analyzed to assess its role, and that of neighboring nucleotides, in frameshifting and AUU initiation (Fig. 5A). SD_B was replaced by two other initiation-proficient regions derived from T7 gene 10. One contains only the GGAG core sequence and the 8 nt downstream (Fig. 5A, lane 7, construct *m233*). The other (Fig. 5A, lane 8, *m234*) carries in addition the 13 upstream nucleotides, including the epsilon sequence (in bold), **uuuacuuu**aagAAG GAG, which enhances translation (33, 34). In another set of plasmids, complementarity with the 3' end of 16S RNA was

FIG. 3. Effect on frameshifting of variations in the design of the stimulatory stem-loop of the IS911 recoding region. The frameshifting frequency determined by pulse-labeling is indicated below each structure.

progressively increased from 1 to 8 nt (Fig. 5A, lanes 9 to 15). With the exception of the first two (no SD sequence or 3 consecutive matches), these changes, even to a potentially better SD sequence, have only a minor, if any, effect on frameshifting and initiation. Changing the SD_B to a less-efficient version, aAuGGAaa (which does not affect the amino acid sequence), reduces both frameshifting and initiation 7.1- and 6.5-fold (Fig. 5A, lanes 10 and 11, *m235* versus *m201*), respectively. Substitution by a totally non-SD sequence, aAuccuca, led to a further reduction in frameshift product synthesis and a total disappearance of the AUU-initiated product (Fig. 5A, lane 9, *m202*). In a third set of constructs, six other SD variations were tested (Fig. 5A, lanes 16 to 21). The first two have 4 (*m240*; UcAGGAaa) and 6 (*m241*; UAAGGAaa) consecutive matches, respectively; they both display a severe reduction in initiation and a moderate but significant (3- and 2-fold) reduction in frameshifting. The four others correspond to the aAuGGNGG series (the capitalized nucleotides are those which have Watson-Crick pairing potential with the CCUC Cuaa end of 16S RNA). Among them, only aAuGGcGG, and

to a lesser extent aAuGGuGG, are affected in initiation and, less severely, in frameshifting.

These results confirm that SD_B has a dual role of frameshift stimulator and essential element for AUU initiation. They also provide hindsight on tolerance of SD_B to sequence variation. As is, SD_B is optimal for frameshifting and for initiation. Both roles rely on the ability of SD_B to pair, at least partly, with the CCUCCUUA sequence at the 3' end of 16S RNA. Further evidence for that was obtained using "specialized" ribosomes (47), i.e., ribosomes in which the terminal sequence of 16S RNA has been mutated to GGAGGUUA (see Materials and Methods). These ribosomes initiate only on messages in which SD sequences are modified to restore the possibility of pairing, partially or totally; consequently, the optimal SD sequence becomes UAACCUCC instead of UAAGGAGG. This SD change was introduced into pAN127 in front of *lacZ* to generate vector pOFX305 (see Table S1 in the supplemental material). In addition, it proved necessary to change the AUG start codon to AUU in order to render initiation strictly SD dependent (otherwise, in this context at least, the AUG codon

FIG. 4. Position and integrity of the stem-loop differentially affect frameshifting and AUU initiation. A pulse-labeling analysis was carried out on two sets of constructs. The samples from the *IS911* wild-type (wt) region are shown in panels A and B, lanes 1 (noninduced), and in panel A, lane 3, and panel B, lane 2 (induced). In the constructs of panel A, the spacer between the stem-loop and the motif (sp2) was reduced to 0 nt (lane 2) or increased to 12 (lane 4) or 18 (lane 5) nt. In the constructs shown in panel B, the stem-loop was progressively deleted from the 3' side (see Table S1 in the supplemental material for the sequence of each construct).

is sufficient to promote initiation). *IS911* frameshift regions, modified in the SD_B region, were then cloned into pOFX305. Frameshifting frequency was determined by a β-galactosidase assay, using an in-frame construct as a reference (*m297*). The results indicate that frameshifting is correlated with the extent of pairing between SD_B and the 3' ends of specialized ribosomes. It ranges from 30.6% (*m298*; perfect match of 8 nucleotides) to 7.7% (*m299*; 5 matches, 4 of which are consecutive, like in the wild-type *IS911* region) and goes down to 2.5% when there is no significant pairing (*m300*).

In the *dnaX* recoding region, the stimulatory effect of the SD upstream of the A-AAA-AAG motif (Fig. 2B) depends on its position: it is maximal when the GGAG core sequence is within 10 to 14 nt from the motif (23). Since the equivalent SD sequence in the *IS911* signal has a dual function, it was important to perform a similar analysis, taking into account this specificity. The SD_B to shift motif spacing is 11 nucleotides (Fig. 1 and 2A). It was either reduced to 5 or 8 nt (by removing nucleotides between the SD sequence and the AUU codon) (Fig. 5B, lanes 2 and 3) or increased up to 35 nt (Fig. 5B, lanes 5 to 9) by inserting nucleotides between the AUU codon and the A-AAA-AAG motif. Reduced spacing negatively affects both frameshifting and initiation. Increased spacing, while keeping constant the SD_B-AUU interval, diminishes frameshifting notably only above 17 nt. Meanwhile, AUU initiation undergoes a progressive increase and reaches a stable level of 12%, with a spacing of 23 or more nucleotides. Thus, the SD_B region of *IS911* is optimally positioned to stimulate frameshifting. The SD_B-to-AUU spacing is close to the optimal SD-AUG initiator distance (41). However, proximity to the branched stem-loop impairs initiation, whereas presence of the AUU codon does not affect frameshifting (Fig. 5A, lanes 4 and 6).

FIG. 5. Incidence on frameshifting and AUU initiation of the sequence and position of the upstream stimulatory SD sequence. Mutations were introduced in the SD_B of *IS911* (A), or the SD_B-frameshift motif distance was modified (B), and cultures of the various constructs were analyzed by pulse-labeling with [³⁵S]methionine (see Table S1 in the supplemental material for sequence details). In panel A, the samples correspond to the following constructs: wild type (noninduced [lane 1] or induced [lanes 4 and 11]), in-frame control (lane 2), AUU changed to AUG (serves as a 100% reference for an AUU-initiated product [lane 3]), no SD_B (lane 5), AUU changed to ACU (lane 6), SD_B replaced by the SD sequence of gene 10 (lane 7), SD_B replaced by an extended SD sequence of gene 10 (lane 8), no SD_B (lane 9), SD_B reduced to 3 contiguous bases (lane 10), SD_B increased to 5 contiguous bases (lane 12), SD_B with 6 contiguous bases (lane 13), SD_B with 7 contiguous bases (lane 14), SD_B with 8 contiguous bases (lane 15), SD_B with 4 contiguous bases (lane 16), another type of SD_B with 6 contiguous bases (lane 17), SD_B with 5 contiguous bases (*m236* [lane 18]; same mutant as that shown in lane 12), mutant derived from *m236* by changing central consensus A to C (lane 19), mutant derived from *m236* by changing central base to G (lane 20), and mutant derived from *m236* by changing central base to U (lane 21). In panel B, samples were prepared from strains containing constructs where the distance between SD_B and the A-AAA-AAG motif varies from 5 to 35 nt. The wild-type samples are shown in lanes 1 (noninduced) and 4 (induced). Note that the SD_B-to-AUU codon distance is shortened in mutants *m245* (lane 2) and *m246* (lane 3) but remains the same as that in the wild-type configuration in all other mutants (lanes 5 to 9).

FIG. 6. Detailed analysis of the effect on -1 frameshifting of the position of an SD sequence relative an A-AAA-AAG frameshift motif. (A) The top and middle diagrams illustrate how to determine the “corrected spacing” either between an SD sequence and an AUG initiation codon (41) or between an SD sequence and the XXZ codon (AAA in this example) of an X-XXZ-ZZN heptameric frameshift motif. The bottom diagram shows the distribution of corrected spacings upstream of the proposed start codon of 2,928 *E. coli* genes (see Materials and Methods). (B) The top diagram shows the corrected spacing for SD sequences upstream of potential frameshift motifs present in 198 IS3 family members. The middle and bottom diagrams show the variation of frameshifting frequency when the corrected spacing varies from 5 to 20 nt in the IS911 and *dnaX* frameshift regions (see Table S1 in the supplemental material for the sequence of each plasmid construction). Frameshifting frequency was assessed by LacZ activity assays using two in-frame constructs (*m200* and *m205*) as 100% references (the standard deviation for each measurement is indicated by a vertical bar).

Frameshift stimulation by SD_B is required for high-level transposition of IS911. The above-described analyses with the IS911 frameshift cassette clearly established the stimulatory role of SD_B, at least within the context of reporter plasmids. To test if this means that SD_B influences IS911 transposition, a mutation which diminished the stimulatory role of SD_B without affecting the amino acids encoded by *orfA* (mutant *m235*, in which the SD_B a-AtG-GAG-a is changed to aa-AtG-GAa-a) (Fig. 5A, lane 10), was introduced into the entire IS carried by the pOFT139 multicopy plasmid (38). The frequency of IS-mediated recombination between this plasmid and a conjugative plasmid was subsequently determined (39). In this assay, the wild-type IS transposed at a frequency of 2.10^{-5} , whereas the mutated derivative presented a 7-fold-lower activity. Thus, probably because of its role in frameshift stimulation, the SD_B region is one of the *cis* factors that determine the level of transposition of IS911.

Distance but not phasing of SD_B is important for frameshift stimulation. A more detailed study of the effect on frameshifting of the distance between the stimulatory SD_B and the A-AAA-AAG motif was carried out in view of an intriguing finding revealed by a comparative analysis of 355 transposable elements of the IS3 family. Among these, 56% have an SD-like sequence preceding a potential frameshift site (16). In order to compare the position of the SD sequence, we calculated for

each IS the “corrected spacing” (41) between the SD and the frameshift motif (Fig. 6A). The corrected spacing for initiation is the number of nucleotides between the ribosomal P site, which contains the AUG start codon, and the last 3' nucleotide of the SD, the one paired to (or in apposition to, if not complementary) nucleotide 1535 of 16S RNA: thus in the two mRNA examples shown in Fig. 6A, the corrected spacing is 8 nt, even though the two SD sequences are not identical. By analogy, we define the corrected spacing for -1 frameshifting as the distance between the P site occupied by the XXZ codon of an X-XXZ-ZZN shifty heptamer (i.e., AAA in the example of Fig. 6A) and the last 3' nucleotide of the SD sequence, the one paired to (or in apposition to, if not complementary) nucleotide 1535 of 16S RNA. For example, the two mRNAs shown in the middle part of Fig. 6A have the same spacing of 11 nt. We first carried out a search of initiator SD sequences in front of *E. coli* ORFs (Fig. 6A, bottom): it appears that most ORFs have an SD sequence located 3 to 11 nt upstream of the start codon, with a preferred spacing of 6 nt. The same search performed on the IS frameshift regions revealed a strikingly different pattern (Fig. 6B, top): there is a marked preference for a discrete number of spacings with a phasing of 3 nt. The highly prevalent corrected spacings (86%) are 8, 11, and 14 nt. Such a strong bias among IS suggested either (i) that the phasing of an SD sequence with a shift site could be important

for optimal stimulation of frameshifting or (ii) that the 3-nt periodicity could result from constraints at the protein level. The previous studies, with the *dnaX* frameshift signal (23) or the IS911 signal (Fig. 5B), were not comprehensive enough to reveal the correct alternative. We therefore carried out a more complete analysis by increasing the corrected SD spacing by one nucleotide at a time, from 5 to 20 nt, using the IS911 and *dnaX* frameshift regions (see Table S1 in the supplemental material). The results presented in the middle and bottom panels of Fig. 6B show that there is in both cases a gradual increase and then a steady decrease in frameshifting frequency. The maximum is for a spacing of 10 nt for *dnaX* and around 10 to 12 nt for IS911. Thus, this lack of correlation between the prevalent spacings in the IS and any peculiarity in frameshifting frequency invalidate the hypothesis that SD phasing with respect to the shift site is important. To explore the second possibility, we carried out an analysis of codon usage around the frameshift motif in 355 selected IS3 family members (see Materials and Methods). Among those with an upstream SD, there is a clear bias for a glutamic acid GAG codon, constituting the core of a good SD sequence, as the fourth (10%), fifth (39%), or sixth (17.5%) codon upstream of the XXZ codon of the X-XXZ-ZZN motif; note that, when GAG is preceded by an NNG codon or followed by a GNN codon, the result is an SD sequence with a corrected spacing of 8, 11, or 14 nt (Fig. 6A). In the IS without SD, a glutamic acid codon is also nearly as frequent at the same three positions (10%, 39%, and 21%), but it is more often encoded by GAA rather than GAG (5.6%, 17%, and 1.2%). In conclusion, the preferred positions for frameshift-stimulating SD sequences in the IS3 family most likely reflect a constraint for glutamic acid in that region of the OrfA and/or OrfAB proteins.

DISCUSSION

The function of frameshifting in IS911 and other related IS is to provide a fusion protein, the OrfAB transposase, required for IS mobility (16, 30, 36, 44, 52). Reducing the stimulatory capacity of SD_B (this study) or of the branched stem-loop (39) brings a similar diminution of transposition frequency. Thus, the level of frameshifting directly determines the level of transposition. Obviously, transposition has to be kept within a certain range to ensure maintenance of the element without deleterious effects to its host. To do so, IS911 evolved toward a compromise by joining an upstream SD sequence, inherently as efficient as that of *dnaX*, to a downstream stem-loop structure barely stimulatory by itself.

SD_B, an optimally designed and positioned frameshift stimulator. Our analyses of variations in the sequence and position of SD_B suggests that the critical factors for maximal frameshift stimulation are (i) 4 consecutive interacting nucleotides from the core GGAGG sequence (Fig. 5) and (ii) the SD sequence-to-motif distance (Fig. 6). Thus, the wild-type SD_B of IS911 appears optimal according to these two criteria. The main difference between SD sequences used for regular initiation and those used for frameshifting stimulation is positioning relative to the ribosomal P site. The preferred and functionally optimal corrected spacing for initiating SD sequences in *E. coli* is 6 nt (Fig. 6A) (11). For frameshifting, the optimal spacing is around 9 to 12 nt (Fig. 6B). The purpose of the anti-SD-SD

interaction is to position the initiation codon in the P site. A longer-than-optimal spacing (when the XXZ codon of an X-XXZ-ZZN motif is in the P site) should therefore tend to push the mRNA so as to reduce the anti-SD-SD helix to P-site distance and thus stimulate frameshifting in the minus direction. This raises an interesting possibility which remains to be investigated: in the case of IS911 and *dnaX*, where corrected spacing is, respectively, 5 and 4 nt longer than optimal for initiation, can the readjustment of the mRNA position be more than one nucleotide?

A surprising finding was the marked preference for three positions, relative to the A6G motif, for stimulatory SD sequences within the IS3 family (Fig. 6): most are located at a corrected spacing of 8, 11, or 14 nt. The reason for that is at the protein level, namely, the presence of a leucine zipper motif in the region 5' of the shift site (19). The three preferred SD spacings correlate with the presence of a glutamic acid residue (GAG or GAA codon) positioned to interact with a positively charged residue located on the parallel α -helix of the coiled-coil motif (see Fig. S2 in the supplemental material). Thus, many IS sequences exploited this constraint by using a GAG codon to specify glutamic acid and by adding an upstream NNG triplet or a downstream GNN triplet to obtain a strong GGAG or GAGG frameshift stimulatory SD sequence.

Atypical initiation codon of OrfB, a protein of unknown function. Use of AUU as a start codon for the OrfB protein by IS911, and possibly by a few other IS, is intriguing since it is only known to be used elsewhere in bacteria for this purpose by the IF3 gene and the *pcnB* gene. At high levels of IF3 there is little, if any, IF3 translation initiation, whereas at low levels the opposite pertains (42). Use of an AUU initiator by IS911 likely modulates the OrfB level. This was shown with the wild-type construct (*m201*) and a pair of strains (an *infC*⁺ strain and a derivative containing the *infC19* mutation impairing the ability of IF3 to prevent AUU initiation). We indeed observed that AUU initiation is increased 3-fold in the *infC* mutant, whereas frameshifting is unaffected (data not shown). Determining whether there is regulatory significance for IS911 of IF3 influencing OrfB product synthesis awaits knowledge of its function. However, AUU may not have been selected as the initiator for IS911 *orfB* because of an advantage conferred by IF3 regulation. The dual use of SD_B for initiation and frameshifting is likely, for spacer reasons, responsible for the initiator and shift site being adjacent. The first base of the shift site is A, which, with a 5'-adjacent AUG or GUG, would introduce a UGA stop codon in frame 0. This would lead to premature termination of *orfA* and prevent synthesis of the frameshift product. AUUA does not generate a 0 frame stop codon (Fig. 1).

The ability to synthesize an OrfB product on its own is a frequent feature of transposable elements of the IS3 family, though the mechanism involved in IS911 is distinctive. Synthesis of an *orfB* product was shown for IS150 (52), IS3 (44), and IS3411 (30). Sequence analysis of the other members of that group suggests that it should occur in at least 20% of them (16). In most cases where OrfB is made, as illustrated by IS3 (44), or is likely to be made (as judged from sequence analysis), its synthesis proceeds via translational coupling. The stop codon of the upstream gene *orfA* overlaps with the *orfB* initiator codon. Even though the function of the OrfB protein

remains elusive, the frequent conservation of its expression suggests that it must play a role in transposition, but perhaps only in certain physiological conditions or for a particular type of recombination event (this protein, as the full-length OrfAB transposase, contains an integrase-related domain [15]). In the case of IS3, OrfB was shown to enhance the inhibitory activity of OrfA on OrfAB-mediated transposition (45). Analyses carried out using *E. coli* with *IS911* showed that it is dispensable for the known types of transposition events and that its over-expression cannot affect these reactions or replace the OrfAB frameshift product (37).

A regular stem-loop located 3' from an AUG initiation codon is inhibitory up to a distance of 9 nt but without adverse effects when it is more distantly located (see Fig. 2B in reference 35). Similarly, the *IS911* stem-loop, at the wild-type distance of 13 nt from the 3' end of the AUU codon, is partially inhibitory for initiation (Fig. 4 and 5B). Decreasing the spacing further reduces initiation by 2-fold, whereas increasing it to 19 and 25 nt augments initiation up to 4-fold. In contrast, the branched stem-loop stimulates frameshifting by about 2.5-fold at the wild-type distance of 6 nt from the 3' end of the shift site. When positioned 6 nt closer or farther, it loses its frameshifting stimulation capacity. When initiation occurs on the *IS911* mRNA, the AUU codon is in the P-site and the branched stem-loop is close to, and perhaps just abutting, the entrance to the mRNA channel of the ribosome. On the contrary, when the AAA and AAG codons of the shift motif are in place for frameshifting (i.e., in the P and A sites, respectively), the structure, being 4 nt closer to the ribosome, must be engaged in the entrance of the mRNA tunnel, wedged into the aperture lined by proteins S3, S4, and S5 (54) and about 1 nt away from the ribosomal helicase active site (48). The operating distance of the stem-loop is therefore not the same for inhibition of initiation and frameshifting stimulation. One reason could be that the stem-loop acts on an initiating ribosome (and perhaps only on the 30S subunit) in the former case, while it acts on an elongating ribosome in the latter case; one possibility is that it interferes with binding of initiation factors and another is that it may hamper binding of the 50S subunit (46).

The branched stem-loop, an inefficient frameshift stimulator by design. While its plan may appear more sophisticated than that of the *dnaX* structure, the *IS911* branched stem-loop evolved to be a rather inefficient stimulator of frameshifting. It is probably the addition, on top of the potent *IS911* basal stem, of the 38-nt-long segment forming the two loosely structured upper arms of the final Y-shaped structure (Fig. 2A) (39) that results in a weak stimulation capacity (about 2.5-fold).

Different designs, especially those in which the structure is a single stem-loop, improve the frameshifting potential of the *IS911* region and even turn it into a stimulator just as good as, or even better than, the *dnaX* stem (Fig. 3). This is also the case with the *dnaX* and HIV-1 recoding regions when the stability of the stem-loop structures is increased (5, 12, 24). Some of our artificially designed regular stem-loops led to frameshifting levels close to 80% when associated with the upstream SD (Fig. 3) (24). However, we failed to generate signals that direct 100% efficient frameshifting. It is perhaps a design problem or it may hint at the existence of an upper limit (one can also wonder about the advantage in real life of such a signal over a standard single gene, unless this signal is the target of a regu-

lation). What in the ribosome causes this limit is still an open question.

Synergistic action of the two frameshift stimulators of *IS911* and *dnaX*. While it is clear that the two stimulators of *IS911* (as well as those of *dnaX*) can act independently, they also have a synergistic action when both are present (Fig. 2). This suggests their concerted action occurs during the same step of the translation elongation cycle. Even if the step in question still is a matter of debate (26) and if the exact molecular mechanism of their action is not known, they likely both act on the realignment of the mRNA within the ribosome. The SD, trapped by the anti-SD sequence for more than one elongation cycle (Fig. 6) (22), probably pushes the mRNA forward (as explained above in the section on SD_B). The stem-loop, when brought to the proper distance by translocation (5 to 7 nt from the ribosomal A site) (24), likely becomes transiently blocked within the entrance of the mRNA tunnel, thus inducing tension in the message segment inside the ribosome (9, 18). One way to interpret the synergy is that after the anti-SD–SD pairing forms and translocation continues, the resulting conformational change makes the ribosome more sensitive to the stimulatory effects of the 3' structure blocked at or very near the unwinding site (48, 54). In the case of the *dnaX* stimulators, the synergy is apparently less important than with those of *IS911* (Fig. 2B). The *dnaX* stimulators, being similar to those of *IS911*, likely possess a similar synergistic potential. As suggested in the previous section, this potential is probably counterbalanced by frameshift-limiting ribosomal features, the efficiency of which increases with that of frameshifting.

Modular architecture and plasticity of prokaryotic –1 frameshift signals. This study, together with a recent comparative analysis of 355 transposable elements from the IS3 family (16) and previous analyses of a few cases (13, 25, 30, 36, 44, 50, 52), highlight the modular architecture and the high flexibility in design of PRF-1 regions of bacterial origin. By the choice of the mandatory frameshift motif, and of the nature and position of the optional upstream and downstream stimulators, a wide range of frameshifting frequencies is probably attained. The outcome of –1 frameshifting is the generation of protein diversity through rather simple signals (e.g., synthesis of two proteins out of one gene in *dnaX* and synthesis of up to three proteins out of two genes in *IS911*); thus, one would expect it to be widespread. However, most known examples of bacterial programmed –1 frameshifting are found in bacteriophages or transposable elements (2), and intriguingly, *dnaX* still remains the only clear example of a housekeeping gene using –1 frameshifting for its expression. At a time where over a thousand complete bacterial genomic sequences are available, a thorough search of candidate genes using this mode of expression still remains to be carried out to determine whether or not this strong bias is indeed biologically significant.

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