The Plant Cell, Vol. 5, 1555–1566, November 1993

Genetic Isolation, Cloning, and Analysis of a *Mutator*-Induced, Dominant Antimorph of the Maize *amylose extender1* Locus

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We report the genetic identification, molecular cloning, and characterization of a dominant mutant at the *amylose extender1* locus, *Ae1-5180*. The identities of our clones are corroborated by their ability to reveal DNA polymorphisms between seven wild-type revertants from *Ae1-5180* relative to the *Ae1-5180* mutant allele and between four of five independently derived, *Mutator* (*Mu*)-induced recessive *ae1* alleles relative to their respective wild-type progenitor alleles. The *Ae1-5180* mutation is associated with two *Mu1* insertions flanked by complex rearrangements of *ae1*-related sequences. One of the *Mu1* elements is flanked by inverted repeats of *ae1*-related DNA of at least 5.0 kb in length. This *Mu1* element and at least some of this flanking inverted repeat DNA are absent or hypermethylated in six of seven wild-type revertants of *Ae1-5180* that were analyzed. The second *Mu1* element is flanked on one side by the 5.0-kb *ae1*-specific repeat and on the other side by a sequence that does not hybridize to the *ae1*-related repeat sequence. This second *Mu1* element is present in revertants to the wild type and does not, therefore, appear to affect *ae1* gene function. A 2.7-kb *ae1* transcript can be detected in wild-type and homozygous *ae1-Ref* endosperms 20 days after pollination. This transcript is absent in endosperms containing one, two, or three doses of *Ae1-5180*. This result is consistent with a suppression model to explain the dominant gene action of *Ae1-5180* and establishes *Ae1-5180* as an antimorphic allele. Homozygous wild-type seedlings produce no detectable transcript, indicating some degree of tissue specificity for *ae1* expression. Sequence analyses establish that *ae1* encodes starch branching enzyme II.

INTRODUCTION

Although dominant mutations occur much less frequently than recessive mutations, they are more easily identified. Approximately a dozen dominant mutants have been isolated in maize (Coe, 1993), spontaneously or via transposon or ethyl methanesulfonate mutagenesis experiments (for example, see Freeling, 1985). The molecular features of the dominant gene action of only a few of these alleles have been elucidated. However, using various genetic and molecular criteria, it has been concluded that three of these dominant mutants, *A1-b*, *C1-I*, and *C2-Idf* (which are alleles of the *anthocyaninless1*, *colored aleurone1*, and *colorless2* loci, respectively) have antagonistic effects on their respective wild-type progenitor alleles (Brink and Greenblatt, 1954; Laughnan, 1961; Paz-Ares et al., 1990; Wienand et al., 1991). According to the nomenclature of Muller (1932), these alleles would therefore be classified as antimorphs. The molecular mode of an antimorph's action can be envisioned to occur at various levels. For example, it has been proposed that *C1-I* inhibits the expression of the wild-type allele at the level of protein function (Paz-Ares et al., 1990).

This report concerns a dominant mutant of the *amylose extender1* (*ae1*) locus. This locus was defined by the recessive, mutant reference allele *ae1-Ref* (Vineyard and Bear, 1952). Additional recessive mutants have been isolated (Moore and Creech, 1972; Garwood et al., 1976; Hedman and Boyer, 1983). All of these recessive mutants confer a glassy, tarnished endosperm phenotype and increase the proportion of straight-chain (amylose) starch in the endosperm from ~25% (wild type) to as high as 70% (homozygous mutant) (Shannon and Garwood, 1984). In addition, the branched amylopectin starch that is present in mutant endosperms has fewer α-1,6-branch points than normal amylopectin.

The α-1,6-linkages of amylopectin are catalyzed by starch branching enzyme (SBE) ([1 → 4]-α-D-glucan:[1 → 4]-α-D-glucan 6-glucosyltransferase, EC 2.4.1.18). Multiple forms of this enzyme have been purified from maize endosperms (Boyer and Preiss, 1978). Immunological studies of the SBEs established that SBE I is immunologically distinct from SBE IIa and SBE IIb, and that SBE IIa and SBE IIb are either similar, but

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distinguishable (Fisher and Boyer, 1983), or identical (Singh and Preiss, 1985). These three forms of SBE do have different catalytic properties (Takeda et al. 1993).

SBE IIb activity is absent from kernels homozygous for *ae1-Ref* and several other *ae1* mutant alleles (Boyer and Preiss, 1978; Hedman and Boyer, 1983) but increases in a near-linear fashion as the number of doses of *Ae1+* is increased (Hedman and Boyer, 1982). The levels of the other branching enzymes, SBE I and SBE IIa, are unaffected by alterations in endosperm dosage of *Ae1+*. Hedman and Boyer (1982) therefore hypothesized that *ae1* is the structural gene coding for SBE IIb, and that *ae1-Ref* is a null allele. In contrast, Singh and Preiss (1985) hypothesized that SBEs IIa and IIb are identical enzymes coded for by a single gene, but they differ chromatographically because of differences in the amount of glucan noncovalently bound to the enzyme molecules. According to this hypothesis, homozygous *ae1* endosperms still produce SBE II (presumably in the form of SBE IIa), but they lack the IIb form because of differences in the amount and type of glucan bound due to alterations in starch structure by the *ae1* mutation. Thus, under this model, *ae1* does not code for SBE IIb but codes for another enzyme that affects the amount of the IIb form of SBE II. The molecular isolation of the *ae1* locus would resolve the open question of whether this locus codes for SBE II.

Transposon tagging has become established as an ideal method for cloning maize genes for which mutants can be identified. Approximately a dozen transposon systems have been identified in maize (reviewed by Peterson, 1988). Lines carrying one of them, *Mutator* (*Mu*), exhibit a mutation rate 50-fold higher than the spontaneous rate and the rate observed in lines carrying other transposon systems (Robertson and Mascia, 1981; Robertson, 1983). Most of the new *Mu*-induced mutants arise via the insertion of *Mu* transposons (Brown et al., 1989). At least eight classes of *Mu* elements are present in maize (reviewed by Chandler and Hardeman, 1992). The common feature of these elements is long terminal-inverted repeat sequences. The DNA sequences internal to the long terminal-inverted repeats are distinct among the eight classes, and it is these internal sequences that are used as element-specific probes. The high transposition rate of *Mu* elements makes the *Mu* transposon system an efficient tool for "gene tagging"; many loci have been tagged with *Mu* elements and subsequently cloned by using the inserted *Mu* elements as molecular probes (reviewed by Walbot, 1992).

This report describes the genetic isolation, molecular cloning, and analysis of a dominant *Mu*-induced mutant allele of the *ae1* locus, *Ae1-5180*. Using *Mu1* as a molecular probe, we were able to clone the *Mu1*-tagged *Ae1-5180* allele. Analysis of this mutant allele established that *ae1* is indeed the structural gene for SBE II. Additional analyses established that this allele represents a complex rearrangement of the *ae1* locus. This complex allele suppresses the expression of the *ae1*-specific transcript from wild-type alleles, thereby accounting for the dominant gene action of *Ae1-5180* and establishing the mutant as an antimorph.

RESULTS

Genetic Isolation and Characterization of *Ae1-5180*

A single glassy/tarnished kernel was identified from among 388,688 progeny derived from cross 1 (see Methods). Subsequent crosses demonstrated that this ae1-like phenotype was transmitted normally through both the male and female gametophytes as a dominant mutant (data not shown). Genetic analyses established that the epistatic interactions of this mutant with mutant alleles of the *sugary1* (*su1*) and *waxy1* (*wx1*) loci were similar to those of the *ae1* mutant (Robertson and Stinard, 1991). Mature mutant endosperms are similar to *ae1-Ref/ae1-Ref/ae1-Ref* endosperms in that both contain ~65% amylose (T. Kasemsuwan, J. Jane, P.S. Stinard, D.S. Robertson, and P.S. Schnable, unpublished observation), in contrast to the 25% found in wild-type endosperms. Because the phenotype conferred by this mutant was indistinguishable from that of *ae1-Ref* when assayed by visible, epistatic, and compositional criteria and because it mapped to the same chromosomal position as *ae1* (data not shown), we concluded that this mutant was a dominant allele of the *ae1* locus, *Ae1-5180*. *Ae1-5180* exhibits complete dominance: mature endosperms containing one, two, and three doses of *Ae1-5180* all exhibited the glassy/tarnished phenotype conferred by recessive *ae1* mutants only in three doses. In addition, the amylose percentage in endosperms did not vary as the number of doses of *Ae1-5180* was changed from one to three (T. Kasemsuwan, J. Jane, P.S. Stinard, D.S. Robertson, and P.S. Schnable, unpublished observation).

Cloning of *Ae1-5180*

To understand the nature of the dominant gene action of *Ae1-5180* and the function of the *ae1* gene, experiments were initiated to clone *Ae1-5180*. Because *Ae1-5180* was isolated from cross 1 in coupling with the *Gl8* allele (of the *glossy8* locus, data not shown), which is 11 centimorgans distal to *ae1* on the long arm of chromosome 5, *Ae1-5180* must have arisen in the *Mu* parent of cross 1 and may therefore be associated with a *Mu* element insertion. DNA flanking such a *Mu* element would have a high probability of representing *Ae1-5180* sequences. *Ae1-5180* was backcrossed to a non-*Mu* line that is wild type for the *ae1* locus (Standard Q60) for 10 generations (cross 2), before conducting cosegregation analyses designed to identify the specific *Mu* element responsible for this mutation.

The final generation of this backcrossing scheme (cross 2) yielded ears that segregated 1:1 for mutant (*Ae1-5180/Ae1+*) and wild-type (*Ae1+/Ae1+*) kernels. An example of such an ear is shown in Figure 1. As a result of the extended backcrossing program, these progeny carried few *Mu* elements. DNAs were isolated from seedlings grown from mutant and wild-type kernels from cross 2 and were used for cosegregation analyses.

Figure 1. Phenotype Associated with *Ae1-5180*.

Portion of an ear segregating for *Ae1-5180* and derived from the cross: *Ae1-5180/Ae1+* × *Ae1+/Ae1+*. The tarnished, glassy kernels have the endosperm genotype *Ae1-5180/Ae1-5180/Ae1+*; the plump wild-type kernels have *Ae1+/Ae1+/Ae1+* endosperms.

The DNAs were digested with the restriction enzyme XhoI, which does not cleave within the *Mu1* element, and subjected to DNA gel blot analysis using a *Mu1*-specific hybridization probe. As shown in the gel blot in Figure 2A, two *Mu1*-homologous DNA fragments, 3.0 and 2.3 kb in length, were identified that cosegregated with the *Ae1-5180* mutant phenotype in 26 progeny of cross 2. Other restriction enzymes (EcoRI, SacI, BamHI, and XbaI) also released two cosegregating, *Mu1*-containing fragments in populations as large as 48 individuals (data not shown).

To determine which of the two (if not both) cosegregating *Mu1* elements is associated with *Ae1-5180*, blots of XhoI-cleaved DNAs from *Ae1-5180* individuals and independent wild-type revertants from *Ae1-5180* (obtained from cross 3, as described in Methods) were hybridized with a *Mu1*-specific probe. Although the *Mu1* copy number was high in the active *Mu* lines from which the revertant alleles were isolated, Figure 3A demonstrates that the 3.0-kb XhoI fragment was clearly absent in at least one of the revertant individuals. It was this fragment that was initially cloned.

Size-fractionated, XhoI-digested DNA fragments that ranged in length from 2.8 to 3.2 kb and that had been isolated from an *Ae1-5180/Ae1+* mutant individual were ligated into λ ZAPII (Stratagene). Three *Mu1*-homologous clones were identified in the resulting library. Further subcloning and restriction mapping, as shown in Figure 4B, revealed that these three clones carried identical 3.0-kb XhoI fragments containing a *Mu1* element. The similarity of these three clones at the level of resolution afforded by restriction mapping establishes that they accurately represent genomic sequences. The *Mu1* element in these clones is flanked by symmetrical restriction sites, thereby suggesting that this *Mu1* element is flanked by inverted repeats. Partial DNA sequence analysis (sequencing inward toward the *Mu1* element from the two flanking XhoI sites) confirmed this hypothesis (Figure 4B; data not shown). Based on the restriction map, a small region of asymmetry (150 bp or less) immediately flanks the left side of the *Mu1* element in Figure 4B.

Ae1-5180 Gene Structure

To obtain further information about the structure of *Ae1-5180*, we cloned two HindIII fragments (12.0 and 12.5 kb) that encompass the two *Mu1*-containing XhoI fragments that cosegregated with *Ae1-5180*: the 3.0-kb fragment with inverted *ae1*-related repeats and the 2.3-kb fragment, respectively (Figures 2A and 4C). The data presented here represent the analysis of two independent clones of the 12.0-kb fragment and a single clone of the 12.5-kb fragment. Restriction mapping revealed that the inverted repeats of *ae1*-related DNA that flank the *Mu1* element present on the 12.0-kb fragment extend at least to the HindIII sites of this clone. DNA gel blotting experiments and restriction mapping established that the *Mu1* element in the 12.5-kb fragment is flanked on one side by a copy of this *ae1*-related sequence and on the other side by a sequence that is not sequence similar (Figure 4C). This arrangement was confirmed by DNA sequence analyses of the 12.5-kb fragment, beginning at the XhoI sites flanking the *Mu1* element, and sequencing inward toward the *Mu1* element (Figure 4C; data not shown). Also, based on our limited sequence data, the inverted *ae1*-related repeats in the 12.0-kb fragment appear to be identical to the corresponding repeat in the 12.5-kb fragment.

Confirmation of the Identity of the *Ae1-5180* Clone

A hybridization probe derived from both of the 700-bp XhoI-NotI fragments flanking the *Mu1* element (probe AE700; Figure 4A; Methods) was hybridized to DNA gel blots derived from the progeny of cross 2 (Figure 2B) that had been used in the

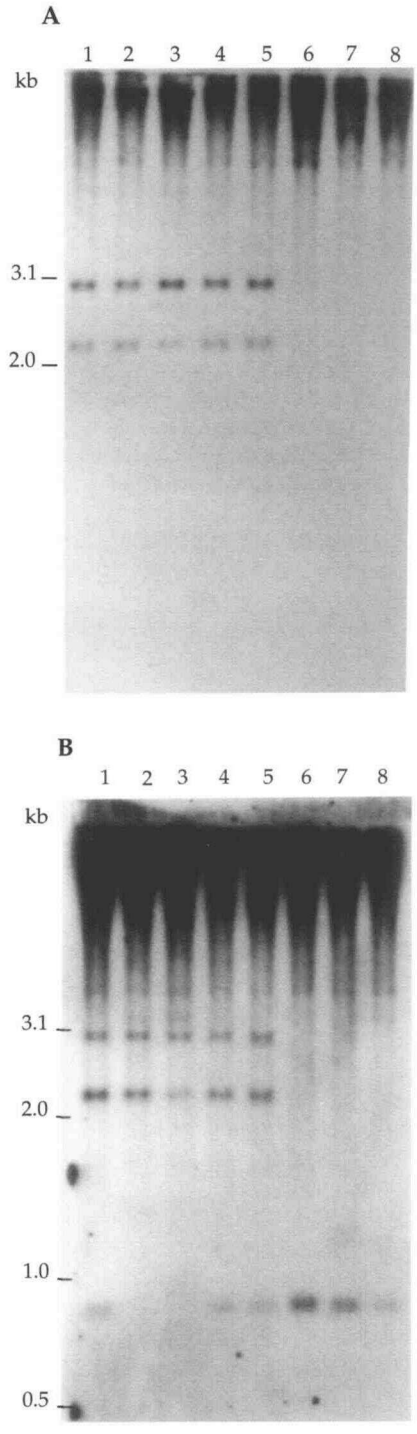

Figure 2. DNA Gel Blot Comparisons of Mutant (*Ae1-5180*) and Wild-Type Siblings.

(A) DNA gel blot analysis of XhoI-cleaved DNAs from five mutant plants (*Ae1-5180/Ae1+*; lanes 1 to 5) and two sibling wild-type (*Ae1+/Ae1+*; lanes 6 to 7) plants all from the cross *Ae1-5180/Ae1+* × *Ae1+Q66/Ae1+Q67*. Lane 8 contains XhoI-digested DNA from the recurrent wild-type parent of the cross, Standard Q60. This blot was hybridized with a *Mu1*-specific probe. This analysis identified two *Mu1*-containing bands of 2.3 and 3.0 kb that cosegregated with the mutant phenotype. Length markers are given at left in kilobases.

(B) DNA gel blot analysis of the same DNAs as shown in **(A)** hybridized with the flanking 700-bp XhoI-NotI probe isolated from the 3.0-kb XhoI fragment (probe AE700; see Figure 4B). In addition to hybridizing to the 2.3- and 3.0-kb *Mu1*-containing DNA fragments that cosegregate with *Ae1-5180*, this probe also hybridizes to a segregating 900-bp DNA fragment in both mutant and wild-type individuals (see text for details). Probe AE700 does not cross-hybridize to *Mu1* (data not shown). Contents of lanes are as given in **(A)**. Length markers are given at left in kilobases.

XhoI cosegregation analyses (Figure 2A). AE700 hybridized to two DNA fragments associated with the *Ae1-5180* allele. In addition to hybridizing to the 3.0-kb DNA fragment from which it was derived, AE700 also hybridized to the other (2.3-kb) *Mu1*-containing DNA fragment that cosegregated with *Ae1-5180* in Figure 2A. Thus, Figure 2B reveals *Ae1-5180* as a complex mutant allele containing at least a duplication of AE700-related sequences and two *Mu1* elements.

Probe AE700 also hybridized to a 900-bp fragment carried by some of the mutant and wild-type individuals in this family derived from cross 2. The 900-bp fragment is derived from a wild-type *Ae1+* allele (*Ae1+Q66*) carried by the F_1 hybrid (Standard Q60) that was used as the male parent of cross 2. The other wild-type allele carried by Standard Q60 (*Ae1+Q67*), which is expected to be present in at least some lanes of Figure 2B, yields an AE700-hybridizing XhoI fragment with a high molecular weight that is not visible in Figure 2B (data not shown). In this blot, AE700 hybridizes strongly to high molecular weight DNA fragments. This is quite typical of even single-copy probes that are hybridized to maize DNA digested with a methylation-sensitive restriction endonuclease such as XhoI. Hybridizations of AE700 to maize DNA digested with restriction endonucleases such as EcoRI and HindIII, which are not methylation sensitive, reveal one to two hybridizing fragments per allele, as shown in Figure 5B (and data not shown). This demonstrates that AE700 is composed of low-copy DNA sequences.

Figures 3B and 5A present the hybridization of AE700 to a DNA gel blot of XhoI-cleaved DNAs from heterozygous mutants (*Ae1-5180/ae1-Ref*) and sibling wild-type revertants (*Ae1-rev/ae1-Ref*) derived from cross 3 (see Methods). AE700 hybridized to both the 2.3- and 3.0-kb fragments in the heterozygous mutant individuals (*Ae1-5180/ae1-Ref*) but to only the 2.3-kb band in six of seven independent revertants (*Ae1-rev/ae1-Ref*). We concluded that in these revertants, the DNA from the 3.0-kb fragment has been lost, rearranged so it is now on a much larger XhoI fragment, or its terminal XhoI sites have become methylated. If either of the latter two explanations is correct, the 3.0-kb fragment DNA would be present in the high molecular weight DNA that hybridizes to AE700 and therefore

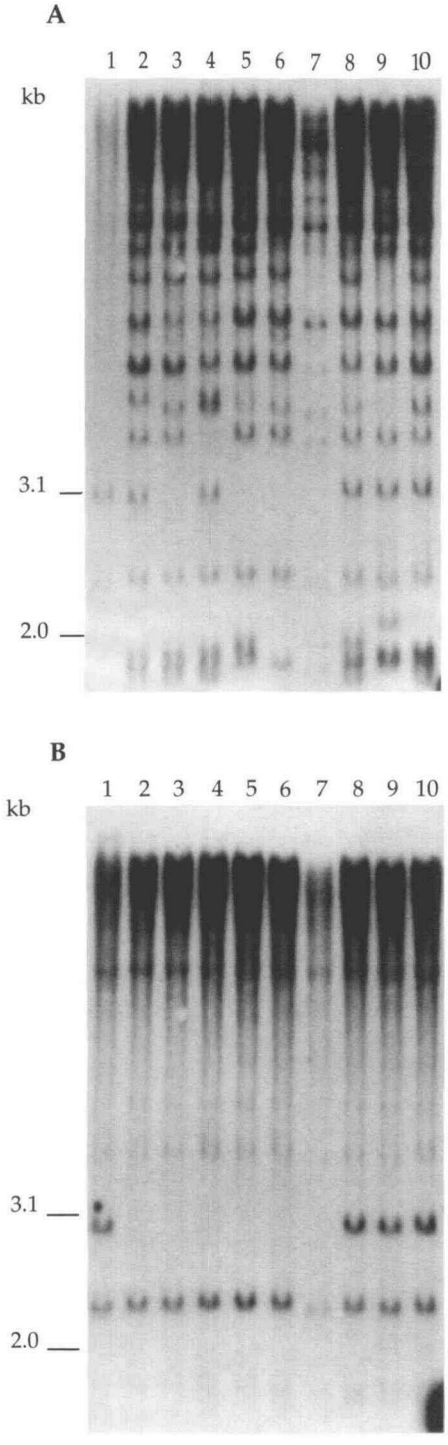

Figure 3. DNA Gel Blot Comparisons of XhoI-Digested DNAs from Wild-Type Revertants and Mutant (*Ae1-5180*) Siblings.

(A) These revertants were produced on an ear from the cross: *Ae1-5180/Ae1-5180 Mutator* × *ae1-Ref/ae1-Ref.* Lane 1 contains DNA isolated from a heterozygous mutant *Ae1-5180* control (*Ae1-5180/Ae1+*) with low *Mu1* copy number. Lanes 2 to 4 contain DNAs isolated from seedlings grown from plump sibling progeny kernels from the cross *Ae1-rev1/ae1-Ref* × *ae1-Ref/ae1-Ref.* Lanes 5 to 7 contain DNAs isolated from seedlings grown from plump sibling progeny kernels from the cross *Ae1-rev2/ae1-Ref* × *ae1-Ref/ae1-Ref.* Lanes 8 to 10 contain DNAs from mutant kernels from the same ear on which *Ae1-rev1* and *Ae1-rev2* arose. This blot was hybridized with a *Mu1*-specific probe. All individuals carry the 2.3-kb cosegregating XhoI fragment, but the revertants lack the 3.0-kb fragment. The fragments in lanes 2 and 4 that appear to be approximately the same length as the 3.0-kb cosegregating fragment are in fact somewhat smaller than 3.0 kb.
(B) DNA gel blot analysis of the same DNAs as shown in **(A)**, hybridized with probe AE700. Note that the 2.3-kb cosegregating fragment is present in all individuals but that the 3.0-kb fragment is missing in the revertants. The 900-bp hybridizing fragment contributed by the *ae1-Ref* allele has been cropped off in this blot but can be seen in Figure 5. Contents of the lanes are as given in **(A)**.
Length markers are given in kilobases at left.

would be masked. The seventh revertant exhibited no alterations in its XhoI fragments (*Ae1-rev4*, Figure 5A, lane 3). The 900-bp band that is present in all individuals was contributed by the male parent of cross 3 and is derived from the *ae1-Ref* allele. This 900-bp band is visible in Figure 5A, but not in Figure 3B (which was cropped).

Results of a similar analysis of revertants using HindIII are shown in Figure 5B. As discussed above, the *Ae1-5180* allele releases two AE700-hybridizing HindIII fragments of 12.0 and 12.5 kb. The 3.0- and 2.3-kb XhoI fragments are entirely contained within the 12.0- and 12.5-kb HindIII fragments, respectively. These two HindIII fragments comigrated on this DNA gel blot. The 10-kb fragment that hybridizes to AE700 and that is present in all lanes is derived from the *ae1-Ref* allele. Most wild-type revertants give the identical hybridization pattern produced by the *Ae1-5180* allele; no novel AE700-hybridizing fragments appear. We interpreted this to mean that the loss of the 3.0-kb fragment observed in Figures 3B and 5A does not occur via a DNA rearrangement. Rather, either the entire 12.0-kb fragment is lost or this fragment remains unchanged in length during reversion events. The latter explanation would be consistent with hypermethylation of the XhoI sites being the cause of the apparent disappearance of the 3.0-kb fragment following reversion. The 12.5-kb fragment is apparently unchanged during reversion. In contrast, both *Ae1-5180* HindIII fragments of the wild-type revertant (*Ae1-rev4*) that did not exhibit altered XhoI fragments have been reduced in size (Figure 5B, lane 3).

Probe AE700 also reveals polymorphisms between four independent *Mu*-induced recessive *ae1* mutant alleles and their corresponding wild-type progenitor alleles. These mutant alleles were isolated, as described in the Methods section. DNAs from seedlings homozygous for five independent *Mu*-induced *ae1* alleles (*ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, *ae1-Mu7*, and *ae1-Mu10*) were isolated, digested with XhoI, and electrophoresed on agarose gels alongside XhoI-cleaved DNAs isolated from

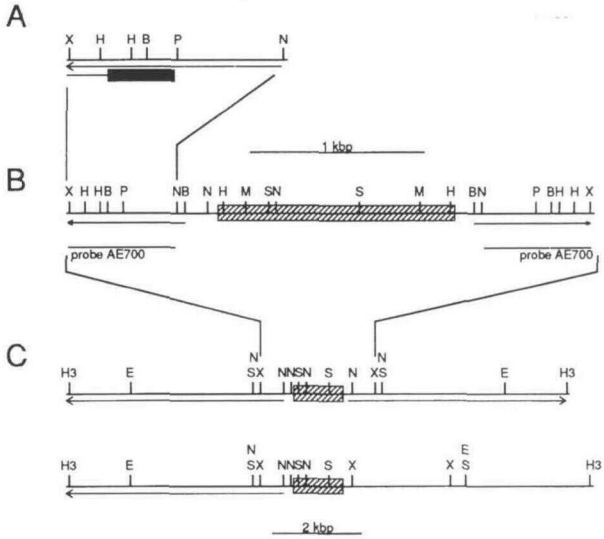

Figure 4. Restriction Mapping of *Ae1-5180* Clones.

(A) Restriction map of probe AE700, which has been partially sequenced. The region that shows identity to the sequence of SBE II (the first exon of *ae1*) is indicated by a thick black bar. A portion of the first intron is indicated by a thin line. The restriction sites indicated are XhoI (X), HinfI (H), BanII (B), PstI (P), and NotI (N). Probe AE700 was used to make hybridization probes for the DNA and RNA gel blots in Figures 2B, 3B, 5A, 5B, and 7. Arrow indicates inverted and repeated *ae1* sequences.

(B) Restriction map of the 3.0-kb XhoI *Ae1-5180* clone. Restrictions sites indicated are XhoI (X), HinfI (H), BanII (B), PstI (P), NotI (N), SacII (S), and MluI (M). This map is shown in relation to the 12.0-kb HindIII fragment of which it is a portion, as shown in **(C)**. The segment representing the 1.4-kb *Mu1* insert is indicated by a hatched box. Arrows indicate the inverted repeats of the *ae1*-related DNA flanking the *Mu1* element.

(C) Restriction map of the 12.0- (above) and 12.5-kb (below) HindIII *Ae1-5180* cloned fragments. Restriction sites indicated are HindIII (H3), EcoRI (E), SacII (S), NotI (N), and XhoI (X). The segments representing the 1.4-kb *Mu1* inserts are indicated by hatched boxes. Arrows indicate the regions of *ae1*-related DNA flanking the *Mu1* element that are inverted and repeated.

seedlings segregating for the corresponding wild-type progenitor alleles (see Methods). DNA gel blot analyses prepared using probe AE700, as shown in Figure 6, revealed that *ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, and *ae1-Mu10* contain ~1.4-kb inserts relative to the alleles present in families expected to be segregating for the corresponding progenitor alleles (Figure 6 and data not shown). Two wild-type alleles (one of which is the progenitor allele) are expected to be present in each of these segregating families. To be conclusive, these allelic cross-referencing experiments must demonstrate that the mutant allele is different from both wild-type alleles present in the *Mu* parent of cross 4, and we did not determine which wild-type allele represented the progenitor. Digestions with HindIII revealed that the families carrying the progenitor alleles of

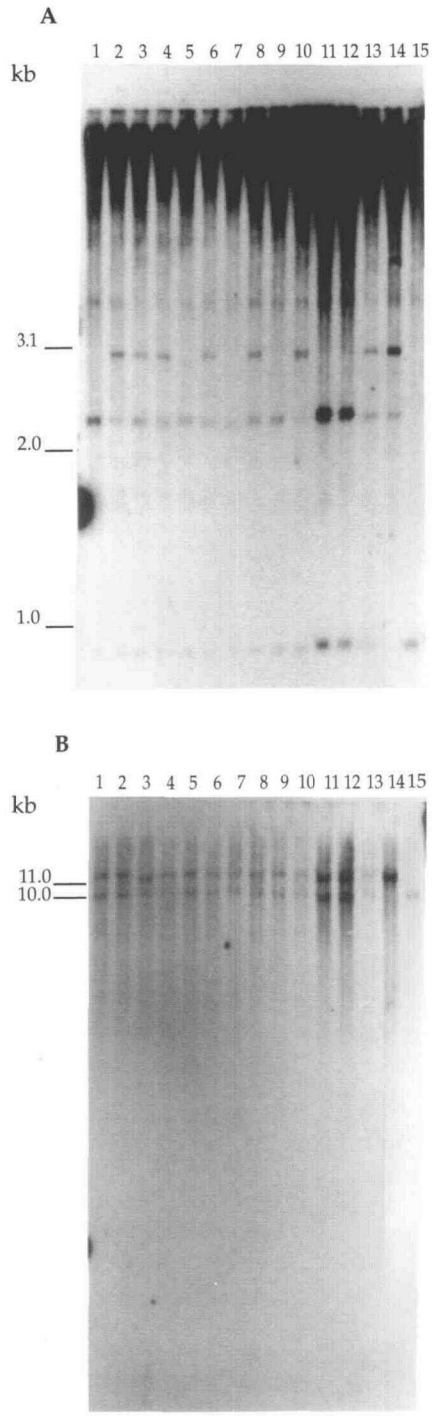

Figure 5. DNA Gel Blot Comparisons of HindIII-Digested DNA from Wild-Type Revertants and Their Mutant (*Ae1-5180*) Siblings.

(A) DNA gel blot analysis of XhoI-cleaved DNAs from seven independent *Ae1-5180* revertants to wild type (*Ae1-rev/ae1-Ref*; lanes 1, 3, 5, 7, 9, 11, and 12), sibling heterozygous mutant *Ae1-5180* plants grown from kernels from the same ears on which the revertants arose (lanes

ae1-Mu3 and *ae1-Mu5* were indeed segregating for two *Ae1+* alleles (data not shown), both of which are distinct from the corresponding *ae1-Mu* alleles.

The finding of DNA alterations in the AE700-hybridizing sequences coincident with mutations at the *ae1* locus confirms that probe AE700 hybridizes to the *ae1* locus. The 1.4-kb inserts in *ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, and *ae1-Mu10* are likely *Mu1* inserts. The *ae1-Mu7* allele showed no change relative to its progenitor in this analysis; the DNA sequence alteration that caused this mutation is either undetectable at this level of resolution or it occurred outside the region revealed using this probe/restriction enzyme combination.

Together, these data (polymorphisms between *Ae1-5180* and revertant alleles and polymorphisms between independent recessive mutant alleles and their progenitor wild-type alleles) establish that probe AE700 contains at least a portion of the *ae1* locus.

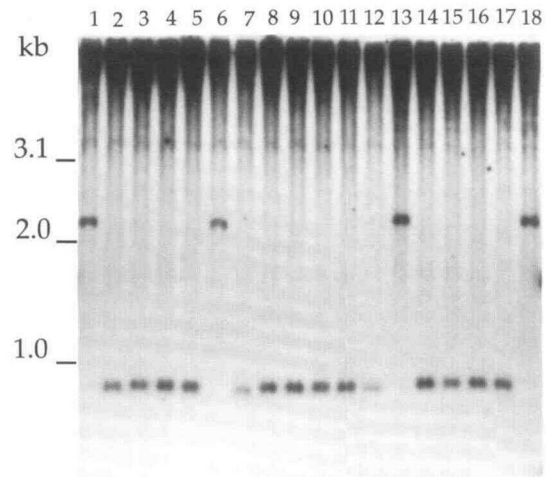

Figure 6. DNA Gel Blot Analysis of *Mu*-Induced Recessive *ae1* Alleles.

DNA gel blot analysis of XhoI-cleaved DNAs isolated from plants homozygous for three different *ae1-Mu* alleles and plants segregating for the corresponding wild-type progenitor alleles: *ae1-Mu6* (lanes 1 and 6) and its progenitors (lanes 2 to 5); *ae1-Mu7* (lanes 7 and 12) and its progenitors (lanes 8 to 11); and *ae1-Mu10* (lanes 13 and 18) and its progenitors (lanes 14 to 17). This blot was hybridized with probe AE700. The *ae1-Mu6* and *ae1-Mu10* alleles have AE700-hybridizing bands 1.4 kb greater than their corresponding progenitor alleles. These 1.4-kb shifts could represent *Mu1* inserts. *ae1-Mu7* showed no change relative to the progenitor in this analysis. Length markers are given at left in kilobases.

ae1 Gene Function

A cDNA corresponding to a type II class of SBE has been isolated via its sequence similarity to SBE I of pea and sequenced (EMBL accession No. L08065; Fisher et al., 1993). Comparison of the sequence of this SBE II cDNA with the partial sequence of AE700 reveals 98% identity over a length of 201 bp (Figure 4A). Identity begins at nucleotide 3 in the cDNA sequence and ends at nucleotide 203. Discrepancies occur at nucleotides 77 (before the first ATG), 100, 101, and 127. AE700 contains sequences 5′ and 3′ to the region of identity. Thus, we concluded that *ae1* is the structural gene for a SBE of the type II class and that AE700 contains sequences 5′ to the transcribed region, the first exon, and portions of the first intron of *ae1*. This result establishes that both *Mu1* elements are inserted 5′ of the *ae1* coding regions present in the *Ae1-5180* allele (Figure 4C).

Figure 5. (continued).

2, 4, 6, 8, 10, and 13), a homozygous *Ae1-5180* control (lane 14), and a homozygous *ae1-Ref* control (lane 15). This blot was hybridized with probe AE700. Revertants were produced on ears from the cross *Ae1-5180/Ae1-5180 Mutator* × *ae1-Ref/ae1-Ref*. The paired *Ae1-rev* revertants and their sibling *Ae1-5180* controls are as follows: *Ae1-rev3* (lanes 1 and 2), *Ae1-rev4* (lanes 3 and 4), *Ae1-rev5* (lanes 5 and 6), *Ae1-rev6* (lanes 7 and 8), *Ae1-rev7* (lanes 9 and 10), and *Ae1-rev1* and *Ae1-rev2* (lanes 11 to 13). All revertants arose on separate ears, with the exception of *Ae1-rev1* and *Ae1-rev2*, which are from opposite ends of the same ear, and probably of independent origin. All revertants lack the 3.0-kb band, with the notable exception of *Ae1-rev4* (lane 3). The 900-bp AE700-hybridizing fragment is derived from the *ae1-Ref* allele contributed by the male parent.

(B) DNA gel blot analysis of the same HindIII-digested DNAs as shown in **(A)**. This blot was hybridized with AE700. Note that *Ae1-rev4* (lane 3), which did not reveal a difference in the XhoI analysis shown in **(A)**, exhibits a size shift between the revertant and its mutant progenitor (lane 4). HindIII does not detect differences between the remaining revertants and progenitors because both AE700-homologous fragments in *Ae1-5180* are approximately the same length (12.0 and 12.5 kb). The 10-kb fragment is released by the *ae1-Ref* allele.

Length markers are given at left in kilobases.

Transcription of the *Ae1-5180*, *Ae1+*, and *ae1* Alleles

As a preliminary step toward investigating the expression of the *ae1* gene and the basis of the dominant gene action of *Ae1-5180*, RNA gel blots were performed using RNAs isolated from endosperms with the following genotypes: *Ae1+/Ae1+/Ae1+*, *Ae1+/Ae1+/Ae1-5180*, *Ae1+/Ae1-5180/Ae1-5180*, *Ae1-5180/Ae1-5180/Ae1-5180*, *ae1-Ref/ae1-Ref/ae1-Ref*, and from seedlings with the genotype *Ae1+/Ae1+*. All alleles in this analysis had been introgressed into the inbred background A636 four or more generations to minimize differences in expression patterns caused by genetic background effects. Single-stranded RNA hybridization probes were prepared from AE700 in both sense and antisense orientations with respect to the cDNA sequence of SBE II. The sense probe, which would detect an antisense transcript, did not hybridize to any detectable transcript in any of the sample lanes (data not shown). However,

as shown in Figure 7, the antisense probe hybridized with a 2.7-kb transcript in RNA samples from endosperms homozygous for *ae1-Ref* and homozygous for *Ae1+*. The transcript identified with AE700 in the endosperm-derived RNAs is virtually the same length as the SBE II cDNA clone (2725 bp). Significantly, the endosperm samples containing one, two, or three doses of *Ae1-5180* produced no detectable transcripts, demonstrating that the *Ae1-5180* allele suppresses expression of *Ae1+* alleles. Seedling RNA samples from *Ae1+/Ae1+* individuals produced no detectable transcripts, suggesting that expression of *ae1* may be endosperm specific.

DISCUSSION

Even more rare than dominant mutants per se are allelic series that include both dominant and recessive mutant alleles of the same locus. The identification of the *Ae1-5180* mutant establishes *ae1* as the fourth such locus in maize (in addition to *c*, *c2*, and *oil yellow1* (*oy1*) loci; see Coe, 1993).

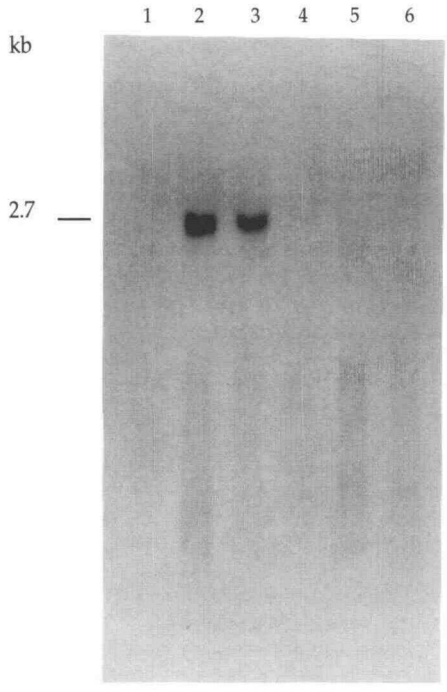

Figure 7. Analysis of *Ae1-5180* RNA Expression.

Gel blot analysis of total RNAs isolated from wild-type (*Ae1+/Ae1+*) seedlings (lane 1), 20-DAP homozygous *ae1-ref* endosperms (lane 2), and 20-DAP endosperms containing 0, 1, 2, and 3 doses of *Ae1-5180* (lanes 3 to 6). The blot was hybridized with an antisense (with respect to SBE II) RNA probe prepared from probe AE700. All genotypes were introgressed into the inbred line A636. Probe AE700 hybridizes to a 2.7-kb transcript in *ae1-ref* and homozygous wild-type (*Ae1+/Ae1+/Ae1+*) endosperms (lanes 2 and 3).

Confirmation of the *Ae1-5180* Clones

We have used a *Mu1*-specific hybridization probe to identify and clone two *Mu1*-homologous DNA sequences that cosegregate with *Ae1-5180*. Allelic cross-referencing data demonstrate that these cloned sequences represent portions of the *Ae1-5180* allele. DNA gel blot analyses of four of five independent *Mu*-induced recessive *ae1* mutants and of seven of seven wild-type revertants of *Ae1-5180* provide proof that the cloned DNA flanking the *Mu1* elements in our 12.0- and 12.5-kb HindIII clones includes *ae1*-specific sequences.

Structure of the *Ae1-5180* Allele

Ae1-5180 has a complex structure, comprising two *Mu1* element insertions coupled with wild-type *Ae1+* sequence repeats. Based upon the evidence presented here, we concluded that the 12.5-kb HindIII fragment includes at least a portion of a wild-type *Ae1+* DNA sequence with a *Mu1* element inserted 5′ to the coding sequences. This *Mu1* insert probably does not disrupt *ae1* gene function. The evidence for this conclusion comes from the analysis of wild-type revertants of *Ae1-5180* that still contain this transposon insertion and that (depending upon the correct model for the loss of the 3.0-kb XhoI fragments following reversion) either lack other copies of the first exon of *ae1* or have additional copies of the first exon that are hypermethylated (and therefore presumably inactive). However, insertions in this region can cause mutations. For example, the apparent insertion of *Mu1* elements into a 900-bp XhoI fragment of a wild-type progenitor allele generated 2.3-kb fragments that are associated with mutant alleles (*ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, and *ae1-Mu10*). These insertions are presumably located in different positions within the XhoI fragment than is the *Mu1* element of the 12.5-kb HindIII fragment of *Ae1-5180*. This would account for the mutant phenotype associated with the former insertions but not the latter.

The 12.0-kb fragment containing the inverted and duplicated *ae1* sequences is probably essential to the dominant mutant expression of *Ae1-5180*, because most revertants either lack this *Mu1* insertion and the associated flanking DNA or carry a hypermethylated form of these sequences. The 12.5- and 12.0-kb fragments are tightly linked genetically, but it is not possible to determine the corresponding physical separation between these fragments because a DNA fragment that spans this distance has not been isolated.

Because the wild-type progenitor allele for *Ae1-5180* has been lost, we can only speculate about the origin of *Ae1-5180*. One model involves a two-step process. In the first step, a *Mu1* element transposed into the 5′ region of *Ae1* but did not cause a phenotypically visible mutation. The 2.3-kb XhoI fragment could reasonably have arisen in this way because the *Ae1+Q66* allele, one of the wild-type alleles present in the *Mu* stocks from which *Ae1-5180* was derived (and therefore a likely progenitor allele of *Ae1-5180*), has a 900-bp XhoI fragment that hybridizes to AE700. The insertion of a 1.4-kb *Mu1* element into this

900-bp XhoI fragment could have generated the 2.3-kb XhoI fragment associated with *Ae1-5180*. According to this model, the second step in the origin of *Ae1-5180* would involve chromosome breakage and repair or aberrant transposition of the first *Mu1* element, thereby resulting in a duplication of the first *Mu1* element and a triplication of its flanking *ae1* DNA.

Reversions to the wild type from *Ae1-5180* are usually associated with the disappearance of one of the *Mu1* elements and two of the three *ae1* repeats found on the 3.0-kb XhoI fragment associated with this allele. One of the two models to explain this disappearance involves the loss of these DNA sequences. Similar losses of repeated sequences have been observed at other loci. Athma and Peterson (1991) found that one of the two direct repeats present in the *P* locus of maize can be lost from an *Activator* (*Ac*) insertion mutant (*P1-ovov*) at high rates. Three models were evoked to explain these losses: unequal sister chromatid exchange, double-strand breaks, and intrachromatid homologous recombination (Figure 7 in Athma and Peterson, 1991). *Ae1-5180* shares several similarities with some *Mu* transposon insertion derivatives of the *Kn1-0* mutant (of the *knotted1* locus) of maize (Lowe et al., 1992). Like *Ae1-5180*, these dominant mutants are associated with additional copies of genic sequences that are lost following reversion to the wild type.

In addition to the unequal sister chromatid exchange and intrachromatid homologous recombination models proposed by Athma and Peterson (1991), Lowe et al. (1992) proposed that reversion of the *Kn1-0* derivatives to the wild type could occur via gene conversion (see Figure 5 in Lowe et al., 1992). With certain modifications necessitated by the structural differences displayed by *Ae1-5180* relative to the *P1-ovov* allele and the *Kn1-0* derivatives, these models could also explain reversion of *Ae1-5180*. However, these models were based upon the observations that losses of repeated sequences from *P1-ovov* and the *Kn1-0* derivatives are not associated with meiosis (in the case of *P1-ovov*) or the exchange of flanking markers (in the case of the *Kn1-0* derivatives). It is not known whether exchange of flanking markers is associated with reversion of *Ae1-5180*. If it is, reversion of *Ae1-5180* could also be explained by oblique pairing followed by unequal crossing over. Such a model has been used to explain at least some of the losses of repeated elements at the *a1*, *red1* (*r1*), and *resistance to Puccicinia sorghi1* (*rp1*) loci of maize (Laughnan, 1961; Robbins et al., 1991; Sudupak et al., 1993).

Mode of *Ae1-5180*'s Dominant Gene Action

Muller (1932) classified dominant mutant alleles as amorphs (and hypomorphs), hypermorphs, neomorphs, and antimorphs depending on their mode of action. As far as can be determined, *ae1* is not a dose-sensitive locus; *Ae1+/Ae1+*, *Ae1+/Ae1+/Ae1+*, and *Ae1+/Ae1+/Ae1+/Ae1+* kernels produced using the TB5La B-A translocation are all wild type (data not shown). It would therefore have been unlikely for *Ae1-5180* to be an amorph, a hypomorph, or a hypermorph. As discussed above, endosperms homozygous for *Ae1+* produce a 2.7-kb *ae1* transcript. However, RNA gel blots have established that one or two endosperm doses of *Ae1-5180* completely suppresses the accumulation of transcript from wild-type *Ae1+* alleles. This suppression probably accounts for the dominant gene action of *Ae1-5180*. As such, *Ae1-5180* can be classified as an antimorph allele. The molecular mechanisms associated with *Ae1-5180*'s antimorphic character are not yet defined. However, the inverted repeats located on the 12.0-kb HindIII fragment associated with the *Ae1-5180* allele (Figure 4) probably play an important role in this suppression phenomenon because at least portions of these repeats are lost (or become hypermethylated) in all seven revertants that were analyzed. In six of these revertants, the affected region included the entire 3.0-kb XhoI fragment; in the remaining instance (*Ae1-rev4*), the 3.0-kb XhoI fragment was left intact, but other sequences within at least the 12.0-kb HindIII fragment were lost or rearranged. Hence, some or all of the sequences critical to the dominant mutant phenotype may lie outside the 3.0-kb XhoI fragment.

The finding that the *ae1*-related duplications present in the *Ae1-5180* allele apparently play a role in suppressing wild-type alleles is reminiscent of "cosuppression." It has been observed that the introduction of chimeric and intact chalcone synthase (*CHS*) and dihydroflavonol-4-reductase genes into wild-type petunia can sometimes (up to 25% of transformants) result in the reversible suppression of the expression of both the ectopic gene and its endogenous homolog (Napoli et al., 1990; van der Krol et al., 1990; additional examples have been reviewed by Jorgensen, 1990). Reversion of cosuppression occurs without loss of the ectopic sequences; this would be similar to the methylation-based model for *Ae1-5180* reversion.

The ability of *Ae1-5180* to suppress in *trans* the expression of wild-type *Ae1+* homologs is similar to the effect of the semidominant *niv-525* and *niv-571* alleles of the *nivea* locus of Antirrhinum (and several derivatives of the latter) on wild-type *Niv+* alleles (Coen and Carpenter, 1988; Bollmann et al., 1991). *Niv+* is the structural gene for *CHS*. The level of *CHS* transcript in heterozygotes carrying one of these mutant alleles and a wild-type allele is greatly reduced relative to wild-type homozygotes. Like *Ae1-5180*, these alleles arose via the action of a transposon, Tam3, and are associated with inverted duplications of *Niv* sequences. Coen and Carpenter (1988) originally presented three models to explain the ability of *niv-525* to suppress expression of *Niv+* alleles: (1) antisense RNA production by *niv-525*, (2) direct or indirect physical interactions between the two alleles that somehow influence transcription, and (3) titration of transcription factors (Bollmann et al., 1991).

We have attempted to address directly the antisense model by assaying for *ae1* antisense transcript. Although the AE700 sense probe failed to detect any antisense transcript in *Ae1-5180* endosperms, antisense transcripts are often difficult to detect via RNA gel blot analyses; hence, a negative result is inconclusive. Although Bollmann et al. (1991) favored the interaction model, such a model makes it difficult to explain the three-way interactions that would be required in *Ae1-5180/Ae1+/Ae1+* cells. We have no data relevant to Coen and

Carpenter's third model (titration). Hence, the molecular mechanism responsible for *Ae1-5180*'s ability to suppress the accumulation of *Ae1+* transcript remains to be elucidated.

ae1 Gene Function

Hedman and Boyer (1982) have proposed that *ae1* is the structural gene coding for SBE IIb. It is still not clear whether there are two forms of SBE II (SBE IIa and SBE IIb) (Singh and Preiss, 1985); however, our data support the view that *ae1* is the structural gene for a SBE II. This conclusion comes from the finding that the transcript detected in homozygous *Ae1+* endosperms by AE700 is the same length as a SBE II cDNA clone (2.7 kb) and from sequence data, which indicate 98% identity between 201 bp of AE700 and the 5′ portion of a SBE II cDNA sequence published by Fisher et al. (1993).

If indeed there are two forms of SBE II, they would be expected to cross-hybridize based on the reported immunological and amino acid sequence similarities (Fisher and Boyer, 1983) between SBE IIa and SBE IIb. However, AE700 detected only a single transcript. This result suggests several possibilities: (1) there is only one form of SBE II, (2) SBE IIa and SBEIIb encode transcripts of identical length, or (3) SBE IIa transcript accumulation is below the level of detection in these experiments. If there are two forms of SBE II, the *ae1* locus probably encodes SBE IIb because the tissue specificity of transcription of *Ae1+* matches the tissue distribution of SBE IIb activity (Dang and Boyer, 1989); the 2.7-kb transcript and SBE IIb are both present in developing wild-type endosperms but absent in seedling leaves.

The 2.7-kb SBE II transcript is observed in homozygous *ae1-Ref* endosperms even though SBE IIb activity is absent in these endosperms (Boyer and Preiss, 1978). This suggests that the lesion in *ae1-Ref* is small (e.g., a point mutation or a frameshift) and does not interfere with transcription but either blocks translation or results in the production of a defective SBE IIb.

METHODS

Genetic Stocks

The *amylose extender1* reference allele (*ae1-Ref*) was obtained from the Maize Genetics Cooperation Stock Center at the University of Illinois, Urbana. *Mutator* (*Mu*) stocks are maintained at Iowa State University by alternately crossing them to the F_1 hybrids Standard Q60 and Standard B70 in successive generations. Standard Q60 (which was also used as the recurrent parent in the *Mu1* copy-number reduction backcrossing program and the male parent of cross 2) is an F_1 hybrid of the two inbred lines Q66 and Q67 (Hallauer, 1967). Standard B70 is an F_1 hybrid of the two public inbred lines B77 and B79. Standard B70 is distinct from the inbred line B70. *Ae1+* is a generic term for a wild-type allele. *Ae1+Q66* designates the wild-type allele from the inbred line Q66. Similar designations are used for wild-type alleles from other inbreds.

Genetic Crosses

The crosses used in this study are as follows.

Cross 1: *gl8 Ae1+/gl8 Ae1+* × *Gl8 Ae1+/Gl8 Ae1+ Mutator*
Cross 2: *Ae1-5180/Ae1+* × *Ae1+Q66/Ae1+Q67* (Standard Q60)
Cross 3: *Mutator Ae1-5180/Ae1-5180* × *ae1-Ref/ae1-Ref*
Cross 4: Standard Q60 or Standard B70 (*Ae1+/Ae1+*) × *Mutator Ae1+/Ae1+*
Cross 5: *Ae1+/Ae1+* (or rarely *ae1-Mu/Ae1+*) selfed

Isolation of Revertants from *Ae1-5180*

Revertants of *Ae1-5180* to the wild-type phenotype were obtained as follows. Lines homozygous for *Ae1-5180* in an active *Mu* background were developed by crossing *Ae1-5180* to active *Mu Ae1+* lines, followed by backcrossing of the resulting heterozygous *Ae1-5180 Mu* plants to homozygous *Ae1-5180* plants to achieve homozygosity for *Ae1-5180*. The homozygous *Ae1-5180 Mu* plants were crossed by homozygous *ae1-Ref* plants, and exceptional wild-type revertant kernels were obtained from the resulting ears (cross 3). With one exception, only revertant kernels arising on separate ears were deemed to be independent of each other. *Ae1-rev1* and *Ae1-rev2* were recovered from the same ear, but they almost certainly represent independent events because they arose at opposite ends of the ear. The clonal nature of maize ear development is such that it would be nearly impossible for one reversion event to produce two wild-type kernels at opposite ends of an ear composed of otherwise mutant kernels. *Ae1-5180* reverts to wild type in the presence of an active *Mu* system at a rate of ~0.1% (data not shown).

Isolation of Recessive *ae1* Mutant Alleles from *Mu* Stocks

Five recessive *ae1-Mu* alleles (*ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, *ae1-Mu7*, and *ae1-Mu10*) were isolated via random *Mu* mutagenesis by selfing (cross 5) *Mu* stocks derived from cross 4. The resulting ears were examined for the segregation of glassy kernels with an ae1-like phenotype. When found, such kernels were allele tested with *ae1-Ref* to determine if they carried an *ae1-Mu* allele. The resulting independently derived recessive *ae1* alleles were used in allelic cross-referencing experiments to confirm the identity of the *Ae1-5180* clone.

However, allelic cross-referencing experiments require that the progenitor allele of each mutant be identified. To assign a progenitor allele to each of the five recessive *ae1* alleles identified from cross 5, we made the assumption that these *ae1* alleles arose in their respective *Mu* parents (cross 4). Progeny of these five *Mu* parents should therefore segregate for the appropriate wild-type progenitor alleles. In addition, each of these progeny should segregate for a second nonprogenitor wild-type allele, because as a consequence of the crossing scheme used to maintain *Mu* stocks at Iowa State Univeristy, any given *Mu* plant is usually heterozygous. Families segregating for the progenitor alleles of *ae1-Mu3*, *ae1-Mu5*, *ae1-Mu6*, and *ae1-Mu10* were obtained from selfed ears of the corresponding *Mu* parents. Families segregating for the progenitor alleles of *ae1-Mu7* were obtained from the appropriate cross 4.

Plant Tissues

Plant materials for RNA gel blot analyses were obtained as follows. For the endosperm dosage series of *Ae1-5180* in A636 genetic

background, plants heterozygous for *Ae1-5180* were crossed to the inbred A636 for six generations and then self-pollinated for two generations to achieve homozygosity for *Ae1-5180*. Endosperms carrying the appropriate doses of *Ae1-5180* were obtained as follows: for zero endosperm doses of *Ae1-5180*, inbred A636 plants were self-pollinated; for one dose, A636 was crossed as a female by the homozygous A636 *Ae1-5180* line; for two doses, the homozygous A636 *Ae1-5180* line was crossed as a female by A636; for three doses, homozygous A636 *Ae1-5180* plants were self-pollinated. In all instances, the endosperms were harvested on the ear 20 days after pollination (DAP), quick frozen in liquid nitrogen, and stored at −70°C. Homozygous *ae1-Ref* endosperms in the A636 background (four generations of backcrossing) were also harvested as described. Greenhouse-grown seedlings of the inbred A636 were harvested 7 days after emergence, quick frozen, and stored at −70°C.

DNA Isolation and DNA Gel Blot Analyses

Maize DNAs for the purpose of DNA gel blot analyses were isolated from seedlings or immature ears either by the method of Dellaporta et al. (1983) or Saghai-Maroof et al. (1984). Maize DNAs for genomic cloning were isolated from seedlings or immature ears by the method of Dellaporta et al. (1983). Maize DNAs were digested using commercially available enzymes according to manufacturers' specifications. Digested DNAs were electrophoresed on agarose gels and transferred to nylon membranes (Magnagraph; Micron Separations Inc., Westboro, MA) according to methods described by Sambrook et al. (1989). Hybridization probes were prepared by random hexamer priming using ^{32}P-labeled dCTP (Feinberg and Vogelstein, 1983). Membranes were hybridized and washed according the manufacturer's specifications and exposed to x-ray film using standard protocols (Sambrook et al., 1989).

Probes

The internal 960-bp MluI fragment of pMJ9 was used as a *Mu1*-specific hybridization probe (Barker et al., 1984; Bennetzen et al., 1984). The AE700 probe consists of both of the apparently identical 700-bp XhoI-NotI fragments flanking the *Mu1* element in the 3.0-kb XhoI clone from *Ae1-5180*. AE700 includes sequences 5′ to the coding sequence of *ae1*, the first exon, and at least a portion of the first intron (Figure 4A).

Genomic Library Preparation and Screening

To obtain the 3.0-kb XhoI clone of *Ae1-5180*, DNA from the immature ear of a heterozygous *Ae1-5180* plant was isolated as described. The DNA was digested to completion with XhoI and electrophoresed on an agarose gel (GTG grade Sea-Kem agarose; FMC, Rockland, ME), and DNAs in the size range of 2.8 to 3.2 kb were recovered by electroelution (Sambrook et al., 1989). Size fractionated DNAs were ligated into the XhoI site of the insertion vector λ ZAPII (Stratagene), packaged using commercial packaging extracts (Stratagene), and plated on the *Escherichia coli* strain SURE (Stratagene). Plaques were lifted on nylon membranes and hybridized using a *Mu1*-specific probe. Hybridizing plaques were picked and purified. Phage DNAs were isolated from three independent positive clones, and the 3.0-kb maize genomic inserts were isolated and subcloned into pBluescript KS+ (Stratagene). All phage and plasmid DNA isolations were performed using the techniques of Sambrook et al. (1989).

HindIII genomic clones of *Ae1-5180* were isolated in a similar manner with the following changes. DNAs were isolated from bulked homozygous *Ae1-5180* seedlings. HindIII-digested DNAs were isolated in the size range of 11 to 14 kb and ligated into sucrose gradient-purified HindIII arms of tho replacement vector Charon 33 (Loenen and Blattner, 1983), a gift from Xiaojie Xu of the P.S. Schnable laboratory. Two independent clones of the 12.0-kb HindIII fragment and one clone of the 12.5-kb HindIII fragment were obtained using AE700 as a hybridization probe.

Restriction Mapping and DNA Sequence Analysis

Restriction maps of the clones were prepared following electrophoresis of plasmid or phage DNAs cleaved with commercially available enzymes. For the purposes of sequencing, the relevant DNA fragments were subcloned into pBluescript KS+ and SK+ (Stratagene), and plasmid DNAs were isolated according to standard procedures. The plasmid subclones were sequenced at the Iowa State University Nucleic Acid Facility using the double-stranded dye terminator technique (Applied Biosystems, Foster City, CA).

RNA Gel Blot Analyses

Total RNAs were isolated from seedlings and 20-DAP endosperms according to the method of Dean et al. (1985). RNAs were electrophoresed on agarose gels and blotted onto nylon membranes (Magnagraph) according to the method of Dean et al. (1985). Single-stranded RNA probes were prepared from pBluescript (KS+ and SK+) subclones of the 700-bp XhoI-NotI flanking fragment using the Riboprobe system (Promega) and ^{32}P-labeled UTP. Hybridizations, washes, and autoradiography were performed as described for DNA gel blot analyses.

ACKNOWLEDGMENTS

We thank Shane Heinen (of the P.S. Schnable laboratory) for technical assistance. We also thank Jack Girton and Tom Peterson for helpful discussions, Mike Lee for a critical reading of the manuscript, and the Jim Colbert laboratory for advice regarding the RNA experiments. This is journal paper No. J-15475 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 2623.

Received August 2, 1993; accepted September 16, 1993.

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