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DNA Methylation of Maize Transposable Elements is Correlated with Activity

Elizabeth S. Dennis and R. I. S. Brettell

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DNA methylation of maize transposable elements is correlated with activity

By Elizabeth S. Dennis and R. I. S. Brettell
CSIRO Division of Plant Industry, GPO Box 1600, *Canberra, ACT* 2601, *Australia*

Transposition of the maize transposable elements *Ac*, *Spm* and *Mu* is correlated with a lack of methylation. For both *Ac* (Schwartz & Dennis 1986) and *Spm* (Fedoroff *et al.* 1988) methylation of a region upstream and downstream of the site of initiation of transcription results in a loss both of mRNA encoding for the transposase and the ability to excise. We have studied the activation of an inactive, methylated derivative of *Ac* by using a cycle of tissue culture. A passage through tissue culture results in activation of the element and is correlated with demethylation of the 5′ end of the transposase gene. Demethylation and activation may occur as a multistep process.

We propose a model in which binding of the *Ac* transposase to the 5′ region of the *Ac* element prevents methylation following replication. Consequently, demethylation and reactivation occurs.

1. Introduction

Maize has provided geneticists with a visual assay for the effects of transposable elements on gene activity. The colour genes of the anthocyanin pathway (e.g. *bronze*), expressed in the outer or aleurone layer of the endosperm, can be used as targets for transposable element insertions. Two other endosperm markers, *waxy*, coding for a UDPG-glucosyl transferase catalysing the synthesis of amylose from amylopectin, and *shrunken*, which encodes sucrose synthase and gives a 'collapsed' kernel phenotype, are also useful.

A colour-marker gene interrupted by a transposable element capable of excising from the gene generates a kernel with a variegated phenotype; cells in which the gene has been inactivated by insertion of the element look colourless, but upon somatic excision of the element, spots due to colour production by the active gene will appear. The frequency and size of the coloured spots are a measure of the time and frequency of excision.

Transposable elements appear to encode a transposase responsible for the specific transposition of that element. When deletions are introduced into the region of a transposable element coding for the transposase, an active transposase is not produced; the element may still transpose if the transposase is made by another element present in the genome. The non-autonomous but receptive mutant form of the *Activator* (*Ac*) element of maize is called *Ds*.

In several transposable-element systems an autonomous element can be inactivated reversibly. In three maize transposable-element systems, *Activator* (*Ac*), *Suppressor–Mutator* (*Spm*) and *Robertson's Mutator* (*Mu*) the reversible loss of transposition ability has been correlated with methylation of the element.

2. *Ac* and methylation

(*a*) *Activity of the Ac element in the wx-m9 locus is correlated with the degree of methylation*

The transposable element *Activator* (*Ac*) is 4.5 kilobases (kb) long and has the structure typical of a plant transposable element – 11 base pair (b.p.) inverted repeats on the ends of the

element, with a single mismatch at the end of the repeats. *Ac* generates an 8 b.p. direct duplication of the flanking sequences upon insertion into genomic DNA. *Ac* encodes a single protein of 807 amino acids, which is presumably the transposase (Kunze *et al.* 1987). S_1-nuclease mapping defined two major initiation sites and a scatter of minor 5′ initiation sites over a 90 b.p. region (Finnegan *et al.* 1988). The 5′ untranslated region is 600–690 bases long and has a base sequence that is CpG-rich relative to the rest of the element.

From an allele of the *waxy* gene in which *Ac* is active, *wx-m9 Ac*, a derivative allele, *wx-m9 Ds-cy*, was isolated in which the *Ac* had become inactive (Schwartz & Dennis 1986). In this allele *Ac* is no longer able to catalyse its own transposition or that of non-autonomous *Ds* (*Dissociation*) elements.

Revertants to fully active *Ac* elements were isolated from the *wx-m9 Ds-cy* (inactive *Ac*) allele. Cloning of the parent *Ac* element, the inactive allele and the active revertant allele showed that there were no changes in the sequence resulting from deletions or insertions greater than about ten base pairs. No differences between the alleles were detected with restriction enzymes that cut frequently (Schwartz & Dennis 1986).

Two other features of reversion of the inactive *Ac* were noted: firstly, that the frequency was increased by the presence of an active *Ac* on the genome, and secondly, that reversion to an active *Ac* is a multistep process (Schwartz & Dennis 1986). Reversions to the active form occur late in development and spots are clustered in a small sector of the aleurone; the sectored revertant kernels tend to occur in sectors on the ear.

In the *Ac* element there are 13 *Hpa* II restriction enzyme sites; three sites in the 3′ untranslated region of the transposase gene, ten in the 5′ region and none in the coding region of the gene. *Hpa* II does not cleave its target site, CCGG, if the internal C is methylated, so the methylation status of *Hpa* II sites can be determined. The three 3′ sites are methylated in the parental active *Ac* element but the ten 5′ sites are unmethylated. In the mutant allele (*wx-m9 Ds-cy*) where *Ac* resembles a non-autonomous *Ds* element, all the *Hpa* II sites are methylated, both at the 3′ and 5′ ends of the element. In alleles that have reverted to full *Ac* activity, a majority of the 5′ *Hpa* II sites are unmethylated (figure 1) (Schwartz & Dennis 1986).

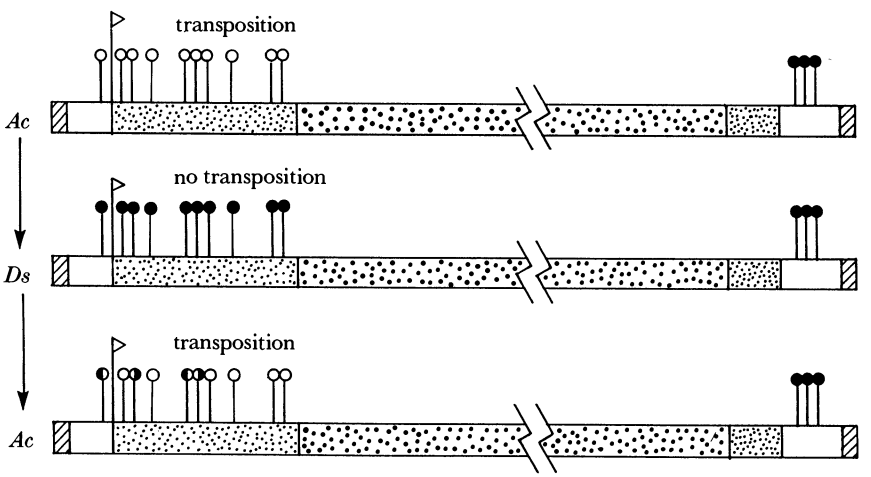

FIGURE 1. Methylation and *Ac* activity. Diagram shows the *Hpa* II sites in the *Ac* element and the effect of their methylation on transposition. The *Ac* element coding and intron region is shown dotted; the GC rich region stippled. *Hpa* II sites are indicated by circles, filled if the site is methylated and open if not. The start of transcription of the *Ac* transposase is indicated by a flag.

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There are two *Pvu* II sites in the *Ac* element, located in the coding region and intron IV of the gene, which are unmethylated in the active *Ac* in *wx-m9 Ac*. In the inactive *wx-m9 Ds-cy*, the site at position 3243 (in the coding region) is always methylated, whereas the *Pvu* II site at position 718 (in intron IV) is only sometimes methylated. Upon reversion to an active *Ac* element the *Pvu* II sites remain methylated (Schwartz & Dennis 1986).

There is therefore a correlation between the activity of the *Ac* element and the methylation of the 5′ region of the gene as judged by *Hpa* II digestion but not with methylation of the coding region as judged by *Pvu* II digestion. The genomic regions of the *waxy* gene flanking the *Ac* sequence were not methylated, even sites within 200 b.p. of the element.

The activity of the element is also correlated with the presence of a transcript of *Ac*, i.e. in inactive *wx-m9 Ds-cy* there is no transcript, whereas both the *wx-m9 Ac* and revertants to *Ac* activity have an *Ac* transcript (figure 2; Kunze *et al.* 1988).

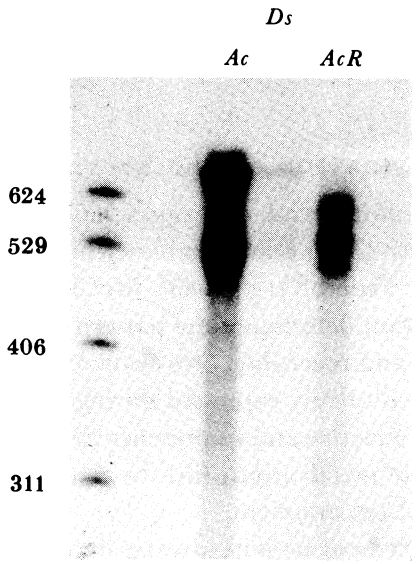

FIGURE 2. Detection of an *Ac* transcript by S_1 nuclease mapping in active and inactive alleles of *Ac*. RNA was extracted from seedlings of an active *Ac* (*wx-m9 Ac*), an inactive *Ac* (*wx-m9 Ds-cy*) and a revertant to active *Ac* (*AcR*). The upper band is undigested probe, two bands at 570 and 510 result from the protection by the RNA of a uniformly labelled probe (probe 5, Finnegan *et al.* (1988)) overlapping the 5′ end of the transcript.

The same *wx-m9 Ds-cy* allele was used by Schwartz in an assay for variation in methylation during development (Schwartz 1988). He showed that there is differential methylation of the *Ac* element in the *wx-m9 Ds-cy* allele when transmitted through the male or female gametes. Reciprocal crosses were made in which the *wx-m9 Ds-cy* allele was used as either the female or male parent and DNA then extracted from the endosperm. (Endosperm is triploid tissue with two copies of the genome of female origin and one of male.) Analyses of the restriction patterns of the endosperm DNA indicated a striking difference in the methylation of the *Ac* element in the *wx-m9 Ds-cy* allele depending upon its origin. Cytosine residues in the *Pvu* II site at position 718 in the male-derived *wx-m9 Ds-cy* DNA appear to be completely methylated; only *Pvu* II sites in the flanking DNA are cut. The same *Pvu* II site in the female-derived *wx-m9 Ds-cy* allele is only methylated occasionally. This difference is not found in the embryo, as DNA extracted from germinating seedlings has the same methylation pattern of this

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Pvu II site, whether the allele is derived from the male or female parent. Schwartz suggests that maintenance methylation of *Ac* during endosperm development accounts for the difference between alleles.

(*b*) *Activity of the Ac element in the wx-m7 locus is correlated with methylation*

McClintock described a reversible type of *Ac* inactivation in which an *Ac* element cycles between an active and inactive phase during plant and kernel development (McClintock 1963, 1964). Initial studies of this phenomenon centred on the *Ac*-induced *waxy* mutation *wx-m7 Ac*. When *Ac* at *wx-m7* is inactive the *Ac* element cannot transpose autonomously or transactivate a *Ds* element. Chomet *et al.* (1987) examined active and inactive alleles of *wx-m7 Ac* and showed that the *Ac* element was methylated at the *Pvu* II sites in the inactive but not the active alleles; this parallels the situation in the *wx-m9* allele. Several *Eco*R II and *Sst* II sites located in the 5′ region of the gene were also methylated in the inactive but not the active allele. Because this allele is cycling there are no revertants to full *Ac* activity that can be examined, but the results obtained are in agreement with those obtained from the *wx-m9* allele.

3. Modification of the *Spm* element

In a series of experiments, Fedoroff and colleagues have studied the inactivation and activation of the *Suppressor–Mutator* (*Spm*) transposable element at a molecular level (Banks *et al.* 1988; Fedoroff & Banks 1988; Federoff *et al.* 1988). McClintock (1961) first discovered the existence of a genetic mechanism that determines the pattern of *Spm* element expression in the maize plant that is both heritable and reversible. *Spm* elements can exist in one of three states: (i) a state when the element is constitutively expressed during development; (ii) a cryptic state in which the element is genetically inactive and its presence can only be detected following rare spontaneous or induced instances of reactivation; (iii) the element is programmed to be active in a particular temporal or spatial environment.

The mechanism that controls element activity during development has been divided into two components. One component determines whether the element is expressed or not (phase setting) and the other determines the stability of the level of expression in development, its ability to be inherited and activity in the next generation (phase programme). The programming mechanism is correlated with the developmental determination of apical and lateral meristems.

When the ability of the programme to be inherited is examined it is seen that the inactive state is less frequently transmitted through tiller gametes than stalk gametes, i.e. the element is more likely to be activated if gametes come from a tiller. Tillers, which develop from axillary buds at the bottom of the plant, have a developmental history different to that of the main inflorescence that gives rise to the stalk gametes. The *Spm* element also appears to remain inactivated more frequently on transmission through the male than through the female gametes.

The forms of the *Spm* element are differentially methylated but, as with *Ac*, the flanking genome sequences are not methylated. The different forms of the element can be distinguished by the pattern of cytosine (C) methylation immediately up- and downstream of the start of transcription of the element in a 0.35 kb long, 80% G+C region. The active, inactive (programmable) and cryptic forms are unmethylated, partly methylated and fully methylated

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respectively (Banks *et al.* 1988). The stability of the inactive phase is also correlated with the pattern of methylation of the multiple C residues in this region. An active *Spm* element in the genome with a cryptic element promotes its partial demethylation but not its activation. In contrast, an active *Spm* element promotes both demethylation and activation of an inactive element.

4. Modification of the *Robertson's Mutator* system

Robertson's Mutator (*Mu*) system was first identified in a line of maize with an abnormally high mutation rate (50- to 150-fold above that of controls) (Robertson 1978). This high mutation rate was shown to be due to the presence of a transposable element. The most common forms of the element are either 1.4 or 1.76 kb in length with inverted repeats on the ends. However, the elements do not appear to be autonomous (i.e. capable of catalysing their own transposition). There are approximately 50 copies of the element in the genome of *Mutator* lines, and lower numbers in the genome of non-*Mutator* lines. The element that controls the transposition of *Mu* has not been identified. *Mu*-induced unstable mutants in aleurone markers (such as *bronze*) have allowed mutator activity to be studied genetically. Stable mutants of the colour gene, resulting from loss of the transposition ability of *Mu*, occur mainly when *Mutator* lines are inbred, but also in some out-crosses. Loss of somatic instability of a mutant, i.e. loss of *Mu* excision, measured as a loss of variegation on the kernel, correlates with an increase in the level of DNA methylation of the *Mu* elements (Chandler *et al.* 1986; Bennetzen *et al.* 1988). Methylation can be progressive during the process of inactivation. Analysis of bulk DNA methylation showed that upon inactivation the level of C methylation of *Mu* sequences increased from 47 % of all C residues (70 % of all CG and CNG sites) in an active element, to 70 % of all C residues (100 % of substrate sites).

Once established, the inactive state of the *Mutator* line is relatively stable. The state of DNA methylation shows maternal dominance in crosses between active and inactive lines. When used as the female parent, inactive lines can suppress the activity of *Mu* elements in *bronze2* transmitted through the pollen. In reciprocal crosses the *bronze2* allele can be unaffected by the modified *Mu* elements introduced from the pollen (Walbot *et al.* 1988).

When the *Mu* element becomes genetically inactive, all copies of *Mu* in the genome are similarly modified (reviewed in Bennetzen *et al.* 1988; Walbot *et al.* 1988). As in *Ac*, only the sequences within the element, and not the flanking DNA sequences, become modified. When seeds of inactive *Mutator* lines were irradiated (15000 R), 0.1 % of progeny kernels showed reactivation of *Mu* and lost the methylation of the *Mu* elements (Walbot 1988).

5. Activation of transposable elements in genomes that do not possess transposable element activity

Multiple copies of sequences related to each of the transposable elements are present in the genomes of all maize plants; most frequently these are genetically silent. A central question is whether these sequences are identical to functional transposable elements but are inactive because they are modified in some way, e.g. by methylation, or whether they are inactive because of sequence alterations in the element.

Transposable elements were first identified in maize stocks undergoing chromosome breakage. McClintock obtained evidence for the activation of *Ac*, *Spm* and *Dt* in plants

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undergoing a breakage–fusion–bridge cycle (McClintock 1950, 1951). Further evidence for chromosome breakage causing activation of transposable elements came from studies showing that UV- or X-ray irradiation induced activation of both *Ac* and *Spm* (Nueffer 1966). More recently, Rhoades & Dempsey (1982) detected three new mutable systems in lines undergoing chromatin loss, suggesting that chromosome breakage had activated these previously silent elements.

Burr & Burr (1988) used ethylmethane sulphonate (EMS) treatment to activate cryptic *Spm* elements in the genome. This agent is thought to cause point mutations but is known also to cause chromosome breakage. Molecular comparison of the activated *Spm* element with the silent *Spm* precursor has not been made, and is critical for determining whether a point mutation can activate a transposable element. It is also possible that EMS treatment induces an error-prone DNA repair mechanism similar to the SOS system in *E. coli*.

A third way of inducing cryptic transposable elements is via a cycle of tissue culture. The plant tissue culture process generates genetic variation among cultured cells and regenerated plants. Nearly all common types of chromosomal aberration have been observed in tissue culture or among regenerated plants. There is a relatively high frequency of structural alterations, as up to 20% of maize plants regenerated from culture can contain visible chromosome interchanges, inversions or duplications (Phillips & Peschke 1988). These observations led Phillips and Peschke to search for activation of cryptic transposable elements in maize plants after a cycle of tissue culture. Regenerated plants were crossed to a stock that contained a test allele for *Ac* activity. Ninety-four embryo cell lines gave rise to 301 plants, and of these, 11 (coming from three embryo cell lines) or 3.7% of plants showed an active *Ac*. Control plants, grown from mature seed on the same ears from which the embryos were taken to initiate cultures, were uniformly negative in the test crosses. Active *Ac* elements were not present in the donor plants but must have been present in inactive or cryptic forms that were reactivated in some regenerated plants. The mechanism of activation was not identified, nor was there a molecular analysis of the newly activated *Ac* elements.

6. Molecular analyses of a cycle of tissue culture reactivated *Ac*

We have recently used the *wx-m9 Ac*/*wx-m9 Ds-cy* system to investigate whether tissue-culture conditions can lead to activation of a particular *Ac* element that had been inactivated by methylation (*wx-m9 Ds-cy*).

Plants heterozygous for the *wx-m9 Ds-cy* allele were self-fertilized, and 12 days after pollination immature embryos were dissected out and placed into tissue culture. The remainder of the kernels on the cob were allowed to mature. Plants were regenerated from tissue culture after various times in culture, commencing after 6 weeks and continuing for 9 months. Regenerated plants were allowed to flower, and pollen was used either to self-fertilize plants or to fertilize a stock containing an *Ac* tester allele. In the presence of an active *Ac* element, kernels from this cross appear variegated, owing to excision of the *Ds* element from the bz_2 gene. In the absence of an active *Ac* (e.g. in *wx-m9 Ds-cy*) the kernels appear yellow as there is no excision of the *Ds* element. The *wx-m9 Ds-cy* line is homozygous for the tester bz_2m allele, so self-fertilization can be used as a test for activation. Pollen from plants grown from kernels from the same cob but that had not had a cycle of tissue culture was used on similar tester plants as a control pollination.

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Twelve different embryos gave rise to plants that produced pollen. In most cases several different plants were regenerated from the same embryo. All embryos gave rise to some plants containing an activated *Ac* (table 1). In some plants from nearly all embryos there was medium to high spotting of the progeny of the selfed plants (50–75 % of kernels) and medium spotting of the cross to the *Ds* tester stock (about 25 % of kernels), indicating that the *Ac* element had become reactivated to near full activity. The general trend was that plants regenerated after a longer time in tissue culture showed a higher level of activity (table 2). When regenerants from within a single subculture are assayed for activation of *Ac*, later plants appear to have *Ac* elements that have become more activated. Second or later plants from the same culture appear to resemble those derived from the process of tillering seen in maize plants. The increased activation may parallel the increased activation of *Spm* seen with tiller transmission.

TABLE 1. ACTIVATION OF *wx-m9 Ds-cy* BY A CYCLE OF TISSUE CULTURE

(Summary of results obtained from a tissue culture cycle. Control seeds were seeds grown from the same cob as the embryos that were placed into tissue culture. Stressed plants were grown from control seed in small tubes.)

number of embryos introduced into culture	number of plants that produced pollen	number of plants with active *Ac*
12 control seeds	54	43[a]
7 stressed plants grown from seed	7	0
10	10	1 (low *Ac* activity)

[a] Some from each embryo. High activity, 22; medium activity, 10; low activity, 11.

Because the embryos put into culture were the product of self-fertilization, some embryos contained one copy of *Ac* and some two. Both genotypes showed activation. Data from the four families of plants Q_o, Q_p, Q_r and Q_u that were homozygous for the *wx-m9 Ds-cy* allele are shown in table 2. Similar data were obtained with the eight heterozygous embryos. Plants derived from embryo Q_o show a low level of activation that increases both within and between subcultures. There is one aberrant plant, $Q_o.3*3$, which showed no activity. This could be a result of chromosomal rearrangement affecting the *Ac* element.

Plants regenerated from Q_p embryos were the only family of the 12 not to display a high level of activation. The $Q_p.1$ regenerants showed an early trend of activation increasing with time, but subsequent plants showed little activity. The Q_r and Q_u families showed increasing levels of activation with time and achieved high levels of activity.

In control plants grown from seven seeds from the same cob used to initiate the embryo culture, no reactivation above the 1 % level generally seen with this allele (Schwartz & Dennis 1986) was detected.

Plants regenerated from tissue culture have an abnormal morphology. They are generally smaller, and flower earlier than plants grown from seed. The same morphological conditions can be induced by germinating seeds and growing them in the same small tubes used when plants are regenerated from culture. Ten seedlings containing the *wx-m9 Ds-cy* allele were grown in this way; the resulting plants had a morphology similar to, but not as extreme as, that seen for regenerants. When pollen from these plants was used to fertilize the tester (bz_2m *Ds*)

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TABLE 2. PERCENTAGE ACTIVATION OF THE *wx-m9 Ds-cy* ALLELE IN REGENERANTS

(The regenerated plant numbering is as follows: the name, e.g. Q_o, refers to the embryo that was introduced into callus. The number immediately following this refers to the subculture number, the number after the * is the plant number; high, H; medium, M; low, L and zero, 0 refer to the levels of activation; percentage activation is the proportion of kernels that were variegated.)

	regenerated plants	self-pollinated	pollination of tester stock	
1.	Q_o.2*1	24	11	L
	2	—	30	M
	3	—	40[a]	H
	5	73	—	H
	Q_o.3*1	64	23[a]	M
	2	—	53	H
	3	56[a]	54	H
	4	0	0[b]	0
	Q_o.4*1	78	—	H
2.	Q_p.1*1	5	14	L
	2	33	30	M
	3	—	0	0
	5	—	0[a]	0
	Q_p.2*1	4	7	L
	2	12	0[a]	L
	3	—	0	0
	5	0	0[a]	L
	6	0[a]	—	0
3.	Q_r.1*1	30	19	M
	2	46	—	M
	4	80[a]	—	H
	5	79[a]	—	H
4.	Q_u.1*1	12	4	L
	2	12	14	L
	3	0	—	0
	5	—	0	0
	Q_u.2*4	97	39	H
	Q_u.3*1	73	52	H
	2	—	90	H
	Q_u.4*2	100	—	H
	3	99	—	H

[a] Small sample, poor seed set.
[b] Cross was made reciprocally with pollen of tester stock.

stock, evidence of a low level of activation in one plant was seen. Nine plants did not show reactivation.

To determine whether the tissue culture-induced activation of the *Ac* element has involved changes in the methylation pattern of the *wx-m9 Ds-cy* allele, DNA was extracted from callus cultures and regenerant plants, cut with the methylation-sensitive enzymes *Hpa* II and *Pvu* II and hybridized to probes from the internal sequence of *Ac*. DNA was prepared from five different callus cultures that contained *Ac*, three containing one copy and two, two copies. Plants obtained from all these callus cultures showed *Ac* activity. The callus cultures had been propagated for a long time – five subcultures over nine months. Many of the plants had been regenerated after one, two or three subcultures. When the callus DNA was digested with *Hpa* II, evidence for demethylation was obtained (figure 3). The lowest band corresponded to that of the active (demethylated) *Ac*, and there were longer bands indicating partial methylation of the *Hpa* II sites at the 5′ end of the gene. This pattern is very similar to that seen in revertants to an active *Ac* selected genetically, i.e. with no tissue-culture cycle (figure 3).

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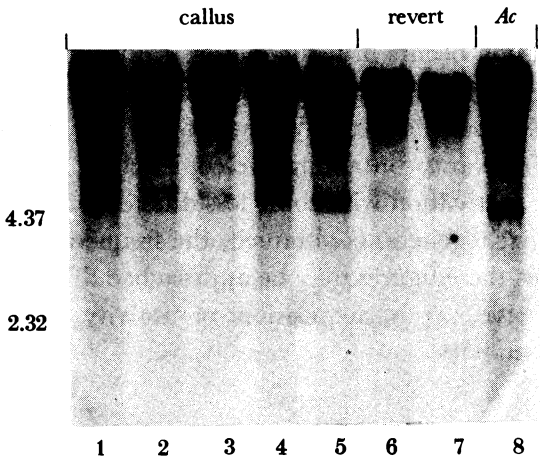

FIGURE 3. Demethylation of *Ac* occurs in tissue culture. Southern analysis of DNA extracted from callus culture: 10 μg of DNA was digested with *Hpa* II, analysed by electrophoresis, transferred and probed with radioactively labelled internal *Hind* II fragment from *Ac*. Callus cultures (lanes 1–5) were from embryos $Q_{af}.5$, $Q_q.5$, $Q_n.5$, $Q_e.6$ and $Q_u.6$. Lanes 6–7 were from plants selected genetically as revertants to full *Ac* activity; *Ac* is the active *wx-m9* *Ac* allele.

Pvu II also showed some evidence of demethylation, with callus cultures Q_n and Q_u showing most. These two cultures gave rise to plants with very active *Ac* elements by the third subculture. The pattern of methylation seen with *Pvu* II resembles that seen in plants grown without a tissue-culture cycle. *Pvu* II demethylation is not necessary for *Ac* activity. The 718 *Pvu* II site is sometimes methylated and the 3243 site is completely methylated.

It is clear that demethylation occurs in tissue culture and is probably a function of the time spent in culture. Two regenerants from different stages of the same callus (figure 4) show progressive demethylation with time (cf. lanes 5 and 6) resulting in a higher proportion of DNA in the lower band and paralleling the increase in activity. Most plants regenerate from a single

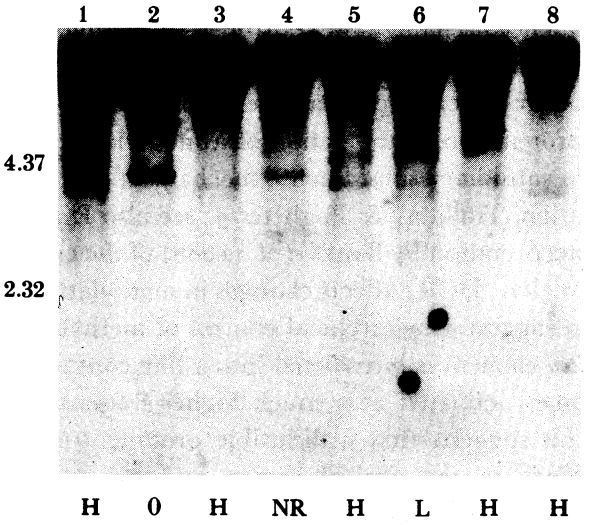

FIGURE 4. Southern analysis of the methylation status of the *Ac* element. DNA was extracted from regenerant plants and subjected to Southern analysis by using *Hpa* II digestion as in figure 3. Lane 1 is plant $Q_u.3*1$; lane 2, $Q_p.2*3$; lane 3, $Q_n.3*1$; lane 4, $Q_e.2*2$; lane 5, $Q_o.3*3$; lane 6, $Q_o.2*1$; lane 7, $Q_{af}.3*1$; lane 8, $Q_n.3*2$. The letters at the bottom indicate the degree of reactivation of the plants; H is high (i.e. > 40%); L is low (i.e. < 15%); 0 is 0% of kernels showing an active *Ac*. Lane 4 (NR) did not produce pollen.

cell. Thus the similarity between different plants regenerated from tissue culture at a given stage suggests that the activation occurs in tissue culture rather than during regeneration. If activation of the *Ac* element were complete, the expected frequency of *Ac* activation for a line containing a single copy of *Ac* would be 50%. When selfed, 75% of plants could contain one or more active *Ac* elements. For plants containing two copies of *Ac*, if both have been activated, out-crosses should give 100% activation with some loss due to excision and segregation of *Ac*. Our data show that in the first regenerants obtained, the frequencies are lower than this; in later regenerants from a callus these figures may be approached. Thus demethylation in tissue culture is probably progressive, as is acquisition of activity, because an incompletely demethylated *Ac* has partial activity.

7. Discussion

The nuclear DNA of higher plants is extensively methylated, primarily as 5-methylcytosine in the sequences 5′–CG–3′ and 5′–CNG–3′ (Gruenbaum *et al.* 1981). In wheat, C residues are methylated in over 90% of 5′–CG–3′ sites and 80% of 5′–CNG–3′. The situation in maize (another graminaceous monocot related to wheat) is similar, but it has been shown that the methylation is not evenly distributed and that sequences repeated more than 1000 times are more than 90% methylated. Single-copy genes are essentially unmethylated.

The data reviewed here show that transposable elements (*Ac* and *Spm*) need to be hypomethylated, at least in the 5′, CpG-rich, region up- and downstream from the site of initiation of the transcript for the transposase if they are to be active.

A specific methylation signal must exist because only sequences within the element, not the flanking genome sequences, are methylated. Either there is a sequence-specific methylase, which recognizes *Ac*, *Spm* or *Mu* sequences, or methylation is controlled within a domain. Such a domain would have one or more initiation sequences controlling *de novo* or maintenance methylation of other sequences within the domain in a hierarchical manner, and 'punctuation' sequences delimiting the domain and preventing 'spread' of methylation outside it. The activity of the initiation sequence itself might be controlled by methylation, and communication of a signal for methylation from the initiation sequence to the subsidiary methylation sites within the domain could occur either by cooperative binding, from the initiation sequence, of regulatory or chromatin proteins or by the action of a methylase moving processively along the DNA from initiation to subsidiary sites. Such a mechanism has been proposed in the case of X-chromosome inactivation (Holliday & Pugh 1975; see also Riggs, this symposium, for a further discussion). The interpretation by Banks *et al.* (1988) of the control methylation in *Spm*, where methylation in one region (DCR) affects changes in methylation at another (UCR) and programmability, seems to suggest a hierarchical control of methylation.

When an active *Ac* or *Spm* element is introduced into a line containing an inactive element, the inactive element becomes activated at a much higher frequency than when the active element is not present. This suggests that a diffusible product (the transposase is the only known product of *Ac*) binds to the critical region and prevents methylation following replication. Deletions in this GC-rich leader region of *Ac* are completely active and may have a higher frequency of transposition (Coupland *et al.* 1988).

As well as inactive forms of the transposable elements that can be readily reactivated, there

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appear to be cryptic copies that can only be reactivated with much more difficulty. An active element cannot transactivate these cryptic elements.

Studies on the timing of transposition show that an active *Ac* element transposes at approximately the same time as replication occurs. Active *Ac* and *Spm* elements mostly occur in fractions of the genomic DNA that are hypomethylated and therefore probably transpose from one site of low methylation to another. These active *Ac* elements give rise, on rare occasions, to the inactive *Ac* element (Chen *et al.* 1987).

The multiple copies of the genetically silent *Ac* sequences occur in highly methylated DNA (Chen *et al.* 1987). Perhaps chromosome breakage is required to activate these elements. We are cloning these sequences to determine how closely they are related to active *Ac* elements.

Chromosome breakage occurred in the experiments of both McClintock (1950, 1951) and Rhoades & Dempsey (1982), where *de novo* activation of these elements is seen. Breakage and rejoining at an inactive or cryptic *Ac* element could provide a new promoter, permitting transposase synthesis and activation of inactive (but not cryptic) *Ac* elements elsewhere in the genome. However, the circumstances producing chromosome breaks may also induce DNA repair enzymes. DNA repair occurring close to DNA replication may result in loss of methylation from both strands of a DNA molecule, because of the lag in methylation after DNA synthesis (Pugh & Holliday 1978). Alternatively, repaired DNA may be less accessible for methylation, or DNA damage may directly inhibit the DNA methylase (Riggs & Jones 1983).

Both *Ac* and *Spm* possess 5′ regions of the element, close to the long 5′ untranslated leader region of the transposase that are critical for activity. This region is rich in methylation targets, for example, in *Ac*, of the 13 *Hpa* II sites in the element, none is in the coding or intron region, which is 3.4 kb in length, three are in the 3′ untranslated region and ten are in the 600 b.p. 5′ region of the gene. *Spm* has a similar distribution of methylation sites. Such an asymmetric distribution of methylation target could lead to rapid inactivation of the element by methylation, if the region became accessible to a methylase. For activation, transposase molecules may bind to this 5′ region and prevent methylation following replication. It also appears that demethylation can be a multistep process; we have evidence of intermediate levels of demethylation of *Ac* that are not active (e.g. lane 2, figure 4). This may reflect the multistep reversion process seen genetically.

8. Conclusions

The three plant-transposable elements systems *Ac*, *Spm* and *Mu* can be reversibly inactivated. This inactivation is correlated with methylation of the element. For *Ac* and *Spm* the methylation is of the 5′ region of the transposase gene up- and downstream of the site of initiation of transcription. Only the element itself and not the flanking DNA sequences are methylated.

Multiple copies of transposable element sequences occur in all lines, whether or not they contain a genetically active element. These might represent cryptic copies of the element that are in methylated regions of the genome and are not readily activated.

Activation of cryptic elements can occur by chromosome breakage induced by bridge–fusion breakage cycles, late replication and UV- or X-irradiation. A cycle of tissue culture can also induce activation of cryptic elements at low frequency and reactivation of inactive elements at

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high frequency. Reactivation of inactive elements is accompanied by demethylation, particularly of the 5′ region of the *Ac* element. The frequency of reactivation of inactive but not cryptic elements is increased by the presence of an active element in the genome; presumably the transposase made by the active element binds to the inactive element and prevents methylation of the critical region.

Transmission of the state of the inactivated element is different between male and female gametes. In *Ac* the male gamete is more highly methylated. In a similar fashion there is a higher probability of an inactive *Spm* element remaining inactive when transmitted through the male gamete.

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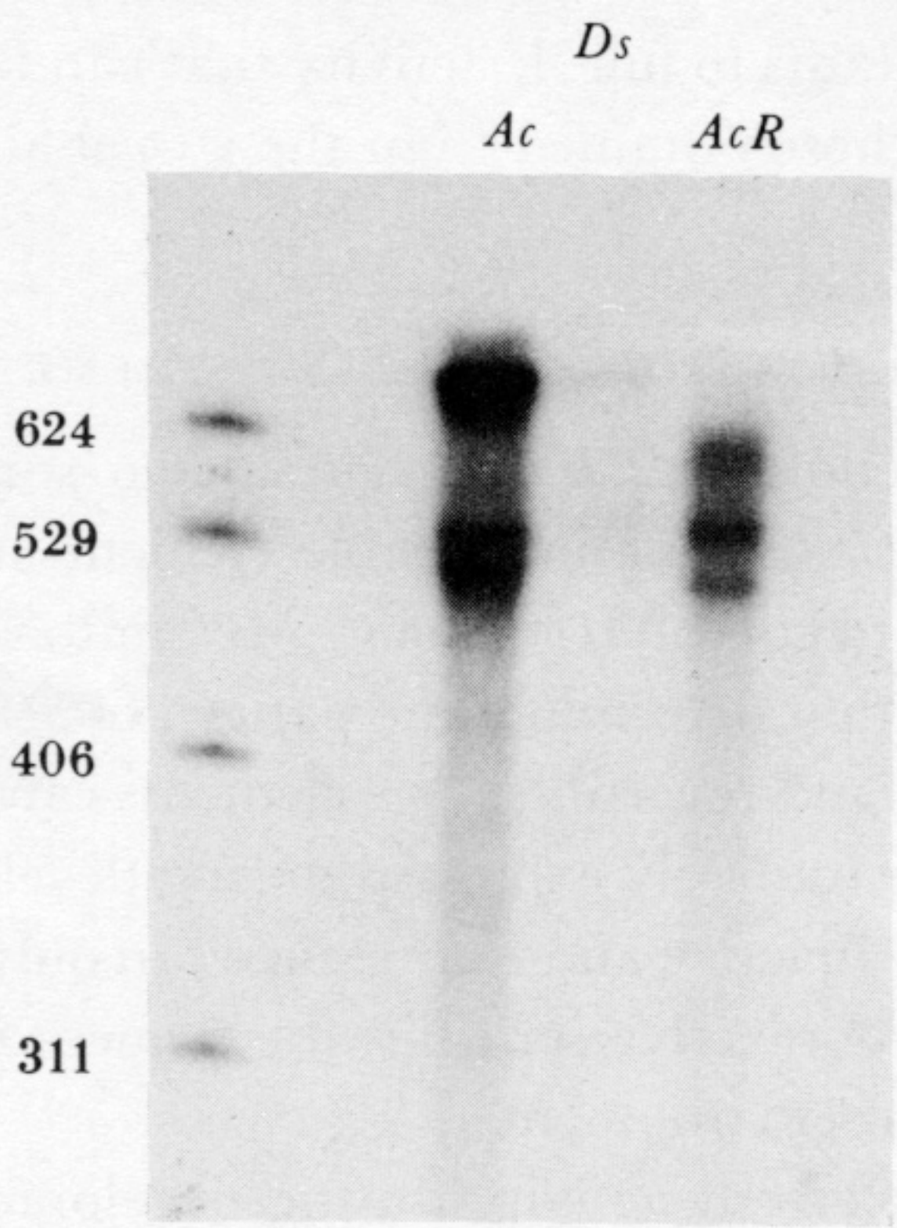

GURE 2. Detection of an *Ac* transcript by S_1 nuclease mapping in active and inactive alleles of *Ac*. RNA was extracted from seedlings of an active *Ac* (*wx-m9 Ac*), an inactive *Ac* (*wx-m9 Ds-cy*) and a revertant to active *Ac* (*AcR*). The upper band is undigested probe, two bands at 570 and 510 result from the protection by the RNA of a uniformly labelled probe (probe 5, Finnegan *et al.* (1988)) overlapping the 5′ end of the transcript.

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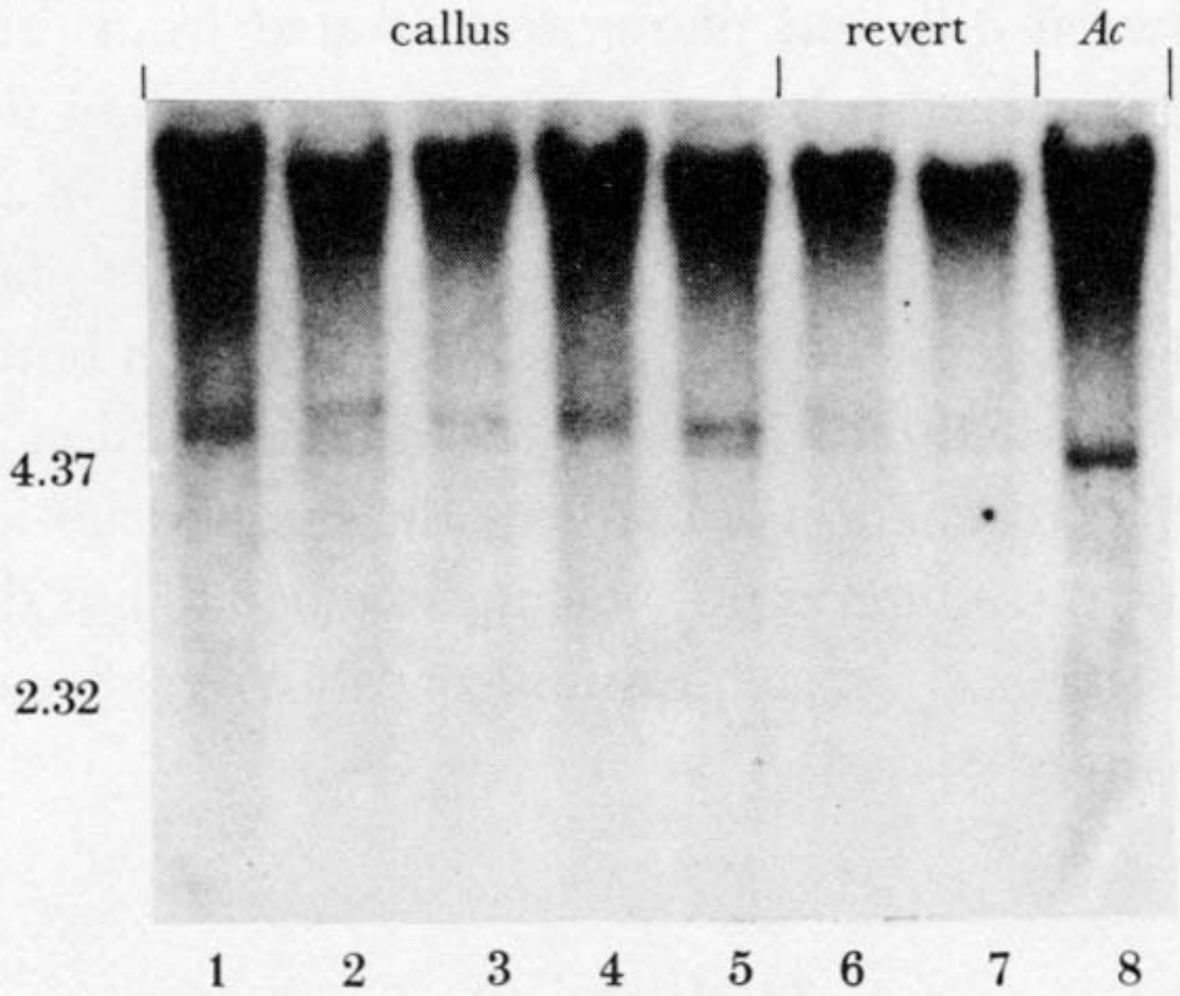

GURE 3. Demethylation of *Ac* occurs in tissue culture. Southern analysis of DNA extracted from callus culture: 10 μg of DNA was digested with *Hpa* II, analysed by electrophoresis, transferred and probed with radioactively labelled internal *Hind* II fragment from *Ac*. Callus cultures (lanes 1–5) were from embryos $Q_{af}.5$, $Q_{q}.5$, $Q_{n}.5$, $Q_{e}.6$ and $Q_{u}.6$. Lanes 6–7 were from plants selected genetically as revertants to full *Ac* activity; *Ac* is the active *wx-m9 Ac* allele.

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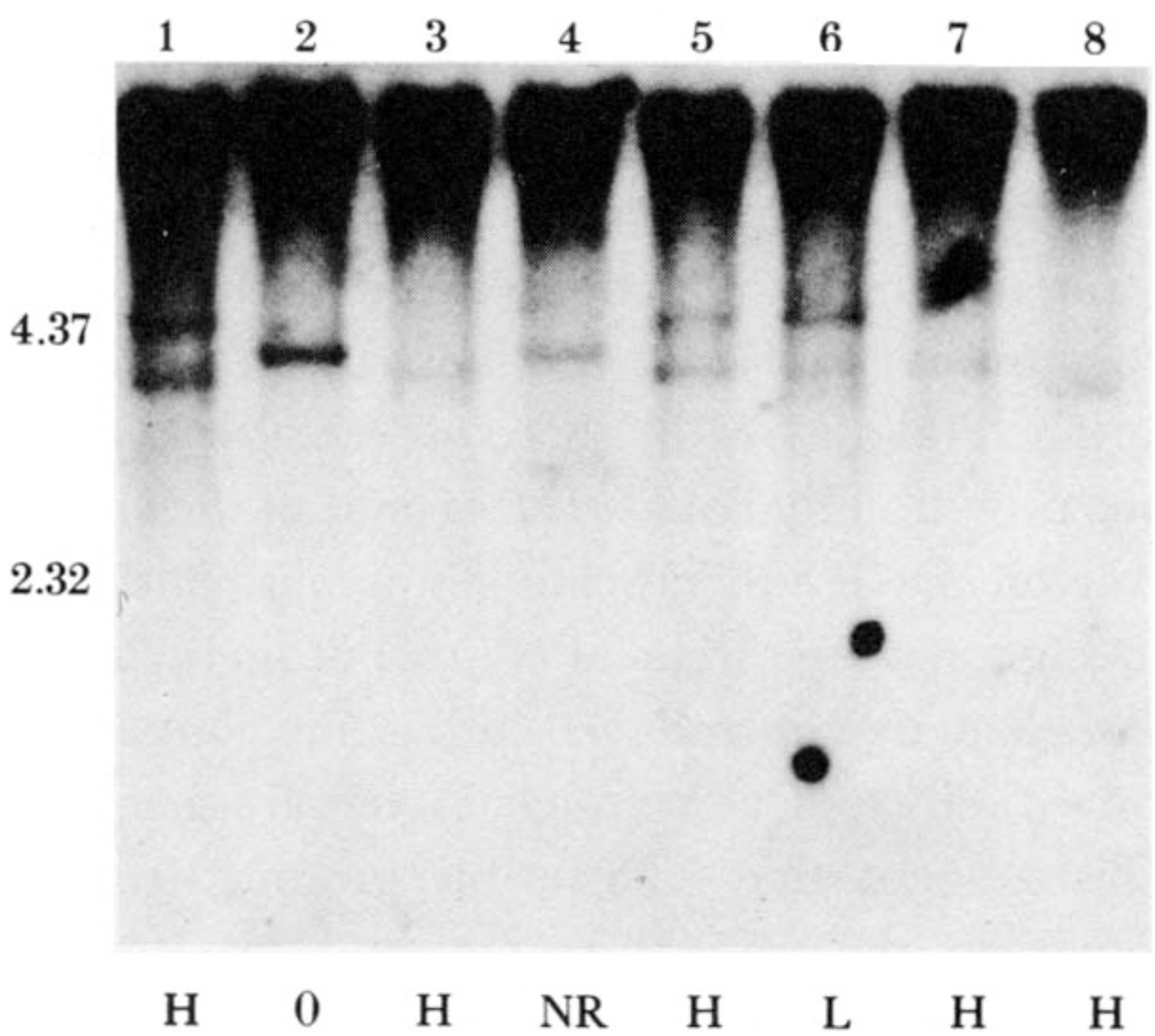

GURE 4. Southern analysis of the methylation status of the *Ac* element. DNA was extracted from regenerant plants and subjected to Southern analysis by using *Hpa* II digestion as in figure 3. Lane 1 is plant $Q_u.3*1$; lane 2, $Q_p.2*3$; lane 3, $Q_n.3*1$; lane 4, $Q_e.2*2$; lane 5, $Q_o.3*3$; lane 6, $Q_o.2*1$; lane 7, $Q_{af}.3*1$; lane 8, $Q_n.3*2$. The letters at the bottom indicate the degree of reactivation of the plants; H is high (i.e. $> 40\%$); L is low (i.e. $< 15\%$); 0 is 0% of kernels showing an active *Ac*. Lane 4 (NR) did not produce pollen.