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Transposase-induced excision and circularization of the bacterial insertion sequence IS*911*

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We have investigated the role of three IS*911*-specified proteins in transposition *in vivo*: the products of the upstream (OrfA) and downstream (OrfB) open reading frames, and a transframe protein (OrfAB) produced by −1 translational frameshifting between *orf*A and *orf*B. The production of OrfAB alone is shown to lead both to excision and to circularization of the element and to be sufficient for intermolecular transposition into a plasmid target. Simultaneous and independent production of OrfA is shown to stimulate OrfAB-mediated intermolecular transposition while greatly reducing the appearance of transposon circles. We have not been able to detect a role for OrfB. Although under certain conditions, the vector plasmid undergoes precise resealing after IS*911* excision, the data suggest that this is not normally the case and that the donor plasmid is not generally conserved. The use of IS*911* derivatives carrying mutations in the terminal 2 bp suggested that circle formation represents a site-specific intramolecular transposition event. We present a model which explains both intra- and intermolecular transposition events in terms of a single reaction mechanism of the 'cut and paste' type.

Key words: IS*911*/IS*3*-family/non-replicative transposition/ transposon circles

Introduction

Transposable elements in bacteria exhibit a disparate set of recombinational properties which, in addition to simple insertion, include the ability to provoke inversions, deletions and replicon fusions (see Berg and Howe, 1989). Not all elements, however, share all these properties nor do they undergo these reactions with similar frequencies.

At the molecular level the transposition process can be described by a limited number of reactions: recognition of the ends of the element and their cleavage by an element-specific transposase, entrapment of a target DNA molecule, cleavage of the target, DNA strand transfer, and religation. In addition, certain elements may undergo replication during transposition. Differences in mechanism from element to element can be viewed to result from permutations of the order in which these reactions occur (see for example Galas and Chandler, 1989; Kleckner, 1990). Although in all cases so far studied a 3'-OH of the transposon is joined to a 5'-P of the target DNA (Craigie and Mizuuchi, 1987; Morisato and Kleckner, 1987; Benjamin and Kleckner, 1989; Brown *et al.*, 1989; Fujiwara and Craigie, 1989; Eichinger and Boeke, 1990; Bainton *et al.*, 1991), the number and order of strand cleavages at the transposon ends can have profound effects on the reaction product. Cleavage can occur simultaneously on both strands at each end, as seems to be the case for IS*10* (Morisato and Kleckner, 1984; Benjamin and Kleckner, 1992), and Tn*7* (Bainton *et al.*, 1991), or on opposite strands at each end, as has been demonstrated for bacteriophage Mu (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987). In the former case, this can give rise to free linear or circular copies of the element. In the latter, an intervening strand transfer and ligation to target DNA physically joins donor and target molecules. Second-strand cleavage then transfers the element to the target molecule with accompanying loss from the donor. Replication of the element without second-strand cleavage generates a replicon fusion.

Previous results obtained with the insertion sequence IS*911* (Figure 1), a member of the widespread IS*3* family, suggested that its transposition may occur without accompanying replication (Prère *et al.*, 1990). This 1250 bp element was originally isolated from *Shigella dysenteriae* as an insertion into the *cI* gene of bacteriophage λ. It carries 36 bp terminal inverted repeats (IRL and IRR) and generates 3 bp (or more rarely 4 bp) direct target repeats on insertion. IS*911* was shown to provoke replicon fusion without duplication. When cloned together with its flanking λ target DNA in a plasmid vector, cointegration is mediated by an active junction composed of IRR and directly adjacent λ sequences which appear to mimic a second end in inverted orientation (see Figure 1A; Prère *et al.*, 1990). Moreover, the IS*911*-mediated integration reaction does not require the left end of IS*911*. A cloned DNA fragment composed of the terminal 50 bp of IRR together with the flanking sequences is proficient in mediating plasmid integration (Polard *et al.*, 1991).

Two functional open reading frames, *orf*A and *orf*B arranged consecutively in the 0 and −1 reading phases respectively are required for this transposition activity and can function *in trans* (Polard *et al.*, 1991). Products of the first (OrfA) and second (OrfB) open reading frames have been detected. A third protein, OrfAB, derived from both reading frames is produced by a programmed −1 translational frameshift on an A_6G heptanucleotide localized in the 3' end of *orf*A (Figure 1B). Although the functions of these proteins have yet to be defined in detail, the product of the downstream frame, OrfB, shows significant similarities to a well conserved region of retroviral integrases (Fayet *et al.*, 1990; Khan *et al.*, 1991) while OrfA exhibits an α-helix−turn−α-helix motif which may be involved in recognition of and binding to the ends of the element. Moreover, OrfAB, which is composed of both 'domains', has been shown to be sufficient for IS*911*-mediated integration when provided *in trans* (Polard *et al.*, 1991). OrfAB can be produced constitutively by the introduction of an additional A residue in the A_6G string (the A_7G

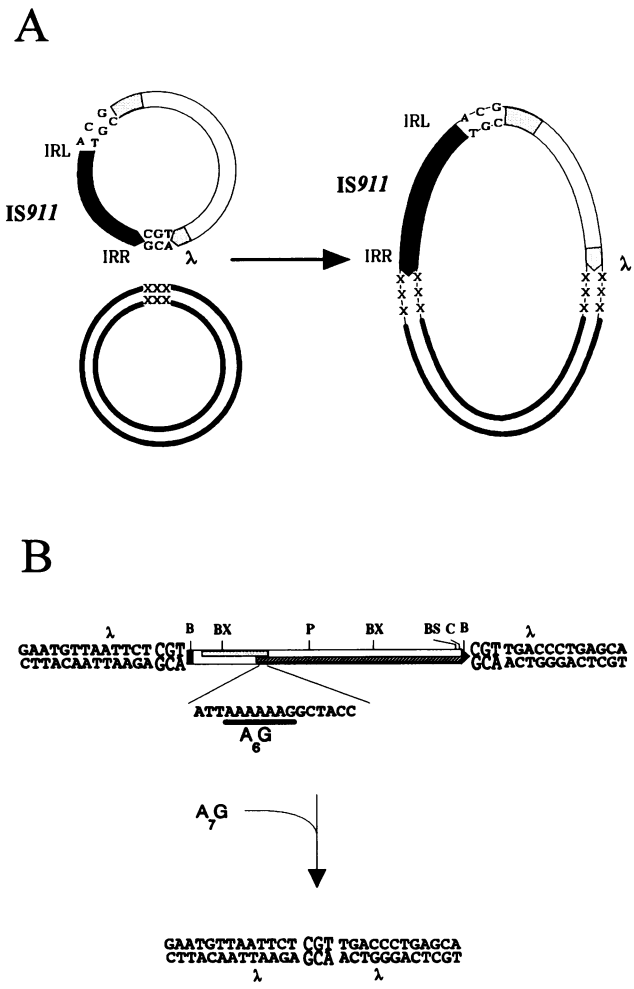


Fig. 1. (**A**) Non-replicative integration mediated by the IS*911* IRR−λ active junction. The original isolate of IS*911* was obtained by insertion into a fragment of bacteriophage λ. The element (shaded box) was subsequently cloned into the pBR322 derivative plasmid, pAT153 (open segment) with flanking λ sequences (stippled boxes). The left (IRL) and right (IRR) inverted repeats are indicated. The 3 bp nucleotide sequence duplicated by insertion (CGT) into the target is indicated. The nucleotide sequence of DNA flanking IRR (pointed end of the box) resembles an inverted IS*911* end. Cointegrate formation between this plasmid (pOFT139) and pOX38Km is shown to occur using IRR and the λ end. It results in loss of one copy of the repeated CGT sequence and generation of a 3 bp duplication (XXX) in the target plasmid. (**B**) Organization of IS911 and flanking sequences: left (IRL) and right (IRR) terminal inverted repeats are indicated as a black box and arrow respectively. Pertinent restriction sites are shown above the figure (B, *Bal*I; BS, *Bst*EII; BX, *Bst*XI; C, *Cla*I; P, *Pvu*II). The *orf*A and *orf*B frames are denoted by shaded and cross-hatched boxes within the IS. The flanking phage λ sequences and the 3 bp target duplication 5′CGT 3′ are indicated. The relative location of the DNA sequence carrying the heptanucleotide permitting frameshifting (A_6G) is included below. The junction sequence in plasmids recovered following attempts to clone the A_7G mutation is shown at the bottom of the figure.

mutation), which does not modify the amino acid sequence but eliminates the production of both OrfA and OrfB.

In the work presented here, we have studied the role of the three IS*911*-encoded proteins in transposition involving both natural ends of the element. We show that supplying OrfAB alone to derivatives of IS*911* results in their excision and the production of high levels of supercoiled circular copies. Excision activity is also accompanied by a stimulation of intermolecular transposition. OrfA and OrfB produced individually or together cannot substitute for OrfAB nor do they specify any detectable transposition activity. However, simultaneous and independent production of OrfA not only reduces OrfAB promoted circle formation but can lead to a further stimulation of intermolecular transposition.

These results, together with those obtained from analysis of the behaviour and structure of circles generated from IS*911* derivatives carrying a defective mutant left (IRL) or right (IRR) end, supports the notion that IS*911* transposes by a non-replicative mechanism involving cleavage of the element from its donor site.

Results

Consequences of constitutive OrfAB production from a functional copy of IS911

Constitutive production *in trans* of OrfAB from the A_7G mutant is sufficient to mediate IS*911* integration (Polard *et al.*, 1991). In order to analyse further the role of OrfAB, we have determined the effect of this mutation in an intact copy of IS*911* (see Figure 1B). The internal *Bst*XI fragment of an IS*911* derivative deleted for both inverted terminal repeats and carrying the A_7G mutation (pAPT86; Polard *et al.*, 1991) was substituted for the corresponding wild-type fragment carried by the plasmid pOFT139. This plasmid is a derivative of pAT153 which carries the original IS*911* isolate together with its flanking phage λ target sequences (Prère *et al.*, 1990). Suprisingly, transformation gave rise only to microcolonies following overnight growth on selective medium. These took a further 24 h to reach a normal size. A parallel control experiment using the unmutagenized wild-type fragment gave colonies of normal size after overnight growth. Twelve microcolonies were isolated after 48 h of growth and their plasmid content analysed by agarose gel electrophoresis. A single plasmid species having a size and restriction pattern consistent with a deletion of IS*911* was observed in all clones (data not shown), suggesting that excision of the element had occurred in all cases.

Insertion of IS*911* into the original phage λ target fragment generated a 3 bp direct duplication (CGT) of the target DNA on each side of the element at the point of insertion. The nucleotide sequence across the λ−IS*911* 'junction' of four deletion derivatives was determined and found to be composed of the original target sequence (carrying a single copy of the CGT repeat; Figure 1B). This therefore confirms that IS*911* had excised precisely.

Since the λ sequences adjacent to IRR were previously shown to be involved in integration (as indicated in Figure 1A), it was important to determine whether they also play a role in this phenomenon. The active IRR and flanking sequences were therefore exchanged for a DNA fragment containing a second right junction with different flanking sequences, derived from an IS*911* insertion into pOX38Km, to generate pAPT28 (Materials and methods). This junction is inactive in integration (P.Polard, unpublished results). While the transformation efficiency of potential A_7G mutants was similar to that obtained above and again only microcolonies were observed, the plasmids isolated from transformants were heterogeneous in size. Of four that were analysed at the nucleotide sequence level, all had retained part of the left IS*911* end and had undergone a deletion across the right end and into the pBR322 vector sequence (data not

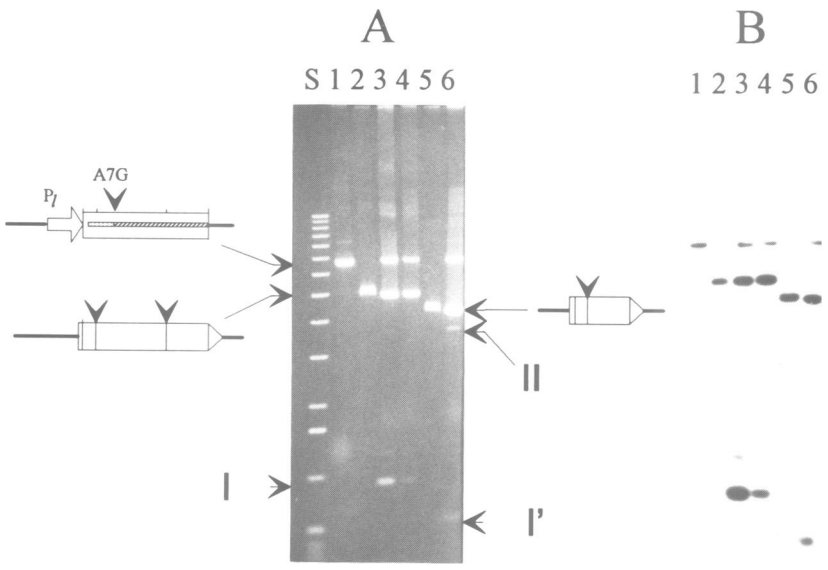


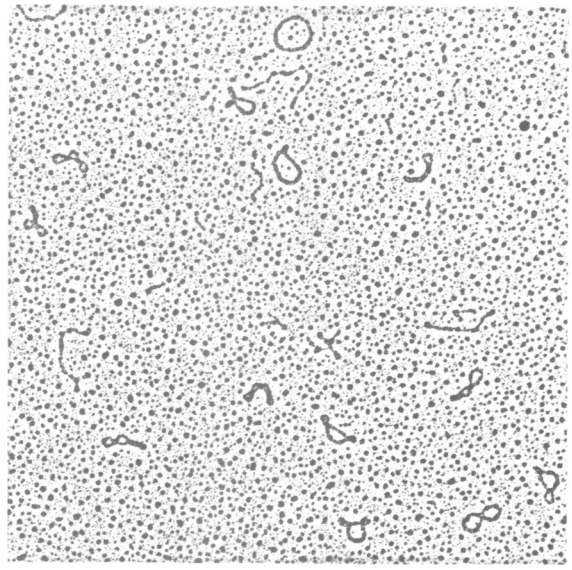
Fig. 2. Effect of supplying OrfAB *in trans* to inactivated IS*911* derivatives. (**A**) The left of the figure shows the structure of the p15A-based plasmid used to supply OrfAB (a *BalI* internal fragment carrying the A_7G mutant reading frame) under the control of a phage λ P_I promoter (shown as arrow). The inactivated IS*911* excision substrate, pOFT159 (mutated at the two *Bst*XI sites; vertical arrowheads) is shown below. The inactivated IS*911* excision substrate, pOFT158 (deleted between the two *Bst*XI sites; vertical arrowhead) is shown on the left. The nature of forms I, I′ and II are described in the text. A 0.8% ethidium bromide stained agarose gel in which each lane contains the equivalent of 0.5 ml of an overnight culture is presented. Plasmid preparations were linearized with *Eco*RI, which does not cleave within the transposable element, prior to electrophoresis. S, 1 kb standard linear ladder; lane 1, pAPT66; lane 2, pOFT159; lane 3, pAPT66 + pOFT159 37°; lane 4, pAPT66 and pOFT159 30° +IPTG; lane 5, pOFT 158; lane 6, pAPT86 + pOFT158 37°. (**B**) A Southern transfer of the gel shown in (A) using the internal *BalI*−IS*911* fragment as probe.

shown). This indicates that the flanking phage λ DNA is important for correct vector reconstitution but not for the instability provoked by the IS*911* A_7G mutation.

Precise excision of IS911 in trans

The high frequency of IS*911* excision implied by the results presented above suggests that this phenomenon is induced by OrfAB acting *in cis* on the two correctly oriented IS*911* ends of the mutated element. It seemed possible that such behaviour might occur if OrfAB were supplied *in trans*, a property which would facilitate further analysis of this activity.

To examine this possibility we used a system in which the 'substrate', a copy of IS*911* mutated by 4 bp deletions in both the A and B frames at the two *Bst*XI sites (Figure 1B), is carried by a pBR322-based plasmid, pOFT159 (Table III). This inactive IS*911* derivative was chosen to avoid possible complications due to the expression of endogenous IS*911* gene products. The various IS*911* reading frames are driven *in trans* by the λ-P_I promoter on a compatible p15A-based backbone which also carries the λ cI857 repressor gene (Table III). It should be noted that these derivative plasmids were generated using the internal *BalI* IS*911* fragment (Figure 1B) and therefore do not carry active IS*911* ends. The general structure of the vector plasmids and the IS*911* derivatives used are shown in Figure 2. In an initial experiment we used plasmid pAPT66 (Table III) which produces the OrfAB fusion protein constitutively due to the presence of the A_7G mutation. Plasmid DNA isolated from the equivalent of 0.5 ml overnight cultures was analysed by agarose gel electrophoresis and visualized by ethidium bromide staining. The results (Figure 2A, lanes 3 and 4) show the presence of an intense band (form I) and a second faint band (form II) in addition to those of the parental plasmids (lanes 1 and 2).



IRR IRL

CAGTTTTTGTTGACCACTTCA CGT TGAAGTGGCACTGAATTTGGCC
GTCAAAAACAACTGGTGAAGT GCA ACTTCACCGTGACTTAAACCGG

Fig. 3. Characterization of IS911 excision products. The top of the figure shows an electron micrograph of gel-purified form I isolated from DNA (pAPT66 + pOFT159 37°) shown in Figure 2, lane 4. The lower part of the figure shows the nucleotide sequence at the circle junction. This was determined using the oligonucleotide primers: 5′ TGGAAAAAACTCTAACTC 3′ and 5′ GTGAGCATATCACCTC-TG 3′ from the left (i.e. for IRR as shown in the figure) and right (for IRL) respectively.

Although these results were obtained from alkaline minipreparations of DNA, essentially identical results were obtained using DNA prepared under neutral conditions (data

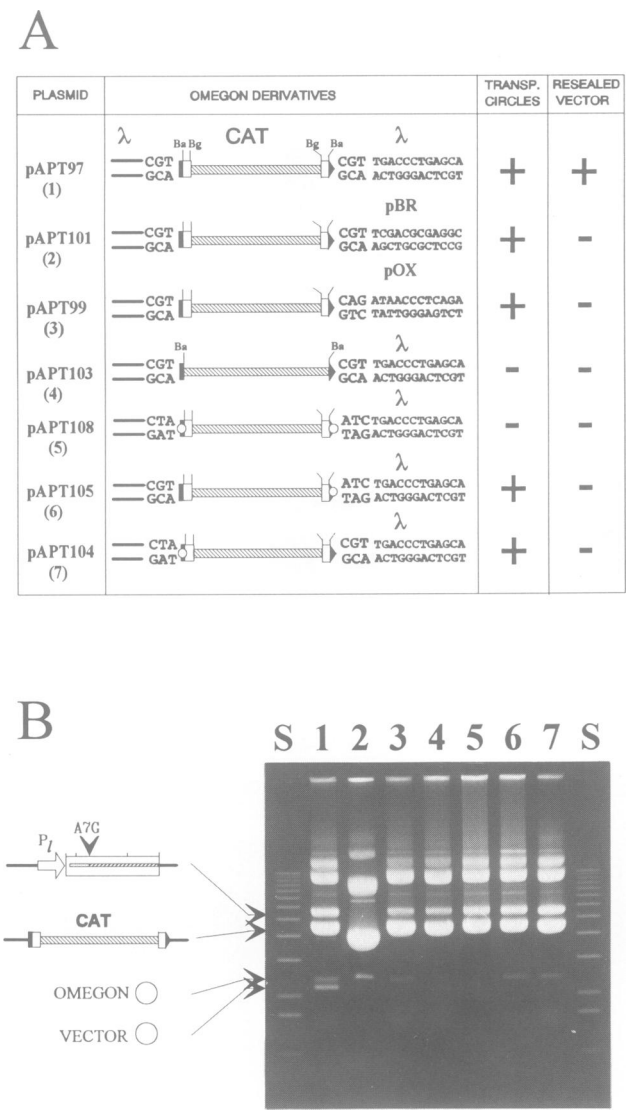


Fig. 4. Circle formation with various Ω-on derivatives. (**A**) The general structure of the Ω-on-carrying plasmids is shown together with the flanking nucleotide sequences. The plasmid designation together with the corresponding lane number in (**B**) are indicated in the right-hand column. The chloramphenicol resistance gene (CAT) delimited by two hybrid *Bgl*II−*Bam*HI sites (Bg) is indicated by cross-hatching; IS*911* left (box) and right (pointed box) terminal inverted repeats are shown; circles superimposed on these ends indicate the terminal dinucleotide mutations; the external 23 bp of each repeat delimited by *Bal*I (Ba) sites is shown as a filled box; flanking 3 bp 'repeats' are highlighted; the nucleotide sequence of the IRR junctions with λ, pBR322 and pOX38Km are also indicated. A summary of the results of the circle assay (from data such as those shown in B) is included on the right of the figure. (**B**) A 0.8% ethidium bromide stained agarose gel. Note that since the samples shown here were not cleaved with restriction enzymes, both multimeric and monomeric plasmid species are present. The positions of monomeric Ω-on-carrying plasmids, co-resident transposase producing plasmid [pAPT66 (~7 kb) in the case of lane 2, and pAPT86 (~9 kb) in the case of the other lanes] and the Ω-on and resealed vector products are shown at the left. Plasmid pAPT101 (lane 2) is significantly smaller (~7 kb) than the other Ω-on-carrying plasmids since it was derived by deletion of λ and pBR322 sequences flanking IRR. S, 1 kb standard ladder; lane 1, pAPT97; lane 2, pAPT101; lane 3, pAPT99; lane 4, pAPT103; lane 5, pAPT108; lane 6, pAPT105; lane 7, pAPT104.

not shown). Form I was more pronounced in cultures grown at 37°C, a temperature at which partial induction of the P_I promoter occurs, than at 30°C (compare lanes 3 and 4 respectively).

The size of form I suggested that it could represent an excised IS*911*. This was supported by the observation that substitution of the mutated IS*911* (1242 bp) excision substrate carried by pOFT159 for a smaller derivative (620 bp) obtained by deletion between the *Bst*XI sites (pOFT158) generated a band identical in size to form II together with a faster migrating band (Figure 2A lane 6). The results of Southern transfer experiments using IS-specific probes (Figure 2B) demonstrate that form I carries IS-specific sequences and that form II does not. An identical experiment using a vector-specific probe (data not shown) demonstrated that form II carries vector-specific sequences while form I does not.

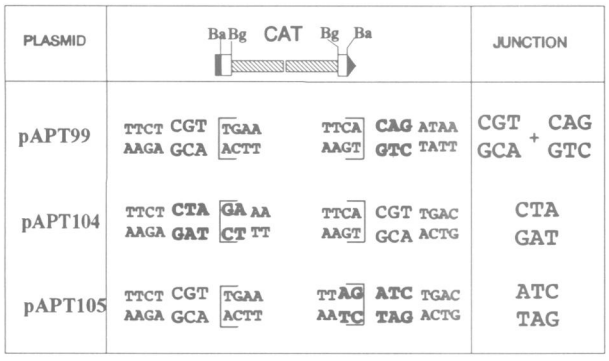
Form II, like the product obtained during our attempts to introduce the A_7G mutation into the entire IS*911*, migrates with an apparent size identical to that expected of a circular vector molecule deleted for IS*911*. The nature of this product was confirmed, after transformation of the gel-purified species, by suitable restriction analysis, and by determination of the nucleotide sequence across the λ−IS*911* junction (data not shown).

Restriction analysis (data not shown) of gel-purified excision products from the experiment shown in Figure 2 (lane 3) indicated that form I is a circular form of the IS which has undergone no detectable rearrangements. Furthermore, a sample of gel-purified form I examined under the electron microscope, contained a unique species of double stranded supercoiled circles (Figure 3) having a contour length of 1.2 ± 0.1 kb (n = 25), the size expected for the IS*911* derivative. Finally the nucleotide sequence of the circle junction was determined from gel-purified form I DNA. It is composed of the left and right IS*911* ends flanking one of the original CGT direct target repeats (Figure 3).

Effect of flanking sequences on circularization

The plasmids used in the experiments described above carry the reactive junction involved in IS*911*-mediated plasmid integration (Figure 1A). To determine whether this active IRR junction plays a role in circle formation, we constructed an IS*911* derivative (omegon; Ω-on) carrying 56 bp of IRL and 51 bp of IRR separated by a 3.6 kb DNA fragment which specifies resistance to chloramphenicol (Fellay *et al.*, 1987; Materials and methods).

When supplied with OrfAB *in trans*, this plasmid (pAPT97, Figure 4A, line 1) like those described above, gave rise to a species with the size (Figure 4B, lane 1) and restriction pattern (data not shown) expected for a closed circular copy of the excised omegon with normal levels of supercoiling (3.8 kb) and a species exhibiting the size of the precisely resealed vector. The right end of the Ω-on carried by pAPT97 was then substituted for an end that retains the homologous target repeat, CGT, but in which pBR322 sequences replace those of λ (pAPT101; Figure 4A, line 2). This exchange has been shown to eliminate the activity of the IRR junction in the IS*911*-mediated integration reaction (P.Polard, unpublished observations). While this did not affect the production of transposon circles, the resealed vector species was absent (Figure 4B, lane 2). Precise resealing of the vector backbone but not circularization of the transposon therefore requires the λ sequences which flank the right end. This result is consistent with the observation that, in the absence of the flanking λ sequences, attempts to introduce the A_7G mutation into an intact IS*911* resulted in the appearance of resealed vector molecules of



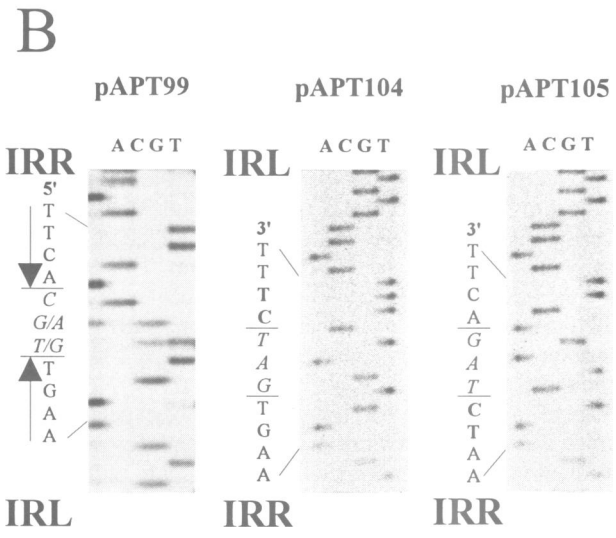
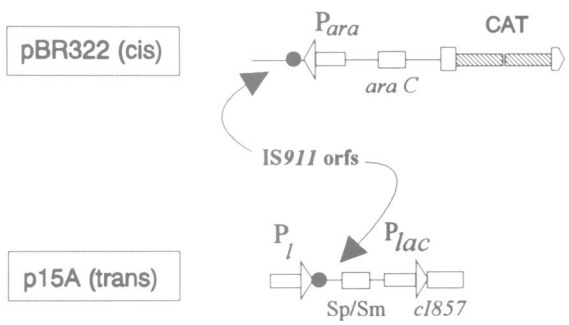


Fig. 5. Junction sequences obtained from Ω-on circles. (**A**) Plasmid designations are shown in the left-hand column. The nucleotide sequences across IRL and IRR junctions are shown for each of the Ω-on derivatives. The flanking 3 bp together with the mutated terminal dinucleotides are shown in bold. The terminal base pairs of IRL and IRR are outlined. The nucleotide sequence of the transposon circle junctions is shown in the right-hand column. (**B**) Sequencing gels showing the junction on one strand of the excised Ω-ons. The terminal nucleotides of IRR and IRL are indicated by vertical arrows. Mutant terminal dinucleotides are shown in bold. The three intervening nucleotides at the junctions are italicized. Oligonucleotides used as sequencing primers were as described in the legend to Figure 3. The sequence shown for pAPT99 was from IRL towards IRR whereas that for pAPT104 and pAPT105 was from IRR towards IRL.

heterogeneous size. It implies that circularization of vector and transposon are not reciprocal events.

IS*911* generates a 3 bp target duplication on insertion. The transposon circles described above retain one copy of the target repeat at the circle junction. It is possible that these repeats are directly involved in the excision pathway. They could, for example, give rise to complementary single strand extensions, analogous to those generated by many restriction enzymes, which might facilitate circle closure. To determine whether they participate in generating transposon circles, the IRR-CGT target repeat of the Ω-on was changed to CAG (Figure 4A, line 3; Materials and methods). The results of excision assays with the heterologous flanking sequences (pAPT99) are presented in Figure 4B (lane 3) and demonstrate that transposon circularization still occurred at high levels. Thus complementarity of the flanking sequences



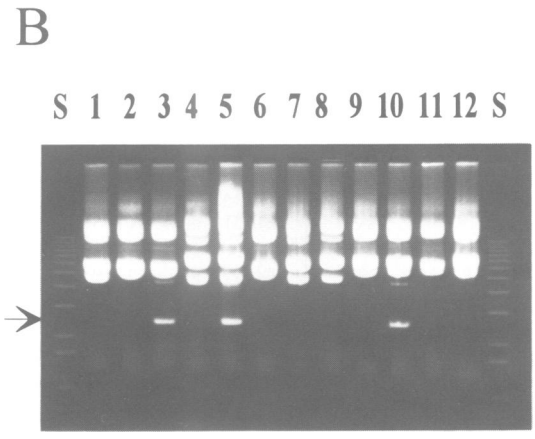


Fig. 6. *Cis*/*trans* complementation. (**A**) Structure of plasmids used in the *cis*/*trans* complementation assay. See text for a description. (**B**) 0.8% ethidium bromide stained agarose gel. The position of the Ω-on circles is indicated by an arrow on the left of the figure. S, 1 kb standard ladder; lane 1, pAPT21(*trans* none) + pAPT137(*cis* none); lane 2, pAPT23(A,B,AB) + pAPT137; lane 3, pAPT86(AB) + pAPT137; lane 4, pAPT21 + pAPT138(A,B,AB); lane 5, pAPT21 + pAPT140(AB); lane 6, pAPT81(A) + pAPT137; lane 7, pAPT21 + pAPT141(A); lane 8, pAPT21 + pAPT139(B); lane 9, pAPT81(A) + pAPT139(B); lane 10, pAPT86(AB) + pAPT139(B); lane 11, pAPT86(AB) + pAPT141(A); lane 12, pAPT81(A) + pAPT140(AB).

does not play a crucial role in circle formation. Interestingly, when the junction sequence in a population of gel-purified circles from pAPT99 was determined (Materials and methods) it proved to be composed of a mixture of both 3 bp heterologous flanking sequences (Figure 5A, line 1 and 5B). This observation indicates that there is no preference for one or other of the flanking sequences to form the circle junction.

Dependence of circle formation on the inverted terminal repeats

The results presented above suggest that the 36 bp terminal inverted repeats may be sufficient for circle formation. This is supported by the observation that an Ω-on derivative carrying only the terminal 22 bp of each end together with the λ sequences flanking IRR (pAPT103, Figure 4A, line 4) failed to exhibit either circularized transposons or the precisely resealed vector (Figure 4B, lane 4).

To examine further the nucleotide sequences at the ends of IS*911* necessary for circle formation, we generated Ω-on derivatives carrying mutations in the terminal two base pairs.

Table I. Effect of wild-type Orfs and OrfAB on Ω-on transposition and circularization

Configuration			Activities		
	trans	*cis*	Circles	Transposition	*n*
1.	–	–	–	5×10^{-8}	4
2.	WT	–	–	2×10^{-6}	6
3.	AB	–	+	7×10^{-6}	6
4.	–	WT	–	2×10^{-4}	6
5.	–	AB	+	3×10^{-4}	6
6.	A	–	–	3×10^{-8}	4
7.	–	A	–	3×10^{-8}	4
8.	–	B	–	3×10^{-8}	4
9.	A	B	–	3×10^{-8}	4
10.	AB	B	+	6×10^{-6}	5
11.	AB	A	–	7×10^{-5}	6
12.	A	AB	±	1×10^{-3}	5

Line 1, pAPT21(*trans* none) + pAPT137(*cis* none); line 2, pAPT23(A, B, AB) + pAPT137(*cis* none); line 3, pAPT86(AB) + pAPT137; line 4, pAPT21 + pAPT138(A,B,AB); line 5, pAPT21 + pAPT140(AB); line 6, pAPT21(*trans* none) + pAPT139(B); line 7, pAPT21(*trans* none)+ pAPT141(A); line 8, pAPT81(A) + pAPT137(*cis* none); line 9, pAPT81(A) + pAPT140(B); line 10, pAPT86(AB) + pAPT139(B); line 11, pAPT86(AB) + pAPT141(A); line 12, pAPT81(A) + pAPT140(AB). The numbers shown on the left of the figure indicate the lane numbers of Figure 6B. *n* is the number of independent mating assays. WT indicates production of A,B and AB.

These nucleotides are highly conserved among the 20 members of the IS*3* family and are therefore likely to be important for transposition activity. We constructed Ω-on derivatives in which these residues were mutated at both ends (pAPT108), in IRR (pAPT105) or in IRL (pAPT104) (Figure 4A, lines 5, 6 and 7 respectively). The mutations were also designed to introduce differences in the 3 bp flanking sequence. The nucleotide sequences of these derivatives are shown in Figures 4A and 5A. The results of the circularization assay are shown in Figure 4B. Mutation of both ends (Figure 4A, line 5 and 4B, lane 5) abolished transposon circle formation whereas mutation of either IRR (Figure 4A, line 6 and 4B, lane 6) or IRL (Figure 4A, line 7 and 4B, lane 7) had no perceptible effect. These mutant ends are thus presumably deficient in at least one of the steps in the circularization reaction since they cannot function together. However, a single defective end can be rescued by the presence of a wild-type end. Moreover, although the original λ sequences are present flanking IRR, no resealed vector plasmid species could be detected with either the single- or double-ended mutants. This implies that precise vector resealing mediated by the λ sequences, in contrast to integration, requires both ends of IS*911*. The reasons for this are as yet unclear.

The consequent asymmetry introduced into the transposon circularization reaction by a single defective mutant end might be reflected in the nature of the junction sequence of the transposon circle. This was determined for both single mutant derivatives and is presented in Figure 5A (lines 2 and 3) and 5B. Significantly, in both cases the 3 bp retained at the junction were exclusively those associated with the mutant end. Thus in contrast to the mixed junction sequence obtained with two wild-type ends and heterologous flanking sequences (Figure 5A, line 1), mutation of one end resulted in asymmetric ligation and/or strand transfer. This strongly suggests that circle formation can result from a single-ended attack of the active end on the second defective end.

Effects of OrfA, OrfAB and OrfB in circularization and in the transposition process

Previous results (Polard *et al.*, 1991) have demonstrated that OrfAB alone is sufficient to promote intermolecular transposition. In order to assess the possible roles played by OrfA and OrfB in both transposition and circularization, we have used a two-plasmid system in which IS*911* gene products can be supplied from independently regulated promoters *in cis* or *in trans* in various combinations (Figure 6A). The p15A-based compatible plasmid used to supply functions *in trans* carries the temperature regulated λ P_l promoter together with the λ *cI857* gene. The pBR322-based plasmid used to drive expression of the various reading frames *in cis* carries the promoter of the *araBAD* operon of *Escherichia coli* (P_{araBAD}) whose expression can be regulated by arabinose. In order to standardize expression in this series of plasmids, the ribosome binding site of the cloned *orf*B gene was replaced by that of *orf*A and its AUU initiation codon was changed to AUG (Materials and methods). The translation initiation signals of OrfA, OrfAB and OrfB are thus identical. The plasmids also carry an Ω-on in which the λ sequences at the active IRR junction have been removed. This not only prevents precise resealing of the vector backbone but also eliminates integration mediated by the IRR−λ active junction, thus simplifying interpretation of Ω-on transposition frequencies.

Table I and Figure 6B show the results of experiments in which the intermolecular transposition frequency and circle production were determined after induction of both the P_{araBAD} and P_l promoters. Intermolecular transposition was measured by the mating-out assay (Materials and methods) using the conjugal plasmid pOX38Km as target replicon. In the absence of IS*911* transposition functions, no transposon circles were observed and only low levels of 'cointegrate' transfer could be detected (Table I, line 1; Figure 6B, lane 1). This background level was characterized by transfer of both the antibiotic resistance gene associated with the transposon (Cm^R) and that carried by the donor plasmid (Ap^R). It is presumably the result of low efficiency site-specific recombination between the pBR322 derivative plasmid and the target replicon pOX38 as observed elsewhere (O'Connor and Malamy, 1984). In cases where transposition was observed to occur, the frequency of doubly resistant exconjugants was increased but was always at least two orders of magnitude lower than Cm^R exconjugants. This suggests that the majority of transposition events occur as direct insertions whereas the doubly resistant clones could result either from true cointegrate formation, or reflect direct transposition from low levels of multimeric plasmids in the population (see for example Berg, 1983).

Circles were not detected if all three proteins were provided *in trans* from the wild-type IS*911* sequence (Table I, line 2; Figure 6B, lane 2), but were clearly visible when the transframe protein OrfAB was supplied 'constitutively' from an IS*911* sequence carrying the A_7G mutant reading frame (Table I, line 3; Figure 6B, lane 3). This relative increase in circle formation was not, however, accompanied by a large increase in intermolecular transposition (Table I, compare lines 2 and 3).

Constitutive production of OrfAB *in cis* also resulted in

Table II. Effect of changes in the levels and ratio of OrfA and OrfAB

Configuration		Activities		
trans	*cis*	Circles	Transposition	*n*
–	WT uninduced	–	3×10^{-6}	4
–	WT	–	2×10^{-4}	6
WT	–	–	2×10^{-6}	6
WT	A	–	2×10^{-6}	6

Line 1, pAPT21(*trans* none) + pAPT138 (A,B,AB) (no arabinose); line 2, pAPT21 + pAPT138; line 3, pAPT23(A,B,AB) + pAPT137; line 4, pAPT23(A,B,AB) + pAPT141(A).

transposon circle production (Figure 6B, lane 5) and in an increase in transposition (40- to 50-fold) compared with that observed *in trans* (compare Table I, lines 5 and 3). This may reflect more efficient action *in cis*, as has been shown for other transposons (see Derbyshire *et al.*, 1990; Kleckner, 1990), but could also be the result of differences in the level of protein production from the two promoters. Transposition was stimulated to similarly high levels with the wild-type IS*911* functions *in cis* (Table I, line 4) compared with the *in trans* configuration (line 2) but, again, no transposon circles can be detected in the presence of wild-type functions (Figure 6B, lane 4). Thus the level of circle production is not correlated with intermolecular transposition frequencies. These results suggest that transcription of *orfA* and *orfB* in the wild-type configuration may supply a function, absent in the constitutive frameshift mutant, which blocks circle production but not intermolecular transposition.

Two candidates for this effect are OrfA and OrfB. We therefore examined the effect of both proteins *in cis* or *in trans* on OrfAB-mediated transposition and circle formation. The results indicate that OrfA or OrfB, alone or together, did not promote transposition (Table I, lines 6, 7, 8 and 9) or circle formation (Figure 6B, lanes 6, 7, 8 and 9). However, simultaneous production of OrfA *in cis* or *in trans* not only eliminated or reduced OrfAB-mediated circle formation (Figure 6B, lanes 11 and 12) but also stimulated intermolecular transposition (Table I, compare line 3 with 11 and 5 with 12) whereas OrfB had no effect (Table I, compare line 3 with 10; Figure 6B, lane 10).

These results indicate that, alone, OrfAB is sufficient to induce transposon circularization and to promote intermolecular transposition at high levels and are consistent with its proposed role as the IS*911* transposase. Furthermore, they suggest that OrfA may affect the balance between transposition and circle formation.

Importance of the ratio of OrfA and OrfAB in determining the outcome of transposition

The stimulatory effect of OrfA on OrfAB-mediated transposition may simply reflect the fact that its presence inhibits OrfAB from acting intramolecularly therefore permitting the protein to fulfil its intermolecular role. This view implies that transposition activity is determined primarily by the quantity of OrfAB and that optimal intermolecular transposition requires a concomitant level of OrfA to prevent sequestration of the transposon substrate into circular forms. Intermolecular transposition should thus be insensitive to excess production of OrfA.

In order to address the first prediction, we have measured the intermolecular transposition frequency mediated by proteins produced from wild-type open reading frames *in cis* at different levels of induction of the P_{araBAD} promoter. The results, presented in Table II, show that increasing transcription promoted increasing transposition frequencies (lines 1 and 2) without accompanying production of transposon circles (data not shown). This demonstrates that transposition activity is determined by the quantity of IS*911* proteins.

We have also determined the effect of production of additional OrfA *in trans* or *in cis* on transposition mediated by wild-type reading frames in which the ratio of OrfA and OrfAB, determined by translational frameshifting, is normally in favour of OrfA (Polard *et al.*, 1991). The level of intermolecular transposition observed with the wild-type configuration was unaffected by additional OrfA (Table II, lines 3 and 4).

Although these results do not formally rule out the possibility that OrfA plays an active role in transposition, they suggest that overall transposition activity is determined by the quantity of OrfAB and that the presence of OrfA may simply channel transposition into an intermolecular pathway.

Discussion

The available data support the idea that IS*911* transposes using a 'cut and paste' mechanism. The element promotes non-replicative insertion (Prère *et al.*, 1990; Polard *et al.*, 1991) and is excised from its donor replicon when provided with high levels of the OrfAB protein (Figure 1). Moreover, in all the intermolecular transposition assays presented in Tables I and II, the transposon-(Cm^R) and donor plasmid-(Ap^R) resistance markers were found associated with the target plasmid at frequencies which were at least two orders of magnitude lower than Cm^R alone. Since it is possible that such 'cointegrates' arise from transposition from plasmid multimers, intermolecular transposition might result almost exclusively from insertion of the transposon alone (direct transposition) into the target molecule rather than from transposon-mediated replicon fusion (cointegration).

Supplying the IS*911* OrfAB protein *in trans* to various derivatives of IS*911* provokes transposon excision to a level which can be readily detected in DNA from small overnight cultures in ethidium bromide-stained agarose gels (Figures 2, 4B and 6B). The transposon products are double-stranded supercoiled closed-circular molecules as judged by electrophoretic mobility, restriction mapping (data not shown) and electron microscopy (Figure 3).

Since there appears to be no correlation between the level of circles and the frequency of intermolecular transposition (Tables I and II) and transposon circles are relatively stable during a 2 h chase in the absence of transposase synthesis (O.Fayet, unpublished results), we do not think that the circles represent obligatory transposition intermediates. This does not necessarily mean that they cannot participate as substrates in transposition. Indeed the active junction composed of IRR separated by 3 bp from flanking λ sequences promotes relatively efficient integration if supplied with OrfAB (Polard *et al.*, 1991) and formally resembles the transposon circle junction. Moreover, in the case of IS*21*, whose transposase, like that of IS*911*, exhibits the characteristic retroviral-like integrase domain (Fayet *et al.*, 1990; Khan *et al.*, 1991), transposition has been shown to

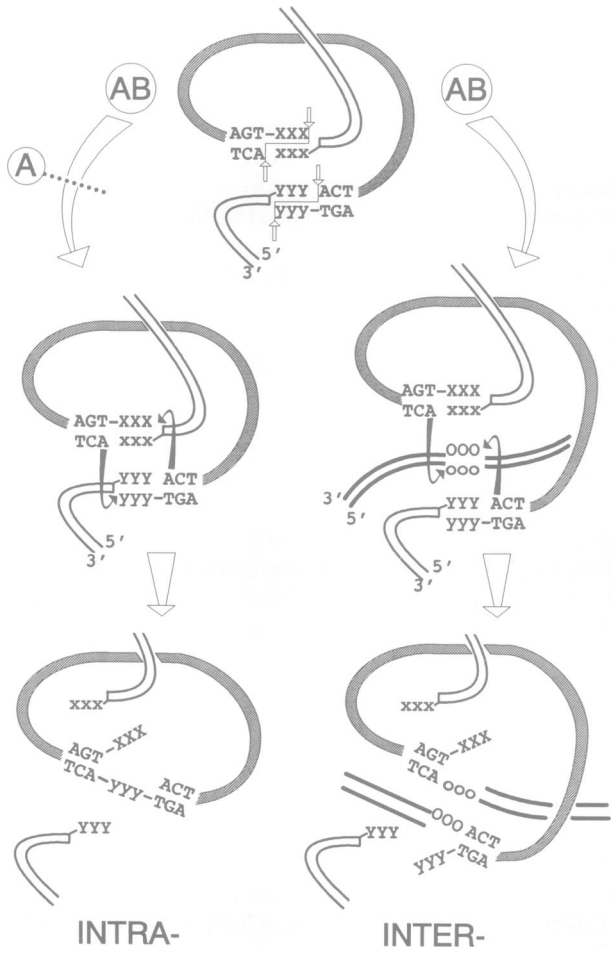


Fig. 7. Model for circle formation and intermolecular transposition. The proposed circle-formation pathway is shown on the left of the figure and that for intermolecular transposition on the right. Transposon DNA is shaded; DNA outside the transposon is shown as an unfilled box; target DNA is shown as bold lines; transposase-mediated cleavages are represented by unfilled vertical arrows; filled curved arrows indicate attack by the -A-OH-3′ on target phosphates; the 3 bp external to the transposon are shown as XXX (xxx) and YYY (yyy); the 3 bp destined to be duplicated in the intermolecular target are shown as OOO (ooo); AB and A indicate OrfAB and OrfA proteins; for simplicity, OrfA is shown as blocking the OrfAB-mediated circularization reaction (upper left).

occur using the abutted ends of two tandem copies separated by 2 or 3 bp (Reimann *et al.*, 1989). Similar observations have been made in the case of IS*3* (Spielmann-Ryser *et al.*, 1991). These structures are also formally similar to both the IS*911* IRR−λ active junction and the transposon circle junction.

We argue below that excision of IS*911* can result in the formation of transposon circles and in intermolecular transposition and we provide a model which explains both these events in terms of a single reaction mechanism. We discuss the fate of the donor molecule during transposition and we examine the manner in which OrfA and OrfAB may intervene to determine the outcome of the reaction.

Circle formation and intermolecular transposition reactions

IS*911* normally generates a 3 bp (or more rarely 4 bp) target duplication at its point of insertion (Prère *et al.*, 1990; Polard *et al.*, 1991). Transposase-induced excision results in a circle whose junction is composed of abutted IRL and IRR flanking an adjacent 3 bp from the original target sequence (Figures 3 and 5). The efficiency of circle formation, however, does not appear to depend on the nature of the adjacent sequences. A transposon with direct 3 bp target repeats generates circles carrying a single copy of the repeat whereas circles generated from an element with heterologous flanking sequences carry intervening 3 bp composed of both sequences on both strands (Figure 5). These results imply that OrfAB may introduce 3 bp staggered cuts at both transposon ends in a similar way to that observed *in vitro* in the case of Tn*7* (Bainton *et al.*, 1991). Moreover, the nucleotide sequence of the junction generated from derivatives which carry one wild-type and one defective IS*911* end suggests that circles are formed by strand transfer of the active end into the defective end. A model depicting these results and underlining the similarity of the inter- and intramolecular reactions is presented in Figure 7.

In view of the similarity of IS*911* ends to those of certain retroviruses (Fayet *et al.*, 1990) and the known mechanism of retroviral integrase-mediated cleavage and integration (Fujiwara and Mizuuchi, 1988; Brown *et al.*, 1989; Katzman *et al.*, 1989; Craigie *et al.*, 1990; Katz *et al.*, 1990), we propose that OrfAB binds and cleaves both ends. We have depicted staggered cuts producing a 5′ overhang of 3 bp containing the target 'repeats', which would separate IS*911* from its donor molecule. It should be noted, however, that a single strand cut would be sufficient to produce a 3′OH end (3′-OH.ACT-5′) which could then attack 5′ phosphates in a target site. This would be consistent with the known activity of retroviral integrases (Craigie *et al.*, 1990; Katz *et al.*, 1990). It would leave single strand connections to vector DNA which could subsequently be processed by OrfAB or cellular components before or after strand transfer.

Intermolecular transposition is proposed to occur with simultaneous attack by each end of an uncleaved target on opposite strands at positions staggered by 3 bp (Figure 7, right). This leaves the original 3 bp flanking target sequences unpaired. Repair at the unjoined ends removes the accompanying target sequences and generates flanking repeats in the new target.

To generate transposon circles, strand transfer is proposed to link the 3′-OH-AC of one end to the second end at a distance of 3 bp from the terminal GT-5′dinucleotide (Figure 7 left). Although attack could occur on an uncleaved 5′ end, we have depicted a precleaved second end for reasons outlined below. This could generate a circle which is covalently closed on only one strand. Repair of the second unjoined strand would then create a complementary junction sequence. Such a mechanism explains the observation that the junction sequence in such mutants derives uniquely from the external 3 bp of the mutated end (Figure 5). It also explains the heterologous junction sequences generated from derivatives carrying two non-mutant ends but with different external 3 bp at each end.

Although mutation of the terminal 2 bp clearly results in polarized strand transfer, it is not clear from these *in vivo* experiments whether this is due to impairment of the cleavage reaction, blockage of strand transfer, or religation of the mutated end. We suggest that cleavage of the mutated end can still occur because Ω-ons carrying one wild-type and one mutant end not only retain the capacity for circle formation, but are also proficient in transposition whereas the double

Table III. List of plasmids used

Plasmid	Backbone	Promoter	Insert	Transposon	Junction	Source
pAPT28	pBR322	none	none	IS*911*	IRR−pOX	this study
pOFT139	pBR322	none	none	IS*911*	IRR−λ	Prére *et al.* (1990)
pOFT159	pBR322	none	none	IS*911* A^-B^-	IRR−λ	this study
pOFT158	pBR322	none	none	IS*911* ΔAB	IRR−λ	this study
pAPT97	pBR322	none	none	Ω-on Cm	IRR−λ	this study
pAPT99	pBR322	none	none	Ω-on Cm	IRR−pOX	this study
pAPT101	pBR322	none	none	Ω-on Cm	IRR−pBR	this study
pAPT103	pBR322	none	none	Ω-on Cm IRR−IRL Δ*BalI*	IRR−λ	this study
pAPT104	pBR322	none	none	Ω-on Cm IRL−CA	IRR−λ	this study
pAPT105	pBR322	none	none	Ω-on Cm IRR−CA	IRR−λ	this study
pAPT108	pBR322	none	none	Ω-on Cm IRL−CA:IRR−CA	IRR−λ	this study
pAPT21	p15A	P_l	none	none	none	this study
pAPT23	p15A	P_l	*orf*A *orf*B	none	none	Polard *et al.* (1991)
pAPT81	p15A	P_l	*orf*A *orf*B$^-$	none	none	Polard *et al.* (1991)
pAPT66	p15A	P_l	*orf*AB	none	none	Polard *et al.* (1991)
pAPT86	p15A	P_l	*orf*AB	none	none	Polard *et al.* (1991)
pAPT137	pBR322	P_{ara}	none	Ω-on Cm	IRR−pOX	this study
pAPT138	pBR322	P_{ara}	*orf*A *orf*B	Ω-on Cm	IRR−pOX	this study
pAPT139	pBR322	P_{ara}	*orf*B	Ω-on Cm	IRR−pOX	this study
pAPT140	pBR322	P_{ara}	*orf*AB	Ω-on Cm	IRR−pOX	this study
pAPT141	pBR322	P_{ara}	*orf*A	Ω-on Cm	IRR−pOX	this study

Construction of the plasmids is described in the text.

mutant shows neither of these activities (P.Polard, unpublished results). The rescue of the mutant by a wild-type end suggests that both ends are cleaved and transferred in intermolecular transposition, but this remains to be confirmed at the nucleotide sequence level. These results are in apparent contradiction to those obtained *in vitro* with retroviral ends where mutation of the equivalent terminal base pairs eliminates integrase-mediated cleavage (Craigie *et al.*, 1990; Bushman and Craigie, 1991; LaFemina *et al.*, 1991; Vink *et al.*, 1991; Sherman *et al.*, 1992). This is perhaps not suprising since the retroviral studies are performed with a single isolated mutant end (i.e. in the absence of a wild-type end on the same DNA molecule).

Vector resealing

An important question in the scheme shown in Figure 7 is the fate of the donor molecule during transposition. Initial experiments in which the A_7G mutation was introduced into a wild-type copy of IS*911* resulted in the appearance of donor plasmids which had undergone precise resealing. This depends on the presence of phage λ sequences flanking IRR. The λ DNA resembles a terminal inverted repeat of IS*911* located at a distance of 3 bp from IRR in an inverted configuration and together with IRR is active in integration (Prère *et al.*, 1990; Polard *et al.*, 1991). However, resealed vector plasmids carrying deletions of different sizes were observed when the DNA was exchanged for unrelated sequences. Thus precise vector resealing appears to be a special reaction determined in part by specific λ sequences.

The importance of this λ sequences in precise vector resealing is confirmed by experiments in which OrfAB was supplied *in trans* to the Ω-on derivatives (Figure 4). The results demonstrate that although the λ DNA is necessary for precise resealing of the vector molecule it does not detectably affect transposon circle formation. Moreover, the results obtained with the truncated terminal inverted repeats and with the terminal dinucleotide mutations (Figure 4) indicate that precise vector resealing requires an intact IRL and IRR. Although the mechanism leading to precise vector resealing is at present unclear, it could be related to the deletion and deletion/inversion events previously described in the cases of IS*903* and IS*10* (Weinert *et al.*, 1984; Benjamin and Kleckner, 1989). It is clear, however, that precise vector resealing is not obligatorily coupled to transposon circle formation. It seems probable that imprecise vector resealing, observed following attempts to introduce the A_7G mutation into an intact IS*911*, may be an event which was detected only because of the selection imposed to maintain the donor plasmid backbone. Further experiments are required to clarify these points.

Control of inter- and intramolecular transposition

The three major IS*911* gene products (Polard *et al.*, 1991), OrfA, OrfB and OrfAB, were originally detected using an expression system based on the bacteriophage T7 ϕ10 promoter and phage specified RNA polymerase. The data presented here provide evidence for a role in transposition of two of these proteins, OrfA and the transframe protein, OrfAB. We have been unable to find evidence for a role of the third protein, OrfB. The absence of an apparent role for OrfB may be due to our assay systems. On the other hand, it is possible that visualization of OrfB is an artefact of the T7 expression system (see for example Iost *et al.*, 1992) and that it is the OrfB translation initiation signals which are important, for example in influencing the frameshifting event. This latter possibility is under investigation.

OrfAB alone is sufficient to promote both circle formation

and intermolecular transposition and therefore exhibits the properties of the IS*911* transposase. Simultaneous production of OrfA, however, reduces or eliminates the production of circles (Tables I, II and III; Figure 6B). This activity is not the result of inhibition of transposition activity. Indeed, under conditions where OrfAB-mediated intermolecular transposition is limited (by providing the protein *in trans*) the effect of OrfA is to stimulate transposition by a factor of 10 (Table I). These results suggest that OrfA may act in concert with OrfAB by blocking circle formation and channelling OrfAB activity into intermolecular transposition events (Figure 7).

In its natural state, the ratio of OrfA and OrfAB specified by IS*911* is maintained by translational frameshifting. Increasing transcription of these reading frames, which presumably results in an increase in both proteins, is accompanied by an increase in intermolecular transposition without the appearance of transposon circles (Table II). Moreover, under these conditions, increasing the availability of OrfA, by synthesis from a second cloned gene, has no effect on transposition. This behaviour can be understood if transposition activity is dependent on the amount of OrfAB protein produced and if OrfA acts stoichiometrically as an accessory protein in OrfAB activity. A given level of OrfAB would therefore require an equivalent minimum level of OrfA to block circle formation and channel activity into intermolecular transposition. Additional OrfA would not then be expected to affect the process.

Functional relationship with other insertion sequences

The unrelated insertion sequence, IS*1*, exhibits a large cointegration component in transposition (Chandler and Galas, 1983) and presumably can undergo replicative transposition. It exhibits a strikingly similar arrangement of open reading frames to those found in IS*911* (Ohtsubo and Ohtsubo, 1978; Sekine and Ohtsubo, 1989; Escoubas *et al.*, 1991). In contrast to IS*911*, however, although the IS*1* transframe protein, InsAB′, fulfills the role of transposase, the upstream protein, InsA, appears to inhibit transposition (Machida and Machida, 1989; Zerbib *et al.*, 1990a), presumably by binding specifically to the terminal inverted repeats of IS*1* (Zerbib *et al.*, 1987, 1990b) and competing with the InsAB′ transposase for the same binding sites (Escoubas *et al.*, 1991). Thus the determining factor in IS*1* transposition activity is the ratio InsA/InsAB (Escoubas *et al.*, 1991; J.M.Escoubas, unpublished observations) whereas in the case of IS*911* the above results suggest that transposition activity is determined by the level of OrfAB. In addition, InsA behaves as a repressor of its own, and of transposase, expression (Machida and Machida, 1989; Zerbib *et al.*, 1990a; Escoubas *et al.*, 1991). In the case of IS*911*, it remains to be determined whether OrfA binds to IRL and IRR. Moreover, in the experiments reported above, the IS*911* proteins were systematically placed under the control of external promoters. We are thus unable to establish from these results whether OrfA is able to repress its own and OrfAB expression from the natural IS*911* promoter located in IRL.

Another interesting parallel is Tn*10*. This element has been shown to transpose via a non-replicative 'cut and paste' mechanism (Kleckner, 1990) and, like IS*911*, can generate circular forms (Morisato and Kleckner, 1984). Tn*10* circles are also the products of intramolecular transposition. They differ from IS*911* circles, which are generated by precise strand transfers at the ends, however, since they have proved to be rearrangements resulting from insertion of the cleaved ends within the excised element (Benjamin and Kleckner, 1989).

At least one other member of the 20 insertion sequences comprising the IS*3* family, IS*150*, has been shown to be organized in a similar way to IS*911* (Vögele *et al.*, 1991). Further analysis both *in vivo* and *in vitro* is obviously necessary to define their pattern of expression and transposition pathway(s) and to determine to what extent these elements, identified in widely different bacteria, share a common mechanism of transposition.

Materials and methods

Bacterial strains

The bacterial strains used were: JS219 [*srlC*::Tn*10*, *recA1 malP*p::lacI^Q *araD*, Δ(*ara leu*), *galU*, *galK*, *hsdS*, *rpsL* Δ(*lacIOPZYA*) X74] (Cam *et al.*, 1988), a derivative of MC1061 (Casabadan and Cohen, 1980), XA103; (Miller, 1972); MGC182(λ*clind*$^-$) [C600 *thr-1*, *leu-6*, *lacY1*, *tonA21*, *supE44*, *recA*1 *hsdR* (λ*clind*$^-$)]; DH5α (BRL, Inc); and RZ1032 (Kunkel, 1985). Media were supplemented, where necessary, with ampicillin (Ap; 100 μg/ml), chloramphenicol (Cm; 20 μg/ml); kanamycin (Km; 20 μg/ml), nalidixic acid (Nal; 20 μg/ml), methicillin (Met; 1 mg/ml), rifampicin (Rif; 50 μg/ml), streptomycin (Sm; 20 μg/ml) and spectinomycin (Sp; 30 μg/ml).

Mating out assay

The transposition frequencies of the IS*911* Ω-on derivatives were determined by a standard mating out assay (Galas and Chandler, 1982) using the conjugal plasmid pOX38Km (Chandler and Galas, 1983) as a target replicon. Donor strains DH5α/pOX38Km, containing the pBR322 derivatives (with the Ω-on and the P_{araBAD} promoter) together with one of the p15A derivative plasmids to be tested in complementation, were first grown overnight at 30°C without arabinose in L Broth supplemented with Ap, Sp, Sm, Km and Cm. IPTG (0.5 mM) was included to assure high level production of the cI857 repressor driven by P_{lac} promoter. Overnight cultures were diluted into L Broth with 1% arabinose but without antibiotics at an OD_{540} of 0.05 at 37°C with shaking. Growth was continued for ~2 h until an OD_{540} of 0.4−0.5, followed by 1 h incubation without agitation. The *rec*A donor cells were mixed at an approximate 1:1 ratio with the recipient XA103 (Nal^R, rif^R) also grown at 37°C with arabinose. Suitable dilutions were plated on L agar plates supplemented with Km, Nal and Rif (for scoring transfer), Rif, Nal, Cm (for scoring transposition of the Ω-on and Rif, Nal, Met, Ap (for scoring cointegration).

Circularization assays

For circularization assays, plasmid-containing cells were grown overnight at 37°C in L Broth supplemented with Ap, Sp, Sm, Km, Cm and arabinose (1%) when required. Plasmid DNA was prepared from 3 ml cultures and the equivalent of 0.5 ml (in 10 μl) was subjected to electrophoresis in 0.8% agarose gels using TEA buffer (Sambrook *et al.*, 1989). The gels were then stained with ethidium bromide before photography.

DNA techniques

Large scale or minipreparation of plasmid DNA and of transposon circles were as described (Sambrook *et al.*, 1989) using the alkaline/phenol extraction procedure. Restriction enzymes, *E.coli* DNA polymerase Klenow fragment, mung bean nuclease, T4 and T7 DNA polymerases and T4 DNA ligase were purchased from Amersham, New England Biolabs or Bethesda Research Laboratories, and used as recommended.

Purification of DNA restriction fragments and of the transposon circles (Geneclean, Bio101), and DNA sequencing (USB Sequenase kit), were performed as recommended by the suppliers.

Directed mutagenesis was accomplished on a matrix cloned into the plasmid pSK$^+$ (Stratagene) using the *dut/ung* system described by Kunkel (1985) and T7 DNA polymerase as described by Zhou *et al.* (1989).

Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer.

Electron microscopy of DNA circles was performed by standard methods.

Southern hybridizations were performed with Biodyne nylon membrane (PALL) using the protocols provided by the manufacturer. Labelling of the purified IS*911* internal *Bal*I fragment prepared from pOFT139 was achieved

by nick-translation in the presence of [α-^{32}P]dATP; the labelling kit and radioactive products were from Amersham.

Plasmids and plasmid construction

Table III summarizes the plasmids retained for use in these studies.

Plasmids carrying IS911 derivatives used in circularization and transposition assays. The IS*911* derivative carried by pAPT28 was derived by IS*911*-mediated integration using the active IRR−λ junction of pOFT139 (Prère *et al.*, 1990) into pOX38Km. The entire IS*911* sequence was excised with *Eco*RI which cleaves to the left of IRL within neighbouring pBR322 sequences of pOFT139 and with *Eco*RV which cleaves 40 bp away from IRR in pOX38Km. This fragment was inserted into *Eco*RI/*Eco*RV-cleaved pAT153 (Twigg and Sherratt, 1980) to give pAPT28.

Plasmid pOFT159 is a derivative of pOFT139 in which 4 bp of *orf*A and of *orf*B were deleted by treatment of the two *Bst*XI sites (Figure 1) with mung bean nuclease. Plasmid pOFT158 is a derivative of pOFT139 in which the internal *Bst*XI fragment has been removed following treatment with mung bean nuclease.

The omegons were constructed as follows: *Bgl*II sites were introduced into IS*911* close to each terminal inverted repeat in two steps. A *Bgl*II site was introduced into IS*911*, 51 bp from the right end. This was accomplished by insertion of two complementary oligonucleotides 5′ CGATACTGGA-AAAAACTCTA**AGATCT** 3′ IRR 3′ TATGACCTTTTTTGAGA**TTC-TAG**AGC 5′ IRR into the *Cla*I site (Figure 1) in pOFT139 and pAPT28 (in the orientation shown) to give plasmids pAPT55 and pAPT56 respectively. This results in a concomitant duplication of 26 bp in the element.

In a second construction, a *Bgl*II site located at 56 bp from the left end was created by site directed mutagenesis. The *Eco*RI−*Bam*HI fragment of pOFT139 containing the entire IS*911* sequence and flanking λ DNA was first cloned between the *Eco*RI and *Bam*HI sites of pSK$^+$ (Stratagene Inc) to give pAPT32. Site directed mutagenesis was performed using the following oligonucleotide to give pAPT47 (the *Bgl*II site is shown in bold and the mutated bases are underlined): 5′ CTCACCTC**AGATCT**ACACAGGT-GCTC 3′

To combine both *Bgl*II sites, the *Eco*RI−*Bst*EII fragment of pAPT47 was then substituted for the equivalent fragments in pAPT55 and pAPT56 to give pAPT71 and pAPT72 which differ in the sequences flanking IRR including the 3 bp target repeat (λ and pOX38Km respectively). Finally the internal *Bgl*II fragment was substituted by a *Bam*HI fragment carrying a Cm resistance gene flanked by two transcription termination signals (Fellay *et al.*, 1987) to generate plasmids pAPT97 and pAPT99.

Plasmid pAPT103 was constructed by insertion of the *Bam*HI Cm fragment (after treatment with Klenow fragment) between the *Bal*I sites of pOFT139 (Figure 1). This results in an Ω-on which carries only 22 bp of the 36 bp inverted terminal repeats at each end.

In order to delete the λ sequence flanking IRR while retaining the original external target repeat, an *Hpa*I site was introduced close to IRR by site directed mutagenesis of pAPT32 using the following oligonucleotide which overlaps the IRR−λ junction (shown as]): 5′ IRR CCACTTCA]C**GT-TA**ACCCTGAGCAGGC λ 3′

The *Eco*RI−*Hpa*I fragment carrying the entire IS*911* was then cloned between the *Eco*RI and *Nru*I sites of pAT153. Finally, the internal *Bal*I fragment was substituted for that of pAPT97 (carrying Cm^R) to give pAPT101 in which IRL and IRR are reconstituted.

The same cloning steps were used to mutate the terminal IRL and IRR nucleotides and flanking target repeats by site directed mutagenesis of pAPT32 with the following oligonucleotides: 5′ λ GTTAATTCT**CTA**[**G**-AAAGTGGCACAC IRL 3′ 5′ IRR GTTGACCACTTAG]**ATC**TGACC-CTGAGC λ 3′. In these cases the *Eco*RI/*Bam*HI fragment was recloned into pAT153 before substitution of the internal *Bal*I fragment carrying Cm to give pAPT104 (IRL) and pAPT105 (IRR). Plasmid pAPT108 carrying the Ω-on mutated at both ends was obtained by substituting the wild-type IRR of pAPT105 by the mutated IRR of pAPT104 using *Bst*EII (which cleaves once in the Cm gene) and *Pst*I (which cleaves once in the Ap gene).

The nucleotide sequences of all IS*911* fragments retained in the final plasmids (Table III) after site-directed mutagenesis were verified to assure that only the desired mutation was present. The sequences spanning IRR and IRL in these plasmids are shown in Figures 4 and 5.

Plasmids used to drive IS911-protein expression in trans of IS911 derivatives. These plasmids have been described previously (Polard *et al.*, 1991). They are derived from p15A and are therefore compatible with the pBR322 vectors carrying IS*911* derivatives. Their general structure is as follows: the internal IS*911* fragment carrying different combinations of *orf*A and *orf*B is under the control of the P_I promoter regulated by the *cI857* gene present on the same plasmid which is in turn under control of the P_{lac} promoter. Except for pAPT66, they also carry an Sp^{R}Sm^R determinant (Prentki and Krisch, 1984). These plasmids were introduced into suitable recipient strains by tranformation at 30°C in the presence of IPTG (0.5 mM) to optimize repression of P_I.

In this study we have used four derivatives: pAPT21, carrying the wild-type *orf*A *orf*B reading frames; pAPT81, mutated in *orf*B; pAPT86, carrying the A_7G mutation; and pAPT23, containing no IS*911* sequences.

Plasmids used to drive IS911-protein expression in cis of IS911 derivatives. We have used a pBR322 derivative carrying the P_{araBAD} promoter of *E.coli* and the *ara*C gene which encodes the arabinose-inducible repressor (pBAD18, a gift from Guzmán and Beckwith via C.Gutierrez). Before cloning the IS*911* open reading frames, we first modified the sequences surrounding the initiation codons of *orf*A and *orf*B to create an *Nde*I site without changing the amino acid sequences. This was done by site directed mutagenesis of pAPT32 using the following oligonucleotides for *orf*A and *orf*B respectively (the *Nde*I site is shown in bold and the mutations are underlined): 5′ ACAGGTGCTC**AT**ATGAAAAAAGAAAT 3′ 5′ TGG AGAATGA**C**ATAT**G**AAAAAAGGCT 3′. The first oligonucleotide was used to generate pAPT130 which carries the mutation in *orf*A alone. A mixture of both oligonucleotides was used to generate a double mutant which was subsequently deleted between the two newly created *Nde*I sites giving pAPT131. This mutation changes the AUU initiation codon of OrfB to AUG. Thus both OrfA and OrfB are placed in the same translational context in these plasmids. The internal IS*911* *Bal*I fragment of these plasmids was then cloned into the unique *Sma*I site of pBAD18 downstream of the P_{araBAD} promoter: pAPT130 was used to generate pAPT132 (containing the OrfA *Nde*I site and the entire wild-type IS*911* orfs) and pAPT131 was used to generate pAPT133, which carries *orf*B alone.

To construct a plasmid which drives expression of OrfAB alone, the internal *Bst*XI fragment of pAPT132 was substituted for that of pAPT93 (constitutive fusion; Polard *et al.*, 1991) to generate pAPT135.

Plasmid pAPT136 which expresses only OrfA was obtained as follows: a similar substitution between pAPT132 and pAPT91 (in which the heptanucleotide A_6G was exchanged for CA_5G, a mutation which greatly reduces frameshifting without changing the amino acid sequence of OrfA; Polard *et al.*, 1991) generated plasmid pAPT134. The *orf*B frame of this plasmid was then deleted between the *Pvu*II site (Figure 1) and the *Xba*I site in the neighbouring vector sequences to generate pAPT136.

In order to contruct the Ω-on Cm-carrying derivatives of these plasmids, the *Eco*I−*Bam*HI fragment of pAPT99 (carrying the Ω-on Cm) was introduced at the unique *Nsi*I sites of pBAD18, pAPT132, pAPT133, pAPT135 and pAPT136, after treatment with T4 DNA polymerase, to give pAPT137, pAPT138(OrfA,OrfAB,OrfB), pAPT139(OrfB), pAPT140 (OrfAB) and pAPT141(OrfA) respectively. The orientation of the Ω-on Cm is identical in all these constructions.

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Note added in proof

Recent experiments have demonstrated that both OrfA and OrfAB, but not OrfB, specifically bind to IS*911* IR sequences.