Apical Loop-Internal Loop RNA Pseudoknots

A NEW TYPE OF STIMULATOR OF – 1 TRANSLATIONAL FRAMESHIFTING IN BACTERIA

Received for publication, April 14, 2008, and in revised form, May 8, 2008. Published, JBC Papers in Press, May 12, 2008, DOI 10.1074/jbc.M802829200

Marie-Hélène Mazauric1, Patricia Licznar1,2, Marie-Françoise Prêre, Isabelle Canal, and Olivier Fayet3
From the Laboratoire de Microbiologie et Génétique Moléculaire, UMR5100, Centre National de la Recherche Scientifique and Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 9, France

Nearly all members of a widespread family of bacterial transposable elements related to insertion sequence 3 (IS3), therefore called the IS3 family, very likely use programmed –1 ribosomal frameshifting to produce their transposase, a protein required for mobility. Comparative analysis of the potential frameshift signals in this family suggested that most of the insertion sequences from the IS31 group contain in their mRNA an elaborate pseudoknot that could act as a recoding stimulator. It results from a specific intramolecular interaction between an apical loop and an internal loop from two stem-loop structures. Directed mutagenesis, chemical probing, and gel mobility assays of the frameshift region of one element from the IS31 group, IS3411, provided clear evidences of the existence of the predicted structure. Modeling was used to generate a three-dimensional molecular representation of the apical loop-internal loop complex. We could demonstrate that mutations affecting the stability of the structure reduce both frameshifting and transposition, thus establishing the biological importance of this new type of RNA structure for the control of transposition level.

The expression of a minority of genes requires a localized nonstandard decoding event, also called recoding (1–4). Among the various type of recoding events, programmed –1 ribosomal frameshifting (PRF-1)4 was first described more than 20 years ago (5); it involves the backward slippage by one nucleotide of the ribosome at a given point on the message. Even though PRF-1 examples are found in organisms from all three domains, most of the authenticated cases are in viruses (notably in retroviruses), in bacteriophages, and in bacterial DNA transposable elements called insertion sequences (IS). The mandatory short sequence on which the ribosome shifts, the frameshift motif, can be either a heptamer of the form X-XY-YYZ (6, 7) or a Y-YYZ tetramer (8–11).

In many cases the frequency of frameshifting is greatly stimulated by a structure formed by the mRNA on the 3′ side of the shift site; this structure can be either a pseudoknot or a stem-loop (12). The combination of the shift site and of a 3′ stimulator leads to frameshifting frequencies well above the spontaneous level of error (13). The design of viral 3′ stimulatory structures elicited much interest as a source of potentially new RNA motifs with the ability to target the ribosome (12, 14). Another reason is their presence in pathogenic viruses such as HIV-1 or the severe acute respiratory syndrome (SARS) coronavirus. It has been proposed that a 3′ structural element stimulates shifting at the slippery site by constituting a physical barrier to mRNA translocation, thus causing ribosomal pausing while the frameshift motif is in the P and A sites (15–18). In retroviruses and coronaviruses, the slippery sequence is almost always associated with a pseudoknot (Recode data base) (19, 20). A notable exception is HIV-1, where the stimulator is a stem-loop (21–23). In contrast with the viral situation, there are no published statistics on the bacterial stimulatory structures. However, stem-loops are predominant over pseudoknots among the few analyzed cases, i.e. dnaX (24), IS150 (25), IS911 (26), IS1221 (27), and IS1222 (28) versus IS3 (8).

To have a better knowledge of the repertoire of stimulatory structures in bacterial frameshift regions, we carried out a comparative analysis of a class of transposable elements, the IS of IS3 family (29), because its members very likely use PRF-1 to express the transposase, a protein required for their mobility. The ISFinder data base currently contains 354 members of the IS3 family. Nearly all members of this ubiquitous family share a common genetic organization where two open reading frames, orfA in frame 0 followed by orfB in frame –1, partially overlap (see Fig. 1). The overlap region invariably contains a potential heptameric or tetrameric frameshift motif generally accompanied by a frameshift stimulator. Detailed analysis of transposase expression has been performed on a few ISs: IS150 (25), IS911 (26, 30), IS3 (8), and IS629 (11). In each case, the OrfAB transposase is indeed synthesized via PRF-1 on the predicted motif. Our comparative analysis revealed one particularly interesting group among the IS3 family, the IS31 group, because many of its members appeared to contain a new type of stimulatory struc-

1 These authors contributed equally to this work.
2 Present address: IFR QUASAV 149, Laboratoire RCIM UPRES EA 2647/USC INRA, UFR Sciences, Université d’Angers, 2 boulevard Lavoisier, 49045 Angers cedex, France.
3 To whom correspondence should be addressed: Université Paul Sabatier, CNRS UMR5100, bat. IBGC, 118 route de Narbonne, 31062 Toulouse cedex 9, France. Tel.: 33-5-6133-5875; Fax: 33-5-6133-5886; E-mail: Olivier.Fayet@ibcg.biotoul.fr.
4 The abbreviations used are: PRF-1, programmed –1 ribosomal frameshifting; ALIL, apical loop-internal loop; PK, pseudoknot; CMCT, 1-cyclohexyl-3-morpholinooethyl) carbodiimide metho-p-toluene sulfonate; DMS, dimethyl sulfate; nt, nucleotide; IS, insertion sequence(s); HIV-1, human immunodeficiency virus, type 1; nt, nucleotide(s); SL, stem-loop; wt, wild type.

This work was supported by Research Grant NT05-1.44848 from the Agence National de la Recherche (Programme Blanc) and by funds from the Centre National de la Recherche Scientifique and the Université Paul Sabatier of Toulouse. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
5 The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Table S1, and Figs. S1–S3.
ALIL RNA Pseudoknot and − 1 Frameshifting

ture. This structure is a pseudoknot more elaborate than those previously analyzed. It is formed by the interaction an apical loop and an internal loop; we called it an ALIL–PK by analogy with similar structures obtained by in vitro selection of RNA aptamers against stem-loops from the 5′- and 3′-untranslated regions of hepatitis C virus (31). It has also been suggested that a related structure may be involved in −1 frameshifting and replication of the Barley yellow dwarf virus (32).

To carry out a detailed analysis of this potentially new frameshift stimulator, we chose an Escherichia coli transposable element from the IS51 group, IS3411 (33). In the present work, we have tested by an extensive genetic analysis the contribution of each element of the PRF-1 region of IS3411 (see Fig. 1). These studies as well as chemical probing experiments and gel shift mobility assays have confirmed the existence of the ALIL–PK structure, and molecular modeling provided clues on its architecture. Through its role as a stimulator of PRF-1, this intricate structure clearly determines the level of transposition of IS3411. From a structure/function point of view, it widens our knowledge on the strategies used by biological systems to promote RNA–RNA interactions for control of gene expression.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—The enzymes were provided by New England Biolabs, by MP Biochemical (Taq DNA polymerase), by Promega (T7 RNA polymerase), and by InvitroGen (SuperScript II reverse transcriptase).

Bacterial Strains and Plasmids—Bacteria were grown in LB medium (34). Ampicillin (40 μg/ml) plus chloramphenicol (200 μg/ml) and spectomycin (200 μg/ml) were added when necessary. Anhydrotetracycline (50 ng/ml) was used for induction of the P_{L_{56O-1}} promoter (35).

The E. coli K12 strain JS238 (MC1061, araDΔ(ara leu) galU galK hisD5 rpsL Δ(lacOPZYA)X74 malP::lacF β rlC::Tn10 recA1) was used for all experiments except the transposition assays. Strain OFB572 (C600, thr1 leu6 lacY1 tonA21 supE44 rpsL) was derived from pMPM-K6 (36) by insertion of an EcoRI-PstI-BsiWI-NheI linker between its BglI and EcoRI sites followed by insertion in the BstEII and EcoRI sites of an 81-bp XhoI-[P_{L_{56O-1}}]-EcoRI fragment from plasmid pZA31 (35).

Measurement of Frameshifting Frequency—Frameshifting was assessed using the pOFX302 reporter plasmid either by measuring β-galactosidase activity or by in vivo labeling of proteins with [35S]methionine (37).

Bacterial strains containing each plasmid construct were grown for 18 h at 37 °C in LB medium, and their LacZ activity of was measured by determining the rate of hydrolysis ortho-nitrophenyl-galactoside at 28 °C (34, 37). Four clones per strain were assayed on three consecutive days. The activity of mutant m1 (Fig. 2), in which the g10 and lacZ genes are in the same frame, was used as 100% to calculate absolute frameshifting frequencies. All of the results were normalized by setting as 1 the activity of the mutant used as wild type (denoted mutant m0).

Transposition Assays—Transposition frequencies of wild type IS3411 and of its three mutated derivatives were determined by a mating out assay as previously described (26).

Electrophoretic Mobility Shift Assay—DNA segments containing the SLI or SLII region were PCR-generated and transcribed with T7 RNA polymerase as recommended by Promega. DNA was eliminated by treatment with RQ1 DNase (Promega). The RNA was purified by gel filtration (MicroSpin G50 from GE Healthcare) and checked for purity and quantity on an 8% polyacrylamide gel in TBE buffer (100 mM Tris base, 83 mM boric acid, 1 mM Na2EDTA, pH 8.0; 28/1 acrylamide/bis-acrylamide ratio). SLI or SLII RNA was labeled with 100 μCi of [α-32P]UTP during the transcription reaction. The interaction between SLI and SLII was assayed by mixing a fixed amount of the [32P]RNA with different quantities of the other partner, in a final volume of 15 μl in TC buffer (10 mM HEPES, pH 7.3, 10 mM NaCl, 70 mM CH3COOK, and 1.5 mM [CH3COO]2Mg). Before mixing, each type of RNA was heated at 75 °C for 5 min and allowed to cool down to 20 °C over a 30-min period. After mixing, each sample was incubated 30 min at 22 °C and then analyzed by electrophoresis for 90 min at 80 V on 8% polyacrylamide gels (TBM buffer served for gel preparation and as running buffer; migration was carried out at 22 °C). The amount of radioactivity present in the gels was determined using phos-
Chemical Probing of RNA—The IS3411 frameshifting region was amplified by PCR with the sense primer OFD3475 (TAA TAC GAC TCA CTA TAG AGC) and the antisense primer OFD3536 (CTG CTCA CGC AGC TTA TCC), or with the sense primer OFD3475 and the antisense primer OFD3476 (CTG CTC AGC AGC TTA TCC), or with the sense primer OFD2463 (TAA TAC GAC TCA CTA TAG AGC TTA TCC) and the antisense primer OFD3476. To produce the DNA fragments containing either stem-loop I or stem-loop II, PCRs were performed on the same plasmid. To produce the DNA fragments containing the frameshift region, PCRs were performed on the same plasmid.

50 pmol of mRNA were renatured in 50 μl of buffer A (50 mM potassium cacodylate, pH 7.2, and 300 mM KCl) or buffer B (50 mM borate-KOH, 100 mM NH₄Cl) by warming at 90 °C for 1 min followed by incubation at 4 °C for 10 min. The magnesium concentration was then adjusted to 10 mM. Chemical probing of mRNA was performed on samples (10 pmol in 50 μl) by the addition of 1.5 μl of DMS (2:10 dilution in 95% ethanol), 3 μl of kethoxal (19 mg/ml in H₂O), or 20 μl of CMCT (84 mg/ml in H₂O). After incubation at 37 °C for 10 min, all of the modification reactions were stopped by the addition of 150 μl of 0.6 M potassium borate, pH 7.0. The pellets were resuspended in 20 μl of H₂O (for DMS and CMCT samples) or in 10 μl of 25 mM potassium borate for kethoxal-treated samples. Modified bases were detected by primer extension reactions with 5'-end-labeled oligonucleotides and reverse transcriptase (38). Fragments were separated on a 7 M urea, 8% (w/v) polyacrylamide gel. For lead mapping, a freshly prepared 40 mM lead acetate solution (pH 5.5) in H₂O was used. The reaction mixture (25 μM potassium borate, pH 7.0) was mixed. The lead-modified samples were adjusted to 25 mM potassium borate, pH 7.0. The pellets were resuspended in 10 μl of H₂O (for DMS and CMCT samples) or in 10 μl of 25 mM potassium borate for kethoxal-treated samples. Modified bases were detected by primer extension reactions with 5'-end-labeled oligonucleotides and reverse transcriptase (38). Fragments were separated on a 7 M urea, 8% (w/v) polyacrylamide gel. For lead mapping, a freshly prepared 40 mM lead acetate solution (pH 5.5) in H₂O was used. The reaction mixture (25 μM potassium borate, pH 7.0) was mixed.

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RESULTS

A Potential Elaborate Pseudoknot (ALIL-PK) Characteristic of the Frameshift Region within the ISS1 Group—Transposable elements of the IS3 family correspond to about 20% of all known ISSs. Its 354 members, found in all eubacterial phyla, were sorted into five groups by comparison of their OrfB protein (ISFinder data base) (40). Our own analysis revealed interesting features in 28 elements of one group, the ISS1 group (supplemental Table S1). The shift site is either U-UUU or U-UUC (U-UUU-UUC in three cases), which is in contrast with the rest of the IS3 family where A-AAA-AA(A/G) motifs are

FIGURE 1. Genetic organization of IS3411 and predicted secondary structure of its frameshift region. ir1 and irr, respectively, indicate the left and right terminal inverted repeats of the IS. The two partially overlapping genes orfA and orfB are in the 0 and −1 translational frames, respectively. The three proteins synthesized by the IS are represented by A, B, and AB. The region from nt 336 to 454 is the fragment cloned into the reporter plasmid and into which various mutations were introduced. The base pairing interaction between the apical loop (Loop 1) of the stem-loop I region (SLI) and the internal loop (Loop 3) of the stem-loop II element (SLII) leads to formation of a fourth stem, stem 2, and the ensemble constitutes what we called an ALIL pseudoknot.
largely predominant. It is separated from a first stem-loop (SLI) by a spacer sequence (sp1) generally 5 nt long. A second spacer (loop 2), whose length varies between 44 and 104 nucleotides, separates SLI from a second, more complex, potential stem-loop (SLII). Remarkably, a kissing loops type of interaction is possible between the apical loop of SLI and an internal loop of SLII (stem 2, in supplemental Table S1 and Fig. 1). The end result is an ALIL pseudoknot. The group can be separated into two subgroups according to phylogenetic origin: type 1 ISs are found in proteobacteria, whereas type 2 ISs originate from actinobacteria.

The sequence of five base pairs constituting the SLI stem, nearly exclusively composed of C and G, is highly conserved in type 1 ISs (5'-GGCGG-3') and less conserved among type 2 ISs. In a few IS the SLI stem contains four base pairs (ISRme15, ISNeu_1, ISAfa_1, IS1137, and IS994) or more than five base pairs (ISMysp3, ISMyna3, ISRru1, ISGwe_1). Except for IS986, IS1137, IS6110, and ISMyna3, in which the SLI loop contains eight nucleotides, most of the IS possess an apical loop of seven nucleotides whose sequence is well conserved (5'-A-[G/a]2-[C/a/g/a]1-[U/g]1-C-[G/a]2-A-[T/A]-3'). In all ISs except ISAfa_1 the junction between stem 1 and the paired region of loop 1 is an adenine.

The size of SLII varies from 38 to 63 nucleotides. The ISs that possess a longer loop 2 generally have a longer SLII region. This is the case for all type 2 ISs except ISTespl. In most ISs stem 3 at the base of SLII is composed of five to six paired bases (with extremes of 4 and 14 base pairs), which are mostly 5'-GC-3' pairs. An argument in favor of the proposed ALIL interaction is the observed covariation for the three nonstrictly conserved bp of stem 2; a substitution in the apical loop of SLI is always accompanied by a complementary variation in the internal loop of SLII; covariation also appears to operate in the case of stem 3. In the three ISs with an 8-nt apical loop, the PK could possibly be extended to 7 base pairs.

**Genetic Dissection of the Frameshift Region of IS3411 Provides Evidence for the Proposed ALIL-PK**—One insertion sequence, IS3411, was selected to determine experimentally whether the conserved structural elements identified by comparative analysis were indeed related to control of expression by translational frameshifting (Fig. 1). This IS was used because it was originally found in *E. coli* and shown to transpose in this organism (33). A recent study on a nearly identical element, IS629, showed that frameshifting is required for transposition (11). Intriguingly, expression of IS629 would give two transframe products by frameshifting either on the predicted U-UUU

![FIGURE 2. Genetic analysis of the IS3411 frameshift region. A, set of 30 mutants generated into the pOFX302 reporter plasmid (“Experimental Procedures” and B). The boxed nucleotides in the sequence were replaced by those indicated on the left. The PRF-1 frequency of the wild type (denoted mutant *m0*, which contains two mutations abolishing expression from the two consecutive AUG codons in frame 1) is 0.66%. Its activity was set as 1 and used to calculate the relative PRF-1 frequency of the others. B, protein expression profile from a selected set of the mutants from A. *isopropyl* β-D-thiogalactopyranoside-induced cultures were pulse-labeled with [35S]methionine, and total proteins were analyzed as described under “Experimental Procedures.” The size and position of the three expected proteins is indicated on the left. The vector plasmid (first lane) produces the G10 protein. The *m1* mutant (second lane) synthesizes only a G10-LacZ fusion similar to the one generated by PRF-1 (FS species). The *m4-5* clone (true wild type, third lane) allows synthesis of 3 plasmid-encoded proteins, the G10 and FS species plus the one resulting from initiation at the orfB AUG codons (IN). The fourth lane corresponds to the *m0* mutant, which synthesizes the G10 and FS proteins. The fifth construct (fifth lane) has a mutation in the slippery sequence and consequently produces only the G10 species. Sixth and seventh lanes, respectively, show the pattern generated by similar plasmid constructions containing the frameshifting signals of dnoX (24) and IS911 (26). The apparent frameshifting frequency for the *m0* mutant is about three times higher than obtained by dosage because for physiological reasons the value used as 100% (amount of radioactivity in the FS band for the *m1* in-frame mutant) is an under evaluation.
motif (OrfAB\textsubscript{629} transposase protein) or on an A-AAA motif in loop 2 (OrfAB\textsubscript{629} modulator protein). The OrfAB\textsubscript{629} protein would interact with and stabilize the OrfAB\textsubscript{H11032}/H11032 transposase, thus allowing a higher frequency of transposition. In the IS\textsubscript{629} study, the authors did not use the full-length frameshift region; the distal part of SLII starting three nucleotides 3\textsubscript{H11032} of stem 2 (nt 428–454) was omitted. This very likely affected quantitatively but probably not qualitatively the results from the expression analysis.

To assess the role of the various elements potentially involved in IS\textsubscript{3411} frameshifting (Fig. 1), mutations were introduced into the region from nt 336 to 454 cloned into a lacZ-based reporter plasmid (Fig. 2A). β-Galactosidase expression requires a 1 frameshift event or an initiation event in the 1 frame within the cloned region. The level of expression in the −1 frame was monitored by LacZ assay and by protein pulse-labeling for some mutants. As shown in Fig. 2B (third lane), the cloned wt fragment of IS\textsubscript{3411} (m4–5) indeed allows synthesis of the protein species expected from a 1 frameshifting event (FS band); however, the amount is much lower than for two other PRF-1 regions: dnaX (24) and IS\textsubscript{911} (26). A second protein is also synthesized (IN band); it results from an initiation event, in the −1 frame, on one of the two consecutive AUG codons overlapping the UGA stop in frame 0. Similar translational coupling was also demonstrated for IS\textsubscript{629} (11) and IS\textsubscript{3} (8). To make sure the LacZ activity reflects only frameshifting, we used as a basis the m0 mutant in which translational coupling is abolished (Fig. 2B, fourth lane). This mutant contains two other mutations in loop 2. Primarily intended for conservation of a potential stem-loop, these mutations have no significant effect on PRF-1 (see m5 versus m0), and no evidence was obtained for the potential structure within loop 2. According to folding programs, stems 1 and 3 could each be extended by an A-U pair at their basis (supplemental Table S1). However, no evidence could be obtained by genetics and probing for their existence. Consequently, they were not taken into account; spacers 1 and 2 were considered to be 5 and 45 nucleotides long, respectively.

The cloned IS\textsubscript{3411} segment leads to only 0.66% frameshifting (Fig. 2). Even if low, this level is significant and depends on the presence of the first PRF-1 motif, the U-UUU tetramer. Its replacement by the nonshifticr C-UUU sequence leads to a 6-fold...
reduction in activity (m2 and m2–3 mutants). In contrast, mutation of the second motif, from A-AAA to C-AAA, has very little or no detectable impact on PRF-1 (mutant m3). Thus, in our conditions where a segment of IS3411 longer than the one tested for IS629 (11) was analyzed, the U-UUU motif appeared to be the only efficient frameshift motif. As expected, substitution of this motif by the highly shifty A-AAA-AAG sequence (mutant m6), found in the dnaX and IS911 signals, resulted in a strong stimulation (6-fold) of PRF-1. The evaluation of IS629 frameshifting using another lacZ-based reporter system gave a value of 4.5% despite the fact that most of SLII was missing (11). The discrepancy probably originates from an under evaluation of the amount of LacZ from the in-frame plasmid in the IS629 study. We noted that full derepression of P_{lac}-type promoters carried by multicopy plasmids often lead to abortive translation and aggregation of LacZ for highly expressed constructions.

The U-UUU motif is not the sole determinant of frameshifting frequency. First, its position relative to the SLII region was shown to be important; a 5-nt spacing is three to four times more efficient than a spacing of 2, 8, or 11 nt (supplemental Fig. S1C). Then the implication of each of the four predicted stems was established by mutating one base pair (or two), first on one side or the other to disrupt Watson-Crick pairing and then on both sides to restore pairing (e.g. mutants m7, m8 and m9 in stem 1; Fig. 2). In all cases, frameshifting was reduced 2–3-fold for the disruption mutants and restored to nearly wt level for mutants reinstating pairing (in Fig. 2, mutants m7 to 9 for stem 1, m21 to 25 for stem 2, m27 to 30 for stem 3, and m16 to 18 for stem 4). In the case of stem 2, the m23 mutant harbors the perfectly matched sequence found in other ISs of the group (see supplemental Table S1). This modified version of stem 2 turned out to be a 3.2 times more efficient recoding stimulator. Starting with this mutant, we derived a series of ten stem 2 mutants with perfect Watson-Crick pairing in which only one base pair was changed (supplemental Fig. S1A). Consequently, PRF-1 frequency varies over a 7-fold range. This suggests that perfect pairing of 6 base pairs is not sufficient to ensure efficient PRF-1 stimulation but that the sequence itself plays a role. The size of loop 1 is also important; the addition of three nucleotides on one side or the other of the six paired nucleotides (mutants m14 and m15) or deletion of A356 (m13) reduces frameshifting severely. The effect of the identity of the unpaired nucleotide of loop 1 (nt 356) was determined; replacement by U or C is detrimental, but substitution by G is not (m10, m11, and m12). Increasing the length of stem 1 by 3 bp results in a notable reduction of PRF-1 (supplemental Fig. S1B, mutants m57 and m59).

FIGURE 4. Sensitivity to Pb^{2+} of the entire IS3411 frameshift region. The 5′ end of mRNA-0 was labeled with [γ-^32P]ATP and treated with the following concentrations of Pb^{2+}: 0.6 mM (lane 1), 1.25 mM (lane 2), 2.5 mM (lane 3), and 5.0 mM (lane 4). A control without Pb^{2+} treatment is presented in lane T. The sites of cleavage were identified by comparison with a ladder of G created by hydrolysis with ribonuclease T1 (lane T1). C, summary of the cleavage sites (arrow) within the IS3411 frameshift signal. For simplification, the nucleotides were renumbered, and nt 343 of the IS becomes the +1 position.

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The architecture of the SLII region is also constrained. The insertion of three nucleotides between stems 2 and 3 results in a 5-fold reduction (m26). The series of stem 4 modifications suggests that a stem-loop, even a short one, is absolutely required to obtain PRF-1 stimulation at the wt level (supplemental Fig. S1D). On the other hand, increasing the size of stem 3 by 3 bp augments frameshifting nearly 2-fold, provided stem 1 is not simultaneously altered (supplemental Fig. S1B, mutants m58 and m59).

To conclude, the genetics approach carried out on IS3411 strongly support the four-stem structure deduced from alignment and secondary structure prediction of frameshift regions from the IS57 group. In addition, several spatial constraints were revealed which suggest that the six nucleotides from loop 1 and their six pairing partners from loop 3 have to be properly positioned to interact and form a structure capable of interfering with the ribosome.

**Formation of an ALIL-PK Revealed by Chemical Probing of the IS3411 Frameshift Region**—We investigated the conformation of an RNA molecule, mRNA-0, containing the entire frameshift region of IS3411. It contains an IS segment from nt 336 to 454, plus a few nucleotides from the pOFX302 vector on both sides. The nucleotides were renumbered; the +1 position corresponds to nt 343 of IS3411 (Fig. 3). The analysis was carried out with DMS, which reacts with the N1 position of adenines and the N3 position of cytosines, with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds.

To conclude, the genetics approach carried out on IS3411 strongly support the four-stem structure deduced from alignment and secondary structure prediction of frameshift regions from the IS57 group. In addition, several spatial constraints were revealed which suggest that the six nucleotides from loop 1 and their six pairing partners from loop 3 have to be properly positioned to interact and form a structure capable of interfering with the ribosome.

**Formation of an ALIL-PK Revealed by Chemical Probing of the IS3411 Frameshift Region**—We investigated the conformation of an RNA molecule, mRNA-0, containing the entire frameshift region of IS3411. It contains an IS segment from nt 336 to 454, plus a few nucleotides from the pOFX302 vector on both sides. The nucleotides were renumbered; the +1 position corresponds to nt 343 of IS3411 (Fig. 3). The analysis was carried out with DMS, which reacts with the N1 position of adenines and the N3 position of cytosines, with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds. Probing data suggest that nucleotides 9–25 of positions of uridines, when those positions are not engaged in CMCT, which modifies the N1 position of guanines and the N3 position of cytosines, and with kethoxal, which reacts with the N1 and N2 positions of guanines, and with CMCT, which modifies the N1 position of guanines and the N3 positions of uridines, when those positions are not engaged in hydrogen bonds. Probing data suggest that nucleotides 9–25 of SLI fold into a short stem-loop because G9, G10, G12, and G13 hydrogen bonds.

**Figure 5. Electrophoretic mobility shift assay of the SLI/SLII ALIL complex.** A, magnesium is required to obtain a stable complex. 50 fmol of radiolabeled SLII mRNA was incubated without (lanes 1 and 3) or with (lanes 2 and 4) 300 fmol of nonlabeled SLII mRNA in the absence (lane 1 and 2; Mg2+ replaced by 1 mM EDTA in the buffer) or in the presence of 3 mM Mg2+ (lane 3 and 4). B, determination of the apparent Kd for an optimized ALIL interaction (m23 mutant). Radiolabeled SLII mRNA was incubated with an increasing amount of nonlabeled SLII mRNA RNA (2-fold increase per step). C, determination of the apparent Kd for an wild type (wt) ALIL interaction (m23 mutant). Radiolabeled SLII mRNA RNA was incubated with an increasing amount of nonlabeled SLII mRNA RNA.

The bases of the internal loop (loop 3) separating on the 5’ side the lower and upper stems are only weakly accessible to the probes. Lead cleavage confirms the probing data; nucleo-
mRNA-I suggests that nucleotides 9–25, like in the longer transcript (SLII region). As shown in supplemental Fig. S2, probing of the reactivity of stem 3 guanines (G72 to G75) to kethoxal is very decreased from the probes. The reactivity of the loop 1 nucleotides protected from the probes. The reactivity of the loop 1 nucleotides to kethoxal, and CMCT, whereas C18, G15, and A14 are more protected from the probes. The reactivity of the loop 1 nucleotides is therefore increased in the shorter transcript. The probing of stem 4 (G91, G88, U87, A86, U85, A100, G98, and U97). In loop 4, the N1 positions of two adenine bases (A94 and A95) as well as the N1 and N2 positions of G93 are very reactive, but the N3 position of C92 is more protected against DMS. A80, C77, and C81 in the loop 3 bulge are accessible to DMS as G78, G79, G83, U76, U103, and U104 are to kethoxal and CMCT.

Taken together, these results are consistent with the formation in solution of the ALIL-PK by base pairing between the apical loop 1 and the internal loop 3. An interesting aspect is that in the absence of SLI, we observe a global destabilization of the SLII structure, suggesting that formation of stem 2, by the loop 1-loop 3 interaction, has an overall stabilizing effect.

Formation of an SLI-SLII Complex in Vitro and in Vivo—The length of loop 2 and the fact that it contains no conserved structure suggested that it was dispensable for SLI-SLII interaction. We therefore anticipated the formation in vitro of a stable SLI-SLII complex by mixing short RNA molecules containing one or the other region. As shown in Fig. 6, this is indeed the case; when 33P-labeled SLI RNA was incubated with unlabeled SLI RNA (or the other way round) and separated by electrophoresis on a nondenaturing polyacrylamide gel, a retarded band was observed provided Mg2+ was present (Fig. 6A). A quantitative analysis was carried out using mutants in the four stems of the IS3411 structure. Fig. 5 (B–D) illustrates the case of stem 2. Having the wt sequence on both sides led to formation of a complex at 22 °C with an apparent Kd of 3035 nM, whereas the m23 sequence increased about 60-fold the SLI-SLII affinity (apparent Kd of 48 nM). When stem 2 was destabilized (wt sequence in SLI and m25 sequence in SLII), no complex was observed within the range of SLII concentrations used. Similar results were obtained for the three other stems. From this, we conclude that SLI-SLII trans complex formation relies on the same structural elements as frameshift stimulation. In contrast with the probing experiments, detection of complexes by electrophoretic mobility shift assay necessitates the constant presence of magnesium. This suggests that although complexes can be formed in an Mg2+-independent manner in solution, this cation is essential for their stability during electrophoresis; similar observations were obtained in the case of other kissing loop complexes (41).

The high affinity of SLI and SLII displayed in vitro by the m23 mutant prompted us to test whether SLII m23 was also capable of acting in trans on SLI m23 when provided in vivo. The SLI m23 region was cloned into the pOFX302 reporter plasmid, and SLII m23 was inserted into a compatible plasmid under control of a Pkm23 promoter; the noninteracting SLII m25 mutant was similarly cloned to test whether SLII m25 was derepressed, whereas it remained unaffected when SLII m25 was expressed. Thus, SLII m23 RNA can act in trans on its normal target, the SLI m23 RNA embedded in a 4-kb-long mRNA. However, to obtain this effect, the SLII RNA had to be inserted into the anticodon loop of a tRNA, presumably to provide a stabilizing environment.

**FIGURE 6. Trans-activity of the SLII RNA in vivo.** A, cloning of the SLI and SLII RNAs into two compatible plasmids. The SLI m23 region was cloned into the pOFX302 reporter plasmid (left), and the SLII m23 RNA was inserted into a compatible plasmid under control of an inducible Pm23 promoter (right). The noninteracting SLII m25 mutant was similarly cloned to test the effect of the disruption of the ALIL interaction. The two types of SLII RNA were themselves inserted into the anticodon loop of an RNA identical to the E. coli lysine-tRNA. Note that short sequences not shown on the figure are also present at the 3′ and 3′ ends of the tRNA-like sequence; GCAGAUAUGUCGGAGAUAC at the 5′ end and CUUGCUAUUCUAGCGAAAGCUAAGGAUUUUUUUU at the 3′ end (the latter correspond to the mnt terminator region where transcription stop somewhere within the run of 8 U). B, frameshifting frequency measured in the absence or presence of an inducer of the P m23 promoter.
Destabilization of Stem 2 and Stem 3 Decreases the in Vivo Transposition Activity of IS3411—PRF-1 was shown to be required for synthesis of the OrfAB transposase in a few members of the IS3 family (8, 11, 30). To determine whether it is the case for IS3411, we introduced mutations affecting frameshifting negatively while not changing the sequence of the OrfA and OrfB proteins; mutation U342C renders the U-UUU motif non-shifty (m2 in Fig. 2), mutation G416A destabilizes stem 3 (m27 in Fig. 2), and mutation C419A destabilizes stem 2 (m24 in Fig. 2). These mutations were transferred into the IS3411 copy carried by plasmid pOFX515 (Fig. 7A). In this plasmid, the right end of the IS (irr) is followed, at a distance of 3 base pairs, by a copy of the left end of the IS (irl). This creates an irr-irl junction that is an efficient substrate for the OrfAB transposase (42), thus leading to integration of pOFX515 into the pOX38Kan target plasmid at high frequency (Fig. 7B). When frameshifting is prevented by mutating the shifty motif, there is a dramatic 2500-fold reduction in cointegrate formation; this is the background frequency of recombination observed with an IS-less plasmid. If frameshifting is made less efficient by destabilizing the ALIL-PK with mutations in either stem 2 or stem 3, the reduction is still important because it is 250-fold. These results suggest that frameshifting frequency and transposition level are directly correlated.

DISCUSSION

The ALIL-PK, a Moderate but Essential Stimulator of Frameshifting and Transposition—The elaborate pseudoknot of IS3411, which also exists in 27 other ISs, acts on the ribosome to increase its frequency of slippage on the U-UUU sequence located 5 nt upstream (Fig. 1 and supplemental Table S1). The resulting OrfAB protein is absolutely required for transposition (Fig. 7) (11). Surprisingly, such a sophisticated structure as the IS3411 ALIL-PK leads to a rather inefficient recoding signal. Only 0.55% of the ribosomes change frame on the U-UUU motif, after deduction of 0.11% corresponding to the background activity of mutants without frameshift motif (Fig. 2). However, such a low level of frameshifting does not prevent IS3411 from being active in transposition (Fig. 7) (11, 33). Like other transposable elements, IS3411 evolved to produce the amount of transposase necessary to maintain transposition frequency at a level ensuring propagation while not generating too many mutations of the bacterial host. To achieve this goal an IS could either produce a relatively large amount of a low efficiency transposase or synthesize a small quantity of a more efficient one. In the IS3 family, IS150, with its PRF-1 frequency of 50%, is an example of the former strategy (25), whereas IS3411 constitutes an illustration of the latter. The intracellular concentration of the OrfABIS3411 transposase is controlled at the translational and post-translational levels. A low amount of OrfAB synthesis results from the use of a moderately efficient slippery motif (U-UUU) is 5.4 times less efficient than A-AAA-

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**TABLE 7.** Transposition frequency

| Plasmid     | Transposition frequency |
|-------------|-------------------------|
| wt          | 22.600 ± 6.300 (× 10⁻⁵) |
| U342C       | 0.009 ± 0.004 (× 10⁻⁵)  |
| G416A       | 0.086 ± 0.025 (× 10⁻⁵)  |
| C419A       | 0.107 ± 0.044 (× 10⁻⁵)  |

The transposition frequency was calculated by dividing the number of recipient bacteria that received a transposition-generated pOX38:pOFX515 plasmid (kanamycin- and ampicillin-resistant clones) by the number of bacteria that received pOX38 or pOX38:pOFX515 (kanamycin-resistant clones). wt, wild type.
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AAG; see m6 mutant in Fig. 2) combined to an intrinsically inefficient frameshift stimulator. Even if stem 2 is improved, as illustrated by the m23 mutant, the stem 2 of which is identical with that of many other members of the IS51 group (supplementary Table S1), the efficiency of frameshifting remains moderate (Fig. 2). The intricate design of the ALIL-PK is perhaps what limits its capacity to interfere with ribosome progression (either because of a loose direct interaction and/or because *in vivo* its formation is impaired by ribosome traffic?). If so, it would appear as an unnecessarily convoluted way to proceed because a regular short stem-loop of the same efficiency would have been simpler to establish. An interesting alternative is that the ALIL-PK evolved for a different purpose; whether or not it is to adjust transposase expression (hence transposition) to cell physiology remains to be investigated. In addition, once synthesized, the OrfAB protein is probably rather unstable as shown by the analysis of IS629 (11). The same study suggested synthesis through frameshifting on a second slippery motif (A-AAA in loop 2; Fig. 1) of an alternate OrfAB protein differing by a few amino-acids. It has no transposase activity but is capable of kissing loops to generate and stabilize a relatively rigid ALIL-PK, as suggested by the modeling analysis (Fig. 8 and supplemental Fig. S2). This similarity maybe related to the necessity of obtaining in both cases a rapid interaction by the proper presentation of two short particular sequences. One unpaired base is also present in loop 1 of IS3411, but none exists in the other loop (loop 3), which is instead closed by a stem on its 3′ side (stem 4). This difference in design of the second partner is probably due to a difference in the fate of the dimerization initiation site and ALIL initial kissing complexes. Whereas the former is stabilized by extending the number of paired nucleotides, such an option does not exists for the latter. IS3411 uses the three helices flanking the kissing loops to generate and stabilize a relatively rigid ALIL-PK, as suggested by the modeling analysis (Fig. 8 and supplemental Fig. S3 and below).

RNA aptamers (30–40 nt in size) binding to one or the other of three RNA hairpins present at the 5′- and 3′-untranslated regions of the hepatitis C virus mRNA were selected (31, 46). Interestingly, with the three targets, the aptamers with the strongest binding turned out to be stem-loops quite similar to the hepatitis C virus ALIL complexes having one unpaired nucleotide on the 5′ side (stem 4). At variance with the IS, the internal loop is on the 3′ side and contains one or three unpaired nucleotides. In two cases, unpaired nucleotides (one or three) are also present on the 5′ side of the aptamer stem, across the internal loop. The exact significance of these differences remains to be determined. One possibility is that the aptamers were selected from random sequences, whereas the IS structure had to emerge (by slow reciprocal adjustment of the two partners through natural evolution) in a more constrained background because it is embedded within a

![FIGURE 8. Molecular models of the ALIL pseudoknot of IS3411. A, cylinder representation of the Md1 structure, a model in which stacking of stem 4 over stem 2 has been maximized. B, ribbon drawing of the same Md1 model; all base pairs closing the four stems are shown.](image-url)
coding sequence (even coding in two frames for a part of it). The end result is the formation of complexes of similar stability with apparent Kd in the nanomolar range (at least for optimized stem 2, like in the m23 mutant; see Fig. 5).

The biological importance of ALIL RNA complexes is substantiated by several examples such as the Drosophila bicoid mRNA dimerization signal (47, 48), the bacteriophage phi29 pRNA multimerization (49), and the loop-loop interaction in the 5’-untranslated region of the avian leukemia virus (50). However, the case most akin to the IS3411 and the in vitro selected ALIL complexes is the frameshift stimulator of the Barley yellow dwarf virus (32). It is constituted by a 6-nt internal loop present on the 3’ side of a stem-loop situated 6 nt downstream of a G-GGU-UUU shift site that interacts with an apical loop located 4 kb downstream in the 3’-untranslated region. Thus, there is an inversion in the positions of the SL1 and SLII equivalents, and like in the in vitro selected ALIL complexes (31), the internal loop is on the 3’ side rather than on the 5’ side. Even if the size, design and distance of the two interacting stem-loops differ from their IS3411 counterparts, some features are nevertheless the same. The Barley yellow dwarf virus loop 1 homolog is also 7 nt long, the unpaired base is also an adenine on the 5’ side and the long range interaction between the two loops implicates 6 base pairs, as well. In addition, the internal loop does not contain unpaired bases and is flanked by two stems, which are longer versions of stems 3 and 4 of IS3411. However, the final structure is going to be presented to the ribosome in a different manner because the stem 3 homolog is on the 5’ side of the mRNA, and the stem 1 homolog is on the 3’ side. This led to a 10-fold stimulation of PRF-1 frequency (compared with a mutant where the loop-loop interaction is disrupted), which attains 1.1%; this moderate level of recoding is similar to the mutant where the loop-loop interaction is disrupted, which is also 7 nt long, the unpaired base is also an adenine on the 5’ side. Perhaps because it restricts their mobility. In model Md1 base stacking between stem 4 and stem 2 has been maximized. Supplementary Fig. S3 shows its superposition with two other structures in which stacking of stems 2 and 4 has been minimized. The tip of stem 4 can be displaced by 2.6 nm at most, the angle between stem 2 and stem 4 increases from 14 to 50°, and the 5’ end of stem 3 moves on one side or the other by about 1.5 nm. A feature of the structure, and what perhaps makes it able to impede ribosome progression, may therefore be its relatively important rigidity. As shown in Fig. 8B, it is conferred by an interlinked network of 4 base pairs acting as restraining struts, namely the ones that close stem 2 (A-U top and G-C bottom), stem 3 (G-C top), and stem 4 (C-G bottom).

To conclude, it appears that the insertion sequences of the IS51 group evolved a quite remarkable RNA structure ensuring a strong interaction of two separate stem-loops present within the coding part of their mRNA. This lends further support to the notion that apical loop-internal loop complexes constitute a mode on their own of intra- and intermolecular RNA interaction and as such are probably widely used for control and structural purposes.

Acknowledgments—We thank Dr. Agamemnon Carpoassios for critical reading of the manuscript and Dr. John Atkins for the same courtesy as well as for many enthusiastic and stimulating discussions.

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