Assembly of a strong promoter following IS911 circularization and the role of circles in transposition

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When supplied with high levels of the IS911-encoded transposase, IS911-based transposons can excise as circles in which the right and left terminal inverted repeats are abutted. Formation of the circle junction is shown here to create a promoter, p_{junc} , which is significantly stronger than the indigenous promoter, p_{IRL} , and is also capable of driving expression of the IS911 transposition proteins. High transposase expression from the circular transposon may promote use of the circle as an integration substrate. The results demonstrate that IS911 circles are highly efficient substrates for insertion into a target molecule *in vivo*. Insertion leads to the disassembly of p_{junc} and thus to a lower level of synthesis of the transposition proteins. The observation that normal levels of IS911 transposition proteins supplied by wild-type copies of IS911 are also capable of generating transposon circles, albeit at a low level, reinforces the idea that the transposon circles might form part of the natural transposition cycle of IS911. These observations form the elements of a feedback control mechanism and have been incorporated into a model describing one possible pathway of IS911 transposition.

Keywords: circularization/IS911/promoter assembly/
transposase expression/transposition cycle

Introduction

Bacterial insertion sequences (ISs) are ubiquitous and exert a strong impact on the plasticity of the bacterial genome. More than 400 ISs have been identified to date from all the major bacterial species. In addition to their role as mutagenic agents, they play an important role in the dissemination and acquisition of what might be considered as 'accessory' functions. They are associated with pathogenicity and virulence functions, with genes involved in catabolic pathways as well as with antibiotic resistance genes (for review see Berg and Howe, 1989).

Transposition of bacterial insertion sequences is generally maintained at low levels, presumably to limit the potentially detrimental effects of excessive genome rearrangements on the host cell. Various mechanisms

which restrict IS activity have been described. Many of these act at the level of transposase expression. They include intrinsically weak transcription or translation initiation signals as well as mechanisms to repress transcription or translation either by host- or IS-encoded factors (for review, see Kleckner, 1990). Regulation can also occur at the level of translation elongation where a transposase molecule may be assembled by programmed translational frameshifting between two consecutive open reading frames (*orf*) (for review, see Chandler and Fayet, 1993). This permits the expression, from a single transcript, of alternative proteins carrying different combinations of functional domains and therefore having different properties.

A second level of regulation can occur by modulation of transposase activity. This may involve an inherent instability of the protein which limits its functional life (e.g. IS903; Derbyshire *et al.*, 1990), direct inactivation by oligomerization or interaction with another protein (e.g. IS50; see Reznikoff, 1993), or competition with other IS- or host-encoded proteins for binding to the ends of the element (e.g. IS1; Escoubas *et al.*, 1991).

In spite of these negative strategies of control, there is evidence that certain elements may undergo transient bursts of transposition activity (Naas *et al.*, 1994). One of the clearest examples of this type of phenomenon occurs with IS10 where both the efficiency of the transposase promoter and the activity of one transposon end is enhanced on hemi-methylated DNA due to dam methylation sites in each end. Since this is produced transiently following the passage of a replication fork, transposition is coupled to the replication cycle of the host cell (Kleckner *et al.*, 1996).

We have been interested in the regulation of IS911 transposition. This insertion sequence, isolated from *Shigella dysenteriae* (Prere *et al.*, 1990), belongs to the extensive IS3 family (Schwartz *et al.*, 1988). The IS911 transposase, OrfAB, like those of other members of the family, is a single polypeptide expressed from two consecutive and partially overlapping open reading frames, *orfA* and *orfB*, by a -1 frameshift. This occurs at a relatively high frequency (15% of translation initiated in the A frame proceeds into the B frame). OrfAB and the product of the upstream frame, OrfA, carry the same N-terminal domain, including a putative helix-turn-helix DNA-binding motif (Prere *et al.*, 1990). The C-terminal domain of OrfAB carries a conserved DD(35)E motif (Fayet *et al.*, 1990) known to be within the catalytic centre of many enzymes of this type (see Grindley and Leshchiner, 1995; Polard and Chandler, 1995a). Independent translation of this domain as OrfB, the product of the *orfB* frame, has also been demonstrated under certain conditions (Polard *et al.*, 1991) but no *in vivo* function in IS911 transposition has yet been attributed to this protein (Polard

et al., 1992). Although the exact role of OrfA has not been determined in detail, when produced in the presence of OrfAB, it is capable of stimulating intermolecular transposition (Polard *et al.*, 1992).

OrfAB exhibits recombinase activity. When supplied alone, IS911 derivative transposons give rise to excised supercoiled closed-circular molecules at high frequency (Polard *et al.*, 1992). More recently, a second OrfAB-induced form has been detected in which only one of the transposon strands has undergone cleavage and transfer to the opposite end, resulting in circularization of a single strand. With a circular plasmid donor, this generates a molecule in the form of a figure-of-eight with the two transposon ends joined by a single-strand bridge. Kinetic studies suggest that the figure-of-eight may be a precursor of the circle (Polard and Chandler, 1995b). Circle formation may therefore occur by a two-step process involving consecutive circularization of each transposon strand. In addition, the fact that figure-of-eight molecules, but not circles, can be generated *in vitro* using partially purified preparations of OrfAB has suggested that circularization of the second strand may require host functions (Polard *et al.*, 1996). The role of the circular transposon in the IS911 transposition cycle remains to be defined.

In the present study, the activity of the IS911-encoded proteins in regulating transposition was investigated. High levels of the wild-type complement of IS911 proteins or of OrfA alone supplied *in trans* was found to slightly reduce IS911 gene expression, while OrfB showed no effect. On the other hand, expression of OrfAB alone resulted in pronounced stimulation of expression of the *orfA* open reading frame. This was shown to be a direct result of transposon circularization. Formation of the circle junction, composed of the two abutted IS911 ends, IRR and IRL, generates a promoter, p_{junc} , whose sequence is distributed between both IRs. Assembly of this promoter increases the basal level of expression of an *orfA-lacZ* gene fusion at least 40-fold. In addition, evidence is presented that the circle junction is a highly active substrate for OrfAB-mediated deletion and for integration. These properties provide the elements of a novel feedback control system for regulation of IS911 transposition. This type of mechanism would also be consistent with the behaviour of several other IS elements.

Results

Regulation of IS911 gene expression by IS911-encoded proteins

In an initial investigation of the effect of IS911-encoded proteins on *orfA* expression *in vivo*, a two-plasmid system was used in which the proteins were supplied *in trans* to a reporter plasmid carrying an *orfA-lacZ* translational fusion within an IS911-based transposon. This latter plasmid, pAPT166, is a ColE1-based replicon carrying the first 86 bp of IS911 including the entire IRL sequence together with the indigenous promoter (p_{IRL}), ribosome binding site (rbs_{IS911}), and the first 10 codons of *orfA* fused to a *lacZ* gene. The plasmid also carries the terminal 52 bp of the right end of IS911, together with a correctly oriented copy of IRR, inserted at the 3' end of the gene fusion (Table I; Polard and Chandler, 1995b).

To provide IS911-encoded proteins, various combin-

Table I. Effect of IS911-encoded proteins on *orfA* expression

PLASMID	rbs	IS911 orfs	Mc-lac	β -gal	
				-IPTG	+IPTG
pAPT110		none	-	71.5	69.6
pAPT112	IS911	A+AB+B	-	69.5	67.0
pAPT155	ϕ 10	A+AB+B	-	71.0	25.5
pAPT156	ϕ 10	A	-	67.0	49.8
pAPT157	ϕ 10	B	-	67.5	71.0
pAPT111	IS911	AB	+	570	3340
pAPT158	ϕ 10	AB	++	1308	(300)

The cartoons above show the structure of pAPT166 (right) employed as the reporter plasmid and the generic plasmid used to supply different IS911-encoded proteins (left). IRL and IRR (white box and pointed box): right and left terminal inverted repeats; p_{IRL} , indigenous IS911 promoter; $plac$, lacUV5 promoter (the position and orientation of both are indicated by an arrow); unfilled ovals, type of origin of replication of the plasmid (ori pBR and ori p15A); small white circles, transcription terminators of the *rrnB* operon of *E.coli*; small white boxes, phage T4 transcription and translation terminators associated with the streptomycin/spectinomycin resistance cassette (SS); Km, *lacI*^q and *bla*, genes for resistance to kanamycin, the *lacI*^Q allele, and β -lactamase respectively; rbs, ribosome binding site. The cross-hatched box represents the gene fusion between the 5' part of *orfA* and *lacZ* from the eighth codon (*orfA-lacZ*). The box containing 'orf' indicates the position of the various cloned IS911 reading frames. The direction of transcription is indicated by curved half-arrows within the circle. The left hand column presents the different plasmids used to supply IS911 proteins followed by the type of ribosome binding site (rbs). IS911 indicates the resident IS911 rbs and ϕ 10, the rbs of gene ϕ 10 of bacteriophage T7. A + AB + B indicates the wild-type open reading frame configuration in which OrfAB must be produced by translational frameshifting. A, B and AB indicate individual reading frames. Mc-lac: MacConkey lactose indicator plates: -, white colonies (Lac⁻ phenotype); +, pink/red colonies (Lac⁺ phenotype); ++, bright red colonies (strong Lac⁺ phenotype). The right hand columns indicate β -galactosidase activities in Miller units obtained from liquid cultures in the absence or presence of IPTG (- or +IPTG). These values represent the average of three independent cultures with a standard error of $\pm 10\%$. In the case of pAPT158, the brackets signify that the value obtained was highly variable.

Fig. 1. Levels of IS911-encoded proteins produced by the transposase donor plasmids. Total lysates of cells carrying pAPT166 and pAPT110 (lane 1), pAPT112 (lane 2), pAPT111 (lane 3), pAPT156 (lane 4), pAPT155 (lane 5), pAPT158 (lane 6). The positions of OrfAB and OrfA together with a species that is probably produced by premature termination or degradation of OrfAB (our unpublished results) are indicated with unfilled arrow heads. Note that to avoid overexposure, approximately one-tenth the quantity of total protein was used from pAPT158-carrying cells compared with the other samples.

ations of *orfA* and *orfB* reading frames were cloned into a p15A-based replicon (pAPT110) under the control of the p_{lacUV5} promoter with either the indigenous *orfA* ribosome binding site, rbs_{IS911} , or the strong ribosome binding sites of the $\phi 10$ gene of bacteriophage T7 ($rbs_{\phi 10}$; Table I). These plasmids were used to provide IS911-encoded proteins *in trans* at different levels as illustrated in Figure 1. This figure shows total cell lysates of each plasmid-carrying strain fractionated by SDS-PAGE and probed in a Western blot using polyclonal antibodies raised against OrfA. Note that the anti-OrfA serum is not entirely specific and revealed some proteins unrelated to IS911 (vector plasmid pAPT110; lane 1). IS911 proteins were hardly detectable in cells carrying pAPT112 (rbs_{IS911} -A+B+AB; lane 2). However, OrfAB was clearly detectable with pAPT111 (rbs_{IS911} -AB; lane 3). The transframe protein was present in significantly higher quantity with pAPT155, even though it must be produced by translational frameshifting ($rbs_{\phi 10}$ -A+B+AB; lane 5). As expected, plasmid pAPT158 ($rbs_{\phi 10}$ -AB), produced the highest levels of OrfAB (lane 6). Note that it was necessary to use a 10-fold dilution of this sample to avoid overexposure. This reduces the apparent level of the non-specific proteins. Although OrfA was found to co-migrate with one of these (lane 1) it is clearly distinguishable with pAPT156 ($rbs_{\phi 10}$ -A; lane 4) and pAPT155 (lane 5). As expected, no OrfA could be detected with pAPT158. Note that an additional band appeared when OrfAB was expressed in high quantities (lane 6). This probably represents premature termination or a degradation product of OrfAB (OrfAB*).

In the presence of the vector plasmid pAPT110, plasmid pAPT166 promoted a low level of β -galactosidase expres-

sion in a standard β -galactosidase assay (Table I, line 1) which was insufficient to specify a Lac^+ phenotype on MacConkey lactose indicator plates (Table I, line 1). This presumably reflects the low efficiency of the indigenous IS911 expression signals. A similar low level was also observed in the presence of pAPT112 (p_{lacUV5} - rbs_{IS911} -A+AB+B) and remained unaffected by IPTG-induced expression of IS911-encoded proteins (Table I, line 2).

Plasmid pAPT155, like pAPT112, carries the IS911 reading frames in a wild-type configuration but includes a strong $rbs_{\phi 10}$ in place of the indigenous rbs_{IS911} and supports a higher level of OrfA and OrfAB synthesis (Figure 1, compare lanes 2 and 5). Replacing pAPT112 by pAPT155 resulted in a small but reproducible reduction in β -galactosidase activity in the presence of IPTG (Table I, line 3). Introduction of *orfA* alone under the control of $rbs_{\phi 10}$ (pAPT156; Table I, line 4) was found to result in only a small reduction in expression in the presence of IPTG. Plasmid pAPT157, which expresses the *orfB* frame alone, had no detectable influence (line 5). These effects are small but may indicate that one or a combination of the IS911 proteins is capable of repressing expression.

The effect of OrfAB alone on IS911 gene expression was investigated using plasmids pAPT111 (rbs_{IS911}) and pAPT158 ($rbs_{\phi 10}$). Both carry a mutation (ATTA₆G to ACTCA₆G) which artificially fuses the *orfA* and *orfB* reading frames to generate *orfAB*. This allows contiguous translation of OrfAB, and eliminates synthesis of both OrfA and OrfB (Polard *et al.*, 1991). These plasmids gave somewhat surprising results. Instead of reducing expression of *orfA-lacZ*, both yielded a strong Lac^+ phenotype on MacConkey lactose indicator plates. Host cells carrying either of the two plasmids together with pAPT166 gave homogenous Lac^+ colonies without sectors (see later) indicating that the majority of cells in the colony must contribute to the phenomenon. A large stimulation of β -galactosidase activity was also observed in cultures in the presence or absence of IPTG (Table I, lines 6 and 7). However, the massive production of OrfAB obtained with pAPT158 (Figure 1, lane 6) appeared toxic for the cells since colonies of this strain grew poorly on MacConkey lactose. This is also reflected in the low (and variable) β -galactosidase activity observed on transposase induction with IPTG (Table I, line 7). Analysis of the plasmid content of the host revealed that a high proportion of cells rapidly lose the plasmid coding for transposase following its induction with IPTG (data not shown).

It is interesting to note that, while pAPT111 activates expression, pAPT155 does not, even though it produces levels of OrfAB (by frameshifting) higher than those obtained with pAPT111 (Figure 1, compare lanes 3 and 5). Since pAPT155 also produces OrfA whereas pAPT111 does not, the results suggest that OrfA might modulate the stimulatory effect of OrfAB on expression of the *orfA-lacZ* reading frame. Such an effect would parallel the previously described modulatory role of OrfA on the level of transposon circles (Polard *et al.*, 1992).

Activation of *orfA* expression and the capacity for transposon circularization

To determine whether activation of *orfA-lacZ* gene expression and the capacity of OrfAB to generate transposon circles are indeed correlated, the behaviour of a circulariz-

Table II. Effect of inactivation of the terminal IRs and of gene orientation

PLASMID		TRANSPOSOME CIRCLES		Mc-lac	
				+OrfAB	-OrfAB
pAPT166		+	+	+	-
pAPT180		+	+	+	-
pAPT181		+	+	+	-
pAPT182		-	-	-	-
pAPT116		+	+	+	-
pAPT117		+	-	-	-

The physical map of the transposase donor plasmid pAPT111 is presented at the top of the table. Symbols are identical to those presented in Table I. Transposase substrate plasmids are indicated in the left-hand column. The general structure of substrate plasmids is shown in the next column. The *lacZ* gene is presented by cross-hatched box and its direction of transcription is indicated by an arrow. The part of *orfA* fused with *lacZ* is shown as stippled box. Left and right terminal inverted repeats are shown by white boxes; circles superimposed on these ends indicate the terminal dinucleotide mutations. For pAPT116 and pAPT117 the *lacZ* coding sequence does not carry a promoter but includes the resident translational initiation signals. The third column indicates the capacity of the plasmids to produce excised transposon circles in a standard agarose gel assay. The two columns on the right present the results obtained on MacConkey plates in presence or absence of IS911 transposase supplied by pAPT111.

ation-deficient mutant of the IS911-based transposon carried pAPT166 was analysed (Polard *et al.*, 1992; Polard and Chandler, 1995b).

IS911 circles carry abutted left and right transposon ends separated by 3 bp initially flanking the transposon in the donor plasmid. Although this intervening sequence is not important for circularization, the terminal CA-3' dinucleotide at both ends plays a key role. If both termini carry mutations of this dinucleotide, circularization is no longer observed whereas if only one end is modified, circles continue to be produced (Polard *et al.*, 1992). These mutations were introduced into the IS911-based *lac*-transposon carried by pAPT166 and their influence on the activation of *orfA* expression by OrfAB was tested on MacConkey indicator plates. The results are summarized in Table II. Plasmids carrying a mutated left or right

CA-3' (pAPT180 and pAPT181 respectively) retain the capacity to be activated by pAPT111 (Table II, lines 2 and 3) whereas pAPT182, which carries mutations in both ends, does not (line 4), even in the presence of high levels of OrfAB (provided by pAPT158; data not shown). Although pAPT166 alone is phenotypically Lac⁻ on MacConkey indicator plates, it gives rise to pale blue colonies on X-gal indicator plates, reflecting the low basal level of expression from the resident p_{IRL} promoter (data not shown). As judged by this colour test, the activity of p_{IRL} is unaffected by the terminal mutations. These results demonstrate a clear correlation between activation of *orfA*-*lacZ* expression and the capacity to undergo circularization.

To examine this phenomenon further, the importance of orientation of the expressed gene with respect to the terminal inverted repeats was determined. The complete *lacZ* coding sequence without its promoter but including the translation initiation signals was introduced between IRL and IRR (see Materials and methods) to generate a transposon similar to that carried by pAPT166. This resulted in pAPT116 and pAPT117 in which transcription of *lacZ* would occur in opposite orientations. Both plasmids generate circular transposons in the presence of OrfAB (supplied by pAPT111). Activation of β -galactosidase expression is observed only when the gene fusion is placed in the original *orfA* orientation (pAPT116; Table II, lines 5 and 6) and does not rely on natural *orfA* translation initiation sequences.

Gene activation requires the presence of the circle junction but not transposase

Several explanations for the correlation between the capacity to generate circular transposon copies and increased β -galactosidase expression are evident: (i) the presence of a promoter within the cloned *lacZ*-carrying DNA fragment which drives transcription across the IRR-IRL circle junction and thence through the *lac* gene; (ii) generation of a new promoter or activation of p_{IRL} by formation of the circle junction; (iii) binding of OrfAB to abutted IS911 ends to activate a promoter; or finally (iv) an increase in the number of *lacZ* genes produced by continued replication of the donor plasmid accompanied by continued excision of transposon circles.

To address these questions, a derivative of pAPT120 was made in which IRR was deleted and IRL was replaced by the circle junction (Figure 2A; see Materials and methods). Plasmid pAPT120 differs from pAPT166 only by the nucleotide sequence flanking IRR (see Materials and methods). When introduced into a suitable host in the absence of OrfAB, this plasmid, pMIT130, conferred a strong Lac⁺ phenotype on MacConkey lactose indicator plates and specified a 45-fold higher β -galactosidase activity than its parent pAPT120 (3500 compared with 80 units). Thus, activation of β -galactosidase synthesis cannot be attributed to the presence of a cryptic promoter within *lacZ*, nor does it directly require OrfAB.

These results therefore strongly support the notion that the β -galactosidase activity observed here is due to the presence of the junction sequence itself. They raise the possibility that the assembly of the junction sequence either creates a new promoter or activates the resident expression signals.

Fig. 2. Transcription initiation sites. (A) Schematic representation of plasmids pAPT120 and pMiT130. Symbols are identical to those used in Table I. The direction of transcription of the gene fusion of *orfA* with *lacZ* is shown as a half-arrow placed on the outside of the plasmid circle. *pjunc*: probable promoter assembled in the abutted terminal IRs. (B) Results of primer extension analysis of *lac* transcripts specified by each plasmid. A 5' end-labelled primer complementary to nucleotides 90–111 was extended using MMLV reverse transcriptase and products were fractionated on a 8% sequencing gel. A sequencing ladder using the same unlabelled oligonucleotide as primer and a corresponding DNA fragment covering the region is included in the left of the figure. Lanes 1 and 2 show the primer extension products obtained from two independent cultures of a host carrying pAPT120. Lanes 3 and 4 show primer extension products obtained from identical quantities of total RNA isolated from two independent cultures of a host carrying pMiT130. The major products are indicated by unfilled arrowheads on the right. The scheme at the left of the figure indicates the relative position of the IRR–IRL junction. It should be noted that the products of reverse transcription migrate slightly above the corresponding band in the nucleotide sequencing ladder due to the additional phosphate group on the labelled oligonucleotide used as a primer (compared with the same, unlabelled oligonucleotide sequencing primer), and the absence of an OH group on each band of the sequence ladder due to the 3' terminal dideoxy nucleotide. (C) Schematic representation of the junction region. The proposed –35 and –10 regions are boxed and the corresponding nucleotides are indicated in large script. Those indicated below show *pjunc* while those above are *pIRL*. The nucleotides G in bold and marked +1 locate the transcriptional start for *pjunc* at nucleotide 22 and for *pIRL* at 56. The relative positions of IRR and IRL are also shown. Nucleotide coordinates referred to in the text are shown above the sequence initiating at the terminal T of IRL.

Activation results from the creation of a new promoter

To ascertain whether the large difference in β -galactosidase activity between pMiT130 and pAPT120 was due to activation of p_{IRL} in pMiT130, or whether it reflected the use of an alternative promoter, the transcriptional start position of the *lac* mRNA was determined in both. Total RNA extracted from exponentially growing cultures of strains carrying pAPT120 or pMiT130 was reverse-transcribed using an end-labelled oligonucleotide primer complementary to coordinates 90–111 (downstream from the sequence shown in Figure 2C). The results are shown in Figure 2B and presented schematically in Figure 2C. In the case of pAPT120 (Figure 2B, lanes 1 and 2), a weak signal can be detected, whereas that from pMiT130 (lanes 3 and 4) is clearly much stronger. The transcript from pAPT120 starts eight bases downstream from the –10 hexamer of the predicted OrfA promoter at nucleotide #54, whereas that from pMiT130 is initiated within IRL, eight bases downstream from a potential –10 hexamer and within the –35 hexamer of the indigenous OrfA promoter (Figure 2B and C; Prere *et al.*, 1990; Polard *et al.*, 1994). Use of a second oligonucleotide complementary to sequences located 44 bases downstream confirmed these locations (data not shown). The results therefore demonstrate that a new promoter has been created in pMiT130.

Figure 2C shows that the IRR sequence carries a well-conserved –35 region which is accompanied by a rather poor potential –10 region located in IRL. Since identical quantities of total RNA were loaded in each case, the result is consistent with the conclusion drawn from β -galactosidase measurements, that this upstream promoter, p_{junc} , is significantly stronger than p_{IRL} .

Effect of IS911 proteins on controlling p_{junc} activity

The identification of a promoter which is assembled in this unusual way raises the question of its biological relevance to transposition. With its high activity and the consequent potential to produce high levels of IS911-encoded proteins, p_{junc} might be expected to be subject to strict regulation. To assess the effects of the IS911 proteins on p_{junc} activity, host strains carrying pMiT130 were transformed with pAPT156 (rbs₉₁₀–A), pAPT158 (rbs₉₁₀–AB) or pAPT155 (rbs₉₁₀–A+AB+B) and spread directly on MacConkey lactose indicator plates. The effects of pAPT111 (rbs_{IS911}–AB) and pAPT112 (rbs_{IS911}–A+AB+B) were also analysed to obtain information on the effect of different levels of these proteins. Clones carrying pAPT156 gave rise to a Lac⁺ phenotype indicating little or no repression of p_{junc} by OrfA alone. On the other hand, while transformants carrying pAPT158 or pAPT155 also gave rise to Lac⁺ clones, they generated Lac[–] segregants at high frequencies in the absence of IPTG-induction of p_{lacUV5} . Segregant colonies of these primary transformants appeared either white or red with various degrees of white 'sectors' (Figure 3A). When cultures were grown overnight in the presence of IPTG before plating, this instability was more pronounced with expression from the wild-type configuration of reading frames than with expression of OrfAB alone and gave rise to ~53% (pAPT112) and ~4% (pAPT111) segregants.

The pronounced instability might reflect OrfAB-induced transposition activity of the abutted IRs. To examine this,

both IRs carried by pMiT130 were inactivated by mutation of the terminal 5'CA 3' dinucleotides. The resulting plasmid, pBST2, specifies a high β -galactosidase activity comparable with that of its parent (data not shown), demonstrating that the terminal mutations do not notably affect the inherent activity of p_{junc} . On the other hand, unlike the parent plasmid, pMiT130, pBST2 is quite stable in the presence of either of the two transposase donor plasmids, pAPT158 or pAPT155, supporting the idea that the instability reflects transpositional rearrangements.

This was confirmed by determining the structure of plasmid DNA from cultures carrying pMiT130 with pAPT112. The DNA was digested with *Apa*I (specific for the transposase donor plasmids), transformed into the original plasmidless host strain, and spread directly on X-gal indicator plates rather than the less-sensitive MacConkey lactose indicator plates. All clones from pBST2-containing cells retained a strong Lac⁺ phenotype, whereas those derived from pMiT130 showed significant variation (Figure 3B). Three distinct phenotypes were observed for pMiT130: intense blue (identical to those obtained with pBST2 and characteristic of p_{junc} driven activity), pale blue, and white colonies. Plasmid DNA isolated from a selection of these colonies is shown in Figure 3C. Plasmids from individual white colonies (lanes 3–6) are smaller than the parental plasmid (lanes 2 and 12) and show a significant variation in size. Lane 7 contains DNA from a large pool of white colonies and provides a clearer indication of the size range. Plasmid DNA from individual pale blue colonies is shown in lanes 8–10. Further restriction analysis (data not shown) demonstrated that all carry small deletions. The limited extent of these deletions is illustrated in the DNA isolated from a large pool of these colonies (lane 11).

To characterize the deleted plasmids, the nucleotide sequences surrounding the IRR–IRL junction region were determined. Plasmids from white clones systematically exhibited a deletion of IRL extending from IRR into the *lacZ* gene and removing p_{IRL} (type I, Figure 3D) while plasmids giving rise to the pale blue colonies generally carried deletions of IRR extending from IRL and leaving p_{IRL} intact (type II, Figure 3D). Their restricted size presumably reflects the fact that selection for resistance to ampicillin was maintained throughout. Examples of such new junction sequences are shown in Figure 3D.

The pronounced instability of the IRR–IRL junction in the presence of OrfAB indicates that it acts as a transposase substrate in the formation of adjacent deletions.

Integration activities of the IRR–IRL junction

Since the adjacent deletions are presumably the result of intramolecular transposition (see Discussion), it seemed possible that the IRR–IRL junction might also serve as an efficient substrate for intermolecular transposition. Such intermolecular events are expected to lead to integration. This was tested by 'mating-out' assays with the conjugative pOX38Km plasmid as a target. In addition to pOX38Km, donor strains carried pAPT111 (rbs_{IS911}–AB) or pAPT112 (rbs_{IS911}–A+AB+B) and pMiT130 or pBST2 as transposase substrates. The results are shown in Table III. In the presence of OrfAB alone (pAPT111), pBST2 exhibited a low background level of transfer which was unchanged when a wild-type complement of proteins was supplied

Fig. 3. OrfAB-mediated deletions of the circle junction. **(A)** MacConkey lactose indicator plates with transformants derived from clones carrying pMiT130 with pAPT110 (left) and pAPT155 (right). **(B)** X-gal indicator plates with purified clones, derived from cells carrying pBST2 (left) or pMiT130 (right) following passage through a host carrying pAPT112. **(C)** A 0.7% ethidium bromide-stained agarose gel (in TAE buffer) showing undigested plasmid DNA extracted from clones derived from pMiT130. Lanes 2 and 12: parental pMiT130; lanes 3–6: plasmid DNA from individual white clones; lane 7: DNA extracted from a large pool of white colonies; lanes 8–10: DNA from individual pale blue colonies; lane 11: DNA extracted from a large pool of pale blue colonies; lanes 1 and 13: 1 kb ladder. I and II represent the deletions in white and pale blue clones. **(D)** Schematic representation of the type of adjacent deletions extending from IRL or IRR on the junction. **(E)** Nucleotide sequence of examples of novel junctions in white i) and pale blue ii) colonies. The wild-type circle junction is presented with the terminal dinucleotides 5'CA3' (TG) inverted repeat underlined and shown in bold. The location of IRR and IRL is indicated. In i) IRR is retained and IRL is deleted, whereas in ii) IRL is retained and IRR is deleted.

(pAPT112). On the other hand, pMiT130 was transferred at a 40-fold higher frequency than pBST2 in the presence of OrfAB and was stimulated ~100-fold by pAPT112 to attain a level of 0.1%. Thus, a functional IRR–IRL junction appears highly efficient in promoting intermolecular transposition. Moreover, OrfA appears to play a significant stimulatory role in the process.

Sixty transconjugants were initially tested for their Lac phenotype on MacConkey indicator plates. About 60% gave rise to white colonies typical of p_{IRL} activity (data not shown) and 30% exhibited an intermediate Lac⁺ phenotype (pink colonies). The occurrence of white colonies is consistent with the notion that insertion of pMiT130 occurs using the IRR–IRL junction resulting in

separation of IRL and IRR and consequent disassembly of *p_{junc}*. In the pink colonies, insertion might have occurred into a highly transcribed region of the target. A single colony exhibited a strong Lac⁺ phenotype. This could have been generated by insertion of a dimer or higher order multimer in which one IRR–IRL junction and thus a copy of *p_{junc}* is retained.

To confirm this interpretation, the structure of plasmids from 25 transconjugants was determined by restriction mapping and Southern hybridization using *pMiT130* as a probe (Materials and methods). DNA was digested with *Bam*HI which cleaves *pOX38Km* three times and *pMiT130* only once. For insertion of a single copy of *pMiT130*, a single *pOX38Km* fragment should disappear to give two new fragments each showing homology to the probe. Moreover, because of the asymmetry of the *Bam*HI site in *pMiT130* (105 bp from IRL), insertion using the IRR–IRL junction would result in a large difference in intensity of hybridization: one fragment would carry only 105 bp and the other 6505 bp of homology to the probe. In addition, insertion of a dimer would give rise to a supplementary band migrating as a linear monomeric *pMiT130* plasmid. Using these criteria, 24 samples gave the expected pattern for a simple insertion of *pMiT130* into *pOX38Km*, while that exhibiting the strong Lac⁺ phenotype appeared to carry an insertion of multimeric *pMiT130* (data not shown). These structures were confirmed using additional restriction enzymes (Materials and methods; also see Galas and Chandler, 1982).

To determine whether IRL and IRR remain functionally intact in the *pOX38Km::pMiT130* plasmids (and therefore potentially capable of undergoing further cycles of transposition), and to verify the nature of the insertion into *pOX38Km*, their capacity to regenerate excised plasmid circles in the presence of OrfAB was examined. Plasmid *pAPT111* (*rbs_{IS911}*–AB) was introduced into individual transconjugant clones and the resulting transformants were spread on MacConkey indicator plates. All primary transformant colonies exhibited a strong Lac⁺ phenotype and contained a plasmid identical to the parental donor (data not shown). This result therefore demonstrates that, after integration, IRL and IRR retain the capacity to excise in a transposase-dependent manner to reconstitute *p_{junc}*.

Circle formation, *p_{junc}* and the *IS911* transposition cycle

These and previous results support the idea that *IS911* derivative transposons are capable of transposition using a pathway involving excision of circular transposon molecules and their subsequent insertion into a new target molecule. The relative importance of such a cycle of excision and insertion in *IS911* transposition is, however, difficult to assess. The circles may simply be the result of unnaturally high levels of OrfAB or of artificial changes in the relative abundance of the different *IS911* proteins.

The effects of different levels and combinations of *IS911*-encoded proteins on plasmid excision was therefore examined. Figure 4A shows colonies of the *recA* host carrying a *pOX38Km::pMiT130* plasmid exhibiting a Lac[−] phenotype. Introduction of *pAPT111* (*rbs_{IS911}*–OrfAB) yielded primary transformant colonies exhibiting relatively homogenous of Lac⁺ phenotype but with edges showing an irregular Lac phenotype (Figure 4B). This may reflect

Table III. Integration frequency of the IRR–IRL junction

TRANSPOSASE DONOR		<i>pAPT111</i>	<i>pAPT112</i>
INTEGRATION SUBSTRATE		(AB)	(wt)
<i>pMiT130</i>		1.8×10^{-5} (0.34)	1.2×10^{-3} (0.45)
<i>pBST2</i>		0.5×10^{-7} (0.09)	0.6×10^{-7} (0.12)

The cartoon at the top of the table represents integration of *pMiT130* into the conjugative *pOX38Km* plasmid. Symbols are identical to those used in Table I. The direction of transcription of the gene fusion of *orfA* with *lacZ* is shown as a half-arrow. *p_{junc}*: probable promoter assembled in the abutted terminal IRs. The unique *Bam*HI site carried by *pMiT130* is located within the *lacZ* gene at a distance of ~100 bp from the IRR–IRL junction. Plasmids tested in the integration assay are shown on the left. Symbols are identical to those in Table I. Integration frequencies in the presence of OrfAB alone (*pAPT111*) or with the wild-type configuration of open reading frames (*pAPT112*) are included in the two right-hand columns. The values represent the average of between five and seven separate experiments. Standard errors are shown between brackets.

instability of the excisants due to the high OrfAB levels. As shown in Figure 4E, lane 3, this is accompanied by high-efficiency excision of the integrated *pMiT130* (compare with lane 7). On the other hand, *pAPT112* generated sectorized colonies (Figure 4C), indicating that excision occurred at a significantly lower level. This is confirmed by analysis of the plasmid content of cells in the red sectors (Figure 4E, lane 4). Note that, as described above, the presence of *pAPT112* results in deletion formation (as judged by the fine bands below *pMiT130*). To provide some indication of the relative frequencies of excision, the proportion of colonies exhibiting a Lac⁺ phenotype or Lac⁺ sectors was scored after 24, 48 and 72 h of incubation. The results are shown in Figure 4F. Cells carrying the *pOX38Km::pMiT130* derivative together with the vector plasmid, *pAPT110*, show no detectable Lac⁺ sectors even after 72 h (<0.1%) whereas in the presence of *pAPT111*, 100% of the colonies were Lac⁺ after only 48 h. On the other hand, 25% of the colonies carrying *pAPT112* exhibited Lac⁺ sectors after 72 h. The difference between the effect of *pAPT112* and that of *pAPT111* may be due to lower levels of OrfAB (produced by natural frameshifting from *pAPT112*) or to the presence of OrfA (which significantly reduces the level of OrfAB-mediated circles and increases intermolecular transposition; Polard *et al.*, 1992; also this work).

Although *pAPT112* provides lower levels of OrfAB than does *pAPT111* (Figure 1), expression from both is driven by the strong *p_{lacUV5}* promoter. Protein levels are thus presumably still significantly higher than those provided by an unmodified *IS911* element. The excision

Fig. 4. Excision. The cartoon at the top of the figure shows schematically the excision of pMiT130 from the pOX38Km::pMiT130 and the reconstitution of the strong p_{junc} promoter. Symbols are the same as those described in Tables I and III. MacConkey lactose indicator plates of primary transformants of pOX38Km::pMiT130 derivatives after 48 h incubation in the presence of: (A) pAPT110 (no IS911 reading frames); (B) with pAPT111 (*plac*-*rb*_{IS911}-AB); (C) pAPT112 (*plac*-*rb*_{IS911}-A+AB+B); and (D) pBST5 (unmodified native IS911). (E) A 0.7% ethidium bromide-stained agarose gel (in TAE buffer) showing the plasmid DNA content of pOX38Km::pMiT130 derivatives (lane 7) transformed with different transposase donors: lane 3: pAPT111 (*Lac*⁺ clone), lane 4: pAPT112 (*Lac*⁺ sector), lanes 5 and 6: pBST5 (*Lac*⁻ and *Lac*⁺ sectors respectively), lane 1: pAPT111 and lane 2: pMiT130. The blank and filled arrowheads show the position of transposase donor plasmid and excised pMiT130 respectively. (F) Proportion of colonies containing *Lac*⁺ sectors after 24, 48 and 72 h of incubation on MacConkey lactose indicator plates.

assay described above is *a priori* exquisitely sensitive since a single excision event should generate a strong, easily detectable, and relatively stable phenotype. This raised the possibility of examining a more natural situation and determining whether an unmodified IS911 element was itself capable of provoking excision *in trans*. The pOX38Km::pMiT130-carrying *recA* strain was transformed with plasmid pBST5 which contains an entire unmodified copy of IS911 with its natural promoter

(Materials and methods). The results of growth on MacConkey indicator plates are shown in Figure 4D. Colonies carrying *Lac*⁺ sectors are clearly visible. Moreover, analysis of the plasmid content of clones purified from the *Lac*⁺ sectors confirmed that excised copies of the parental plasmid pMiT130 were present (Figure 4E, lane 6) whereas plasmid DNA from *Lac*⁻ clones showed no indication of excised pMiT130 (Figure 4E, lane 5). The proportion of colonies carrying *Lac*⁺ sectors as a

function of incubation time was found to be ~5% after 72 h (Figure 4F), measurably lower than that obtained with pAPT112. These data provide strong evidence that IS911 is capable of excising as a circular molecule under conditions of normal and balanced expression of its transposition proteins.

Discussion

The experiments presented here were designed to determine whether the various IS911-encoded proteins, OrfA, OrfB and OrfAB were involved in regulating IS911 gene expression. This was prompted by the fact that OrfA and the transposase OrfAB share a common domain conferring binding specificity to the terminal inverted repeats (our unpublished observations) and by the observation that a protein equivalent to OrfA from another similarly organised, but otherwise unrelated, IS element, the InsA protein of IS1, behaves as a repressor (Zerbib *et al.*, 1990). An *orfA-lacZ* reporter system contained within a proficient IS911-based transposon or an identical transposon in which both IRs had been inactivated by mutation were used for these purposes. When a wild-type complement of IS911 proteins was supplied at relatively high levels *in trans*, expression of *orfA-lacZ* was reduced by a factor of 3. If IS911 proteins were supplied individually, only OrfA was found to reduce expression and this only by a factor of 1.5. One interpretation of these results is that while OrfA is capable of providing a low level of regulation—presumably by repressing the resident IS911 promoter, P_{IRL} , partially located in IRL (Prere *et al.*, 1990)—the wild-type complement of proteins act synergistically to increase the level of repression. These effects are smaller than those observed for the related element, IS2, where high expression of the OrfA protein alone *in cis* to the transposase promoter resulted in a 4.7-fold reduction of expression (Hu *et al.*, 1994). Clearly, further analysis is necessary to explore these effects.

Assembly of a strong promoter by formation of the circle junction

A remarkable result obtained from these experiments was that OrfAB alone greatly stimulated β -galactosidase synthesis. This required intact IRs and was correlated with the capacity of the reporter transposon to undergo circularization. Moreover, the effect was greatly reduced when OrfA was produced concomitantly, a configuration which reduces or eliminates free transposon circles (Polard *et al.*, 1992). Subsequent experiments demonstrated that joining IRR and IRL to create the junction of the excised transposon circle generates a new promoter, P_{junc} , which is significantly stronger than the resident promoter, P_{IRL} . This was implied by the observation that the cloned junction fragment drives expression of an *orfA-lacZ* gene fusion at a level 40-fold higher than P_{IRL} . It was confirmed by demonstrating that the point of initiation of transcripts driven from the IRR–IRL junction occur upstream of those driven by P_{IRL} and that they are significantly more abundant.

Although we have not addressed the nature of the promoter in detail, analysis of the nucleotide sequence of IRR and IRL reveals the presence of a potential –10

hexamer in IRL and a corresponding –35 hexamer in IRR. These are separated by 17 bp in the circle junction by virtue of a 3 bp linker sequence derived from the sequences flanking IS911 in the parental plasmid. The 3 bp represent one of the two copies of the 3 bp target sequence duplicated during the initial insertion event. The spacing of 17 bp is that found in a majority of known promoters (see Harley and Reynolds, 1987). The –35 hexamer, TTGACC, is a relatively good match to the consensus TTGACA, and transcription initiates at a purine, G, localized 8 bp downstream from the –10 hexamer. On the other hand, the proposed –10 hexamer, CACACT, is somewhat distant from the consensus TATAAT although it differs from several known promoters by only 1 nucleotide (Harley and Reynolds, 1987). The presence of outwardly directed –35 hexamers in the ends of insertion elements is relatively common and several ISs have been shown to activate neighbouring genes on insertion by forming hybrid promoters composed of a –10 hexamer in the neighbouring DNA and the IS-associated –35 element (for review, see Galas and Chandler, 1989).

This arrangement of –10 and –35 hexamers within the terminal inverted repeats is not restricted to IS911. It has been observed in IS21 (Reimann *et al.*, 1989) and IS30 (Dalrymple, 1987). More recently, it has also been proposed for IS2 (Szeverenyi *et al.*, 1996) which, like IS911, is a member of the IS3 family. In these cases the authors have suggested that the promoter is assembled by the formation of tandem dimers of the ISs. In each case, the distance between the –35 and –10 elements includes several additional base pairs located between the IRs. For IS911 and IS30, the number of base pairs included in the spacer is equal to the length of the target duplication generated upon integration. This is not, however, a general rule since in the case of IS21 and IS2, the spacer is shorter (3 bp for IS21 and 1 bp for IS2) than the normal target duplication (4 and 5 bp respectively).

Scanning the terminal inverted repeats of insertion sequences in our database of over 400 (J.Mahillon and M.Chandler, unpublished results) suggests that many other IS elements may be capable of assembling promoters in a similar way. Figure 5 presents abutted IR sequences from a selection of insertion sequences. Since most of these elements have not been analysed experimentally, we have not included a spacer sequence of specific length. The selection includes only easily recognisable hexamers. In view of the wide range in promoter sequences (Harley and Reynolds, 1987; Lissner and Margalit, 1994; Helmann, 1995; Sabelnikov *et al.*, 1995), and variations associated with different groups of bacteria from which the IS sequences are drawn, such an arrangement may prove to be quite common.

Circle formation and the reactivity of abutted junction sequences

In addition to promoting increased transposase synthesis, the abutted copies of IRR and IRL are excellent substrates for various types of transpositional recombination. The IS911 circle junction undergoes OrfAB-dependent adjacent deletion at high frequencies. Moreover, mating-out assays using a donor plasmid carrying the junction demonstrate efficient insertion into a suitable target plasmid. In view of the high frequencies obtained, it is probable that the

IRR --- IRL

a) Characterized (at least partially).

IS21 ACATCGTGGCG**TTGACAXX**TGTCAGCGCCAGTGAT**TATAAG**ACGGTAATT
 IS30 GGGGTGTTGCA**TTGACA**GATTGAATCTAC**XX**TG**TAGATT**CAATTGGTC
 IS2 CAGATGTCT**TGAAA**TATAGGGGCAAATCC**AXTAGACT**TGGCCCCCTG
 IS911 GCCAGTTTTTGT**TTGACC**ACTTC**AXXX**TGAAGTGG**CACACT**GAAATTTGGC

b) Potential promoters.**IS3 family:**

IS120 AAAAACTGTCT**TTGACA**ATGGGGAGCATTATA **TATAAT**GATACCAAA
 IS1068 CTAGTTTTTCG**TTGACT**TTCCA TGGAAAGGT**TATAAT**AACTAGAC
 ISL1 CCGAAAAAGTG**TTGACT**TCCC GGGTCTGTACT**TAGCCT**TTTCGGACAG
 ISBT1 TGTTACGTTTT**TTGTAT**TTTTTCA TGAAAAAT**TATAA**GAATTTAGT

IS30 family:

IS1070 TCGGTTAATTC**TTGCAAT**CTACC GGTAGATTG**TAAAT**TAAACCGAA

IS21 family:

IS232 ACATGTTTATC**TTGACA**TTTACAC GTATAAATGCT**TAACT**TAAATATG

IS256 family:

IS256 GGAGTCTGGACT**TTGACT** GATAAAGTCCG**TATAAT**TGTGTAA
 IS406 CAGAAAAGAGG**TTGCACA**ATC AGTTGTG**TATCGT**TCTTTCT
 IS1201 ACACAAAAGAT**TTGACA**GTTTC GAGACTG**TAAAT**AGTTTGT

Fig. 5. Identified and potential promoters in the ends of IS elements. The DNA sequences have the following sources and accession numbers: IS30, *Escherichia coli*, X00792; IS21, (*E.coli*), Z11878; IS2, *E.coli*, M18426; IS911, *Shigella dysenteriae*, X17613; IS120, *Clostridium thermocellum*, NONE; IS1068, *Lactococcus lactis*, X52273; ISL1, *Lactobacillus casei*, X02734; ISBT1, *Bacillus thuringiensis*, L29100; IS1070, *Leuconostoc lactis*, U17353; IS256, *Staphylococcus aureus*, M18086; IS406, *Pseudomonas cepacia*, M83145; IS1201, *Lactobacillus helveticus*, L26311. Potential -35 and -10 hexamers are shown in bold type; where underlined, these are perfect matches to the consensus. X represents the number of base pairs separating IRR and IRL.

intact IRR-IRL junction is responsible for this activity. An alternative explanation is that transposition of a single plasmid molecule may occur from dimeric plasmids in the population using the flanking copies of IRR and IRL. However, this seems less likely since the activity of the junction as a substrate in intermolecular transposition has been confirmed, by initial results using an *in vitro* system which indicate that the junction undergoes efficient integration into a circular target molecule (unpublished results).

It should be noted that the junctions formed by IS21, IS30, IS3 and IS2 are also highly unstable in the presence of their respective transposases (Reimann *et al.*, 1989; Spielmann-Ryser *et al.*, 1991; Olasz *et al.*, 1993; Szeverenyi *et al.*, 1996). Moreover, in the case of IS21, direct evidence has been presented that the IS21-encoded IstA protein specifically cleaves both the 3' transposon ends at the junction (Reimann and Haas, 1990). Similar cleavage activities are catalysed by OrfAB both *in vivo* and *in vitro* with the IS911 circle junction (B.Ton-Hoang, P.Polard and M.Chandler, manuscript in preparation).

Measurements of transposition activity of a preformed IRR-IRL junction revealed that in mating-out assays pMit130 exhibits a 60-fold higher frequency of insertion into the target pOX38Km plasmid in the presence of pAPT112 (rbs_{IS911}-A+AB+B) than with pAPT111 (rbs_{IS911}-AB). A similar effect was observed on the

formation of adjacent deletions from the IRR-IRL junction. Although, for simplicity, we have only compared here plasmids supplying OrfAB with plasmids carrying the wild-type configuration of *orfs* (and we cannot therefore rule out an effect of OrfB) these results are consistent with a stimulatory role for OrfA. The results of a previous study indicated that simultaneous production of OrfA together with OrfAB greatly stimulates intermolecular transposition while OrfB has no effect (Polard *et al.*, 1992). In that study, a large reduction in the abundance of excised transposon circles was also observed. The results presented here confirm this observation. We find that neither pAPT112 (rbs_{IS911}-A+AB+B) nor pAPT155 (rbs_{φ10}-A+AB+B) generate detectable levels of free circles or significant levels of β-galactosidase with the pAPT166 reporter plasmid, whereas pAPT111 (rbs_{IS911}-AB) and pAPT158 (rbs_{φ10}-AB) do.

The decrease in levels of excised transposon circles and the increase in intermolecular transposition observed in the presence of OrfA might be interpreted in two ways. Transposition into an intermolecular target and circularization could be viewed as two separate branches of IS911 transposition (Figure 6, see below). OrfA could channel the transposon into the intermolecular pathway by inhibiting circularization. IS911 circles could then simply be viewed as side-products of transposition. Alternatively, transposon

Fig. 6. Model for IS911 transposition pathways. The transposon (heavy double line) is shown resident in a target DNA sequence (lighter double line) at the top of the figure. The open circles represent the transposon termini. The pathway on the right involves consecutive circularization of each transposon strand. Top to bottom: First-strand circularization occurs at a low level, using proteins produced from the weak p_{IRL} , by OrfAB-induced single-strand cleavage and transfer to an intramolecular target at the opposite transposon end; second-strand circularization may require a host function and could occur either by replication (thus reconstituting the donor molecule) or second-strand cleavage. The formation of p_{junc} provokes high level synthesis of transposition proteins which introduce two single-strand cleavages (one at each end) and insertion into a suitable target (double dotted line). Insertion results in disassembly of the strong promoter and consequent reduction in the levels of transposition proteins. The left hand of the figure shows a classic pathway which involves double-strand or single-strand cleavage at the ends which could respectively liberate the transposon from the vector backbone in a 'cut and paste' mechanism or lead to the formation of a Shapiro intermediate.

circles could be precursors for intermolecular transposition and OrfA might reduce the level of circles by promoting their insertion into target DNA. Circle integration would result if the target were located on a second molecule and adjacent deletions would be expected if the target were on the same molecule. While these results are consistent with the idea that OrfA stimulates circle integration and adjacent deletions, the possibility that OrfA directly inhibits circularization remains to be determined. Further analysis using OrfA and OrfAB proteins produced concomitantly from individual *orfs* or by analysis in an *in vitro* transposition system will be required to clarify their exact roles.

Model for an IS911 transposition pathway

Two, non-exclusive, pathways for intermolecular transposition of IS911 are presented in Figure 6. The main difference between these pathways resides in the initial

strand cleavage(s) introduced by the transposase. In the 'circular intermediate' pathway shown on the right, an initial single-strand cleavage occurs at one transposon end and the strand is transferred to the opposite end. In more 'classical' transposition models (Figure 6, left), both transposon ends undergo simultaneous single- or double-strand cleavage.

The asymmetric circularization reaction has been observed both *in vivo* and *in vitro* and the cleaved strand has been shown to be transferred precisely 3 nucleotides from the second transposon end (Polard and Chandler, 1995a; Polard *et al.*, 1996). On a circular substrate, it gives rise to a product in the form of a figure-of-eight in which the transposon ends are connected by a single-strand bridge. We propose that this step is catalysed by the low levels of OrfAB, synthesized from the weak indigenous promoter, p_{IRL} . Although kinetic data obtained *in vivo* are consistent with the notion that the figure-of-eight molecules are precursors of transposon circles, the reaction does not proceed further in the present *in vitro* system. It is possible that missing host factors are necessary for further processing of the figure-of-eight form into a circle in this system. Once formed, however, the circle junction becomes an efficient substrate for integration.

A second fundamental difference between the circular intermediate model and the mechanisms involved in the transposition of other, well-studied, elements lies in the nature of the synaptic complex. Rather than a complex between two physically distant ends and a third DNA segment (the target), the model invokes prior joining of the transposon. This would be similar to the transposition strategy adopted by the conjugative transposons (for a review, see Scott and Churchward, 1995). This mechanism circumvents the apparent inability of the IS911 OrfAB recombinase to generate double-strand breaks at the ends of the transposon (Polard *et al.*, 1996). Two appropriate OrfAB-mediated single-strand cleavages on the circular transposon, one at each 3' terminal end in the IRR-IRL junction, would be sufficient to generate a configuration capable of undergoing productive strand transfer of both ends into a suitable target.

Circularization of the element assembles the relatively strong junction promoter, p_{junc} , in turn leading to increased expression of the IS911 transposition proteins which could act on the IRR-IRL junction to promote efficient insertion into an intermolecular target. Separation of IRR and IRL would disassemble p_{junc} , reducing the level of transposase synthesis, and restoring transpositional recombination to a low level. Like the *dam* methylation regulation of IS10 transposition (Kleckner *et al.*, 1996), this two-step mechanism would ensure that high levels of transposase are present only when the appropriate substrate (in this case the transposon circle) is available and could couple transposition to the physiological state of the cell. Moreover, it represents one way of solving the problem engendered by a transposase only capable of single-strand cleavage and transfer.

Finally, since the transposition proteins are supplied here to the transposon substrate *in trans*, an additional consideration must be the problem of an appropriate feedback control. High levels of expression of OrfAB from transposon circles might be expected to induce transposition of other copies of the transposon in the cell.

However, for wild-type copies of IS911, the transposase circles would direct the synthesis of high levels of OrfA in addition to producing OrfAB by frameshifting. If OrfA were to inhibit circle formation *in trans* in addition to stimulating circle integration, this would tend to reduce further circle formation while favouring integration of free circles. In view of its instability, we have been unable to construct a suitable molecule carrying the wild-type open reading frames and the circle junction in the configuration expected for a 'natural' IS911 circle.

Although most of the steps proposed in this model have been tested experimentally, serious consideration must be given to the fact that the majority of the results were obtained under unnatural conditions in which transposition proteins are supplied to the transposon substrate *in trans* and under artificial conditions of expression. To determine what influence this may have it will be necessary to devise a more natural transposition system. To this end, we have eliminated one obvious objection to the relevance of the scheme shown in Figure 6: that of the effect of artificial expression. We have shown that a wild-type copy of IS911, from which transposition proteins are supplied using the resident expression signals, is capable of generating excised circular copies of pMiT130 from a pOX38Km::pMiT130 derivative at low levels. In the alternative transposition pathway (shown on the left of Figure 6), the transposase introduces concerted cleavages at both ends of the transposon. These could be either double- or single-stranded cleavages, leading respectively to separation of the element from the donor backbone before integration or to the formation of a branched 'Shapiro' intermediate (Arthur and Sherratt, 1979; Shapiro, 1979). The fact that we have been unable to demonstrate transposase-induced double-strand breaks at the ends of IS911-derivative transposons either *in vivo* (Polard and Chandler, 1995a) or *in vitro* (Polard *et al.*, 1996) leads us to disfavour a double-strand cleavage model. It should be noted, however, that *in vivo* evidence for double-strand cleavage and excision of a linear molecule has recently been presented for the related element, IS3 (Sekine *et al.*, 1996).

A mechanism in which control of transposition is exercised by transient assembly of a 'strong' transposase promoter may be quite common. As described above, many IS elements exhibit the potential to generate promoters by abutting left and right ends. Various elements have also been shown to generate circles similar to those obtained with IS911-derivative transposons. These include the IS3-family members, IS2 (L. Lewis and N. Grindley, personal communication), IS3 itself (Sekine *et al.*, 1994) and IS150 (C. Weltz and B. Rak, personal communication), as well as the unrelated element, IS1 (Turlan and Chandler, 1995). It will be interesting to determine whether elements such as IS21 and IS30, whose transposition has been postulated to proceed via the formation of a head-to-tail dimer, are also capable of undergoing circle formation.

Materials and methods

Bacterial strains and media

JS219 (MC1061, *recA1 lacI^q*; Cam *et al.*, 1988); MC240 (XA103, F⁻, *ara*, *del(lac pro)*, *gyrA (nal^R)*, *metB*, *argE^{am}*, *rpoB*, *thi*, *supF*). Cultures were grown in L broth supplemented, where necessary, with ampicillin (Ap; 100 mg/ml), kanamycin (Km; 25 mg/ml), nalidixic acid (Nal;

20 mg/ml), methicillin (Met; 1 mg/ml), rifampicin (Rif; 50 mg/ml), streptomycin (Sm; 20 mg/ml) and spectinomycin (Sp; 30 mg/ml). Standard MacConkey indicator plates (Miller, 1972) were supplemented with 1% lactose. X-gal plates were prepared using VB medium (Vogel and Bonner, 1956) and 1.2% agarose supplemented with 0.5% glucose, 0.5% casamino acids and 100 mg/ml X-gal.

Plasmids

Transposase substrates. Plasmid pAPT166 has already been described (Polard and Chandler, 1995b). Briefly, it is a ColE1-based replicon carrying the first 86 bp of IS911 including the entire IRL sequence together with the indigenous promoter (*P_{IRL}*), ribosome binding site (*rbs_{IS911}*), and the first 10 codons of *orfA* fused to a *lacZ* gene. The plasmid also carries the terminal 52 bp of the right end of IS911, including a correctly oriented copy of IRR, inserted at the 3' end of the gene fusion (Table I).

Plasmid pAPT120 is similar to pAPT166, with the exception that the sequence flanking IRR is different. It was constructed using pAPT35 and pAPT72 (Polard *et al.*, 1992). Plasmid pAPT72 carries a derivative of IS911, in which two *Bgl*II sites flank *orfA* and *orfB*. Plasmid pAPT35 carries the IRL-*orfA-lacZ* fusion without IRR. The gene fusion was cloned as an *Eco*RI-*Bam*HI fragment between the *Eco*RI and *Bam*HI sites of pAPT71 to give pAPT120.

The construction of pAPT180, pAPT181 and pAPT182 which carry the IS911-derived transposon from pAPT166 and mutations in the terminal dinucleotide in the left, right or both ends respectively, has also been described (Polard and Chandler, 1995b).

Plasmids pAPT116 and pAPT117 were constructed using pAPT72 and pAPT35 [both described in (Polard *et al.* (1992))]. In a first step, an (*rbs_{lac}-lacZ*) expression module was assembled. Plasmid pAPT35 carries the same fusion *orfA-lacZ* as pAPT166 but is deleted for IRR. The *Hind*III-*Nde*I IRL-*orfA-lacZ* fragment of pAPT35 was replaced by a *Hind*III-*Nde*I fragment of plasmid pCB267 (Schneider and Beck, 1986) which carries the complete *lacZ* coding sequence together with translational initiation sequences (*rbs*) but without a promoter. In the resulting plasmid the (*rbs_{lac}-lacZ*) expression module is carried on a *Bam*HI fragment. In a second step this fragment was introduced between the *Bgl*II sites which flank *orfA* and *orfB* in plasmid pAPT72. This resulted in pAPT116 and pAPT117 in which the fragment is inserted in opposite orientations.

Plasmid pMiT130 was assembled by isolating circular transposon DNA from a derivative of pAPT120, linearizing the transposon circle to generate a molecule in which the intact circle junction is located upstream from the *orfA-lacZ* gene fusion, and substituting the IS911-based transposon carried by pAPT120 for this linearized circle. In order to provide suitable restriction sites within the transposon circle, pAPT174 was used. This plasmid is identical to pAPT120 but carries *p_{lacUV5}* inserted proximal to IRR, downstream of and in the same direction as the *orfA-lacZ* gene fusion (the exact construction of this plasmid is available on request). The *lac* promoter is flanked on its IRR-proximal side by an *Xba*I site and on the other by an *Xho*I site. Transposon circles were generated *in vivo* using the compatible pAPT111 to supply OrfAB protein (Polard and Chandler, 1995b) and the excised transposon DNA was isolated as described (Polard *et al.*, 1992). The circle was digested with *Xba*I and *Xho*I, the cohesive ends filled in by treatment with DNA polymerase Klenow fragment and the resulting molecule was inserted between similarly treated *Bam*HI and *Hind*III sites of pAPT120. One clone in which the direction of transcription of the *orfA-lacZ* gene fusion is the same as that in pAPT120 was retained as pMiT130.

Plasmid pBST2, carrying a mutation of the terminal CA3' of IRL and IRR in the junction, was constructed in two steps. The first step involved, replacing the *Pst*I-*Bal*I fragment of pAPT35, containing the outer part of IRL (from the *Bal*I site), by the same fragment of plasmid pAPT180 in which IRL carries the mutated terminal dinucleotide (together with an *Xba*I site). This gave rise to pBST1. Thus, this plasmid contains in pAPT180 and the adjacent *orfA-lacZ* gene fusion without IRR. A copy of IRR abutting IRL was then introduced by insertion of two complementary oligonucleotides:

5'-AGCTCTCTAACTCGGTGGCCAGTTTTGTGACCACTTAG-3'
3'-GAGATTGAGCCACCGGTCAAAAACAACCTGGTGAATCGATC-5'

between the *Xba*I site and an adjacent *Hind*III site distal to IRL. The letters in bold indicate the position of mutated CA3' dinucleotide.

Plasmids supplying transposition functions. Plasmids pAPT110, pAPT111 and pAPT112 have been described previously (Polard and Chandler, 1995b). Briefly, pAPT110 is a p15A-based plasmid carrying

the P_{lac} promoter, *lacI^q* and an Sp-Sm resistance cassette. Plasmids pAPT111 and pAPT112 are derived from pAPT110. They carry *orfAB* and the wild-type open reading frame configuration respectively together with indigenous translation initiation signals under the control of P_{lacUV5} (Polard and Chandler, 1995b).

Plasmid pAPT155 was constructed in two steps as follows. It derives from pAPT132 (Polard *et al.*, 1992) a plasmid carrying wild-type *orfA* and *orfB* open reading frames but in which an *NdeI* site was introduced at the *orfA* translation initiation codon. The natural *orfA* rbs was then substituted (between this *NdeI* site and an upstream *XbaI* site) by an *XbaI*-*NdeI* fragment from pAR30-39 which carries the rbs of the T7 $\phi 10$ gene (Studier and Moffatt, 1986). This gave pAPT150. The entire expression module (*rbs₁₀*-*orfA* *orfB*) was purified as an *XbaI*-*BamHI* fragment and cloned downstream from the P_{lacUV5} promoter in pAPT110 (Polard and Chandler, 1995b) to give pAPT155. Plasmids pAPT156, pAPT157 and pAPT158, which express OrfA, OrfB or OrfAB respectively, were constructed in a similar way, starting from plasmids pAPT136, pAPT133 and pAPT135 (Polard *et al.*, 1992).

Plasmid pBST5 was constructed by transferring the *EcoRI*-*BamHI* fragment carrying the entire *IS911* from pAPT28 (Polard *et al.*, 1994) into the *EcoRI*-*BamHI* site of pAPT110.

β -galactosidase assays

For measurement of β -galactosidase activity, cultures of MC1061(*recA*) carrying the constructions to be tested were grown overnight at 37°C in VB medium supplemented with 0.5% glucose, 0.5% casamino acids, Km and Ap and diluted into fresh medium at an OD_{600} of 0.05 with or without IPTG. After 2 h of growth ($OD_{600} = 0.3$ – 0.4), β -galactosidase was measured according to the method of Miller (1972) except that lysis was accomplished by SDS–chloroform treatment and BSA (1 mg/ml) was included. The lysates were centrifuged before measurement of absorbance at 420 nm.

Circularization assays

The alkaline lysis procedure for minipreparations of plasmid DNA was as described (Polard *et al.*, 1992). OrfAB was induced overnight with 0.1 mM IPTG at 37°C in LB medium. Plasmid DNA extraction used 3 ml of culture and one-fifth of the final preparation was analysed by agarose gel electrophoresis and detected by ethidium bromide staining.

Deletion assay

DNA extracted by alkaline lysis of the relevant strains was digested with *Apal*, to eliminate the plasmid which provides transposase, and used to transform MC1061(*recA*). Transformants were spread on X-gal plates supplemented with Ap.

RNA preparation and primer extension

An overnight culture of cells grown in L broth supplemented with ampicillin (100 mg/ml) was diluted to $OD_{540} = 0.05$ into fresh medium and grown to $OD_{540} = 0.4$. RNA was extracted using the hot phenol method according to Gordia and Gutierrez (1996).

Primer extension was performed using two oligonucleotides:

5'-GGGATCCCCAACTCTGCGCTG-3' (primer 2)
5'-TGGGTAACGCCAGGGTTTCCCC-3' (primer 3)

These were 5' end-labelled using polynucleotide kinase (Biolabs) before use. Primer extension reactions (Ausubel, 1995) using MMLV reverse transcriptase (BRL) were performed in a final volume of 20 μ l containing ~10 mg total RNA. After hybridization of the RNA with kinased primers, the mixture was incubated with dNTPs each at 200 μ M and 300 units of enzyme at 42°C for 30 min.

The products were fractionated on an 8% sequencing gel. A sequencing ladder generated using the same unlabelled oligonucleotide as primer was also effected using T7 sequencing kit (Pharmacia).

Mating-out assays

The frequency of *IS911* junction-mediated integration was determined by a standard mating-out assay (Galas and Chandler, 1982) using the conjugal plasmid pOX38Km (Chandler and Galas, 1983) as a target replicon. MC1061(*recA*)/pOX38Km, containing the transposon donor plasmids was transformed with the different p15A derivatives as source of transposase. After phenotypic expression at 42°C, the transformants were spread on L agar plates at 42°C (this temperature was used in order to reduce OrfAB activity which is temperature-sensitive; L.Haren, Betermier, M., Polard, P. and Chandler, M., submitted). Overnight cultures grown from a single colony in L broth supplemented with Ap, Km, Sp

and Sm at 42°C were diluted into L broth with 0.1 mM IPTG but without antibiotics at an OD_{540} of 0.05 at 37°C with shaking. Growth was continued for ~3 h to an OD_{540} of ~0.5, followed by 1 h incubation without agitation. The donor cells were mixed at an ~1:1 ratio with the recipient MC240 (*Nal^R*, *Rif^R*) also grown at 37°C without IPTG. Suitable dilutions were plated on L agar plates supplemented with *Nal*, *Rif*, *Km* (for scoring transfer) and *Nal*, *Rif*, *Ap*, *Met* (for scoring integration).

For further study, the derivatives of conjugal plasmid pOX38Km with integrated circles carried by primary transconjugants were transferred into the host strain MC1061(*recA*) by cross-streaking on suitable selective plates.

Structure of pOX38Km::pMiT130 derivatives

DNA of pOX38Km::pMiT130 was prepared by the cleared lysate method as described by Polard and Chandler (1995b). Samples were digested with *BamHI*, separated on 0.7% agarose gels in TAE buffer and subjected to electrophoresis at 60 V for 18 h at room temperature. The gels were dried for 1 h at 70°C. DNA was denatured by incubation of the gel for 20 min in 0.5 M NaOH, 0.15 M NaCl, neutralized by incubation in 0.5 M Tris, pH 8, 0.15 M NaCl. Hybridization was accomplished after 2 h of pre-hybridization in 50% formamide, 5 $\times$ SSC, NaH_2PO_4 / Na_2HPO_4 (0.05 M) pH 6.5, SDS 0.1%, 100 mg/ml of calf thymus DNA and 2% non-fat milk for 15 h at 42°C. The probe was prepared using the Megaprime labelling system (Amersham) with the entire pMiT130 plasmid. The gel was washed three times and exposed for autoradiography using Kodak XR5 film. The structures were confirmed by additional digestions using *EcoRI* and *BglII* (Chandler and Galas, 1983).

Western blotting

The relative level of *IS911*-encoded proteins (OrfA and OrfAB) in strains carrying different constructions were analysed by immunoblot analysis of crude extracts using polyclonal anti-OrfA antibodies raised against denatured OrfA protein. Cells were grown as described for β -galactosidase assays in the presence of 0.4 mM IPTG. Cell lysates were prepared by mixing exponentially growing cells (2 h after induction) in sample buffer and boiling for 2 min. Samples were fractionated by SDS–PAGE on 12.5% gels (Laemmli, 1970) and transferred to a protran–nitrocellulose membrane (Schleicher & Schull) by electroblotting using a semi-dry transfer system (Touzart Matignon). The membranes were treated as described by (Sambrook *et al.*, 1989). After preincubation, the membrane was incubated at 4°C overnight with anti-OrfA antiserum diluted 1:5000, washed, incubated with an anti-rabbit horseradish peroxidase-coupled secondary antibody and revealed using an ECL detection kit (Amersham).

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