Circularized Ac/Ds Transposons: Formation, Structure and Fate

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ABSTRACT

The maize Ac/Ds transposable elements are thought to transpose via a cut-and-paste mechanism, but the intermediates formed during transposition are still unknown. In this work we present evidence that circular Ac molecules are formed in plants containing actively transposing elements. In these circles, transposon ends are joined head-to-head. The sequence at the ends' junction is variable, containing small deletions or insertions. Circles containing deleted Ac ends are probably unable to successfully reintegrate. To test the ability of circles with intact transposon ends to integrate into the genome, an artificial Ds circle was constructed by cloning the joined ends of Ac into a plasmid carrying a plant selectable marker. When such a circular Ds was introduced into tobacco protoplasts in the presence of Ac transposase, no efficient transposase-mediated integration was observed. Although a circular transposition intermediate cannot be ruled out, the findings of circles with deleted transposon ends and the absence of transposase-mediated integration of the circular Ds suggest that some of the joined-ends-carrying elements are not transposition intermediates, but rather abortive excision products. The formation of Ac circles might account for the previously described phenomenon of Ac loss. The origin of Ac circles and the implications for models of Ac transposition are discussed.

THE maize Ac/Ds elements were the first discovered transposons (McClintock 1948). Since then, transposable elements of a similar type have been found in a wide range of prokaryotic and eukaryotic species. Extensive studies provided a detailed description of the transposition process for bacterial elements such as Tn7 and Tn10 (for reviews see Saedler and Gierl 1996), but the precise mechanism by which Ac/Ds elements move from donor to recipient site remains unclear.

Ac belongs to the DNA-DNA type of transposable elements (Finnegan 1992). It is flanked by 11-bp terminal inverted repeats and generates an 8-bp target site duplication upon insertion (Muller-Neumann et al. 1984; Pohlsman et al. 1984; Sutton et al. 1984). Ac encodes transposase, a protein required for transposition (Kunze et al. 1987). Ds elements are considered mutated Ac's that retain the terminal Ac regions required for transposition but contain either deletions or insertions in their internal part that abolish transposase production (for review see Fedoroff 1989). Ac transposes autonomously, whereas Ds transposes only in the presence of Ac (McClintock 1951) or Ac transposes. It has been shown that Ac is active in plant species other than maize (reviewed in Kunze 1996), when introduced into the genome via Agrobacterium-mediated transformation. It was also demonstrated that, following protoplast transformation, Ds can transpose from extrachromosomal DNA vectors into plant chromosomes in the presence of transposase (Houba-Herin et al. 1994; Sugimoto et al. 1994).

It is thought that Ac transposes by a conservative (cut-and-paste) process in which the element excises from a donor site and reinserts into a recipient site (Greenblatt and Brink 1962; Chen et al. 1992). Yet, the intermediates formed in the course of transposition are unknown. One possibility, proposed for Ac (Greenblatt 1984) and for Tn3 (Robbins et al. 1989), is that Ac transposes without extrachromosomal intermediates. According to this model, one transposon end is cut first and the second transposon end is cut only after the first end is ligated to the recipient site. Alternatively, extrachromosomal intermediates might be formed during transposition. Extrachromosomal circular DNA molecules were identified for the MuI transposon of maize (Sundaresan and Freeling 1987), and extrachromosomal linear molecules were found for the Tat1 element of Arabidopsis thaliana (Peleman et al. 1991) as well as for Tc3 of Caenorhabditis elegans (Van Luenen et al. 1993). For the Tc1 element of C. elegans, both linear and circular forms were detected (Rose and Snutch 1984; Ruan and Emons 1984). However, the ability of extrachromosomal putative intermediates to integrate into the genome has not been tested for any eukaryotic transposon. By contrast, linear extrachromosomal species that were detected for the bacterial transposons Tn7 (Bainton et al. 1991) and Tn10 (Hancock et al. 1991; Benjamin and Kleckner 1992) were shown to integrate into the chromosome, as expected for true transposition intermediates.

In this work, we show that joined Ac ends are formed in plants containing active Ac elements. The sequence at the junction of the transposon ends is variable, containing small deletions or insertions. Several pieces of
evidence indicate that joined Ac ends are derived from circularized transposons. The ability of transposon circles to reintegrate into the genome was examined using a synthetic Ds circle transformed into plant protoplasts. Analysis of the transformed plants failed to identify insertions with the pattern characteristic of Ac transposition. The implications of these findings on the origin of Ac circles and on models for Ac transposition are discussed.

**MATERIALS AND METHODS**

**Plant material:** The transgenic tobacco plants used for detection of joined Ac ends consisted of T2 plants of *Nicotiana tabacum var. Xanthi*. T2 plants were obtained from independent T1 transformants, each containing a single T-DNA insertion, as deduced from the segregation of T2 plants. Transformation was performed either with the Ac-carrying construct pAGS411 (DOONER et al. 1991) or the Ds-carrying construct pAGS4081 (KELLER et al. 1993) by an Agrobacterium-mediated procedure (HORSCH et al. 1985). Constructs were kindly provided by H. DOONER. Transposition activity in these plants was tested on streptomycin-containing media as described (JONES et al. 1989). For protoplast transformation experiments, two types of *N. tabacum var. Samsun* plants were used: either wild type or transgenic plants carrying the pMF006 construct (FRIELENDEER et al. 1996), containing the Ac transposase open reading frame (ORF) under the control of the cauliflower mosaic virus 35S promoter. Two maize lines were used as Ac/Ds sources. One line, by the maize cooperative, carried Ac within the *Plocus* (*P,-VV/P,-W*). The other line, given by V. WALBOT, carried a Ds2 element at the B2 locus and an active Ac element in the background as deduced by the aleurone variegation of the *dz2:*Dsx allele (THERES et al. 1987).

**Plant DNA extraction:** DNA was isolated from young leaves of tobacco or maize immature cobs as described (DELLA-PORTA et al. 1985). To extract DNA from protoplasts the same protocol was used with the following modifications: the volume of all buffers was scaled down to perform the procedure in an Eppendorf tube, and the isopropanol precipitation step was omitted.

**PCR and IPCR, cloning of the PCR products and sequencing:** All PCR amplifications were performed with a cycle of 1 min denaturation at 94°, 1 min of annealing at 55°, and 1 min of extension at 72° repeated 30 times. Sequencing was done by Sanger’s method using an Applied Biosystems 373A DNA sequencer. For joined ends detection, ~1 μg of genomic DNA was used as a template for two nested rounds of amplification, as shown in Figure 1, with primers 2 (5' GGCGGCCATTACGGTAATCG-3') and 3 (5' CTAACTGCAATCGGACGGAG-3') in the first round. In the second round, 1.5 μl of the reaction from the first round was used as a template for amplification with primers 1 (5’ TCCGGAGATTTAGAAAATACCGG-3’) and 4 (5’ GGTGGCGTCCAGATTATATG-3’). PCR products were cloned as follows: clone 3 (Table 1) was obtained by direct cloning of the PCR product into the pcRII TA cloning kit (Invitrogen); all other clones were obtained by digestion of the PCR products with BamHI and PstI, and cloning into the corresponding sites of the pNEB193 vector (New England Biolabs). The resulting plasmids were sequenced with primers 5 (5’ GTTTTTTATCTCGGTTTCG-3’) and 13 (5’ TTTCTGTTTTCCGAGTTAAAATA-3’).

To amplify empty donor sites following Ds excision from pVG5, two nested rounds of PCR were done with standard T3 and T7 primers in the first round, and in the second round, with two primers from the Ac-flanking region of the maize Waxy gene: W1 (5’ GAGGCTGTTACGTCGCCGAGCTCAG-3’) and W2 (5’ AGTGTCAGCCCGCAGCGGCGCGG-3’). Resulting PCR products were cloned directly into a pGEM-T vector (Promega) and sequenced with the T7 primer.

For IPCR 1 μg of genomic DNA was digested with one-sixth unit of Sau3A, extracted with phenol-chloroform, ethanol precipitated, and circularized in a total volume of 500 μl with 10 units of T4 DNA ligase. After ethanol precipitation, one-half of the reaction was amplified to amplify the Ds end in the second round of PCR with primers 5 and 6 (5’ CGAAAAAGCAGGGAGGACGCGG-3’) in the first round, and primers 7 (5’ TTTCCGGTTTGGCTCCCAGTAA-3’) and 8 (5’ TGGTTCTCCGCGCTTGGCACG-3’) in the second. The 3′ end of the element was amplified with primers 9 (5’ ACCGGTGTAAGAAAAAAGCAG-3’) and 10 (5’ TTTCGTTTCCGCTCCCAGTAA-3’), in the first round, and primers 11 (5’ GGGAATAAACCTGGTTAGC-3’) and 12 (5’ GAAAATGAAACCGGTAGGC-3’) in the second round. IPCR products were cloned directly into the pGEM-T vector (Promega) and sequenced with the primers 7 and 12 for the 5′ and 3′ transposon ends, respectively.

**Plasmids:** Plasmid pVG2 was constructed as follows: the Ac-containing 4.8-kb *KpnI-BamHI* fragment of pAGS411 (DAVIS et al. 1984) was cloned into the KpnI-BamHI sites of Bluescript II KS (Stratagene), and Ac was converted into Ds by deletion of the internal 1.6-kb *HindIII* Ac fragment. Plasmid pNT804 (HOURA-HERIN et al. 1990), the transposase producer, was kindly provided by Dr. N. HOURA-HERIN. Plasmid pVG6 was derived from pNT804 by deleting a 3.5-kb *BamHI* fragment, containing the transposase ORF. The synthetic Ds circle (Figure 3A) was constructed as follows: the PCR fragment containing joined Ac ends (clone 3, Table 1) was cloned into the pcRII vector (Invitrogen) into the *pRw1* plasmid using the *KpnI* and *XbaI* sites. *pRw1* is a Bluescript II KS (Stratagene) derivative containing the 2.5-kb *Cad-sEd* fragment from pGA992 (AN 1987); the latter carries the plant kanamycin-resistance gene under the nopaline synthase promoter. pVG5 is schematically described in Figure 4A. It contains a *Ds* element cloned into the *KpnI-BamHI* sites of Bluescript II KS. The *Ds* element contains an 5′ end derived from an ~0.3-kb *KpnI-MstI* fragment from pAGS4411, followed by the plant kanamycin-resistance gene of *pRw1*, a bacterial chloramphenicol-resistance gene isolated as a 2.7-kb *Acl* fragment from pACYC184 (New England Biolabs), and as a 3′ *Ac* end, the *PstI-BamHI* fragment from pAGS4411.

**Protoplast transformation and plant regeneration:** Protoplasts were isolated from sterile leaves (AVIV and GALUN 1985). Polyethylene glycol-mediated transformation was done as described (VARDI et al. 1990) with some modifications: plasmids were uncut and calf thymus DNA was not added. For stable transformation aliquots of 106 protoplasts were cotransformed with 5.4 pmol of *Ds* circle and either 2.7 pmol of pNT804 or 2.7 pmol of pVG6. After transformation, protoplasts were resuspended in 0.5 M sucrose-containing VM medium (AVIV and GALUN 1985) at a density of 104 per ml, and kept for 4 days in the dark, and 2 days at low light at 25°. The protoplasts were then transferred under light, and the sucrose concentration was gradually reduced to 0.2 M within 10 days. Sixteen days after transformation microcalli were embedded in 0.6% Seaplaque LMT agarose (Marine Colloids, Rockland, USA) in 5-cm Petri dishes. The agarose with embedded microcalli was cut into pieces, which were inverted upon transferring into two 5-cm Petri dishes, each with 6 ml of liquid containing equal volumes of MS (MURASHIGE and SKOOG 1962) and VMK and 50 μg/ml kanamycin. One week later the liquid medium was replaced by liquid MS with 100 μg/ml kanamycin. The medium was replaced each week with
a fresh one. After 2 months kanamycin-resistant calli were scored and transferred into solid MS medium supplemented with 2 μg/ml naphthaleneacetic acid and 0.5 μg/ml benzylaminopurine. After 1 month of growth, calli were transferred to regeneration medium (MS containing 2 μg/ml kinetin and 0.8 μg/ml indoleacetic acid).

Southern analysis of tobacco plants transformed with Ds circle: For Southern analysis, 5 μg of genomic DNA was digested with Pael and EcoRV, fractionated on 0.8% agarose gels, and transferred to nitrocellulose purchased from MSI. Hybridization was performed according to manufacturers instructions. A 2.5-kb XbaI-SacI fragment from the Ds circle plasmid, containing the kanamycin-resistance gene, was radiolabeled by the random priming method (Feinberg and Vogelstein 1983) and used as a probe.

RESULTS

Joined Ac ends are formed in plants containing actively transposing elements: To test for the presence of Ac circles, an attempt was made to physically isolate such circles by CsCl-EtdBr gradient centrifugation and Southern blotting. Several DNA extractions were done from Ac-containing tobacco leaves, and 200–500 μg of DNA, depending on the extraction, was run on a gradient. A band of the size expected for the circularized Ac was seen only in some of the gradients and was always at the limit of detection (data not shown). This approach was not practical for further analysis, therefore a PCR strategy was used to identify circular Ac molecules. Two nested pairs of PCR primers were constructed that anneal within Ac and prime DNA synthesis toward the ends of the element (Figure 1A). Thus, only molecules containing nearby or adjacent right and left ends of Ac could serve as amplification templates (Figure 1B). Such molecules could be either extrachromosomal Ac circles or tandem genomic Ac in direct orientation.

Circularized Ac/Ds Transposons

Figure 1.—A PCR assay designed to detect the formation of joined Ac ends. The filled and empty arrow heads correspond to the 5' and 3' terminal inverted repeats of Ac, respectively. (A) A set of PCR primers (arrows) designed to amplify joined Ac ends. (B) Possible amplification templates for the primers 2 + 3 and 1 + 4.

Figure 2.—Detection of joined Ac/Ds ends by PCR. An ethidium bromide-stained agarose gel displaying the PCR products obtained with the primers designed to amplify joined Ac ends (Figure 1A). Primers 2 and 3 were used in the first round of amplification, and 1.5 μl of the reaction mixture was then used as a template for the second round of amplification with primers 1 and 4. The arrow indicates ~540-bp PCR products expected for a circularized Ac or Ds element. The template in lanes 1–10 was the genomic DNA extracted from leaves of tobacco containing Ac (lanes 1–5) or Ds (lanes 6–10) elements. In lane 11, the template was the genomic DNA extracted from maize containing actively transposing elements. Lanes 12 and 13 show the results of the experiment designed to detect joined ends upon transient transformation of tobacco protoplasts with Ds-containing plasmid (pVG2). pVG2 was transformed into protoplasts that express stable transposase (lane 12) and into wild-type protoplasts (lane 13). Transformation was done by CaCl2/polyethylene glycol technique (Vardi et al. 1990). After overnight incubation protoplast DNA was extracted and analyzed by PCR.

Transgenic tobacco: We used transgenic tobacco plants containing an Ac or Ds element cloned into the leader sequence of the streptomycin-resistance gene (constructs pAGS411 or pAGS4081). To confirm that Ac was actively excising and that Ds was stable, progeny seeds of the transgenic plants were germinated on streptomycin-containing media where excision can be monitored in cotyledons by the appearance of green (streptomycin resistant) sectors. The template in lanes 1–10 was the genomic DNA extracted from leaves of tobacco containing Ac (lanes 1–5) or Ds (lanes 6–10) elements. In lane 11, the template was the genomic DNA extracted from maize containing actively transposing elements. Lanes 12 and 13 show the results of the experiment designed to detect joined ends upon transient transformation of tobacco protoplasts with Ds-containing plasmid (pVG2). pVG2 was transformed into protoplasts that express stable transposase (lane 12) and into wild-type protoplasts (lane 13). Transformation was done by CaCl2/polyethylene glycol technique (Vardi et al. 1990). After overnight incubation protoplast DNA was extracted and analyzed by PCR.
Ds-containing plants (lanes 6–10). On the other hand, out of 17 Ae-containing plants tested, all but Ae-4 gave rise to PCR products of the size expected for circularized Ac (~540 bp) as shown for a sample of plants in lanes 1–5. The absence of the 540-bp PCR product with Ds, and with Ae-4, which apparently carries an inactive Ac, demonstrates that transposition activity is involved in the formation of joined Ac ends. Occasionally PCR products of variable sizes, bigger than 540 bp, were observed (data not shown). They might originate from tandem Ac copies that arose as a result of somatic nearby jumps. The major reaction product always had the size expected for joined Ac ends and is therefore likely to be derived from extrachromosomal Ac circles; tandem genomic Ac’s would be expected to give rise to PCR products of various sizes depending on the length of the sequence between the two elements.

Maize: To test whether joined Ac or Ds ends are formed in maize, plants were grown from variegated kernels (i.e., kernels carrying actively transposing elements). One line carried Ds within the B2 locus (Theres et al. 1987), and another carried Ac within the PI locus (Greenblatt and Brink 1962). DNA was extracted from immature cobs and amplified with the primers designed to detect joined Ac ends. Unlike in transgenic tobacco, PCR amplification yielded several products (Figure 2, lane 11), one of which had the size expected for joined Ac ends. The multiplicity of bands in maize was expected as various active and defective Ac/Ds elements are probably present in the genome.

Protoplasts: To rule out the possibility that joined Ac ends originate from tandem genomic Ac’s, we used transient assays to test for the formation of joined ends in tobacco protoplasts. Protoplasts obtained from transgenic plants that express stable Ac transposase, but do not carry any functional Ac or Ds elements, were transformed with the Ds containing plasmid (pVG2). Wild-type protoplasts were used in the control treatment. Twelve hours after transformation total DNA was extracted from the protoplasts and tested by PCR for the formation of joined ends. Occasionally PCR products of variable sizes, bigger than 540 bp, were observed (data not shown). They might originate from tandem Ac copies that arose as a result of somatic nearby jumps. The major reaction product always had the size expected for joined Ac ends and is therefore likely to be derived from extrachromosomal Ac circles; tandem genomic Ac’s would be expected to give rise to PCR products of various sizes depending on the length of the sequence between the two elements.

**Table 1**

<table>
<thead>
<tr>
<th>Conceptual head-to-head joining of Ac ends</th>
<th>Sequences at the junction of joined Ac ends</th>
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<tr>
<td>-CATCCTACCTTTGACATCCCTCG</td>
<td>TAGGGATGAAAAAGGTCG-</td>
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Sequences flanking Ac in pAGS411 construct

\[
\ldots GTGGGGCGCGTTGCGTGACCAcGCGTGACCCGGCGTGACCCGGCCGCGCGGG. \]

"The B2::Ds2 allele of maize, which generated sequence 4, contains a Ds2 element with perfect TIRs.

Clones 11–15 contain insertions of the indicated size; sequences are shown below: clone 11, AGCATAA; 12, CCCGGGCGCGCGGTACGCG; 13, TATACTATCTATCTGAGAC; 14, GGTACGACCGCGCCGGTGACGAC; 15, GAGTTCACTTCGATGATCAGGTTCACAAAGGAGGTAAAATTCTTATGAGGACAC.

joined ends. All the sequences contained either two intact Ac ends with a short intervening insertion, or small deletions in one or both ends. Clones with the deletions from both sides (five sequences out of 26) gave an additional evidence in favor of extrachromosomal Ac circles. They could not arise from tandem Ac copies, because if one transposon inserts at the position adjacent or even within the second element, at least one of the transposon ends must remain intact. Molecules that had one end intact and a deletion in the other end (four sequences out of 26) could be interpreted either as transposition of Ac into itself near its termini, or as a circular Ac. Deleted circles are not thought to reintegrate successfully as all de novo Ds insertions found so far have perfect terminal inverted repeats, or in the case of Ac, imperfect terminal inverted repeats with one single mismatch at the 5' end. Sequences with intact Ac ends are of two types: those with insertions
resembling the flanking sequences of the donor site in the original construct (clones 1, 2, 5, 12, 14, and 16), and those with insertions unrelated to the donor. Insertions resembling the flanking donor site could be caused in principle by tandem jumps, or alternatively, insertions unrelated to the donor site could be derived from secondary transposition events, or could be generated upon circularization. Ac circles with two intact transposon ends might serve as transposition intermediates.

Are Ac/Ds circles functional transposition intermediates? To find, whether Ac/Ds circles are functional transposition intermediates, we tested their ability to reinsert into the genome. We constructed an artificial Ds circle (Figure 3A) that carried intact joined Ac ends from clone 3 (Table 1) with >200 bp of Ac sequence flanking the junction from each side [this length of subterminal regions is sufficient for transposition (COUPLAND et al. 1989)] and a plant selectable marker (kanamycin resistance). Such a Ds circle was cotransformed into tobacco protoplasts, with the transposase producing plasmid (pNT804), or with the control plasmid (pVG6) lacking the transposase ORF. The number of kanamycin-resistant calli was scored. The transposase source was identical to that used in experiments where Ds was shown to reinsert from a plasmid into the tobacco genome (HOURA-HERIN et al. 1994).

Two types of events might lead to the formation of kanamycin-resistant calli: (1) random insertion, which should also occur in the absence of transposase, and (2) insertion via the transposition pathway. Results of three independent experiments are summarized in Table 2. In all three experiments, transposase increased the efficiency of transformation with Ds circles.

To study whether Ds circles integrated via the transposition pathway, insertion patterns were analyzed. In the case of transposition, the circle is expected to open precisely between the Ac ends and to generate an 8-bp target site duplication upon insertion. Plants were regenerated from kanamycin-resistant calli, which were obtained by cotransformation of Ds circle and the transposase-expressing plasmid. Genomic DNA from 34 plants was digested with PaeI and EcoRV enzymes that cut within the Ds circle close to the joined ends (Figure 3A) and hybridized with a probe to an internal part of the Ds circle. Integration of the Ds circle via transposition, i.e., upon linearization at the ends junction, is expected to give a 5.2-kb band in a Southern blot. Figure 3B shows the Southern blot of 18 plants out of 34 analyzed; the remaining 16 plants gave a similar band pattern (data not shown). Most plants showed multiple bands with sizes corresponding to random plasmid integration; no prominent 5.2-kb band was observed among the different plants.

Four plants that showed a band of ~5.2 kb and contained a low number of insertions (plant 14 in Figure 3B, and another three that gave similar pattern on the Southern blot) were chosen, and the regions flanking Ac ends in these plants were analyzed by inverse PCR. Genomic DNA of these plants was partially digested with Sau3A, circularized and amplified at both Ds borders. Two nested pairs of PCR primers were used that anneal within the Ds circle, with one primer of each pair pointing toward the transposon end and the second primer pointing toward the proximal part of Ds (see Figure 3A and MATERIALS AND METHODS). When
TABLE 2
Number of kanamycin-resistant calli obtained in three independent experiments

<table>
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<tr>
<th>Plasmids used for transformation</th>
<th>Experiment</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$D_s$ circle + pNT804 (+transposase)</td>
<td>12</td>
</tr>
<tr>
<td>$D_s$ circle + pVG6 (-transposase)</td>
<td>1</td>
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Values are number of kanamycin resistant calli. $D_s$-circle was cotransformed into tobacco protoplasts with a plasmid that expresses transposase (pNT804) or with a control plasmid (pVG6) that does not express transposase.

PCR products were cloned and sequenced, all of them were found to carry the same joined Ac ends as in the $D_s$ circle, indicating that in these plants $D_s$ circles integrated in a random (illegitimate) manner.

To confirm that experimental conditions were adequate to detect transposition, we tested whether a "standard" $D_s$ element (not a $D_s$ circle) cloned into a Bluescript vector could excise in the presence of transposase. Plasmid pVG5, containing a $D_s$ element with 2.7 kb of DNA (the vector) separating the element ends, was cotransformed into tobacco protoplasts, with the transposase-producing plasmid (pNT804), or with the control plasmid (pVG6) lacking the transposase ORF. Twenty-four hours after transformation, protoplast DNA was extracted and used as a template to detect empty donor sites by PCR (see MATERIALS AND METHODS). A DNA fragment with the size expected for empty donor sites was found only in cotransformation with pNT804. This fragment was cloned and independent clones were sequenced. The sequences obtained (Figure 4) contain typical Ac-excision footprints (SCOTT et al. 1996). This suggests that the experimental conditions used were adequate for transposition to occur.

Nevertheless, the $D_s$ circle used in this experiment seemed not to be an efficient substrate for transposase-mediated integration.

**DISCUSSION**

*Ac* joined ends are derived from circular DNA molecules: *Ac* joined ends were detected, using a PCR assay, in maize and transgenic tobacco plants. Joined ends were found only in plants that contained actively transposing elements, which demonstrates that the transposition machinery was involved in their formation and argues strongly against the possibility of a PCR artifact. The following evidence indicates that the joined ends were derived from *Ac* or $D_s$ circles, rather than from tandem genomic copies of the transposon. (1) Based on migration in agarose gels, the major amplification product had the size expected for precise joining of the transposon ends, whereas tandem copies of the transposon were expected to give rise to PCR products of various sizes depending on the length of the sequence separating the two elements. It must be mentioned that *Ac* was not reported to have a preference for transpositions within only a few bp from the original site. Among more than 15 reports corresponding to hundreds of transposon insertions mapped relative to the original site, only one event has been found where *Ac* transposed just 6 bp from its original position (ATHMA et al. 1992). (2) Several sequences of the PCR products containing joined ends had deletions at both ends, which cannot be explained by tandem transposition. (3) Joined ends were formed 12 hr after protoplast transformation with $D_s$-containing plasmid, making the possibility of formation of tandem genomic elements very unlikely.
**Interpretation of the Ac end junctions:** We have presented the structure of 26 amplification products containing joined Ac ends (Table 1), obtained from nine plants (eight tobacco and one maize). Different clones were obtained from the same plant, indicating the population of transposon circles is heterogeneous. No molecules were found with either perfect joined ends, or with an eight-nucleotide insertion. This is consistent with the observations that the type of excision that leaves the host duplication unchanged (Scott et al. 1996) and precise excision (Baran et al. 1992) are rare. All the sequences contained either two intact Ac ends with a short insertion in between, or a few nucleotides deleted from one or both ends. Deletions at the transposon ends in Ac circles could have been caused by exonucleolytic attack that occurred before circularization or by imprecise excision. Additional nucleotides inserted between the transposon ends might have been derived from the flanking sequences as a result of imprecise excision. In this case, the absence of molecules with perfect ends joining would suggest that Ac is not excised by flush cleavages at the transposon ends but rather by some kind of a staggered cut, as previously proposed (Saedler and Nevers 1985; Coen et al. 1989). Excision with staggered cuts was observed upon Tn7 transposition (Bainton et al. 1991). Alternatively, additional nucleotides may reflect the use of “filler DNA” to repair deletion (Wessler et al. 1990; Nassif et al. 1994) or untemplated synthesis, similar to “N” bases at V(D)J junctions (Gellert 1992).

**Are Ac circles transposition intermediates?** Although transposon circles have been previously described in eukaryotes, they have never been assayed for their ability to integrate. In this work, we tested the ability of a putative Ds circular transposition intermediate to reinsert into the genome. When Ds circles were transformed into tobacco protoplasts, transposase increased the efficiency of transformation. Analysis of the transformed plants, however, did not show efficient transposase-mediated integration, but rather patterns of illegitimate recombination typical of direct DNA transformation. We cannot exclude the possibility that circles used in our experiments could occasionally reinsert via transposition-mediated integration, since in plants with multiple insertions (Figure 3B), some inserts might be due to transposition events. However, only a minority of the integrations could be transposition-mediated based on the size of hybridizing fragments in S blots (Figure 3B). The effect of transposase on transformation efficiency might be explained by an overall stimulation of recombination activity in the cell, or by a role in targeting Ds circles into the nucleus.

Experimental conditions used here were probably adequate to detect transposase-mediated integration, because when similar transformation experiments were performed (Houba-Herin et al. 1994) using Ds cloned into a plasmid in a conventional way (i.e., with the transposon ends separated by a long enough stretch of plasmid DNA), quite efficient integration was reported. Moreover, we confirmed that a standard Ds element could excise from a plasmid donor (Figure 4) under the experimental conditions used. Therefore one interpretation of the lack of reinsertion of a joined-ends-containing circle is that circular transposons are not transposition intermediates, but rather abortive products. Alternatively, it is possible that real intermediates are circular. Such intermediates might have perfect joined ends, or ends spaced by 8 bp corresponding to the host duplication. As we did not find such molecules, it might be implied that the real circular intermediates occur so transiently in the cell that they were not detected even though a sensitive PCR assay was used. Another possibility is that circular intermediates are not able to reinsert in our system if, for example, a transposase-transposon complex needed for reinsertion can be formed only upon excision. In summary, we cannot rule out a circular intermediate in the process of Ac transposition. However, it is likely that some of the circles, such as the ones with deletions at both ends, are not able to reinsert.

**Model for Ac transposition:** A question raised by this work is how do circular Ac molecules fit into the models of transposition? One transposition model, which is still speculative, has been proposed for Ac (Greenblatt 1984) and for the Tam3 element of Antirrhinum majus (Robbins et al. 1989). According to this model, there is no full excision of the transposon, rather, transposition is a step by step process in which one end of the element is first excised and ligated to the recipient site and only after that the second end is cut (Figure 5B). In such a pathway, circles can be formed only if the transposition process is aberrant and, following cleavage of the first end of Ac (end A in Figure 5B), the second end (end B) is cleaved, yielding a fully excised element, and then the two free ends are joined. The resulting circles might be abortive products, as suggested here for some of the circles, or might reinsert. In any case, our findings of circles suggests that at some point there is full excision of the element. An alternative model to the step by step pathway can provide a simpler explanation for circle formation. According to this model, Ac normally transposes via a full excision pathway (Figure 5C) with cleavage at both ends (C/A and B/D) and formation of a linear intermediate as was shown for prokaryotic transposons Tn7 (Bainton et al. 1991) and Tn10 (Haniford et al. 1991). Such linear molecule could transpose directly and/or be circularized (Figure 5C).

Circularization of extrachromosomal linear DNA molecules by nonhomologous end-joining has been shown for several species including mammalian (Woodworth-Gutai 1981; Wilson et al. 1982; Roth et al. 1985), avian (Miller and Temin 1983) and plant cells (V. Gorbunova and A. A. Levy, unpublished), and
is often associated with deletions and insertions reminiscent of those shown here (Table 1). Hence, host-mediated circularization of a linear transposition intermediate might be a mechanism that prevents an excised transposon to reinsert. Such mechanism might enable eukaryotic hosts to cure themselves from transposons.

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LITERATURE CITED


Aviv, D., and E. Galun, 1985 An in vitro procedure to assign pigment mutations in Nicotiana to either the chloroplast or the nucleus. J. Hered. 76: 135–136.


Baran, G., G. Echard, T. Bureau and S. Wessler, 1992 Molecular analysis of the maize waxy3 allele indicates that precise excision of the transposable Ac element is rare. Genetics 130: 577–584.


Fernberg, A. P., and B. Vogelstein, 1983 A technique for radiola-


ROBBENS, T., R. CARPENTER and E. S. COEN, 1991 A chromosome rearrangement suggests that donor and recipient sites are associated during Tn3 transposition in Antirrhinum majus. EMBO J. 10: 5–13.


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