

Molecular Analysis of *Ds* Controlling Element Mutations at the *Adh1* Locus of Maize

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Transposable genetic elements of the *Ac-Ds* system in maize have been studied by McClintock (1, 2). The autonomous *Ac* (activator) element controls its own movements and those of dependent *Ds* (dissociation) elements. These movements can be mapped through the inhibitory effects of *Ac* and *Ds* elements on the activity of adjacent genes. In addition, some activated *Ds* elements can cause

codes the maize enzyme sucrose synthase. Several restriction site mapping and cloning studies have been reported, both for *Sh* alleles and for *Ac*-controlled mutable alleles and revertants resulting from the activity of a nontransposable *Ds* element (8–14). These studies show that several of the mutable alleles are interrupted within or near the *Sh* coding region by long DNA sequences (> 20 kb)

Abstract. An active maize *Adh1-F* gene, a *Ds*-induced mutant of this gene, and two independent *Ac*-induced revertant alleles have been isolated. The *Ds* mutant differs from the progenitor allele in having a 405-base pair insertion flanked by a direct repeat of 8 bp. The repeat is a duplication of the 8 bp existing at the point of insertion in the 5' untranslated region of the gene. The insertion sequence is AT-rich (A, adenine; T, thymine) and has 11-bp inverted repeat sequences at its termini. In the revertants the insertion with its inverted repeats is deleted, but the 8-bp direct repeats remain in modified form. These results establish that the 405-bp sequence is a *Ds* element. The *Adh1* messenger RNA level is low in the *Ds* mutant, and it appears that new sites for transcription initiation or RNA processing or both are used. There are at least 30 sequences in the maize genome related to the *Ds* element.

chromosome breaks, giving rise to breakage-fusion-bridge cycles that, in appropriately marked stocks, allow mapping of the initial breakpoint. The *Ac-Ds* system has many complexities, and different states of *Ds* that differ in their ability to respond to *Ac* have been described. Some *Ds* elements induce few if any chromosome breaks, and some are no longer transposable although they still cause mutations and reversions. Both *Ac-Ds* and the other maize transposable element systems show obvious similarities to other transposable element systems in both prokaryotes and eukaryotes (3, 4); nevertheless, a full characterization requires a detailed analysis of *Ac-Ds* at the molecular level.

Fine structure mapping (5) and the characterization of mutant proteins (6, 7) indicated that at least some *Ac-Ds*-induced mutations are located within the coding regions of the genes affected. Attempts to characterize *Ds*-induced mutations at the DNA level have focused on the shrunken locus (*Sh*), which en-

that appear to involve large, dissimilar insertions (9) and, possibly, more complex chromosomal rearrangements (13).

We have used a set of *Ac-Ds*-induced mutations at the *Adh1* locus on chromosome 1 (7, 15). A complementary DNA (cDNA) probe for *Adh1* (16) was used to isolate clones containing the *Adh1* gene from lines containing a *Ds*-induced mutable allele, the wild-type progenitor of this allele, and two revertant alleles. These clones have been analyzed by restriction site mapping, hybridization studies, and DNA sequencing.

Cloning and Mapping of *Ds*-Associated *Adh1* Mutants

Partially overlapping *Adh1* clones (λ PR.1 and λ PR.2) were selected from a Sau 3AI fragment library (17) of DNA from the progenitor of the mutant line by hybridization with a *Adh1* cDNA probe. Restriction site mapping and Southern hybridization (17, 18) showed that the

inserts in these clones covered a 20-kb region at the *Adh1* locus, extending more than 6 kb upstream and 10 kb downstream from the *Adh1* structural gene (Fig. 1). The progenitor gene was also isolated as a 7-kb Bam HI fragment in another clone, λ PR.3, selected from a Bam HI fragment library.

Southern hybridizations to Bam HI-digested maize DNA indicated that while the *Adh1* gene was located on 7-kb fragments in both progenitor and revertant stocks (19), the *Adh1-Fm335* allele was located on a fragment of about 7.4 kb (Fig. 2A). Digestion with Bgl II in addition to Bam (Fig. 2B) also showed that a particular fragment had increased in size by 0.4 kb in the *Adh1-Fm335* allele. This was confirmed by mapping restriction sites in the *Adh1* clones λ DS.2 and λ DS.5, each of which contains DNA from the *Adh1-Fm335* mutant, as well as λ RV.5 and λ RV.10 isolated from Bam HI fragment libraries of two independent revertants of *Adh1-Fm335* (Fig. 1). The only differences between the progenitor, mutant, and revertant clones were that, in the progenitor and revertants, the Pst I-Hind III segment at the 5' end of the gene was approximately 0.35 kb, whereas in the *Ds*-controlled mutant it was approximately 0.75 kb. These segments were subcloned into the pUC8 plasmid vector—giving plasmids designated as pPR.1A, pDS.2A, pRV.5A, and pRV.10A—and then excised; their sizes were then determined by acrylamide gel electrophoresis to be 340 and 750 bp.

Nucleotide Sequences of the Mutant Regions

The Pst I-Hind III inserts from pPR.1A, pDS.2A, pRV.5A, and pRV.10A, were subcloned into m13 vectors and sequenced. The sequence of the progenitor fragment (Fig. 3a) was identical (except for two substitutions) to that of the homologous fragment from *Adh1-S* (20), and therefore we used the same numbering system; nucleotide 1 is the site of transcription initiation, nucleotide 101 is the start of translation, and nucleotides TATAAA at –32 to –37 and CCAAA at –92 to –96 are the presum-

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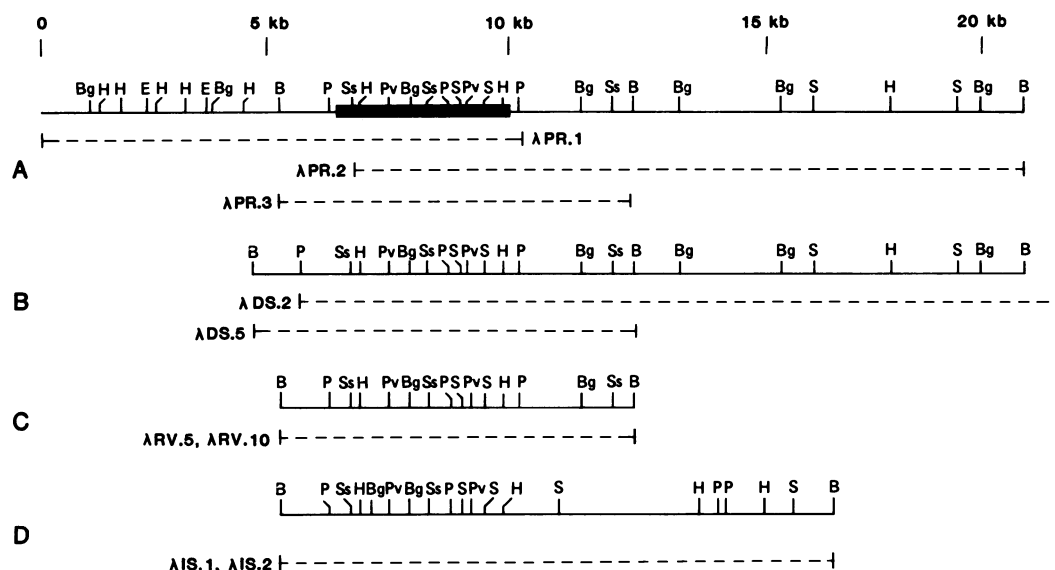


Fig. 1. Restriction maps of maize DNA clones containing *Adh1* genes. (A) Progenitor *Adh1-F* allele. The solid bar indicates the transcribed region, with the direction of transcription being from left to right (20). Subsidiary bars indicate the maize DNA segments isolated in individual λ clones. Abbreviations: B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; P, Pst I; Pv, Pvu I; S, Sal I; and Ss, Sst II. (B) *Adh1-Fm335* allele. (C) Revertant alleles. (D) *Adh1-S* allele (data from 20).

tive "TATA" and "CCAAT" boxes for the *Adh1* promoter (T, thymine; A, adenine; C, cytosine).

The sequence of the mutant fragment contained an additional 413 bp inserted between progenitor nucleotides 45 and 46 (Fig. 3b) but no other changes. The sequence of the extra nucleotides can be represented as a 405-bp insertion with 11-bp inverted repeat sequences at its termini, followed by an 8-bp direct repeat of progenitor nucleotides 38 to 45. The insertion sequence has a high AT content (76 percent).

The sequences of the two revertant fragments did not contain the 405-bp insertion, but retained the duplicated 8-bp sequences derived from the duplication of progenitor nucleotides 38 to 45 with slightly different sequences in each revertant allele (Fig. 3c). The pRV.10 fragment had a single additional change with the run of six GC pairs (G, guanine) at nucleotides 93 to 98 occurring as seven GC pairs. There is no reason to suggest that this change is related to the excision of the 405-bp sequence in the revertant.

Other Maize Sequences Related to the *Adh1-Fm335* Insertion Sequence

Southern hybridizations to Bam HI-digested maize DNA with the pDS.2A Pst I-Hind III fragment as a probe revealed 30 to 40 bands in DNA's from different maize stocks including teosinte, a primitive relative of maize (Fig. 4). Similar patterns, differing only in the intensity of the bands, were obtained when the hybridization conditions were either more stringent or more relaxed. In control experiments, a probe made from

the pPR.1A Pst I-Hind III fragment hybridized only to the expected 7-kb fragment in Bam HI digests of DNA from the progenitor stock and to a 0.35-kb fragment in a Pst I-Hind III digest of the same DNA.

Inspection of sequence data showed that no sequences homologous to the 405-bp insertion sequence occur in the *Adh1-S* structural gene. In order to test whether copies of this sequence are present within or close to the *Adh1-F* progenitor allele, we carried out Southern hybridizations to Pst I-Bgl II digests of λ PR.1, λ PR.2, and λ DS.2 DNA's, using

as a probe a 361-bp Sau 3AI fragment containing the first 329 bp of the *Ds* insertion sequence and 32 bp of adjacent *Adh1-F* 5' leader sequences. As expected, the probe gave strong hybridization to the *Ds*-containing fragment of the *Ds* allele. The only hybridizations seen with the *PR* allele fragments were those expected from the 32 bp of *Adh1-F* 5' leader sequence.

Discussion

Our restriction site map for the *Adh1-F* progenitor gene (Fig. 1) revealed a 4-kb region of close structural homology to the standard *Adh1-S* allele (20), with 12 of the 13 sites compared being present at similar positions (± 0.1 kb) in both. In contrast to this, in the 7.4-kb flanking region compared in our *Adh1* clones, none of the 14 sites were present at similar positions in both *Adh1-S* and *Adh1-F*. These results are consistent with observations (21) that there may be a high level of restriction site polymorphism adjacent to the *Adh1* locus. All 21 restriction sites downstream from the *Ds* insertion site mapped at similar positions in our progenitor and *Ds* clones as expected.

Osterman and Schwartz (7) isolated the *Adh1-Fm335* mutation from a maize line known to contain an active *Ds* element in the long arm of chromosome 1, approximately 20 map units from *Adh1*. They demonstrated that the *Adh1* mutation was caused by a *Ds* element because in the absence of *Ac* elements the phenotype was stable but in the presence of *Ac* reversions to normal levels of enzyme activity occurred in high frequencies (> 2 percent).

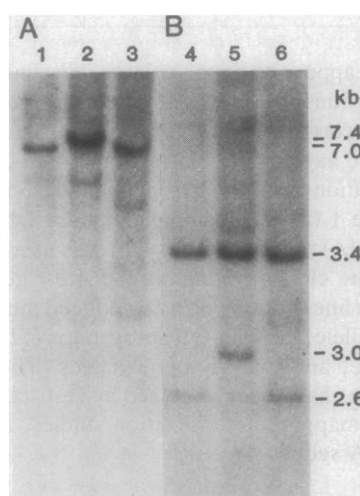


Fig. 2. Blot hybridization of maize DNA's with an *Adh1* cDNA probe. Restriction digests of maize DNA were fractionated by agarose gel electrophoresis and hybridized with labeled insert sequences from pZML793 (20). (A) Bam HI digests. (B) Bam HI-Bgl II double digests. (Lanes 1 and 4) Progenitor stock; (lanes 2 and 5) *Adh1-Fm335* stock; (lanes 3 and 6) revertant stock. Sizes of *Adh1* bands hybridizing with the cDNA probe are shown. Minor bands arise from cross hybridization of probe with *Adh2* gene sequences.

The only *Ac-Ds*-induced changes detected at the *Adh1* locus by restriction site mapping and DNA sequencing were the insertion and excision of a 405-bp sequence not present in the progenitor allele, an 8-bp duplication of the progenitor sequence at the insertion site, and relatively minor changes at or near this site associated with individual revertants.

Although we cannot exclude the possibility that other sequence changes may have occurred in our stocks at positions flanking the *Adh1* locus, there is no evidence to support such changes, and the alterations already found appear capable of explaining the known characteristics of the mutant and its revertants. It is clear that these *Ds*-induced alterations at *Adh1* differ markedly from those de-

scribed by others at *Sh* (8, 13). It appears possible that the *Ds* elements responsible for mutations at the *Adh1* and *Sh* loci may exhibit significant differences. Our results establish that the *Ds* element involved in the *Adh1-Fm335* mutation functions as a 405-bp transposable insertion sequence.

The small size of the *Ds(Adh1-Fm335)* sequence, and the presence of 11-bp inverted repeats at its termini, are reminiscent of bacterial *IS* elements (22) and the repeat sequences that flank a number of eukaryotic transposons (23). The 8-bp duplication of progenitor nucleotides at the insertion site, resulting in short direct repeats flanking the insertion, and the presence of multiple *Ds(Adh1-Fm335)*-related sequences in the maize genome, are features shared with other eukaryotic

transposable elements (4). In certain other respects, discussed in more detail below, *Ds(Adh1-Fm335)* appears to be an exception.

It is not established whether the multiple *Ds(Adh1-Fm335)*-related sequences in maize DNA also function as transposable elements. Genetic studies of *Ds* have generally been based on the assumption that the loss of a *Ds* element from one locus and the simultaneous recovery of a *Ds* element at a nearby locus represents the transposition of a single element. In fact, provided that they do not initiate obvious breakage-fusion-bridge cycles and do not reside near loci where they cause obvious phenotypic effects, multiple *Ds* elements may be present and undetectable in the maize genome.

A -148 -96 -92
CTGCAGCC CCGGTTTCGC AAGCCGCGCA CGTGGTTTCG TTGCCACAG CGGGCAAAAC CGCACCTCC

-37 -32
TTCCCGTCGT TTCCCATCTC TTCCTCCTTT AGAGCTACCA CTATATAAAT CAGGGCTCAT TTTCTCGCTC

-1 +1 +38 +45
CTCAGAGCT CATCTCGCTT TGGATCGATT GGTTCGTAA CTGGTGAGGG ACTGAGGGTC TCGGAGTGGA
(A)

+101
TTGATTTGGG ATTCTGTTTCG AAGATTTGCG GAGGGGGGCA ATGGCGACCG CGGGGAAGGT GATCAAGTGC
(C)

AAAGGTCCGC CTGTTTCTC CTCTGTCTCT TGATCTGACT AATCTTGGT TATGATTCTG TGAGTAATTT

+182
TGGGGAAAGC TT

B +38 +45
gggactgaTAGGGATGAAACGGTCGAAATCGGTATTTTTTCGGTAATCAGTTTTTATGTCGTTTTTCTTTGATT

GCGAATAAATAGGATATAGAATATACAATAAATTTGTATTCTTGTGTTTTTAACATCCAGCTTGTTAAGATTCATA

AAAGTAAACCTCAAATTCATCCTATATTTCTCAAATAATAGATATAAATTCGGTATGGATTCGGAATAAATTC

GGTAATTTTTTCAACCTTTTATGTTGTAGGGAGCAAATAATACATAAAACAATGTATGTAATTTTATTCATATTT

CTACTAATGTGCTTGATAACATAAGAAAAGATCAATATCAAATTTATATATATCTATTTTAAATATTAATTTA

+38 +45
TCCTAACAGTTCGGATTACCACTTTTCATCCCTAGggactga

C (AG)
pRV.5A CGTAAGTGGTGAGGGACTGTCCGACTGAGGGTCTCGGAGT

(AGG)
pRV.10A CGTAAGTGGTGAGGGACTGTCCGACTGAGGGTCTCGGAGT

quencing gels. (B) The 413-bp insertion from the *Adh1-Fm335* allele. Nucleotides 200 to 413 were sequenced with the use of an mp8 clone containing the Pst I-Hind III fragment from pDS2A in the orientation shown. Nucleotides 1 to 240 were sequenced with an mp9 clone containing the same fragment in the opposite orientation. The terminal 11-bp inverted repeats and four related sequences at internal sites are underlined. The 8 bp of duplicated genomic DNA flanking the DS element sequence are shown in lowercase. (C) Nucleotide sequences adjacent to the insertion-deletion site in two relevant alleles, as determined from mp8 clones containing the Pst I-Hind III fragments from pRV.5A and pRV.10A in the orientations shown. The synonymous *Ac* elements used to obtain these revertant alleles were M (27) for pRV.5A and Mp (28) for pRV.10A. The remnants of the duplicated sequences related to progenitor residues 38 to 45 are underlined, with brackets showing the bases which were present in the original duplications but which have changed in the revertants. Fig. 4 (right). Hybridization of maize DNA's with probes containing the DS (*Adh1-Fm335*) insertion sequence. Bam HI digests of maize DNA were blot-hybridized with labeled insert sequences from pDS2A. (Lane 1) "Berkeley fast" standard line; (lane 2) "knobless Wilbur Flint" standard line; (lane 3) teosinte.

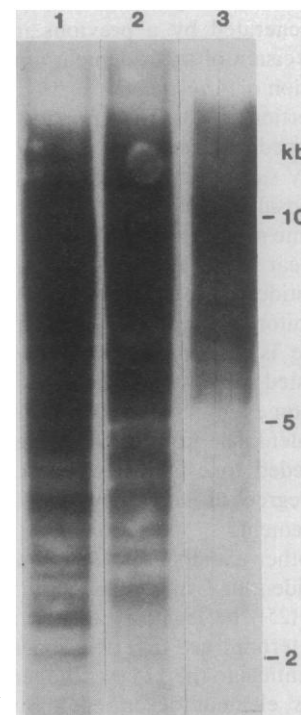


Fig. 3 (left). Nucleotide sequences at the 5' end of the maize *Adh1* gene. (A) Progenitor allele. The sequence was determined using an mp8 clone containing the Pst I-Hind III fragment from pPR.1A in the orientation shown. Nucleotides -148 to +72 were sequenced with an mp8 clone containing the 5' Pst I-Bgl II fragment from λPR.1 in the opposite orientation. The two differences from the corresponding sequence of the *Adh1-S* allele (20) are shown in brackets; the A at -121 is uncertain in analysis of

Although the entire *Ds*(*Adh1-Fm335*) element was excised in our revertants, there were allele-specific changes at the site of *Ds* excision. These changes could be the result of DNA breakages associated with excision, followed by an error-prone rejoining process. According to this working hypothesis, relatively accurate rejoining events would result in reversion to wild-type activity, less accurate events would result in deletions, and complete failure of the rejoining process would result in chromosome breakage.

The revertants had transversions on each side of the excision site. If this is a common pattern for the reversion of *Ds*-induced mutations, it may be significant that nucleotides 34 to 49 of the progenitor sequence, 5'-GTGAGGGA CTGAGGGT-3', can be represented as a duplication of an 8-bp sequence with 1 bp inverted on each side of the central junction. Possibly this near duplication was generated by a previous insertion and excision of a *Ds* element during the evolution of *Adh1*.

Insertions of *Ds*(*Adh1-Fm335*) may be influenced by sequence homology, as already suggested for some prokaryotic transposable elements (24), since the first nine nucleotides of the 11-bp inverted repeat sequence show homology with nucleotides 35 to 45 and 43 to 49 at the progenitor insertion site. The latter sequence is also present at the site we suggested was involved in a previous *Ds* insertion event. Sequencing of other independent insertions of *Ds* elements will be needed to establish whether or not this degree of homology is a general requirement.

Another example of a *Ds* element has been identified in mutant sh-m5933 of maize (25). Its length is 2.04 kb and the 11-bp termini are inverted repeats that are identical to the 11 bp at the termini of the *Ds* element described above. The sequence between the 11-bp termini has little or no homology with the *Ds* element inserted in the *Adh1-Fm335* allele. An 8-bp duplication is formed at the site of insertion in the sh-m5933 mutation.

The effects of the *Ds*(*Adh1-Fm335*) element on *Adh1* expression were characterized (7) as a sevenfold reduction in ADH activity and a reduction in ADH thermal stability when mutant extracts were compared with progenitor extracts diluted to a comparable specific activity using extracts from an *Adh1*-null mutant.

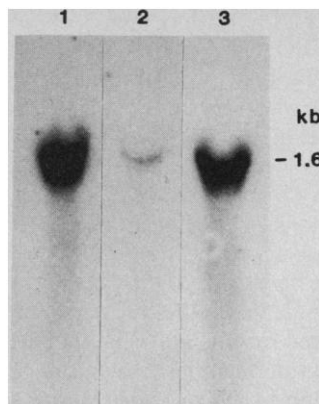


Fig. 5. Hybridization of maize RNA's with an *Adh1* cDNA probe. RNA (20 μ g) from anaerobic maize seedling roots was fractionated by formaldehyde-agarose gel electrophoresis and blot-hybridized with labeled insert sequences from pZML84 (29). (Lane 1) Progenitor stock; (lane 2) *Adh1-Fm335* stock; (lane 3) revertant stock.

They suggested four possible explanations for these effects. (i) The *Ds* element might be transcribed and translated but less than 15 bp long; (ii) *Ds* might be located near the 3' terminus of the *Adh1* coding region; (iii) *Ds* might be located in an intron and modify RNA processing; (iv) the *Ds* element might itself function as a new intron but result in a modified *Adh1* messenger RNA (mRNA) coding sequence. The first three are ruled out by our data. The remaining suggestion is supported by results of blot hybridizations of RNA from the appropriate stocks (Fig. 5). The size of the *Adh1*-specific mRNA from the mutant is close (± 100 bases) to that of the progenitor, and the level of mRNA can account for the lower ADH contained by the mutant. If transcription started at the same position, then most, perhaps all, of the extra 413 bases transcribed from the mutant gene must be deleted during RNA processing. Also, the high AT content of the *Ds* insertion sequence is close to that reported for the introns of several plant genes including *Adh1-S* (20) and soybean *Lb* (26). There is, however, a further possibility that the *Ds* insertion may contain a new site for transcription initiation. This suggestion is supported by the homology already noted between the downstream margin of the *Ds*(*Adh1-Fm335*) insertion and residues 35 to 48 in the progenitor gene, and by the presence of several TATA box sequences at appropriate positions within the insertion.

References and Notes

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17. Plant DNA was isolated by a modification of the method of W. D. Sutton [*Biochim. Biophys. Acta* 366, 1 (1974)]. Fragments of 6 to 9 kb were recovered from agarose gels [J. Langridge, P. Langridge, P. L. Bergquist *Anal. Biochem.* 103, 264 (1980)] from complete Bam HI digests. Fractions (12 to 20 kb) were selected from partial Sau 3A1 digests. This DNA was cloned in the Bam HI sites of λ 1059 vector [J. Karn, S. Brenner, L. Barnett, G. Cesareni, *Proc. Natl. Acad. Sci. U.S.A.* 77, 5172 (1980)] and screened by plaque hybridization [after W. D. Benton and R. W. Davis, *Science* 196, 180 (1977)] with the use of an *Adh1* cDNA probe. Subclones of appropriate restriction fragments were prepared in the M13 vectors mp8 and mp9 [J. Messing and J. Vieira, *Gene* 19, 269 (1982)] for sequencing by the method of F. Sanger *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977)].
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