Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2789–2793, April 1989
Genetics

Gene-controlled cytosine demethylation in the promoter region of the *Ac* transposable element in maize

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Communicated by M. M. Rhoades, January 25, 1989

ABSTRACT **The transposase product of the *Ac* transposable element causes demethylation of the *Bam*HI site in the promoter region of *Ac*. This site remains methylated in *Ds9*, the deletional derivative of *Ac* that fails to make an active transposase. In the presence of an active *Ac* element the *Bam*HI site of *Ds9* is demethylated. Transcription of the element is correlated with the demethylation of the promoter region. Evidence is presented that supports the model that the trans-acting protein causes site-specific demethylation by inducing a configuration change in chromatin structure making the site inaccessible to the methylase. A chromosome alteration that involves the insertion of the 4.5-kilobase *Ac* element results in the methylation of *Pvu* I target sites about 1 kilobase removed from the point of insertion.**

Methylation plays an important role in gene regulation. A good correlation is observed in vertebrates between the degree of cytosine methylation and gene activity. Genes have been shown to be active when hypomethylated and inactive when hypermethylated (1, 2). In mammals many genes that are inactive and hypermethylated in the embryo become hypomethylated in those tissues and developmental stages where they are activated (3, 4). The critical sites are in the promoter regions of the genes (5, 6). In plants, evidence for a correlation between methylation and gene activity has been presented for maize transposable element systems *Ac* (7, 8), *Spm* (9), and *Mu* (10, 11). Conversions from an active to inactive condition in which transposase function is lacking and the reversion to the active condition were shown to involve methylation and subsequent hypomethylation. As with mammals, it appears that hypomethylation of the promoter region is critical for gene activity. This is clearly shown for the *Ac* element in maize (7). An active *Ac* element in the *Wx* gene (*wx-m9*::*Ac*) is, for the most part, unmethylated as far as can be determined by digestion of the DNA with restriction enzymes such as *Hpa* II, *Pvu* II, *Hae* II, *Eco*RII, and *Hha* I, which do not cut targets in which particular cytosine residues are methylated. There are 12 *Hpa* II target sites in *Ac*. The 9 clustered near the promoter end are not methylated. Only the 3 downstream *Hpa* II sites close to the nonpromoter end of the element are methylated. An inactive derivative of this *Ac* in the allele *wx-m9*::*Ds-cy*, still at the same position in the *Wx* gene, is hypermethylated. Active revertants are hypomethylated at the promoter end but still show considerable methylation at the two downstream *Pvu* II sites (7). A similar conclusion can be drawn from the studies of Chomet *et al.* (8) on *wx-m7*::*Ac*. In contrast to mammalian genes, the *Wx* gene of maize, which shows a striking tissue specificity and functions only in the gametophyte stage and endosperm, is always hypomethylated, even in tissues where it does not function.

In maize, hypomethylation and associated reactivation of inactive transposable elements *Spm* (9, 12) and *Mu* (10, 11) are promoted by crosses to plants that carry the active forms of the elements. Another example, described in this paper, involves the *Ac* transposable element. In the proper genetic background 100% reactivation of the inactive methylated element in *wx-m9*::*Ds-cy* can be achieved by the introduction of an active *Ac* into the genome. The associated hypomethylation of this *Ds* appears to be site specific and there is no detectable change in plant growth or overall cytosine methylation in the genome. In this paper evidence is presented in support of the hypothesis that specific, gene-controlled demethylation in the promoter region of *Ac* is mediated by the production of a trans-acting product of *Ac* that protects or alters the configuration of the chromatin in the promoter region of the element so that it cannot be acted upon by the methylase.

METHODS AND MATERIALS

DNA extraction, purification, electrophoresis, transfer, and Southern hybridization procedures were as described (7).

DNA was extracted from plumules of 4-day seedlings grown in the dark at 30°C. The transposable elements used were *wx-m9*::*Ac*, *wx-m9*::*Ds*, and *wx-m9*::*Ds-cy*. The *wx-m9*::*Ac* allele (13–16) has a 4.5-kilobase (kb) *Ac* element inserted in an exon near the 3′ end of the waxy gene on chromosome 9 that controls the synthesis of amylose in the endosperm. *wx-m9*::*Ds* is a derivative of *wx-m9*::*Ac* with an internal 194-base deletion in the element (13, 14, 17). This element has no transposase function and behaves as a *Ds* element in that it responds to and transposes in the presence of an active *Ac*. *wx-m9*::*Ds-cy* (7) is a transposase-inactive, highly methylated derivative of *wx-m9*::*Ac*. *bz2-m* is a *Ds* suppressed allele of the *Bz2* (bronze) gene on chromosome 1 (18). *Bz2* controls anthocyanin pigmentation in the plant and the aleurone layer of the endosperm. The *Wx* vs. *wx* phenotypes are scored by the differential staining of the endosperm starch grains with iodine/potassium iodide.

The transposable elements in *wx-m9*::*Ac*, *wx-m9*::*Ds*, and *wx-m9*::*Ds-cy* occupy identical positions in the waxy gene. The elements in *wx-m9*::*Ac* and *wx-m9*::*Ds* are referred to as *Ac9* and *Ds9*, respectively.

Ac acts to cause autoexcision as well as excision of *Ds* elements in trans. The presence of *Ds* elements, the transposase-inactive derivatives of *Ac*, is detected by their excision in response to an active *Ac*. For example, kernels carrying the *wx-m9*::*Ds* or *wx-m9*::*Ds-cy* alleles are mutant and waxy in phenotype but show reversions to starchy (*Wx*), blue iodine-stained sectors in the presence of an *Ac* element in the genome.

The *Sal* 3 segment of the *Wx* gene used as a probe in the hybridization was kindly supplied by Susan Wessler (University of Georgia).

RESULTS AND DISCUSSION

The 4.5-kb *Ac* element contains a single *Bam*HI site about 150 bases upstream from the point of initiation of transcription

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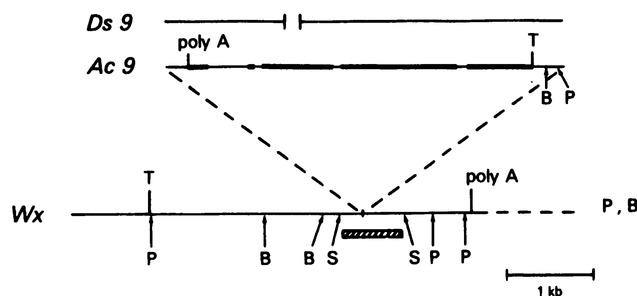

FIG. 1. Structure and partial restriction map of *wx-m9::Ac* constructed from sequences presented by Fedoroff *et al.* (14) and Klosgen *et al.* (16). Restriction enzymes: P, *Pvu* I; B, *Bam*HI; S, *Sal* I. T indicates the transcription start points in *Wx* and *Ac9*. The dotted line indicates the unsequenced region outside the limits of the *Wx* gene. The hatched bar designates the region of the *Wx* gene represented in the *Sal* 3 probe used in this study. The exons in the *Ac* element are indicated by heavy lines and the gap in *Ds9* shows the position of the 194-base deletion.

(19) at position 4379 (Fig. 1). When genomic DNA extracted from seedlings carrying the transposase-inactive *Ds9* element is restricted with *Bam*HI and hybridized with the unique 750-base *Sal* 3 probe of the waxy gene that flanks *Ds9* on both sides, a prominent band of ≈9 kb appears with two faint smaller bands, ≈4.6 and ≈4.2 kb in length (Figs. 2 and 3). The predominance of the 9-kb band indicates that in most molecules the *Bam*HI site in *Ds9* is not cut by the endonuclease. The waxy gene has been sequenced and the *Ds9* element has been shown to be inserted at position 2466, counting from the presumed start of transcription of the waxy gene (16). There is a *Bam*HI site 461 bases upstream from the *Ds9* site, at position 2005. The *Bam*HI target in *Ds9* is 4185 bases from the left end of the element. Thus one of the two smaller bands is of the size expected from restriction at the *Bam*HI sites. The predicted size is 4646 bases (4185 + 461) and a 4.6-kb band is observed. The downstream *Bam*HI site lies outside the waxy gene and the DNA segment between the *Bam*HI sites inside and outside the gene is highly polymorphic. In different non-*Ac*-containing strains distances of 3.3, 4.8, and 11.3 kb were measured between the two sites.

The presence of the prominent 9-kb band in the *Bam*HI digests indicates modification of the target site in *Ds9* making it resistant to digestion by the restriction enzyme. This modification is probably cytosine methylation since the *Bam*HI target is protected against the corresponding endonuclease by methylation (20).

The *Bam*HI site is hypomethylated in *Ac9* and the two smaller bands predominate (Fig. 3). Both of the smaller bands

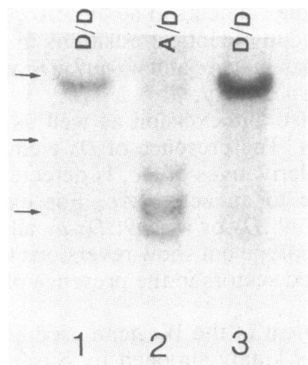

FIG. 2. Southern blot of seedling DNA restricted with *Bam*HI and probed with the *Sal* 3 fragment of the *wx* gene. A, *wx-m9::Ac*; D, *wx-m9::Ds*. Lane 3, *wx-m9::Ds* homozygous progeny from a backcross of *wx-m9::Ac/wx-m9::Ds* by *wx-m9::Ds*. The arrows indicate the positions of the 9.4-, 6.7-, and 4.4-kb markers.

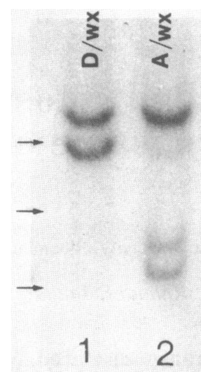

FIG. 3. Same as Fig. 2 but DNA extracts were from progeny of the cross by *wx/wx*. Lane 1, *wx-m9::Ds-wx*; lane 2, *wx-m9::Ac/wx*. The large *Bam*HI fragment is derived from digestion of the *wx* allele that is present in both heterozygotes.

in *wx-m9::Ds* differ from the smaller bands in the *wx-m9::Ac* blots. This is a reflection of the 194-base deletion in *Ds9*, which decreases the size of the 4.6-kb band, and a small insertion about 1.4 kb downstream in the flanking DNA of the *wx-m9::Ac* allele.

In the presence of an active *Ac* the methylation status of the *Bam*HI site of *Ds9* is almost completely reversed. In Southern blots (21) of *wx-m9::Ac/wx-m9::Ds* heterozygotes, the four smaller bands are prominent and the large 9-kb band is very faint, indicating digestion and thus demethylation of the *Bam*HI site in the *Ds* element as well as the *Ac* (Fig. 2). When these two alleles in the heterozygote were separated, by backcrossing to *wx-m9::Ds* homozygotes or by crossing to *wx* testers, the *Bam*HI site in the *wx-m9::Ds* homozygotes (Fig. 2) or in the *wx-m9::Ds/wx* heterozygotes (Fig. 3) reverted back to the methylated condition, resistant to digestion by the restriction endonuclease.

Ac acts in trans to cause cytosine demethylation in the *Bam*HI site in a *Ds* element located on another chromosome. This could be effected by a trans-acting product of *Ac* or by some form of transvection involving paired *m9Ac* and *m9Ds* elements. Transvection (22) can be ruled out since reversal in methylation status is detected in F_1 heterozygous seedlings before the alleles come in contact by meiotic pairing. Even mitotic pairing, which has never been found in maize, can be ruled out since the demethylation of the *Bam*HI site is effected by *Ac* elements that are not linked to the *Wx* locus (Fig. 4). Conceivably the trans-acting product could be the protein product—i.e., transposase—or the RNA transcript. It is unlikely that the protection against methylation is afforded by the 3.5-kb RNA transcript since S1 nuclease mapping of *Ac* (19) showed that transcription is initiated 49 bases downstream from the *Bam*HI site and hence the transcript has no sequence homology with that site.

I propose that the protein product of *Ac* binds to the promoter region of the element and blocks methylation of that region by an alteration in chromatin structure. *Ds9* differs from *Ac* by a small 194-base deletion 2.6 kb downstream from the point of initiation of transcription. The deletion in *Ds9* is in exon 3 (19). The amino acids specified by the 64 codons excluded in the deleted transcript would be lacking in the protein product and since the length of the deletion is not a multiple of three, the shift in reading frame would cause misreading of the remaining 203 bases of the message. In addition to being defective in transposase activity, the product of *Ds9* is unable to block methylation of the promoter region.

An active *Ac* element can reactivate *wx-m9::Ds-cy*, the highly methylated, inactive derivative of *wx-m9::Ac*. *wx-m9::Ds-cy* reverts back to the active state at a low sponta-

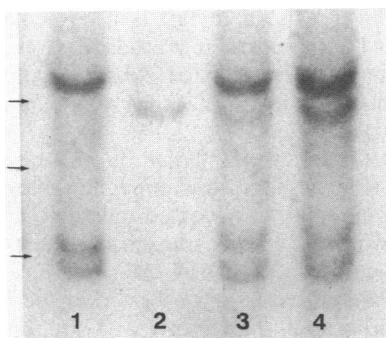

FIG. 4. Southern blot of *Bam*HI-restricted DNA hybridized with the *Sal* 3 probe. Lane 1, DNA from *wx-m9*::*Ds*/*wx*, *Ac* heterozygotes carrying an unlinked *Ac*; lane 2, *wx-m9*::*Ds*/*wx-m9*::*Ds*; lanes 3 and 4, DNAs of seedlings grown from *wx*, *bz2* variegated kernels on class B and class A ears, respectively, of the genotype *wx-m9*::*Ds-cy*, *bz2-m*/*bz2-m*, *Ac* (see cross 2 in Fig. 5). DNAs in lanes 1, 3, and 4 have the same *Ac*. Arrows indicate the positions of the 9.4-, 6.7-, and 4.4-kb markers.

neous rate (7), but reactivation is greatly enhanced by the presence of an active *Ac*. In contrast to the demethylation of the *Bam*HI site in *Ds9*, this reactivation persists in the absence of *Ac*. Tests were performed to determine if demethylation of the *Bam*HI site in *wx-m9*::*Ds-cy* is responsible for the reactivation. The test was made feasible by the finding that a second unlinked gene is involved in the reactivation in addition to an active *Ac* element. If reactivation results from demethylation of the promoter region of *wx-m9*::*Ds-cy*, differences in the degree of demethylation of the *Bam*HI site would be expected in genotypes that possess or lack the second gene. The genetic analysis that indicated the involvement of the second gene follows.

Twelve heterozygous plants of genotype *wx-m9*::*Ds-cy*/*wx*, *bz2-m*/*bz2-m* were crossed to *wx*/*wx*, *bz2-m*/*bz2-m*, *Ac* plants. This *Ac* has not been mapped but it is not linked to the waxy locus. From each ear, kernels variegated for *Bz*(*bz2-m*→*Bz*) and *Wx*(*wx-m9*::*Ds-cy*→*Wx*) were scored and planted as separate families. The plants must have carried an active *Ac* and the *wx-m9*::*Ds-cy* allele and thus were of the genotype *wx-m9*::*Ds-cy*/*wx*, *bz2-m*/bz2-*m*, *Ac*. Plants in each family were fertilized by *wx*, *bz2-m* tester pollen and the ears were scored for *Bz* and *Wx* variegation. These series of crosses are outlined in Fig. 5.

The 12 families are divided into two classes, A and B. In the final crosses to the *wx*, *bz2-m* testers, the 7 families designated A produced approximately equal proportions of ears (51:58) that showed 50% *Bz* variegated kernels, half of which were *Wx* variegated, indicating the presence of only a single active *Ac* element, and ears with 75% *Bz* variegated kernels, of which two-thirds were *Wx* variegated, indicating reversion of the *wx-m9*::*Ds-cy* to active form. No reactivations of *wx-m9*::*Ds-cy* occurred in the 5 families classed as B since they showed the presence of only a single *Ac* element unlinked to the *Wx* gene and produced ears with 50% *Bz* variegated, 25% *Wx* variegated kernels. These results implicate the segregation of a second unlinked gene, shown in parentheses in the diagram of the crosses, with the + allele required for *Ac*-induced reversion of *wx-m9*::*Ds-cy* to an active *Ac*. In the presence of both an active *Ac* and the second gene, the methylated element in *wx-m9*::*Ds-cy* appears to always revert to the active form.

The *Bam*HI sequence of the *wx-m9*::*Ds-cy* is modified and not cut by the restriction endonuclease. However, the intro-

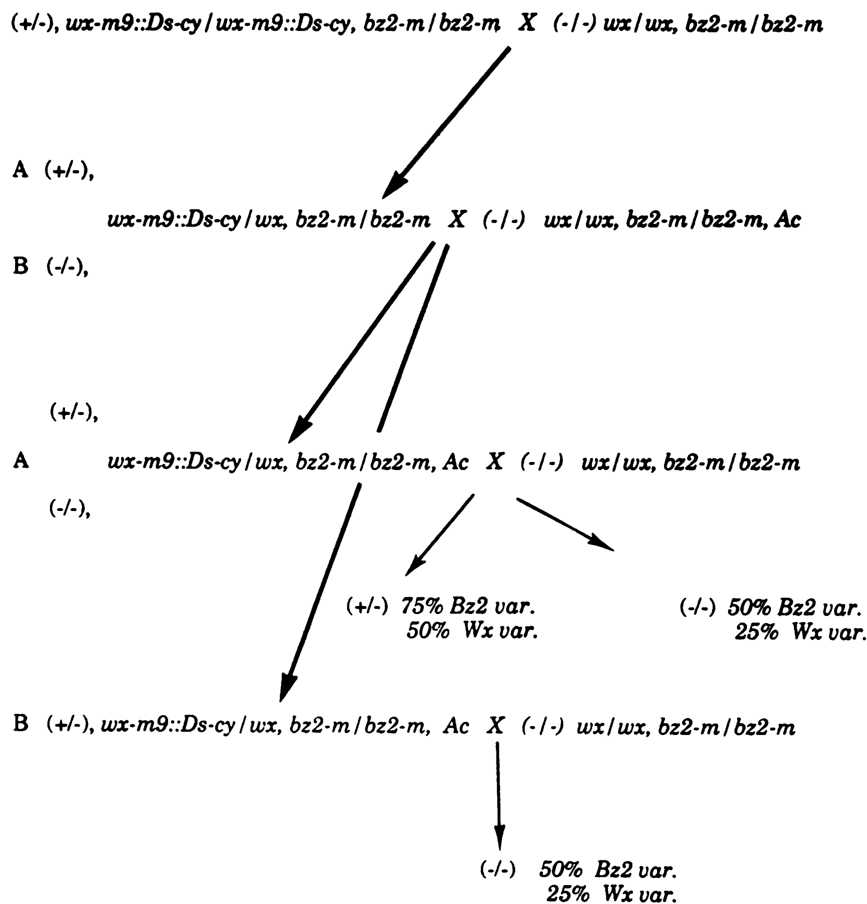

FIG. 5. Diagram of crosses that indicate the involvement of a second gene, designated in parentheses, in the *Ac*-induced activation of the *wx-m9*::*Ds-cy* allele.

duction of the same unlinked *Ac* that produces almost complete demethylation of the *Bam*HI target in *Ds9* results in a lesser degree of demethylation of that site in the highly methylated *wx-m9*::*Ds-cy* element (Fig. 4). This suggests that the demethylation of the *Bam*HI site is influenced by the methylation state of the rest of the element and offers support for the hypothesis presented below that the *Ac*-induced site-specific demethylation results from an alteration in chromosome structure. The second gene does not appear to be involved in the demethylation of the *Bam*HI site of the *wx-m9*::*Ds-cy* element. Seedlings of the presumed genotype *wx-m9*::*Ds-cy*/*wx*, (−/−), *Ac*, grown from *Wx* variegated kernels on ears in class B, which have no reactivation of the inactive methylated element, do not show a reduction in the degree of demethylation of the *Bam*HI site as compared to the (+/−), class A material (Fig. 4).

The *Ac* element makes a trans-acting product that is responsible for the demethylation of the *Bam*HI site in the promoter region of the *Ds9* element. Since the sequences of *Ac9* and *Ds9* are identical in the promoter region, the *Bam*HI site of the *Ac* element is most likely also kept in the unmethylated condition by the action of its transposase product binding to its own promoter region. This cyclic process would produce a steady-state condition that could be responsible for maintenance of *Ac* activity. According to this model, methylation of the promoter region of *Ac* shuts off transcription. An active *Ac* makes an RNA transcript that produces a transposase that keeps the element in the actively transcribed state by binding to the promoter and blocking the action of the methylase. Any interruption of this cycle should lead to methylation of the promoter and gene inactivation. The 194-base deletion in *Ds9* causes a shift in the reading frame resulting in the formation of defective protein that cannot bind to the promoter region. Support for this hypothesis comes from the observation of Kunze *et al.* (19) that only a very small amount of the 3.3-kb transcript is made by *Ds9* in comparison with the amount of the 3.5-kb transcript made by *Ac9*. A small proportion of DNA of *Ds9* is cut by *Bam*HI endonuclease and this fraction of DNA that is unmethylated in the promoter region could account for the small amount of transcript produced.

The demethylation of the cytosine residues induced by the active *Ac* appears to be specific, affecting only *Ac*. No change in overall cytosine methylation is observed associated with a presence of an active *Ac* in the genome, when comparing the degree of restriction of DNA by *Hpa* II, *Pvu* II, and other methylation-sensitive endonucleases. Furthermore, demethylation appears to be specific for a particular site within the element. There are two methylated regions at the termini of the *Ac* element, the *Pvu* I site about 71 bases from the terminus at the promoter end of the element (Fig. 1) and a cluster of three *Hpa* II sites within 181 bases of the downstream end of *Ac* (7). In *Ac*, the cytosine residues in these sites remain methylated, whereas the cytosine of the *Bam*HI site, only 112 residues downstream from the *Pvu* I, is unmethylated. Both the *Bam*HI and the *Pvu* I sites are methylated in the *Ds9* element. Introduction of an unlinked *Ac* into the genome results in almost total demethylation of the *Bam*HI target, whereas the *Pvu* I target remains methylated and is not cut by the restriction enzyme (Fig. 6).

Razin and Riggs (1) have proposed several mechanisms for specific demethylation. The possibility that the transposase product of *Ac* is a demethylase is considered unlikely. The ability of the transposase to excise and transpose elements of the *Ac* system must be unrelated to the demethylation of the *Bam*HI and promoter region of *Ac* since this region is not present in the small 405-base *Ds1*-type element (23, 24), which is readily transposed in the presence of an *Ac*. The *Ds1* element does not contain a *Bam*HI target sequence.

A limiting level of the methylase is also ruled out since the *Bam*HI site in the *Ds9* plants that produce no *Ac* transposase is almost completely methylated, whereas that site in *Ac9* plants, which differ only in that they make an active transposase, is demethylated.

Blockage of the *Bam*HI target by the binding of the transposase to that site, thus preventing access to the methylase, is possible but deemed unlikely in view of the following consideration. The transposase must bind to the ends of the element since it causes excision by cleavage at the termini. The *Bam*HI site, 185 bases from the terminus, is demethylated whereas the closer *Pvu* I site, only 71 bases from the terminus, remains methylated.

I propose that the demethylation of the *Bam*HI results from an alteration in chromatin structure in the promoter region as a result of the binding of the transposase to the termini of the *Ac* element. The altered configuration renders the site inaccessible to the methylase. Support for this model comes from experimental results that indicate that a structural alteration of one site can affect methylation of DNA at a distant site. The *Pvu* I site in the 3′ end of the waxy gene at position 3258 (16) is not methylated. The upstream *Pvu* I site is at position 25. The expected 3.2-kb *Pvu* I band is observed in Southern blots of DNA extracted from *wx*/*wx* plants and probed with the unique *Sal* 3 fragment (15) of the waxy gene (Fig. 7). However, in *wx-m9::Ac*, with the 4.5-kb transposable element inserted at position 2466 of the waxy gene, the *Pvu* I site at 3258 is methylated and not cut by the corresponding restriction endonuclease. A large band of ≈9 kb is seen in the Southern blots. This is the *Pvu* I fragment size expected when the *Pvu* I site at 3258 as well as the nearby *Pvu* I site at position 3644 are methylated (Figs. 1 and 7). These sites are also methylated in the *wx-m9*::*Ds* and *wx-m9*::*Ds-cy* alleles. This is not a result of methylation of flanking DNA by the presence of a DNA segment that is methylated at both ends. There are two *Pst* I sites and a *Sal* I site between the point of insertion of *Ac* and the downstream *Pvu* I site, at positions 2584, 2758, and 2974, respectively. These sites are unmethylated in the waxy allele containing the *Ac* insert, whereas the *Pvu* I sites that are farther downstream, 870 and 1186 bases from the insert, are methylated. To determine if it is the presence of the *Ac* insertion that is responsible for the methylation of the *Pvu* I site, I have analyzed three *Ac* transpositions where the element moved out of the waxy locus to nearby positions, within three map units. In all three cases the *Pvu* I target at position 3258 reverted to the unmethylated condition and the 3.2-kb *Pvu* I band reappeared in the Southern blots (Fig. 7).

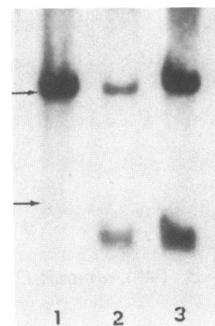

FIG. 6. Southern blot of seedling DNA restricted with *Pvu* I and probed with the *Sal* 3 fragment. Lane 1, *wx-m9*::*Ds*/*wx-m9*::*Ds*; lane 2, *wx-m9*::*Ds*/*wx*, *Ac* (the same DNA sample digested in Fig. 4, lane 1); lane 3, *wx-m9*::*Ds-cy*/*wx*. The arrows indicate the 9.4- and 4.4-kb size markers. Note that the size of the *Pvu* I fragment in the three digests is the same, indicating that the *Pvu* I site in *Ds9* is not demethylated in the presence of an active *Ac* (lane 2).

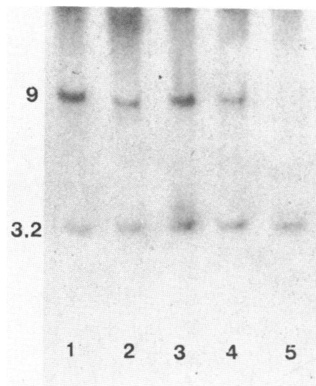

FIG. 7. Southern blot of seedling DNA restricted with *Pvu* I and probed with the *Sal* 3 fragment. Lane 1, *wx-m9*::*Ac*/*wx*; the 9-kb band is derived from the *wx* allele. Lanes 2–4, digests of seedlings grown from *Wx* variegated kernels of the genotype *Acwx**/*wx-m9*::*Ds*. The *wx** alleles are derivatives of *wx-m9*::*Ac* resulting from excisions of the *Ac* element and reinsertion into closely linked sites. They represent independent transpositions. Lane 5, *wx*/*wx*. Note the similarity in size (indicated in kb) of the smaller fragment in the five lanes.

The assistance of Kim McManus is greatly appreciated. This work was supported by National Science Foundation Grants PCM 83-19544 and DCB 87-07071.

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