Two Frameshift Products Involved in the Transposition of Bacterial Insertion Sequence IS629*

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IS629 is 1,310 bp in length with a pair of 25-bp imperfect inverted repeats at its termini. Two partially overlapping open reading frames, orfA and orfB, are present in IS629, and two putative translational frameshift signals, TTTTG (T₄G) and AAAAA (A₄T), are located near the 3′-end of orfA. With the lacZ gene as the reporter, both T₄G and A₄T motifs are determined to be a −1 frameshift signal. Two peptides representing the two transframe products designated OrfAB’ and OrfAB, are identified by a liquid chromatography-tandem mass spectrometric approach. Results of transposition assays show that OrfAB’ is the transposase and that OrfAB aids in the transposition of IS629. Pulse-chase experiments and Escherichia coli two-hybrid assays demonstrate that OrfAB binds to and stabilizes OrfAB’, thus increasing the transposition activity of IS629. This is the first transposable element in the IS3 family shown to have two functional frameshifted products involved in transposition and to use a transframe product to regulate transposition.

The insertion sequence IS629 is a member of the IS3 family of transposable elements. It was initially isolated from the chromosome of Shigella sonnei (1) and has been detected in many other enteric bacteria, including S. dysenteriae, S. flexneri, S. boydii, Escherichia coli C, E. coli O157:H7, Enterobacter cloacae MD36, and Serratia marcescens (2). IS629 is 1,310 bp in length and has a pair of 25-bp imperfect inverted repeats at its termini (3). Similar to the genetic organization of other members of the IS3 family, two consecutive and partially overlapping open reading frames, designated orfA and orfB, are present in IS629 (see Fig. 1). The coding potential of orfA (nucleotides 55–381) is 108 amino acids, and that of orfB (nucleotides 378–1,268) is 296 amino acids (3). The stop codon (TGA) of orfA overlaps the initiation codon (ATG) of orfB (see Fig. 1). A putative promoter and the Shine-Dalgarno sequence are found upstream from the initiation codon of orfA, but no such sequences are present in the upstream region of orfB (3).

Two putative −1 translational frameshift signals, TTTTG (T₄G) and AAAAA (A₄T), are located near the 3′-end of the orfA at nucleotide positions 342–346 and 375–379, respectively. The lacZ gene was fused to orfA (nucleotides 55–425) containing the two motifs (3), suggesting the existence of two frameshifted products. In this study, we demonstrated that both of these two putative frameshift signals are functional, causing a −1 translational frameshift and resulting in the production of two transframe products designated OrfAB’ and OrfAB. OrfAB’ was shown to be the transposase of IS629, and OrfAB was demonstrated to bind and stabilize OrfAB’.

EXPERIMENTAL PROCEDURES

Cloning of IS629—A fragment containing both orfA and orfB sequences of IS629 was amplified by PCR from the chromosome of S. sonnei (ATCC 9290) or E. coli O157 with primers Fₙd₁₋₅₅ and Rₑc₁₋₁₅₆₆⁻Term (Table 1). The PCR product thus generated was cloned into pGEM-Easy (Promega) to produce pGEM629. Subsequently, the 1.2-kb NdeI-Ecl1136II fragment containing orfA and orfB without terminal repeats was isolated from pGEM629 and then inserted into the corresponding sites of pET-29a(+) (Novagen), generating pET629. DNA fragments containing different portions of IS629 for various experiments were generated from pGEM629 or pET629 by PCR using oligonucleotide primers listed in Table 1. Recombinant plasmids used in this study are described below in Table 2.

Detection of Translational Frameshifting—To detect −1 translational frameshifting in IS629, the lacZ gene was fused to a DNA fragment containing the two putative frameshift motifs, referred to as the frameshift window (fsw),2 so that the lacZ gene is expressed only when a −1 frameshift occurs and that the frameshifting can be detected by measuring β-galactosidase activity. The 3.2-kb Smal-PstI fragment containing the lacZ gene from pMC1871 (5) was cloned into the corresponding sites of pUCD1752X (6) to generate pUCDlacZ. To investigate the function of the two putative frameshift motifs, a DNA fragment (IS629 nucleotides 55–425) containing the two motifs was amplified from pGEM629 using primers Fₓba₁₋₅₅ and Rₑc₁₋₁₅₆₆ and cloned between XbaI and SmaI sites of pUC18, generating pUC629-21. The 450-bp XbaI-Acc65I fragment from pUC629-21 was then inserted into the corresponding sites of pUCDlacZ to generate pF₁wF₁wIw, thus making the function of the two putative frameshift motifs possible. Two frameshift products, designated orfA and orfB, are present in IS629 (see Fig. 1). The coding potential of orfA (nucleotides 55–381) is 108 amino acids, and that of orfB (nucleotides 378–1,268) is 296 amino acids (3). The stop codon (TGA) of orfA overlaps the initiation codon (ATG) of orfB (see Fig. 1). A putative promoter and the Shine-Dalgarno sequence are found upstream from the initiation codon of orfA, but no such sequences are present in the upstream region of orfB (3).

Two putative −1 translational frameshift signals, TTTTG (T₄G) and AAAAA (A₄T), are located near the 3′-end of the orfA at nucleotide positions 342–346 and 375–379, respectively.

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2 The abbreviations used are: fsw, frameshift window; IPTG, isopropyl 1-thio-D-galactopyranoside; MBP, maltose-binding protein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; IRL, left terminal inverted repeat; IRR, right terminal inverted repeat; RNAP-α, a subunit of RNA polymerase.
"w" denotes wild-type sequence, and "m" means mutated sequence. Because this 450-bp XbaI-Acc65I fragment contained the initiation codon of orfB, which may render the lacZ gene constitutively expressed, this initiation codon was mutated, generating pF1mF2mIm. To investigate the function of the first frameshift motif T₄G (F1), the sequence AAAAT (F2) was mutated to TCGAT to create pF1mF2mIm. Similarly, the sequence TTTTG (F1) was changed to TACTG to investigate the function of the second frameshift motif, generating pF1mF2mIm. The plasmid that contained mutations of both motifs and the orfB initiation codon was called pF1mF2mIm, whereas the one containing the two mutated motifs with the wild-type orfB initiation codon was referred to as pF1mF2mIm.

Purification and Detection of Transframe Products—To detect and identify the frameshifted products of IS₆₂₉, the gene encoding a portion (amino acids 147–402) of the E. coli maltose-binding protein (MBP) with a His₆ tag at the 3'-end was fused to a DNA fragment containing either one of the two frameshift motifs so that MBP-His₆ is produced only when a -1 frameshift occurs. IS₆₂₉ fragment (nucleotides 334–375) containing the T₄G motif was amplified from pET629 with primers FBamHI-334 and RAscI-375 and then inserted between BamHI and Ecl136II sites of pET-29a(H11001) to yield pET629T₄G. The 767-bp BssHII-HindIII fragment containing the malE gene encoding MBP from pMAL-C2 (New England Biolabs) was then inserted between AscI and HindIII sites on pET629T₄G to obtain pET629T₄GMBP, thus fusing MBP to the T₄G motif. The hybrid gene was driven by the T7 promoter and regulated by the lac operator. To fuse the A₄T motif with MBP-His₆, IS₆₂₉ fragment (nucleotides 367–458) containing the A₄T motif was amplified from pGEMT629 with primers FIGURE 1. The organization of IS₆₂₉. Left and right terminal inverted repeats, IRL and IRR, are indicated by solid triangles. The putative frameshift window (fsw) is shown as a gray box. The two partially overlapping open reading frames (OrfA and OrfB) are indicated; they are in phase 0 and -1 translational frames, respectively. Two putative frameshifted products (OrfAB' and OrfAB) are depicted. The nucleotide sequence of the putative frameshift window and amino acids encoded by this sequence are shown. The two putative frameshift signals (T₄G and A₄T) are indicated in boldface and are underlined. Partial amino acid sequences of OrfA, OrfB, and the two putative frameshifted products are shown with boldface letters. The numbers above the nucleotide sequence denote the IS₆₂₉ nucleotide positions. IS₆₂₉ is 1,310 bp in length.

TABLE 1
Oligonucleotide primers used in this study

| Primer | Sequence (5' → 3') |
|--------|-------------------|
| FNdeI-55 | CATATGACTAAAAATACTCGTTTTTCCCCCG |
| REcl136II-Term | GAGCTCAGGCTCATCATCGTTTTCCGATGGAAC |
| FXbaI-55 | CTCTAGAATGACTAAAAATACTCGTTTTTCCCCCG |
| RRsrII-425 | CGGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGCA |
| RA4TAATC | CGGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGgattaTTTTTTCCAGA |
| RClaI-TAATC | GGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGGATatcgaTTCCAGAGG |
| F316-Bst1107I | CGATATCCTTCGCCAGGCTTCCGCGTAtactgCGAAGGCGGAGTT |
| RRsrII-ClaI | CGGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGCATCatcgaTTCCAGAGGCGGTCA |
| RAgeI-375 | GACCGGTGAATTTCCAGAGGCGGTCAAACTCCGCCTTCGcagtaTACGCGG |
| FBamHI-367 | CGGATCCCTCTGGAAAAAATGATGCCTCTGCTGGAT |
| RAscI-458 | AGGCGCGCCCTCGGTACCGTTGACGGGGCAATATGCA |
| FIRL-BamHI | TGAACCGCCCCGGGAATCCTGGAGACTAAGGATCCTGAGA |
| RIRR-BamHI | TGAACCGCCCCGGGTTTCCTGGAGAGTGTGGATCCTGTGAACTCA |
| RT4A5 | CGGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGCATCAtttttTTCCAGAG |
| FT5 | AACGATATCCTTCGCCAGGCTTCCGCTTAtttttGCGAAGGC |
| RA5 | CGGTCCGACCCCGTACTGCTCACGCAGCTTATCCAGCAGTGGGATAtttttTTCCAGAG |
| FNotI-55 | GCGGCCGCGATGACTAAAAATACTCGTTTTTCCCCCG |
| FP-1 | ACCTACAACAAAGCTCTCATCAACC |
| PRP-1 | ACGTTTCCTCGTAGATAGTGCGCAT |
The occurrence of frameshifting was confirmed by detecting peptides that were produced by a \(-1\) frameshift by matching the mass spectra of the peptides against two databases created based on the frameshift model of Jacks et al. (8). These two databases contained computer-generated nucleotide sequences of different peptides that may be produced by trypsin or Asp-N digestion of fsw(T\(_{G}\))-MBP-His\(_6\) and fsw(A\(_{T}\))-MBP-His\(_6\), respectively, translated in both the 0 and \(-1\) frames. Because the region located between the most downstream peptide sequence derived from the 0 translation frame and the most upstream peptide sequence derived from the \(-1\) translation frame is where frameshifting may occur, this region is referred to as the “frameshift region.” Two additional new databases were then created. The first one contained the nucleotide sequences of different peptides derived from a \(-1\) frameshift that occurs at each codon within the first frameshift region (fsw(T\(_{G}\))-MBP-His\(_6\)), and the second one contained those derived from the second frameshift region (fsw(A\(_{T}\))-MBP-His\(_6\)). Each nucleotide sequence corresponded to a resultant peptide from one \(-1\) frameshifting event within a frameshift region.

The collision-induced dissociation spectra of a peptide were acquired as three successive scans as described by Tsay et al. (9). The acquired collision-induced dissociation spectra were interpreted using a ThermoFinnigan software package, the TurboSEQUEST browser, which matches the tandem mass spectrum with those in the databases described above. The MS/MS data that matched the peptide sequences with appropriate cleavage sites at the right positions were subjected to manual analysis using another computer program (EverNew Biotech) to confirm the results.

Transposition Assays—To investigate effects of IS629-encoded proteins on IS629 transposition in vivo, a mini-IS629 with the kanamycin resistance gene was first constructed, and proteins that may affect IS629 transposition were supplied in trans. The left terminal repeat (IRL) sequence of IS629 was amplified from the chromosome of S. sonnei ATCC 9290 with primers F\(_{I\text{RL}}\)-BamHI and R\(_{A\text{scl-375}}\) and the PCR product was ligated into pGEMT-Easy (Promega) to generate pGEMT-IRL. The right terminal repeat (IRR) was amplified with primers F\(_{316}\)-Bst1107I and R\(_{IJ\text{R}}\)-BamHI and similarly cloned into pGEMT-Easy to generate pGEMT-IRR. The 1.9-kb Scal-BamHI fragment containing the IRL from pGEMT-IRL and the 1.2-kb Scal-BamHI fragment containing the IRR from pGEMT-IRR were joined together to obtain pGEMT-mini629. The 1.3-kb BamHI fragment containing the kanamycin resistance gene from pUC4K (Amersham Biosciences) was then inserted into the BamHI site of pGEMT-mini629 to generate pGEMT-mini629Km. Finally, pMini629 was constructed by inserting the 1.3-kb NotI fragment containing the mini-IS629 with the kanamycin resistance gene from pGEMT-mini629Km into pET-22b(+) (Novagen).

The 1.2-kb Ndel-Ecl136II fragment containing the orfA and orfB sequences of IS629 from pGEMT629 was then inserted into the corresponding sites of pMini629 to generate pMini629AB’-AB-A-B, which would express OrfAB’, OrfAB, OrfA, and OrfB. The 370-bp Ndel-RsrII DNA on pMini629AB’-AB-A-B was then replaced with the 370-bp PCR-generated Ndel-RsrII DNA fragment encoding OrfAB’, OrfAB, and OrfA to produce pMini629AB’-AB-A. Similarly, pMini629AB’-A that would express OrfAB and OrfA was constructed by replacing the same Ndel-RsrII DNA on pMini629AB’-AB-A-B with the 370-bp PCR-generated Ndel-RsrII DNA fragments encoding OrfAB’ and OrfA (Table 2).

The transposition activity of IS629 was determined by the standard mating-out assay as described previously (10). Derivatives of pMini629 (Km\(^{\text{r}}\)) carrying various IS629 genes were transformed into E. coli DH1(DE3) cells (Str\(^{\text{r}}\)) harboring an F-derived conjugative plasmid pCJ105 (Cm\(^{\text{r}}\)), which served as the target for IS629 transposition. Because pCJ105 carries a chloramphenicol resistance gene, transposition of mini629Km onto pCJ105 will render the host resistant to both kanamycin
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and chloramphenicol. To determine the transposition frequency of IS629, pCJ105::mini629Km was mated out from *E. coli* DH1(DE3) to *E. coli* HB101(Str') at 37 °C for 90 min. Appropriate dilutions of the conjugation mix were plated on LB agar plates containing both chloramphenicol (50 µg/ml) and streptomycin (150 µg/ml) as well as on plates containing kanamycin (50 µg/ml), chloramphenicol (50 µg/ml), and streptomycin (150 µg/ml). Colonies that appeared on these plates were counted, and the transposition frequency was determined as the ratio of the number of Cmr Kmr Strr colonies to that of the Cm' Km' Str' colonies. To confirm transposition, some of the transposition products (pCJ105::mini629Km) were isolated and examined for direct repeat sequences flanking the mini-IS629. The direct repeat sequence adjacent to IRR was identified by nucleotide sequencing using primer FP-1 (Table 1), which anneals to the 3'-end of the kanamycin resistance gene 140 bp upstream from IRR. To detect the direct repeat sequence adjacent to IRL, primer PRP-1 (Table 1), which anneals to the 5'-end of the kanamycin resistance gene 164 bp downstream from IRL, was used for sequencing.

**Pulse-Chase Experiments**—Pulse-chase experiments were performed to investigate the half-life of OrfAB' and OrfAB. To express OrfAB', the 1.2-kb NdeI-Ecl136I1 fragment from pMini629AB'-A was cloned into the corresponding sites on pET-29a (+) (Novagen) to generate pET629A-AB'. Similarly, the NdeI-Ecl136I1 fragments from pMini629AB-A and pMini629AB'-AB-A were inserted between NdeI and Ecl136I sites on pET-29a (+) to generate pET629A-AB and pET629A-AB'-AB that express OrfAB and OrfAB'+OrfAB', respectively.

Overnight cultures of *E. coli* DH1(DE3) cells containing pET629A-AB', pET629A-AB, or pET629A-AB'-AB were diluted 1:50 with fresh M9 minimal medium containing kanamycin (50 µg/ml) and grown to an A600 of 0.3. The cells in the culture were pelleted, washed with M9 buffer (11), and suspended in M9 minimal medium containing 2% methionine assay medium (Difco Laboratories). After 100-min incubation at 37 °C, IPTG was added to the culture to a final concentration of 1 mM to induce the synthesis of the T7 RNA polymerase. Forty minutes later, rifampin (200 µg/ml) was added, and the culture was incubated for another 40 min. The cells were then labeled with [35S]methionine (20 µCi/ml, Amersham Biosciences) for 10 min and subsequently chased with an excess amount of non-radioactive methionine (final concentration, 2.5 mg/ml). Samples were taken at different time points, pelleted, and suspended in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 7 mM β-mercaptoethanol, 10% glycerol, 0.1% bromphenol blue). The samples were boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel. The gels were scanned with a PhosphorImager (Amersham Biosciences), and quantification of the protein was performed with the program ImageQuant (Amersham Biosciences).

**Bacterial Two-hybrid Assay**—To assess the interaction between OrfAB' and OrfAB, an *E. coli* two-hybrid experiment was performed. In this system (BacterioMatch Two-Hybrid System, Stratagene), the bait plasmid pBT (Cm') encodes the bacterial phage λcl protein under the control of the lac-UV5 promoter. This λcl protein contains the N-terminal DNA-binding domain and the C-terminal dimerization domain. The protein of interest, the bait, is fused to the λcl protein. The target plasmid pTRG (Tc') contains an RNAγα gene, which is driven by the *E. coli* lipoprotein promoter (lp) and regulated by the lac operator. The target protein is fused to the N-terminal domain of the RNA polymerase α subunit.

DNA fragments encoding OrfAB' or OrfAB were cloned into these plasmids to fuse OrfAB' or OrfAB to λcl or RNAγα to generate λcl-OrfAB', λcl-OrfAB, RNAγα-OrfAB', and RNAγα-OrfAB. When interaction between λcl-OrfAB' and RNAγα-OrfAB', λcl-OrfAB and RNAγα-OrfAB, λcl-OrfAB' and RNAγα-OrfAB, or λcl-OrfAB and RNAγα-OrfAB' had occurred, these complexes would bind to the λ operator (O₂) located upstream from the reporter cassette containing the lacZ genes in *E. coli* XL-1 Blue MRF' (Km'). This binding would recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of the reporter gene. Thus, the protein-protein interaction between OrfAB and OrfAB' or between themselves could be determined by the levels of β-galactosidase activity.

The bait and target plasmids were constructed as follows. The IS629 fragment containing an insertion of a thymine residue within the T₄G motif was amplified from pET629A-AB' with primers F7 and R₇₋₁₋₁₋₁₋₁₋₁₋₁₋₁. The PCR product was digested with EcoRV and HindIII and then cloned into the corresponding sites of pGEMT629, resulting in pGEMT629T5. A 1.2-kb NotI-EcoRI fragment containing IS629(T5) without the terminal repeats was then amplified from pGEMT629T5 with primers F₇₋₁₋₁₋₁₋₁₋₁₋₁₋₁ and R₇₋₁₋₁₋₁₋₁₋₁₋₁₋₁ and then cloned into the corresponding sites of pBT and pTRG to yield pBT-AB' and pTRG-AB'. For construction of pBT-AB and pTRG-AB, the IS629 fragment (nucleotides 55–425) with an adenine insertion within the A₅T motif was amplified from pET629A-AB with primers F₇₋₁₋₁₋₁₋₁₋₁₋₁₋₁ and R₇₋₁₋₁₋₁₋₁₋₁₋₁₋₁. This PCR product was ligated into pGEMT-Easy vector (Promega) to generate pGEMT629A5NR. The RssIl-SphI fragment containing the IS629 nucleotides 420–1,269 from pGEMT629 was then inserted into the corresponding sites of pGEMT629A5SR to obtain pGEMT629A5. The NotI-EcoRI fragment of pGEMT629A5 was inserted between NotI and EcoRI sites on pBT and pTRG to generate pBT-AB and pTRG-AB, respectively.

*E. coli* XL-1 Blue MRF' was co-transformed with various combinations of recombinant bait and target plasmids to examine interaction between OrfAB' and OrfAB or with non-recombinant pBT and pTRG vectors to serve as negative controls for the interaction analysis. Transformants were selected on LB agar plates containing 12.5 µg/ml tetracycline, 34 µg/ml chloramphenicol, and 50 µg/ml kanamycin. In the presence of 20 µM IPTG, cells were cultured at 30 °C to mid-log phase, and then assayed for β-galactosidase activity by the method of Miller (12) using o-nitrophenyl-β-d-galactopyranoside as the substrate.

**RESULTS**

**Two Functional −1 Frameshift Signals**—To determine whether the T₄G or the A₅T motif was able to provoke a −1 frameshift, a
lacZ reporter gene was fused to the 3'-end of a DNA fragment (nucleotides 55–425) containing the entire orfA, the first 48 bp of orfB, and the wild-type or mutated putative frameshift motifs. The lac promoter and E. coli RNA polymerase were used to express the hybrid gene on these constructs (Table 2). Translation of mRNA derived from each of these plasmids would start at frame 0 of orfA, and the β-galactosidase would be expressed only when a 1 frameshift had occurred, because lacZ was fused to the 1 reading frame of orfA. The β-galactosidase activity in cells harboring a certain construct after IPTG induction was measured (Fig. 2). As the positive control for β-galactosidase production, the lacZ gene on pF1mF2mLacZ was fused in-frame to orfA. The β-galactosidase activity conferred by this plasmid was determined to be 3,896 ± 243 units (Fig. 2) and designated as 100%. pF1wF2wIw, which contained the wild type of both T₄G and A₄T sequences

### Table 2: Plasmids used in this study

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pGEMT629     | IS629 sequence in pGEMT-Easy | FNdel-55+RRec136II-Term |
| pET629       | IS629 sequence in pET-29a(+)  |              |

**Plasmids for detection of translational frameshifting**

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pUCDlacZ     | pUCD1752X: lacZ          |              |
| pF1mF2mLacZ  | orfA-lacZ                | Fxbal-55+RAgeII-375 |
| pF1wF2wIw    | T₄G (F1), A₄T (F2), ATGATG for orfB initiation (I) | Fxbal-55+RRecII-425 |
| pF1wF2wIm    | T₄G, A₄T, ATGATG→ATAATC  | Fxbal-55+RATAC |
| pF1fF2mIm    | T₄G→TCAG, A₄T, ATGATG→ATAATC | Fxbal-55+RRecII-Cl |
| pF1fF2mIm    | T₄G→TACTG, A₄T, ATGATG→ATAATC | F316-Bst1107 FRecII-425 |
| pF1fF2mIw    | T₄G→TACTG, A₄T→TCAG, ATGATG→ATAATC | F316-Bst1107+RRecII-Cl |
| pF1fF2mIw    | T₄G→TACTG, A₄T→TCAG, ATGATG for orfB initiation | F316-Bst1107+RRecII-Cl |

**Plasmids for identification of frameshift sites**

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pET629T₄GMBP | IS629 (nt 334 to 375), fsw(T₄G)-MBP-His₄ | FBalH3-334+RAscII-375 |
| pET629AT₄MBP | IS629 (nt 367 to 458), fsw(A₄T)-MBP-His₄ | FBalH3-367+RAscII-458 |

**Plasmids for in vivo transposition assays**

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pMini629     | mini-IS629::Km'          | FIRI-BalH3+RAscII-375, F316-Bst1107+RRecII-BalH3 |
| pMini629AB(T4A5) | mini-IS629::Km', OrfAB without frameshifting | FNdel-55+RRecII-425 |
| pMini629AB'(T5A4) | mini-IS629::Km', OrfAB' without frameshifting | FSt,RRecII-425 |
| pMini629AB(A5)  | mini-IS629::Km', OrfA without frameshifting | F316-Bst1107+R5 |
| pMini629AB'(T5)  | mini-IS629::Km', OrfAB' without frameshifting | FSt,RRecII-425 |
| pMini629AB'-AB-A-B | mini-IS629::Km', OrfAB', OrfAB, OrfA, OrfB | FNdel-55+RRecII-425 |
| pMini629AB'-AB-A  | mini-IS629::Km', OrfAB', OrfAB, OrfA | FNdel-55+RRecII-425 |
| pMini629AB'-A  | mini-IS629::Km', OrfAB', OrfA | FNdel-55+RRecII-425 |

**Plasmids for protein expression**

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pET629A-AB   | production of OrfA and OrfAB | FNdel-55+RT4A5 |
| pET629A-AB   | production of OrfA and OrfAB | FSt+RRecII-425 |
| pET629A-AB'AB | production of OrfA, OrfAB and OrfAB | FNdel-55+RRecII-425 |

**Plasmids for bacterial two-hybrid analysis**

| Plasmid name | Critical characteristics | Primers used |
|--------------|--------------------------|--------------|
| pBT          | Bait vector              |              |
| pTRG         | Target vector            |              |
| pBT-AB'      | bait plasmid, a thymine insertion within the T₄G motif | FSt,RRecII-425, FNdel-55+RT7 |
| pBT-AB       | bait plasmid, an adenine insertion within the A₄T motif | FNdel-55+R5 |
| pTRG-AB'     | target plasmid, a thymine insertion within the T₄G motif | FSt,RRecII-425, FNdel-55+RT7 |
| pTRG-AB      | target plasmid, an adenine insertion within the A₄T motif | FNdel-55+R5 |
fused to the lacZ gene, conferred 665 ± 50 units (17.1% of control) of β-galactosidase activity, suggesting that a frameshift had occurred. To determine whether both T_G and A_T motifs were essential for frameshifting, they were mutated to TACTG and TCGAT, respectively. Surprisingly, pF1mF2mIw, which harbors these mutations, still conferred 504 units (12.9% of control) of β-galactosidase activity (Fig. 2). A careful analysis of the nucleotide sequence revealed the presence of two tandem translation initiation codons (78ATGATG83) located at the beginning of orfB. To determine whether the lacZ activity conferred by pF1mF2mIw was due to translation initiated from one of these two codons, the sequence ATGATG on pF1mF2mIw was changed to ATAATC to generate pF1mF2mIm. As expected, pF1mF2mIm with both the two putative frameshift signals and both the two ATG codons mutated conferred very little β-galactosidase activity (1.69 ± 0.15 units, 0.04% of control). This result indicates that the majority of β-galactosidase activity derived from frameshifting was ~4.5%.

Identification of Frameshift Sites—To confirm that frameshifting indeed occurred, the transframe products were identified. A DNA fragment containing nucleotides 334–375 of IS629 nucleotides 55–425 containing the wild-type or mutated frameshift motifs were fused with the lacZ gene. The nucleotide sequence of the frameshift window (fsw) and the deduced amino acids encoded by 0 and −1 reading frames in the window are shown. The stop codon of orfA is indicated by asterisks. The wild-type (T_G and A_T) or mutated frameshift motifs are underlined. The two ATG initiation codons for orfB and the mutated sequences are boxed. The β-galactosidase levels in Miller units derived from each construct are shown. The value derived from pF1mF2mIw on which the lacZ gene is fused in-frame to the fsw sequence is set as 100%, and the relative values of those derived from other constructs are shown in parenthesis.

FIGURE 2. The function of the putative frameshift motifs. DNA fragments (IS629 nucleotides 55–425) containing the wild-type or mutated frameshift motifs were fused with the lacZ gene. The nucleotide sequence of the frameshift window (fsw) and the deduced amino acids encoded by 0 and −1 reading frames in the window are shown. The stop codon of orfA is indicated by asterisks. The wild-type (T_G and A_T) or mutated frameshift motifs are underlined. The two ATG initiation codons for orfB and the mutated sequences are boxed. The β-galactosidase levels in Miller units derived from each construct are shown. The value derived from pF1mF2mIw on which the lacZ gene is fused in-frame to the fsw sequence is set as 100%, and the relative values of those derived from other constructs are shown in parenthesis.
the sequence GSoMADIGSAYFCEGGVRPPLEIHR was identified (Fig. 3C). This sequence was the resultant product from a −1 frameshift that took place at the T₄G motif.

A similar experiment was performed to examine whether a −1 frameshift indeed occurred at the A₄T motif. Nucleotides 367–458 containing the A₄T motif of IS629 was fused out-of-frame to the sequence encoding MBP-His₆ to generate pET629A₄TMBP (Fig. 4A). pET629A₄TMBP was then introduced into E. coli, and the expressed protein was analyzed by gel electrophoresis (Fig. 4B, left panel), immunoblotting (Fig. 4B, right panel), and LC-MS/MS analysis. When the expected 34-kDa band was analyzed, a peptide with the sequence DIGSLWKK-MMPLL was found (Fig. 4C), indicating that a −1 frameshift had occurred at the A₄T motif.

Effects of IS629-encoded Proteins on IS629 Transposition— The results described above indicate that both the T₄G and A₄T motifs can mediate a −1 frameshift, suggesting that in addition to OrfA and OrfB, two transframe proteins, OrfAB' and OrfAB, are also produced. Experiments were then performed to investigate effects of these proteins on IS629 transposition. A plasmid (pMini629) carrying a mini-IS629 composed of the kanamycin resistance gene flanked by terminal repeats of IS629, IRL (nucleotides 1–29) and IRR (nucleotides 1,280–1,310), was first constructed. DNA fragments containing various IS629 genes without terminal repeats, including OrfA, OrfB, OrfAB, and OrfAB', were then inserted immediately upstream from the mini-IS629 (Fig. 5).

To determine the function of OrfAB', the T₄G sequence was changed to T₅G by inserting an extra thymine residue to generate pMini629AB'(T₅A₄) so that OrfAB' would be produced without frameshifting. Similarly, an adenine residue was inserted into the A₄T motif, changing it to A₅T to generate pMini629AB(T₄A₅) so that OrfAB would be produced without frameshifting. No other changes in IS629 sequence on these two plasmids were made; therefore, the OrfAB' and OrfAB proteins produced by pMini629AB'(T₅A₄) and pMini629AB(T₄A₅), respectively, were of native form.

The plasmids containing the mini-IS629 with various IS629 genes were introduced into E. coli DH1(DE3) that harbors an F-derived conjugative plasmid pCJ105. Transposition of the mini-IS629 onto pCJ105 was assessed by mating pCJ105 out to E. coli HB101 and confirmed by detecting direct nucleotide sequence repeats flanking the mini-IS629 that was transposed onto pCJ105. The mini-IS629 alone (pMini629) had no transposition activity with a background frequency of (0.4 ± 0.1) × 10⁻⁷ (Fig. 5). In the presence of OrfAB (pMini629AB(T₄A₅)), this transposition frequency was not significantly increased ((0.6 ± 0.1) × 10⁻⁷) (Fig. 5), suggesting that OrfAB alone has no role in IS629 transposition, although it differs from
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OrfAB’ in sequence by only ten amino acid residues. In contrast, a 109-fold increase \((43.5 \pm 3.8) \times 10^{-7} \) versus \((0.4 \pm 0.1) \times 10^{-7}\) (Fig. 5) in transposition frequency was observed when OrfAB’ (pMini629AB’(T5A4)) was present, indicating that OrfAB’ is the transposase of IS629.

In both pMini629AB(T4A5) and pMini629AB’(T5A4), the translation initiation codon for orfB is intact and may produce the OrfB protein. To avoid effect of OrfB on IS629 transposi-
tion, the two ATG codons located at the beginning of orfB in these two plasmids was changed to ATAAATC. In addition, the \(A_{1}\)T motif was mutated to TCGAT, resulting in pMini629AB’(T5) so that OrfAB’ would be produced without frameshifting. Similarly, the \(T_{1}\)G motif was changed to TACTG, generating pMini629AB(A5), which would produce OrfAB without frameshifting. The transposition frequency of the mini-IS629 on pMini629AB’(T5) was determined to be \((42.2 \pm 5.9) \times 10^{-7}\) very similar to that of pMini629AB’(T5A4), which had a transposition frequency of \((43.5 \pm 3.8) \times 10^{-7}\) (Fig. 5). The mini-IS629 on pMini629AB(A5) transposed at a frequency of \((0.8 \pm 0.1) \times 10^{-7}\) very similar to that on pMini629AB’(T4A5), which had a transposition frequency of \((0.6 \pm 0.1) \times 10^{-7}\) (Fig. 5). These results indicated that these mutations did not affect the functions of OrfAB and OrfAB’ and confirmed that OrfAB’ plays a major role in IS629 transposition and that OrfAB alone does not mediate IS629 transposition.

In the experiments described above, OrfAB and OrfAB’ were artificially produced without frameshifting. To investigate effects of these two proteins that were produced by frameshifting on IS629 transposition, the transposition frequency of the mini-IS629 on pMini629AB’-AB-A, which expresses all four IS629 proteins, was examined and determined to be \((70.3 \pm 12.2) \times 10^{-7}\) (Fig. 5). To abolish the synthesis of OrfB, the two ATG codons located at the beginning of orfB was changed to ATAAATC, generating pMini629AB’-AB-A. The transposition frequency of the mini-IS629 on pMini629AB’-AB-A was increased by 1.8-fold (from \((70.3 \pm 12.2) \times 10^{-7}\) to \((128.9 \pm 20.9) \times 10^{-7}\) (Fig. 5) when the OrfB protein was not expressed, suggesting that OrfB negatively regulates IS629 transposition. To abolish the synthesis of OrfAB, the \(A_{1}\)T motif was mutated to TCGAT, resulting in pMini629AB’-A. Similarly, the \(T_{1}\)G motif was changed to TACTG to prevent the synthesis of OrfAB, generating pMini629AB-A. The transposition activity of the mini-IS629 on pMini629AB’-A was profoundly diminished (from \((128.9 \pm 20.9) \times 10^{-7}\) to \((2.6 \pm 0.9) \times 10^{-7}\) (Fig. 5) when the OrfAB protein was not expressed. This result

![FIGURE 4. Verification of the A_{1}T motif as a -1 frameshift site. A, structure of the fsw(A_{1}T)-MBP-His\textsubscript{6} reporter gene. The amino acid sequences of the putative products in the 0 and -1 frames are shown below the diagram. The boldface letters indicate the amino acid sequence of the transframe peptide determined by LC-MS/MS analysis. The A_{1}T motif is boxed. Abbreviation of restriction sites: Ba, BamH\textsubscript{i}; As, Ascl; Hd, Hind\textsubscript{III}; T\textsubscript{5}, T\textsubscript{7}, promoter; fsw, frameshift window; His\textsubscript{6}, histidine hexamer tag; MBP, E. coli maltose-binding protein. B, the -1 frameshifted fsw(A_{1}T)-MBP-His\textsubscript{6} protein. Left panel, proteins purified by nickel column chromatography were separated by electrophoresis on a 10% SDS-polyacrylamide gel and then stained with Coomassie Blue dye. Right panel, the immunoblot of the gel was probed with MBP-specific monoclonal antibody and developed with chemiluminescent reagents. Arrowheads indicate the frameshifted product. C, LC-MS/MS spectrum of the transframe peptide DIGSLWKKMMMPPLL. y-ions are peptide fragments carrying the positive charge at the N terminus, and b-ions are those with the positive charge at the C terminus, and b-ions are those with the positive charge at the C terminus when a protein is fragmented between carbonyl and nitrogen groups of a peptide bond, a-ions are peptide fragments derived from breaking that takes place between amino acid side chain and carbonyl group.](image-url)
suggests that OrfAB enhances IS629 transposition, although OrfAB itself did not mediate transposition as evidenced by a background transposition frequency \((0.6 \pm 0.1) \times 10^{-7}\) (Fig. 5) when the transposition activity of the mini-IS629 on pMini629AB-A was assayed.

**Stabilization of OrfAB by OrfAB**—The experiments described above demonstrated that the OrfAB protein is not a transposase but has the ability to enhance transposition of IS629. Because transposase stability may affect the transposition activity of a certain transposable element (13, 14), the effect of OrfAB on the stability of OrfAB was examined by pulse-chase experiments. After IPTG induction, IS629 proteins encoded by pET629A-AB, pET629A-AB', and pET629A-AB'-AB were labeled with \(^{35}\text{S}\)methionine for 10 min and then chased with excess amounts of non-radioactive methionine. The half-life of OrfAB' in the presence and absence OrfAB was then measured by determining the ratio of radioactive OrfAB' to that of OrfA every 30 min up to 120 min (Fig. 6). OrfA was used as the reference, because it is constitutively expressed from all plasmids used in this experiment.

In the absence of OrfAB, the ratio of OrfAB' to OrfA was 0.53% at the zero time point, and 0.2, 0.17, 0.15, and 0.14% at the 30-, 60-, 90-, and 120-min time points, respectively (Fig. 6A). From these data, the half-life of OrfAB' was determined to be \(~30\) min. The ratios of OrfAB' to OrfA were 2.0, 1.99, 1.38, 0.9, and 0.69% at 0-, 30-, 60-, 90-, and 120-min time points (Fig. 6B), indicating that the half-life of OrfAB was \(~90\) min. Because OrfAB' and OrfAB are identical in size, they cannot be separated by the gel electrophoresis used in these experiments.
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FIGURE 6. Half-life of OrfAB’ and OrfAB. The proteins encoded by pET629A-AB’ (A), pET629A-AB (B), and pET629A-AB-AB (C) were labeled for 10 min with [35S]methionine and chased with excess nonradioactive methionine for 0-, 30-, 60-, 90-, or 120 min. [35S]Methionine-labeled proteins were resolved on a 12% SDS-polyacrylamide gel, and the gel was subjected to autoradiography followed by imaging analysis. The ratio of the amount of frameshifted product(s) to that of OrfA is shown below each panel. Numbers on the right-hand side of each panel denote molecular sizes markers.

FIGURE 7. Bacterial two-hybrid analysis of OrfAB’-OrfAB interaction. β-galactosidase activities in E. coli strain XL-1 Blue MRF’ containing (+) or not containing (−) bait (pBT-) or target (pTRG-) plasmids were determined by the standard method using o-nitrophenyl-β-D-galactopyranoside as the substrate. Enzyme activity is given in Miller units. Data with standard deviation were derived from six different colonies of each co-transformation.

Therefore, the ratio of OrfAB’+OrfAB to OrfA was measured and determined to be 4.53, 4.38, 4.3, 3.5, and 2.7% at 0-, 30-, 60-, 90-, and 120-min time points, respectively (Fig. 6C). From this result, the half-life of OrfAB’+OrfAB was determined to be 120 min. Therefore, the half-life of OrfAB’ was much longer in the presence of OrfAB, indicating that OrfAB has the ability to stabilize OrfAB’. The results suggested that OrfAB may bind to OrfAB’ to form hetero-multimers that are more stable than homo-multimers of OrfAB’ or OrfAB.

Interaction of OrfAB with OrfAB’—The possibility that OrfAB binds and stabilizes OrfAB’ was then examined by an E. coli two-hybrid assay in which interaction between bait and target proteins activates the lacZ reporter gene enabling the cells to produce β-galactosidase. In this experiment, OrfAB’ or OrfAB was fused to the bait protein on pBT or to the target protein on pTRG. To allow expression of OrfAB’, the T5G sequence was changed to T5G, and the 1.2-kb DNA fragment containing this mutation without the terminal repeats of IS629 was cloned into the NotI and EcoRI sites on pBT or pTRG fusing OrfAB’ to the lacI protein or to RNAPα, generating pBT-AB’ or pTRG-AB’. Similarly, the A5T sequence was changed to A5T to allow expression of OrfAB without frameshifting, and the 1.2-kb DNA fragment containing this mutation was cloned into the NotI and EcoRI sites on pBT or pTRG to generate pBT-AB or pTRG-AB. Different pairs of pBT- and pTRG-derived plasmids were then introduced into E. coli XL-1 Blue MRF’, and the co-transformants were assayed for β-galactosidase activity. As shown in Fig. 7, negative control cells containing no plasmids had a basal level of β-galactosidase activity of 17.5 ± 0.4 units. Cells containing plasmid pairs (pBT/pTRG, pBT/pTRG-AB’, pBT-AB'/pTRG, and pBT-AB/pTRG) that did not express both OrfAB’ and OrfAB or expressed only one of the two did not have significantly elevated levels of β-galactosidase activity. In contrast, cells containing plasmid pairs (pBT-AB’/pTRG-AB’, pBT-AB'/pTRG-AB’, pBT-AB/pTRG-AB’, and pBT-AB/pTRG-AB) that expressed OrfAB’, OrfAB, or both OrfAB’ and OrfAB had ~3 times as much β-galactosidase activity as the negative controls. These results indicate that OrfAB’ and OrfAB can bind to each other or to themselves.

DISCUSSION

In this study, we showed that the two putative translational frameshift signals, T5G and A5T, located near the 3’-end of orfA of IS629 are functional. Using the lacZ gene as a reporter, we demonstrated that each of these two motifs can mediate a −1 translational frameshift (Fig. 2), resulting in the production of two transframe proteins OrfAB and OrfAB’. This −1 translational frameshift mediated by the T5G or the A5T motif was confirmed by the existence of the transframe products (Figs. 3 and 4). Therefore, IS629 has the potential to encode four different proteins, including OrfAB, OrfAB’, OrfA, and OrfB. These proteins were expressed either alone or in combinations in E. coli, allowing examination of their ability to mediate IS629 transposition. The transframe protein OrfAB’ alone was sufficient for IS629 transposition (Fig. 5), indicating that OrfAB’ is the transposase of IS629. Simultaneous production of both OrfAB’ and OrfAB increased the transposition activity of IS629, whereas production of OrfAB alone did not mediate IS629 transposition. These results suggest that the OrfAB protein is not a transposase but can enhance the transposition of IS629. OrfAB was shown by the bacterial two-hybrid assay to have the ability to bind OrfAB’ (Fig. 7), and binding of OrfAB to OrfAB’ was shown to increase the half-life of OrfAB’ (Fig. 6). Increase in the half-life of the transposase has been shown to enhance the transposition of IS903 (15). Therefore, the stabilization of OrfAB’
by OrfAB would be a mechanism by which IS629 positively regulates its transposition. This type of regulation has not been found in other members of the IS3 family.

The ability of OrfAB and OrfAB’ to bind to each other or to themselves would allow them to form multimers. In many transposable elements, multimerization of transposase forms a stable transpososome, which is essential for transposition (16–20). For phage Mu, the transposase MuA is a monomer in solution, but its transpososome, which is essential for transposition (16–20). For transposable elements, multimerization of transposase forms a stable themselves would allow them to form multimers. In many trans-

orfs of IS629. In IS629, OrfA forms hetero-multimers with the transposase OrfAB via a leucine zipper to enhance the inter-molecular transposition (34). Because IS629 OrfA contains the same leucine zipper as OrfA, it is possible that OrfA functions similarly to that of IS911 OrfA to stabilize IS629 transposition.

Analyses of nucleotide sequences of IS629 from various organisms revealed two different types of IS629 sequences. These two sequences differ by one base located at nucleotide position 360 between the T₆G and A₆T motifs. The presence of a T residue at this position creates a TGA stop codon in the –1 reading phase, which will render IS629 unable to produce the transposase OrfAB’. Such IS629 would not be transposable. Among the 60 IS629 sequences we have analyzed, 55 sequences have a C and 5 sequences have a T residue at this position (35–42). Therefore, the majority of IS629 elements in nature are transposable. The significance for the existence of non-functional IS629 elements remains to be investigated.

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