RecA-independent ectopic transposition in vivo of a bacterial group II intron

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ABSTRACT

RmInt1 is a group II intron of Sinorhizobium meliloti which was initially found within the insertion sequence ISRm2011-2. Although the RmInt1 intron-encoded protein lacks a recognizable endonuclease domain, it is able to mediate insertion of RmInt1 at an intron-specific location in intronless ISRm2011-2 recipient DNA, a phenomenon termed homing. Here we have characterized three additional insertion sites of RmInt1 in the genome of S. meliloti. Two of these sites are within IS elements closely related to ISRm2011-2, which appear to form a characteristic group within the IS630-Tc1 family. The third site is in the oxi1 gene, which encodes a putative oxidative reductase. The newly identified integration sites contain conserved intron-binding site (IBS1 and IBS2) and 5′ sequences (14 bp). The RNA of the intron-containing oxi1 gene is able to splice and the oxi1 site is a DNA target for RmInt1 transposition in vivo. Ectopic transposition of RmInt1 into the oxi1 site occurs at 20-fold lower efficiency than into the homing site (ISRm2011-2) and is independent of the major RecA recombination pathway. The possibility that transposition of RmInt1 to the oxi1 site occurs by reverse splicing into DNA is discussed.

INTRODUCTION

Group II introns are a unique class of introns initially found in organellar genes of lower eukaryotes and plants but recently also found in bacteria (1). These introns are large catalytic RNAs which splice via a lariat intermediate, similar to the mechanism of spliceosomal introns (1). Some group II introns are mobile genetic elements that insert site specifically in intronless alleles, a process known as homing. In addition to homing, some introns are able to transpose to novel (ectopic) sites at low frequency (2–4). Recent studies with the Lactococcus lactis L1.ltrB intron and the yeast mitochondrial aI1 and aI2 introns have established a mechanism for group II intron retrohoming (5–7). Mobility occurs by a target DNA-primed reverse transcription mechanism involving intron-encoded reverse transcriptase (RT) and a site-specific DNA endonuclease (5–10). The endonuclease is a ribonucleoprotein (RNP) complex containing both the intron RNA and the intron-encoded protein (IEP). The intron RNA cleaves the sense DNA strand by a partial or complete reverse splicing reaction at the intron insertion site, while the IEP cleaves the antisense strand after the +10 position of the 3′ exon, or +9 in the case of the L1.ltrB intron. The 3′-end of the cleavage site is then used as the primer for reverse transcription of either the unspliced RNA precursor or the intron RNA that had reverse spliced into the sense strand of the recipient DNA. Group II intron endonucleases use both their RNA and protein components to recognize specific sequences in their DNA target sites. In the case of the L1.ltrB intron the RNP recognition site spans from –25 to +10, while intron RNA base pairs over positions –13 to +1 (5). Retrohoming of the L1.ltrB intron occurs via complete reverse splicing of the intron RNA into DNA, independent of homologous recombination (5).

In contrast to retrohoming, the mechanism(s) of ectopic transposition is still poorly understood and different pathways have been proposed (2–4). Recently, data obtained with the L1.ltrB intron suggest that the major retrotransposition pathway can proceed by reverse splicing of the intron into an ectopic site in RNA followed by reverse transcription and homologous recombination of the resulting cDNA. This retrotransposition occurs in an endonuclease-independent and recombinase-dependent fashion (11). On the other hand, for yeast intron aII in vitro data show that it reverse splices directly into ectopic DNA transposition sites (6).

RmInt1 is a group II intron from Sinorhizobium meliloti, the IEP of which lacks a recognizable endonuclease domain (12,13). Nevertheless, this intron has been shown to be mobile (13). Although the homing pathway of this intron has yet to be determined, the fact that the IEP lacks a HNH motif suggests that its mobility may involve a different mechanism. RmInt1 is abundant and widespread within S. meliloti native populations, where it appears located mostly within the insertion sequence ISRm2011-2. Nevertheless, some S. meliloti strains harbor copies of RmInt1 at different locations. From sequence analysis data it was previously suggested that one of these heterologous sites was another IS element closely related to ISRm2011-2 (12).

In the present study we have characterized three additional insertion sites of RmInt1 in the genome of S. meliloti. We show that RmInt1 is able to splice at the most divergent of these locations (the oxi1 gene) and that it transposes in vivo to this ectopic site independently of homologous recombination.

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Bacterial strains, media and growth conditions

*Escherichia coli* was routinely grown at 37°C on Luria–Bertani medium (14) and rhizobial strains at 28°C on TY (15) or defined minimal medium (16). Antibiotics were used as required at the following concentrations: ampicillin, 200 µg/ml; kanamycin, 180 µg/ml for *S.meliloti* and 50 µg/ml for *E.coli.*

**Sinorhizobium meliloti** strains used were GR4 and RMO17 (this laboratory), GR4 RecA⁻ (13), 2011 (Nod⁺, Fix⁺ SU47 derivative strain; J. Denarié), CE31A and LPU119 (17)

**DNA sequencing and analysis**

Sequencing was performed with an Automatic Laser Fluorescent DNA Sequencer (Applied Biosystems). DNA sequence edition, translation and analysis were performed with the GenWorks software package (Oxford Molecular Group). The program FASTA and the BLAST Network Server at the National Center for Biotechnology Information (NCBI) were utilized for homology searching.

**DNA hybridization and fingerprinting**

Total DNA was isolated according to standard protocols (14). After restriction enzyme digestion, 2 µg of DNA was electrophoretically separated in a 0.8% Tris–borate agarose gel and vacuum blotted onto nylon filters (Hybond-N; Amersham) for 18 h at 100 V. DNA probes were DNA templates. Oligonucleotides used in the amplification reaction (Boehringer Mannheim), using plasmid pRmNT40 (18) as template. Oligonucleotides used in the amplification reaction according to the manufacturer’s instructions. DNA probes for IS*Rm101-2* and *RmInt1* were obtained by PCR amplification of internal fragments, in the presence of digoxigenin-11-dUTP (Promega), resulting in the RNA annealing primer. PCR reactions were carried out in a Robocycler 40 (Stratagene) using primers ORA (positions 49–64 in the *oxil* + strand) and ORB. The cycling conditions were 3 min initial denaturation at 94°C, followed by 35–40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2.5 min, with an extension of 15 min at 72°C. PCR samples (8 µl) were analyzed by agarose gel electrophoresis. The RT–PCR product of 0.5 kb was isolated, purified from the agarose gel and sequenced using primers ORA and ORB.

**In vivo analysis of transposition to the oxil gene**

The region encompassing –415 to +98 of the oxil gene insertion site was amplified and subcloned into pGEM-T (Promega), resulting in plasmid pTOR. Plasmid pTOR was then digested with Apal and Sall and the *oxil*-derived fragment was cloned into plasmid pBBCRMS-2 (19), to generate plasmid pBBTOR. Plasmid pBB0.6+, containing a 640 bp fragment encompassing the oxil gene insertion site of IS*Rm101-2* and the derivative pBBA129, carrying an internal deletion of the target site, were used as controls for homing (13). Plasmids were transferred from *E.coli* cells to *Sinorhizobium* by triparental mating using the helper plasmid pRK2013 (20). Plasmid isolation from *S.meliloti* was from cells grown in TY medium until stationary phase. The DNA was digested with Sall and Southern hybridization with the 5'-end of the oxil gene probe was performed at high stringency. Detection of pBBTOR*Intron (pBB2.4) required overexposure of the film, which resulted in non-specific hybridization signals from the intron recipient plasmid (pBB0.6+). The heavy hybridization signals observed at the bottom of Figure 4B (lanes 1 and 6) correlate with the level of intron-invaded plasmids and are due to undigested supercoiled forms. Densitometric scanning of the bands of ethidium bromide stained gels was performed with an Imagequant AQ system (Amersham). The percentage of invasion of recipient plasmids containing the homing target site (pBB0.6+, Fig. 4A, lanes 1 and 6) was calculated from the ethidium bromide stained gel data, corrected by taking into account the differences in size between the two plasmids analyzed (intron-invaded and recipient). However, the percentage of invasion of plasmid containing the oxil target (pBBTOR, Fig. 4, lanes 2 and 4) could only be estimated by comparing the specific hybridization signal intensities displayed by the *RmInt1*-invaded plasmid forms.
RESULTS

The insertion sites for intron RmInt1 in the genome of S. meliloti

Even though in S. meliloti intron RmInt1 is mostly located in ISRm2011-2, some strains carry copies of the intron at a different location. We investigated these additional insertion sites in strains 2011, CE31A and LPU119 (see Materials and Methods).

Consistent with our previous findings (12), DNA and amino acid sequence analyses of the 3' exon showed that the additional insertion site in strain 2011 is an IS element, hereafter referred to as ISRm10-1. Similarly, in strain CE31A the additional insertion site is another IS element, hereafter named ISRm10-2. Interestingly, in strain LPU119 the insertion site appears to be a gene coding for a putative oxidoreductase (hereafter referred to as oxi1; Table 1), which shows similarity (39% identity) with DlE protein of Bacillus subtilis (21). The intron integration site in oxi1 is located close to the end of the coding region. DNA hybridization studies indicated that ISRm10-1 and ISRm10-2 are less abundant than ISRm2011-2 in S. meliloti (Table 1). In addition, it was found that oxi1 is present as a single copy in the genome of S. meliloti, where it mostly appears as an oxi1 intronless gene (Table 1; N.Toro, unpublished data). All the insertion sites identified are in the sense orientation of the respective genes.

Table 1. Presence of RmInt1 and host elements in the genome of S. meliloti

<table>
<thead>
<tr>
<th>Strain</th>
<th>ISRm2011-2</th>
<th>ISRm10-1</th>
<th>ISRm10-2</th>
<th>oxi1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR4</td>
<td>12/9</td>
<td>2/1</td>
<td>0/0</td>
<td>1/0</td>
</tr>
<tr>
<td>2011</td>
<td>12/2</td>
<td>1/1</td>
<td>0/0</td>
<td>1/0</td>
</tr>
<tr>
<td>CE31A</td>
<td>4/2</td>
<td>1/0</td>
<td>1/1</td>
<td>1/0</td>
</tr>
<tr>
<td>LPU119</td>
<td>5/4</td>
<td>2/1</td>
<td>0/0</td>
<td>1/1</td>
</tr>
</tbody>
</table>

*Total copy number of the host element/number of copies of the host element invaded by RmInt1.

The intron copies in the above insertion sites were partially sequenced (from 165 to 584 bp, including the EBS1 and EBS2 motifs) and each was shown to have an identical DNA sequence to that of the intron found within ISRm2011-2 in pRMNT40 (12). Furthermore, the intron inserts between the same A and C nucleotides (Fig. 1). Identification of these new intron insertion sites enabled us to propose the sequence UUUCGGC (nt 256–263) as the EBS1 sequence of intron RmInt1, instead of the sequence previously suggested (12).

The three ISs harboring RmInt1 in S. meliloti are closely related and, together with an IS found in Spingomonas aromaticivorans (22), appear to constitute a particular group within the IS630-Tc1 family (12,23), hereafter referred to as the ISRm2011-2 group (Fig. 2). Proteins encoded by ISRm10-1, ISRm10-2 and the IS found in S. aromaticivorans (SaISA) show 45, 43 and 42% amino acid identity, respectively, with ISRm2011-2 ORF AB. The ISs have a similar size (1042 bp for ISRm10-1, 1049 bp for ISRm10-2, 1053 bp for ISRm2011-2 and 1056 bp for SaISA) and identical terminal direct repeats (TA) and encode two ORFs (A and B) with a potential translational frameshifting site (AAAAAAAG) at a similar distance upstream of the intron insertion site (109 nt for ISRm2011-2, 108 nt for ISRm10-1 and 109 nt for ISRm10-2). The ISRm2011-2 group, in addition to the characteristic ‘DE, D35E’ motif (23) has the conserved motif FIDET encompassing the dipeptide Asp–Glu (DE), which results from translation of the IBS1, IBS2 and δ’ sequences (Fig. 1). The oxi1 gene also contains the IBS1, IBS2 and δ’ sequences and its encoded product contains in its C-terminal region the FIDET motif.
The DNA target site for intron RmInt1

A deletion analysis of the wild-type DNA target site (ISRm2011-2) defined a minimal region of 25 bp (20 bp of homology in the 5′ exon and 5 bp of homology in the 3′ exon) that supports homing to wild-type levels (F.M.García-Rodriguez and N.Toro, unpublished results). Hence, the DNA target sites compared in Figure 1 encompass only 25 bp (5′ exon −20 to 3′ exon +5). Within the IBS (positions −1 to −13) and δ′ (position +1) sequences, which base pair with complementary intron RNA EBS and δ sequences, only positions −2, −5 and −11 show nucleotide changes that represent transitions –5 and –11 show nucleotide changes that represent transitions –5 and –11 show nucleotide changes that represent transitions –5 and –11 show nucleotide changes that represent transitions.

Forward splicing of RmInt1 at the oxil insertion site

RmInt1 splices at the ISRm2011-2 wild-type insertion site (12,24). We investigated whether RmInt1 is also competent for splicing at the oxil site. RNA from S.meliloti strain LPU119 was reverse transcribed and amplified by PCR with primers specific for both exons of oxil. The 0.5 kb RT–PCR product obtained (Fig. 3, lanes 3 and 4) was reverse transcribed and amplified by PCR with primers RmInt1 (positions 528–546 in the oxil strand) and ORB (positions 528–546 in the oxil strand) primers annealing to both exons. Arrows indicate the PCR products derived from DNA of the oxil gene invaded by RmInt1 (2.3 kb) and from mRNA of the ligated exons (0.5 kb) or native oxil gene (not invaded by RmInt1).

Intron invasion of pBBTOR was compared with that of pBB0.6+ carrying the wild-type insertion site of ISRm2011-2 (13). Plasmid pool analysis and DNA hybridization indicated that intron invasion occurred in all transconjugants analyzed (two independent experiments; data not shown) but not in those where the target (pBBΔ129) lacks the insertion site (Fig. 4, lanes 3 and 5; 13). DNA sequence analysis of invaded pBBTOR in strain GR4 shows that intron insertion occurs at the exon junction and that the flanking regions are identical to those of the wild-type oxil intronless gene. Moreover, the DNA sequence of the intron inserted into pBBTOR was identical to the sequence already published for intron RmInt1 (12; data not shown). These results indicate that the transposition of RmInt1 to the oxil gene is a site-specific event, like homing (13). To test if the ectopic transposition of RmInt1 follows a RecA-dependent pathway as described for L1.LtrB (11), transposition assays were carried out in parallel with a GR4 recA− derivative strain (Fig. 4, lanes 1–3). In both recA+ and recA− hosts the oxil target site was invaded by the intron with similar efficiencies of 1.3 and 1.1%, respectively (Fig. 4C), which represents a 20-fold lower efficiency compared with that of the homing site (ISRm2011-2). This result indicates that ectopic transposition of RmInt1 to the oxil gene is independent of homologous recombination and suggests that it may involve a DNA rather than an RNA target.

DISCUSSION

Here, we report on group II intron RmInt1 natural insertion sites and transposition events in S.meliloti. We have characterized three additional insertion sites, two of them corresponding to

Figure 3. Splicing of RmInt1 at the oxil site. Electrophoretic analysis of PCR products obtained using 10 ng total DNA from LPU119 (lane 1) or GR4 (lane 2) or LPU119 cDNA (lanes 3 and 4). PCR amplification was carried out for 35 (lane 3) and 40 (lanes 1, 2 and 4) cycles using the ORA (positions 49–64 in the oxil + strand) and ORB (positions 528–546 in the oxil − strand) primers annealing to both exons. Arrows indicate the PCR products derived from DNA of the oxil gene invaded by RmInt1 (2.3 kb) and from mRNA of the ligated exons (0.5 kb) or native oxil gene (not invaded by RmInt1).

Figure 4. RecA-independent transposition of RmInt1 into the oxil gene. (A) Plasmid pool analysis from GR4 (lanes 4–6) and its recA− derivative (lanes 1–3) were performed as specified (see Materials and Methods). Lanes 1 and 6, pBB0.6+ pool; lanes 2 and 4, pBBTOR pool; lanes 3 and 5, pBBΔ129 pool. (B) DNA hybridization blot performed with the intron-specific probe. Arrowheads indicate the novel plasmids that migrate 1.8 kb above the cognate target pBB0.6+ and pBBTOR plasmids. Molecular markers are indicated. T, recipient plasmid. (C) Levels of invasion of the respective recipient plasmids at the indicated host. The percentage intron invasion was determined as indicated (see Materials and Methods). Rel WT indicates relative to 100 for the pBB0.6+ recipient in a RecA+ host. The data is the mean of two independent experiments.
IS elements closely related to ISRm2011-2, while the third shows similarity to genes of the family of oxide reductases.

Most of the bacterial group II introns identified to date have been localized within genes likely to be involved in DNA mobility (25–30). However, the association of RmInt1 with a specific group of IS elements is remarkable and unprecedented for bacterial group II introns. The target site within these IS elements and the flanking regions are conserved, exhibiting a high similarity between the corresponding encoded ORFs and inverted repeats. Interestingly, in addition to the DE and D35E motifs characteristic of the IS630-Tc1 family (23), the host elements for intron RmInt1 contain in the ORF B portion of the transposase the motif FIDET, which results from translation of the IBS1, IBS2 and δ′ sequences (Fig. 1). All these features suggest that they form a subgroup within the IS630-Tc1 family (Fig. 2). Recently, an IS element of this group (ISRm2011-2) has been reported in plasmid pNL1 of S. aromaticivorans (22). However, it is not known whether this bacterial species contains more copies of this element and if any of them is interrupted by an RmInt1 homolog. ISRm2011-2 is considered an ancestral IS element of S. melliloti (31), but similar mobile elements have been detected in the closely related species Sinorhizobium medicae, where each appears as a single copy and is free of introns (E. Muñoz and N. Toro, unpublished results). On the other hand, a group II intron similar to RmInt1 (+95% nt identity) has been identified in other Sinorhizobium species within a putative mobile element of the ISRm2011-2 group (E. Muñoz and N. Toro, unpublished results). Thus it is likely that the presence of specific DNA target sites provided by the abundance of these IS elements, particularly of ISRm2011-2, in S. melliloti has facilitated the successful spread of the intron in these bacterial species.

In addition to the former ISRm2011-2 group of IS elements, RmInt1 was found in a gene, oxil, which codes for a putative oxide reductase that is not related to genes involved in DNA mobility. This ectopic site contains the IBS1, IBS2 and δ′ sequences (Fig. 1) recognized by the intron RNA, which allows sufficient base pairings with intron sequences for forward splicing (Fig. 3). Hence, it is unlikely that insertion of the intron into the oxil gene would interfere with the putative function of this locus. Although the oxil gene encoded product does not show similarity to the transposases of the ISRm2011-2 group, it does carry the FIDET motif as a consequence of possession of the IBS1, IBS2 and δ′ sequences. Whether this particular translated frame has any meaning in either splicing or intron invasion remains to be elucidated.

All the insertions found for intron RmInt1 in the genome of S. melliloti are in the sense orientation of the respective transposase and oxil genes. Similarly, the ectopic insertions described for intron L1.ItrB were in the sense orientation of the gene (11), which was taken to support the hypothesis of retrotransposition into an RNA target. This work will be required to elucidate this question.

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