

Methylation Pattern of *Activator* Transposase Binding Sites in Maize Endosperm

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The maize transposable element *Activator* (*Ac*) transposes after replication from only one of the two daughter chromatids. It has been suggested that DNA methylation in conjunction with methylation-sensitive transposase binding to DNA may control the association of *Ac* transposition and replication. We present here a detailed genomic sequencing analysis of the cytosine methylation patterns of the transposase binding sites within both *Ac* ends in the *wx-m9::Ac* allele, where *Ac* is inserted into the tenth exon of the *Waxy* gene. The *Ac* elements in *wx-m9::Ac* kernels exhibit intriguing methylation patterns and fall into two distinct groups. Approximately 50% of the elements are fully unmethylated at cytosine residues through the 256 nucleotides at the 5' end (the promoter end). The other half is partially methylated between *Ac* residues 27 and 92. In contrast, at the 3' end, all *Ac* molecules are heavily methylated between residues 4372 and 4554. The more internally located *Ac* sequences and the flanking *Waxy* DNA are unmethylated. Although most methylated cytosines in *Ac* are in the symmetrical CpG and CpNpG arrangements, nonsymmetrical cytosine methylation is also common in the hypermethylated regions of *Ac*. These results suggest a model in which differential activation of transposon ends by hemimethylation controls the chromatid selectivity of transposition and the association with replication.

INTRODUCTION

The maize transposable elements of the *Activator/Dissociation* (*Ac/Ds*) family can transpose throughout development of the endosperm tissue. However, timing and frequency of transposition events are influenced by their copy number and genomic locations. Maize kernels carrying one, two, or three copies of an *Ac* element exhibit distinct variegation phenotypes (McClintock, 1951; Heinlein and Starlinger, 1991; Heinlein, 1995). This enables the fate of an excised *Ac* to be traced (McClintock, 1951; Greenblatt and Brink, 1962; Greenblatt, 1984). By using elegant genetic experiments, Greenblatt and Brink (1962) have demonstrated that *Ac* transposes frequently during mitosis. The appearance of "twin mutations," which are generated by excision of *Ac* from the *P-vv* locus ~80% of the time (Greenblatt, 1974), has led to the conclusion that *Ac* transposes during or shortly after the S phase of the cell cycle. Moreover, there is genetic and molecular evidence that after replication of *Ac*, only one of the two daughter elements is competent for transposition (Greenblatt and Brink, 1962; Greenblatt, 1984; Chen et al., 1987, 1992; Fedoroff, 1989). Although these results indicate a very strong correlation between chromosomal replication and transposition of *Ac* at the *P* locus, they do not imply a general requirement for DNA replication in the *Ac* transposition process.

The autonomous *Ac* consists of sequences required in *cis* and in *trans* for transposition. The *trans*-acting region contains the transcription unit (starting ~300 nucleotides from the 5' end and terminating 264 nucleotides from the 3' end), which encodes the gene for the 807–amino acid transposase (TPase) protein (Kunze et al., 1987). The TPase is the key protein and is essential for the transposition process. The *Ac* element, like the maize *Enhancer/Suppressor-Mutator* (*En/Spm*) element (McClintock, 1957), can cycle between an active and an inactive state (McClintock, 1951). The inactive state of *Ac* is correlated with methylation of several methylation-sensitive restriction enzyme sites in the 5' untranslated sequence and the putative promoter region and a lack of transcription (Schwartz and Dennis, 1986; Chomet et al., 1987; Kunze et al., 1988). Also, with maize *En/Spm* and *Mutator* transposons, a strong correlation of reversible inactivation, Tase–gene silencing, and hypermethylation of the promoter and downstream control regions is found (reviewed in Fedoroff and Chandler, 1994).

The *Ac* sequences that are required in *cis* for transposition of the element consist of the 11-bp terminal inverted repeats and of subterminal regions that are ~250 bp long at both ends (Coupland et al., 1988, 1989; Varagona and Wessler, 1990). The subterminal regions are very GC rich, and there is a strong bias toward the arrangement of these two bases in potential cytosine methylation sites, CpG or CpNpG (Gruenbaum et al., 1981). The 5' and 3' subterminal regions contain 26 and 24 CpG dinucleotides but only one GpC sequence each (Kunze et al., 1988). Many of these potential methylation sequences

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are part of TPase binding sites that are related to the sequence AAACGG and are found in multiple copies throughout the 250-bp subterminal regions at both ends of the element (Kunze and Starlinger, 1989; Feldmar and Kunze, 1991).

Binding of TPase to AAACGG sequences *in vitro* is dependent on the methylation state of these motifs. TPase binds to unmethylated double-stranded AAACGG and hemimethylated 5'-AAACGG-3'/5'-m^cmCGTTT-3' motifs, whereas sequences methylated on the other strand and on both strands (5'-AAA^mCGG-3'/5'-CCGTTT-3' and 5'-AAA^mCGG-3'/5'-m^cmCGTTT-3') are not recognized (Kunze and Starlinger, 1989; Kunze et al., 1991). These binding properties of the TPase suggest that, in addition to TPase gene silencing, methylation could have a second regulatory function during transposition, that is, the coupling of transposition to replication and providing the differential transpositional competence of the two daughter elements after replication. A precedent for such a mechanism is the bacterial transposon IS10, whose transposition is regulated by DNA adenine methylation of the IS10 TPase binding sites. After passage of the replication fork, one hemimethylated IS10 is more active than the other and at least 1000-fold more active than the fully methylated element (Roberts et al., 1985).

A prerequisite for a similar, methylation-dependent regulation of *Ac* transposition is that the TPase binding sites within *Ac* are methylated before replication. By using restriction analysis of genomic maize DNA with methylation-sensitive enzymes, it has been shown that the three HpaII sites within the *cis*-acting subterminal region at the 3' end of *Ac* in the *wx-m9::Ac* allele, where *Ac* is inserted into the tenth exon of the *Waxy* gene, are methylated (Schwartz and Dennis, 1986; Schwartz, 1989). The HpaII sites are close to the TPase binding sites, but the subterminal AAACGG TPase binding sites themselves are not accessible by any restriction enzymes. In contrast to the 3' end, no methylation could be detected by

restriction analysis at the 5' end of *Ac*. However, at this *Ac* end, with only one exception, all analyzed restriction sites are located within the 5' untranslated sequence of the *Ac* messenger RNA. This sequence is neither in *cis* nor in *trans* required for transposition.

In this study, we determined the methylation state of the TPase binding sites at both *Ac* ends in the *wx-m9::Ac* allele by genomic sequencing. We used the positive display protocol, which is based on the conversion of unmethylated cytosine residues to uracil by bisulfite treatment. This procedure allows the methylation state of individual molecules to be determined (Frommer et al., 1992; Clark et al., 1994). We found that at the 3' end of *Ac*, essentially all TPase binding sites are always fully methylated. In contrast, the 5' end is totally unmethylated in ~50% of the sequenced *Ac* molecules. The other half of the *Ac* molecules are partially methylated in a region that contains several putative TPase binding sites; however, the role of these binding sites in transposition has not yet been investigated. Based on these results, we present a model to explain the association of transposition with replication and the strand selectivity of transposition.

RESULTS

Experimental Design

Sodium bisulfite treatment of single-stranded DNA results in the conversion of cytosine to uracil, whereas 5-methylcytosine is not deaminated (Frommer et al., 1992). DNA from *wx-m9::Ac* kernels was denatured and reacted with sodium bisulfite (for details, see Methods). The modified *Ac* ends were polymer-

Table 1. PCR Primers for Amplification of Genomic *wx-m9::Ac* DNA and the pJAC Control Plasmid

Primer	Gene	5' Residue ^a	Sequence ^b	3' Residue ^a
a1	<i>Waxy</i>	2539	CGATACACGCATGTAGTAGCCG	2518
a2	<i>Ac</i>	283	CCTCAGTGGTTATGGATGGGAGTT	260
b1	pBR322	1046	GCAACGCGGCCATCCCGATG	1027
c1	<i>Ac</i>	4218	AGCCAAGAGCCCAAGACTTATCAC	4241
c2	<i>Waxy</i>	2371	GGACCGGAACATCCCGCTGGTGG	2393
Bb2	pBR322	1046	GGGGTACCGTAATGTGGGTATTTTATG	1027
Bd2	<i>Ac</i>	281	GGGGTACCTCAA/GTAATTATAAATAAAAATTTAAAAC	255
Bd1	<i>Waxy</i>	2537	GGGGTACCT/CGATATAT/CGTATGTAGTAGT/CT/CG	2518
Be1	<i>Waxy</i>	2491	GGGGTACCCAA/GAACA/GATCTA/GCACA/GTCCTCCACC	2467
Be3	<i>Ac</i>	278	GGGGTACCGTGGTTATGGATGGGAGTTGG	258
Be2	<i>Ac</i>	363	GGGGTACCATGT/CTGTTATTT/CGTGTGGTGT/CTGG	339
Bf1	<i>Ac</i>	4219	GGGGTACCGTTAAGAGTTTAAAGATTATTATTATG	4246
Bf2	<i>Waxy</i>	2414	GGGGTACCAAACAA/GAAAAACCCA/GACA/GTCATAAC	2439
Bg1	<i>Ac</i>	4246	GGGGTACCATACTACATTAATACTATATACTCC	4271
Bg2	<i>Waxy</i>	2414	GGGGTACCGAGT/CAGAAGGGTTT/CT/CGAT/CGTTATGG	2438

^a The numbers relate to the corresponding positions in pBR322, *Ac*, and the *Waxy* gene (Klößgen et al., 1986). The B primers carry a 10-nucleotide extension with a KpnI cloning site at their 5' ends.

^b Some B primers are degenerate (T/C or A/G) at positions that correspond to the C residues in CpG and CpNpG sequences.

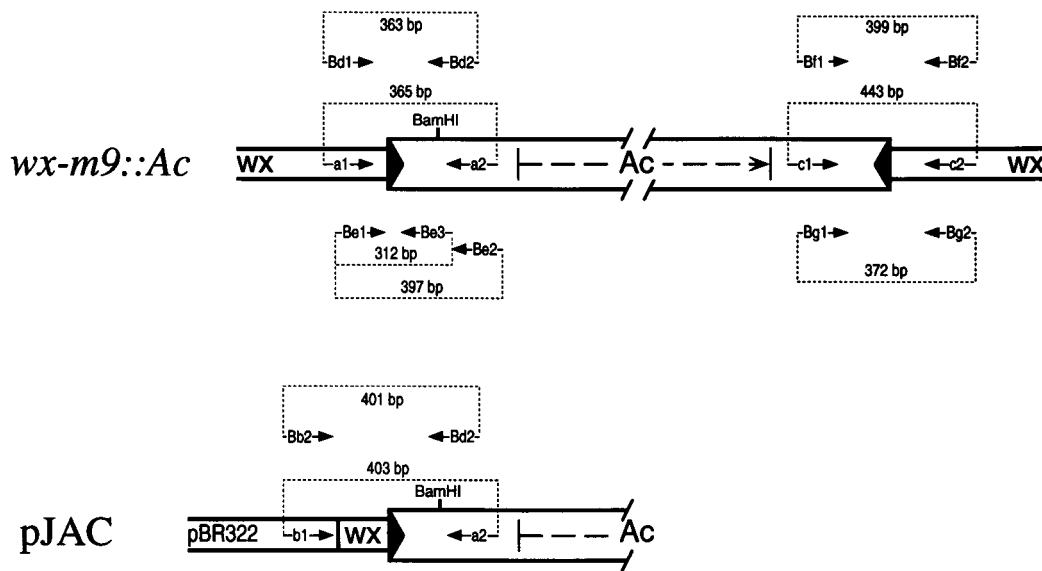


Figure 1. Strategy for PCR Amplification of Genomic *wx-m9::Ac* DNA and the pJAC Control Plasmid before and after Bisulfite Modification.

Primers drawn within the bars represent *Waxy* (*wx*), *Ac*, and pBR322 sequences that hybridized with unmodified DNA. Primers shown above amplified specifically bisulfite-modified DNA of the upper strand, and those drawn below amplified bisulfite-modified lower strand DNA. For the amplification of the *Ac* left end (5' end) lower strand, two successive rounds of amplification with a nested primer (Be3) were necessary, whereas in all other cases, a single amplification yielded sufficient amounts of PCR product for cloning and sequencing. The arrow between the vertical lines within *Ac* indicates the direction of *Ac* transcription.

ase chain reaction (PCR) amplified, and the products were cloned and sequenced. As occurs after sodium bisulfite treatment, the two DNA strands are no longer complementary at the former cytosine positions. Consequently, two different sets of PCR primers are required to amplify the upper and the lower strand of the reacted DNA, respectively (Table 1 and Figure 1).

The maize genome contains hundreds of sequences with homology to *Ac* (Geiser et al., 1982; Theres et al., 1987). Many of these are *Ds* elements and have the same terminal sequences as *Ac*. Therefore, to generate PCR products exclusively from the *Ac* element in the *wx-m9::Ac* allele (referred to as *Ac9*), we defined one primer in the ends of *Ac9* and the second one in the flanking single-copy *Waxy* gene (Figure 1).

The primers should preferably contain no potential methylation sites (CpG or CpNpG). Due to the high frequency of these sites in the *Waxy* gene and the *Ac* ends, this was not always possible; thus, at the respective positions, some primers are degenerate for G and A or T and C, respectively (Table 1).

The reaction conditions for the bisulfite treatment of DNA must be carefully adjusted to ensure complete conversion of all cytosines to uracil but to prevent too much degradation of the DNA (Clark et al., 1994). As a control, a single-copy gene equivalent of plasmid pJAC, grown in an *Escherichia coli* host that was either the wild type (*dcm*⁺) or deficient for the Dcm methylase (*dcm*⁻), was mixed with maize endosperm DNA before sodium bisulfite treatment (see Methods). pJAC contains the *Ac* element plus flanking sequences from the *wx-m7::Ac* allele, where the element is inserted into the *Waxy* gene in

a different place than in the *wx-m9::Ac* allele. The *Ac* termini can be amplified from this plasmid by using the same *Ac* internal primers as used for amplification of genomic *wx-m9::Ac* DNA and a second set of primers hybridizing with the pBR322 DNA (Figure 1). In all experiments, the completeness of the bisulfite reaction was verified by two criteria. First, before bisulfite treatment, PCR bands of the expected length were generated with the respective wild-type primer pairs for genomic *wx-m9::Ac* DNA and pJAC, whereas no products were obtained with the primer pairs specific for bisulfite-treated DNA (B primers). After bisulfite treatment, PCR bands were obtained with the B primers but not with wild-type primers (data not shown). Second, the amplified pJAC fragment contains two Dcm recognition sites (C^mCATGG), one in the *Waxy* sequence flanking the *Ac* 5' end and the other overlapping with the *Ac* 5' terminal inverted repeat. If pJAC/*dcm*⁻ DNA was mixed with the genomic DNA, all cytosines of the amplified fragment were converted to uracil (Figure 2). If pJAC/*dcm*⁺ DNA was included, as expected all but the two C residues in the Dcm sites were converted (Figure 2).

Due to the amplification of the modified genomic DNA, it is possible that multiple clones with identical C methylation patterns are derived from the same genomic *Ac* molecule. Therefore, to ensure that we obtain sequences from different genomic *Ac* molecules, the strategy outlined in Figure 3 was used. We started with three independent *wx-m9::Ac* endosperm DNA preparations (D1, D2, and D3). Several aliquots of each DNA preparation were individually treated with sodium bisulfite

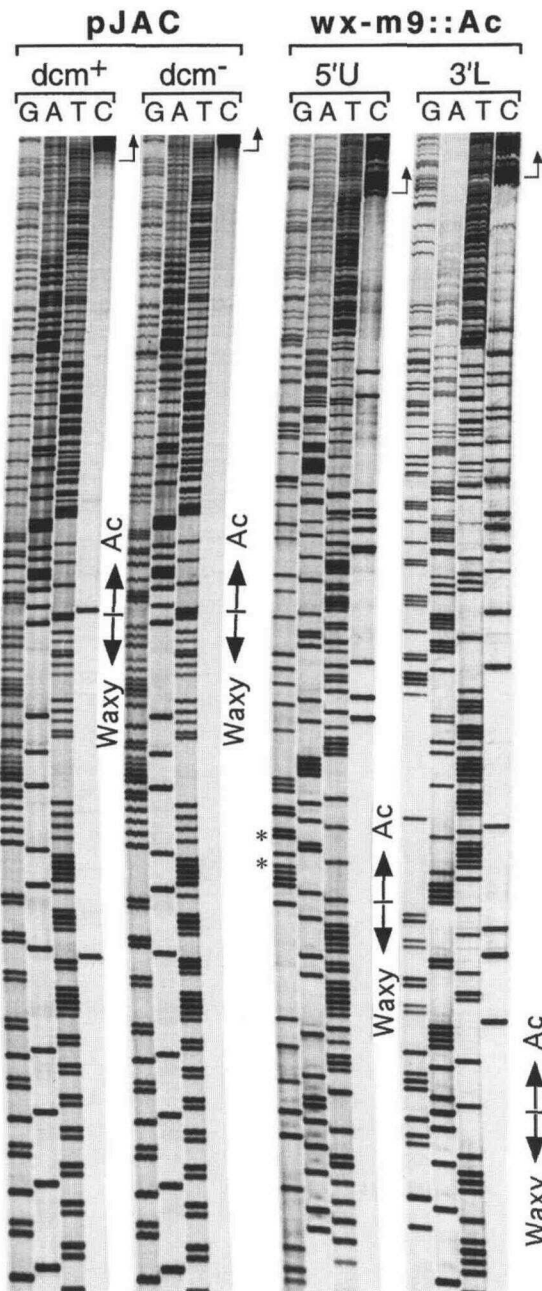


Figure 2. Ac Sequences Obtained from Bisulfite-Modified Genomic Maize *wx-m9::Ac* DNA and the pJAC Control Plasmid.

Shown are representative methylation patterns of the upper strand DNA of the control plasmid pJAC, grown in a *dcm*⁺ host (*dcm*⁺) or a *dcm*⁻ host (*dcm*⁻), of a partially methylated *Ac9* 5' end upper strand DNA (5'U) and of a methylated *Ac9* 3' end lower strand DNA (3'L). To the right of lanes C, the border between *Ac* and the flanking *Waxy* sequence is indicated. Near the top of the gel, arrows mark the break point between cloned insert and vector DNA. The two asterisks beside the *wx-m9::Ac* 5'U sequence denote two A → G misincorporations by Taq polymerase during PCR.

(B1 to B14), and between one and four PCRs were performed on each aliquot (designated P1 to P4 in Figure 3). Subsequently, between two and 38 cloned product molecules from each PCR were sequenced (termed C1 to C38). If multiple sequences with identical methylation patterns were obtained from the same PCR, they were listed only once in Figures 4 and 5 because they could possibly be derived from the same genomic *Ac* molecule.

To reduce the risk of misincorporations by the *Taq* DNA polymerase, we optimized the reaction conditions to generate in only one round of amplification sufficient PCR product for cloning. This was successful for both *Ac* 3' end DNA strands and the 5' end upper strand. However, amplification of the 5' end lower strand proved to be more difficult and required a second round of amplification, using a nested primer (Figure 1).

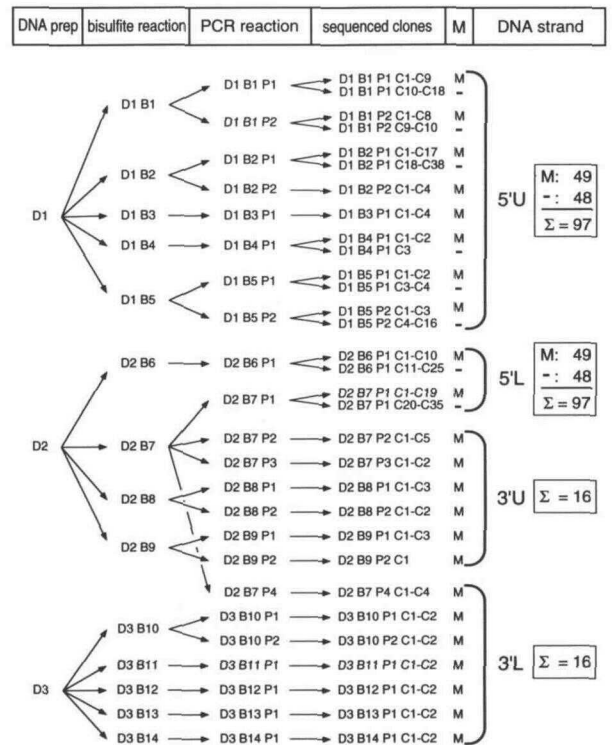
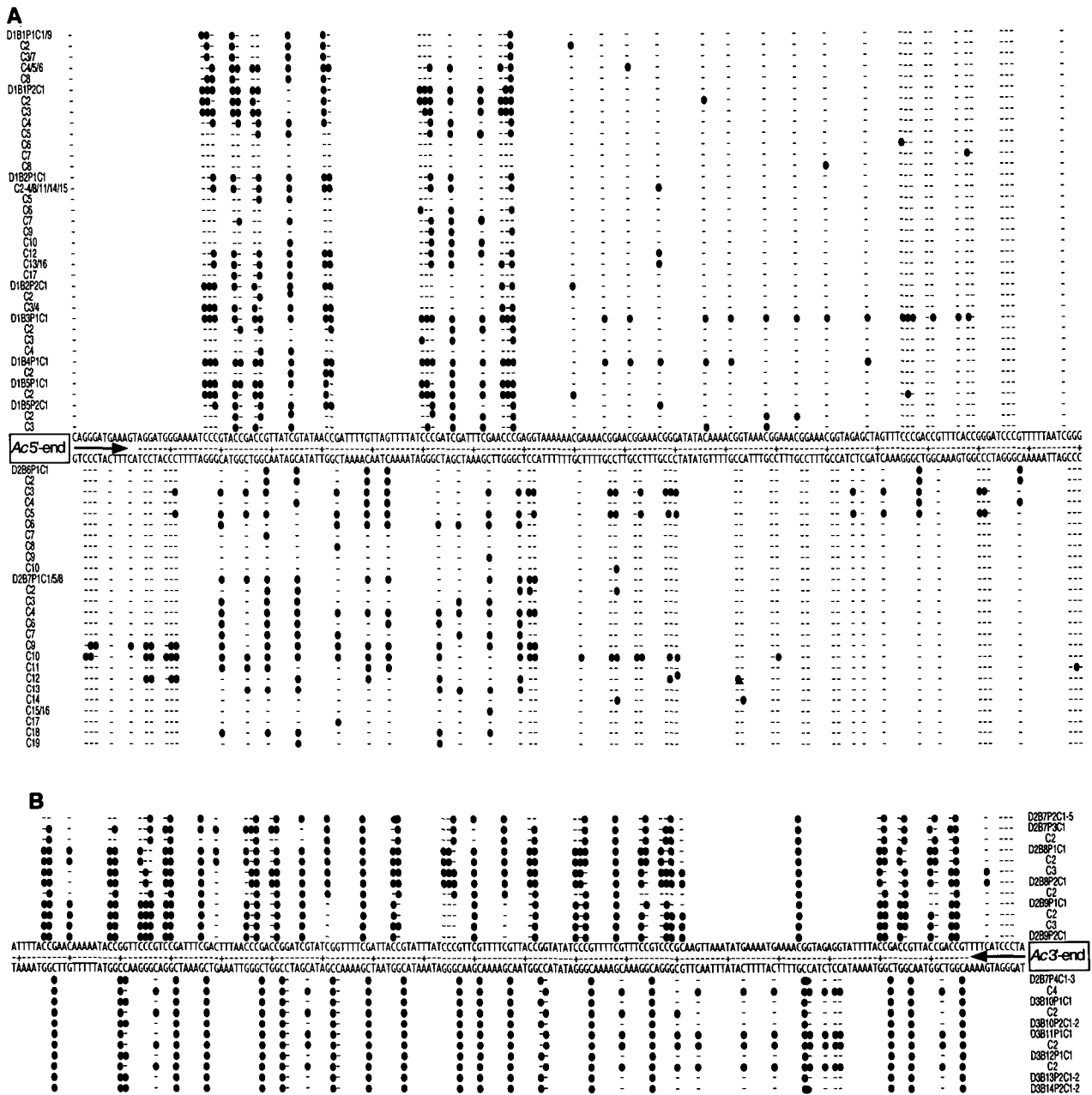


Figure 3. Experimental Strategy to Determine the Methylation Patterns of Independent *Ac* Molecules.

On each of three genomic *wx-m9::Ac* DNA preparations, D1, D2, and D3 (under DNA prep), several independent modification reactions were performed (B1 to B14; under bisulfite reaction). One to four PCR amplifications were run on each modified DNA sample (P1 to P4; under PCR reaction), and subsequently, the PCR products were purified and cloned. Between two and 38 clones from each PCR were sequenced (C1 to C38; under sequenced clones). Under DNA strand, the numbers of sequenced clones are provided. M, clones with methylated cytosines; (-), unmethylated clones; Σ, sum of sequenced clones; 5'U, *Ac* 5' end upper strand clones; 5'L, *Ac* 5' end lower strand clones; 3'U, *Ac* 3' end upper strand clones; 3'L, *Ac* 3' end lower strand clones.



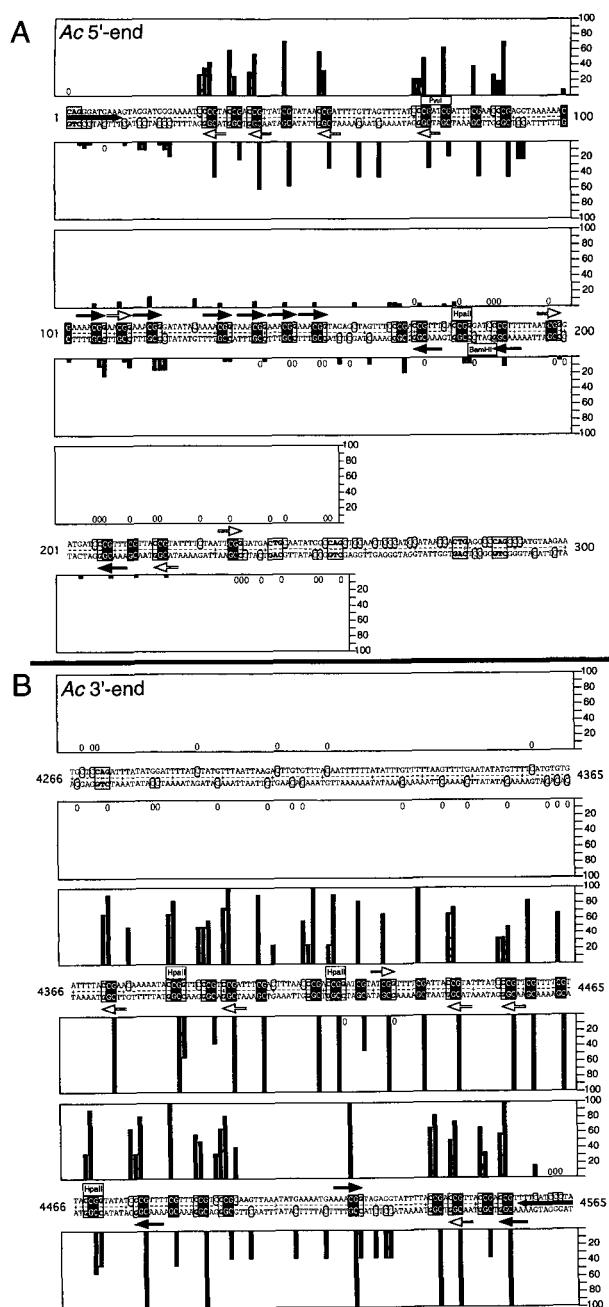


Figure 5. Cytosine Methylation Patterns of the 5' and 3' Ends of Ac9.

For each cytosine, the percentage of methylated residues is shown as a closed bar above or below the sequence.

(A) At the Ac 5' end, the average degree of methylation of only the partially methylated molecules is shown; the 50% unmethylated molecules are not included.

(B) The average degree of C methylation at the Ac 3' end is shown. A 0 instead of a bar indicates that the respective cytosine was not methylated in any of the analyzed molecules. CpG sequences are highlighted by white letters in black boxes, and CpNpG sequences are highlighted by boldface letters in both DNA strands. Nonsymmetrical cytosine

Methylation Pattern of the 5' End of Ac9

We determined the methylation pattern of the 5' terminal 255 residues of the Ac9 element in mature *wx-m9::Ac* kernels. In total, 157 cloned PCR products, representing 97 upper and 60 lower strand DNA molecules, were sequenced (Figure 3).

Interestingly, 48 of the 97 upper strand clones were totally unmethylated. However, the other 49 clones were derived from partially methylated Ac molecules that carried between one and 31 5-methylcytosine residues. A representative sequence of a partially methylated Ac 5' end is shown in Figure 2. Twelve clones had methylation patterns identical with other clones derived from the same PCR and thus were possibly derived from the same genomic Ac molecule. This assumption is supported by the fact that all the remaining 37 independent molecules differed in their methylation patterns. The methylation maps of these molecules are shown in Figure 4A.

Very similar results were obtained from the lower strand. Here, 31 of 60 sequenced clones were unmethylated. The remaining 29 clones were partially methylated. Of these, 26 had different and unique methylation patterns containing between one and 27 5-methylcytosines (Figure 4A).

If we assume that equal fractions of clones are derived from individual, methylated, and unmethylated genomic DNA molecules, respectively, the Ac elements in 50% of the *wx-m9::Ac* endosperm cells are totally devoid of methylation throughout their 5' ends, including the TPase binding sites. Figure 5A shows for each C residue at the Ac 5' end the percentage of methylated residues in the partially methylated molecules (the unmethylated molecules were not included in this calculation).

Between individual, partially methylated molecules, the distribution of 5-methylcytosine residues differs considerably. Yet, in the majority of Ac9 5' ends, methylation is restricted to the region between residues 27 and 92. Remarkably, with only five exceptions (D1-B3-P1-C1, D1-B4-P1-C1, D2-B6-P1-C3, D2-B6-P1-C4, and D2-B7-P1-C10), almost no methylation is found within the more internal Ac region. This region contains clusters of TPase binding motifs between positions 103 to 152, which were shown to be important for transposition (Coupland et al., 1989; Kunze and Starlinger, 1989; Chatterjee and Starlinger, 1995). The only HpaII site in the analyzed region (at position 179) and the overlapping BamHI site are unmethylated in 97% of molecules, which is consistent with the results of earlier DNA gel blot analyses (Schwartz and Dennis, 1986; Schwartz, 1989). However, in our experiments, the PvuII site at Ac position 76 was methylated in only 28% of molecules, whereas Schwartz (1989) estimated this site to be fully methylated in Ac9 as determined by DNA gel blot analysis.

residues are individually boxed on each DNA strand. Putative AAACGG- and A/TA/TCGG-TPase binding sites are highlighted by closed and open arrows, respectively, above or below the Ac sequence. The 11-bp terminal inverted repeats of Ac are indicated by the arrows between the DNA strands.

Methylation Pattern of the 3' End of Ac9

To determine the methylation pattern at the 3' end of Ac, we sequenced the ~300 terminal residues in 16 upper strand and 16 lower strand clones. Twelve upper strand and 11 lower strand clones displayed different methylation patterns and thus are derived from individual genomic Ac9 elements. The C methylation maps of these molecules are compiled in Figure 4B.

The methylation pattern at the 3' end of Ac differs markedly from that at the 5' end. In the most internal region between residues 4246 and 4371, which is devoid of any TPase binding sites, all 274 cytosines were unmethylated. However, in sharp contrast to the 5' end, all 3' end molecules are heavily methylated throughout the terminal 194 Ac residues, except for the terminal inverted repeat. This region contains all 3' terminal TPase binding sites. The average degree of methylation at each cytosine residue in the Ac 3' end is shown in Figure 5B. At some positions, the cytosines are methylated in 100% of molecules. The three HpaII sites in the 3' end of Ac are methylated in 91 and 97% of the sequenced molecules, respectively. Methylation of these sites in the active Ac9 element has also been detected by DNA gel blot analysis (Schwartz and Dennis, 1986).

Methylation Pattern of Flanking Waxy Sequences

In the *wx-m9::Ac* allele, the Ac element is inserted 37 bp upstream of intron 10 into a very GC-rich region of exon 10 (Klößgen et al., 1986). The cloned PCR products contained up to 60 bases of Waxy DNA flanking Ac at either end. At both ends, the flanking Waxy DNA is 99% unmethylated (data not shown), which is consistent with the results of Schwartz and Dennis (1986), who have shown that the HpaII sites located 49 bp beside the 5' end and 92 bp beside the 3' end of Ac9 are unmethylated.

Methylation of Nonsymmetrical Cytosine Residues

In plants, the symmetrical CpG and CpNpG sequences are considered to be the exclusive methylation sites (Gruenbaum et al., 1981). In addition to these canonical methylation sequences, both ends of Ac contain several nonsymmetrical cytosine residues. Remarkably, a significant fraction of these cytosines is methylated in Ac, and at some positions they are methylated as frequently as cytosines that are embedded in a CpG or CpNpG context. We could not determine a correlation between methylation frequency and distance to the closest 3' located G residue. Interestingly, methylation of nonsymmetrical cytosines is mostly restricted to the same regions at both Ac ends that are methylated at symmetrical sequences. In only four cases, twice at the 5' end and twice at the 3' end, methylation of nonsymmetrical cytosine residues in the terminal inverted repeats was observed. The two respective 5' end

molecules D2-B7-P1-C9 and D2-B7-P1-C10 had an overall exceptional methylation pattern, however.

DISCUSSION

Ac Is a Methylation Island with Distinct Local Methylation Patterns

By using genomic sequencing, we investigated the methylation pattern of the ~250-bp subterminal regions of the active Ac element in the *wx-m9::Ac* allele that are required in *cis* for transposition. We found that the pattern and degree of methylation are strikingly different in the two ends. In half of the molecules analyzed, the Ac 5' end is absolutely unmethylated. In the other partially methylated molecules, cytosine methylation is mostly restricted to Ac positions 27 to 92. Because the DNA preparations contained embryonic and endosperm tissue, it is possible that the methylation patterns of the Ac 5' ends differ in these two tissues. In contrast, 100% of the Ac molecules are hypermethylated throughout the 194 3' terminal Ac residues, except for the terminal inverted repeat. The Waxy sequences flanking Ac are always unmethylated. The observed methylation pattern suggests that the Ac element may be a methylation island that contains certain regions whose methylation (or demethylation) is governed by signals within the Ac sequence. These signals seem to act specifically on Ac, because the hypermethylation of the Ac 3' end remains restricted to Ac and does not extend into the flanking CpG-rich Waxy DNA.

Recently, by using a different genomic sequencing protocol with transgenic tobacco, the 5' end of Ac was found to be unmethylated between residues 73 to 200 (Ott et al., 1992). Possibly, the partial methylation between residues 73 and 92 remained undetected due to the lower sensitivity in these experiments. Alternatively, the methylation pattern of Ac in tobacco differs from that in maize.

Both ends of Ac have a very similar sequence composition, that is, they contain 26 and 24 CpG motifs versus only one GpC sequence. Thus, one would ask what mechanism prevents the 5' end from being methylated to a degree similar to that of the 3' end. One possibility would be that steric hindrance by protein factors excludes the methyltransferases from the Ac 5' end, as has been suggested for CpG islands in mammals (Bird, 1986). Because the 5' end contains the Ac promoter in addition to the TPase binding sites, it is conceivable that transcription factors bound to this region interfere with methylation. Interestingly, the architecture of the Ac 5' end is somewhat similar to that of the *Eni/Spm* 5' end, in which the promoter and binding sites of the element-encoded TnpA protein overlap. Recently, it was shown that TnpA can activate the inactive, methylated *Eni/Spm* promoter and cause its demethylation (Schläppi et al., 1994). A similar mechanism seems to be common in mammalian genes. For example, peripherally located

GC boxes, the binding sites for the Sp1 transcription factor, protect the CpG islands in the mouse and hamster *aprt* gene promoters from de novo methylation (Brandeis et al., 1994; Macleod et al., 1994). Another model suggests that CpG islands in mammals are subject to de novo methylation but that the modification is subsequently removed by an island-specific demethylating activity (Frank et al., 1991).

A Model for the Strand Selectivity of *Ac* Transposition

The analysis of variegation patterns generated by excision of *Ac* from the *P* locus has led to the conclusion that *Ac* transposes during the mitotic cycle. In most cases, the element excises after replication of the donor locus exclusively from one of the two daughter chromatids (strand selectivity) and reinserts into both unreplicated and replicated target sites (Greenblatt and Brink, 1962; Greenblatt, 1984; Chen et al., 1987, 1992). This implies that the enzyme required for excision of *Ac* is not active on the unreplicated transposon and differentiates between the two daughter elements after replication (chromatid selectivity). It has been suggested that hemimethylation of transposon sequences after replication could provide a mechanism for the cell cycle-dependent regulation of *Ac* transposition (Schwartz and Dennis, 1986) and the distinction of the daughter chromatids (Fedoroff, 1989). The *E. coli* mobile element IS10 provides a precedent for this mechanism of transposition regulation. Hemimethylation of its TPase binding sites activates IS10 for transposition and preferentially one hemimethylated form of IS10 transposes (Roberts et al., 1985).

The *Ac* transposase specifically binds *in vitro* to AAACGG and similar sequence motifs that occur in multiple copies in both ends of *Ac*. Interestingly, the protein binds to DNA that is unmethylated or hemimethylated on one strand (5'-AAACGG-3'/5'-^mC^mCGTTT-3'), whereas cytosine methylation on the other strand almost completely inhibits TPase binding (5'-AAA^m-CGG-3'/5'-CCGTTT-3' and 5'-AAA^mCGG-3'/5'-^mC^mCGTTT-3') (Kunze and Starlinger, 1989; Kunze et al., 1991). These binding properties suggest indeed that methylation of TPase binding sites is of functional importance for transposition. To date, methylation of *Ac* sequences has been investigated by using methylation-sensitive restriction enzymes. Hypermethylation of several restriction sites within *Ac* has been correlated with an inactive state of *Ac* and the lack of TPase gene expression (Schwartz and Dennis, 1986; Chomet et al., 1987; Kunze et al., 1988; Schwartz, 1989; Brettell and Dennis, 1991; Brutnell and Dellaporta, 1994). However, because *Ac* in the inactive state (*Ds-cy*) can be mobilized by an active *Ac* element (Schwartz and Dennis, 1986), methylation of *Ac* and the lack of TPase expression do not inhibit the activity of the sub-terminal TPase binding sites and the terminal inverted repeats of *Ac*.

The TPase binding sites in the *Ac* 5' end are distributed between residues 28 and 235, and at least four of them are located in the partially methylated region (Figure 5A). However, most TPase binding sites are located in the unmethylated region

between *Ac* residues 100 and 235, including a prominent cluster extending from position 103 to 152. This region is essential for transposition because a deletion retaining 166 5' terminal *Ac* residues decreases the transposition frequency to 19% and a mutant retaining only 116 bp is virtually immobilized (0.4% transposition frequency) (Coupland et al., 1989). The significance of the TPase binding sites in the *Ac* 5' end has also been demonstrated *in vivo* by showing that their substitution in the unmethylated region between residues 97 and 243 with unrelated sequences of identical length abolishes transposition (Chatterjee and Starlinger, 1995).

The 3' end of *Ac* exhibits an entirely different methylation pattern from the 5' end. Between residues 4266 and 4371, the element is unmethylated and devoid of CpG dinucleotides and TPase binding sites. This region is dispensable for transposition (Coupland et al., 1989; Varagona and Wessler, 1990). In contrast, the 3' terminal 194 bases are heavily C methylated at all 24 CpG sequences. All of the 3' TPase binding sites are located in this region and are methylated in 80 to 100% of the molecules (Figure 5B). This finding confirms the results of Schwartz and Dennis (1986), who have used DNA gel blot analysis to show that the three HpaII sites in the 3' end of *Ac*9 are modified. The 3' terminal 194 *Ac* residues are essential for transposition (Coupland et al., 1989), and even minor sequence alterations may severely disturb the transposition activity. An insertion of four bases between residues 4387 and 4392 causes a 10-fold decrease in transposition activity in tobacco (Keller et al., 1993), and a single C→G substitution in the outermost AAACGG motif at position 4553 results in a fivefold lower transposition frequency in petunia protoplasts (Chatterjee and Starlinger, 1995).

Based on the differential binding affinities of TPase to hemimethylated DNA *in vitro* and the *in vivo* methylation pattern of *Ac* reported in this study, we suggest a model to explain the strand selectivity of *Ac* transposition (Figure 6). Before replication, TPase presumably binds to unmethylated 5' ends (Figure 6A) and, in a different mode, to *Ac* elements with partially methylated 5' ends (Figure 6B), whereas the fully methylated 3' ends are not recognized. Immediately after replication, previously methylated recognition sites become hemimethylated. Because these sites occur in both orientations in either end of *Ac*, both ends of each pair of daughter elements can now be bound by TPase. However, the arrangement of TPase proteins differ at the 3' ends of the daughter transposons and also at the 5' ends of those *Ac* elements that were partially methylated before replication (Figure 6B). We suggest that the TPase arrangement on the 3' end of one daughter chromatid renders the element competent for transposition, whereas that on the other one does not promote transposition.

At the 3' end, seven of nine putative TPase binding sites are in the same orientation (Figure 5B). Therefore, one of the two differentially hemimethylated 3' ends binds more TPase than will the other. One would then ask whether TPase binding or the lack of binding to certain sites activates the 3' end for transposition. *Ac* and *Ds* elements that are transfected as naked DNA into petunia protoplasts readily excise from the

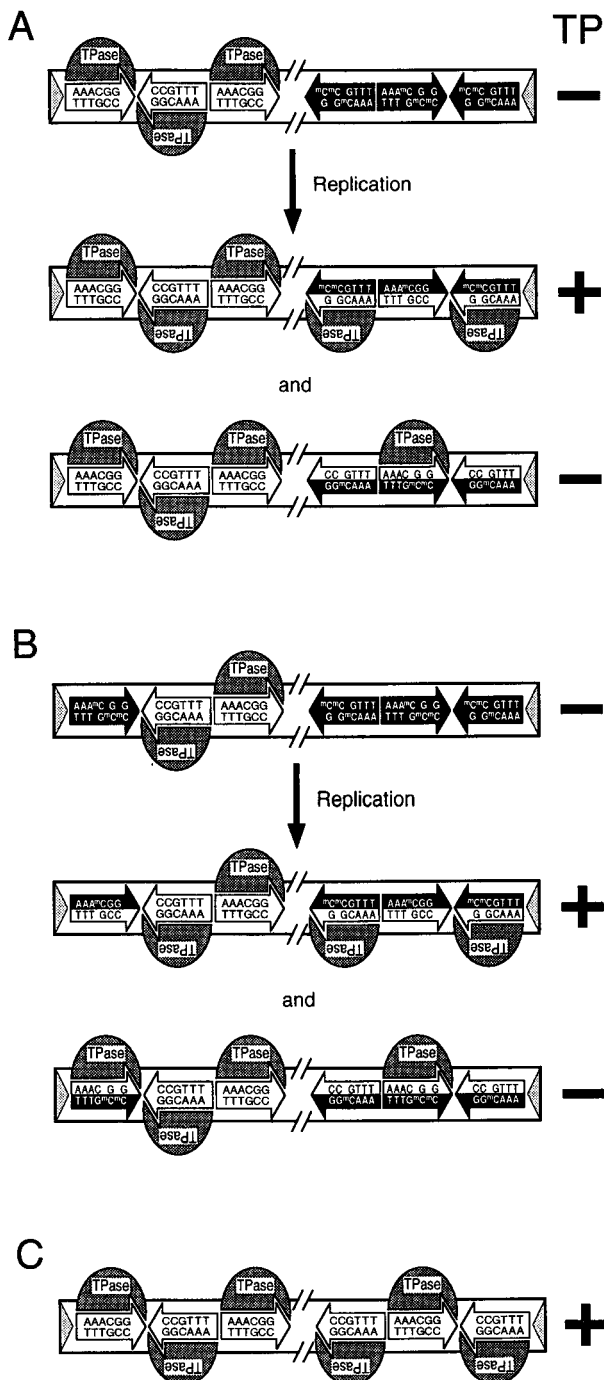


Figure 6. Model for the Chromatid Selectivity of Ac Transposition.

Three AAACGG-TPase binding sites at each end of Ac are shown schematically. Unmethylated DNA strands are shown as black letters in open arrows, and methylated DNA strands are shown as white letters in closed arrows. TPase molecules are symbolized by shaded half spheres. TPase binds *in vitro* to unmethylated double-stranded AAACGG sequences and with increased affinity to 5'-AAACGG-3'/5'-m^CmCGTTT-3' motifs, whereas cytosine methylation on the other

strand (Houba-Hérin et al., 1990). Because bacterial plasmids are not methylated at CpG sequences, it must be assumed that the transposon that is unmethylated at both ends excises from the plasmid DNA (Figure 6C). According to our model, TPase could bind to all recognition sites of unmethylated 3' ends. Hence, it seems unlikely that TPase binding to certain sites inhibits transposition. Rather, TPase binding to particular sites in the Ac 3' end activates the element for transposition.

In half of the Ac elements, the 5' ends are unmethylated. Consequently, after replication, the occupation of the two daughter elements with TPase should be identical and the 5' ends of both daughter elements should be competent for transposition. In this situation, it is theoretically possible that the 5' end on one daughter transposon engages erroneously in a transposition complex with the transposition competent 3' end on the other chromatid. However, during extensive genetic and molecular analysis of Ac transposition, no such events have been detected (Greenblatt and Brink, 1962; Greenblatt, 1968, 1974, 1984; Chen et al., 1987, 1992). Possibly, a mechanism exists that prevents an interchromatid reaction at simple (state II) *Ds* or Ac elements. This could be achieved if both ends of the element were marked differentially after replication.

Recently, evidence for a differential activation of both Ac ends was obtained by the analysis of chromosome breakage events induced by aberrant transposition in tobacco. The aberrant intrachromatid transposition or SIN model (Döring et al., 1984, 1989; English et al., 1993) proposes that two directly oriented *Ds* ends on the same chromatid undergo aberrant transposition. In contrast, the aberrant interchromatid transposition or SIS model suggests that, after replication, a 5' end on one and a 3' end on the other daughter chromatid combine to form an aberrant transposition complex (Greenblatt, 1984; Fedoroff et al., 1989; English et al., 1993, 1995; Weil and Wessler, 1993). English et al. (1995) have consistently shown that the SIS mechanism predominates in tobacco. Unlike the SIN model, the SIS model requires that both ends of the transposon are asymmetrically activated for transposition. On the other hand, the fact that unmethylated Ac elements can excise from plasmid DNA in petunia cells (Houba-Hérin et al., 1990; Becker et al., 1992) indicates that hemimethylation is not essential for transposition. Because the TPase has a higher affinity for hemimethylated DNA, we hypothesize that in the presence of unmethylated and hemimethylated transposon ends, the TPase has a strong preference to establish a productive transposition complex involving the hemimethylated ends, whereas in the absence of

strand (5'-AAA^mCGG-3'/5'-CCGTTT-3' and 5'-AAA^mCGG-3'/5'-m^CmCGTTT-3') inhibits TPase binding (Kunze and Starlinger, 1989; Kunze et al., 1991). The presumed presence (+) or lack (-) of transposition activity is indicated to the right of each element (TP).

(A) Model for Ac₉ elements with unmethylated 5' ends.

(B) Model for Ac₉ elements with partially methylated 5' ends.

(C) Model for unmethylated Ac elements.

hemimethylation the unmethylated transposon ends may be accepted.

Similar to *Ac*, several other plant transposable elements have highly structured subterminal regions containing repetitive sequence motifs. Because some of these partially resemble the *Ac* TPase binding motifs, including the methylation sites (Table 2), it is intriguing to speculate that these repeats may be the targets of transposition enzymes whose DNA binding properties are influenced by the methylation state of their binding sites. Indeed, the *En/Spm* element transposes frequently during replication (Dash and Peterson, 1994), and it was shown that binding of the *En/Spm*-encoded TnpA protein to the hemimethylated target site is reduced by a factor of 5 to 10. Holomethylation results in an even more severe reduction (Gierl et al., 1988).

Methylation of Nonsymmetrical Cytosine Residues

In plants, the symmetrical CpG and CpNpG sequences are the canonical methylation sites, and it is generally supposed that C residues in other sequence contexts (nonsymmetrical C residues) are not methylated (Gruenbaum et al., 1981). Meanwhile, there is growing evidence for nonsymmetrical C methylation in plants. In the inactive, hypermethylated state, maize transposons *Ac* and *Mutator* are not cleaved by the 5-methylcytosine-sensitive enzymes BamHI and SstI, respectively, although their sensitive C residues are not in the CpG or CpNpG context (Schwartz, 1989; Martienssen and Baron, 1994). Recently, the methylation pattern of the maize *A1* gene in transgenic petunia plants has been investigated. In a variant with a silenced, hypermethylated *A1* transgene, >90% of Cp(Np)G sequences but also 32% of the nonsymmetrical cytosines were methylated (Meyer et al., 1994). Significant methylation of nonsymmetrical cytosines was also observed in an endogenously duplicated, partially silenced copy of the maize *R* gene (Ronchi et al., 1995).

Table 2. Subterminal Repeats of Plant Transposable Elements with Putative Asymmetrical Methylation Sites

Element ^a	Subterminal Repeat	Number ^b
<i>Ac</i>	AAACGG	12
<i>Tpn1</i>	GACAACGGTT	55
<i>Tgm1</i>	ACATCGG	>12
<i>Tpc1</i>	TTCGG	>4
<i>En/Spm</i>	TAAGAGTGTCGG	24
<i>Tat1</i>	T/ACGGT/A	7
<i>Tam3</i>	T/ACGGC	14

^a Maize *Ac* (Kunze and Starlinger, 1989); Japanese morning glory *Tpn1* (Hoshino et al., 1995); soybean *Tgm1* (Rhodes and Vodkin, 1988); parsley *Tpc1* (Herrmann et al., 1988); maize *En/Spm* (Gierl et al., 1988); Arabidopsis *Tat1* (Peleman et al., 1991); snapdragon *Tam3* (Hehl et al., 1991).

^b Number of repeats at both ends of the transposon.

We have found that in *Ac*, methylation of nonsymmetrical cytosines occurs but is restricted to regions with a high density of methylated CpG and CpNpG sequences. This finding could indicate that the methyltransferase, which establishes symmetrical methylation patterns, is also active on nonsymmetrical cytosines in the vicinity of hemimethylated symmetrical sites. Alternatively, the maintenance methylase could recruit and direct a second, different methyltransferase to nonsymmetrical cytosine residues.

METHODS

Plant Material and DNA Preparation

DNA was extracted from mature maize kernels that were either homozygous for the *wx-m9::Ac* allele, where *Activator (Ac)* is inserted into the tenth exon of the *Waxy* gene (preparations D2 and D3), or originated from the cross *wx-m9::Ac* × *wx* (preparation D1), thus being heterozygous for *Ac* in the embryo and carrying two doses of *Ac* in the endosperm (*wx-m9::Ac/wx-m9::Ac/wx*). The kernels were ground in liquid nitrogen to a fine powder, and the DNA was extracted by CTAB precipitation, according to Murray and Thompson (1980).

Bacterial Strains and Plasmids

Escherichia coli strains used for the propagation of control plasmids for the bisulfite reaction were DH5α (*dcm*⁺) and RP41-82 (*dcm*⁻) (a gift of Wolfgang Gläsner, Georg-August University, Göttingen, Germany). The control plasmid was pJAC. pJAC contains the complete *Ac* element from the *wx-m7::Ac* locus plus 72- and 30-bp *Waxy* sequences flanking *Ac* at the 5' and 3' end, respectively, in pBR322 (Kunze et al., 1987). Polymerase chain reaction (PCR) products were cloned into pUC19 or pT7T3 (Pharmacia).

Sodium Bisulfite Modification

Genomic DNA and pJAC control plasmid were digested with EcoRI before bisulfite treatment. Bisulfite modification was performed as described by Frommer et al. (1992) and Clark et al. (1994). Briefly, 5 μg of genomic DNA was mixed with 60 pg of pJAC (approximately one single-copy gene equivalent), and the volume was adjusted to 100 μL with water. The DNA was denatured for 10 min at 95°C, quickly cooled to 0°C, mixed with 1.2 mL of 3.1 M sodium bisulfite (Sigma), 0.5 mM hydroquinone, pH 5.5, and incubated for 15 to 20 hr at 50°C under mineral oil. Subsequently, the DNA solution was diluted 1:5 with water and precipitated with 0.7 volumes of isopropanol. After washing the pellet with 70% ethanol, the DNA was resuspended in 200 μL of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, mixed with 86 μL of 1 M NaOH, and incubated for 30 min at 37°C (Wang et al., 1980). Subsequently, the DNA was precipitated once more by adding 0.1 volumes of 3 M sodium acetate and 2 volumes of ethanol, washed with 70% ethanol, and resuspended in 100 μL of 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

PCR Amplification, Cloning, and Sequencing

Bisulfite-treated DNA was amplified in a total volume of 100 μL containing 5 to 10 μL of the DNA solution, 100 pmol of each primer (Figure

1), 0.2 mM deoxynucleotide triphosphates, 50 mM KCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, and 1 unit of Taq polymerase (Promega). Amplification cycles were for 3 min at 95°C, followed by 5 cycles (1 min at 95°C, 1 min at 48°C, and 1 min at 72°C), 35 cycles (1 min at 95°C, 1 min at 50°C, and 2 min at 72°C), and a final 20 min at 72°C. Reamplification of the 5' end lower strand was performed under the same conditions, except that the annealing temperature was always 50°C. The PCR products were digested with KpnI and size fractionated on a 1% agarose gel. The band was excised, eluted using a GlassMAX kit (Life Technologies, Gaithersburg MD), and subsequently ligated into KpnI-linearized pUC19 or pT7T3. Individual clones were either manually sequenced with T7 DNA polymerase (Pharmacia) or automatically sequenced on an ABI 373A sequencer, using a dideoxy terminator kit (Perkin-Elmer).

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REFERENCES

- Becker, D., Lütticke, R., Li, M.-g., and Starlinger, P. (1992). Control of excision frequency of maize transposable element *Ds* in *Petunia* protoplasts. *Proc. Natl. Acad. Sci. USA* **89**, 5552–5556.
- Bird, A.P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213.
- Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994). Sp1 elements protect a CpG island from de novo methylation. *Nature* **371**, 435–438.
- Brettell, R.I.S., and Dennis, E.S. (1991). Reactivation of a silent *Ac* following tissue culture is associated with heritable alterations in its methylation pattern. *Mol. Gen. Genet.* **229**, 365–372.
- Brutnell, T.P., and Dellaporta, S.L. (1994). Somatic inactivation and reactivation of *Ac* associated with changes in cytosine methylation and transposase expression. *Genetics* **138**, 213–225.
- Chatterjee, S., and Starlinger, P. (1995). The role of subterminal sites of transposable element *Ds* of *Zea mays* in excision. *Mol. Gen. Genet.* **249**, 281–288.
- Chen, J., Greenblatt, I.M., and Dellaporta, S.L. (1987). Transposition of *Ac* from the *P* locus of maize into unreplicated chromosomal sites. *Genetics* **117**, 109–116.
- Chen, J., Greenblatt, I.M., and Dellaporta, S.L. (1992). Molecular analysis of *Ac* transposition and DNA replication. *Genetics* **130**, 665–676.
- Chomet, P.S., Wessler, S., and Dellaporta, S.L. (1987). Inactivation of the maize transposable element *Activator (Ac)* is associated with its DNA modification. *EMBO J.* **6**, 295–302.
- Clark, S.J., Harrison, J., Paul, C.L., and Frommer, M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990–2997.
- Coupland, G., Baker, B., Schell, J., and Starlinger, P. (1988). Characterization of the maize transposable element *Ac* by internal deletions. *EMBO J.* **7**, 3653–3659.
- Coupland, G., Plum, C., Chatterjee, S., Post, A., and Starlinger, P. (1989). Sequences near the termini are required for transposition of the maize transposon *Ac* in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* **86**, 9385–9388.
- Dash, S., and Peterson, P.A. (1994). Frequent loss of the *En* transposable element after excision and its relation to chromosome replication in maize (*Zea mays* L.). *Genetics* **136**, 653–671.
- Döring, H.-P., Freeling, M., Hake, S., Johns, M.A., Kunze, R., Merkelbach, A., Salamini, F., and Starlinger, P. (1984). A *Ds* mutation of the *Adh1* gene in *Zea mays* L. *Mol. Gen. Genet.* **193**, 199–204.
- Döring, H.-P., Nelsen-Salz, B., Garber, R., and Tillmann, E. (1989). Double *Ds* elements are involved in specific chromosome breakage. *Mol. Gen. Genet.* **219**, 299–305.
- English, J., Harrison, K., and Jones, J.D.G. (1993). A genetic analysis of DNA sequence requirements for *Dissociation* state I activity in tobacco. *Plant Cell* **5**, 501–514.
- English, J.J., Harrison, K., and Jones, J.D.G. (1995). Aberrant transpositions of maize double *Ds*-like elements usually involve *Ds* ends on sister chromatids. *Plant Cell* **7**, 1235–1247.
- Fedoroff, N.V. (1989). Maize transposable elements. In *Mobile DNA*, D.E. Berg and M.M. Howe, eds (Washington, DC: American Society for Microbiology), pp. 374–411.
- Fedoroff, N.V., and Chandler, V. (1994). Inactivation of maize transposable elements. In *Homologous Recombination and Gene Silencing in Plants*, J. Paszkowski, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 349–385.
- Fedoroff, N., Masson, P., and Banks, J.A. (1989). Mutations, epimutations, and the developmental programming of the maize *Suppressor-Mutator* transposable element. *Bioessays* **10**, 139–144.
- Feldmar, S., and Kunze, R. (1991). The ORFa protein, the putative transposase of maize transposable element *Ac*, has a basic DNA binding domain. *EMBO J.* **10**, 4003–4010.
- Frank, D., Keshet, I., Shani, M., Levine, A., Razin, A., and Cedar, H. (1991). Demethylation of CpG islands in embryonic cells. *Nature* **351**, 239–241.
- Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827–1831.
- Geiser, M., Weck, E., Döring, H.-P., Werr, W., Courage-Tebbe, U., Tillmann, E., and Starlinger, P. (1982). Genomic clones of a wild-type allele and a transposable element-induced mutant of the sucrose synthase gene of *Zea mays* L. *EMBO J.* **1**, 1455–1460.
- Gierl, A., Lütticke, S., and Saedler, H. (1988). TnpA product encoded by the transposable element *En-1* of *Zea mays* is a DNA binding protein. *EMBO J.* **7**, 4045–4053.
- Greenblatt, I.M. (1968). The mechanism of *Modulator* transposition in maize. *Genetics* **58**, 585–597.

- Greenblatt, I.M.** (1974). Movement of *Modulator* in maize: A test of an hypothesis. *Genetics* **77**, 671–678.
- Greenblatt, I.M.** (1984). A chromosomal replication pattern deduced from pericarp phenotypes resulting from movements of the transposable element, *Modulator*, in maize. *Genetics* **108**, 471–485.
- Greenblatt, I.M., and Brink, R.A.** (1962). Twin mutations in medium variegated pericarp maize. *Genetics* **47**, 489–501.
- Gruenbaum, Y., Naveh-Mary, T., Cedar, H., and Razin, A.** (1981). Sequence specificity of methylation in higher plants. *Nature* **292**, 860–862.
- Hehl, R., Nacken, W.K.F., Krause, A., Saedler, H., and Sommer, H.** (1991). Structural analysis of *Tam3*, a transposable element from *Antirrhinum majus*, reveals homologies to the *Ac* element from maize. *Plant Mol. Biol.* **16**, 369–371.
- Heinlein, M.** (1995). Variegation patterns caused by excision of the maize transposable element *Dissociation (Ds)* are autonomously regulated by allele-specific *Activator (Ac)* elements and are not due to *trans*-acting modifier genes. *Mol. Gen. Genet.* **246**, 1–9.
- Heinlein, M., and Starlinger, P.** (1991). Variegation patterns caused by transposable element *Ac*. *Maydica* **36**, 309–316.
- Herrmann, A., Schulz, W., and Hahlbrock, K.** (1988). Two alleles of the single-copy chalcone synthase gene in parsley differ by a transposon-like element. *Mol. Gen. Genet.* **212**, 93–98.
- Hoshino, A., Inagaki, Y., and Iida, S.** (1995). Structural analysis of *Tpn1*, a transposable element isolated from Japanese morning glory bearing variegated flowers. *Mol. Gen. Genet.* **247**, 114–117.
- Houba-Hérin, N., Becker, D., Post, A., Larondelle, Y., and Starlinger, P.** (1990). Excision of a *Ds*-like maize transposable element (*AcΔ*) in a transient assay in *Petunia* is enhanced by a truncated coding region of the transposable element *Ac*. *Mol. Gen. Genet.* **224**, 17–23.
- Keller, J., Jones, J.D.G., Harper, E., Lim, E., Carland, F., Ralston, E.J., and Dooner, H.K.** (1993). Effects of gene dosage and sequence modification on the frequency and timing of transposition of the maize element *Activator (Ac)* in tobacco. *Plant Mol. Biol.* **21**, 157–170.
- Klößgen, R.B., Gierl, A., Schwarz-Sommer, Z., and Saedler, H.** (1986). Molecular analysis of the *Waxy* locus of *Zea mays*. *Mol. Gen. Genet.* **203**, 237–244.
- Kunze, R., and Starlinger, P.** (1989). The putative transposase of transposable element *Ac* from *Zea mays* L. interacts with subterminal sequences of *Ac*. *EMBO J.* **8**, 3177–3185.
- Kunze, R., Stochaj, U., Laufs, J., and Starlinger, P.** (1987). Transcription of transposable element *Activator (Ac)* of *Zea mays* L. *EMBO J.* **6**, 1555–1563.
- Kunze, R., Starlinger, P., and Schwartz, D.** (1988). DNA methylation of the maize transposable element *Ac* interferes with its transcription. *Mol. Gen. Genet.* **214**, 325–327.
- Kunze, R., Coupland, G., Fusswinkel, H., Feldmar, S., Courage, U., Schein, S., Becker, H.-A., Chatterjee, S., Li, M.-g., and Starlinger, P.** (1991). Structure and function of the maize transposable element *Activator (Ac)*. In *Plant Molecular Biology 2*, NATO ASI Series, Life Sciences. R.G. Herrmann and B.A. Larkins, eds (New York: Plenum Press), pp. 285–298.
- Macleod, D., Charlton, J., Mullins, J., and Bird, A.P.** (1994). Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. *Genes Dev.* **8**, 2282–2292.
- Martienssen, R., and Baron, A.** (1994). Coordinate suppression of mutations caused by Robertson's *Mutator* transposons in maize. *Genetics* **136**, 1157–1170.
- McClintock, B.** (1951). Chromosome organization and genic expression. *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13–47.
- McClintock, B.** (1957). Genetic and cytological studies of maize. *Carnegie Inst. Wash. Year Book* **56**, 393–401.
- Meyer, P., Niedenhof, I., and ten Lohuis, M.** (1994). Evidence for cytosine methylation of non-symmetrical sequences in transgenic *Petunia hybrida*. *EMBO J.* **13**, 2084–2088.
- Murray, H.G., and Thompson, W.F.** (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.
- Ott, T., Nelsen-Salz, B., and Döring, H.-P.** (1992). PCR-aided genomic sequencing of 5' subterminal sequences of the maize transposable element *Activator (Ac)* in transgenic tobacco plants. *Plant J.* **2**, 705–711.
- Peleman, J., Cottyn, B., Van Camp, W., Van Montagu, M., and Inze, D.** (1991). Transient occurrence of extrachromosomal DNA of an *Arabidopsis thaliana* transposon-like element, *Tat1*. *Proc. Natl. Acad. Sci. USA* **88**, 3618–3622.
- Rhodes, P.R., and Vodkin, L.O.** (1988). Organization of the *Tgm* family of transposable elements in soybean. *Genetics* **120**, 597–604.
- Roberts, D., Hoopes, B.C., McClure, W.R., and Kleckner, N.** (1985). */S10* transposition is regulated by DNA adenine methylation. *Cell* **43**, 117–130.
- Ronchi, A., Petroni, K., and Tonelli, C.** (1995). The reduced expression of endogenous duplications (REED) in the maize R gene family is mediated by DNA methylation. *EMBO J.* **14**, 5318–5328.
- Schläppli, M., Raina, R., and Fedoroff, N.** (1994). Epigenetic regulation of the maize *Spm* transposable element: Novel activation of a methylated promoter by *ThpA*. *Cell* **77**, 427–437.
- Schwartz, D.** (1989). Gene-controlled cytosine demethylation in the promoter region of the *Ac* transposable element in maize. *Proc. Natl. Acad. Sci. USA* **86**, 2789–2793.
- Schwartz, D., and Dennis, E.S.** (1986). Transposase activity of the *Ac* controlling element in maize is regulated by its degree of methylation. *Mol. Gen. Genet.* **205**, 476–482.
- Theres, N., Scheele, T., and Starlinger, P.** (1987). Cloning of the *Bz2* locus of *Zea mays* using the transposable element *Ds* as a gene tag. *Mol. Gen. Genet.* **209**, 193–197.
- Varagona, M., and Wessler, S.R.** (1990). Implications for the *cis*-requirements for *Ds* transposition based on the sequence of the *wxB4 Ds* element. *Mol. Gen. Genet.* **220**, 414–418.
- Wang, R.Y.H., Gehrke, C.W., and Ehrlich, M.** (1980). Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic Acids Res.* **8**, 4777–4790.
- Weil, C.F., and Wessler, S.R.** (1993). Molecular evidence that chromosome breakage by *Ds* elements is caused by aberrant transposition. *Plant Cell* **5**, 512–522.