The Left End of IS2: A Compromise between Transpositional Activity and an Essential Promoter Function That Regulates the Transposition Pathway

Leslie A. Lewis,¹,²* Edruche Cylin,¹ Ho Kyung Lee,¹ Robert Saby,¹ Wilson Wong,³ † and Nigel D. F. Grindley³

Department of Biology, York College of the City University of New York, Jamaica, New York 11451; Program in Cellular, Molecular and Developmental Biology, Graduate Center, City University of New York, New York, New York 11016; and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

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Cut-and-paste (simple insertion) and replicative transposition pathways are the two classical paradigms by which transposable elements are mobilized. A novel variation of cut and paste, a two-step transposition cycle, has recently been proposed for insertion sequences of the IS family. In IS2 this variation involves the formation of a circular, putative transposition intermediate (the minicircle) in the first step. Two aspects of the minicircle may involve its proposed role in the second step (integration into the target). The first is the presence of a highly reactive junction formed by the two abutted ends of the element. The second is the assembly at the minicircle junction of a strong hybrid promoter which generates higher levels of transposase. In this report we show that IS2 possesses a highly reactive minicircle junction at which a strong promoter is assembled and that the promoter is needed for the efficient completion of the pathway. We show that the sequence diversions which characterize the imperfect inverted repeats or ends of this element have evolved specifically to permit the formation and optimal function of this promoter. While these sequence diversions eliminate catalytic activity of the left end (IRL) in the linear element, sufficient sequence information essential for catalysis is retained by the IRL in the context of the minicircle junction. These data confirm that the minicircle is an essential intermediate in the two-step transposition pathway of IS2.

IS2 is a 1.3-kb transposable element which belongs to the IS family of insertion sequences, one of the largest of about 19 families of this group of mobile elements (3). Extensive studies of IS911 (5, 18, 26, 34), confirmed with IS2 (14, 15), have shown that transposition of IS-family elements occurs by a variation of the cut-and-paste pathway, which involves the formation of novel intermediates—first, a figure eight and subsequently an IS minicircle (36) (Fig. 1A). Formation of the figure eight results from the transposase-mediated cleavage of one IS 3’ end and the strand transfer of this end to a site on the same DNA strand a few nucleotides outside the other IS end. Replication and/or host repair systems convert the figure eight into an IS minicircle (and regenerate the initial replicon with the parental IS). The junction formed by the abutted IS ends in the minicircle, e.g., in IS50, IS3, and IS911, is a transpositionally hyperactive substrate (7, 29, 34). In this configuration, both IS ends are readily nicked by the transposase, forming a linear IS with free 3'-OH ends that can insert into a new DNA target in the manner of other cut-and-paste transposons such as Tn10 or Tn5 (25, 27). In several cases it has also been shown that formation of the IS end-to-end junction creates a novel hybrid promoter, composed of elements from both the right and left IS ends, that results in increased expression of transposase (5, 34). The presence of this promoter in IS911 improves the chance of reintegrating the excised IS before the nonreplicating minicircle is diluted from the dividing cells (5). Transpositionally reactive junctions, either in circles or in linear dimers, have also been reported for elements in families other than IS, e.g., IS2 (22–24) IS30 (4, 13, 16), IS1 (31, 35), and IS186 (33). Some but not all of these junctions or those formed by IS7 family members are associated with the formation of hybrid promoters (5), and a generic role for a stronger promoter in their transposition pathways remains unclear.

IS2 is a typical IS3 family member, both in its mechanism of transposition (15) and in the formation of a junction promoter (32). However, while IS911 and other typical IS3-family elements, e.g., IS50 and IS3 (7, 30), use each IS end equally well as the donor or target in the initial cleavage and strand transfer step, IS2 uses only its right end, IRR, as the donor and its left end, IRL, as the target. Furthermore, even as targets, IRL and IRR (which can act as a target if it is artificially paired with an IRR donor) differ from one another—targeting IRL results in a 1- or 2-bp spacer between the abutted IS ends in the minicircle, whereas targeting IRR results in a 2- or 3-bp spacer (14).

We have previously shown that the distinct target characteristics of IRL and its inability to act as a donor in the initial step of transposition result largely from two DNA sequence differences between IRL and IRR—an extra base pair between the conserved transposase binding sequences and the IRL terminus and a change of the terminal dinucleotide from the CA-3’ typical of all IS3 family members to TA-3’ (14) (Fig. 1B). We have hypothesized that these differences between IRL and
IRR (and the asymmetry of the initial strand transfer event that results from them) have been selected because they optimize the minicircle junction promoter, $P_{\text{junc}}$ (Fig. 1C). Here, we have further characterized $P_{\text{junc}}$, shown that its activity indeed depends on these two sequence differences, and demonstrated that $P_{\text{junc}}$ activity is essential for the efficient transposition of IS2. In addition we show that IRL has retained sufficient critical terminal sequences to function in catalysis of minicircle insertion reactions, despite its inactivity as a donor in minicircle formation.

MATERIALS AND METHODS

Bacterial strains and media. The strains of *Escherichia coli* used in these experiments were essentially as previously described (14, 15).

IS2 constructs. (i) Wild-type and mutant linear IS2 elements. pLL17 and pLL18, elements with the frame-fused orfAB sequence, have been described in detail (15), as have pLL40 (with the minitransposon in which the orfA and orfB sequences have been replaced by a Kan resistance gene), pLL44 (containing the wild-type element with its native overlapping orfA and orfB genes), and pLL49 (with the end-less orfAB frame-fused gene in a pACYC vector). (ii) IS2 elements with mutations of the left-end terminal dinucleotide. These constructs, pLL46, pLL50, pLL80-84, and pLL86-93 (see Table 4), were created by mutating the left end of pLL18, which has the frame-fused orfAB gene as described earlier (14).

(iii) lacZ fusion constructs. The lacZ gene from pSV-β-galactosidase (Promega Corp.) was cloned into pUC19 as a HindIII-BamHI fragment. An Xhol NcoI cassette was created by PCR site-directed mutagenesis at the HindIII end of the fragment, with the NcoI site corresponding to the translational start site of the lacZ gene (pLL132). Promoters were created by in vitro site-directed PCR mutagenesis and cloned into the cassette. The 3' ends of the promoters were created with the primer NCOI, which contained the NcoI site and the ribosomal binding site. The lac operator sequence was also included in this primer (1). The sequence was 5'-CATGTGCCATGGTTCCTCC[AATTTGAGAGGGA TAAACATTTCCAAGAGATGC]-3'. The NcoI and ribosomal binding site sequences are in bold, and lacO is shown in brackets. The 5' end of each promoter was created using a primer that contained an Xhol site; the primer varied with the specific lacZ fusion construct created. For pLL148, which contained the hexamers of the lacUV5' promoter, this primer was 5'-CTTAAGTGA TCTGAGATCTGTATATAGCCGCAAAATCCACATATATGCGGCCC CGTAAT-3'. The Xhol site and the $P_{\text{junc}}^{-35}$ and $P_{\text{junc}}^{-10}$ hexamers are shown in bold. The DNA template used in the PCR protocol to create this promoter was a cloned minicircle with a wild-type minicircle junction formed from the abutted IRL$^{TA}$ and IRR$^{CA}$ ends separated by a 1-bp spacer (pLL186). To create

FIG. 1. (A) The two-step transposition pathway of IS2, showing formation of figure eight (i) and minicircle (ii) intermediates and the second step or insertion reaction (iii). (i) Asymmetric single-stranded cleavage of the active IRR donor end and its intramolecular strand transfer to the inactive target end (IRL) creates a figure eight structure. (ii) DNA replication or host repair systems resolve the figure eight and produce the excised minicircle intermediate. (iii) In the second step the minicircle junction is the substrate for the IS2 transposase (Tnp) produced by a strong junction promoter, $P_{\text{junc}}$ (see below), which provides the levels of Tnp needed for the cleavage and strand transfer reactions. Both IRR and IRL, which function as active donors, are cleaved at the minicircle junction and participate in intermolecular strand transfer to the target. (B) Aligned sequences of the IRR and IRL of IS2. IRR (red, upper sequence) is 41 bp long and IR (blue, lower) is 42 bp. Sequences common to both ends are shown as large uppercase letters. Diverged sequences are in lowercase. The Tnp binding domain is indicated in yellow as sequences 10 to 41 for IRR and 11 to 42 for IRL. The basis for the asymmetry of the first-step reaction (14) and for the mechanistic aspects of the second step (this report) are dictated by the divergences in length, sequence, and the terminal dinucleotides seen in the catalytic domain of IRL (blue) relative to IRR (red). (C) Sequence of the IS2 minicircle junction showing the role of the terminal hexamer of the catalytic domain of IRL as the $-10$ motif of the minicircle junction promoter ($P_{\text{junc}}$). The outwardly directed $-35$ hexamer in IRR contributes to the formation of this promoter.
TABLE 1. Promoter strength assayed by β-galactosidase expression from lacZ fusions

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Plasmid</th>
<th>Promoter sequences</th>
<th>β-galactosidase Miller units (±SE)</th>
<th>n*</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>pLL132</td>
<td>TTTACAAGAGCTAGATATATAAT</td>
<td>517.5 (22.5)</td>
<td>7</td>
<td>109.9</td>
</tr>
<tr>
<td>P\text{lacUV5}</td>
<td>pLL148</td>
<td>ATATACAGAGCTAGATATATAAT</td>
<td>34.5 (4.1)</td>
<td>30</td>
<td>0.7</td>
</tr>
<tr>
<td>P\text{IRL}</td>
<td>pLL135</td>
<td>ATATACAGAGCTAGATATATAAT</td>
<td>470.7 (34.9)</td>
<td>15</td>
<td>100.0</td>
</tr>
<tr>
<td>P\text{punc} with 1-bp mcj spacer</td>
<td>pLL136</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>35.6 (9.4)</td>
<td>6</td>
<td>7.6</td>
</tr>
<tr>
<td>P\text{punc} with 2-bp mcj spacer</td>
<td>pLL144</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>45.7 (5.1)</td>
<td>30</td>
<td>9.7</td>
</tr>
<tr>
<td>Mutated P\text{punc} (IRL\text{CA})</td>
<td>pLL143</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>31.3 (1.8)</td>
<td>3</td>
<td>6.6</td>
</tr>
<tr>
<td>Mutated P\text{punc} (IRL\text{TG})</td>
<td>pLL138</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>12.1 (1.6)</td>
<td>6</td>
<td>2.6</td>
</tr>
<tr>
<td>P\text{punc} with 3-bp mcj spacer</td>
<td>pLL145</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>26.9 (6.4)</td>
<td>6</td>
<td>5.7</td>
</tr>
<tr>
<td>P\text{punc} with 6-bp mcj spacer</td>
<td>pLL146</td>
<td>TGGAAGAGCT[ TAAGCTAGACT]</td>
<td>3.0 (1.8)</td>
<td>30</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a Letters in bold identify the substitution mutations in the −10 hexamer of P\text{punc} Square brackets identify the outside ends of IRR (on the left) and IRL at the minicircle junction (mcj); between them, the mcj spacer is shown in lowercase. −10/−35 spacer sizes are listed beneath the hexamers.

b n = number of experiments. Each experiment consisted of the reading from an independently isolated colony. The standard error is of the mean of n experiments.

c Relative to P\text{punc} on pLL136.

P\text{IRL}, the indigenous IS2 promoter in the left end (pLL135), pLL17 was used as the DNA template and a primer with an XhoI site which hybridized upstream of the left end of IS2 was employed: 5′-CTGGCGAAAGGGGCTCGAG-CTATTA-3′. For wild-type and mutant junction promoters, plasmids (pLL136, -138, and -143), a primer containing the IRR sequence which hybridized just upstream of the A site was used. For pLL186, the DNA template was pLL186; for p\text{punc} with the mutant −10 hexamer TGGACT (pLL143), the DNA template was a cloned minicircle isolated from pLL46, the construct with the IRL\text{CA} mutation; for P\text{punc} with the mutant −10 hexamer CAGACT (pLL143), the DNA template was a cloned minicircle isolated from pLL46, the construct with the IRL\text{TG} mutation; and for P\text{punc} with the mutant −10 hexamer CAGACT (pLL138), the cloned minicircle isolated from pLL50, the construct with the IRL\text{TG} mutation was used. Junction promoters with 18 bp (pLL144), 19 bp (pLL145), and 22 bp (pLL146) between the −35 and −10 hexamers (Table 1) were created with a primer composed of the minicircle junction sequence containing 2, 3, or 6 bp in the minicircle junction spacer. For example, the sequence with a 2-bp spacer was 5′-CTTAAGTCAGAGCTGGCGAAATAGTTCGACCATACCACTAGTCAGACCGGCCCTGATTTCAATCC-3′. The abutted ends of IS2 are identified by brackets. The XhoI sequence and the −35 and −10 P\text{IRL} hexamers are shown in bold.

(iv) Constructs designed to test the relative efficiencies of P\text{punc} and P\text{IRL} promoters in promoting autonomous transposition in IS2. pLL440 was created by replacing the wild-type left end of IS2 (IRL\text{TG}), an EcoRI-Aval fragment of pLL4 (15), with the mutant left end (IRL\text{CA}) of pLL46.

(v) Constructs with cloned minicircle junctions. The creation of constructs with closed minicircle junctions, which is achieved by cloning BstEII-digested minicircles into the Avai site of pUC19, has been previously described in detail (14). Cloned minicircle constructs pLL181 and pLL460 (see Table 5) were derived from minicircles produced by pLL18 and pLL46 respectively, which possess the frame-merged orfAB gene. Cloning of their minicircles dispersed the orfAB gene but left orfA intact. Cloned minicircles in constructs pLL1834, -1835, -1859, and -1860 (see Table 5) were generated from elements created by mutation of the left end of pLL18 (see Table 4). It should be noted that all of these constructs, with the exception of pLL181, lacked a functional minicircle junction promoter. Constructs identified as components of the plasmid series and the plasmid series (see Table 3) were derived from minicircles produced by the minitransposons pLL40 and pLL48 (14), respectively, from which the orfA and orfB genes were deleted. Cloned minicircles of the 400 series were pLL407, -408, -412, and -413. Those of the 600 series were pLL602, -605, -608, and -609. The constructs identified as pLL538, -610, -617, and -619 were also derived from minicircles produced by pLL48.

β-Galactosidase assays. lacZ fusion constructs were transformed into the JM105 strain of E. coli and grown overnight in Luria-Bertani broth plus 300 μM isopropyl-β-D-thiogalactopyranoside. Overnight cultures were diluted 1:50 into the same medium and grown to an optical density of 0.4 to 0.5. One-milliliter aliquots were assayed for β-galactosidase as described by Sambrook et al. (28).

Primer extension reactions. Total RNA preparations were made from 20-ml log-phase cultures grown to an optical density of 0.2 to 0.3. RNA was prepared using a Qiagen RNeasy kit combined with the RNAsprint stabilization solution (Qiagen Inc.) RNA concentrations of 2 to 4 μg/ml were obtained in 40-μl preparations. mRNA was enriched from total RNA preparations with the MICROBExpress kit from Ambion Inc. Preparations started with 15 μg of total RNA yielding on average 4.5 μg of mRNA in a 20-μl preparation. Concentrations were determined spectrophotometrically at 260 nm, and purity was assessed from 260-nm/280-nm optical density ratios, which gave a value of 2.0, and by analysis on formaldehyde—1% agarose gels. Primer extension reactions utilized the Omnistrone reverse transcriptase kit of Qiagen Inc. With their protocol we used 1.6 pmol of a 5′ end 32P-labeled primer, specific to the lacZ gene for use with lacZ fusions such as pLL136, and 1.25 pmol of the RNA template in a 20-μl reaction mixture. Eighteen microliters of stop Buffer (U.S. Biochemical) was added to the mixture at the end of the reaction, and the whole was dried down to 8.0 μl. Two microliters of the reaction mixture was loaded onto a denaturing polyacrylamide gel along with a control sequencing reaction mixture which used the same primer employed in the primer extension reaction but without the 5′-end-labeled PO4 .32 . Trial runs of oligonucleotides with and without the 5′ PO4 indicated that those with the phosphate migrate one nucleotide faster than those without it, presumably because of the presence of the extra charge. This adjustment was made in analyzing the data shown in Fig. 2. Quantification of the runoff products was carried out on a Storm 840 PhosphorImager (Molecular Dynamics, Inc.).

Transposition assays. Overall transposition frequencies were determined using a mating-out assay (15). This was based on the transfer of the mini-F-factor pCF105 (11) from a RecA strain (LL235) to a nalidixic acid-resistant F - strain (14R525). For autonomous transposition the RecA strain also contained the IS2 construct cloned into a pUC19 plasmid. For nonautonomous transposition with constructs containing cloned minicircles, the frame-fused transposase OrfAB was provided by the addition of the compatible pACYC derivative pLL49 to the F - strain (15).

DNA manipulations. In vitro site-directed PCR-based mutagenesis, PCR protocols, DNA sequencing, plasmid DNA preparations, cloning reactions, and the specific cloning of minicircles were all carried out essentially as described previously (14, 15).

RESULTS

Analysis of P\text{punc}, the promoter that spans the IS2 minicircle junction. To analyze the two proposed IS2 promoters, we made promoter-lacZ reporter gene constructs in pUC19. We determined the precise locations of the P\text{punc} and P\text{IRL} tran-
scription start sites by using a primer extension assay with a 5'-end 32P-labeled primer annealed about 90 bases downstream of the candidate Pjunc promoter (Pjunc) is shown in lines 1 and 4 (wild type) and lines 3 and 5 (mutated) with their transcriptional start sites. The indigenous wild type promoter (PIRL) is shown in line 2. * and †, primary and secondary transcriptional start sites, respectively. IRR sequences are in black, and IRL sequences are in red. Square brackets indicate the outside termini of IRR and IRL. The curved bracket in line 2 identifies the inside end of IRL (base 42). The red (positions 1 to 20 and 21 to 42) and the purple (positions 43 to 79) sequences in lines 1 and 2 are contiguous and show Pjunc and PIRL 25 bp apart in the minicircle. The purple sequence which begins at base 43 at the inside end of IRL joins PIRL to the orfA gene. The −10 and −35 hexamers of Pjunc, are in blue as is the extended −10 motif of PIRL; the −10/−35 spacer sizes for Pjunc (17 to 22 bp) are indicated below the sequences. Uppercase letters identify the minicircle junction spacer sequences. The mutation in Pjunc in line 3 is the A-to-G transition in the second position of the −10 hexamer (TA-3′ to CA-3′). The mutation in line 5 was created by the addition of 5 bp to the minicircle junction spacer. The five constructs shown are (see also Table 1) as follows: 1, pLL136; 2, pLL135; 3, pLL143; 4, pLL144; and 5, pLL146. (B) Sequencing (GATC) and primer extension (lanes 1 to 5) reactions. Both utilized the same primer, LacRI, located 90 bases downstream of PIRL. Reactions in lanes 1 to 5 were carried out with the correspondingly labeled constructs described in panel A. The template used for the sequencing reaction was pLL136 (panel A, lane 3) with the IRLCA dinucleotide mutation. The black horizontal arrow identifies the Pjunc runoff products; the red arrow identifies the PIRL runoff product.

Analysis of β-galactosidase expression from the same plasmids confirmed and extended the primer extension results (Table 1). These data show that Pjunc is nearly (90%) as strong as PlacUV5 and is 14-fold more efficient than PIRL (compare pLL136, pLL148, and pLL135). Pjunc is inactivated by mutations in either the first or second position of IRL, supporting the hypothesis that positions 1 to 6 of IRL form the conserved −10 element of this strong promoter (pLL138 and -143). The inactivity of the A-to-G substitution at position 2 is particularly instructive, since this corresponds to a change of the 3′ end of IRL from -TA to -CA, which is the usual 3′ end of IS3-family elements. Finally, both sets of data show that Pjunc is surprisingly sensitive to the size of the minicircle junction spacer. An increase from 1 bp (the most frequently observed spacer in vivo [15]) to 2 bp, corresponding to a change in the length of the −35 to −10 spacer from 17 to 18 bp, eliminates Pjunc activity (Fig. 2B, lane 4), as do larger increases (Fig. 2B, lane 5; Table 1, pLL144, pLL145, and pLL146).

Pjunc is critical for IS2 transposition in vivo. To determine the importance of Pjunc for the transposition of IS2 in a normal “wild-type” situation, we compared the transposition frequencies of a pair of marked IS2 derivatives (Table 2). Both derivatives contained the normal arrangement of orfA and orfB.
genes, requiring the natural translational frameshift (19, 30, 37) to produce the active OrfAB transposase. pLL44 contains the Kan'-marked, but otherwise wild-type, IS2, in which transposase is initially expressed from P_{IRL} but, following IS2 minicircle formation, can be expressed from the minicircle junction promoter, P_{junc}. pLL440 contains the A-to-G substitution at position 2 of the IS2 IRL but is otherwise identical to pLL44; this mutation, which changes IRL TA to IRLCA, eliminates P_{junc} activity (Table 1 and Fig. 2B, lane 3) but affects neither the efficiency of forming minicircles (14) nor the substrate activity of preformed minicircle junctions (see the discussion of spacer and sequence requirements below). Thus, in pLL440, transposase expression depends on P_{IRL} both before and after IS2 minicircle formation.

The results of mating-out experiments shown in Table 2 indicate that for the construct lacking a functional P_{junc}, pLL440, the frequency of transposition \( (3.6 \times 10^{-5}) \) was about 10% of that for the wild-type construct (pLL44) \( (3.7 \times 10^{-5}) \). These results are comparable to the relative efficiencies of P_{IRL} and P_{junc} and show that P_{junc} plays an important role in maximizing the reinsertion of excised IS2 minicircles; indeed, in the absence of the P_{junc} promoter about 90% of the excised IS2 minicircles fail to reinsert.

**Spacer and sequence requirements for transpositional activity of IRL in the IS2 minicircle junction.** The IRL of IS2 is completely inactive in the initial strand transfer step that creates the figure eight intermediate (and the minicircle junction) (14). Nevertheless, subsequent IS2 insertion implies that in the context of the minicircle junction, IRL becomes active. What are the constraints on this activation? Specifically, is activation dependent on a particular spacer size and the sequence of the IRL terminal dinucleotide?

The effects of minicircle junction spacer size on transposition were determined using mating-out assays with a transposon-less F plasmid (pCJ105) and a series of Kn' pUC19-derived plasmids with different minicircle junctions (Table 3). IS2 transposase was provided by the chromosomal copies of IS2 present in the donor strain. The results showed that junctions with spacers of 1 or 2 bp were highly active (even with the low endogenous levels of IS2 transposase). However, an increase in spacer size to 6 or 8 bp was strongly inhibitory to transposition. DNA sequences of several insertions revealed the 5-bp target duplications characteristic of IS2 transposition, showing that the events which were scored indeed resulted from the transposition of the entire plasmid into the F factor.

In an earlier study we compared the effects of all possible 16 terminal dinucleotides at the left end on minicircle formation (14). None of the mutations had any effect on the efficiency of minicircle formation, a result that is in accord with the exclusive role of IRL as a target in the initial strand transfer reaction. We have now examined the effects of these same mutations of IRLTA on the frequency of autonomous transposition (Table 4). Note that in these transposition assays the transposase is provided by the fused orfAB gene carried by IS2 (15). The mutants fell roughly into two groups. Those in which only the T was replaced transposed with normal or modestly reduced frequency, while those in which the 3'-terminal A was replaced transposed at frequencies of 0.03 to 1% of the IRLTA control. The results suggest that the 3'-terminal A is the critical residue and that the penultimate T plays little or no role in the cleavage and joining reactions of minicircle integration, unlike the penultimate C of IRR in first-step reactions (14).

Since mutations at the left end of IS2 also alter the sequence of P_{junc}, diminishing the levels of transposase and thereby reducing the overall frequencies of transposition, the specific effects of the mutations of IRLTA on cleavage and joining

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**TABLE 2. Transposition frequencies of IS2-kan constructs that assemble active versus inactive P_{junc} promoters**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptiona</th>
<th>Frequency of transposition (±SE)</th>
<th>n</th>
<th>% Efficiency of transposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLL44</td>
<td>IS2-kan with wild-type IRLTA: active P_{junc}</td>
<td>3.7 \times 10^{-5} (0.5)</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>pLL440</td>
<td>IS2-kan with IRLTA: inactive P_{junc}</td>
<td>3.6 \times 10^{-5} (0.2)</td>
<td>8</td>
<td>9.6</td>
</tr>
</tbody>
</table>

a Both constructs shown here contain the natural conformation of orfA and orfB genes in IS2.

b n = number of mating-out experiments. Each experiment consisted of the mating of a single independently isolated F- clone with the F+ strain. Standard errors were calculated as described in Table 1, footnote h.

c The percent efficiency of transposition is described as a function of that of pLL44 (wild-type IS2).

d The sequence of the mutated 10 hexamer of P_{junc} was TGGACT.

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**TABLE 3. Effect of IS2 minicircle junction spacer size on the frequency of transposition**

<table>
<thead>
<tr>
<th>Plasmida</th>
<th>Junction spacer size (bp)</th>
<th>Sequence of terminal dinucleotides</th>
<th>Frequency of transposition (±SE)</th>
<th>n</th>
<th>% Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLL40</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>pLL400*</td>
<td>1</td>
<td>IRCA3'/IRLTA3'</td>
<td>(1.3 ± 0.3) \times 10^{-4}</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>pLL600*</td>
<td>1</td>
<td>IRCA3'/IRLCA3'</td>
<td>(8.8 ± 1.4) \times 10^{-5}</td>
<td>9</td>
<td>70</td>
</tr>
<tr>
<td>pLL610</td>
<td>2</td>
<td>IRCA3'/IRLCA3'</td>
<td>(3.6 ± 0.9) \times 10^{-5}</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>pLL617</td>
<td>3</td>
<td>IRCA3'/IRLCA3'</td>
<td>(4.9 ± 1.7) \times 10^{-6}</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>pLL619</td>
<td>6</td>
<td>IRCA3'/IRLCA3'</td>
<td>(3.0 ± 1.0) \times 10^{-7}</td>
<td>6</td>
<td>0.2</td>
</tr>
<tr>
<td>pLL538</td>
<td>8</td>
<td>IRCA3'/IRLCA3'</td>
<td>(6.1 ± 1.7) \times 10^{-8}</td>
<td>6</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Construct numbers with asterisks identify individual series of three or four independently cloned minicircles from the same IS2 construct. All minicircles used here lack the orfA and orfB genes (see Materials and Methods). Transposase is provided by endogenous wild-type chromosomal copies of IS2 and requires a −1 translational frameshift between the orfB and orfA genes.

a NA, not applicable, no minicircle present. The pLL40 construct contains a linear IS2 derivative from which the orfA and orfB genes have been deleted.

b n, number of mating-out experiments, as described in Table 2, footnote h.
reactions during the final insertional step are better evaluated by examining transposition frequencies of cloned minicircles generated from the mutant elements, with transposase provided in trans at a constant level (Table 5). These data confirmed that IRL TA and IRL CA are essentially equivalent and have provided strong evidence that the minicircle is an essential intermediate in IS2 transposition and extends earlier reports of IS3-family transposition mechanisms (14, 26).

The minicircle junction is a hyperactive target for the IS2 transposase. We have identified the IS2 minicircle junction itself as the target for the transposase by showing that mutations which increase the size of the junction spacer diminish its innate hyperactivity when transposase is provided merely by endogenous chromosomal copies of the element (Table 3). Similar results are observed when frame-fused OrfAB is provided in trans to cloned constructs with wild-type and mutated ends in preformed junctions (Table 5).

Comparison of the P_{IRL} and P_{junc} promoters. From a comparison of P_{junc} and P_{IRL} sequences (Fig. 3), it is clear that the increased activity of P_{junc} is the result of a closer match to the promoter consensus sequence. Not only does the weak promoter, P_{IRL}, appear to lack a recognizable −35 region but its −10 hexamer is also far from ideal, with only the three most important positions matching the consensus. From its sequence, P_{IRL} may well belong to the group of promoters that rely on an "extended −10 motif," with a TG sequence located 1 bp upstream of the −10 hexamer (12, 20). P_{junc} by contrast, has an improved −10 region with a four out of six match to the −10 consensus, a reasonable −35 hexamer (with four consensus bases), and an optimal 17-bp spacer between these elements. P_{junc} is somewhat unusual in its sensitivity to a 1-bp spacer change and its pyrimidine initiation sites (8). The outwardly directed −35 hexamer at the right end of IS2 has long been implicated in the creation of new hybrid promoters, formed following the insertion of the element at an appropriate distance from a sequence resembling a −10 hexamer in target DNA (6, 10). It is now evident that this −35 hexamer exists so that a strong regulatory promoter can be assembled at the IS2 minicircle junction.

Balancing P_{junc} activity with strand transfer activity: the evolution of IRL. The strength of the P_{junc} promoter is dependent on two features of IRL, each of which compromises its strand transfer activity. First, P_{junc} activity depends upon the unusual 3′ end of IRL, which is TA-3′ rather than the CA-3′.

Discussion

Previous studies have shown that formation of the IS2 minicircle junction creates a promoter, P_{junc}, with each of the abutted ends providing essential and functional promoter elements (5, 32). Here we have confirmed this, showing that P_{junc} is nearly as strong as the P_{lacUV5} promoter and about 14-fold stronger than the endogenous transposase promoter, P_{IRL}. We have precisely identified the initiation sites of P_{junc} and P_{IRL} and have provided strong evidence that the −10 conserved region of P_{junc} is the terminal 6 bp of IRL. In addition, we have shown that the increased activity of P_{junc} is very important for maximizing the efficiency with which IS2 minicircles are cleaved and transferred into new target sites. This work provides strong evidence that the minicircle is an essential intermediate in IS2 transposition and extends earlier reports of IS3-family transposition mechanisms (14, 26).

Table 4. Effects of IRL terminal mutations on the overall transposition frequency of IS2

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>IRL terminal dinucleotide</th>
<th>Frequency of transposition (±SE)</th>
<th>Relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLL18</td>
<td>TA (WT)</td>
<td>4.7 × 10^{-4} (0.5)</td>
<td>8 100</td>
</tr>
<tr>
<td>pLL46</td>
<td>CA</td>
<td>7.1 × 10^{-4} (0.7)</td>
<td>6 150</td>
</tr>
<tr>
<td>pLL91</td>
<td>GA</td>
<td>2.8 × 10^{-4} (1.3)</td>
<td>3 60</td>
</tr>
<tr>
<td>pLL88</td>
<td>AA</td>
<td>1.9 × 10^{-4} (1.6)</td>
<td>3 4</td>
</tr>
<tr>
<td>pLL50</td>
<td>TG</td>
<td>5.1 × 10^{-4} (2.0)</td>
<td>5 0.1</td>
</tr>
<tr>
<td>pLL68</td>
<td>CG</td>
<td>3.0 × 10^{-4} (1.7)</td>
<td>3 0.6</td>
</tr>
<tr>
<td>pL107</td>
<td>GG</td>
<td>4.8 × 10^{-5} (2.6)</td>
<td>5 1</td>
</tr>
<tr>
<td>pLL89</td>
<td>AG</td>
<td>1.7 × 10^{-5} (1.5)</td>
<td>3 0.4</td>
</tr>
<tr>
<td>pLL93</td>
<td>TT</td>
<td>8.3 × 10^{-5} (2.7)</td>
<td>3 0.2</td>
</tr>
<tr>
<td>pLL82</td>
<td>CT</td>
<td>5.5 × 10^{-5} (2.0)</td>
<td>3 0.1</td>
</tr>
<tr>
<td>pLL83</td>
<td>CT</td>
<td>2.9 × 10^{-5} (0.8)</td>
<td>3 0.6</td>
</tr>
<tr>
<td>pLL92</td>
<td>AT</td>
<td>2.4 × 10^{-5} (0.3)</td>
<td>2 0.05</td>
</tr>
<tr>
<td>pLL81</td>
<td>TC</td>
<td>1.5 × 10^{-5} (0.2)</td>
<td>5 0.03</td>
</tr>
<tr>
<td>pLL84</td>
<td>CC</td>
<td>2.4 × 10^{-5} (0.8)</td>
<td>4 0.05</td>
</tr>
<tr>
<td>pLL80</td>
<td>GC</td>
<td>3.3 × 10^{-5} (0.4)</td>
<td>3 0.07</td>
</tr>
<tr>
<td>pLL90</td>
<td>AC</td>
<td>3.3 × 10^{-5} (2.0)</td>
<td>3 0.07</td>
</tr>
</tbody>
</table>

The strength of the P_{junc} promoter is dependent upon the unusual 3′ end of IRL, which is TA-3′ rather than the CA-3′.

Figure 3. Comparison of the indigenous (P_{IRL}) and minicircle junction (P_{junc}) promoters of IS2. The top line presented in uppercase lettering shows the consensus sequences for the −10, extended −10, and −35 promoter motifs. P_{IRL} lacks a recognizable −35 motif and appears to rely on an extended −10 motif (uppercase letters; bases which match the consensus sequence are underlined). P_{junc} with its −10 and −35 motifs is created by the formation of the minicircle junction. The abutment of the right and left ends of IS2 is indicated by square brackets. The −10 hexamer is of IRL origin, and the −35 hexamer is of IRR origin. The junction contains a 1-bp spacer of vector origin. Transcriptional start sites for both promoters are indicated by uppercase letters with hooked arrows.

Table 5. Effects of IRL terminal mutations on the transposition of preformed IS2 minicircles

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>IRL terminal dinucleotide</th>
<th>Frequency of transposition (±SE)</th>
<th>% Efficiency of transposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLL18</td>
<td>TA (WT)</td>
<td>3.3 × 10^{-3} (0.9)</td>
<td>7 100</td>
</tr>
<tr>
<td>pLL46</td>
<td>CA</td>
<td>2.1 × 10^{-3} (0.8)</td>
<td>7 64</td>
</tr>
<tr>
<td>pLL1838</td>
<td>AG</td>
<td>4.9 × 10^{-3} (1.3)</td>
<td>5 1.5</td>
</tr>
<tr>
<td>pLL1508</td>
<td>TC</td>
<td>4.4 × 10^{-4} (0.7)</td>
<td>4 0.01</td>
</tr>
<tr>
<td>pLL1592</td>
<td>AT</td>
<td>1.5 × 10^{-4} (2.4)</td>
<td>3 0.005</td>
</tr>
<tr>
<td>pLL1676</td>
<td>CC</td>
<td>7.5 × 10^{-5} (2.0)</td>
<td>3 0.002</td>
</tr>
</tbody>
</table>

The frame fused orfAB gene is present in these IS2 derivatives.

The efficiency is described as a function of the transposition frequency of the minicircle element in plasmid pLL18.
that terminates IS2 IRR and the vast majority of the ends of other IS3-family elements (3). If the terminal –CA-3’ of IRR is replaced with TA, its donor activity in the initial asymmetric strand transfer step (that forms the figure eight and IS2 minicircle intermediates) is virtually eliminated (14). However, introducing the preferred CA at the 3’ end of IRL destroys \( P_{\text{junc}} \) activity (Fig. 2B, lane 3) and, consequently, nearly eliminates IS2 transposition for lack of sufficient transposase for the second strand transfer step.

Secondly, \( P_{\text{junc}} \) activity depends upon the formation of a 1-bp spacer between IRR and IRL. This spacer size provides the optimal 17-bp spacer between the –35 and –10 regions of \( P_{\text{junc}} \) and remarkably, increasing this to 18 bp also eliminates \( P_{\text{junc}} \) activity (Fig. 2B, lane 4). The 1-bp interend spacer size is very strongly dependent on the “extra” base pair in IRL that increases the distance between the IRL terminus and the transposase binding subdomain (Fig. 1B). The extra base pair in IRL (relative to IRR) not only is responsible for reducing the minicircle junction spacer from 2 or 3 bp (the size that results from using IRR as a target) to 1 bp but also, like the terminal TA, inactivates IRL donor activity in figure eight and minicircle formation (14).

Although the evolution of \( P_{\text{junc}} \) has come at the cost of reducing IRL strand transfer, the overall transposition of IS2 is clearly enhanced by the formation of \( P_{\text{junc}} \). This is because the initial asymmetric strand transfer step is not compromised by IRL donor inactivity (since IRR donor activity suffices for this step), while for the second strand transfer step the initial IRL inactivity is overcome by its juxtaposition with IRR across the IS2 minicircle junction.

The transposase binding activity of IRL is not sufficient for the activation of IRL strand transfer from the IS2 minicircle intermediate—additional sequences directly involved in the catalytic steps are also needed. Our earlier study of the role of IRL in IS2 minicircle formation showed that sequences from positions 11 to 42, the presumed transposase binding domain (see Fig. 1B), were sufficient to provide target function (14). Here we have shown that additional sequences at the tip of IRL, particularly a 3’-terminal A, are required for efficient cleavage and strand transfer of the minicircle junction. Thus, activation of an IS end in the context of the transpositionally hyperactive minicircle junction requires that the features most essential for both binding and catalysis remain intact. Nevertheless, the juxtaposition of ends in the minicircle junction is able to counteract both the CA-to-TA terminal substitution and the increased separation between the binding and catalytic subdomains found in IRL.

The role of \( P_{\text{junc}} \) in regulating transposase levels during the transposition cycle. Our data show that the IS2 \( P_{\text{junc}} \) promoter, like that of IS911, plays an important role in transposition. In the case of IS2 this is most clearly illustrated by the behavior of a derivative in which IRL contains a CA-3’ end. This mutant end inactivates \( P_{\text{junc}} \) and reduces transposition by more than 10-fold (to an approximate background level of \( 4 \times 10^{-5} \)) when present in a marked linear IS2 that provides its own transposase. Note, however, that the IRL CA-3’ end has no effect on the efficiency of minicircle formation (a step requiring \( P_{\text{IRL}} \)) (14) or any significant effect on minicircle integration when transposase is provided in trans (Table 5, pLL460). Thus, the transposition defect of this IRL mutation is entirely due to inactivation of \( P_{\text{junc}} \).

Why have the ends of IS2, IS911, and many other IS elements that form reactive IRR-IRL junctions such as IS21, IS30, and IS492 (2, 5, 13, 17, 21) evolved to form a strong junction-spanning promoter that elevates transposase expression? For transposons such as IS10 and IS50 that transpose via the classical cut-and-paste process, both excision and insertion of an individual element are catalyzed by the same transpososome complex (i.e., a protein-DNA synaptic complex with transposase and two paired transposon ends), with no dissociation between the steps (25, 27). By contrast, in the transposition pathway proposed for IS911 and IS2, transpososomes are required to assemble on two temporally separable occasions. The first transpososome, which catalyzes the initial strand transfer to form the figure eight, must be disassembled in order to process the figure eight into the IS2 minicircle. Once it is formed, the nonreplicating minicircle then has only a limited window of opportunity to assemble a new transpososome and insert into a target before it is lost by dilution or degradation. The strong \( P_{\text{junc}} \) promoter provides a burst of transposase gene expression just when it is most needed to maximize reinsertion and minimize loss. Without the elevated induction of transposase that results from forming the \( P_{\text{junc}} \) promoter, at least 90% of the IS2 minicircles formed fail to reinsert. The induction is temporary, however, since integration of the minicircle into a target separates the two IS ends, destroying \( P_{\text{junc}} \) and returning transposase expression to the control of the weak \( P_{\text{IRL}} \) promoter.

The importance of \( P_{\text{junc}} \) provides strong evidence that IS2 transposition proceeds via a minicircle intermediate most of the time, since \( P_{\text{junc}} \) is created only by covalent linkage of IRR and IRL. Further support is provided by the strand transfer properties of IRL, which is inactive in a linear IS2 insertion but becomes active when abutted to IRR once a minicircle is formed.

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