Regulation of *Activator/Dissociation* Transposition by Replication and DNA Methylation

Francesca Ros and Reinhard Kunze

Institut für Genetik und Mikrobiologie, Universität München, 80638 Munich, Germany

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ABSTRACT

In maize the transposable elements *Activator/Dissociation* (*Ac/Ds*) transpose shortly after replication from one of the two resulting chromatids ("chromatid selectivity"). A model has been suggested that explains this phenomenon as a consequence of different affinity for *Ac* transposase binding to holo-, hemi-, and unmethylated transposon ends. Here we demonstrate that in petunia cells a holomethylated *Ds* is unable to excise from a nonreplicating vector and that replication restores excision. A *Ds* element hemi-methylated on one DNA strand transposes in the absence of replication, whereas hemi-methylation of the complementary strand causes a >6.3-fold inhibition of *Ds* excision. Consistently in the active hemi-methylated state, the *Ds* ends have a high binding affinity for the transposase, whereas binding to inactive ends is strongly reduced. These results provide strong evidence for the above-mentioned model. Moreover, in the absence of DNA methylation, replication enhances *Ds* transposition in petunia protoplasts >8-fold and promotes formation of a predominant excision footprint. Accordingly, replication also has a methylation-independent regulatory effect on transposition.

THE maize transposable element *Activator* (*Ac*) is the prototype element of the class of "*hAT*" eukaryotic transposons. These elements are characterized by common conserved transposase segments including a unique signature motif (Calvi *et al.* 1991; Feldmar and Kunze 1991; Essers *et al.* 2000) and similar terminal inverted repeats (reviewed in Kunze 1996). The *hAT* elements transpose conservatively by a cut-and-paste mechanism and supposedly use the same transposition mechanism.

In the maize sporophyte *Ac* transposes predominantly during or shortly after replication, and only one of the two resulting daughter elements is transposition competent (chromatid selectivity; Greenblatt and Brink 1963; Greenblatt 1984; Chen *et al.* 1987, 1992). In these properties *Ac* closely resembles the bacterial IS*10* element, where differential binding of the IS*10* transposase to hemi- and holomethylated transposon ends mediates these effects (Roberts *et al.* 1985). In maize kernels, the *Ac* TPase binding sites in the subterminal regions of *Ac* and *Dissociation* (*Ds*) elements are heavily C-methylated, and the TPase protein binds *in vitro* with different affinities to holo-, hemi-, and unmethylated target DNA (Kunze and Starlinger 1989; Wang *et al.* 1996; Wang and Kunze 1998). On the basis of these findings a model has been proposed according to which DNA methylation of the transposon ends is responsible for the replication dependence and chromatid selectivity of transposition (Fedoroff 1989; Wang *et al.* 1996).

However, DNA methylation-mediated replication dependence cannot completely explain the behavior of *Ac/Ds* transposition. In several studies it was found that in a transient assay *Ac/Ds* element excision from extrachromosomal geminivirus vectors in maize, barley, wheat, and rice cells is dependent on vector replication, although the transfected DNAs were not C-methylated (Laufs *et al.* 1990; McElroy *et al.* 1997; Wirtz *et al.* 1997). In contrast, *Ds* is able to excise from nonreplicating plasmids in petunia, parsley, and *Nicotiana plumbaginifolia* cells (Houba-Hérin *et al.* 1990, 1994; R. Lütticke and R. Kunze, unpublished results).

In this article we studied the correlation between *Ds* transposition in petunia cells and DNA replication by use of a replicon sequence derived from the genome of the monopartite geminivirus tomato yellow leaf curl virus (TYLCV; Kheyr-Pour *et al.* 1992). This family of geminiviruses infects a variety of dicotyledonous hosts. TYLCV contains two divergently transcribed gene clusters that are separated by an intergenic region (IR). The IR contains the DNA-binding site for the Rep protein, the origin of replication, and eukaryotic promoter signals (Fontes *et al.* 1994). Except for the Rep protein that catalyzes cleavage and joining at the viral origin of replication (Laufs *et al.* 1995), geminivirus DNA synthesis relies entirely on the DNA replication apparatus of the host plant, although host cell division does not appear to be a prerequisite for geminiviral DNA replication (Nagar *et al.* 1995).

We show that transposition of a *Ds* element from an

Corresponding author: Reinhard Kunze, Botanisches Institut II, Universität zu Köln, Gyrhofstrasse 15, 50931 Cologne, Germany.
E-mail: reinhard.kunze@uni-koeln.de

extrachromosomal vector in petunia cells is regulated by DNA replication in a methylation-dependent and independent mode. Holomethylation completely inhibits *Ds* excision from a nonreplicating plasmid, whereas *Ds* transposition is restored by replication. Moreover, *Ds* elements that are hemi-methylated on one DNA strand transpose in the absence of replication, whereas methylation on the complementary DNA strand results in at least 6.3-fold reduced excision frequencies. These data strongly support the transposition model of Wang *et al.* (1996). Beyond that, *Ds* transposition also is strongly promoted by replication in the absence of methylation.

MATERIALS AND METHODS

Plasmid constructs: pMiDsf1 and pMiDs<f1 were derived from pNT150Ds (Becker *et al.* 1992) by replacement of the *Ds* with a smaller *Ds* element that consists of the 246 5′-terminal and the 446 3′-terminal *Ac* residues (Chatterjee and Starlinger 1995). Between the β-glucuronidase (GUS) and the ampicillin resistance genes the M13 f1 origin was inserted as a 414-bp *Bgl*II-*Ssp*I fragment from pT7T3 in the (+) (pMiDsf1) or (−) (pMiDs<f1) orientation, respectively. In pMiDsf1-RI the luciferase gene of pMiDsf1 was replaced with the *cis*- and *trans*-acting TYLCV replication sequences from pTYSst14 (Kheyr-Pour *et al.* 1992; details of the construct are provided upon request). In pMiDsf1-rI the C1 gene (Rep) was mutated by inverting a *Bst*I107I/*Pfl*MI fragment. In pMiDsf1-R, a *Pfl*MI/*Nco*I fragment corresponding to the TYLCV replicon *cis*-acting IR was deleted.

***Ds* excision assay in petunia protoplasts and DNA extraction:** Transpositional activity of *Ds* reporter plasmids in petunia cells was assessed as described by Houba-Hérin *et al.* (1990) and Heinlein *et al.* (1994). Briefly, sterile shoot cultures of *Petunia hybrida* ssp.RL01 × ssp.Blue were grown in Gamborg's B5 medium (basal salts mixture) at 26° under a 16-hr light/8-hr dark cycle. Mesophyll protoplasts from 3- to 5-wk-old shoots were cotransfected with the TPase expression plasmid pNT600-10.ATG and a *Ds* excision reporter plasmid. Two aliquots of the transfected protoplasts were plated on nitrocellulose filters for GUS staining; the remaining cells were sedimented by centrifugation and DNA was extracted by using the DNeasy Plant mini kit (QIAGEN, Hilden, Germany).

Preparation of holo- and hemi-methylated reporter plasmids: To generate holomethylated plasmids, pMiDsf1 and pMiDsf1-RI were treated with M-*Sss*I (New England Biolabs, Beverly, MA) for 16 hr at 37° as recommended by the manufacturer. After phenol extraction, the completeness of the reaction was confirmed by *Hpa*II and *Bsi*EI digestion. The mock-methylated plasmids were prepared under the same conditions except for the presence of M-*Sss*I.

The preparation of locally hemi-methylated plasmid DNA is depicted in Figure 5. Phagemid single-stranded DNA was isolated from pMiDsf1, pMiDs<1f, and pMiDsf1-RI as described by Sambrook *et al.* (1989). Amplification of the *Ds* element was carried out using primers *Ds*2 (GACCCAGGGAT GAAAGTAGGATGGGAAAATCC) and *Ds*3 (CGGTCGGTA ACGGTCGGTAAAATACCTCTA) on pMiDsf1 as a template and with one primer being biotinylated at the 5′ end. The PCR cocktail (50 μl) contained 20 mm Tris-HCl (pH 8.8), 2 mm $MgSO_4$, 10 mm KCl, 10 mm $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA, 0.2 mm of each dNTP (with dCTP facultatively replaced by ^{5m}dCTP), 0.2 μm of each primer, 10 ng of plasmid template, and 1.25 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). After 5 min of initial denaturation at 95°, 30 cycles of amplification were carried out (94°, 30 sec; 55°, 30 sec; 72°, 1:55 min), followed by a final 5-min extension at 72°. The methylated single-stranded *Ds* element was prepared by adsorption of the biotinylated PCR product (50 μl) to 0.7 mg streptavidin-conjugated magnetic beads (Merck, Darmstadt, Germany) in 10 mm Tris-HCl (pH 7.5), 0.5 mm EDTA, and 1 m NaCl for 30 min at room temperature, followed by elution of the nonbiotinylated DNA strand in 0.1 n NaOH for 8 min at room temperature. The eluate was neutralized by adding 0.5 volume of 0.2 m HCl, 77 mm Tris-HCl (pH 8).

Single-stranded methylated *Ds* element was annealed to single-stranded phagemid DNA at a molar ratio 1:1 in 50 mm NaCl, 10 mm Tris-HCl (pH 8), and 5 mm EDTA by heating for 3 min at 95° and allowing to cool down to 25° over 2 hr. The hybridization product was purified by gel filtration in Sephacryl micro spin columns (Amersham-Pharmacia, Freiburg, Germany).

Approximately 60 ng of hybridization product was filled in at 72° for 1 hr, in a 50-μl reaction mixture containing 20 mm Tris-HCl (pH 8.8), 2 mm $MgSO_4$, 10 mm KCl, 10 mm $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA, 0.05 mm of each dNTP, and 1.25 units of *Pfu* DNA polymerase (Stratagene). The product was purified by gel filtration, and the remaining nick was closed by treatment with 2 units T4 DNA Ligase (Roche Biochemicals, Basel, Switzerland) overnight at 16° in a final volume of 50 μl. Each ligation contained ~0.3 μg of DNA. Before transfection into petunia protoplasts the constructs were extracted with phenol and purified by ethanol precipitation.

Determination of *Ds* excision frequency and isolation of excision footprints by PCR: Total DNA was prepared twice from four independent batches of protoplasts that were cotransfected with pNT600-10.ATG and pMiDsf1 or pMiDsf1-RI, respectively. On each DNA preparation two to four PCRs were performed, and the products were ligated into pCR2.1-Topo (Invitrogen, San Diego) and transformed into *Escherichia coli*. For each transformation, the plasmid inserts from 7 to 18 colonies were sequenced. To amplify the empty *Ds* donor site (300-bp excision product), PCRs were performed on 150 ng DNA using primers Pr_1 (GGATACTTACGTCACGTCTT GCGCACTGAT) and Pr_2 (CCACAGTTTTCGCGATCCAG ACTGAA). The reaction mixture (50 μl) contained 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm of each dNTP, 0.4 μm of each primer, and 2.5 units *Taq* DNA polymerase.

Amplification was carried out by incubating for 5 min at 94°, followed by 25 cycles of 94°, 30 sec/60°, 20 sec, and one final step of 5 min at 72°. With the same primer pair a 250-bp fragment was coamplified from plasmid pNT150, which lacks the *Ds* insertion and which was added (1 pg) as a control to each reaction tube.

Sixty-five excision footprints each from pMiDsf1 and pMiDsf1-RI, originating from 20 independent PCR reactions and four transfection assays, were cloned into pCR2.1-TOPO (Invitrogen) and sequenced.

DNA analysis by gel blot hybridization: For gel blot analyses DNA was size fractionated by 1% agarose gel electrophoresis and transferred to positively charged nylon membranes (Hybond-N^+). Hybridization and washing were done using standard conditions (Sambrook *et al.* 1989). Probes were labeled by random priming using [α-^{32}P]dCTP.

Gel retardation assays: Gel retardation assays were performed using renatured $TPase_{103-465}$ essentially as described (Feldmar and Kunze 1991; Becker and Kunze 1997). Target DNAs (Figure 6A) were prepared as follows: unmethylated and holomethylated *Ds* ends were prepared by PCR amplification of the complete *miniDs* in the presence of dCTP or

^{5m}dCTP, respectively, and digestion of the product with *Dra*I, yielding a 0.3-kb 5′ end fragment and a 0.4-kb 3′ end fragment.

Hemi-methylated *Ds* ends were prepared by hybridization of methylated and unmethylated single-stranded *Ds* DNAs, followed by *Dra*I digestion of the hybridization products. Methylated and unmethylated single-stranded *Ds* DNAs were prepared as described in Figure 5, steps two to four, except that ^{5m}dCTP or dCTP were included in the PCR reaction mixture. The fragments were radiolabeled by 5′ phosphorylation using [γ-^{32}P]ATP and T4 kinase. The 5′ end segments I, II, and III were generated by combinatorial hybridization of complementary methylated or unmethylated radiolabeled oligonucleotides.

RESULTS

Replication promotes *Ds* excision: In monocots it was found that excision of *Ds* elements from geminivirus vectors requires replication (Laufs *et al.* 1990; McElroy *et al.* 1997; Wirtz *et al.* 1997). In contrast, in three dicot species *Ds* transposes from plasmids that supposedly do not replicate in the plant cells (Becker *et al.* 1992; Kunze *et al.* 1993). To determine whether in dicots replication has an influence on *Ds* excision, we constructed a set of novel reporter plasmids and controls. pMiDsf1 carries a *uidA* (GUS) gene whose expression is blocked by a *miniDs* transposon (Figure 1A). pMiDsf1-RI carries in addition the *cis*- and *trans*-acting replication sequences from TYLCV (Kheyr-Pour *et al.* 1992). This virus is capable of propagating in various solanaceaeous species (B. Gronenborn, personal communication).

The replication activity of each construct in petunia cells was tested by cotransfecting protoplasts with a TPase expression plasmid and *Dam*-methylated reporter plasmid DNA. At different time points after transfection DNA was reisolated, subjected to *Mbo*I digestion, and analyzed by gel blotting. Plasmid DNA propagated in *Dam*$^+$ bacteria is resistant to *Mbo*I, whereas replication in plant cells leads to *Mbo*I sensitivity due to the loss of *Dam* methylation. The reisolated pMiDsf1 DNA is resistant to *Mbo*I digestion, indicating that the plasmid does not replicate in the plant cells (Figure 1B, lanes 5–7). In contrast, *Mbo*I releases increasing amounts of the 1024-bp digestion fragment from pMiDsf1-RI DNA isolated 30 and 60 hr after transfection (Figure 1B, lanes 2–4). To ensure that the replication activity of pMiDsf1-RI depends on the functionality of the viral replicon, we tested two plasmids with defects in the *cis*- and *trans*-acting TYLCV replication sequences, respectively. In pMiDsf1-rI the C1 gene encoding the *trans*-acting Rep

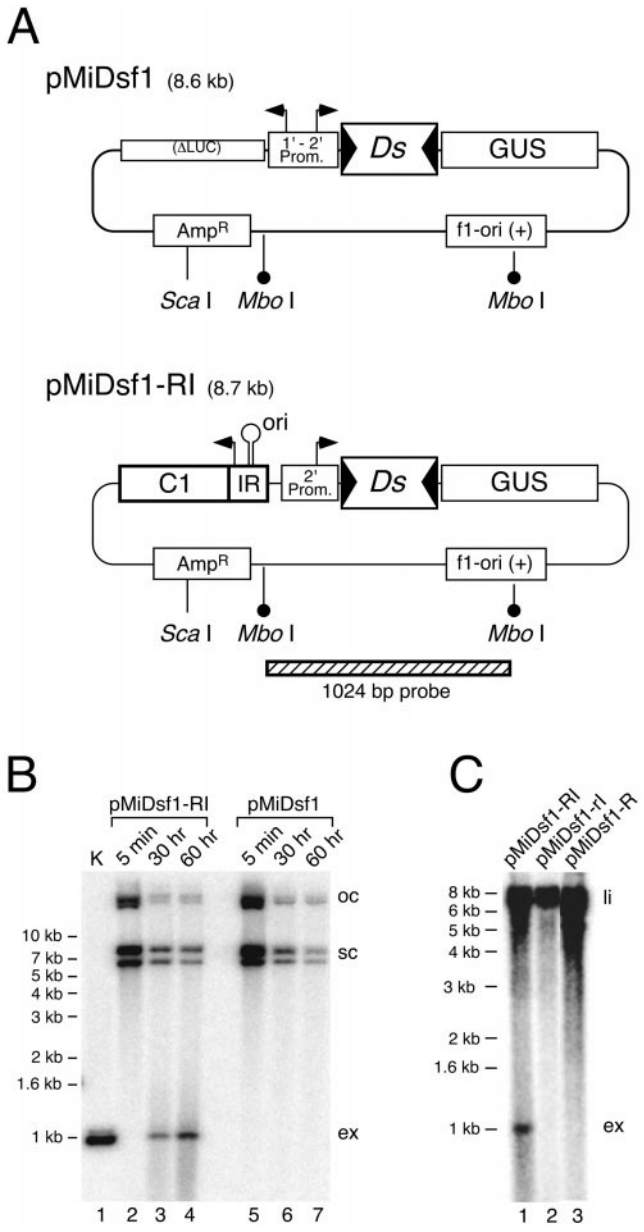

Figure 1.—The TYLCV Rep protein and replication origin are active in petunia protoplasts. (A) Structure of the reporter plasmids pMiDsf1 and pMiDsf1-RI. In both plasmids a nonautonomous *Ds* transposon blocks expression of the GUS gene. In pMiDsf1-RI the *C1* gene for the TYLCV replication initiator protein (Rep) and the origin of viral-strand DNA replication *IR* replace a deleted, nonfunctional fragment of the *Luciferase* gene in pMiDsf1. The arrowheads indicate the T-DNA 1′–2′ promoter and a TYLCV promoter. The hatched bar below pMiDsf1-RI denotes the 1024-bp *Mbo*I fragment used as a hybridization probe on the gel blots shown below. (B) DNA isolated from petunia protoplasts at different time points after cotransfection with the TPase expression plasmid pNT600-10.ATG (7.8 kb) and pMiDsf1-RI (lanes 2–4) or pMiDsf1 (lanes 5–7) was digested with the *dam* methylation-sensitive enzyme *Mbo*I, electrophoresed, blotted, and hybridized with the probe shown in A, which hybridizes with the same *Mbo*I fragment in all reporter plasmids. Lane 1, unlabeled probe fragment (K). oc, undigested, open circle forms of TPase expression plasmid and reporter plasmid; sc, undigested, supercoiled TPase expression and reporter plasmid; ex, 1-kb *Mbo*I digestion product of the reporter plasmid. (C) Gel blot analysis of DNA isolated from petunia protoplasts cotransfected with pNT600-10.ATG and pMiDsf1-RI or the replication-deficient mutants pMiDsf1-rI (8.7 kb) or pMiDsf1-R (8.4 kb), respectively. The DNA was digested with *Mbo*I and *Sca*I, electrophoresed, blotted, and hybridized with the probe shown in A. li, linearized TPase expression and *Ds* reporter plasmids.

TABLE 1

***Ds* excision events of replicating and nonreplicating *Ds* elements in petunia protoplasts**

		No. of GUS-positive cells[a]			
		Exp. I	Exp. II	Exp. III	Exp. IV
pMiDsf1	(−)[b]	53	42	130	127
pMiDsf1-RI	(+)	135	140	319	323
pMiDsf1-rI	(−)	52	60	ND	ND
pMiDsf1-R	(−)	49	40	ND	ND

[a] Petunia protoplasts were cotransfected with 10 μg pNT600-10.ATG and 10 μg pMiDsf1, pMiDsf1-RI, pMiDsf1-rI, or pMiDsf1-R, respectively. The results of four independent experiments are shown. ND, not determined.

[b] (−), Nonreplicating in petunia cells; (+), replicating in petunia cells.

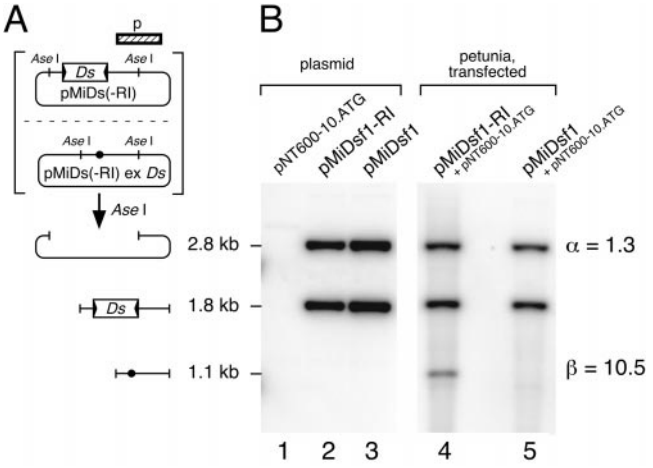

FIGURE 2.—Replication enhances *Ds* excision. (A) Schematic of the restriction and gel blot hybridization analysis. DNA was isolated from petunia protoplasts cotransfected with the TPase expression plasmid pNT600-10.ATG and pMiDsf1-RI or pMiDsf1, digested with *Ase*I, and analyzed by gel blot hybridization. The probe (*p*) hybridizes equally well with the 2.8-kb pMiDsf1(-RI) vector fragment, the 1.8-kb fragment containing the *Ds*, and the 1.1-kb fragment resulting from *Ds* excision. (B) Gel blots of *Ase*I-digested pNT600-10.ATG, pMiDsf1-RI, and pMiDsf1 plasmid DNA as controls (lanes 1 to 3) and DNA from petunia protoplasts cotransfected with pNT600-10.ATG and pMiDsf1-RI or pMiDsf1 (lanes 4 and 5). α, ratio of 2.8-kb vector bands in lanes 4 and 5; β, ratio of 1.1-kb *Ds* excision bands in lanes 4 and 5.

protein is destroyed, and in pMiDsf1-R the intergenic region, containing the promoter and the Rep protein binding sites (IR), is deleted. Both control plasmids are unable to replicate in the plant cells (Figure 1C, lanes 2–3).

Ds excision from the reporter plasmids is accompanied by reversion to GUS activity. Petunia protoplasts cotransfected with a TPase expression plasmid and pMiDsf1, pMiDsf1-RI, pMiDsf1-rI, or pMiDsf1-R, respectively, were spread on filters, stained, and inspected for frequency and staining intensity of GUS-positive (blue) cells. No *Ds* excision is detectable 5 min after transfection. Thirty hours after transfection *Ds* excision is observed with all four plasmids (Table 1). This confirms that in petunia *Ds* is able to transpose in the absence of replication. Remarkably, the frequency of blue staining cells with the replicating plasmid pMiDsf1-RI is two to three times higher compared to the three nonreplicating plasmids, and the staining intensity of the protoplasts is significantly higher (data not shown).

The GUS assay allows no distinction regarding whether these two effects result from an increase in *Ds* excision frequency or from replicative amplification of plasmids before and after *Ds* excision. We therefore performed a quantitative gel blot hybridization analysis. DNA from protoplasts was digested with *Ase*I, blotted, and hybridized with a probe that detects three fragments: a 2807-bp vector fragment whose abundance reflects the total amount of plasmid, a 1798-bp fragment from plasmids that still contain the *Ds*, and a 1089-bp fragment from plasmids where *Ds* has been excised (Figure 2A). The ratio α of the 2807-bp band intensities between pMiDsf1- (lane 4) and pMiDsf1-RI-transfected cells (lane 5) reflects the overall difference in plasmid content due to replication ("copy number effect") times aliquot size. The ratio of the 1089-bp band intensities (β) corresponds to the product of copy number effect times change in excision frequency times aliquot size. The ratio α/β indicates the factor by which the *Ds* excision frequency is enhanced (or reduced) by replication of the reporter plasmid pMiDsf1-RI. Quantification of the respective bands (Figure 2B; upon overexposure, the *Ds* excision band in lane 5 becomes visible as a faint signal) led to the conclusion that the *Ds* excision frequency in petunia cells is promoted at least eightfold by replication of the host plasmid.

To determine whether the reduced *Ds* excision frequency from the nonreplicating host plasmid correlates with *de novo* methylation in the petunia cells of the *Ds* and/or the whole plasmid, DNA was isolated from the cells 30 hr after transfection, digested with the methylation-sensitive enzyme *Bsi*EI, and analyzed by gel blot hybridization. No *de novo* methylation of any *Bsi*EI sites in the *Ds* or the flanking plasmid sequences was detected (data not shown).

Transposon footprint formation is influenced by replication: Excision of *Ac* and *Ds* elements is associated with the formation of characteristic transposon "footprints" that are the products of DNA end joining and repair reactions. We wanted to investigate whether excision footprint formation is also influenced by replication. Using standard conditions, the *Ds* excision products from nonreplicating plasmids were not detectable by gel blot hybridization or by PCR. We therefore developed optimized reaction conditions that allow the selective amplification of rare excision site sequences among a large excess of "wild-type" plasmids (see MATERIALS AND METHODS).

Sixty-five footprints were amplified, cloned, and se-

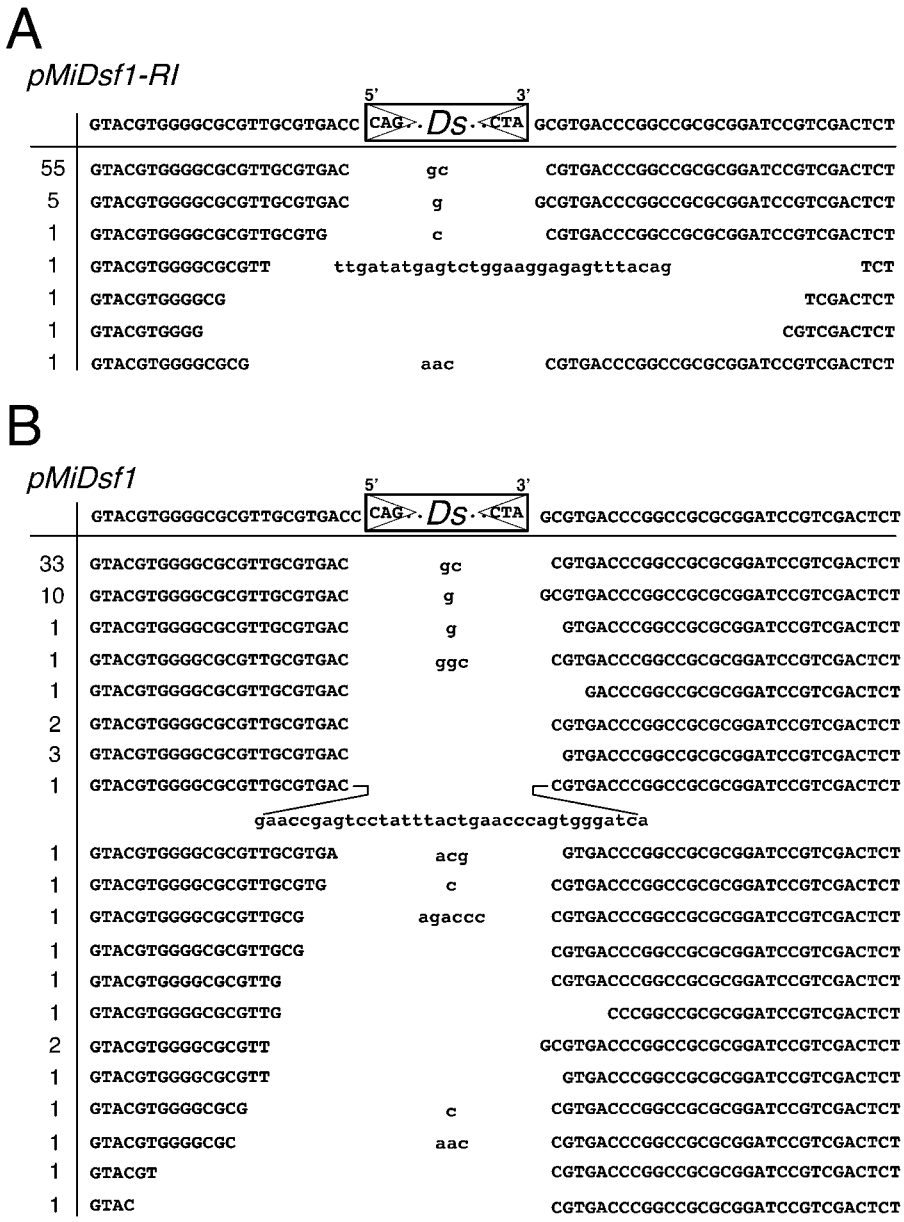

FIGURE 3.—Transposon footprints after *Ds* excision. (A) Footprints from the replicating pMiDsf1-RI plasmid. (B) Footprints from the nonreplicating pMiDsf1 plasmid. The top lines show the sequence at the *Ds* insertion site. Lines below show empty donor site sequences of *Ds* excision events. The numbers to the left indicate the frequency of each excision footprint.

quenced from the replicating and the nonreplicating reporter plasmids, respectively (Figure 3). The sampling strategy for the clones assures that these sequences are derived from independent plasmid molecules (see MATERIALS AND METHODS). However, we cannot exclude the possibility that individual excision products were preferentially amplified in the protoplasts by postexcisional plasmid replication. In the presence of replication, one predominant footprint ("gc") is formed in 84% of excision events, 8% contain a second type ("g"), and 8% of footprints have individual sequences (Figure 3A). The gc and g footprints dominate with frequencies of 51 and 15% also in the absence of replication; however, the frequency of deviating and individual footprints is almost fivefold higher (34%; Figure 3B). If we assume that most of the analyzed products are derived from independent excision reactions, these data suggest that replication not only facilitates transposon excision but also has an influence on excision site repair. Possibly, the efficiency and fidelity of the reactions leading to the formation of the predominant footprints are enhanced.

Methylation of *Ds* inhibits transposition: Genetic and molecular experiments led to the hypothesis that in maize the DNA methylation status of *Ac/Ds* elements determines their transpositional competence (FEDOROFF 1989; KUNZE and STARLINGER 1989; WANG *et al.* 1996; WANG and KUNZE 1998). However, the studies of *Ac/Ds* transposition from geminivirus vectors in monocots and our results in this study suggest that replication can regulate transposition in the absence of methylated cytosines.

We therefore directly tested the effects of *Ds* methylation in the presence and absence of replication. According to the proposed model, C-methylation of TPase binding sites on both DNA strands should inhibit trans-

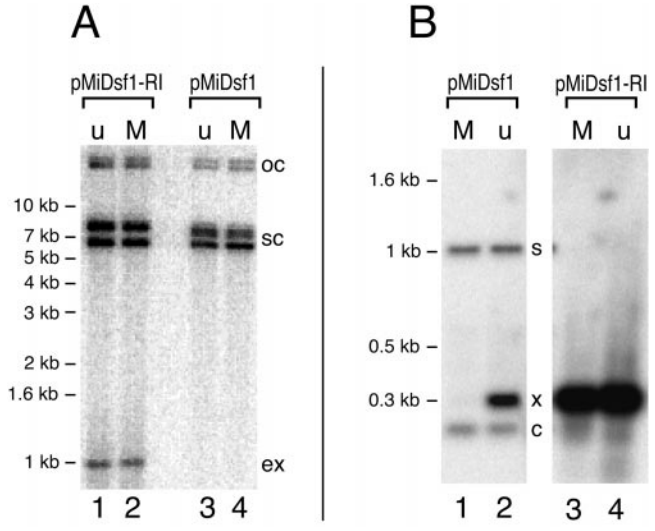

FIGURE 4.—Methylation inhibits transposition of a *Ds* element. (A) Methylation does not impede replication of plasmids. DNA was isolated from petunia protoplasts 30 hr after cotransfection with TPase expression plasmid pNT600-10. ATG and unmethylated (lanes 1 and 3) or methylated (lanes 2 and 4) pMiDsf1-RI or pMiDsf1, digested with the *dam* methylation-sensitive enzyme *Mbo*I, electrophoresed, blotted, and hybridized with the probe shown in Figure 1A. oc, undigested, open circle forms of pNT600-10.ATG and the reporter plasmid; sc, undigested, supercoiled pNT600-10.ATG and reporter plasmid; ex, 1024-bp *Mbo*I digestion fragment from the reporter plasmid. (B) PCR analysis of *Ds* excision from methylated and unmethylated pMiDsf1 and pMiDsf1-RI. DNA extracted from protoplasts 30 hr after transfection was supplemented with ~1 pg pNT150 and subjected to PCR using *Ds*-flanking primers. The PCR products were analyzed by gel blot hybridization using the 0.25-kb PCR product from pNT150 that spans the *Ds* insertion site as probe. s, *Ds*-containing donor fragment; x, empty donor site after *Ds* excision; c, amplified pNT150 fragment as internal control.

position in the absence of replication. pMiDsf1 and pMiDsf1-RI plasmids, isolated from Dam^+ bacteria, were treated with M-*Sss*I that converts all cytosine residues in CpG motifs to ^{5m}C. In *Ac/Ds* elements almost all CpG motifs are located in the *cis*-acting terminal regions that include the subterminal TPase binding sites (KUNZE *et al.* 1988; KUNZE and STARLINGER 1989). Petunia protoplasts were transfected with the methylated and, as controls, mock-methylated plasmids. Restriction analysis with *Mbo*I of DNA reisolated from the cells showed that exclusively in pMiDsf1-RI the *Mbo*I sites become Dam-demethylated (Figure 4A, lanes 1–2), thus showing that C-methylation does not significantly affect the replication activity of pMiDsf1-RI. This conclusion is corroborated by the observation that the frequency of GUS-positive petunia cells, indicating *Ds* excision, is similarly high after transfection with untreated, mock-methylated, and methylated pMiDsf1-RI (data not shown).

To determine whether *Ds* is able to excise from methylated pMiDsf1 a PCR analysis was performed [the GUS reversion assay is not suitable here because methylation by M-*Sss*I inactivates the GUS gene (data not shown)]. A PCR primer pair was used that coamplifies the empty *Ds* donor site (0.3-kb excision product; Figure 4B, x), the *Ds*-containing donor site (1-kb product; Figure 4B, s), and a 250-bp fragment from plasmid pNT150, which lacks the *Ds* insertion in the GUS gene and was added as a control in transfections with pMiDsf1 (Figure 4B, c). Because *Ds* excises only in a small fraction of the transfected plasmids and thus in the reaction the rare empty donor sites have to compete against a large excess of *Ds*-containing donor sites, a PCR cycle was chosen that conditions underrepresentation of the donor site among the PCR products (see MATERIALS AND METHODS). PCR products were visualized by gel blot hybridization and quantified. In contrast to the unmethylated plasmid (Figure 4B, lane 2), with methylated pMiDsf1 no empty donor site is detectable (lane 1). With the replicating pMiDsf1-RI the empty donor site signals from methylated and unmethylated plasmids are similar (Figure 4B, lanes 3–4). In summary, these results prove that C-methylation of a *Ds* element severely inhibits transposition and that this inhibition is overcome by replication.

Hemi-methylation determines the transposition competence of *Ds* elements: It has been suggested that the chromatid selectivity of *Ac/Ds* transposition is caused by differential transposition competence of the two transiently hemi-methylated daughter elements resulting from replication of a holomethylated element (WANG *et al.* 1996). To scrutinize this hypothesis we generated two modified derivatives of the replication-deficient pMiDsf1 DNA, in which only the bottom or the top strand of the *Ds* element, but not the flanking plasmid sequences, were C-methylated. Briefly, phagemid single-stranded DNA was isolated from pMiDsf1 and pMiDs<1f (which carries the M13 f1 origin in opposite orientation from pMiDsf1) and hybridized with the respective complementary, C-methylated or unmethylated *Ds* DNA strand. The residual single-stranded plasmid sequences were filled in and the remaining nick was closed by T4 ligase (Figure 5 and MATERIALS AND METHODS). The completeness of the polymerase filling-in reaction and hemi-methylation of the *Ds* element were confirmed by restriction analysis (data not shown). The hemi-methylated and mock-hemi-methylated plasmids were transfected into petunia protoplasts and after 30 hr the cells were plated and stained for GUS activity.

The results of three independent experiments are shown in Table 2. The *Ds* element that is hemi-methylated on the top strand (pMiDs<f1-hemi) achieves on average a 6.3-fold higher number of GUS-positive cells than the bottom-strand-methylated *Ds* (pMiDsf1-hemi). The apparent excision frequency of the top-strand-methylated *Ds* is as high as that of the mock-hemi-methylated *Ds* (pMiDsf1-mock). These results corroborate the proposed model, and they prove that the top-strand-hemi-methylated *Ds* is fully transposition competent,

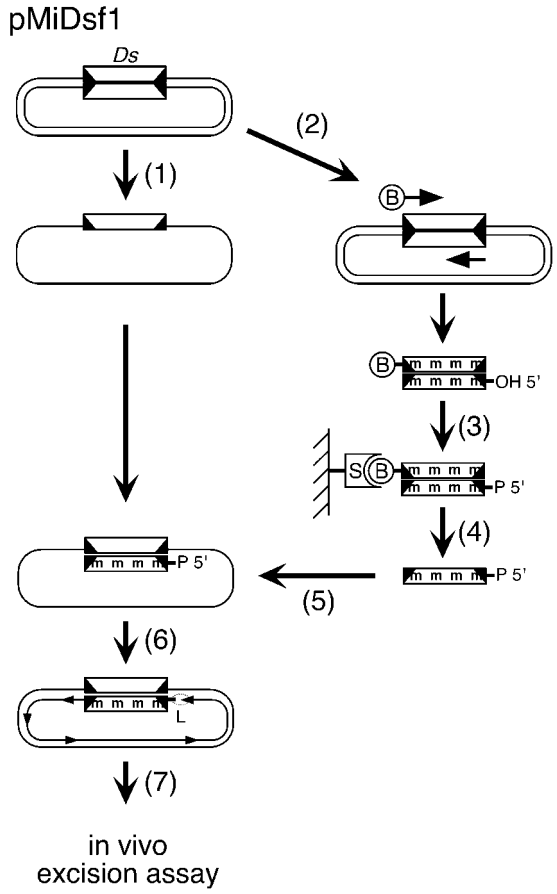

FIGURE 5.—Preparation of locally hemi-methylated reporter plasmids. (1) Isolation of single-stranded phagemid DNA; (2) PCR amplification of the *Ds* element in the presence of ^{5m}dCTP and one biotinylated primer; (3) phosphorylation of the *Ds* PCR product and adsorption to streptavidin-conjugated magnetic beads; (4) elution of a methylated *Ds* single strand by NaOH treatment; (5) hybridization of the methylated *Ds* single strand to the single-stranded phagemid; (6) filling in of the residual phagemid sequences by *Pfu* polymerase; (7) closure of the remaining nick by treatment with T4 DNA Ligase.

whereas C-methylation on the bottom strand severely inhibits transposition. Additional evidence that the C-methylation is responsible for the inactivity of pMiDsf1-hemi was gained by showing that replication completely restores the transposition competence of a bottom-strand-methylated transposon, pMiDsf1-RI-hemi (Table 1).

TPase binding to transposition-competent and -incompetent *Ds* elements: *Ac/Ds* elements contain multiple short TPase binding motifs in both ends that contain a 5′-CCG-3′ sequence (Figure 6A; KUNZE and STARLINGER 1989; BECKER and KUNZE 1997). DNA-binding studies with synthetic oligomers of these motifs have shown that *in vitro* the *Ac* TPase protein binds selectively to such sites that are hemi-methylated on the top strand (5′-^{m}C^{m}CG-3′/5′-CGG-3′), whereas a 5-methylcytosine on the bottom strand (5′-CCG-3′/5′-^{m}CGG-3′) inhibits TPase binding (KUNZE and STARLINGER 1989).

To this end it remained an open question whether TPase binding affinity to these synthetic, hemi-methylated concatemers reflects the binding properties to hemi-methylated transposon ends (*i.e.*, the TPase binding motifs in their native sequence environment). We therefore analyzed the *in vitro* TPase binding reaction to hemi-methylated, transposition-competent or -incompetent *Ds* ends. We separately synthesized the complete *Ds* 5′ and 3′ ends and the three TPase binding site clusters I, II, and III from the 5′ end (Figure 6A) in the unmethylated ("u"), holomethylated ("M"), and both alternate hemi-methylated states ("tm" and "bm") and determined their *in vitro* binding affinities to a TPase$_{103\text{-}465}$ protein fragment by gel shift assays (Figure 6B). TPase$_{103\text{-}465}$ is an N- and C-terminally truncated *Ac* TPase protein containing the complete bipartite DNA-binding domain (BECKER and KUNZE 1997). The binding properties of TPase$_{103\text{-}465}$ to synthetic binding sites and the transposon ends resemble those of the wild-type TPase (FELDMAR and KUNZE 1991), but owing to the lack of a dimerization domain it has a reduced tendency to aggregate and precipitate (ESSERS *et al.* 2000). Because this TPase fragment still contains a multimerization domain (R. ADOLPHS and R. KUNZE, unpublished results), it forms—as the wild-type protein—in solution oligomers of variable sizes and thus the protein/DNA complexes appear as a broad, diffuse band (KUNZE and STARLINGER 1989; FELDMAR and KUNZE 1991; BECKER and KUNZE 1997).

TPase binds efficiently to the unmethylated *Ds* 3′ end (Figure 6B, lane 20). Remarkably, TPase affinity is even increased when the 3′ end fragment is hemi-methylated on the top strand (Figure 6B, lane 19). This is the transposition-competent state (Table 2). In that hemi-methylation state, 12 out of 14 subterminal TPase binding site motifs are in the 5′-^{m}C^{m}CG-3′/5′-CGG-3′ configuration and therefore presumably able to bind TPase protein (Figure 6C, tm). In the alternate hemi-methylation state, which correlates with very low transposition activity, the 3′ end is only weakly bound (Figure 6B, lane 18). This is in accordance with the assumption that in this state TPase can recognize only two widely separated TPase binding site motifs (Figure 6C, bm). If the 3′ end is methylated on both strands, it is bound by TPase only in traces (Figure 6B, lane 17).

At the 5′ end the situation is different. As is the case at the 3′ end, holomethylation prevents TPase binding (Figure 6B, lane 1). However, both alternatively hemi-methylated 5′ ends are bound similarly well (lanes 2–3), a little less efficiently than the unmethylated 5′ end (lane 4). The 5′ end central cluster II has opposite orientations of TPase binding sites relative to clusters I and III. We therefore individually tested the occupation of each of these clusters with TPase. In the unmethylated state, clusters I and II are moderately well bound (lanes 8 and 12), and cluster III is more efficiently complexed (lane 16). Methylation on both DNA strands completely inhibits TPase binding to all three fragments (lanes 5,

TABLE 2

Transposition competence of hemi-methylated *Ds* elements in petunia protoplasts

	No. of GUS-positive cells[a]		
	Exp. I	Exp. II	Exp. III
pMiDsf1-hemi[b]	18	13	14
pMiDs<f1-hemi[c]	90	90	105
pMiDsf1-mock[d]	ND	110	95
pMiDsf1-RI-hemi[e]	ND	90	100

[a] Petunia protoplasts were cotransfected with 10 μg pNT600-10.ATG and 3 μg hemi-methylated or mock-methylated reporter plasmids. The results of three independent experiments are shown. ND, not determined.
[b] Bottom-strand-hemi-methylated *Ds*, nonreplicating.
[c] Top-strand-hemi-methylated *Ds*, nonreplicating.
[d] Mock-methylated *Ds*, nonreplicating.
[e] Bottom-strand-hemi-methylated *Ds*, replicating.

9, and 13). The terminal cluster I is most efficiently bound in the transpositionally competent, top-strand-hemi-methylated state (lane 7). The innermost cluster III is also recognized in this state (lane 15), whereas the central cluster II is not bound at all (lane 11). In the transpositionally inactive, bottom-strand-hemi-methylated state the 5′ terminal cluster I is not recognized by TPase (lane 6), whereas cluster II is efficiently bound (lane 10) and cluster III is weakly complexed (lane14).

These experiments demonstrate that hemi-methylation of the subterminal TPase binding sites is a *cis*-acting determinant of transposase binding that correlates with transposition activity of *Ds* elements. In the active state, both *Ds* ends have a high affinity for the *Ac* TPase protein, whereas the inactive state correlates with much reduced TPase binding to the 3′ end and to the terminal 5′ end TPase binding site cluster I. Figure 6C shows a model of the occupation of the *Ds* terminal fragments with TPase molecules dependent on the methylation state, according to the gel shift assays shown in Figure 6B and binding studies with synthetic target site oligomers (Kunze and Starlinger 1989; Becker and Kunze 1997). Because *in vitro* the TPase readily forms heterogeneous oligomers in the absence of DNA (R. Adolphs and R. Kunze, unpublished results) and binds to DNA in a cooperative manner (Feldmar and Kunze 1991), the protein presumably does not occupy the binding sites in the transposon end with a 1:1 stoichiometry. The *in vitro* TPase/DNA interactions are more compatible with a model where the protein coats the transposon ends completely or in patches as depicted in Figure 6C.

DISCUSSION

The replication dependence of *Ac/Ds* transposition is more pronounced in monocots than dicots: Using four different monocot species (maize, rice, wheat, and barley) it was found that excision of *Ac/Ds* elements from extrachromosomal, nonreplicating geminivirus vectors is extremely rare or even absent (Laufs *et al.* 1990; McElroy *et al.* 1997; Wirtz *et al.* 1997). In contrast, in petunia and other dicots like *N. plumbaginifolia*, tobacco and parsley *Ds* element excision from simple plasmids that supposedly do not replicate in plant cells is readily detectable (Houba-Hérin *et al.* 1990, 1994; R. Kunze, unpublished results). What accounts for this difference between monocots and dicots? Different molecular mechanisms could be involved:

1. In monocots, but not in dicots (this work), extrachromosomal nonreplicating DNA is rapidly methylated, resulting in inactivation of the *Ac* TPase promoter and interference with binding of residual TPase to the transposon ends. This scenario can be dismissed because in the monocot experiments the reporter genes used remained active for several days. However, it cannot be excluded that monocots possess a *de novo* methylation system that specifically acts on transposable elements. In maize, the *Ac* element at the *wx-m9* locus is heavily C-methylated at its termini, whereas the flanking *Waxy* DNA is unmethylated (Wang *et al.* 1996). Similarly, in *En/Spm* both active and inactive elements are extensively methylated, whereas the sequences flanking the transposon at the insertion site are not (Banks *et al.* 1988).
2. It is conceivable that in monocots, but not in dicots, a transposition activator is specifically expressed during S-phase. Alternatively, monocots might contain a cell-cycle-dependent inhibitor of transposition.

Replication facilitates *Ds* excision in the absence of DNA methylation: In petunia cells replication of the reporter plasmid promotes *Ds* excision frequency at least eightfold. As no *de novo* methylation of the transfected plasmid DNA was detectable, this phenomenon does not depend on DNA (de)methylation. On the one hand, the Rep protein is required for reporter plasmid replication; on the other hand, it is known to be severely toxic for prokaryotic and eukaryotic cells (B. Gronenborn, personal communication). This might explain the observation that only a fraction of the TYLCV-containing plasmids has replicated after 30 hr, indicated by the low amplification factor of 1.3. We therefore speculate that this toxicity is a limiting factor for plasmid replication and that without this effect the boost in *Ds* excision frequency would be even more pronounced.

At present we can only speculate about the molecular mechanism underlying the transposition activation by replication. The fact that a mutation in the Rep binding site (IR) abolishes the transposition boost excludes the possibilities of a stimulation by direct interaction of the replicase with the *Ac* TPase (because a Rep/TPase interaction should not be affected) and of an activation of a host cell accessory factor by Rep. It is conceivable that the access of the TPase to its binding sites is facilitated by the conformational change of the transposon ends in the replication fork and/or by the interaction with

replication-specific components. However, such an effect cannot account for the different activation factors in monocots and dicots.

Transposon footprints are influenced by replication: Replication not only promotes *Ds* excision from extrachromosomal vectors, but apparently also affects the formation of the transposon footprints. With and without replication the same two footprints dominate; however, the frequency of aberrant footprints is higher in the absence of replication. This indicates that replication has no influence on the mechanism but rather on the fidelity of the reactions leading to the formation of the predominant footprints. The predominant footprint we obtained is characterized by transversion of the nucleotides immediately flanking the *Ds* on both sides. The same type of footprint also dominates after *Ac* or *Ds* excision in maize and transgenic Arabidopsis plants (Scott *et al.* 1996; Rinehart *et al.* 1997) and from geminivirus vectors in maize, wheat, rice, and barley protoplasts (Laufs *et al.* 1990; Shen *et al.* 1992; Sugimoto *et al.* 1994; McElroy *et al.* 1997). As has been pointed out by Scott *et al.* (1996) and Rinehart *et al.* (1997), these footprints can be explained likewise by a modified "exonuclease" model (Saedler and Nevers 1985) or a "hairpin-intermediate" model (Coen *et al.* 1989). Recently it was demonstrated that *Ac/Ds* elements can transpose in yeast (Weil and Kunze 2000). The *Ds* excision footprints in that system differ from those in plants by the presence of palindromic duplications of the flanking host sequence centered around the 3′-terminal base of the *Ds* element. These footprints clearly indicate that in yeast *Ds* excision follows the hairpin-intermediate model. Indirect support for this mechanism also acting in plants was obtained by the study of extrachromosomal *Ac/Ds* elements (Gorbunova and Levy 2000). Therefore, because it appears unlikely that *Ac/Ds* elements excise with alternate mechanisms in different environments, we assume that the *Ac/Ds* excision reaction in plants also follows the hairpin-intermediate pathway. The hairpin model has also been employed to explain the footprints generated by the transposons *Tam3* from Antirrhinum (Coen *et al.* 1989), hobo from Drosophila (Atkinson *et al.* 1993), Ascot from Ascobulus (Colot *et al.* 1998), Tn*10* from *E. coli* (Kennedy *et al.* 1998), and the coding end joints generated during V(D)J recombination (Roth *et al.* 1992; van Gent *et al.* 1996).

C-methylation of *Ac/Ds* elements is responsible for the chromatid selectivity of transposition: The model for the chromatid selectivity of *Ac/Ds* transposition sug-

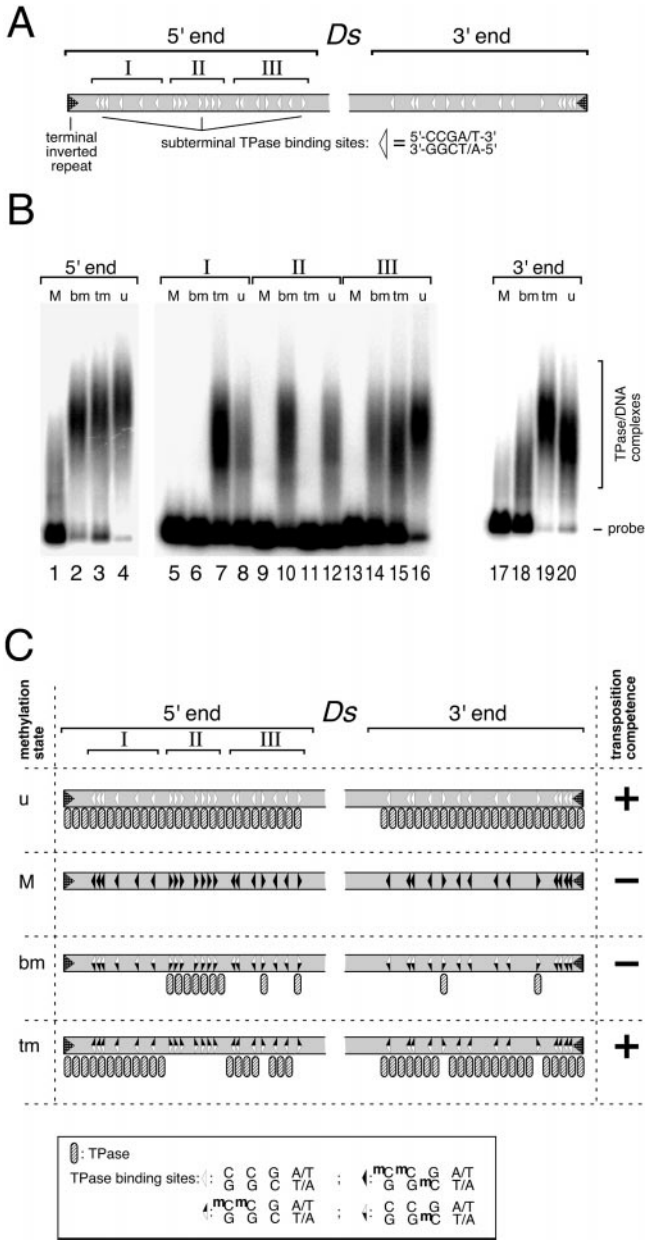

Figure 6.—Gel mobility shift assay of TPase binding to differentially methylated *Ds* end fragments. (A) Schematic of the *Ds* ends. The *Ds* 5′ and 3′ ends comprise 250 bp and 200 bp of the transposon termini, respectively. The three 5′ end segments I, II, and III correspond to positions 30–96, 106–158, and 168–242 on the *Ac* sequence. The distribution of TPase binding sites (open triangles) is drawn approximately to scale. (B) Gel mobility shift assays with the *Ds* end fragments in different methylation states and the $TPase_{103-465}$ protein. u, unmethylated; M, holomethylated; bm, bottom strand methylated; tm, top strand methylated. The diffuse appearance is characteristic of TPase/DNA complexes and is caused by the presence of heterogeneous TPase oligomers in the protein preparation. Free probe DNA migrates at the bottom of the gels. (C) Model for the methylation-dependent TPase binding to the *Ds* 3′ end and 5′ end fragments. Shown is the proposed occupation of the *Ds* terminal fragments with TPase dependent on the methylation state, according to the gel shift assays shown in B and binding studies with synthetic target site oligomers (Kunze and Starlinger 1989; Becker and Kunze 1997). More than one transposase molecule is depicted per binding site, according to the strong cooperativity of the TPase binding to its target sites observed by Becker and Kunze (1997). The four different methylation states are designated at left as in B. The transposition competence of the respective hemi-methylated transposons is indicated at the right.

gested by Wang and coworkers rests on the assumption that holomethylated elements do not transpose because TPase cannot bind to their ends. In addition, it predicts that, following replication, only one of the two daughter transposons is transposition competent (Wang *et al.* 1996; Wang and Kunze 1998). As these hypotheses cannot be scrutinized in transgenic plants, we determined the transpositional activities of fully methylated and hemi-methylated *Ds* elements in the presence or absence of replication in transfected petunia protoplasts and the TPase binding affinities for these differentially methylated target DNAs. Our results conclusively explain the phenomenon of chromatid selectivity of *Ac/Ds* transposition and verify the proposed model in all aspects:

A fully methylated *Ds* element is transposition incompetent and not recognized by the TPase protein.

By replication of a holomethylated *Ds* two differentially hemi-methylated daughter elements are generated, and in our assay one of these is 6.3-fold more active than the other. The hyperactive *Ds* is methylated on the top strand and thus most TPase binding sites, including the ones closest to the terminal inverted repeats, can be efficiently bound by the TPase.

It is unclear whether the 6.3-fold difference in transposition activity between the two daughter elements in the petunia system truly reflects the situation of an element in the maize genome. The plasmid vectors supposedly differ in topology and association with proteins from the chromosomal chromatin structure. Also, in the synthetically methylated *Ds* elements all cytosines are modified to the same extent, whereas the *Ac9* element in maize and its epigenetically inactivated derivative *Ds-cy* display distinct methylation patterns with an uneven distribution of ^{5m}C residues (Wang *et al.* 1996; Wang and Kunze 1998).

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