Transient promoter formation: a new feedback mechanism for regulation of IS911 transposition

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IS911 transposition involves a free circular transposon intermediate where the terminal inverted repeat sequences are connected. Transposase synthesis is usually driven by a weak promoter, pIRL, in the left end (IRL). Circle junction formation creates a strong promoter, punc, with a −35 sequence located in the right end and the −10 sequence in the left. punc assembly would permit an increase in synthesis of transposase from the transposon circle, which would be expected to stimulate integration. Insertion results in punc disassembly and a return to the low pIRL-driven transposase levels. We demonstrate that punc plays an important role in regulating IS911 transposition. Inactivation of punc strongly decreased IS911 transposition when transposase was produced in its natural configuration. This novel feedback mechanism permits transient and controlled activation of integration only in the presence of the correct (circular) intermediate. We have also investigated other members of the IS3 and other IS families. Several, but not all, IS3 family members possess punc equivalents, underlining that the regulatory mechanisms adopted to fine-tune transposition may be different.

Keywords: IS911/promoter/regulation/transposase/transposition

Introduction

Extensive analysis of a number of transposable elements has revealed that they have adopted a variety of different strategies to control their displacement. Even elements that use related transposases (Tpass) carrying a conserved triad of amino acids (the DDE motif; Fayet et al., 1990; Rowland and Dyke, 1990; Kulkosky et al., 1992; Rezsohazy et al., 1993) as part of their active site, and which therefore presumably share identical chemistry, show a wide diversity in the details of their transposition cycle (Mizuuchi, 1992; Haren et al., 1999). DDE Tpases catalyse the cleavage of a single DNA strand to generate a 3′-OH at the transposon end. This then acts as a nucleophile in the attack of the target DNA backbone. The major defining difference between the elements encoding such Tpases is the way in which they deal with the second strand (see Turlan and Chandler, 2000).

In one major strategy adopted by IS911 and other members of the IS3 family [IS911 (Ton-Hoang et al., 1997), IS3 (Sekine et al., 1994), IS2 (Lewis and Grindley, 1997) and IS150 (Welz, 1993)], a single-strand cleavage occurs initially at one end of the insertion sequence (IS). The free 3′-OH generated is then directed to attack the opposite end on the same DNA strand to generate a figure-of-eight form in which both ends are joined by a single-strand bridge (Polard and Chandler, 1995a). This is converted into a transposon circle (Polard and Chandler, 1995a; G.Duval-Valentin, unpublished results), which then undergoes the final step of integration. The transposon circle carries abutted left and right ends (IRL and IRR). Since such junctions have been observed for several ISs, including IS21 (Reimmann et al., 1989; Berger and Haas, 2001), IS30 (Olasz et al., 1993), IS256 (Lyon et al., 1987; M.Prudhomme, C.Turlan, J.-P. Claverys and M.Chandler, submitted) and ISL3 (Kallastu et al., 1998), members of these families may also transpose using a similar mechanism. In all cases IRL and IRR are separated by a short spacer whose length is a characteristic of the particular IS. These structures are several orders of magnitude more active as substrates in transposition than are plasmid donor molecules in which the two ends are distant (i.e. in their normal configuration). Transposition of these elements is thus also likely to pass through a circularization step.

In the case of IS3 family members, the transposition reactions are catalysed by two proteins, OrfAB and OrfA. The Tpass, OrfAB, is produced as a fusion protein by translational frameshifting between two consecutive and partially overlapping reading frames, orfA and orfB. OrfA is the product of the upstream frame, orfA. OrfAB alone is capable of generating the bridged figure-of-eight form while both proteins are required for the reactions at the IRL–IRR junction leading to efficient insertion of the transposon circle (Ton-Hoang et al., 1997).

In addition to their inherently high recombination activity, the IRR–IRL junctions of several of these IS elements have been shown to constitute relatively strong promoters (punc) positioned to direct expression of the Tpass. The −35 element is located in IRR while the −10 hexamer is located in IRL (Ton-Hoang et al., 1997). This is illustrated for IS911 in Figure 1. The assembly of punc has lead to the notion of a novel regulatory circuit where the indigenous promoter, pIRL, maintains low levels of Tpass synthesis sufficient to generate low levels of figure-of-eight junctions. Processing these forms into transposon circles assembles the punc promoter leading to extensive expression of IS-encoded proteins, efficient Tpass-catalysed single-strand cleavage at each end and insertion into a target site. Insertion then separates the two promoter elements and again renders Tpass expression dependent on pIRL. This novel feedback model is illustrated in Figure 1.
In the work described here we have addressed the role of \( p_{\text{junc}} \) in IS911 transposition. We have demonstrated that an IS911 derivative carrying a native \( p_{\text{junc}} \) junction promoter capable of driving Tpase expression in cis shows significantly higher transposition activity than a derivative in which \( p_{\text{junc}} \) has been inactivated. This difference is not observed if Tpase is provided in trans independently of \( p_{\text{junc}} \). Since \( p_{\text{junc}} \) activity could be modulated by binding of IS911 transposition proteins, we have also analysed the effect of IS911-encoded proteins on expression from \( p_{\text{junc}} \). We find that neither OrfA nor OrfAB is capable of repressing expression from \( p_{\text{junc}} \). In contrast, an artificial OrfAB derivative, OrfAB[1–149], truncated at its C-terminal end and previously demonstrated to strongly bind isolated IRR and IRL sequences (Haren et al., 1998, 2000; Normand et al., 2001) is able to repress \( p_{\text{junc}} \). Interestingly, while our results demonstrate that junction sequences of the IS3 family members IS911, IS2 and IS600 include a strong promoter, not all members of this family appear to share this property. Thus even though these elements form part of a highly homogenous family, the result underlines the diversity in strategies adopted by individual elements to fine-tune their transposition activities.

Results

**Inactivation of IS911 \( p_{\text{junc}} \) by directed mutagenesis**

To determine the role of \( p_{\text{junc}} \) in regulating IS911 transposition, the putative −10 hexamer (CACACT) located in IRL (Figures 1 and 2A) was subjected to directed mutagenesis in order to reduce or eliminate \( p_{\text{junc}} \) activity. We chose to introduce transversions at the most conserved bases in the canonical sequence TATAAT. Five single point mutants were generated (Figure 2B) at positions that are not conserved in IRR. The mutant IRL ends were substituted for the wild-type end in an IS911 derivative carrying a lacZ reporter gene (pCN100; Normand et al., 2001). Promoter activity was measured after transposon circle formation in the presence of OrfAB (Materials and methods) using a standard β-galactosidase assay. Three of the mutations exhibited only a slight reduction in activity compared with the wild-type promoter (mutants 2, 3 and 6; see Figure 2), while three others showed significantly lower activities (17%, mutant 1, CACACC; 14%, mutant 4, CCCACT; and 30%, mutant 5, GACACT) compared with the wild-type \( p_{\text{junc}} \). In an attempt to further reduce activity, all three mutations were introduced into a single −10 hexamer. The resulting triple mutant (IRL145) exhibited a residual activity of ~3% compared with the wild-type \( p_{\text{junc}} \) promoter. This residual expression is presumably due to the presence of the weak \( p_{\text{IRL}} \) promoter in IRL in these plasmid constructs (Figure 2A), which has previously been shown to have an activity of ~2–3% that of \( p_{\text{junc}} \) (Ton-Hoang et al., 1997).

**Effect of the mutations on the inherent recombination activity of IRL**

Since the mutations introduced into \( p_{\text{junc}} \) lie within IRL, it is possible that they directly affect the inherent transposition activity in addition to their effect on promoter activity. Although no obvious difference in the global amounts of transposon circles produced by the different mutants could be detected (data not shown), it was possible that they exerted more subtle effects. To examine this, the possible direct effect on transposition activity was determined in detail for several steps in the transposition cycle. These included the efficiency of production of transposition intermediates (figure-of-eight and transposon circles), circle junction integration and the overall transposition activity when transposition enzymes were supplied in trans to the mutated ends.

**Kinetics of figure-of-eight formation in vitro.** To test their possible effect on the presumed first step of the transposition cycle, figure-of-eight formation of an artificial transposon carrying the mutant IRL145 and a correctly...
orientated wild-type IRR sequence was measured in vitro. In this system, which is dependent on OrfAB alone, the recombination reaction does not lead to transposon circles but is arrested after figure-of-eight formation. Plasmids pAPT56.2 (carrying wild-type IRR and IRL sequences) and pDV27 (carrying a wild-type IRR and IRL1.4.5) (Figure 3A) were used in a standard in vitro reaction (Materials and methods) in the presence of Mg\(^{2+}\). Samples were removed at intervals, digested with EcoRV, to convert the figure-of-eight into a \(\chi\) form, and analysed by electrophoresis through a 0.8% agarose gel. This allows separation of the figure-of-eight from the parent plasmid (Materials and methods; Polard and Chandler, 1995b). Figure 3B shows a SyBr green-stained gel. Quantification of the bands by fluorimager showed that both mutant and wild-type ends display indistinguishable activities in figure-of-eight formation (Figure 3C).

**Figure-of-eight and transposon circle formation in vivo.** Recombination activity of IRL1.4.5 was also examined in vivo by determining the level of transposition intermediates (figure-of-eight and transposon circle molecules) produced under standard growth conditions. In these experiments two IS911 derivatives carried by pDV19 and pDV28 were used (Figure 4A). These plasmids carry the terminal 52 bp of the wild-type (pDV19) or mutated left end (pDV28) together with a wild-type right end flanking an orfA–lacZ translational fusion. OrfAB was provided in trans under control of the pRacUV5 promoter from a second compatible plasmid, pAPT111. The plasmid content of cultures grown in increasing levels of IPTG (to induce OrfAB synthesis; Materials and methods) was analysed by agarose gel electrophoresis following digestion with XhoI to convert the figure-of-eight into an \(\chi\) form and to linearize the transposon circle (Figure 4A). No significant difference in levels of transposition intermediates could be detected between IS911 derivatives carrying the wild-type (pDV19) or triple mutant (pDV28) ends (Figure 4B and C).

**Circle junction integration activity in vivo.** Although the IRL1.4.5 mutant had no measurable effect on the production of figure-of-eight intermediates and transposon circles, it was possible that it affects the final step in the transposition cycle: integration of the circle.

In a first set of experiments, the effect of the mutant end on insertion activity of the junction was determined. Transposon donor plasmids were constructed by cloning the IRR–IRL junction into a suitable plasmid backbone. Purified transposon circles carrying the wild-type or mutant IRR–IRL junctions derived from pCN100 (Normand et al., 2001) and pDV11 respectively, were cloned into pAPT120 as described previously (Ton-Hoang et al., 1997; see Materials and methods). IS911 proteins were provided in trans under the control of a pRacUV5 promoter either from reading frames with a wild-type configuration in which OrfAB is produced by frameshift-
Fig. 4. Figure-of-eight and circle formation in vivo as a function of increasing concentrations of IPTG. (A) Relevant features of pAPT111, which was used to produce OrfAB under control of the p<sub>lacUV5</sub> promoter in trans and of the synthetic omega-carrying plasmids pDV19 (p<sub>lacUV5</sub> with IR<sub>UV</sub>-IT) and pDV28 (p<sub>lacUV5</sub> with IR<sub>UV</sub>-IT<sub>1.45</sub>) are shown. Also indicated are: the position of the XhoI sites used in the analysis (X); the positions and direction of transcription of the transposase, orfAB, the spectinomycin-streptomycin (Sp-Sm), kanamycin (Km), chloramphenicol (Cm) and ampicillin (Ap) resistance genes and the lac repressor gene (lack) used to control p<sub>lacUV5</sub> (which drives OrfAB expression); and the two compatible origins of replication (ori P15A and pBR322). The transposable element carried by pDV19 and pDV28 is drawn in bold. (B) DNA samples were prepared by the cleared lysate procedure and digested by XhoI to separate all intermediates and plasmids. Samples were separated by electrophoresis on a 0.8% TAE-agarose gel. Concentrations of IPTG added in lanes 1-6 were: 0, 0.025, 0.1, 0.2, 0.4 and 0.6 mM, respectively. (C) Detection and quantitation of figure-of-eight molecules and circles were performed by fluorimaging after Sybr green I staining.

Fig. 5. Analysis of the effects of mutation on the integration step and overall transposition activity. (A) Integration frequencies using a preformed IRL–IRR junction. The general structure of the donor plasmids pDV13 (p<sub>lacUV5</sub>+) and pDV23 (p<sub>lacUV5</sub>-) is shown in the left hand column. (B) Overall transposition using a substrate with distant ends. The general structure of the donor plasmids pDV19 (p<sub>lacUV5</sub>+) and pDV28 (p<sub>lacUV5</sub>-) is shown in the left hand column. Symbols are those used in the legends to Figures 3 and 4. IS911 proteins were supplied in two different configurations: a wild-type configuration of the open reading frames in which the transposase was produced by translational frameshifting (OrfAB + OrfA) or with OrfAB alone. The values obtained represent the average of six distinct experiments (n = 6). Standard errors are shown in parentheses.

Overall transposition activity in vivo. In the experiments described above, the two transposon ends were retained close together in a preformed junction structure. This configuration of ends is highly active but represents only the final step in the transposition cycle. In the preceding steps, IRL and IRR must be assembled into a paired end synaptic complex (catalysed by OrfAB alone; Haren et al., 1998, 2000; Normand et al., 2001), strand cleavage and transfer must take place and the resulting figure-of-eight molecules must be resolved into transposon circles carrying the junction. To determine whether the triple mutation in IRL affects these initial steps in the transposition cycle, an artificial IS911 derivative, including IRL<sub>1.45</sub> at one end and a wild-type IRR at the other, was constructed in a plasmid donor and its behaviour in the mating-out assay was measured. The derivative used was composed of a chloramphenicol resistance gene (Cm<sup>R</sup>) introduced between the wild-type IRL and IRR ends (pDV19) or between the triple mutant IRL<sub>1.45</sub> and a wild-type IRR (pDV28). Again, IS911 proteins were supplied in trans. The results are presented in Figure 5B. No significant effect of the IRL mutations on transposition frequency was observed with either OrfAB alone or with the wild-type configuration of proteins.

Moreover, these results confirm previous findings (Ton-Hoang et al., 1997) which showed that overall transposition frequencies are significantly lower than circle junction-mediated integration (~10-fold with OrfAB and 5–6 × 10<sup>6</sup>-fold with a wild-type complement of proteins). This presumably reflects the fact that synopsis...
between IRR and IRL, figure-of-eight formation or resolution into transposon circles are limiting steps in IS911 transposition relative to integration using the circle junction.

**Effect of supplying IS911 proteins in cis and the role of pUnc**

If pUnc is indeed involved in regulating IS911 transposition activity, it would be expected to exhibit its effects only when present in cis, i.e. where it would drive OrfA and OrfAB synthesis from the element whose transposition is being monitored. In order to determine whether this is the case, elements were constructed in which the natural configuration of orfA and orfB, or a fused-frame configuration, was placed under control of pIRL. In these derivatives a CmR gene was also included between the ends as a selectable marker. It was cloned downstream of orfB in such a way as not to disrupt the orfB frame, which normally continues into IRR (Polard et al., 1994). Activity was measured using the mating-out assay.

The results obtained with a wild-type complement of proteins supplied in cis from the weak pIRL promoter are presented in Figure 6A. Under these conditions, a mutant IRL_{1-4,5} end (pDV27) produced a marked effect on overall activity compared with the wild-type end (pAPT56.2). It resulted in a ~20-fold reduction in transposition frequency. This is in contrast to the results obtained when a similar configuration of transposition proteins were supplied in trans (Figure 5B). Thus, pUnc clearly provides a regulatory function in its natural context when driven expression of transposition proteins in cis.

To extend this analysis, the effect of pUnc-driven OrfAB alone rather than the wild-type protein complement was also measured. Previous attempts to introduce a mutation which fuses orfA and orfB into IS911 have been unsuccessful (Polard et al., 1992). Constitutive synthesis of OrfAB (i.e. without frameshifting) from its native promoter within the IS resulted in a high level of deletions and rearrangements in the vector plasmid. In order to facilitate construction of this type of IS911 derivative, we exploited the observation that OrfAB is inactive at 42°C (Haren et al., 1997). Bacterial hosts were therefore maintained at 42°C during the appropriate stages of plasmid construction and at all times preceding the mating-out assays. The results of mating-out assays are presented in Figure 6B. They clearly show an effect of pUnc on transposition. Comparison of transposition frequencies obtained with the wild-type IRL (pDV22) and IRL_{1-4,5} (pDV29) showed a 54-fold decrease in the case of the mutant. This confirms the transient pUnc promoter plays an important stimulatory role in the transposition cycle, which operates only in the presence of the correct (circular) intermediate.

It should be underlined that the cis effect of pUnc is to amplify Tpase production. This is distinct from the cis preference observed for other ISs (see Mahillon and Chandler, 1998, for a review) as well as for IS911 (Polard et al., 1992), which implies that Tpase acts preferentially on the element from which it is synthesized.

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### Table 1: Transposase substrates

<table>
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<th>Transposase substrates</th>
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<td></td>
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<td>2.6</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>5100</td>
<td>94</td>
</tr>
</tbody>
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### Figure 6

**Role of pUnc, with IS911 proteins supplied in cis.** Transposition frequencies were analysed by mating-out. (A) Analysis performed with the wild-type configuration (OrfA + OrfAB) or (B) OrfAB alone. The values obtained represent the average of six distinct experiments (n = 6). Symbols are those used in the legends to Figures 3 and 4. Standard errors are shown in parentheses.

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### Figure 7

**Effect of IS911-encoded proteins on pUnc activity measured by β-galactosidase assays.** (A) The proteins. Four protein configurations were tested: OrfA, OrfAB, a truncated derivative of OrfAB, OrfAB[1–149], deleted for the C-terminal catalytic domain and OrfA + OrfAB (wild-type configuration). The proteins are shown schematically illustrating the relative position of the catalytic DDE motif and of a helix–turn–helix motif potentially involved in DNA binding. The proteins were supplied in trans under control of pUnc from plasmids pAPT156 (OrfA), pAPT111 (OrfAB), pLH14 (OrfAB[1–149]), and pAPT112 (OrfA + OrfAB). pAPT110 was used as a control without IS911 proteins. (B) The test plasmids. pBS2 carries mutations in the terminal dinucleotides at both ends in the IRR–IRL junction. pDV23 carries a wild-type IRR–IRL junction. Symbols are as described in Figures 3, 4 and 5. (C) β-galactosidase activities. The right hand column indicates β-galactosidase activities as a percentage relative to pUnc activity in the absence of any proteins in trans (100%). These results are the average of three independent experiments with a standard error of ~15%. pDV23 (active junction) was tested in the absence of proteins supplied in trans to ensure that the terminal dinucleotide mutations (5’-CA to 5’-AG) introduced to inactivate integration activity of the junction did not affect pUnc activity. 100% = 17 500 Miller units.
Effects of IS911 proteins on \( p_{\text{junc}} \) activity

Since IS911 transposase must bind to the IRR–IRL junction, it is possible that the transient activity of \( p_{\text{junc}} \) could be further modulated by these proteins. To determine whether OrfA or OrfAB might modify \( p_{\text{junc}} \) activity, their effect on \( p_{\text{junc}} \)-driven expression of a lac reporter gene was determined. The reporter plasmid was similar to pDV23 described above, but the wild-type IRR–IRL junction was replaced with a junction in which both terminal \( 5'\)-CA-3' dinucleotides were mutated to \( 5'\)-AG-3'. This modification inactivates the ends as donors for transposition and was introduced to prevent OrfAB-catalysed cleavage of the junction and therefore loss of \( p_{\text{junc}} \) (Ton-Hoang et al., 1997). These mutations, localized in the \( p_{\text{junc}} \) spacer, have no effect on the activity of the promoter as previously shown (Ton-Hoang et al., 1997) and confirmed here by \( \beta \)-galactosidase dosage (Figure 7). IS911 proteins were supplied in trans under the control of \( p_{\text{laczUV5}} \).

Three IS911-derived proteins were tested in these studies: the full-length transposase, OrfAB (pAPT111); a derivative deleted for the C-terminal catalytic domain, OrfAB(1–149) (pLH114); and OrfA (pAPT156). Previous \textit{in vitro} studies have shown that OrfAB binds poorly to IRR and IRL, but that OrfAB(1–149) binds strongly and generates a paired-end complex. OrfA showed no significant binding (Haren et al., 1998, 2000).

The results of these experiments are presented in Figure 7. OrfA alone had no detectable effect on \( p_{\text{junc}} \) efficiency. On the other hand, a clear inhibition of \(-90\%\) of \( \beta \)-galactosidase levels was observed in the presence of OrfAB[1–149]. This is consistent with the observation that OrfA fails to bind either IS911 end \textit{in vitro}, whereas OrfAB[1–149] binds strongly (Haren et al., 2000). An inhibition of \( 70\% \) was observed in the presence of OrfAB. Although we cannot rule out the possibility that part of this may be due to trace levels of a truncated OrfAB derivative protein, OrfAB*, which is often detected in these preparations (Ton-Hoang et al., 1997; Haren et al., 1998), this result is compatible with the low but measurable binding activity of OrfAB. The wild-type configuration of IS911 proteins (OrfA + OrfAB; pAPT112) showed a small but reproducible inhibitory effect (30%). It should be noted that the level of OrfAB in this configuration is significantly lower than that of OrfA. These results raise the possibility that transposase exerts a feedback control on \( p_{\text{junc}} \) activity in addition to its catalytic activity.

\( p_{\text{junc}} \) formation with other ISs?

The presence of a junction promoter has been described in IS elements belonging to other families including IS21 (Reimmann et al., 1989) and IS30 (Dalrymple, 1987). To determine how general this phenomenon is, we surveyed the sequence of the ends of known ISs for the capacity to form such promoters. Although putative \(-35\) and \(-10\) elements may be present in IRR and IRL, respectively, two limitations of this analysis are: whether the IS naturally generates a structure with two abutted ends and, if so, whether the spacer length is compatible with an active promoter. Several of these are presented in Figure 8B. Some have been demonstrated to generate IRR–IRL junctions (IS1, IS2, IS3, IS21, IS30, IS911, IS492), whereas others have not (IS600, IS987).

These junctions were synthesized and cloned into a standard \( \text{lacz} \) reporter gene system (Figure 8A). The \( \beta \)-galactosidase activities specified by the resulting plasmids were measured and are presented in Figure 9. The results show that the IS3 family members, IS2 and IS600, exhibit a promoter activity comparable to \( p_{\text{laczUV5}} \). IS911 has an activity 3-fold greater than \( p_{\text{laczUV5}} \), while two
other members of the family, IS3 and IS987, have no detectable promoter activity. Thus junction promoter formation does not appear to have been chosen as a control strategy for all members of this family.

On the other hand, members of other families also show strong junction promoter activities. These include IS21 (IS21 family; 3-fold \( p_{\text{lacUV5}} \), IS30 (IS30 family; 3-fold \( p_{\text{lacUV5}} \)) and IS492 (IS110 family; >6.5-fold \( p_{\text{lacUV5}} \)). IS1 (IS1 family), on the other hand, does not appear to exhibit junction promoter activity.

Discussion

The experiments described here were designed to determine whether the \( p_{\text{junc}} \) promoter, assembled by circularization of IS911, plays a role in regulating transposition activity as proposed in the model presented in Figure 1. Although the \( p_{\text{junc}} \) −10 and −35 hexamers do not entirely fit the consensus *Escherichia coli* promoter elements, the promoter does include an extended −10 TG motif (Burr et al., 2000). This presumably contributes to the relatively high activity of this promoter (2.8-fold greater than that of \( p_{\text{lacUV5}} \); Figure 9). In order to significantly reduce \( p_{\text{junc}} \) activity, it was found to be necessary to introduce three mutations into the probable −10 hexamer located in IRL. The residual activity was found to be 3% that of wild-type \( p_{\text{junc}} \), a value similar to that maintained by the resident \( p_{\text{IRL}} \) (Ton-Hoang et al., 1998), also present in these constructions. To minimize any direct effects on transposition activity of the mutated end, these mutations were chosen to modify base pairs that are not conserved between the two IRs. No detectable difference in recombination activity of the triple mutant, IRL\( _{1,4,5} \) and its wild-type counterpart was observed either *in vitro*, in figure-of-eight formation (Figure 3), or *in vivo* in both figure-of-eight and transposon circle formation (Figure 4).

Using a more sensitive genetic assay to measure integration activity of the preformed IRR–IRL junction (Figure 5), the mutated IRL\( _{1,4,5} \) exhibited at most a 1.5-fold lower activity compared with the wild-type IRL. This small effect may indeed be due to slight differences in transposition activity or in the environment of the IR, but is minimal compared with the reduction of at least 97% in promoter activity.

\( p_{\text{junc}} \) regulation of transposition

To determine whether \( p_{\text{junc}} \) plays a role in regulating overall transposition activity, the mutant IRL was placed at its natural position where it would be capable of driving transposase synthesis and its activity was compared with that obtained with a wild-type end. Two types of derivative were constructed. In one, \( p_{\text{junc}} \) would drive a wild-type reading frame configuration following transposon circularization, while in the other, the promoter would drive a fused-frame derivative producing OrfAB alone. The derivative carrying the fused-frame mutant had previously been shown to be particularly unstable (Polard et al., 1994). This problem was overcome here by exploiting the temperature-sensitive nature of OrfAB activity (Haren et al., 1997). The corresponding plasmid was constructed and maintained by imposing a high growth temperature (42°C) at all times.

The results of mating experiments using these donor plasmids (Figure 6) capable of forming a functional \( p_{\text{junc}} \) indicated that the activity of the element expressing the OrfAB fusion protein was significantly higher than that expressing the wild-type complement of proteins (\( p_{\text{junc}} ^{2+} \), \( 5 \times 10^{-4} \) compared with \( 6 \times 10^{-3} \)). In both cases, the presence of the mutared \( p_{\text{junc}} \) reduced activity by a factor of 22 for the wild-type complement of proteins and 54-fold for the fused-frame derivative. This difference in overall transposition activity compares well with the ratio of 45 obtained by measurement of \( p_{\text{junc}} \) and \( p_{\text{IRL}} \) activities in a standard \( \beta \)-galactosidase assay (Ton-Hoang et al., 1997). Suppression of \( p_{\text{junc}} \) activity therefore decreases the transposition frequency by a factor corresponding to the basal level of \( p_{\text{IRL}} \) activity compared with \( p_{\text{junc}} \). These results demonstrate an important role of \( p_{\text{junc}} \) in the regulation of IS911 transposition in its natural configuration.

**Regulation of expression in cis and preferential cis activity**

\( p_{\text{junc}} \)-Mediated expression *in cis* as observed here is distinct from the previously described preferential cis activity of various transposases (see Mahillon and Chandler, 1998). Although preferential cis activity has been concluded to occur in some circumstances for IS911, the experiments described here do not address this question directly. However, even in the absence of \( p_{\text{junc}} \), OrfAB alone retained a 5-fold higher activity *in cis* (94 × 10^{-7}; Figure 6B) under the control of the weak \( p_{\text{IRL}} \) compared with its activity *in trans* under control of the strong \( p_{\text{lacUV5}} \) (18.6 × 10^{-7}; Figure 5B). In the case of the wild-type protein complement, it is possible that the absence of a measurable effect results from a combination of inherently low activity together with the difference in promoter strength. A true comparison would necessitate the use of promoters with identical strengths in both situations.

**Differential activities of OrfAB alone and the wild-type protein complement**

The results presented in Figures 5 and 6 also reiterate previous observations that OrfAB alone does not have the same effect at different steps of the transposition process as does the wild-type complement of proteins. This has been described in situations where these proteins were supplied *in trans* (Ton-Hoang et al., 1997). Thus the frequency of integration of a preformed junction is 12- to 13-fold lower with OrfAB alone (Figure 5A) whereas, while globally lower, OrfAB alone sustains a 4-fold higher level of transposition in a situation requiring prior formation of the junction (Figure 5B). The stimulatory effect of OrfAB in this situation is more pronounced when it is provided *in cis* either with a functional \( p_{\text{junc}} \) (82-fold) or in the absence of \( p_{\text{junc}} \) (36-fold; Figure 6A and B).

**Regulation of \( p_{\text{junc}} \) activity by transposition proteins**

Although regulation of \( p_{\text{junc}} \) activity presumably occurs primarily by promoter assembly and disassembly, a more subtle modulation could occur by repression involving binding of IS911 proteins to the abutted IRs. The effect on \( p_{\text{junc}} \) expression of different IS911 proteins supplied *in
trans (Figure 7) clearly indicated that a truncated derivative of the OrfAB transposase (OrfAB[1–149]) represses punc activity. This is consistent with the DNA-binding properties of the protein (Haren et al., 2000; Normand et al., 2001). Full-length transposase, which appears to bind less well, exhibits a measurably lower but significant effect. Although part of this repression could be due to the presence of truncated derivatives of OrfAB often found following partial purification of the protein (Ton-Hoang et al., 1997; Haren et al., 1998), this observation raises the possibility that punc activity could be controlled by feedback repression in addition to assembly and disassembly.

The presence of punc in other elements

IS911 is not unique in carrying a junction promoter. These have been described for several elements belonging to different IS families including IS21 (Reimmann et al., 1989), IS30 (Dalrymple, 1987) and IS492 (Perkins-Balding et al., 1999). In a limited survey of IS3 family members, three, including IS911, exhibited a strong punc and two did not (Figures 8 and 9). It remains possible that the absence of promoter activity in certain cases is due to the choice of an incorrect spacer length between IRL and IRR or that the sequences of the ends themselves were incorrect. For two members of the IS3 family, IS987 and IS600, the formation of an IRR–IRL junction has yet to be formally demonstrated. Indeed, more extensive studies on IS3 in which the length of the spacer was varied also failed to reveal a punc activity (Sekine et al., 1999). Of those elements from families other than IS3 whose transposition activity includes the formation of an IRR–IRL junction, IS492 exhibits exceptionally high activity (see also Perkins-Balding et al., 1999), whereas punc activity of IS30 and IS21 is similar to that of IS911 (Figures 8 and 9) and ISJ (with an 8 bp spacer as found in ISJ transposon circles; Turlan and Chandler, 1995) shows no activity. Again, more extensive results using 6, 7, 8 and 9 bp spacers also failed to reveal an ISJ punc (Shiga et al., 1999). Although it has not been examined here, it is interesting to note that IS256 also generates transposon circles and tandem IS dimers (M.Prudhomme, C.Turlan, J.-P.Claverys and M.Chandler, submitted). Inspection of such junctions (which carry a 5 or 6 bp spacer) reveals the presence of a nearly canonical –35 element together with a canonical –10. These elements are separated in a majority of cases by the optimal 17 bp spacing and, like the IS911 punc, many include the conserved extended –10 TG motif (Burt et al., 2000).

Taken together, these results clearly demonstrate that IS911 has adopted a novel strategy for modulating its transposition activity via the assembly of a strong promoter intimately linked to the formation of a circular transposition intermediate and its disassembly upon integration of the circle. This assures that high levels of transposase expression occur only in the presence of the correct integration substrate, transposon circles. Once integrated, such circles lie ‘dormant’ and are activated only rarely to generate circles. Thus, the system is set up to ensure integration once these rare circles are formed and to limit transposition following their integration. Moreover, the strategy of activation of transposition by creation of a transitory promoter able to boost transposase expression appears to be widespread, since several other IS elements tested also share this property. It is not universal, however, even within a single IS family. These observations therefore underline the fact that although different elements may share an overall transposition mechanism, the strategies that have been chosen to fine-tune transposition activities are not necessarily identical. This further emphasizes the diversity of transposable elements.

Materials and methods

Bacterial strains and media

JS219 (MC1061, recA1, lacφ) has been described previously (Cam et al., 1986) and was used as a donor strain in mating-out experiments. The recipient strain for mating-out experiments was MC240 [XA103, F′, ara- leu(lac pro), gyrA (nalR), meB, argE<sup>E</sup>, rpoB, thi, supF]. NMS22 mutS<sup>[F, lac8 hAlacZM15 proXB+supE thi h(λlac-proAB)ΔhsDS-mcrB]5 (ΔmcrA mcrBC) (mutS<sup>E</sup>:Thn10)] was used for directed mutagenesis as described in Deng and Nickoloff (1992). DH5<sup>[F, endA1, hsdR17 (λmcrA)] supE44, thi1, relA1, gyrA (Nal<sup>R</sup>), relA1, λΔE2Y-argF</sup> U169, Δ(lacZ)M15) was used for β-galactosidase assays. Cultures were grown in Luria broth supplemented, where necessary, with ampicillin (Ap, 100 μg/ml), oxacillin or methicillin (Ox or Met, 0.8 g/l), chloramphenicol (Cm, 30 μg/ml), kanamycin (Km, 25 μg/ml), nalidixic acid (Nal, 20 μg/ml), rifampicin (Rif, 50 μg/ml), streptomycin (Sm, 20 μg/ml), spectinomycin (Sp, 30 μg/ml) and tetracycline (Tc, 15 μg/ml).

Mutagenesis

Directed mutagenesis was based on the unique site elimination procedure developed by Deng and Nickoloff (1992) using the U.S.E. Mutagenesis Kit (Pharmacia Biotech). This approach utilizes two primers. One is used to introduce the desired mutation, the second eliminates a unique non-essential restriction site in the plasmid permitting selection of the mutant plasmid. A set of oligonucleotides (described in Figure 2B) was used to obtain simple, double or triple mutations in the –10 box of punc located in IRL. The substrate plasmid pCN99 was constructed by replacing the BamHI–PstI fragment from pBR322 by the BamHI–PstI fragment from pAPT66 (Polard and Chandler, 1995b) bearing a wild-type IRL.

Plasmids

Transposase substrates. Plasmids carrying separated pairs of mutant IRL and wild-type IRR ends were obtained by directed mutagenesis of pCN99 as described above. The BamHI–PstI bearing a wild-type or mutant IRL<sub>1.45</sub> was cloned into pCN100 as described in Normand et al. (2001) to generate pVKG13 and pVKG11, respectively. Plasmids carrying an IRR–IRL junction, pDV23 [wild-type IRL (IRL<sub>革新</sub>) and pDV13 (IRL<sub>1.45</sub>) were assembled in two steps. First, transposon circles were generated in vivo from pVK13 and pDV11 using pAPT111 as a source of transposase (Polard and Chandler, 1995b). After agarose gel separation and isolation, the circular transposon DNAs were linearized by XbaI and treated with DNA polymerase I Klenow fragment. This generates a linear molecule carrying the junctions upstream of an OrfA–lacZ fusion. This fragment was then cloned into the gel-purified 3325 bp BamHI–HindIII fragment obtained from pAPT120 (Ton-Hoang et al., 1997), which had been treated with DNA polymerase I Klenow fragment and dephosphorylated with Shrimp phosphatase.

Plasmids carrying IS911 derivatives with the wild-type configuration of transposon genes in cis were constructed as follows. pDV27 (IRL<sub>1.45</sub>) was obtained by partial digestion of pAPT56.2 (Polard et al., 1994) with BclI, purification of the 4640 bp fragment containing the transposon orf<sub>S</sub> and a downstream Cm<sub>革新</sub> gene, and ligation with the purified 3586 bp fragment obtained after BclI restriction of pDV11. Clones in which the insert was in the required orientation were distinguished by PstI–HindIII digestion.

Plasmids carrying pAPT56.2 and pDV27 devoid of transposon genes but retaining the Cm<sub>革新</sub> gene were constructed by deletion between PmeI and SnaBI sites and recircularization of the resulting 7241 bp fragment. This
generated pDV19 and pDV28 containing, respectively, IRLuv and IRLuv+1,5 ends.

pBST2 carrying a mutation on the terminal 5'-CA-3' of IRL and IRR in the junction to inactivate integration has been described previously (Ton-Hoang et al., 1997).

Plasmids supplying transformation functions in trans. pAPT110, pAPT111 and pAPT112, which express no IS911 proteins, OrfAB alone or OrfA + OrfAB (wild-type configuration), respectively, under control of the Phuv promoter have been described previously (Polard and Chandler, 1991). In addition, experiments designed to test the inhibition effect of IS911 proteins on the activity of Phuv also employed pHL114 (OrfAB(1-149); Haren et al., 1998) and pAPT156 (OrfA; Ton-Hoang et al., 1998). More detailed descriptions of the construction of these plasmids are available on request.

Alkaline and cleared lysates

Alkaline lysates were prepared as described previously (Birnboim and Doly, 1979). Cleared lysates were prepared as described previously (Clewell and Helinski, 1969) with the following modifications: the cleared supernatants were collected and incubated with proteinase K (0.5 mg/ml) and SDS (0.5%) for 1 h at 50°C. DNA was concentrated by isopropanol precipitation and resuspended in 100 μl of TE buffer and purified by using a Qiaquick kit as described by the manufacturer (Qiagen).

Mating-out assays

The transposition frequencies of the different IS911 synthetic elements was measured by standard mating-out assay (Galas and Chandler, 1982) using the conjugative plasmid target pOX38Km (Chandler and Galas, 1983). Strain JS219 containing pOX38Km was transformed with the different compatible derivatives as source of OrfAB and/or OrfA, synthetic elements carrying the chloromphenicol acetyltransferase gene (Cm) or plasmids carrying IRR–IRL junction with the β-lactamase gene (Ap) as phenotypic markers. Overnight cultures grown at 42°C to reduce OrfAB activity, which is temperature sensitive (Haren et al., 1997), from a single colony in LB supplemented with Ap, Km, Sp and Sm (and Cm when present), were diluted into LB without antibiotics at an OD600 of 0.05 at 37°C, with shaking. Growth was continued to an OD600 of 0.5 and followed by 1 h incubation without agitation to allow plasmid formation. Donor cells were then mixed at a 1:1 ratio with the recipient XA103 cells (NalR, RifR) also grown at 37°C. Suitable dilutions were plated on LB agar plates supplemented with Nal, Rif, Km (scoring for transfer) and Nal, Rif, Km, Cm (or Ap and Met instead of Cm for plasmids carrying junctions) for scoring transposition or integration, and incubated overnight at 37°C.

Transposase purification and in vitro activity assay

OrfAB was purified following expression from pAPT158 (Ton-Hoang et al., 1998) under the control of the Phuv promoter and bacteriophage T7 10 ribosome-binding site sequence from pAR30–39 (Studier and Moffatt, 1986).

Figure-of-eight formation as a function of time was performed at 30°C for 1–5 min in a final volume of 50 μl containing 2.5 μg of DNA substrates (pDV19 or pDV28) and 2 μg of transposase, in buffer B (20 mM HEPES pH 7.5, 5 mM DTT, 200 mM KCl, 10 mM MgCl2 and 10% glycerol). Aliquots (10 μl) were removed at intervals and the reaction was stopped with 20 nM EDTA on ice. The samples were then treated with 0.5 mg/ml (final concentration) of proteinase K 1 h at 50°C, and purified with Qiaquick purification minicolumns as described by the manufacturer (Qiagen).

Cloning junctions of a selection of ISs

Both previously identified and potential IRR–IRL junctions from various IS elements were analysed by measurement for the presence and strength of an IRR junction. These include the IS3 family members, IS2 (L.A.Lewis and N.D.Grindley, personal communication), IS2 itself (Sekine et al., 1994), IS907 (Hermans et al., 1991), IS600 (Matsutani et al., 1987), IS911 (this work), and members of other IS families such as IS1 (Turlan and Chandler, 1995), IS21 (Reimann et al., 1989; S.Schmid and D.Haas, personal communication), IS30 (Olasz et al., 1993) and IS492 (Perkins-Balding et al., 1999), whose transposition mechanisms have been postulated to proceed via formation of tandem or circles creating junctions of their ends. The junctions were synthesized with an extension of 5–11 bases on either side of the ~35 and ~10 consensus sequences so as to include the complete sequence of potential promoters. Double-stranded fragments were constructed by annealing complementary synthetic oligonucleotides from 30 to ~55 nucleotides with protruding ends compatible with BamHI and XhoI restriction sites. About 50 pmol of single-stranded fragments were mixed with a ratio of β:1.5 (in order to completely anneal one of them) in TEN buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 50 mM NaCl), boiled for 5 min, and annealed by slowly decreasing the temperature for 4–5 h. The double-stranded fragments were then 5'-phosphorylated with T4 kinase and cloned with T4 DNA ligase (Gibco-BRL) into plasmid pCB267 (Schneider and Beck, 1986), linearized by digestion with BamHI and XhoI. This promoter–probe vector carries a multiple cloning site in upstream of the lacZ marker without promoter. DNA of 10 clones were extracted by alkaline lysates and directly sequenced with Amersham kit. The Phuv promoter was also cloned upstream of the lacZ gene and used as reference for β-galactosidase dosage experiments.

β-galactosidase assays

Cultures of DH5α cells transformed with the different constructions to be tested were grown overnight at 37°C in LB medium supplemented with ampicillin (100 μg/ml) and methicillin (1 mg/ml), and then diluted into fresh medium at an OD600 of 0.05. In experiments where IS911 proteins were supplied in trans, IPTG (1 mM final concentration) was added to induce production under control of the lacuv promoter. After ~2 h of growth (OD600 = 0.3–0.4), β-galactosidase was measured according to the method of Miller (1972), except that cultures were performed in LB medium instead of VB supplemented with glucose and casamino acids. BSA (1 mg/ml) was included in the reaction, and lysis was accomplished by SDS-chloroform treatment. The lysates were centrifuged before measurement at OD520.

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References


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