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State II *Dissociation* Element Formation Following *Activator* Excision in Maize

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ABSTRACT

Active *Activator* (*Ac*) elements undergo mutations to become nonautonomous *Dissociation* (*Ds*) elements at a low frequency. To understand the mechanism of *Ds* formation, we have developed high-throughput genetic and molecular screens to identify these rare *Ds* derivatives generated from any *Ac* insertion in the maize genome. Using these methods we have identified 15 new *Ds* elements derived from *Ac* insertions at eight different loci. Approximately half of the *Ds* elements contain filler DNA inserted at the deletion junction that is derived from sequences within or adjacent to *Ac*. In contrast to previous reports, several of these *Ds* elements lack direct repeats flanking the deletion junctions and filler DNA in the donor *Ac*. To accommodate our findings and those of others, we propose a model of slip mispairing during error-prone repair synthesis to explain the formation of state II *Ds* elements in maize. We discuss the use of these lines and molecular techniques developed here to capture somatic *Ds* transposition events in two-component *Ac/Ds* tagging programs in maize.

THE *Activator/Dissociation* (*Ac/Ds*) transposon family has been extensively characterized since its discovery in maize >60 years ago (McClintock 1946; Kunze and Weil 2002). *Ac/Ds* are class II DNA transposons that belong to the *hAT* superfamily of plant transposable elements (Kunze and Weil 2002). *Ac* is a 4565-bp autonomous element capable of catalyzing the transposition of itself and nonautonomous *Ds* elements (McClintock 1949, 1951). *Ac* encodes a 3.5-kb open reading frame (ORFa) that directs the synthesis of an 807-amino-acid transposase (TPase) essential for both *Ac* and *Ds* transposition (Fedoroff *et al.* 1983; Kunze *et al.* 1987). The 11 bp imperfect terminal inverted repeats (TIR) and ~240 bp of subterminal sequences are critical for TPase binding and transposition of both *Ac* and *Ds* (Coupland *et al.* 1988, 1989).

In contrast to the highly conserved structure of *Ac* elements (Fedoroff *et al.* 1983; Behrens *et al.* 1984; Muller-Neumann *et al.* 1984; Pohlman *et al.* 1984), *Ds* elements are structurally diverse. State I or "double *Ds*" elements consist of nested *Ds* insertions or contain multiple *Ds* end sequences in close proximity (Doring *et al.* 1984, 1989, 1990; Weck *et al.* 1984; Ralston *et al.* 1989; Weil and Wessler 1993). State II elements are likely deletion derivatives of *Ac* that differ in size and internal structure (reviewed in Kunze and Weil 2002). For instance, *Ds1*-like elements contain large internal deletions sharing only the 5′ terminal 13 bp and 3′ terminal 26 bp of sequence with *Ac* (Sutton *et al.* 1984). The *Ds9* element is identical to *Ac* with the exception of a 194-bp simple deletion in the element (Rubin and Levy 1997). Other *Ds* family members, such as *wxB4*, share the TIR and subterminal sequence with *Ac* but carry internal sequences with no homology to *Ac* (Varagona and Wessler 1990). State I elements often induce chromosome breakage but rarely transpose whereas state II elements transpose frequently but rarely break chromosomes (McClintock 1946, 1949).

Ac and *Ds* have been used to clone and characterize many genes in maize (for reviews see Kunze *et al.* 1997; Brutnell 2002; Settles 2005). Strategies to utilize these elements in gene-tagging programs have been devised that exploit the tendency of *Ac* and *Ds* to transpose to closely linked sites (Van Schaik and Brink 1959; Greenblatt 1984; Dooner and Belachew 1989). To facilitate the use of *Ac* in tagging programs, several *Ac* elements have been mapped throughout the maize genome (Auger and Sheridan 1999; Cowperthwaite *et al.* 2002), including 60 that have been precisely positioned on genetic and physical maps (Singh *et al.* 2003; Kolkman *et al.* 2005).

Although *Ds* has not been used extensively for gene tagging in maize (Kunze *et al.* 1997; Brutnell and Conrad 2003), it does afford several advantages over *Ac*. Foremost, *Ds*-induced mutations allow for the analysis of either stable or mutable phenotypes. In the absence of an *Ac* transposase source, *Ds* insertions are stable, facilitating physiological studies that would be complicated

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by somatic excision. Stable *Ds* alleles can also be incorporated into breeding programs without the risk of losing the desired allele due to transposon excision. When mutable phenotypes are desirable, for instance, in clonal analysis (Emerson 1917; Dawe and Freeling 1990) or to generate an allelic series (Wessler *et al.* 1986; Moreno *et al.* 1992; Weil *et al.* 1992; Singh *et al.* 2003), the elements can be remobilized by introducing an *Ac* element into the genome through crossing. Unfortunately, relatively few *Ds* insertions have been genetically or physically mapped in the maize genome, limiting the potential of *Ds* in directed or regional tagging programs (Conrad and Brutnell 2005).

The generation of a two-element system from a one-element system was first observed by McClintock when she identified *Ds* derivatives from *Ac* insertions at the *Bronze1* and *Waxy1* loci of maize (McClintock 1955, 1956, 1962, 1963). More recently, Yan *et al.* (1999) described the formation of three *Ds* elements from the *bz1-m2::Ac* allele and one from an *Ac* element inserted 0.05 cM proximal to the *Bronze1* locus, designated *tac2094::Ac*. Two related mechanisms have been proposed to account for the formation of *Ds* elements from *Ac*. Rubin and Levy (1997) concluded that state II *Ds* elements are generated through an abortive gap-repair process following *Ac* excision. However, in some instances, a synthesis-dependent strand-annealing (SDSA) repair mechanism is initiated at the time of this abortive gap repair. In these instances, polymerase slippage during DNA synthesis at the site of direct repeats within *Ac* may result in the insertion of sequences known as filler DNA at the deletion junction (Rubin and Levy 1997). Yan *et al.* (1999) proposed that *Ds* elements were formed through slip mispairing during DNA synthesis resulting both in an internal deletion and in some cases the insertion filler DNA at the deletion junction (Yan *et al.* 1999). Both models propose that slip mispairing of direct repeats at the deletion breakpoints within *Ac* occurs during DNA synthesis.

In addition to *Ac*, the formation of nonautonomous elements via double-strand break (DSB) repair has been studied for several class II transposon families such as *Mutator* in maize (Hsia and Schnable 1996), *P*-elements in Drosophila (O'hare and Rubin 1983; Takasu-Ishikawa *et al.* 1992), and *Tam3* in Antirrhinum (Yamashita *et al.* 1999). Although the formation of these nonautonomous elements is thought to occur through similar DNA repair mechanisms, there are differences in the frequency that repair products arise in each of these transposon families (Yamashita *et al.* 1999). In contrast to *P*, *Mu*, and *Ac* elements, the *Tam3* family of elements is extremely conserved in structure due to a lack of the formation of repair products following excision. Yamashita *et al.* (1999) suggest that hairpin formation at the ends of *Tam3* prevents gap repair from proceeding into the element, precluding the formation of functional nonautonomous *Tam3* elements. Although it has been shown that both *Mu* and *Ac* ends are capable of forming hairpin structures, these elements still produce nonautonomous derivatives (Yamashita *et al.* 1999). These studies suggest there are likely several factors that affect the DSB repair mechanism following excision of an active transposon including secondary structure.

In this report we describe the development of high-throughput genetic and molecular screens to identify newly formed *Ds* elements from previously positioned *Ac* insertions. Using these methods we identified 15 new *Ds* elements derived from *Ac* insertions at eight different sites in the maize genome. Sequence analysis revealed significant differences between these 15 *Ds* elements and previously reported *Ds* derivatives (Rubin and Levy 1997; Yan *et al.* 1999). Specifically, several of these *Ds* elements lack direct repeats flanking the deletion junctions in the donor *Ac*. Approximately half of the *Ds* elements contain filler DNA inserted at the deletion junction and this filler DNA often, but not always, originated from within *Ac*. Thus, we propose a modified version of a model proposed by Dooner and colleagues (Yan *et al.* 1999) that accounts for the formation of all state II derivatives characterized to date. To further exploit these lines in mutagenesis programs, we developed a "*Ds*-casting" procedure to capture local somatic *Ds* transposition events and we discuss the use of these lines in two-component *Ac/Ds* tagging programs in maize.

MATERIALS AND METHODS

Description of maize stocks: All maize stocks used in this study were in the W22 inbred background. All *Ac* lines, with the exception of *bti97156::Ac*, were previously described (Kolkman *et al.* 2005). The *bti97156::Ac* line contains an *Ac* insertion 4 cM from the *Pink Scutellum1* (*Ps1*) gene and has been previously described (Singh *et al.* 2003). The *r1-sc:m3 Ds* tester line contains a *Ds6*-like insertion in the *r1* locus that controls anthocyanin accumulation in the aleurone and scutellar tissues (Alleman and Kermicle 1993). In the absence of *Ac* transposase, this insertion renders the kernels colorless. In the presence of *Ac* transposase, excision of the *Ds* from the *r1* locus can be visualized as purple sectors in the aleurone. Increasing copy numbers of *Ac* in the genome result in fewer and smaller sectors known as the negative dosage effect (McClintock 1951). The *Ac-immobilized* (*Ac-im*) stock contains an *Ac* derivative inserted at 7.02, which is incapable of transposition but still encodes a functional transposase protein (Conrad and Brutnell 2005).

Selection for *Ds* derivatives: *Ac* activity was monitored using the *r1-sc:m3 Ds* tester line described above. Kernels homozygous for an *Ac* insertion were selected on the basis of the canonical negative dosage effect of *Ac* (McClintock 1951). Kernels were sown in the field and mature plants were testcrossed to the *r1-sc:m3 Ds* tester line. Colorless kernels resulting from a loss of *Ac* activity were selected from the progeny ears and plants self-pollinated to generate segregating families. The resulting ears were screened using one of two methods. When the *ps1-m8::Ac* allele was used as a donor *Ac*, colorless progeny ears were screened visually for segregation

of a *ps1* mutant phenotype with a lack of *Ac* activity (Singh *et al.* 2003; Bai *et al.* 2007). Progeny ears from all other donor *Ac* elements were screened using a molecular assay. Ten colorless kernels from self-pollinated F_2 ears were sown in greenhouse sandbenches. DNA was extracted from seedling leaf tissue and a verification PCR assay was performed on DNA pools as described below.

DNA extraction: DNA pools were constructed from ~1 g of seedling leaf tissue that was collected in two-dimensional pools. Seed from each segregating ear was planted in 10-kernel rows. Leaf tissue from these 10 seedlings were pooled and placed in both a row pool, consisting of 10 rows, and a column pool, consisting of one column from each of the 10 rows. Thus, a unique address could be assigned to each of the 100 segregating families using 20 pooled DNA extractions. Positive results were confirmed by extracting DNA from individual family members and repeating the PCR assay. DNA extraction was performed as described by Chen and Dellaporta (1994).

DNA extractions for the *Ds*-casting assay (see below) were conducted in a 96-well format. Two centimeters of leaf tissue were collected from the base of the youngest leaf of field-grown plants ~50 days after planting. The midrib was removed and tissue was placed in a 96-well plate with two ball bearings. Tissue was frozen in liquid nitrogen and pulverized in a paint shaker for 3 min. A phenol-chloroform extraction was performed as previously described (Chen and Dellaporta 1994).

PCR amplification: *Verification PCR:* PCR was performed on pooled DNA extractions described above. PCR primers designed for *Ac* insertion verification described in Kolkman *et al.* (2005) were utilized to amplify a region from the DNA flanking the element into the end of putative *Ds* derivatives (supplemental Table 1 at http://www.genetics.org/supplemental/). Verification PCR was performed using ~6 ng of total genomic DNA in a 50 μl total reaction volume according to reaction conditions described in Conrad and Brutnell (2005). PCR products were fractionated on a 0.8% agarose gel containing ethidium bromide and visualized on a UV light box.

Amplification of Ds elements: The long-range inverse PCR (IPCR-3) protocol described in Kolkman *et al.* (2005) was used to amplify deletion breakpoints from the *Ds* derivatives. PCR was performed on ~6 ng of genomic DNA extracted from five pooled seedling leaf tissue samples from individual segregating ears. Flanking primers presented in supplemental Table 1 at http://www.genetics.org/supplemental/ were used in conjunction with *Ac* primers. For the *ps1-m8::Ds* alleles D1, D4, and D5, sequences were amplified with the Ps1-21 primer (supplemental Table 1) while the *ps1-m8::Ds(D3)* allele was amplified with the Ps1-10 primer described in Singh *et al.* (2003). The *ps1-m8::Ds(D2)* was amplified with two flanking primers Ps1-21 (supplemental Table 1) and Ps1-10 (Singh *et al.* 2003). *Ac* primers used to amplify toward the 5′ *Ac* flanking sequences were: 191.Ac4: 5′-CAATAGCCATATCATCTTGAC TCG, TBp34, Ac14, and TBp41 reported in (Kolkman *et al.* 2005). *Ac* primers used to amplify toward the 3′ *Ac* flanking sequences were: TEB252.35Ac1: 5′-CAGGGATGAAAGTAGGA TGGGAAA, TEB252.35Ac3: 5′-CAAGCTGTTGTGTCATTTG TGTGC, Ac2: 5′-TTCATGTGAGGTGTGCTTGTC, 191.Ac5: 5′-GTGCTAGACTCTGTTATTGCTGCT, TBp38, Tbp40, and JGp2 reported in Kolkman *et al.* (2005). PCR products were fractionated on a 0.8% agarose gel with ethidium bromide and visualized on a UV light box.

Ds casting: A line homozygous for the *bti00252::Ds(D2)* derivative was used to pollinate a line homozygous for the *Ac-im* insertion. The resulting progeny were planted in the field and seedling leaf tissue was collected. DNA extractions were performed according to the protocol described above and a *Ds*-casting protocol was adapted from Singh *et al.* (2003). Two rounds of nested PCR were performed using the IPCR-3 reaction protocol described in Kolkman *et al.* (2005) with nested flanking and *Ds* end primers (supplemental Figure 2A at http://www.genetics.org/supplemental/). The first round of PCR used primers bti00252A.F2: 5′-CTAAGCTGACTGT TGTGTGGGAAG and Ac3: 5′-CATATTGCAGTCATCCCGAA with ~1–3 μg of genomic DNA in a total reaction volume of 20 μl. First round products were diluted 1:200 in dH_2O and 1 μl of this dilution was used as a template for the second round of nested PCR with primers bti00252A.F7: 5′-CGTACGTGTCATA ACTTTTGGAAG and TBp35 (described in Kolkman *et al.* 2005). PCR products were fractionated on a 1% agarose gel with ethidium bromide and visualized on a UV light box (supplemental Figure 2B).

Sequencing: PCR products were separated on a 0.8% agarose gel and purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). DNA was cloned into either pGEM-T Easy (Promega, Madison, WI) or the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) and sequenced as previously described (Singh *et al.* 2003).

RESULTS

Genetic selection for newly formed *Ds* derivatives: *Ac* donor lines were selected from a subset of previously characterized insertions (Kolkman *et al.* 2005) and from an *Ac* insertion at the *pink scutellum1* (*ps1*) locus, *ps1-m8::Ac*, which conditions a weak mutant phenotype when homozygous (Bai *et al.* 2007). *Ac* activity was visualized as purple sectors on the kernels resulting from excision of a *Ds* element from the *r1* locus during kernel development (*r1-sc:m3*; see materials and methods). Thus, a loss of *Ac* activity was scored as the lack of these purple sectors. To identify deletion derivatives of *Ac,* lines homozygous for an *Ac* insertion and the *r1-sc:m3* allele were testcrossed by the *Ds* tester line (Figure 1). The majority of progeny kernels from F_1-testcross progeny are coarsely spotted as a consequence of being hemizygous for the original *Ac* insertion. Approximately 2–4% of the progeny were finely spotted resulting from an increase in *Ac* copy number due to a new transposition (*tr-Ac*) (Kolkman *et al.* 2005). Rare colorless kernels were selected, grown, and self-pollinated to identify ears that were completely colorless in the F_2 generation due to a loss of *Ac* activity. Consequently, *Ds* derivative events that cosegregate with a *tr-Ac* in the genome would not be detected in our screen.

Approximately 0.6% of the F_1-testcross progeny kernels were colorless due to a loss of *Ac* activity. It is likely that in the majority of these cases, the loss of *Ac* activity was due to premeiotic excision of *Ac* followed by an independent assortment of the donor locus and transposed *Ac* (Greenblatt 1984). In rare cases, *Ac* will undergo a mutational event that renders it inactive. Both losses of *Ac* from the genome and putative inactivation events are selected in the genetic scheme outlined in Figure 1. A total of 753 colorless progeny ears were screened representing colorless kernel derivatives from 20 loci containing *Ac* insertions, including the *ps1-m8::Ac* allele. As described in the following sections,

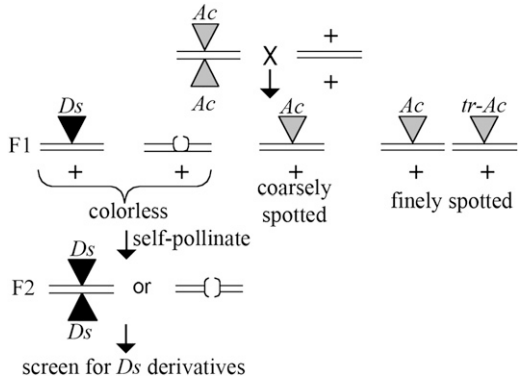

FIGURE 1.—Genetic selection for newly formed *Ds* derivatives. Lines homozygous for an *Ac* insertion (shaded triangles) were pollinated by the *Ds* tester line. All lines are homozygous for the *r1-sc:m3* allele (not shown). Colorless kernels either containing a new *Ds* (solid triangles) or a loss of *Ac* due to excision (parentheses) were selected from the resulting progeny ears. Colorless kernels were sown and mature plants were self-pollinated resulting in an ear segregating for either a new *Ds* or the *Ac* excision site. Ears derived from the *ps1-m8::Ac* lines were visually screened for a mutant phenotype. Kernels from the remaining ears were planted and DNA was extracted from seedling leaf tissue. PCR was used to screen for the insertion of putative *Ds* at the original locus as described in the text.

24 *Ac* inactivation events, or putative *Ds* elements, were identified at 8 loci, including 6 from the *ps1-m8::Ac* allele. These putative *Ds* insertions were further characterized through phenotypic or PCR-based screens to confirm the presence of an insertion at the original locus as detailed below. Importantly, this genetic screen could be applied to detect *Ds* derivatives from any *Ac* insertion in the maize genome.

Identification of *Ds* insertions through a molecular screen: We developed a general PCR assay to distinguish putative *Ds* derivatives from *Ac* excision events. As shown in Figure 1, putative *Ds* derivatives would be expected to segregate in self-pollinated F_2 families. Thus, 10 kernels from colorless F_2 ears were planted in the greenhouse and DNA was extracted from pooled seedling leaf tissue samples (MATERIALS AND METHODS). PCR primers were designed to amplify a fragment immediately adjacent to the original *Ac* insertion using a subterminal *Ac* end- and flanking sequence-specific primer pair (supplemental Table 1 at http://www.genetics.org/supplemental/). Germinal excisions of *Ac* would not result in amplification products whereas those elements that retained the sequences complementary to the primer at the original insertion site would result in an amplification product of a predicted size. We screened 673 families and identified 18 that contained an *Ac*-end sequence at the site of the original insertions but lacked *Ac* activity. These families were selected as carrying putative novel *Ds* insertions.

One limitation of this screen was the requirement for intact *Ac* end sequences at the priming site. In other words, if a more complex rearrangement of *Ac* sequences resulted in the deletion of end sequences containing the primer sequences, the event would not be detected in the screen. Nevertheless, the screen does enrich for events that are likely to generate a functional *Ds* derivative.

Identification of *Ds* insertions through a phenotypic screen: To investigate the diversity of *Ac*-mediated deletions and more complex rearrangements, we utilized an *Ac* insertion at the *ps1* locus (*ps1-m8::Ac*). In contrast to the molecular screen described above, this phenotypic screen did not require intact *Ac* ends or flanking sequences for the identification of a *Ds* derivative at this locus. *Ps1* encodes an enzyme with lycopene β-cyclase activity that catalyses the first committed step in xanthophyll biosynthesis (SINGH *et al.* 2003). Although the majority of mutations in *Ps1* are lethal, *ps1-m8::Ac* kernels germinate at nearly the same frequency as wild type (BAI *et al.* 2007). Seedlings homozygous for the *ps1-m8::Ac* allele have slightly pale green leaf sheath tissue and virescent leaf blade tissue and kernels from self-pollinated ears are slightly pink due to an accumulation of δ-carotene in the endosperm (L. BAI, unpublished results; BAI *et al.* 2007). We reasoned that *Ds* derivatives would condition for a similar mutant phenotype as the donor *Ac* insertion and thus could be identified as slightly pink kernels lacking *Ac* activity.

Genetic selections were performed as described above and colorless F_2 ears were visually screened for segregation of a weak *ps1* mutant phenotype segregating in a 3:1 Mendelian ratio (SINGH *et al.* 2003; BAI *et al.* 2007). Six alleles were selected as having a weak mutant phenotype and lacking *Ac* activity. *Ds* derivatives that restore wild-type *Ps1* function would not be detected in this screen.

***Ds* deletion junction sequence analysis:** To define the deletion breakpoints in each of the putative *Ds* derivatives, a long-distance PCR method was utilized to rapidly survey the structure of the element (MATERIALS AND METHODS). As illustrated in Figure 2A, PCR primers designed to sequences throughout the *Ac* element were used in conjunction with an *Ac*-flanking primer as described by KOLKMAN *et al.* (2005). As shown in Figure 2B, if the primer sequence is contained in the deleted portion of the *Ds*, a PCR product will not be generated (lane 3) while primers flanking this deletion will give a smaller product (lanes 5 and 7) when compared to a full-length *Ac* (lanes 6 and 8). PCR products containing the deletion breakpoint were then sequenced (see MATERIALS AND METHODS). This PCR assay enabled the rapid identification of the sequences deleted from 15 of the 24 putative *Ds* elements.

The nine derivatives that did not appear to result from simple deletions of *Ac* were further characterized. Four contained an apparently full-length *Ac* element inserted at the original locus, based on PCR product size even though no *Ac* activity was detected. There are at least two possibilities that could account for the loss of *Ac* activity in lines that contain an apparently full-length

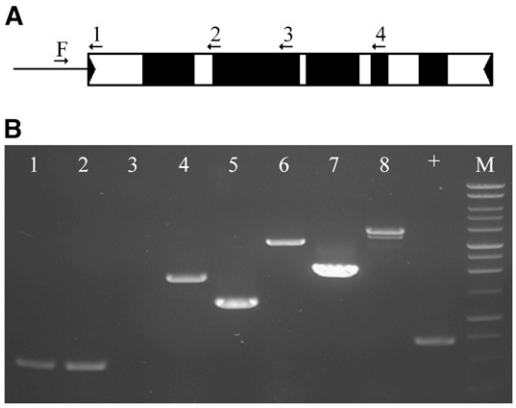

FIGURE 2.—Amplification across *Ds* deletion junctions. (A) *Ac* schematic. Flanking DNA upstream of the *Ac/Ds* represented by a line and *Ac* sequence shown as rectangle with TIRs as solid triangles, exons as solid boxes, and noncoding seqeunces as open boxes. Primers are shown as arrows, F is the flanking primer, and 1–4 are internal *Ac* primers. (B) Ethidium bromide-stained agarose gel showing PCR amplification products derived from a new *Ds* element. Lanes 2, 4, 6, and 8 show PCR products generated using *bti97156::Ac* as a template and lanes 1, 3, 5, and 7 show PCR products derived from *bti97156::Ds* as a template; the + is a positive control, and the M is a Promega 1-kb ladder. Primer F was used in all reactions: lanes 1 and 2, F + primer 1; lanes 3 and 4, F + primer 2; lanes 5 and 6, F + primer 3; lanes 7 and 8, F + primer 4.

Ac. First, small deletions (≤50 bp) would not be detected in the PCR assays, but still disrupt *Ac* activity. Alternatively, epigenetic silencing associated with increased methylation of internal *Ac* sequences (SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987; KUNZE *et al.* 1987, 1988; FUSSWINKEL *et al.* 1991; BRUTNELL and DELLAPORTA 1994; BRUTNELL *et al.* 1997) may have resulted in the loss of *Ac* activity. To examine this latter possibility, DNA gel blot analysis was performed on families that contained an apparently full-length *Ac* element at the original locus. To monitor the methylation status of diagnostic nucleotides within *Ac,* DNA was digested with the methylation-sensitive restriction enzyme *Pvu*II and blots were hybridized with the internal Ac900 or Ac700 fragments described in KOLKMAN *et al.* (2005). This analysis suggests at least one of the putative *Ds* elements was methylated at the internal *Pvu*II sites (data not shown), indicating that the loss in activity in some families was due to epigenetic modification of *Ac* sequences. The methylation of the internal *Pvu*II sites does not appear to be the consequence of *Ds* formation, as derivative *bti00228::Ds* retained two unmethylated *Pvu*II sites. The five remaining putative *Ds* elements retained *Ac* end sequences at the original locus but we were unable to amplify products in the long-distance PCR assay, despite repeated attempts. These insertions may have resulted from more complex rearrangements of *Ac* sequences rather than simple deletions. DNA blot analysis of three of these derivatives indeed suggests a more complex rearrangement of sequences (data not shown).

TABLE 1

Frequency of *Ds* formation

Ac progenitor	Kernels screened	*Ds* identified
mon03077::Ac	9965	1
bti00228::Ac	895	1
bti00252::Ac	6948	2
bti00257::Ac	ND	3
1.07::Ac	ND	1
bti00191::Ac	2115	1
bti97156::Ac	ND	1
ps1-m8::Ac	ND	5

Frequency of *Ds* formation: A total of 15 new *Ds* elements were identified from eight unique *Ac* insertions in the maize genome. Table 1 presents the minimum frequency of *Ds* formation for several of the *Ac*-containing lines screened. Accurate progeny counts were only obtained for four *Ac* donor lines and of these, screens of only one line (*bti00252::Ac*) resulted in the recovery of multiple *Ds* insertions. In this instance, the frequency of *Ds* formation was calculated at 0.03%. However, if we consider the total number of kernels screened regardless of *Ac* donor, we estimate the frequency of *Ds* formation at 0.025% (5/19,923 × 100).

***Ds* elements contain novel deletions:** As predicted from previously published studies (RUBIN and LEVY 1997; YAN *et al.* 1999), a preferential size or location of deleted *Ac* sequence was not observed (Figure 3). The size of deletions ranged from 388 bp in *bti00191::Ds* to 3505 bp in *ps1-m8::Ds(D2)* and were not restricted to any particular region within *Ac* (Table 2, Figure 3). Interestingly, *bti00257::Ds(D1)* carries an in-frame deletion of amino acid residues 331–475 of exon 2. This derivative is associated with a tightly linked *Ac* insertion but conditions a finely spotted aleurone that appeared nearly colorless in initial kernel selections. This aberrant spotting pattern suggests that *bti00257::Ds(D1)* encodes a protein that interacts with *Ac* and alters its activity. We have not yet separated the tightly linked *Ac* element from this derivative and thus, have not further characterized this element in this study. None of the other derivatives encoded proteins with any detectable activity.

Seven of the 15 *Ds* derivatives have filler DNA inserted at the deletion junction (Table 2). Filler DNA refers to extraneous nucleotides that are copied into sequence rearrangements from elsewhere in the genome. It is a common result of DSB repair and has been observed in plants, animals, and fungi (for example, see ROTH *et al.* 1989; SAINSARD-CHANET and BEGEL 1990; WESSLER *et al.* 1990; BUNDOCK and HOOYKAAS 1996; PUCHTA 2005). The size of the filler DNA varied from 1 to 64 bp and was always found at the deletion junctions. All of the filler DNA originated from sequence in close proximity to the deletion breakpoint and the majority of it is present elsewhere within the *Ds* (Figure 3). The one exception,

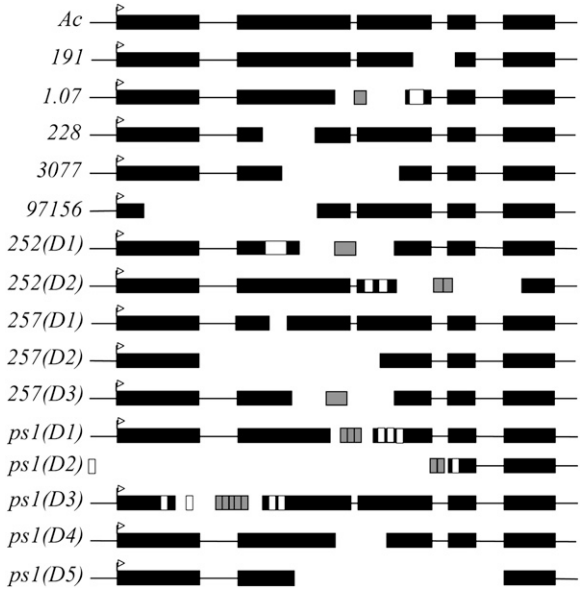

FIGURE 3.—Structures of *Ds* derivatives. The internal structures of 15 *Ds* derivatives characterized in this study are shown in comparison to *Ac* (top). Solid lines represent noncoding sequences and solid rectangles are exons. The arrowheads denote the start of transcription. Deletions within *Ds* elements are shown as a gap. Filler DNA is denoted by shaded boxes and the origin of the filler DNA sequence, when known, is shown as open boxes.

TABLE 2

Deletion derivatives identified in this study

Map position	Derivative	Deletion size (bp)	Presence of filler DNA (bp)	Origin of filler DNA
1.01	*mon03077::Ds*	1553	None	NA
1.03	*bti00228::Ds*	710	None	NA
1.04/1.05	*bti00252::Ds(D1)*	1274	29	*Ac*
	bti00252::Ds(D2)	1345	33	*Ac*
1.05	*bti00257::Ds(D1)*	435	None	NA
	bti00257::Ds(D2)	1848	None	NA
	bti00257::Ds(D3)	1120	4	*Ac*
1.07	*1.07::Ds*	673	10	*Ac*
2.02	*bti00191::Ds*	388	None	NA
5.04	*bti97156::Ds(D1)*	1806	None	NA
5.04	*ps1-m8::Ds(D1)*	1141	38	*Ac*
	ps1-m8::Ds(D2)	3505	53 + 11	*Ps1* + *Ac*[a]
	ps1-m8::Ds(D3)	815	51	*Ac*
	ps1-m8::Ds(D4)	465	None	NA
	ps1-m8::Ds(D5)	1815	None	NA

[a] Fifty-three base pairs of filler DNA originating from flanking DNA and 11 bp originating in the *Ac* sequence.

ps1-m8::Ds(D2), contains filler DNA that originated from the flanking sequence adjacent to the site of *Ac* insertion at *ps1* and is missing one *Ds* end sequence. The derivatives *ps1-m8::Ds(D4)* and *bti00257::Ds(D3)* have 1 and 4 bp of filler DNA, respectively, inserted at the deletion junction. As the sequence of the filler DNA occurs multiple times within *Ac*, it precludes determination of the template DNA. Four of the 7 *Ds* derivatives contain filler DNA that originated from multiple locations and is assembled at the deletion junction (Figure 4 and supplemental Figure 1 at http://www.genetics.org/supplemental/). Interestingly, these filler sequences are not always arranged at the deletion junction in the same order as they occur in their original context in *Ac*. The most extreme example of this is the *ps1-m8::Ds(D3)* element where there are five tandem pieces of filler DNA inserted at the deletion junction plus two additional nucleotides. This mosaic of filler DNA originates from upstream, downstream, and from within the deleted sequences of the *Ds* with the same 8 bp of filler sequence being repeated twice at the deletion junction (supplemental Figure 1). In summary, to the extent the filler origin can be determined, it always originates from sequence in close proximity to the deletion, can arise from within or outside the *Ac* sequence, and may be assembled into tandem arrays.

The current model for the formation of *Ds* derivatives including the insertion of the filler DNA into the deletion junction is based on the observation that short direct repeats flank both the deletion and the origin of filler DNA (YAN *et al.* 1999). However, in this study, several *Ds* derivatives do not have direct repeats flanking the deletion junction and/or filler DNA in the *Ac* donor (Figure 4). We classified the *Ds* derivatives reported here into two types with regard to the presence or absence of these direct repeats. An example of each type is presented in Figure 4. Type I derivatives (8/15) contain direct repeats in the donor *Ac* flanking the deletion junction and filler DNA as predicted by previous models. Type II derivatives (5/15) do not contain direct repeats flanking either the deletion junction or, when present, the filler DNA. As mentioned above, two derivatives, *ps1-m8::Ds(D4)* and *bti00257::Ds(D3)*, contain 1 and 4 bp of filler DNA, respectively, so it is not possible to classify them as either type I or type II. Thus, at least 33% of the deletions identified lack direct repeats flanking filler DNA or deletion breakpoints in the donor *Ac*, indicating that repeat sequences at deletion/filler breakpoints are not prerequisites for state II *Ds* element formation in maize.

***Ds* elements capable of somatic transposition:** With the exception of *ps1-m8::Ds(D2)*, all of the *Ds* derivatives reported here contain internal deletions and are likely to be transposition competent (COUPLAND *et al.* 1988, 1989). To test this prediction and extend the utility of these *Ds* insertions in gene-tagging programs, we developed a PCR-based method to capture somatic transpositions of *Ds*. This method was modified from the "*Ac*-casting" procedure described by SINGH *et al.* (2003). To demonstrate this method, a plant carrying the *Ds* insertion *bti00252::Ds(D2)* was crossed by an individual homozygous for *Ac-im* (MATERIALS AND METHODS). *Ac-im* is a stabilized source of transposase, capable of mediating

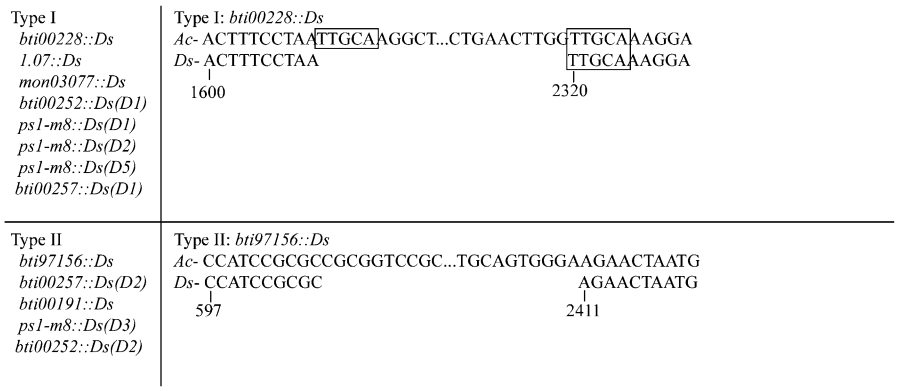

FIGURE 4.—Sequence at *Ds* deletion junctions. Two general classes of *Ds* elements are defined. Type I are defined by the presence of direct repeats of *Ac* sequence at the junction of the *Ds* breakpoint or filler DNA. Type II elements are defined by the absence of direct repeats of *Ac* sequence at the junction of the *Ds* breakpoint or filler DNA. Sequence alignment for an example of each *Ds* type is shown. A single strand of *Ac* sequence (top) and *Ds* (bottom) is shown. Numbering is based on published *Ac* sequence (GenBank accession no. X05424). Direct repeats are surrounded by boxes.

somatic and germinal *Ds* excisions (CONRAD and BRUTNELL 2005). PCR primers were designed to sequences flanking the *Ds* insertion (GenBank accession no. AY559233) and the 5′-*Ds* end (supplemental Figure 2A at http://www.genetics.org/supplemental/) to amplify short-range somatic *Ds* transpositions. Amplification products are expected only when the *Ds* element excises and reinserts upstream of the flanking primer in the opposite orientation as the original insertion. The results of *Ds* casting are shown in supplemental Figure 2. Multiple PCR products were detected and sequenced. Some of these products did not contain *bti00252* flanking DNA and were likely the result of *Ds–Ds* amplification products generated from cryptic *Ac*-like elements present elsewhere in the maize genome. However, one product was recovered that was the result of a local somatic transposition from *bti00252::Ds(D2)* (supplemental Figure 2B). Sequence analysis of this fragment revealed that the *Ds* element excised from the original insertion site generating an 8 bp excision allele and reinserted in the opposition orientation 282 bp upstream of the original insertion. These results demonstrate *Ds* casting as a method to obtain additional genomic sequences flanking *Ds* elements in the genome and shows that *bti00252::Ds(D2)* is transposition competent.

DISCUSSION

Development of two-component *Ac/Ds* tagging systems: *Ac* and *Ds* offer a number of advantages for gene-tagging experiments in maize. Both elements are present at relatively low copy number in the genome facilitating molecular and genetic screens (FEDOROFF *et al.* 1983). They display a high frequency of somatic excision that can be exploited to confirm the identity of a tagged gene (SCHULTES *et al.* 1996; SCHAUSER *et al.* 1999), to create an allelic series (ATHMA *et al.* 1992; MORENO *et al.* 1992), or to generate stable "footprint" alleles (WEIL *et al.* 1992; ALLEMAN and KERMICLE 1993; BAI *et al.* 2007). Of particular value are small in-frame insertions following *Ac/Ds* excision that subtly alter the protein activity (WESSLER *et al.* 1986; ALLEMAN and KERMICLE 1993; GIROUX *et al.* 1996; BAI *et al.* 2007). Finally, both *Ac* and *Ds* display a tendency for linked transposition that can be exploited in regional mutagenesis experiments (HAKE *et al.* 1989; ATHMA *et al.* 1992; MORENO *et al.* 1992; WEIL *et al.* 1992; ALLEMAN and KERMICLE 1993; COLASANTI *et al.* 1998) and to obtain additional gene sequence from closely linked somatic transpositions (SINGH *et al.* 2003).

Although *Ac* and *Ds* share these common features a major difference is that *Ac*-induced mutations are genetically unstable. The use of *Ds* in a two-component tagging approach would allow for the generation of stable or mutable alleles. As outlined above, this can be useful in a range of studies from crop improvement to clonal analysis. Here, we have adapted the *Ac*-casting method to amplify sequences flanking *Ds* insertions following somatic transposition. We have also shown that it is possible to select for novel *Ds* derivatives from multiple *Ac* elements throughout the genome. Each of the *Ds* elements identified carries a unique molecular signature consisting of either filler DNA, a novel breakpoint, or a combination of the two. These unique molecular tags can be readily exploited in PCR-based techniques to selectively amplify DNA flanking each unique *Ds* element. For instance, we are currently amplifying germinal transpositions of the *Ds* resident at the *r1-sc:m3* locus, by exploiting the unique breakpoint in the *Ds6*-like element resident as a molecular signature in IPCR (http://www.plantgdb.org/prj/AcDsTagging/).

Two component systems have been used for gene characterization extensively in Arabidopsis, tomato and rice (for example, BANCROFT *et al.* 1993; JONES *et al.* 1994; JAMES *et al.* 1995; SUNDARESAN *et al.* 1995; CHIN *et al.* 1999; MEISSNER *et al.* 1999). However, in maize only two genes, *Indeterminate1* and *Knotted1*, have been cloned using a two-component directed tagging approach with *Ds* (HAKE *et al.* 1989; COLASANTI *et al.* 1998). One of the primary factors limiting *Ds* tagging in maize is the lack of molecularly mapped *Ds* insertions in the genome. By definition, a *Ds* element does not encode an active transposase, and this has made it difficult to develop genetic screens to mobilize these elements. However, several groups have successfully distributed *Ac*

elements throughout the genome (Auger and Sheridan 1999; Cowperthwaite *et al.* 2002; Kolkman *et al.* 2005). Currently, 171 *Ac* elements distributed throughout the maize genome are available from the Maize Genetics Cooperative Stock Center (http://www.maizegdb.org/cgi-bin/stockcatalog.cgi#tool). Thus, one approach to distributing *Ds* insertions throughout the maize genome is to select for *Ds* derivatives from mapped *Ac* elements using the methods described here.

To demonstrate the utility of a two-component tagging approach, we identified five *Ds* insertions at the *ps1* locus of maize. The *ps1-m8::Ac* allele conditions a weak mutant phenotype that accumulates lycopene in the embryo due to an *Ac* insertion in the 5′-UTR of the lycopene β-cyclase gene (Bai *et al.* 2007). *Ds* insertions at this locus provide a viable stable mutant phenotype that can now be used to move the increased lycopene trait into maize breeding lines. Additionally, these *Ds* insertions can be used as donor elements to create numerous stable *Ds* insertions throughout this gene for further structure/function studies. One derivative, *ps1-m8::Ds(D2),* was identified that contained a single intact end of the *Ac* element, known as a fractured *Ac*. These elements have been shown to induce chromosome breakage and more complex rearrangements when linked in *cis* with a functional *Ac* element (Dooner and Belachew 1991; Zhang and Peterson 2004). Thus, it should now be possible to generate deletion derivatives and a number of stable *ps1* derivatives using this series of *Ds* elements.

Frequency of *Ds* element formation: In this study we identified 15 *Ds* derivatives from *Ac* insertions at eight loci. The frequency of *Ds* formation across these eight loci was ~0.025%. This estimate is similar to frequency of *Ds* formation calculated by Dooner and colleagues at *bz1-m2::Ac* ($2/3867 \times 100 = 0.05\%$ (Yan *et al.* 1999). However, McClintock identified 13 putative *Ds* derivatives from the *wx-m9::Ac* allele in screens of 4737 gametes (McClintock 1963). She conducted further genetic tests on only two of these derivatives and confirmed that both were *Ds* derivatives. Thus, the frequency of *Ds* formation from *wx-m9::Ac* may be as high as 0.27% and is minimally 0.04%. Interestingly, we identified a single *Ds* derivative in screens of only 895 progeny from *bti00228::Ac*, suggesting that the frequency of *Ds* formation can vary as a function of the *Ac* insertion site. However, in the absence of additional *Ds* derivatives from *bti00228::Ac* or an accurate frequency of *Ds* formation from *wx-m9::Ac*, we can only speculate on the significance of these findings. Studies of *Ac* excision in maize suggest that immediately following *Ac* excision, a double-strand break is formed resulting in hairpin formation of flanking DNA (Scott *et al.* 1996; Bai *et al.* 2007). These hairpins are likely resolved through endonucleolytic attack resulting in predominant 7- and 8-bp excision alleles (Bai *et al.* 2007). In rare instances, hairpin formation does not occur and instead the excision site is repaired through DNA synthesis using the sister chromatid as a template (Rubin and Levy 1997; Yan *et al.* 1999). If repair does not execute faithfully, a derivative *Ds* element may be produced. It is possible that local chromatin organization or epigenetic modifications can influence the DNA repair process following *Ac* excision and therefore influence the frequency of *Ds* element formation.

Model for *Ds* formation: All 15 *Ds* elements sequenced in this study have a unique deletion junction with the insertion of filler DNA occurring in about half of them (7/15). The filler DNA originates from sequence near the deletion and in one instance comes from genomic sequences flanking the *Ds* insertion. In previous reports of *Ds* formation, many *Ds* derivatives contain filler DNA at the deletion junction that originated from sequence nearby, within, and outside *Ac* (Rubin and Levy 1997; Yan *et al.* 1999). The occurrence of filler DNA and direct repeats in the template element flanking deleted sequences has also been observed at the deletion breakpoints of nonautonomous transposons derived from *P* elements in Drosophila (O'hare and Rubin 1983; Takasu-Ishikawa *et al.* 1992) and *Mutator* in maize (Hsia and Schnable 1996), suggesting that the insertion of filler DNA is common in the repair of transposon-mediated excision events. As mentioned above, filler DNA results from mispairing during DNA synthesis following *Ac* excision (for reviews see Gorbunova and Levy 1999; Puchta 2005). The common feature of filler DNA within *Ds* elements is that the choice of template is dependent on its position relative to the deletion breakpoint.

Interestingly, all of the newly derived *Ds* derivatives identified by Yan *et al.* (1999), contained short direct repeat sequences in the template *Ac* element at the deletion breakpoints of the *Ds*, suggesting repeats were involved in the mechanism of *Ds* formation. In studies of native *Ds* elements in the maize genome, the majority also carried simple deletions that appear to originate at short direct repeats within *Ac*. However, several *Ds* insertions were also defined with deletion breakpoints that do not appear to have arisen from simple slip mispairing during repair synthesis (Pohlman *et al.* 1984; Dooner 1986; Rubin and Levy 1997). Thus, it was unclear if the lack of repeat sequences in the *Ac* element at the *Ds* deletion junction was due to secondary mutations or simply the outcome of another repair process.

In contrast to these previous reports, several deletion junctions or filler DNA sequences in this study did not include a single copy sequence that is repeated twice in the progenitor element at the deletion junction (minimally 5/15). Moreover, several of these *Ds* derivatives contain filler DNA originating from multiple locations within *Ac* indicating a nonsequential positioning of filler during repair synthesis. For instance, *ps1-m8::Ds(D3)* contains five blocks of filler DNA derived from upstream, downstream, and within the deleted region of *Ac*. This suggests that slip mispairing may be occurring

on both upper and lower template strands during DNA synthesis and that slip mispairing may occur at least five times during the synthesis of one *Ds* element. Alternatively, these complex insertions of filler DNA could be created through multiple, independent rounds of excision and repair. However, since the *Ds* elements in this study formed in a single generation, it is highly unlikely that multiple excision events occurred.

The most recent model for *Ds* formation attributes both deletion formation and insertion of filler DNA to slip mispairing at direct repeat sequences within the template *Ac* (Yan *et al.* 1999). However, this model cannot account for the formation of 5/15 *Ds* elements identified in this study. Figure 5 illustrates a model of *Ds* formation that demonstrates how slip mispairing may occur without direct repeats at the deletion junction in the template DNA. Previous studies have established that *Ds* formation is dependent upon the excision of *Ac* (Rubin and Levy 1997). Thus, the process of *Ds* formation likely begins with *Ac* excision, initiating a DSB (Figure 5A; reviewed in Kunze and Weil 2002). Typically, this double-strand break is repaired through a hairpin intermediate resulting in *Ac* footprint formation (Weil and Kunze 2000; Bai *et al.* 2007). However, in rare instances, the DSB is resolved through 3′ strand invasion that initiates a SDSA repair pathway with as little as 1–6 bp of homology required to initiate synthesis (Rubin and Levy 1997; Salomon and Puchta 1998; Gorbunova and Levy 1999; Puchta 2005).

We envision DNA repair synthesis initiating from both ends of the excised element using the sister chromatid as a template (Figure 5B). Typically, slip mispairing takes place at short stretches of sequences that occur multiple times within the template *Ac* (Rubin and Levy 1997; Yan *et al.* 1999) and it has been suggested that 1–5 bp of homology is sufficient to invoke slippage during DNA synthesis (Yan *et al.* 1999). For simplicity these repeat sequences are represented by letters A, A′, B, and B′ in Figure 5, B–E. Following DNA synthesis of sequence A (*e.g.*, 3-bp sequence), slip mispairing occurs followed by reannealing at A′, resulting in a deletion between the repeat sequence A and A′ in the *Ac* template. This single slip- mispairing event would result in the generation of the type I *Ds* elements shown in Figure 4 and those previously characterized by Yan *et al.* (1999). However, we also observed sequential slippage events, as illustrated in Figure 5D. Here, the short filler DNA sequence B (*e.g.*, 3 bp) is copied into the new *Ds* element adjacent to sequence A (Figure 5C). Immediately following synthesis of sequence B, a second slip mispairing occurs, resulting in reannealing at B′ and another deletion of template DNA in the new *Ds* elements (Figure 5D) generating a novel junction AB. DNA synthesis is concluded when the template disassociates from the newly synthesized DNA and the molecule is ligated to the excision site via nonhomologous end joining (NHEJ) (Figure 5E). As shown in Figure 5E, the end result is a *Ds* element with a deletion flanked by sequences A and B. However, in the template strand these sequences are not contiguous; they appear as A, A′B, and B′. Thus, by invoking a series of tandem slip-mispairing events, this model can accommodate the formation of both type I and type II *Ds* elements observed in our study (Figure 4).

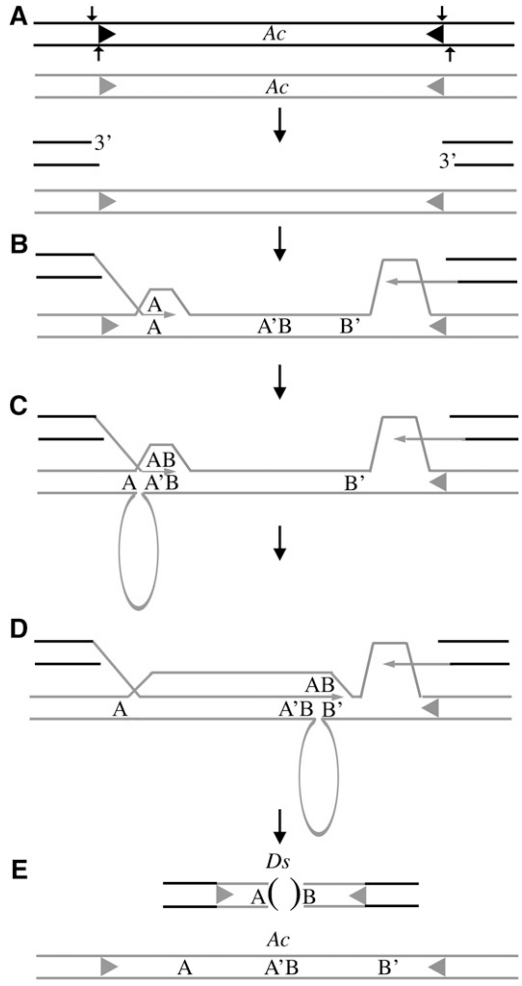

Figure 5.—Model for *Ds* formation. (A) A DSB is created at the donor locus through *Ac* excision. Arrows indicate a staggered cut resulting in a 1-bp 5′ overhang (review in Kunze and Weil 2002). The sister chromatid is shown as shaded. (B) A SDSA mechanism is initiated through a 3′-strand invasion of the sister chromatid. DNA synthesis begins from both excision sites using the sister chromatid as a template for repair. Letters (A, A′ and B, B′) represent 1–5 bp of sequences that occur multiple times within *Ac.* (C) Slippage occurs at sequence motif A with reannealing at A′ and a deleted segment of the *Ac* template (looped-out region) during DNA synthesis. Filler DNA sequence B is copied into the new *Ds* element now adjacent to sequence A rather than A′ as occurs in the template. (D) A second slip mispairing occurs at sequences B with reannealing at B′ resulting in another deletion of template DNA (looped-out region) in the new *Ds* element. (E) Newly synthesized DNA is ligated back into the excision site via NHEJ. The *Ds* product carries a unique AB junction not present in the donor *Ac.*

In summary, our data agree with previous models suggesting that error-prone DNA repair initiated through the SDSA pathway gives rise to *Ds* derivatives following *Ac* excision (Rubin and Levy 1997; Yan *et al.* 1999). However, our data suggest slip mispairing can occur numerous times during DNA repair synthesis, resulting in complex mosaics of filler DNA that are derived from *Ac* sequences located both in front of and behind the replication fork as observed in *ps1-m8::Ds(D1)* and *ps1-m8::Ds(D3)*. Previous models of *Ds* formation do not invoke multiple rounds of slippage nor do they suggest a reordering of template DNA copied into the deletion junction as was observed here. Although we could invoke NHEJ or abortive gap repair to explain some type II *Ds* formation, we prefer the more parsimonious model described above that can account for the formation of all state II *Ds* elements.

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