Ac Transposition From a T-DNA Can Generate Linked and Unlinked Clusters of Insertions in the Tomato Genome

Brian I. Osborne,* Catherine A. Corr,*† James P. Prince,‡ Reinhard Hehl,* Steven D. Tanksley,* Sheila McCormick*§ and Barbara Baker*†

*Plant Gene Expression Center, Albany, California, 94710, †Department of Plant Pathology, University of California, Berkeley, California 94720, ‡Department of Plant Breeding and Biometry, Cornell University, Ithaca, New York, 14853, and §Department of Plant Biology, University of California, Berkeley, California 94720

Manuscript received May 2, 1991
Accepted for publication July 15, 1991

ABSTRACT

We have investigated the distribution of transposed Acs in the tomato genome. Our approach has been to clone the regions flanking the T-DNAs and transposed Acs from two transgenic lines of tomato and place these sequences on the tomato restriction fragment length polymorphism (RFLP) map. The distribution of transposed Acs around the T-DNA and at locations unlinked to the T-DNA indicates that Ac transposes to linked and unlinked sites in tomato as it does in maize. The structure and terminal sequence of these cloned elements shows that Ac remains intact after transposition. We discuss these results and their bearing on gene tagging strategies using Ac and Ds.

The autonomous Activator (Ac) and nonautonomous Dissociation (Ds) elements are members of a family of maize transposons whose properties have been well described in genetic and molecular terms (reviewed in Döring and Starlinger 1986; Fedoroff 1989). These elements were first discovered and then studied by virtue of their action as mutagens in maize (McClintock 1948, 1950). The molecular cloning and characterization of Ac and Ds (Fedoroff, Wessler and Shure 1983; Behrens et al. 1984; Müller-Neumann, Yoder and Starlinger 1984; Pohlman, Fedoroff and Messing 1984; Sutton et al. 1984) has led to cloning of a number of maize genes that had been insertionaly deactivated by members of this family. These efforts required establishing the cosegregation of a mutant phenotype with an Ac- or Ds-hybridizing restriction fragment, then isolating the adjacent, genic DNA by physical methods (Fedoroff, Furtak and Nelson 1984; Dooner et al. 1985; Theres, Scheele and Starlinger 1987; Delaporta et al. 1988; Hake, Vollbrecht and Freeling 1989).

The efficacy of Ac and Ds as insertional mutagens in maize prompted researchers to introduce Ac into other plants. The fundamental conclusion of these experiments is that Ac transposes in a number of dicot species (Baker et al. 1986; Van Sluys, Tempe and Fedoroff 1987; Knapp et al. 1986; Yoder et al. 1988; Houba-Herin et al. 1990). Advantages of using heterologous species as hosts for Ac and Ds include the ability to introduce recombinant transposons bearing marker genes (Hehl and Baker 1989; Lassner, Pals and Yoder 1989; Masterson et al. 1989), the absence of Ac- or Ds-hybridizing sequences in their genomes, and, in principle, the ability to introduce constructions that regulate transposition by fusing the Ac transposase coding region to characterized promoters. However, the success of these strategies depends upon some understanding of the unique properties of the Ac and Ds elements. The primary source of our understanding comes from studies in maize. We must ask whether what holds true for Ac in maize applies to Ac in transgenic plants as well. Certain properties of Ac that are conserved between maize and transgenic tobacco plants include the maintenance of Ac structure and preservation of terminal sequences following transposition, generation of a characteristic 8-bp duplication of target sequences upon integration, and the hypomethylated character of target sites and the correlation between hypomethylation within the element and continued transposition (Hehl and Baker 1989, 1990). Certain properties are not conserved when Ac is introduced into transgenic plants, such as the negative regulation of Ac transposition by Ac copy number observed in maize (McClintock 1948, 1951). This property, if conserved in transgenic plants bearing Ac, would constitute a significant impediment in the utilization of Ac. However, recent work has shown that Ac transposition is positively regulated in tobacco, both for wild-type Ac (Hehl and Baker 1990) and for an Ac that had been modified in vitro (Jones et al. 1989).

Another pertinent feature of Ac in maize is that it often transposes to sites linked to its original location. For example, Greenblatt (1984) showed that 64 of 105 transpositions from the P locus (61%) gave rise to linked insertions. In a study on Ac at h2-m2(Ac) Dooner and Belachew (1989) found that 49 of 94
(52%) of the transposed Acs remained linked to bz. These experiments mapped transposed Acs genetically with respect to their starting positions at P or bz. Although the distributions of transposed Acs around P or bz could be determined in detail, the disadvantage of this approach is that the unlinked, transposed Acs could not be easily mapped.

The conservation of the property of frequent, linked transposition in transgenic plants would have implications for strategies of transposon mutagenesis. If Ac transposed randomly with respect to its starting point then any starting point would suffice as material with which to attempt to mutagenize a specific locus, though the probability of obtaining a specific insertion would be low. Conversely, if Ac transposed to linked sites then the highest likelihood of mutagenizing a specific locus would arise when Ac is first disposed near that locus. In addition, continued linked transposition by Ac would tend to generate a cluster of insertions. Cloning of the regions flanking these transposed Acs would yield a concentration of molecular markers in a given region, an invaluable asset in map-based cloning strategies. As such, the distances that Ac transposes must be considered a critical parameter in describing Ac's activity in transgenic plants. Previous work has described the transposition of an in vitro modified and less active Ac in tobacco where Ac was shown to transpose to a closely linked location in 11 of 14 cases examined (Jones et al. 1990). The genetic assay employed assessed the linkage of a transposed Ac to one T-DNA locus in 14 progeny from a single, tobacco transformant.

In this report we describe the genomic distribution of transposed, wild-type Acs in two independent lines of tomato. We have cloned the sequences flanking eight transposed Acs and the sequences flanking the T-DNA from a primary transgenic plant (R0), A78. In the second line (R0 A9 and selfed RI progeny) we have cloned T-DNA flanking regions and the regions flanking 16 transposed Acs. We describe the overall structure and the sequences of the Ac termini in all Ac clones. Our approach differs from that previously described in Jones et al. (1990) in that we map transposed Acs with respect to the T-DNA and at any other location in the tomato genome. We conclude from these studies that Ac transposes to linked and unlinked sites at roughly equal frequencies in tomato, similar to its behavior in maize.

MATERIALS AND METHODS

Tomato transformation: Cotyledons from Lycopersicon esculentum cv. VF36 were transformed with pGVS350HPT: pKU3 (Baker et al. 1987) as previously described (McCormick et al. 1986) with hygromycin selection at 10 µg/ml. The selection against Agrobacterium was cefotaxime (Calbiochem) at 500 µg/ml.

Southern blot analysis: Genomic plant DNA was prepared from mature leaf tissue and Southern blotting was performed as described previously (Hedeh and Baker 1990). Entire cloned inverse polymerase chain reaction (IPCR) (Ochman, Gerber and Hartl 1988; Triglia, Peterson and Kemp 1985) products or restriction fragments were labeled by random priming according to manufacturer's instructions (Amersham). The T-DNA probe for segregation analyses was the right border HindIII fragment A (Zambryski et al. 1980).

Cloning of T-DNA flanking regions from R0 A9 and R0 A78 in λ Libraries of T of 7 x 10^3 (R0 A9) and 8 x 10^3 (R0 A78) recombinants were screened for T-DNA left border hybridization using the HindIII fragment B (Zambryski et al. 1980). Three (A9; pertinent, mapped clone: #29) and six (A78; pertinent, mapped clone: #6) positive, independent clones were recovered. EcoRI fragments from each clone were screened by Southern blotting for their ability to hybridize to VF36 genomic DNA. λ DNAs were digested and hybridized to fragment B. In this way putative junction fragments that contained genomic DNA and T left border sequences were identified, then subcloned. The identity of putative junction fragments was confirmed by Southern blotting of A9 and A78 DNA. A 5.0-kb HindIII fragment was subcloned from λ #29 (A9) that hybridized to a 1.8-kb HindIII fragment from VF36 as well as to the predicted 5.0-kb HindIII fragment from the DNA of A9 (not shown). A 2.4-kb EcoRI-HindIII fragment was subcloned from λ #8 (A9) that hybridized to a 1.0-kb EcoRI-HindIII fragment from VF36 as well as to a predicted 2.4-kb EcoRI-HindIII fragment in DNA selected progeny of A78 (not shown). EcoRI fragments from three different λ clones (#6, #8, #11) obtained from the A78 λ library hybridized to the same 1.0-kb HindIII fragment in VF36 DNA, indicating that the genomic DNA in these clones was identical. Fragments from λ #6 (A78) and #29 (A9) were used as probes to place the A78 and A9 T-DNAs on the tomato restriction fragment length polymorphism (RFLP) map.

Cloning of Ac elements from λ EMBL4 libraries: DNAs from transformants R0 A9 and R0 A78 were partially digested with XbaI as described (Maniatis, Fritsch and Sambrook 1982). The 10-20-kb fraction was purified as described (Maniatis, Fritsch and Sambrook 1982), and ligated into λ EMBL4 arms (Frischauf et al. 1985), purified as described (Maniatis, Fritsch and Sambrook 1982). Ligation were packaged according to the manufacturer's instructions (Gigapack Plus, Stratagene, or Packagene, Promega), and phage were plated on CES2000 (sheB13 recB21 recC22 hsdR). A library of 1 x 10^6 recombinants constructed from R0 A78 DNA was screened with the Ac 1.6-kb internal HindIII fragment. Fifty nine positive clones were recovered from this library (pertinent, mapped clones: CC5, CC7, CC11, CC25, CC32, CC35, CC50). Two Ac clones were recovered from a library of 1 x 10^6 recombinants constructed from a complete BglII digest of A78 DNA ligated into λ EMBL4 arms (pertinent, mapped clone: CC60). The probe used for screening was the entire Ac element. Pertinent Ac clones were subcloned as BamHI, EcoRI, or BamHI-EcoRI fragments into pUC18.

Cloning of Ac elements from a λ ZAPII library: DNA from R0 A9 was digested with EcoRI and the 5-10-kb fraction was purified with NA45 according to the manufacturer's instructions (Schleicher and Schuell). The fraction was ligated into EcoRI-digested, phosphatase-treated λ ZAPII (Short et al. 1988) according to the manufacturer's instructions (Stratagene). Ligations were packaged according to the manufacturer's instructions (Gigapack Plus, Stratagene), and phage were plated and subsequently screened on E. coli C600. Two recombinants that hybridized to a 5'
Ac BamHI-EcoRI probe were recovered from 2 × 10^5 plaques (9# 59, 9# 160).

Cloning of Ac flanking sequences by IPCR: Two to 4 μg of tomato genomic DNA was digested with 25 units of EcoRII or HinFl, then phenol extracted and resuspended in 4 ml of 10 mm Tris-HCl, pH 7.5, 10 mm MgCl2, 5 mm β-mercaptoethanol with 400 units of T4 DNA ligase (New England Biolabs). After 12-hr incubation at 16°, ligations were heated to 65° for 15 min, cooled, then cut with 50 units of BamHI or PstI. The DNAs were precipitated with 3 volumes of ethanol and 1/10 volume of 4 m NaCl. Precipitates were resuspended in 100 μl of PCR buffer (2 mm MgCl2, 50 mm KCl, 10 mm Tris-HCl, pH 8.3-0.1% gelatin) with 2.5 units of Taq DNA polymerase (Cetus). PCR reactions were carried out in a Cetus Thermal Cycler (1 min/94°, 2 min/50°, 3 min/72°, 35 cycles) with 0.5 pg of oligonucleotides 5'GGTGCGGAGTTTCGCAATCTG-3' (positions 38 to 25 of Ac) and 5'-GGGATCCATAACCCGACG-3' (positions 267 to 288 of Ac). Two microliters from each reaction transferred to 100 μl of PCR buffer for an additional 25 cycles of amplification with the same oligonucleotides. PCR products were digested with BamHI and SalI and cloned into the BamHI-SalI interval of pUC18.

The region flanking the 5' end of Ac in λ cc11 was cloned by IPCR from a DNA using Sau3A digestion followed by RsaI digestion as described above. PCR was for 25 cycles using the positions 23 to 8 primer and 5'-ACGTCC-CTGTTAAACCGATT-3' (positions 36 to 57 of Ac). The sequence of the region flanking Ac in the IPCR clone was identical to the sequence of the flanking region in the 5' BamHI-EcoRII fragment subcloned from λ cc11.

Cloning of Ac flanking sequences from R1 B31 by PCR: Two micrograms of R1 B31 genomic DNA was used in a 35-cycle PCR reaction as described above. The oligonucleotides were 5'-GGGCTACCAACTAACTGG-3' (specific for the 31#2 insertion of Ac) and 5'-ACCGT-CCATGTAACCGATTT-3' (positions 38 to 25 of Ac). The sequence of the region flanking Ac in the IPCR clone contains only the 5' end of Ac. The Ac element in clone CC50 is inserted in the coding region of the 1'-NptII gene.

Assignment of cloned sequences to the tomato RFLP map: Restriction fragments containing sequences flanking Ac (Figure 1 and Table 1) or T-DNA were used as probes to filters bearing L. esculentum cv. VF36 and L.lycopersicum pennellii DNA digested with BstNI, DraI, EcoRI, EcoRV, HaeIII or XbsI. Enzymes that revealed polymorphisms were used to digest DNAs from 56 (laboratory of B. Baker) or 76 (laboratory of S. Tanksley) F2 progeny from an L. esculentum cv. VF36 × L. pennellii F1 hybrid (Bernatzky and Tanksley 1986). The data from the resultant Southern hybridizations were analyzed using the program MapMaker (Lander et al. 1987). MapMaker placed the probe sequence on a tomato RFLP map composed of 76 RFLP markers distributed over the 12 tomato chromosomes. The 76 RFLP markers were isolated either from tomato cDNA libraries or from libraries of PsI-digested tomato genomic DNA (Bernatzky and Tanksley 1986; S. Tanksley, unpublished results). In all cases the assigned location was at least tenfold more likely than the second-most likely location.

Computer model: A program was created to simulate Ac transposition with varying degrees of linkage in the tomato genome using Hypercard on the Macintosh. The program places insertions on a graphic representation of the RFLP map used in these studies (12 chromosomes of specific

![Figure 1](image.png)
TABLE 1

List of molecular probes

<table>
<thead>
<tr>
<th>Marker</th>
<th>T-DNA or Ac</th>
<th>Plant-lineage</th>
<th>Chromosome</th>
<th>Flanking region</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9:</td>
<td>29B T-DNA</td>
<td>A9-R0</td>
<td>2</td>
<td>5.0</td>
<td>λ</td>
</tr>
<tr>
<td>A9:</td>
<td>9r16</td>
<td>A9-R0</td>
<td>11</td>
<td>0.15</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>9H16</td>
<td>A9-R0</td>
<td>1</td>
<td>0.35</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>9r59</td>
<td>A9-R0</td>
<td>5</td>
<td>2.2</td>
<td>λ</td>
</tr>
<tr>
<td>A9:</td>
<td>9r160</td>
<td>A9-R0</td>
<td>2</td>
<td>1.8</td>
<td>λ</td>
</tr>
<tr>
<td>A9:</td>
<td>2r5</td>
<td>A9-R0</td>
<td>2</td>
<td>0.3</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>2r6</td>
<td>A9-R0</td>
<td>2</td>
<td>0.25</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>2r7</td>
<td>A9-R0</td>
<td>2</td>
<td>0.2</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>14#3</td>
<td>A9-R0</td>
<td>6</td>
<td>0.35</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>14#7</td>
<td>A9-R0</td>
<td>1</td>
<td>0.25</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>14#8</td>
<td>A9-R0</td>
<td>2</td>
<td>0.4</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>31#2</td>
<td>A9-R0</td>
<td>2</td>
<td>0.1</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>31#4</td>
<td>A9-R0</td>
<td>11</td>
<td>0.25</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>2r14</td>
<td>A9-R0</td>
<td>1</td>
<td>12 bp</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>2r8</td>
<td>A9-R0</td>
<td>1</td>
<td>1 bp</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>2r10</td>
<td>A9-R0</td>
<td>1</td>
<td>0.1</td>
<td>IPCR</td>
</tr>
<tr>
<td>A9:</td>
<td>14r9</td>
<td>A9-R0</td>
<td>1</td>
<td>0.1</td>
<td>IPCR</td>
</tr>
</tbody>
</table>

**Line A78:**

<table>
<thead>
<tr>
<th>Marker</th>
<th>T-DNA</th>
<th>Plant-lineage</th>
<th>Chromosome</th>
<th>Flanking region</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B T-DNA</td>
<td>A78-R0</td>
<td>6</td>
<td>2.4</td>
<td>λ</td>
<td></td>
</tr>
<tr>
<td>A78:</td>
<td>CC3</td>
<td>A78-R0</td>
<td>2</td>
<td>0.7</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC7</td>
<td>A78-R0</td>
<td>7</td>
<td>1.1</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC11</td>
<td>A78-R0</td>
<td>6</td>
<td>0.2</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC25</td>
<td>A78-R0</td>
<td>7</td>
<td>2.8</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC32</td>
<td>A78-R0</td>
<td>6</td>
<td>8.1</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC35</td>
<td>A78-R0</td>
<td>3</td>
<td>4.0</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC50</td>
<td>A78-R0</td>
<td>6</td>
<td>—d</td>
<td>λ</td>
</tr>
<tr>
<td>A78:</td>
<td>CC60</td>
<td>A78-R0</td>
<td>2</td>
<td>0.2</td>
<td>λ</td>
</tr>
</tbody>
</table>

*These Ac flanking regions were too short to hybridize to genomic DNA.

*The Ac flanking region in this probe hybridized to a moderately repetitive sequence.

*The Ac flanking region in this probe is Ac sequence.

*This transposed Ac resides within the T-DNA.

do not cut between the right border and an EcoRI site that is 3.3-kb internal to the right border in pGV3850HPT::pKU3. The 10.0-kb EcoRI-PstI fragment was detected in 12 of 12 HygR R1 progeny obtained from selfing of A9. Plating of seed obtained from selfing of A9 confirmed that HygR segregated as a single locus: 145 of 187 seedlings (77%) were HygR.

Similar analysis showed that transformant A78 contained a single T-DNA locus. EcoRI-PstI digestion of A78 DNA followed by Southern blotting revealed 4.7- and 3.5-kb fragments hybridizing to the T-DNA right border probe (not shown). Southern blotting of DNA from HygR selfed R1 progeny of A78 was performed to show that these two fragments were linked. The 4.7- and 3.5-kb fragments were present in all 12 HygR progeny examined, consistent with the proposal that the fragments were linked. In addition, Southern analysis of 8 unselected, R1 progeny of A78 showed that 1 individual lacked both characteristic fragments, where the remaining 7 contained both fragments. Plating of seed obtained from selfing of A78 confirmed that HygR segregated as a single locus: 60 of 83 seedlings (72%) were HygR.

Ac transposition in these two lines was investigated by Southern blot hybridization analysis of EcoRI-PstI-digested DNA isolated from A78 and A9 and three KnR R1 progeny of A9. An example of the results of Ac probe hybridization is shown in Figure 2. The Ac probe detects two fragments for each Ac element. The 2.1- and 3.0-kb Ac-hybridizing fragments are diagnostic of Ac in place in the T-DNA (Figure 2, lanes R0 A9 and R1 B14). Ac-hybridizing fragments of different sizes and intensities correspond to transposed Acs. A78 contained several transposed Acs as judged by the novel Ac fragments, and no Ac remained “in place” in the T-DNA, as shown by the absence of the characteristic hybridizing fragments of predicted sizes. The Ac-hybridizing fragments varied in intensity, indicating that Ac was transposing somatically. A9 contained fewer Ac-hybridizing fragments than A78, and Ac remained mostly “in place” in the T-DNA of A9. B14 also contained Ac “in place” and a large number of transposed Acs. B2 and B31 were similar to each other and contained a few transposed Acs and no Ac in the T-DNA.

The identification of transposed Acs in these plants was supported by rehybridization of the same filters with the p1 probe as previously described (Baker et al. 1987; data not shown). This probe hybridizes to a 3.0-kb EcoRI fragment when Ac is located in its original T-DNA location, but hybridizes to a 0.5-kb “in place” T-DNA fragment if Ac has excised. Ac excision was detected in all DNAs (data not shown).

Cloning of T-DNA flanking regions from R0 A9 and R0 A78 in λ: Sequences flanking the T-DNA in

the tomato cultivar VF36 by Agrobacterium transformation (McCormick et al. 1986) using pGV3850HPT::pKU3 (Baker et al. 1986). The T-DNA construction bearing Ac contained a functional hygromycin resistance gene (HygR) for transformation selection, as well as a p1′-NptII transcriptional fusion (Baker et al. 1987) conferring kanamycin resistance (KnR). The p1′-NptII gene had been activated by insertion of Ac into the untranslated 5′ leader. Fourteen independently regenerated (R0) plants were obtained after hygromycin selection. These plants were shown to be transformed by Southern blotting using Ac and p1′ sequences as hybridization probes (data not shown).

Two lines, R0 A9 and three selfed KnR R1 progeny (B2, B14 and B31), and R0 A78 were chosen for this work. Both lines contained single T-DNA loci by genetic and physical criteria. Southern blotting of DNA from A9 revealed a single 10.0-kb EcoRI-PstI fragment hybridizing to a T-DNA right border probe (not shown). This digest should reveal the junction fragments between T-DNA and genomic DNA as PstI
A9 or A78 were cloned from bacteriophage λ libraries. Junction fragments between the T-DNAs and genomic DNA were identified by Southern blotting of λ DNAs with T-DNA left border probes and subcloned. Restriction enzyme fragments bearing genomic DNA flanking the T-DNAs were used as probes to place the A78 and A9 T-DNAs on the tomato RFLP map (see MATERIALS AND METHODS). These clones were digested and analyzed by Southern blotting using probes specific for the T-DNA right border probes. This characterization served to distinguish Ac from those that remained "in place" in the untransposed Ac.

Cloning of genomic DNA flanking transposed Ac from R0 A78 in λ: The sequences flanking transposed Ac were cloned from bacteriophage λ libraries constructed from A78 DNA. Sixty one λ clones were obtained from A78 MboI-partial and A78 BglII-complete libraries by hybridization to an Ac probe (see MATERIALS AND METHODS). These clones were digested and analyzed by Southern blotting using probes specific for the Ac 5' and 3' ends, as well as p1' and T-DNA right border probes. This characterization served to distinguish Ac that had transposed into genomic sequences from those that were contained within the T-DNA. In addition, this analysis distinguishes Ac that had transposed within the T-DNA from those that remained "in place" in the 5' untranslated region of the p1'-NptII gene. Clones representing transposed Ac were divided into eight groups according to restriction map using EcoRI and BamHI digestion to identify independent isolates of the same insertion (Table 1). Single clones from each of the eight groups were examined further by subcloning Ac 5' and 3' ends following EcoRI, BamHI, or EcoRI-BamHI digestion. Figure 1 shows that all clones retained characteristic internal BamHI and EcoRI sites. One clone (CC25) contained only the 5' end of Ac, presumably due to MboI digestion within Ac. Figure 3 shows that these clones retain the the predicted Ac sequences at their 5' and 3' termini. All evidence derived from analysis of these clones shows that these transposed Ac are neither internally nor terminally rearranged. Figure 3 also shows that four of the seven clones containing entire Ac were flanked by the typical 8-base direct duplication generated on Ac insertion (POHLMAN, FEDOROFF and MESSING 1984; MÜLLER-NEUMANN, YODER and STARLINGER 1984; SUTTON et al. 1984).

Cloning of genomic DNA flanking transposed Ac from R0 A9 and progeny by IPCR: Genomic DNA flanking transposed Ac in R0 A9 and three of its selfed R1 progeny (B2, B14 and B31) were cloned by IPCR (OCHMAN, GERBER and HARTL 1988; TRIGLIA, PETERSON and KEMP 1988) as described in MATERIALS AND METHODS. DNAs from each of the four plants were treated separately and the resultant products were cloned in separate reactions. A total of 25 IPCR products were cloned from these plants. In all, 15 distinct sequences were represented among the 25 clones.

We concluded that a thorough characterization of the generated products was essential because we were concerned that PCR artifacts may have arisen during the relatively large number of cycles required to amplify a clone. IPCR products have a predicted structure dictated by the primer sequences and the particular restriction enzyme used to first digest the DNA: the two terminal primer sequences flank the genomic and Ac sequences in the IPCR product and are directed toward each other, with the specific restriction sites flanking transposed Ac.
enzyme site at the junction of flanking and internal Ac sequence. Of the fifteen unique clones examined in detail, fourteen were faithful to the predicted structure, containing (1) a terminal primer sequence, then seven bases of the Ac 5' terminus, (2) flanking sequences of varying length immediately adjacent to the Ac terminus, (3) an EcoRII or HinFI site marking the boundary between flanking sequence and the internal Ac sequence between the 5' terminus and the first EcoRII (position 331 of Ac) or HinFI site (position 396 of Ac), and (4) the Ac sequence between the restriction site and the second, internal primer. The lengths of the flanking regions in the fourteen bona fide IPCR clones varied from one base to roughly four hundred bases long (Table 1). The correspondence between the predicted and actual structures in these fourteen IPCR clones demonstrated that we had isolated the flanking regions to Ac insertions.

Certain IPCR clones were isolated from more than one plant. Two identical clones, 2#12 and 14#4, were isolated from the sibling plants B2 and B14. Since the IPCR reactions on these DNAs were performed simultaneously we conclude that neither of the isolates arose from contamination by a previously cloned product. In fact, the independent cloning of identical products is additional evidence for the veracity of the IPCR method used here. We conclude that this insertion arose in the A9 parent and was transmitted to and isolated from the progeny B2 and B14. Three clones from different plants, 9#3, 2#8 and 14#5, were also identical. However, in this case the flanking regions were one base long. Such a short flanking region is a consequence of Ac insertion 3' to C which will form an EcoRII site at the immediate 5' end of Ac (EcoRII was one of the two enzymes used in the initial digestion for IPCR). We cannot maintain that these three clones represent the same insertion since the flanking region is so short.

Two of the 14 independent IPCR clones, 31#2 and 31#4 from B31, contained a sequence variation in Ac. Figure 4 shows that both products differed from the wild-type Ac sequence at the first base of the Ac 5' end, where C had been replaced by T. To address the possibility that these point mutations were artifactual, we recloned the 5' ends of both insertions by PCR from B31 DNA using primers that were specific to the two different genomic flanking regions (see MATERIALS AND METHODS). The sequence of the cloned PCR products contained the same, terminal point mutation as the IPCR products. These PCR clones could not have arisen from amplification of previously cloned, contaminating IPCR products since the Ac-specific primer sequence used in the PCR amplification was located outside the Ac region amplified by IPCR.

Cloning of genomic DNA flanking transposed Ac from R0 A9 in λ: Two clones (9#59, 9#160; Table 1) were obtained from an A9 EcoRI-complete library that hybridized to an Ac 5' probe (see MATERIALS AND METHODS). Since EcoRI cuts once within Ac at position 2483 we expected that hybridizing clones would contain only those sequences that were 5' to the internal EcoRII site. Both clones were flanked by EcoRI sites as expected and contained internal restriction fragments characteristic of the Ac 5' end (not shown). Figure 4 shows the Ac 5' ends and genomic flanking regions of these two clones. Clone 9#59 contains an intact Ac 5' end, whereas the Ac in clone 9#160 has sustained a 6-base terminal deletion. This deletion is reminiscent of the 4-base 3'-terminal deletion found in the stable Ac element that was cloned from tobacco transformed with wild-type Ac (HEHL and BAKER 1989).

Assignment of flanking regions to the RFLP map: The sequences flanking the T-DNA and the transposed AcS from R0 A78 and R0 A9 were assigned to the tomato RFLP map (BERNATZKY and TANKSLEY 1986; S. TANKSLEY, unpublished results) as described in MATERIALS AND METHODS. The positions of regions flanking the T-DNAs and transposed AcS are shown in Figure 5. Restriction fragments containing the genomic regions immediately adjacent to the 3' or 5' ends of Ac were subcloned from the λ clones isolated.
from A78. One of the two Ac-flanking fragments (Figure 1) was used as a hybridization probe to place the flanking regions on the RFLP map (Figure 5B). The flanking regions to six of the eight cloned Acs were unique sequences in the tomato genome. The two large fragments flanking the 5' and 3' ends of Ac in λ CC11 both hybridized to moderately repetitive genomic sequences (not shown) and thus could not be used to place CC11 on the RFLP map. Therefore, a short region immediately flanking the Ac 5' end was subcloned from λ CC11 by IPCR (see MATERIALS AND METHODS). This subclone hybridized to a unique genomic fragment and was placed on the RFLP map.

Southern blot analysis of the eighth clone (λ CC50) with T-DNA fragments as probes (not shown) indicated that the Ac was inserted within the T-DNA of A78, but not at its starting position in the untranslated leader of the p1'-NptII gene. Subcloning and sequencing of the Ac ends showed that this Ac was flanked by the characteristic 8-base direct duplication (POHLMAN, FEDOROFF and MESSING 1984; MÜLLER-NEUMANN, YODER and STARLINGER 1984; SUTTON et al. 1984), indicating that it had transposed to a new location within the T-DNA. Comparison of these flanking sequences to the GenBank database showed that the Ac in CC50 was inserted within the NptII coding region, 163 bp downstream from the AUG codon.

Ten of the 14 Ac-flanking region clones isolated from A9 and its progeny by IPCR (Table 1) were assigned to the tomato RFLP map (Figure 5A). The four remaining clones contained flanking regions that could not be mapped. Two of these four clones, 2#12 (identical to 14#4) and 2#8 (identical to 9#3 and 14#5), contained flanking regions that were too short to constitute effective probes (12 bases and 1 base long, respectively). The third clone (2#10) could not be placed on the RFLP map as it hybridized to a moderately repetitive sequence (not shown). The fourth unmapped clone (14#9) contained a flanking region greater than 100 bases long, yet no hybridization was detected when the clone was used as a probe in Southern blots with VF36 DNA. Comparison of the cloned sequence with the GenBank database revealed that the flanking region was Ac itself. In this clone the Ac 5' end is inserted within Ac at position 294 in direct orientation.

**Discussion**

**Ac transposition can generate clusters of insertions in the tomato genome:** The maps of the transposed Acs show that Ac transposes to both linked and unlinked sites (Figure 5). Five of 20 insertions are linked to their T-DNA, and Ac has transposed to 4 (R0 A78) and 5 (R0 A9 and progeny) of the 12 tomato chromosomes. However, a large proportion of the Acs are linked to another Ac (14 of 20). This tendency is most clearly shown by the cluster of four linked insertions on chromosome 2 in the A9 and progeny map (Figure 5A). Our point of reference in this work is to the distributions of linked, transposed Acs that have been observed in maize. In those experiments researchers have also detected the presence of both linked and unlinked Acs after Ac transposition from a single locus. These studies have shown that roughly half of the detectable, transposed Acs are linked to their initial locations (VAN SCHAIK and BRINK 1959; GREENBLATT 1984; DOONER and BELACHEW 1989).

In this work we do not select for specific events. Rather, our molecular approach maps transposed Acs with respect to their starting locus, the T-DNA, and at any other site in the tomato genome. We have used both conventional λ cloning and the IPCR method (OCHMAN, GERBER and HARTL 1988; TRIGLIA, PETERSON and KEMP 1988; EARP, LOWE and BAKER 1990) to clone the regions flanking transposed Acs. We reasoned that the use of two cloning approaches would reduce that sampling bias that may arise through reliance on a single method. However, analysis of each line A9 and progeny (by IPCR) or A78 (by λ cloning) yielded the same conclusion, that transposition by wild type Ac can generate clusters of insertions in the tomato genome.

These arrays would not readily arise if Ac transposed according to either of two extreme models: In one model Ac transposes to linked locations at all times. This tendency would generate a single cluster of Ac insertions whose members were all linked to at least one other insertion. This prediction is clearly not applicable to our data. In the other extreme model insertions are randomly distributed about the chromosomes. It is possible that some insertions would be linked to each other according to this model. However, we may exclude this as an explanation of our findings by using the Poisson formula. In this instance the size of the target is 1260 cM, which is the length in centimorgans of the tomato genome within the most distal markers on each chromosome in our RFLP database (BERNATZKY and TANKSLEY 1986; S. TANKSLEY unpublished results). A cluster is defined as a group of insertions in which any member is linked to at least one other insertion. This prediction is clearly not applicable to our data. In the other extreme model insertions are randomly distributed about the chromosomes. It is possible that some insertions would be linked to each other according to this model. However, we may exclude this as an explanation of our findings by using the Poisson formula. In this instance the size of the target is 1260 cM, which is the length in centimorgans of the tomato genome within the most distal markers on each chromosome in our RFLP database (BERNATZKY and TANKSLEY 1986; S. TANKSLEY unpublished results). A cluster is defined as a group of insertions in which any member is linked (within 20 cM) to at least one other member. For example, the distribution of insertions cloned from A78 shown in Figure 5B contains two solo insertions and two clusters with three insertions in each cluster. We have calculated the probabilities for the occurrence of given cluster sizes with the Poisson formula and subjected these probabilities to the G statistic (ZAR 1984) and we conclude that P is less than 0.0001 for each line. That is, the extent to which Ac is clustered on either map would occur by chance roughly once in 10,000 trials if Ac inserted randomly (or 1 in 10⁸
FIGURE 5.—RFLP map positions of transposed Ac's in RO A9 and progeny (R1 B2, R1 B14 and R1 B31) (A) and RO A78 (B). Arrows indicate the positions of the single T-DNA loci (hatched arrows) and transposed Ac's (filled arrows) on a partial RFLP map of tomato. Brackets indicate the maximum separation between loci in centimorgans. The unlabeled hatch marks represent RFLP marker sites. The names of 7 of the 12 tomato chromosomes are shown in the circles.
trials would yield the clustering seen in both maps). Therefore, these Ac's are not arranged randomly in the tomato genome. One simplistic explanation for the observed arrays is that they are the consequence of Ac insertion into genomic hotspots, rather than insertion into linked sites. We consider this explanation to be inadequate as we observe Ac insertions linked to the tomato genome. Rather than supposing that the two T-DNAs have inserted into Ac insertional hotspots (with the implication that T-DNA insertions will be clustered in the tomato genome, although they appear not to be; Chyi et al. 1986), we prefer the interpretation that Ac transposes to linked and unlinked sites in the tomato genome, as it does in maize. The existence of clusters of linked Ac's that are unlinked to a T-DNA indicates that the transposed, unlinked Ac's may remain active and give rise to linked insertions themselves.

An important difference between studies on linked transposition in maize and this study is that we have mapped these without regard to their lineage. In maize, single detectable excision events gave rise to independent stocks, and each new insertion was relatively stable. The experiments in maize mapped the positions of linked, transposed Ac's originating from a single locus, and these linked and unlinked insertions were considered to be the results of primary transposition events. Unlinked, transposed Ac's were not mapped precisely, but the number of linked transpositions divided by the total number of transpositions yielded percent linked transposition by Ac from a given locus (Van Schaik and Brink 1959; Greenblatt 1984; Dooner and Belachew 1989). These estimates are slightly lower than that obtained by Jones et al. (1990) in studies on Ac in tobacco. The difference may be due to a sequence change introduced into Ac in vitro in that study, rendering Ac less active.

Ac transposes into unique or low copy tomato DNA: We have characterized the genomic copy number of the Ac flanking regions in the process of assigning these clones to the tomato RFLP map by Southern blot analysis. We have found that Ac transposes into unique or low copy tomato DNA at a very high frequency, similar to its behavior in tobacco (Hehl and Baker 1990). In fact, only one of twenty genomic flanking regions cloned could not be placed on the tomato RFLP map because of its hybridization to repetitive DNA. However, it is unwarranted to conclude from this study that Ac prefers to integrate into unique DNA rather than repetitive DNA, since the tomato genome is composed primarily of low copy sequences (Ganal, Lapitan and Tanksley 1988; Zamir and Tanksley 1988).

Tightly linked transposition by Ac: The structure of one clone (lambda CC50) is pertinent to a discussion of linked transposition by Ac. The T-DNA introduced into VF36 contains Ac inserted into the 5' untranslated leader of a p1'-NptII fusion gene in direct orientation with respect to NptII transcription (Baker et al. 1987). Clone CC50 contains a single Ac inserted within the NptII coding region in inverted orientation, 163 bp from the AUG of NptII. The insertion is flanked by the characteristic 8-base duplication (Figure 3). The simplest explanation is that the CC50 clone represents a short range, tightly linked transposition by Ac. Similar tightly linked transpositions by Ac Transposition from a T-DNA
Ac have been described at the P locus in maize: three transposition events where Ac had reinserted 160 bases, ~700 bases, or 4 kb away from its original position have been described (PETERSON 1989).

The structure of transposed Ac: We have determined the restriction maps of all cloned, transposed Ac, as well as determined the sequence of the immediate flanking regions and of the Ac termini (Figures 1, 3, and 4). This analysis shows that all transposed Ac from λ clones possess a characteristic 2.3-kb internal EcoRI-BamHI fragment (excluding CC25, which contains only the 5' end of Ac). Furthermore, all termini are intact except for the single, small deletion found in the 5' end of 9#160. These data are in agreement with a conclusion from previous analyses of cloned Ac from transgenic tobacco (HEHL and BAKER 1990), namely, that Ac maintains its structural integrity after transposition. Interestingly, only four of the seven transposed Ac clones that contain both ends are flanked by the typical 8-base direct duplication (POHL-#2 and -3) from R1 plant B31. We would not expect perfect inverted repeats to be dispersed throughout the genome, and therefore Ac may be dispersed throughout the genome, and therefore Ac may be considered a random mutagen. However, this view is incorrect, as a random distribution is statistically distinct from a dispersed, clustered distribution. By definition, clusters exclude regions of the genome as insertion targets. One cannot predict whether a gene or a region of the genome that is unlinked to a T-DNA would reside within an unlinked cluster of Ac. Therefore, if the most predictable tendency of Ac is to transpose to linked sites then the sensible strategy to mutagenize a gene or a genomic region is to first obtain a T-DNA bearing a transposon linked to that region.

Sequence analysis of the Ac 5' ends cloned by IPCR revealed the presence of point mutations in the terminal nucleotide of the transposon (Figure 4). The termini of the cloned Ac element used in this study are imperfect inverted 11-base pair repeats (Ac from wx-m7; BEHRENS et al. 1984; MÜLLER-NEUMANN, YODER and STARLINGER 1984; SUTTON et al. 1984). It is possible that Ac had transposed to generate three of the seven cloned insertions without forming this repeat. Alternatively, Ac insertion may have initially generated a flanking 8-base duplication, followed by a deletion event that would have precisely removed sequences adjacent to one end without deleting Ac terminal sequence. Such a deletion adjacent to Ac has been described at the Bz-m2(Ac) locus in maize (DOONER, ENGLISH and RALSTON 1988). These hypotheses are currently under investigation.

One cannot predict whether a gene or a region of the genome that is unlinked to a T-DNA would reside within an unlinked cluster of Ac. Therefore, if the most predictable tendency of Ac is to transpose to linked sites then the sensible strategy to mutagenize a gene or a genomic region is to first obtain a T-DNA bearing a transposon linked to that region. We suppose that Ds would also transpose to both linked and unlinked sites in the presence of Ac transposase. Linked transposition by Ds is a matter of interest since the stability of a transposed Ds bearing a marker gene may be a desirable feature either in mutagenesis or in using Ds as a mobile RFLP marker. Less experimentation has been done on linked Ds transposition in maize than on linked Ac transposition.

However, there is evidence concerning mutagenesis of the R locus in maize that is consistent with linked transposition by Ds (KERMICLE, ALLEMAN and DEL-LAPORTA 1989). In this work an unlinked Ac was used to trans-activate Ds excision from r-sc::m3 to generate nine fully colored R revertants. Ac was then used to trans-activate Ds insertion back into R. New, unique Ds insertions into R were found in progeny of five of the nine revertant stocks. These new alleles were insertions by a Ds of the same size as that in r-sc::m3 and not by Ac. The five revertants were thought to bear Ds linked to R. In addition, two Ds have been described that have transposed short distances from a form of mismatch repair during pairing of the termini.

The sequence of one IPCR clone, 14#9, was also notable. This clone conforms to the predicted IPCR structure, demonstrating that it represents an Ac insertion. However, the clone would not hybridize to VF36 DNA in Southern analysis (not shown). This observation was explained by comparison of the Ac flanking region to the GenBank database: the region flanking the Ac 5' terminus was Ac sequence. In this clone Ac had inserted into Ac in direct orientation at position 294 of the Ac 5' end. The predicted structure is reminiscent of the well studied double Ds at shrunk in maize (DÖRING, TILLMANN and STARLINGER 1984). However, in that case a Ds is inserted into a Ds in inverted orientation.

Linked Ac transposition and gene tagging: We have shown that Ac transposition can generate clusters of Ac. These clusters may be unlinked to the initial location of Ac, the T-DNA. At first glance one might extrapolate from this finding to say that with sufficient transposition Ac may be dispersed throughout the genome, and therefore Ac may be considered a random mutagen. However, this view is incorrect, as a random distribution is statistically distinct from a dispersed, clustered distribution. By definition, clusters exclude regions of the genome as insertion targets. One cannot predict whether a gene or a region of the genome that is unlinked to a T-DNA would reside within an unlinked cluster of Ac. Therefore, if the most predictable tendency of Ac is to transpose to linked sites then the sensible strategy to mutagenize a gene or a genomic region is to first obtain a T-DNA bearing a transposon linked to that region.

We suppose that Ds would also transpose to both linked and unlinked sites in the presence of Ac transposase. Linked transposition by Ds is a matter of interest since the stability of a transposed Ds bearing a marker gene may be a desirable feature either in mutagenesis or in using Ds as a mobile RFLP marker. Less experimentation has been done on linked Ds transposition in maize than on linked Ac transposition.

However, there is evidence concerning mutagenesis of the R locus in maize that is consistent with linked transposition by Ds (KERMICLE, ALLEMAN and DEL-LAPORTA 1989). In this work an unlinked Ac was used to trans-activate Ds excision from r-sc::m3 to generate nine fully colored R revertants. Ac was then used to trans-activate Ds insertion back into R. New, unique Ds insertions into R were found in progeny of five of the nine revertant stocks. These new alleles were insertions by a Ds of the same size as that in r-sc::m3 and not by Ac. The five revertants were thought to bear Ds linked to R. In addition, two Ds have been described that have transposed short distances from
their starting position in the *brassica* locus in *Zea mays* (Dowe, Roman and Klein 1990).

B.I.O. expresses his gratitude to Salva Luria, an extraordinary scientist and tireless friend who passed away a short time ago. We thank V. Williamson for critical review. This work was supported by the McKight Foundation and the United States Department of Agriculture (Cris. #5335-22230-002-00D).

**LITERATURE CITED**


Communicating editor: B. BURR