Inactivation of the maize transposable element *Activator* (*Ac*) is associated with its DNA modification

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The *Activator* (*Ac*) element at the *waxy* locus (*wx-m7* allele) has the ability to undergo changes in its genetic activity and cycles between an active and inactive phase. Comparison of active *Ac* elements at several loci and the inactive *Ac* at *wx-m7* by Southern blot analysis revealed that the inactive *Ac* sequence was not susceptible to digestion by the methylation sensitive enzyme *PvuII* while active elements were susceptible to *PvuII* digestion. Restriction digest comparisons between the clones of the active and inactive *Ac* elements were indistinguishable. Further analyses with the enzymes *SstII* and the methylation sensitive and insensitive isoschizomers *EcoRII* and *BstNI* showed the inactive *Ac* sequence was methylated at these sites, whereas the active *Ac* was hypomethylated. Although the active *Ac* at the *wx-m7* allele in different genetic backgrounds showed differences in the *Ac* DNA modification pattern, at least a fraction of genomic DNA contained *Ac* sequences that were unmethylated at all of the internal sites we assayed. These data may suggest a role for DNA modification in the ability of *Ac* to transpose from the *waxy* locus and to destabilize unlinked *Ds* elements.

Key words: *Zea mays*/transposable elements/*Activator* element/methylation and activity

Introduction

Controlling elements in maize were so named for their ability to alter gene expression by inserting in or near genes (McClintock, 1956). Among the many transposable controlling element systems described, the *Ac/Ds* system is one of the best characterized at the genetic and molecular level. The *Ac* (*Activator*) transposable element is genetically defined by three functions: (i) its capability of autonomous transposition, (ii) its ability to destabilize non-autonomous *Ds* (*Dissociation*) transposable elements, possibly by supplying a 'transposase function', and (iii) its temporal regulation of *Ac* and *Ds* transposition in response to the dosage of functional *Ac* elements in the genome (McClintock, 1947). *Ds* elements are defined by their ability to respond to an *Ac* element in *trans* resulting in either chromosome breakage at the *Ds* genomic position or by transposition (McClintock, 1947, 1949, 1951a). A number of *Ac* elements have been cloned and sequenced and it is shown to be a 4.56-kb sequence bounded by 11-bp imperfect inverted repeats (Federoff *et al.*, 1983; Pohlman *et al.*, 1984; Behrens *et al.*, 1984; Muller-Neumann *et al.*, 1984). Molecular data indicate that some *Ds* elements contain sequences homologous to *Ac* (Federoff *et al.*, 1983; Pohlman *et al.*, 1984; Doring *et al.*, 1984a,b), whereas others appear

unrelated to *Ac* except for the 11-bp inverted terminal repeats and a 12-bp internal sequence (Sutton *et al.*, 1984), the latter features shared by all elements in the *Ac/Ds* system so far examined.

Heritable changes that affect the activity of *Ac* elements are usually associated with the loss of all *Ac* functions. These events are usually irreversible and are associated with gross structural alterations including internal deletions of *Ac* DNA (Federoff *et al.*, 1983; Pohlman *et al.*, 1984). Such elements behave genetically as *Ds*, i.e. they can be destabilized by autonomous *Ac* elements and they do not contribute to *Ac* dosage effects (McClintock, 1951a, 1962). A second type of yet unexplained phenomenon, termed 'changes in phase' of an *Ac* element (McClintock, 1963), is a reversible type of *Ac* inactivation in which an *Ac* element cycles between an active and inactive phase during plant and kernel development. The initial studies of this phase change involved the *Ac*-induced *waxy* mutation, *wx-m7*, isolated by McClintock (1963, 1964). The *waxy* locus codes for a UDPG: glucosyl transferase (Nelson *et al.*, 1962) that catalyzes the synthesis of amylose from amylopectin in endosperm and pollen cells. When *Ac* at *wx-m7* is inactive, the *Ac* element can no longer autonomously transpose, *trans*-activate a *Ds* element, or contribute to overall *Ac* dosage effects (McClintock, 1964). When *Ac* activity is regained after a shift in phase, each of these functions is restored. The active *Ac* at the *wx-m7* allele has been cloned (Behrens *et al.*, 1984) and shown to be structurally indistinguishable from other *Ac* elements associated with the *wx-m9* and *bz-m2* mutations (Federoff *et al.*, 1983, 1984).

We report here a correlation between the genetic activity of *Ac* and DNA modification. When the inactive *Ac* was examined certain internal restriction sites were shown to be protected from cleavage in genomic DNA but not in the cloned, inactive element. These sites are recognition sequences for methylation sensitive restriction enzymes. Cleavage at these sites was detectable in at least a fraction of genomic DNA from plants containing an active *Ac* element.

Results

Genomic blot analysis of active and inactive Ac elements

We examined *Ac*-homologous sequences in genomic DNA of plants homozygous for an active *Ac* element at the following loci *waxy* (*wx-m7:active*), *bronze* (*bz-m2*), *R* (*r-nj:m1*), *C2* (*c2-m*), *P* (*P-vv*); and also a plant homozygous for an inactive *Ac* at the *waxy* locus (*wx-m7:inactive*). Southern blots of *SstI* digested genomic DNAs were probed with the internal 1.6-kb *HindIII* fragment of *Ac9* (Federoff *et al.*, 1984). Approximately nine *Ac*-homologous bands were observed in each genomic digest (Figure 1). Since *SstI* does not cleave within the *Ac* element, these bands approximate the number of *Ac*-homologous sequences dispersed throughout the maize genome. One of these bands represents the *SstI* genomic fragment that contains the active *Ac* element resident at the particular locus indicated. The additional *SstI* fragments that hybridize to *Ac* in each DNA represent the cryptic *Ac*-like DNA sequences found in all maize genomic DNA exam-

Fig. 1. Genomic blot analysis of active and inactive *Ac* elements. Genomic DNA from plants homozygous for an *Ac* insertion at the following loci: *c2-m*, *P-vv*, *wx-m7:inactive* phase, *wx-m7:active* phase, *r-nj:m1* and *bz-m2* was digested with 3-fold excess units of *PvuII* (P), *PvuII* and *SstI* (P+S) and *SstI* (S) alone, electrophoresed on a 0.8% agarose gel and blotted to nitrocellulose filters. The pACh1.6 insert was used as the probe. **Lane M** (marker) shows a reconstruction of the cloned 4.5-kb *Ac* element digested with *PvuII* and diluted to a single gene equivalent. This internal *Ac* probe detects a single 2.5-kb *PvuII* fragment in plants carrying an active *Ac* element. No 2.5-kb fragment appears in the *PvuII* digests of genomic DNA from plants carrying the *wx-m7:inactive* phase allele. The slight difference in mobility of the 2.5-kb fragment in the reconstruction lane compared with genomic DNA is reproducible and is due to the difference in amount of DNA loaded in each well.

ined to date (Fedoroff *et al.*, 1983). For the *wx-m7* (active and inactive) DNA digests a 7.5-kb *SstI* fragment also hybridized to the pWx1.0 *waxy* gene probe (data not shown).

To further compare the structure of active and inactive *Ac* elements, these genomic DNAs were digested with both *SstI* and *PvuII*. Active *Ac* elements at the *wx-m7*, *bz-m2*, *r-nj:m1*, *P-vv* and *c2-m* alleles have been shown to be structurally indistinguishable based on restriction digest and hybridization analysis (Muller-Neumann *et al.*, 1984; Fedoroff, 1984; data not shown). Each *Ac* element is ~4.56-kb long and contains two internal *PvuII* sites that define an internal 2.5-kb fragment (Pohlman *et al.*, 1984). In each of the DNAs from plants containing an active *Ac* element, *PvuII* digestion caused the loss of the genomic *SstI* fragment containing the active *Ac* element and the appearance of the expected 2.5-kb *PvuII* fragment of *Ac* (Figure 1); the pACh1.6 probe used in this experiment is the internal 1.6-kb *HindIII* fragment of *Ac9* and is contained within the *PvuII* fragment of *Ac*. We have noted that the 2.5-kb *PvuII* fragment in genetic crosses co-segregates with the active *Ac* element (data not shown) supporting the conclusion that the 2.5-kb fragment is derived from the active *Ac* element. In contrast, the 7.5-kb *SstI* fragment in DNA from plants homozygous for *wx-m7:inactive* was not susceptible to *PvuII* digestion. This suggests that one or both *PvuII* sites are missing within the inactive *Ac*, or else are protected from *PvuII* cleavage. Furthermore, it appeared that many of the cryptic *Ac*-like sequences were insensitive to

PvuII cleavage since *SstI* and *SstI/PvuII* digests exhibited quite similar hybridization patterns.

Molecular analysis of phases of *Ac*

Selected stocks carrying the *wx-m7* allele showed differences in the frequency of cycling between the active and inactive phase (see Materials and methods and Figure 2). These stocks were used to determine any correlation between *Ac* activity in the plant and sensitivity of the *Ac* sequences to *PvuII* digestion. The source of *wx-m7:inactive* phase material was from a derivative which showed no *Ac* activity over four generations, whereas the *wx-m7:active* phase derivative rarely underwent a phase change to the inactive phase. DNA from plants homozygous for the *wx-m7:active* (McC and K55 × W23 backgrounds) and *wx-m7:inactive* alleles were digested with *SstI*, *SstI* and *PvuII*, *SstI* and *SstII*, Southern blotted and probed with pWx0.4. Lane 1 shows a 7.5-kb fragment representing the active *Ac* element inserted into a 3.0-kb *Wx SstI* fragment. The *Ac* element from the *wx-m7:active* phase has been previously cloned and characterized (Behrens *et al.*, 1984; Muller-Neumann *et al.*, 1984). The element is located ~200-bp 5' of the *Sall* site (Figure 4). Cleavage with *SstI* and *PvuII* generated two expected fragments from the 7.5-kb *SstI* fragment: a 5' 1.3-kb *Wx-Ac* border fragment, and a 3' 1.9-kb *Ac-Wx* border fragment. The pWx0.4 probe only detects the 3' 1.9-kb fragment (Figure 3). This digest showed that the active *Ac* sequence was cleaved by *PvuII* at the 3' site (see restriction map, Figure 4). The 5' *PvuII* site of *Ac* was also cleaved in the active element (K55 × W23 background) (data not shown). The *SstI-PvuII* cleavage products of the inactive *Ac* included a 5.4-kb fragment, which contains the entire *Ac* sequence (Figure 3B, lane 3). This indicated that the inactive *Ac* DNA was not cleaved at the two *PvuII* sites internal to *Ac*, whereas the *PvuII* sites in the flanking *Wx* DNA were completely cleaved by this enzyme.

We further investigated the DNA modification pattern of *Ac* at *wx-m7* using *Wx* gene probes. We compared the *wx-m7:active* line in the K55–W23 mixed genetic background and *wx-m7:active* stocks in the original genetic background used by B.McCline (McC background) with the *wx-m7:inactive* allele in the McC background. Plant DNA from each strain was digested with *SstI*, *SstI* and *PvuII*, or *SstI* and *SstII*. In each DNA sample the expected 7.5-kb *SstI* fragment was present (Figure 3A). This represents the *Ac* inserted into the 3.0-kb *SstI Wx* fragment. Cleavage with *SstI* and *PvuII* generated a 3' 1.9-kb *PvuII* fragment in DNA samples from both active lines (Figure 3B). This fragment was not detected in the DNA from the inactive line (lane 3), only a 5.4-kb fragment representing a *Wx-Ac PvuII* fragment in which the internal *PvuII* sites of *Ac* remained uncleaved was detected. The 5' 1.3-kb *Wx-Ac* border fragment cannot be detected using this probe. Moreover, the *Ac* element at *wx-m7* appeared to contain *PvuII* sites that were unmodified in a fraction of the DNA from the McC background while cleavage of the *PvuII* sites was nearly complete in the DNA from the K55 × W23 background. Both the 5.4- and 1.9-kb fragments were present in the active McC line (Figure 3B, lane 2). This analysis indicated that while *PvuII* sites immediately flanking the *Ac* element were completely cleaved regardless of the phase of *Ac*, no *PvuII* cleavage of *Ac* DNA was detected when the element was inactive.

What is the basis of the restriction endonuclease site protection?

PvuII does not cleave its recognition sequence CAGCTG when the first cytosine is methylated (McClelland, 1982). *SstII* is also

Fig. 2. Phenotypes of *Ac* action at the *wx-m7* allele and destabilization of *Ds* at the *A1* locus. **Active *Ac* progeny:** the section of the ear shown was from a plant of the genotype *a1-m3 Sh2/a1 sh2, wx/wx* which was pollinated by an *a1-m3 Sh2/a1 sh2, wx-m7:active/wx* male individual. The active *Ac* is detected by the progeny kernels having the variegated aleurone phenotype due to the instability of the *Ds* element in response to a fully active *Ac* at *wx-m7*. Approximately 50% of the plump kernels show full *Ac* activity as expected. **Active kernel:** the genotype of the triploid endosperm is *a1-m3/a1-m3/a1, wx-m7:active/wx/wx*. The aleurone of the kernel contains a uniform pattern of *A1* mutations. The same kernel cut transversely (shown adjacent) and stained with IKI shows somatic mutations to *Wx* uniformly distributed throughout the starchy endosperm tissue due to excision of *Ac*. **Cycling *Ac* progeny:** this ear arose from a plant of the genotype *a1-m3 Sh2/a1 sh2, wx/wx* and was pollinated by an *a1-m3 Sh2/a1 sh2, wx-m7:cycling/wx* male individual. Less than half of the plump kernels show *Ac* activity. The *Ac* action that is apparent appears in sectors on the aleurone and in the starchy endosperm. Those kernels that show no *Ac* activity but carry the *wx-m7* allele have the inactive *Ac* phenotype (see below). **Cycling kernel:** the genotype of the triploid endosperm was *a1/a1-m3/a1-m3* or *a1, wx-m7:cycling/wx/wx*. The *Ac* at the *wx-m7* allele is undergoing detectable changes in genetic activity during kernel development. The upper two thirds of the aleurone layer shows no *A1* somatic mutations due to the lack of the *Ac*-mediated destabilization of *Ds* at *A1*. By this *Ds* test, the *Ac* in this sector has no detectable genetic activity. The lower third of the aleurone contains a clonal sector of *A1* spots. The same kernel cut and stained with IKI (shown adjacent) indicates that *Wx* mutations appear only beneath the clonal aleurone sector that shows *A1* mutations; the sector that shows no transactivation of *Ds* corresponds to the sector lacking *Wx* mutations. A low level of *Wx* expression in the presence of an inactive *Ac* element is characteristic of this phase of *wx-m7* (McClintock, 1964). **Inactive progeny:** this ear arose from a plant of the genotype *a1-m3/a1-m3, wx/wx* and was pollinated by an *a1-m3 Sh2/a1 sh2, wx-m7:inactive/wx* male individual. The *wx-m7:inactive* allele does not transactivate the *Ds* at *A1*. The *a1-m3* allele gives rise to a pale phenotype in the absence of *Ac*. **Inactive kernel:** (The endosperm genotype is *a1-m3* or *a1/a1/a1-m3, wx/wx/wx-m7:inactive*). no *A1* mutations are visible in the aleurone and no clonal *Wx* mutations occur in the underlying starchy endosperm (see the cut and stained inactive kernel), although a low level pattern of staining is associated with the *wx-m7:inactive* allele.

sensitive to cytosine methylation (McClelland, 1982). We tested whether the modification of *PvuII* and *SstII* sites in genomic DNA from plants containing an inactive *Ac* was lost after cloning the inactive element in *Escherichia coli*. *PvuII* or *SstII* digestion of the cloned *wx-m7:inactive* phase allele should then reveal whether the sites in the inactive *Ac* are present. We cloned the *wx-m7:inactive* allele and compared the restriction digestion patterns of cloned active and inactive *Ac* at *wx-m7*. Figure 4 shows a restriction map of a region of the *wx-m7* allele. Both *PvuII* sites and *SstII* sites were present in the cloned inactive *Ac* element. Therefore, the modification that protected these sites from cleavage in the genomic DNA was not present after cloning the *Ac* sequence into *E. coli*. Moreover, all the digest pattern comparisons between the clones of the active and inactive phase *Ac* elements are indistinguishable by agarose gel electrophoresis (data not shown). These data also showed that the site and orientation of the inserted element into the *Wx* gene were identical in both active and inactive phases of *wx-m7*.

We used isoschizomers that differed in their sensitivity to 5-

methyl cytosine to confirm that the basis for the *Ac* DNA modification was cytosine methylation. The DNA from *wx-m7* plants was digested with the enzymes *BstNI* and *EcoRII*, both of which recognize the sequence CC(A or T)GG. *EcoRII* does not cleave this site when the internal cytosine is methylated whereas *BstNI* is insensitive to methylation at this position (Hughes and Hattman, 1975). Genomic DNA from plants carrying *wx-m7:active* and *wx-m7:inactive* alleles was digested with *EcoRII* or *BstNI* and hybridized to the *PvuII*–*SalI* *Wx* fragment of pSalC. Two fragments (4.1 kb and 0.3 kb) are expected to hybridize to this probe when the *Ac* DNA and adjacent *Wx* gene sequences are not methylated at these sites. The expected 4.1-kb fragment was present in the *BstNI* digests of both active and inactive phase DNA (Figure 5, lanes 2, 4 and 6). In contrast, in the *EcoRII* genomic digests, a 4.1-kb fragment was not present in the inactive *Ac* DNA but was present in the digest of active *Ac* DNA (Figure 5, lanes 3, 5 and 7). Instead, a 5.2-kb fragment was detected in the inactive *Ac* DNA digest (lane 5). This indicated that all *EcoRII* sites in the inactive *Ac* sequence were protected from

Fig. 3. Genomic blot analysis of the *wx-m7* alleles. Southern blots comparing *Sst*I (panel A), *Sst*I/*Pvu*II (panel B) and *Sst*I/*Sst*II (panel C) digests of genomic DNA from plants homozygous for the *wx-m7:active* allele (K55 × W23 and McC background), *wx-m7:inactive* allele, and pAc7I. The filters were hybridized to the pWx0.4 insert probe. The weakly hybridizing band in each lane 4 is due to hybridization to the plasmid DNA.

Fig. 4. Restriction map of the *wx-m7* allele. Restriction map of *Pvu*II (P), *Bst*NI/*Eco*RII (E), *Hind*III (H), *Sst*I (S1), *Sst*II (S2), *Sal*I (S) and *Sma*I (Sm) sites in the *Ac* and *Wx* sequence of the *wx-m7* allele. Hatched bars represent the *Ac* sequence and solid lines represent the flanking *Wx* sequence. The solid line below represents the *Sst*I fragment of *Wx* without the *Ac* sequence insertion. The *Pvu*II to *Sal*I fragment (pWx0.7) represents the *Wx* probe used in Figure 5. The *Sal*I to *Sma*I fragment (pWx0.4) represents the *Wx* probe used in Figure 3. The open box above the map represents the pAcH1.6 insert.

digestion and thus methylated, whereas the flanking sites in the *Wx* DNA were cleaved giving rise to the expected 5.2-kb fragment (lane 5). Moreover, the presence of the weakly hybridizing 5.2-kb band (lanes 3 and 7) suggests that the *Eco*RII sites in the active *Ac* element were methylated in a small fraction of the

genomic DNA. Similarly, the presence of a 6.5-kb band in lane 5 suggests that one site in the *Wx* sequence, together with *Ac*, may be methylated in a fraction of the inactive *Ac* DNA.

It appeared that modification of the region defined by the *Pvu*II site at nucleotide position 3247 was correlated with *Ac* inactivation. The restriction endonuclease *Sst*II is sensitive to cytosine methylation and three *Sst*II sites are present between positions 3827 and 3958. We examined whether modification of *Sst*II sites was correlated with *Ac* activity. The *Sst*II–*Sst*I cleavage product of the *wx-m7:active* alleles was a 1.2-kb *Sst*II fragment, as detected by the pWx0.4 probe (Figure 3C, lanes 1 and 2). This represents the *Ac*–*Wx* 3' border fragment (see Figure 4). This fragment was not present in the *Sst*II–*Sst*I digest of the *wx-m7:inactive* DNA. Instead a 6.5-kb fragment was detected (Figure 3C, lane 3) and most likely it represents cleavage of the *Wx* sequence at the 5' *Sst*I and the *Wx* *Sst*II site 3' of *Ac*, but without cleavage of the inactive *Ac* sequence at the *Sst*II sites. This suggests the inactive *Ac* was not cleaved with the cytosine methylation sensitive enzyme *Sst*II at all three sites while the active *Ac* was digested to completion at at least one of these sites.

If *Ac* activity was associated with undermethylation at the *Pvu*II, *Sst*II and *Eco*RII sites, we predicted that transposed active *Ac* elements should be hypomethylated regardless of the genomic position of the element. We investigated the DNA modification of the active *Ac* (McC background) after it had transposed to new genomic positions. Four *Wx*, *a1* mutable kernels (*a1-m3*) shows mutability in the presence of *Ac* were selected from ears of the genotype *wx-m7:active* (McC)/*wx* homozygous for *a1-m3*. These cases were associated with reversion of the *wx-m7* allele to *Wx* and are presumed to be due to the transposition of *Ac* away

Fig. 5. Southern blot analysis of *wx-m7* alleles using methylation sensitive and insensitive isoschizomers. Southern blot analysis of a genomic DNA from plants containing the *wx-m7:active* (K55 $\times$ W23 and McC background) and *wx-m7:inactive* alleles. The blot was hybridized with the pWx0.7 insert probe. **Lane 1** contains pAc7I diluted to a single gene equivalent and digested with *Bst*NI (B). **Lanes 2 and 3** contain *Bst*NI (B) and *Eco*RII (E) digests of DNA from plants homozygous for the *wx-m7:active* (K55 $\times$ W23 background) allele. **Lanes 4 and 5** contain digests of DNA from plants homozygous for the *wx-m7:inactive* allele and **lanes 6 and 7** are digests of DNA from plants heterozygous for the *wx-m7:active* (McC background) allele and a *wx* tester allele. The recessive *wx* allele does not contribute to the banding pattern seen in **lanes 6 and 7**. As a positive control for *Eco*RII enzyme activity, all digests contained *dcm*⁻ λ phage DNA. When the *Wx* probe was removed and the blot was rehybridized to nick-translated λ phage DNA, both *Eco*RII and *Bst*NI digested the unmethylated λ phage DNA to completion giving rise to the same pattern of fragments.

from the *wx-m7* locus. Genetic analysis of plants derived from these seeds showed one *Ac* element linked to the *Wx* allele in three of the four cases (data not shown). DNA was prepared from the four plants containing these transposed *Ac* elements and from the sibling plants carrying the *wx-m7:active* allele. These samples were digested with *Pvu*II, Southern blotted and probed with the pAcH1.6 fragment (Figure 6A). In each case, we detected the cleavage of both *Pvu*II sites within the transposed *Ac*; however, we observed variation in the intensity of the 2.5-kb fragment (compare lane 2 with lanes 3–5). The ability of *Pvu*II to cleave the *Ac* sequence had apparently increased in all four independently derived cases where *Ac* was in a new genomic location. To ensure the band intensity difference between the lanes was not due to

Fig. 6. Southern blot analysis of transposed *Ac* elements from the *wx-m7* locus. (A) Three micrograms of genomic DNA prepared from plants with the alleles indicated (above the lanes) and heterozygous for the *wx* tester allele were digested with *Pvu*II, loaded on a 0.8% agarose gel and separated by electrophoresis. The fragments were transferred to nitrocellulose and hybridized to the pAcH1.6 insert probe. The active *tr-Ac* individuals all had the genotype *Wx-revertants/wx*, and one active *Ac* element. The weakly hybridizing 2.5-kb fragment in **lane 2** is due to only a small fraction of genomic DNA in which both *Pvu*II sites internal to *Ac* were cleaved, a characteristic of the *wx-m7:active* allele in the McC background (see Figure 3). **Lane 7** shows a reconstruction of the cloned 4.5-kb *Ac* element digested with *Pvu*II and diluted to a single gene equivalent. (B) The pAcH1.6 probe was removed from the nitrocellulose filter by washing in 0.05% SDS, $0.05 \times$ SSC at 95°C for 20 min. The blot was hybridized to a nick-translated pSalE clone of *Wx* (Shure *et al.*, 1983). This probe revealed a *Pvu*II fragment of ~ 1.6 kb. The intensity of the fragment in each lane is approximately equal, showing equal amounts of DNA were loaded and digested in each lane.

unequal amounts of DNA in each lane, the pAcH1.6 probe was removed and the blot was rehybridized with the pSalE fragment of *Wx* (Shure *et al.*, 1983). This probe detected a 1.6-kb fragment (Figure 6B) which appeared in equal intensity in all lanes, which indicated each lane had been loaded with the same amount of

DNA and that the *PvuII* enzyme digested each of the samples to completion within the *Wx* sequence. These results showed that active *Ac* elements at new genomic positions derived from the donor *wx-m7* allele were hypomodified at the internal *PvuII* sites.

Discussion

'Changes of phase' refers to the reversible inactivation of *Ac*. When the *Ac* element is in the inactive phase, it no longer carries the transposase function and operationally behaves as a *Ds* element (McClintock, 1963, 1964). Our studies showed that when *Ac* at *wx-m7* was inactive, regions of the *Ac* DNA were modified. This type of modification appeared to involve cytosine methylation and appeared to be specific for *Ac* DNA since restriction enzymes sensitive to methylation cleaved flanking *Wx* DNA but did not cleave internal *Ac* sites when *Ac* was inactive.

Differences in methylation were the only changes we observed between the active and inactive *Ac* elements. The cloned inactive *Ac* was structurally indistinguishable from the active *Ac wx-m7* by restriction analysis. Furthermore, no change in the position or orientation of the *Ac* element in relation to the *waxy* locus was observed when the active and the inactive phase *Ac* element were compared. This suggested that the loss of *Ac* activity when *Ac* cycles to the inactive phase was not due to gross structural rearrangements in the *Ac* element, as was previously shown for other functional changes of an *Ac* to a *Ds* element (Fedoroff *et al.*, 1983). We cannot rule out the possibility that small nucleotide changes are also associated with *Ac* inactivity until sequence data are obtained. However, the basis for phase changes must be reversible since inactivation appears to be a metastable phenomenon.

The reversible nature of phase changes and its association with DNA modification suggests that modification may play a direct or indirect role in determining *Ac* genetic activity. It is not known whether modification directly regulates the expression of *Ac* or whether this modification is consequential and thus only reflects the state of expression of the *Ac* element. Other studies suggest that DNA methylation has a role in regulating gene expression (Doerfler, 1984). Several experiments have shown that there is an inverse correlation between methylation of DNA at specific sites within a gene and the level of gene expression (Doerfler, 1984; Sutter and Doerfler, 1980; Vardimon *et al.*, 1982; Weintraub *et al.*, 1981). Adenine methylation has recently been shown to inhibit the transposition of a prokaryotic transposable element, *IS10* (Roberts *et al.*, 1985). In *dam*⁺ strains, *IS10* appears to preferentially transpose when the DNA is hemimethylated, presumably immediately after the passage of a replication fork.

Studies of DNA modification in eukaryotes have focused on the methylation of the CpG dinucleotide since 5-methyl cytosine (5MeC) occurs in this dinucleotide in vertebrate animals (Vanyushin, 1984). In higher plants only a portion of 5MeC is found in the CpG dinucleotide sequence (Vanyushin, 1984; Doheim *et al.*, 1978; Kirnos *et al.*, 1981). Much of the 5MeC exists in the trinucleotide sequence CAG and CTG (Gruenbaum *et al.*, 1981). This trinucleotide is found in the *SstII*, *PvuII* and *EcoRII* recognition sequences, and methylation of the cytosine residue inhibits DNA cleavage with these enzymes (McClelland, 1982). These enzymes fail to cleave internal *Ac* sites in genomic DNA isolated from plants containing the *wx-m7:inactive* allele. Furthermore, we observed a difference in hybridization pattern between *EcoRII* and *BstNI* digests of genomic *wx-m7:inactive* DNA. Moreover, the *EcoRII* digestions of DNA containing the active or inactive *Ac* clearly showed a difference in the ability of this

enzyme to cleave internal *Ac* sites. We conclude that hypomethylation of *Ac* DNA is associated with the inactive phase of *Ac* whereas the active element is hypomethylated.

Since certain enzymes fail to cleave a fraction of *Ac* sequences even when *Ac* is active, it appears that plant genomic DNA may always contain a portion of methylated *Ac* sequences. Although the *wx-m7:active* stock remained active over a number of generations when assayed in the kernel progeny, we could not ascertain the activity of *Ac* in the somatic tissue used to isolate DNA. If *Ac* became inactive in a sector of leaf tissue from which we prepared the active *Ac* DNA, it would account for the portion of methylated sequences. Alternatively, *Ac* may exist in both states and become demethylated at specific times during development or during chromosome replication. We are currently investigating these possibilities by constructing *wx-m7:active* stocks with a *Ds* marker visible in the sporophyte to detect whether *Ac* is active throughout plant development. We are also analyzing other tissues such as endosperm to study the correlation between *Ac* DNA modification and its inactivity.

Differences in genetic background had an effect on the degree of modification of the active element. We observed a difference in *PvuII* digestion of the DNA from the *wx-m7:active* plants in different genetic backgrounds. This may indicate a genetic basis for the regulation, or the fact that the pattern of *Ac* DNA modification is unlinked to *Ac*. This possibility is currently being investigated by inheritance studies.

Sequence data of *Ac9* and *Ac7* have suggested the presence of a possible AT-rich intergenic control region for at least two possible divergent open reading frames (ORFs) (from nucleotides 3167 to 3739) followed by a region of very high GC content (nucleotides 3739 to 4387) (Pohlman *et al.*, 1984; Muller-Neumann *et al.*, 1984). The high GC region falls within one of the possible ORFs. One of the *PvuII* sites, all of the *EcoRII/BstNI* sites, and all of the *SstII* sites fall into or near these regions. This may be significant in that activity of the element is inversely correlated with the modification of the *PvuII*, *BstNI* and *SstII* sites within this region. The *PvuII* site is 80-bp upstream from the putative ORF 1 (Pohlman *et al.*, 1984). This ORF has been implicated as the putative transposase function since a deletion mutation of this ORF abolishes *Ac* genetic activity (McClintock, 1963; Fedoroff *et al.*, 1983; Pohlman *et al.*, 1984). It is interesting to speculate that the reversible modification of the 5' region of this gene is involved in phase changes.

The degree of *PvuII* site modification appeared to change upon transposition of the active element. In each of the four cases examined where *Ac* had transposed to a new genomic position, it appeared that the extent of modification of the *Ac* DNA at the *PvuII* site(s) was reduced. It is unclear what significance this change in modification may have on the genetic behavior of the *Ac* element. Preliminary findings suggest these newly transposed elements are typical *Ac* elements with regard to genetic activity. Thus, the change in modification of the element may be a reflection of its new genomic position. Alternatively, if lack of modification was a prerequisite for transposition, selecting germinal transposition events of *Ac* may lead to changes to the modification patterns of the element. There is precedent for inheritance of DNA modification patterns. In experiments using DNA-mediated gene transfer, it was shown that foreign, methylated DNA inherits its own methylation pattern when introduced into different locations in the genome of the host cell (Wigler *et al.*, 1981).

Ac is not unique among maize transposable elements in the ability to cycle between inactive and active phases. This behavior has been observed in the *Spm* system (McClintock, 1958), the

Dt system (Doerschug, 1968, 1973) and more recently with Robertson's *Mutator* elements (Chandler and Walbot, 1986). In the latter studies with *Mu* elements, DNA modification of *Mu* has also been correlated with inactive mutator stocks (Chandler and Walbot, 1986). It will be interesting to examine the inactivation of other maize controlling elements to see whether DNA methylation of the inactive element is a general phenomenon.

Materials and methods

Phenotypic assay of *Ac* activity

The activity of the *Ac* element at the *waxy* locus can be assayed genetically by the ability of *Ac* to destabilize a *Ds* element resident at the *A1* locus (*al-m3* allele) (McClintock, 1951b). Somatic instability of *Ds* restores full *A1* gene expression, detected as sectors of fully colored aleurone tissue. The *waxy* gene product controls the amylose content of the endosperm and can be assayed by staining the tissue with an IKI solution (Weatherwax, 1922). Endosperm cells with a functional *waxy* gene stain blue-black, whereas cells homozygous for a null *wx* mutation stain red with IKI. Somatic instability of an active *Ac* from the *wx-m7* allele gives rise to clonal sectors of *Wx* mutations (darkly stained sectors). Endosperm cells carrying the *wx-m7:inactive* allele show a distinct pattern of reduced *Wx* expression (McClintock, 1963, 1964).

Strains

Stocks derived from the original *wx-m7* mutation (McClintock, 1963) show heritable changes in phase of *Ac* activity at *wx-m7*. It is not known if this genetic variation maps at the element or elsewhere in the genome. These stocks have been designated *wx-m7:active*, *wx-m7:cycling* and *wx-m7:inactive* (see Figure 2). *wx-m7:active* lines show rare deviations from the expected *Ac* behaviors; the *wx-m7* phenotype shows proper Mendelian segregation in repeated outcrossing and self matings except for premeiotic transposition events. The *Ac* in these lines also shows the expected dosage response as described earlier. Rare events were studied by McClintock (1963) which showed reversible changes of *Ac* activity (*wx-m7:cycling*). Certain kernels from these *wx-m7:cycling* stocks show *Ac* activity only in somatic sectors during the development of the endosperm or plant. This is seen as either (i) a clonal sector of no *Ac* activity on a kernel showing *Ac* mutability at the *waxy* locus, or (ii) clonal sectors of *Ac* mutability on a kernel showing no *Ac* activity. Simultaneous with the sector change in *Ac* self-transposition at the *waxy* locus is a corresponding change in the response of the *Ds* at unlinked loci. That is, when the *wx-m7:cycling* allele is combined with a *Ds*-suppressed *A1* mutation (*al-m3*), clonal aleurone sectors showing *A1* spotting (*Ds* transposition) are produced. The clonal sector of *waxy* mutability corresponds to the sector of *A1* spotting. The other kernels on these *wx-m7:cycling* ears show either no *Ac* activity at *wx-m7* and *al-m3* or show full *Ac* activity throughout endosperm development. In certain cases the inactive phase of *Ac* was inherited. Plants grown from the kernels which showed no *Ac* activity at *wx-m7* gave rise to all progeny that showed the inactive *Ac* phenotype. One such stock used in these studies was propagated over four generations and showed no *Ac* activity at *wx-m7*, although a distinct pattern of *Wx* expression was observed in the endosperm of these kernels (see Figure 2) (McClintock, 1964).

The *wx-m7:active* allele was originally isolated by McClintock (1963) and propagated within the original genetic background (designated McC background). Homozygous seed was obtained by self-pollinating the original *wx-m7/wx* heterozygous plants. A second *wx-m7:active* stock derived from McClintock's stock by backcrossing into different genetic backgrounds (K55 × W23 hybrid) was obtained from S. McCormick. The *wx-m7:inactive* allele and the *al-m3* allele was kindly supplied by B. McClintock.

The *Ac*-suppressed *c2-m*, *P-vv* allele and *r-nj:m1* allele were kindly supplied by I. Greenblatt. The *Ac*-suppressed *bz-m2* (McClintock, 1951b) allele was kindly supplied by N. Fedoroff.

Plasmids

pAcH1.6 contains the 1.6-kb *HindIII* fragment of *Ac9* (Fedoroff *et al.*, 1984); pWx1.0 is the 1.0-kb *EcoRV*–*SalI* fragment of *Wx*, derived from pSalC (Shure *et al.*, 1983), cloned into the *EcoRV*–*SalI* sites of pBR322. The *SmaI*–*SalI* fragment of *Wx* used as a probe for the blots was purified by gel electroelution (McDonnell *et al.*, 1977) from a *SmaI*, *SalI* digest of pSalD (Shure *et al.*, 1983), and was subsequently cloned into the *SmaI*, *SalI* site of the Blue Scribe vector (Vector Cloning Systems, San Diego, CA) and designated pWx0.4. A clone of *Ac* from the *wx-m7:active* allele (pAc7) (Muller-Neumann *et al.*, 1984) was obtained from P. Starlinger. The inactive *Ac* from *wx-m7* was subcloned from λwxm7I (see below) as a 7.5-kb *SstI* fragment into the *SstI* site of pUC119 and designated pAc7I.

Genomic cloning

Maize genomic DNA was isolated from leaf tissue according to Shure *et al.* (1983). DNA from plants homozygous for the *Ac wx-m7:inactive* allele was digested with

SstI and size selected for the 7.5-kb *SstI Wx* fragment containing *Ac* by glycerol gradient centrifugation (Maniatis *et al.*, 1982). The size selected genomic DNA was cloned into the λ Charon 34 vector (Loenen and Blattner, 1983). Recombinant phage were screened by plaque hybridization (Benton and Davis, 1977) with the pWx1.0 probe. A recombinant phage containing the 7.5-kb *SstI wx-m7* fragment was isolated and designated λwxm7I.

Genomic blot hybridization

Genomic DNA was digested with 3-fold excess units of restriction enzymes according to the manufacturer's recommendations, fractionated on agarose gels, denatured and transferred to nitrocellulose according to the method of Southern (1975). Plasmid DNA was isolated by the method of Holmes and Quigley (1981) and nick-translated as described by Rigby *et al.* (1977). Filters were prehybridized at 65°C in 10% dextran sulfate, 6 × SCP (1 × SCP = 0.2 M NaCl, 60 mM Na₂HPO₄, 10 mM EDTA), 2% sarcosine and 500 µg/ml heparin, and hybridized in the same buffer containing 100 µg/ml denatured salmon sperm and denatured nick-translated probe (final concentration 10 ng/ml). The filters were washed in 0.2 × SCP, 0.2% SDS at 65°C.

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Note added in proof

We have obtained genetic data that suggests the *c2-m* line contains *Ds* at the *C2* locus and *Ac* elsewhere in the genome.