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Characterization of the Maize Gene *sugary1*, a Determinant of Starch Composition in Kernels

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In maize kernels, mutations in the gene *sugary1* (*su1*) result in (1) increased sucrose concentration; (2) decreased concentration of amylopectin, the branched component of starch; and (3) accumulation of the highly branched glucopolysaccharide phytoglycogen. To investigate further the mechanisms of storage carbohydrate synthesis in maize, part of the *su1* gene locus and a cDNA copy of the *su1* transcript were characterized. Five new *su1* mutations were isolated in a *Mutator* background, and the mutant allele *su1-R4582::Mu1* was isolated by transposon tagging. The identity of the cloned element as the *su1* gene locus was confirmed by the cosegregation of restriction fragment length polymorphisms in the same or nearby genomic intervals with three additional, independent *su1* mutations. Pedigree analysis was also used to confirm the identity of *su1*. A 2.8-kb mRNA that is homologous to the cloned gene was detected in maize kernels, and a 2.7-kb cDNA clone was isolated based on hybridization to the genomic DNA. Specific portions of the cDNA hybridized with multiple segments of the maize genome, suggesting that *su1* is part of a multigene family. The cDNA sequence specified a polypeptide of at least 742 amino acids, which is highly similar in amino acid sequence to bacterial enzymes that hydrolyze α-(1→6) glucosyl linkages of starch. Therefore, debranching of glucopolysaccharides is seemingly part of the normal process of starch biosynthesis, and the final degree of branch linkages in starch most likely arises from the combined actions of branching and debranching enzymes.

INTRODUCTION

An important function of endosperm tissue in plant development and reproduction is the storage of carbohydrate reserves in the form of starch. The biochemical mechanisms of starch biosynthesis are of interest for understanding fundamental aspects of plant physiology and also for their potential utility in manipulating plant growth for practical purposes. Starch not only is a critical primary source of dietary carbohydrates but also is used extensively for various industrial purposes, ranging from formation of packaging materials to ethanol production. Despite its wide availability in nature and its many industrial applications, the mechanisms by which starch is formed in plant endosperm tissue are not well understood.

Starch is essentially a mixture of the homopolysaccharides amylose and amylopectin (for review, see Manners, 1985). Amylose typically is a linear chain of glucosyl units joined by α-(1→4) glycosidic bonds, although lightly branched forms of amylose have also been identified (Takeda et al., 1990). Amylose normally constitutes ~25% of the total endosperm starch in maize (White, 1994). Amylopectin is another type of glucose homopolymer that, in normal maize endosperm, constitutes ~75% of the starch. Amylopectin comprises many linear chains of monomers joined by α-(1→4) linkages; these chains are joined to each other by α-(1→6) glycosidic bonds, often referred to as branch linkages. In the amyloplasts of endosperm cells, glucose units are donated from the high-energy carrier compound ADP-glucose to the growing glucan chains by formation of α-(1→4) linkages at their nonreducing end. In maize, two types of ADP-glucose glucosyltransferases (also called starch synthase) are known to catalyze this reaction, the granule-bound starch synthase (GBSS) and the soluble starch synthase (SSS). Multiple forms of both GBSSs and SSSs have been identified (Nelson and Rines, 1962; Boyer and Preiss, 1978; MacDonald and Preiss, 1985; Dang and Boyer, 1988). The α-(1→6) branch linkages are formed by (1→4)-α-D-glucan:(1→4)-α-D-glucan 6-glucosyltransferases, also known as starch branching enzymes (SBEs). Again, multiple forms of SBEs have been reported (Singh and Preiss, 1985; Dang and Boyer, 1988).

The activities of starch synthases and SBEs presumably are combined and regulated so that specific starch forms result. Thus, the distribution of chain lengths in amylose and the frequency of α-(1→6) branch linkages relative to α-(1→4) linkages in amylopectin are both fixed within certain limits. The nature of this enzymatic coordination is not known but is of interest because changes in the regulatory parameters would produce different forms of starch in the endosperm.

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Genetic analysis of maize has allowed isolation of several genes that code for enzymes involved in starch biosynthesis (Preiss, 1991; Lopes and Larkins, 1993; Boyer and Hannah, 1994). The *amylose extender1* (*ae1*) gene codes for the branching enzyme SBEIIb (Stinard et al., 1993). Mutations at this locus result in a decrease in the percentage of amylopectin from the normal value of ~75% of total starch to as low as 30% (Shannon and Garwood, 1984). The *waxy* (*wx*) gene codes for GBSSI (Shure et al., 1983; MacDonald and Preiss, 1985); mutations at this locus result in production of starch granules that lack amylose and contain only amylopectin (Shannon and Garwood, 1984). These data suggest GBSSI produces a polymer that is not acted upon by any SBE and that a different starch synthase produces α-(1→4)–linked linear chains subject to SBE action. A biosynthetic mechanism of this type would require specific coordination of enzyme activities leading to starch production.

Analysis of mutations of the maize *sugary1* (*su1*) gene suggested that an enzyme hydrolyzing α-(1→6) glycosidic bonds, that is, a starch debranching enzyme, is also involved in starch biosynthesis (Pan and Nelson, 1984). The *su1* gene was identified originally by a mutation, termed here *su1-Ref* (indicating the reference allele) (Correns, 1901). When homozygous, *su1-Ref* results in mature dried kernels that have a glassy, translucent, and somewhat shrunken appearance; immature mutant kernels accumulate sucrose and other simple sugars as well as the water-soluble polysaccharide phytoglycogen (Black et al., 1966; Evensen and Boyer, 1986). This combination of traits is referred to as the sugary phenotype. The *su1-Ref* mutation results in reduced kernel dry weight and reduced total starch concentration. There have been conflicting reports regarding the concentration of amylose in starch of *su1-Ref* mutants (Shannon and Garwood, 1984); however, most data indicate that the concentration of amylose is increased in starch from *su1-Ref* mutant kernels relative to the concentration in starch from normal kernels (Ikawa et al., 1981; Yeh et al., 1981; Boyer and Liu, 1985; White, 1994). Concordantly, there is a reduction in the amylopectin present in starch of *su1-Ref* mutant kernels relative to starch in normal kernels (Wang et al., 1993). The total carbohydrate in *su1-Ref* mutant kernels, however, is not significantly reduced compared with its level in normal kernels, because the concentration of disaccharides increases, as does that of phytoglycogen (Sumner and Somers, 1944; Garwood and Creech, 1972). This glucose polymer accumulates in *su1-Ref* kernels to ~25 to 35% of the dry weight but is present in normal kernels at very low or undetectable levels (Creech, 1965; Black et al., 1966; Shannon and Garwood, 1984). Taken together, these observations suggest that phytoglycogen is produced in *su1-Ref* kernels at the expense of amylopectin and, therefore, that the two polysaccharides may share a common biosynthetic pathway.

Other mutations in the *su1* gene locus are known to cause less severe kernel phenotypes in terms of shrunkenness, wrinkling, and phytoglycogen accumulation. For example, the *sugary1-Brawn2* (*su1-Bn2*) mutation results in a kernel phenotype and phytoglycogen content intermediate to those of normal and *su1-Ref* kernels (Garwood and Creech, 1972; Brink, 1984), whereas *sugary1-crown* (*su1-cr*) results in wrinkling just on the crown of the kernel and accumulation of only small amounts of phytoglycogen (Brink, 1984). The *amylaceous sugary* (*su1-am*) mutation is allelic to *su1-Ref* and results in the sugary phenotype in combination with mutations of the *dull1* (*du1*) gene but by itself does not result in a noticeable kernel phenotype (Mangelsdorf, 1947).

Phytoglycogen resembles amylopectin in that α-(1→4)–linked chains are joined by α-(1→6) branch linkages, but the ratio of α-(1→6) to α-(1→4) linkages is significantly higher in phytoglycogen than it is in amylopectin (Manners, 1985). This highly branched polysaccharide is likely to accumulate in *su1* endosperm tissue because of the loss of one or more starch debranching enzymes (Pan and Nelson, 1984). In that study, debranching enzyme activity was found to be significantly reduced in homozygous *su1-Ref* mutants and, in heterozygotes, is proportional to the number of wild-type *su1* alleles present. These data suggest that *su1* codes for a starch debranching enzyme. Pan and Nelson (1984) postulated that synthesis of amylopectin could result from an equilibrium between the actions of SBEs and starch debranching enzymes. According to this hypothesis, a deficiency in starch debranching enzyme activity is expected to result in a more highly branched polysaccharide. Accumulation of phytoglycogen is not proportional to *su1* gene dosage but occurs only in endosperm homozygous for a *su1* mutation (Doehlert et al., 1993; P. Keeling and G. Singletary, personal communication). This observation suggests that relatively small amounts of the *su1* gene product (termed SU1) are sufficient for normal starch biosynthesis.

This report describes the application of the transposon tagging strategy to isolate part of the *su1* gene locus and a cDNA copy of the *su1* mRNA transcript. The amino acid sequence deduced from the nucleotide sequence is significantly similar to that of bacterial isoamylases, enzymes that hydrolyze α-(1→6) glycosidic bonds. These data provide strong evidence in support of the hypotheses that (1) *su1* codes for a starch debranching enzyme and (2) biosynthesis of specific starch forms in maize endosperm occurs by the coordinated activity of three types of enzymes, namely, starch synthases, branching enzymes, and debranching enzymes.

RESULTS

Identification of *Mutator*-Induced *su1* Mutations

The *Mutator* (*Mu*) transposable element system was used to generate five new mutations at the *su1* gene locus (Scanlon et al., 1994). As diagrammed in Figure 1, plants from hybrid lines Q66/Q67 or B77/B79 with no history of *Mutator* activity (see Methods) were crossed to a line in which transposition of *Mu* elements was known to occur frequently (i.e., a *Mutator* line); the resulting F_1 progeny were self-pollinated. In rare instances, the sugary phenotype was observed in approximately

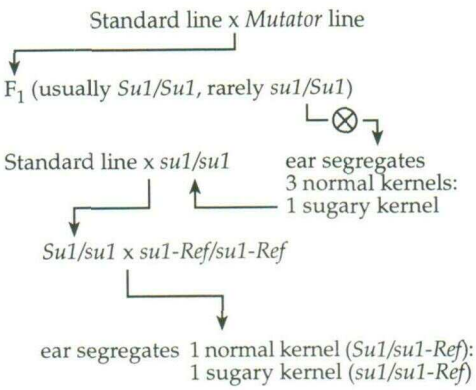

Figure 1. Crossing Scheme for Isolation and Identification of *su1* Mutations.

Mutations at the *su1* gene locus were generated by crossing active *Mutator* plants with standard lines homozygous for the non-mutant allele *Su1* (see Methods). The F_1 progeny were grown and self-pollinated, and the resulting F_2 ears were examined for 3:1 segregation of normal and sugary kernels. Sugary kernels from individual F_2 ears were planted, and the resultant plants were crossed to standard lines. Progeny from this cross (heterozygous for the putative *su1* mutation) were planted and crossed to tester plants homozygous for *su1-Ref.* Allelism of the new mutation with *su1-Ref* was indicated by 1:1 segregation of normal to sugary kernels on the resulting ears.

one-fourth of the kernels on a self-pollinated ear, indicating that the F_1 plant could be heterozygous for a recessive *su1* mutation. In this study, plants grown from the sugary mutant kernels were crossed to a standard line to produce kernels heterozygous for the recessive mutation. As expected for a recessive mutation, all of the heterozygous kernels were wild type in appearance. Mature plants grown from the heterozygous kernels were crossed to a *su1-Ref/su1-Ref* tester. Figure 2A shows a typical ear resulting from this cross, which contained sugary and normal kernels at approximately equal frequencies. These data indicated the new mutation did not complement *su1-Ref* and thus most likely is allelic to mutations at the *su1* gene locus. The one-to-one segregation ratio also confirmed that the sugary phenotype of the original mutant kernel was a single gene trait. Five new *su1* mutations were identified in this way and were designated *su1-R4582::Mu1*, *su1-R2412*, *su1-R7110*, *su1-R3162*, and *su1-R8064* (see Methods).

Figure 2B shows the kernel phenotypes that result from each new *su1* mutation in the homozygous condition. The mutations *su1-R3162* and *su1-R8064* resulted in kernel phenotypes similar to that resulting from *su1-Ref.* The mutation *su1-R2412* resulted in a seemingly less severe phenotype, with only a slight glassiness and wrinkling at the crown of the kernel. Kernels homozygous for *su1-R4582::Mu1* or *su1-R7110* generally appeared more severely shrunken than *su1-Ref* homozygotes. The kernels compared in Figure 2B are from diverse genetic backgrounds; thus, the observed phenotypic variations are not necessarily due to the nature of the mutations themselves but rather could have resulted from genetic background effects.

These *su1* mutations, together with *su1-Ref*, currently are being introgressed into a common inbred line to eliminate potential phenotypic variability resulting from genetic background effects.

Cloning of *su1-R4582::Mu1*

The fact that the five new *su1* mutations arose in the *Mutator* line suggested they were caused by insertion of a *Mu* transposable element at the *su1* gene locus. To test this hypothesis, plants containing *su1-R4582::Mu1* and others lacking this allele were examined for the presence of a specific *Mu* transposon that cosegregated with the putative *Mu*-induced mutation. This analysis utilized the segregating population of sugary kernels (*su1-R4582::Mu1/su1-Ref*) and sibling normal kernels containing the non-mutant allele *Su1* (*Su1/su1-Ref*) indicated in Figure 1. Figure 3A shows part of a DNA gel blot analysis of DNAs isolated from maize seedlings; these data identified a 4.0-kb EcoRI restriction fragment containing sequences homologous to the transposon *Mu1* that cosegregated with *su1-R4582::Mu1.*

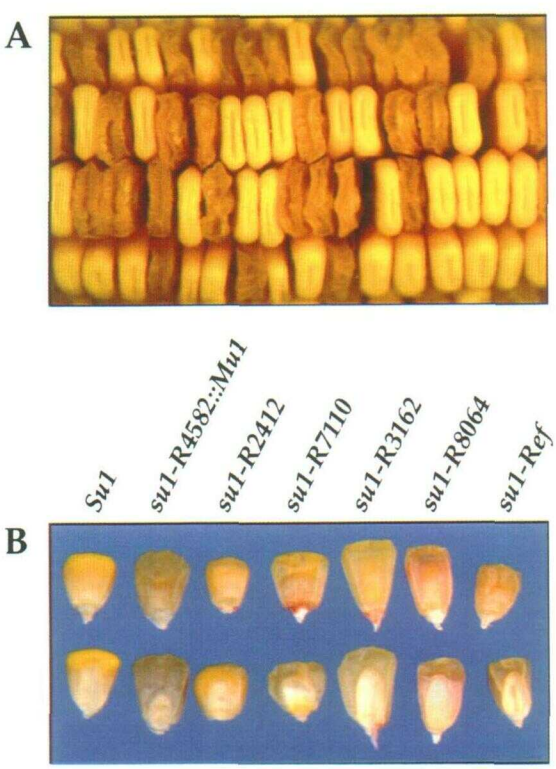

Figure 2. Kernel Phenotypes Resulting from *su1* Mutations.

(A) Allelism test. A plant heterozygous for a putative *su1* mutation (later designated *su1-R4582::Mu1*) was crossed with a plant homozygous for *su1-Ref.* A portion of the mature dried ear is shown.

(B) Kernel phenotypes. Plants heterozygous for the indicated *su1* mutation were self-pollinated. Abgerminal (top row) and germinal (bottom row) views of mature sugary kernels from the resulting ears are shown. The non-mutant *Su1/Su1* kernels are from the F_1 hybrid Q66/Q67.

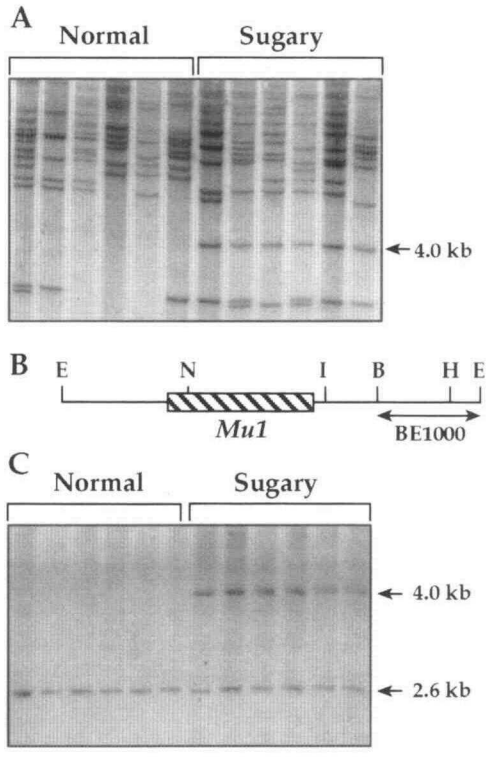

Figure 3. Identification of a Genomic Interval That Cosegregates with *su1-R4582*::*Mu1*.

(A) Detection of genomic fragments containing *Mu1*-homologous sequences. Normal and sugary sibling kernels from a population segregating 1:1 for the non-mutant allele *Su1* and *su1-R4582*::*Mu1* were germinated. (This population was established as shown in Figure 1.) Genomic DNA from the resulting seedlings was digested with EcoRI and probed on DNA gel blots with the internal 960-bp MluI fragment of *Mu1*. The length of the fragment marked with the arrow was estimated based on the mobility in the same agarose gel of standards of known nucleotide sequence.

(B) Map of the cloned 4.0-kb EcoRI fragment containing *Mu1*. The figure is drawn to scale. Restriction sites are indicated for EcoRI (E), NotI (N), HincII (I), HindIII (H), and BamHI (B). The estimated position of the *Mu1* element and the position of probe BE1000 in this fragment are indicated.

(C) Detection in genomic DNA of sequences homologous to the cloned fragment. The analysis is identical to that shown in **(A)**, except that the probe used was BE1000, which is shown in **(B)**. The lengths of the fragments marked with arrows were estimated based on the mobility in the same agarose gel of standards of known nucleotide sequence.

In total, the genomic DNAs of seedlings grown from 60 sugary kernels and 57 normal kernels were examined. This specific *Mu1*-homologous sequence, therefore, either is located within the *su1* gene locus or is tightly linked to that locus.

A 4.0-kb EcoRI restriction fragment containing *Mu1* was isolated from the genome of a *su1-R4582*::*Mu1*/*su1-Ref* plant (see Methods). The fragment initially was identified in a library of size-selected genomic DNA fragments constructed in a bacteriophage λ vector, based on hybridization to a probe internal to *Mu1*. This recombinant phage was single-plaque purified, and the genomic DNA insert was subcloned as part of plasmid pMJ60. Figure 3B shows a physical map of the cloned fragment. The position of restriction enzyme recognition sequences in pMJ60 indicated the transposon present was *Mu1* and predicted the position and orientation of this 1.4-kb element within the 4.0-kb EcoRI fragment. The nucleotide sequences of both termini of the transposon were determined and found to match the known sequence of *Mu1* (Barker et al., 1984). A direct repeat sequence of 9 bp was observed in the genomic DNA immediately adjacent to the transposon at each of its termini, typical of *Mu* transposable elements; in this instance, the particular repeated sequence was 5′-CGCGCTCCG-3′.

The region of DNA adjacent to the cloned *Mu1* transposon was shown to cosegregate with *su1-R4582*::*Mu1*, confirming that this sequence was derived from a genomic interval located within or nearby the *su1* gene locus. The 1.0-kb BamHI-EcoRI genomic fragment flanking *Mu1* in the cloned DNA (Figure 3B) was purified and used as a hybridization probe (termed BE1000). Figure 3C shows sample results of a DNA gel blot analysis in which probe BE1000 was hybridized with genomic DNAs from seedlings in the population, segregating for *su1-R4582*::*Mu1* and *Su1*; in total, 96 plants were examined. This genomic probe detected a 4.0-kb EcoRI fragment that was present in all *su1-R4582*::*Mu1*/*su1-Ref* plants but was missing from all *Su1*/*su1-Ref* plants. Thus, the cloned *Mu* transposon is the same element that is within or tightly linked to the *su1* gene locus. Probe BE1000 identified a second EcoRI fragment of 2.6 kb that was present in all plants examined; presumably, it was representative of the non-mutant progenitor allele. This fragment was the only one observed in the *Su1*/*su1-Ref* plants, indicating the reference mutation is not associated with a discernible deletion or insertion. Presumably, insertion of the 1.4-kb transposon *Mu1* within this 2.6-kb region resulted in the 4.0-kb EcoRI fragment that cosegregated with *su1-R4582*::*Mu1*.

The pedigree analysis diagrammed in Figure 4 suggested the cloned DNA is in fact from the *su1-R4582*::*Mu1* allele, as opposed to the alternative possibility that the isolated transposon is linked to the *su1* gene locus but is not the causative agent of the mutation. This conclusion is based on the observation that the 4.0-kb EcoRI fragment containing *Mu1* arose in the same narrow developmental window as did the *su1-R4582*::*Mu1* mutation. F_1 plants derived from 70 kernels of the F_1 ear produced by crossing the F_0 *Mutator* line to an F_0 standard line were self-pollinated in the original screen for sugary mutants. Only one of these F_1 plants produced sugary kernels on its self-pollinated ear (Figure 4). The *su1-R4582*::*Mu1* mutation therefore could not have been present in the somatic cells of either F_0 parent but must have arisen during gametogenesis in one parent or early in development of the specific F_1 embryo that eventually resulted in the sugary kernels. Consistent with this hypothesis, self-pollination of the *Mutator* F_0 parent yielded an ear without sugary kernels. To determine whether the 4.0-kb *Mu1*-containing fragment was present in

the F_0 progenitor plants, nine more kernels from the F_1 ear were planted, and genomic DNA was extracted from the resulting seedlings. In DNA gel blot analysis using probe BE1000, only the 2.6-kb EcoRI fragment was detected (data not shown). If the 4.0-kb fragment existed in one of the F_0 plants, then by Mendelian segregation the fragment would have been present in approximately one-half of the plants analyzed. Thus, the 4.0-kb *Mu1*-containing fragment was not present prior to the generation in which *su1-R4582*::*Mu1* was formed.

The *su1-R4582*::*Mu1* Clone Identifies Restriction Fragment Length Polymorphisms That Cosegregate with *su1-R2412*, *su1-R7110*, and *su1-R3162*

Mutations of the *su1* gene locus other than *su1-R4582*::*Mu1* were analyzed to determine whether they also cosegregated with physical alterations in the cloned region of the genome. Populations segregating for the non-mutant allele *Su1* and either *su1-R2412*, *su1-R7110*, or *su1-R3162* were established as shown in Figure 1. For each allele, genomic DNA from eight *su1/su1-Ref* plants and eight *Su1/su1-Ref* plants was digested with EcoRI and probed in DNA gel blot analysis with genomic fragment BE1000. Figure 5 shows the 4.0-kb EcoRI fragment that in the *su1-R7110* and *su1-R3162* families was present in all the seedlings grown from sugary kernels but was not observed in any seedlings grown from non-mutant sibling kernels. A different EcoRI fragment, 4.6 kb in length, was found to cosegregate with *su1-R2412* (Figure 5). As observed previously, a 2.6-kb EcoRI fragment was detected by the probe in all plants

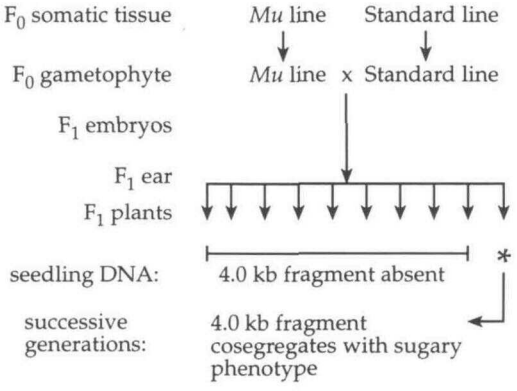

Figure 4. Pedigree Analysis.

The generations that resulted in the mutation *su1-R4582*::*Mu1* are diagrammed. A single F_1 plant, indicated with an asterisk, produced sugary kernels on its self-pollinated ear. The self-pollinated ears of 69 additional F_1 plants were completely wild type with respect to the sugary phenotype. Genomic DNA was isolated from nine other F_1 plant seedlings and examined for the presence of a 4.0-kb EcoRI fragment homologous to genomic probe BE1000. As indicated, none of the F_1 seedling DNAs contained a 4.0-kb EcoRI fragment homologous to probe BE1000.

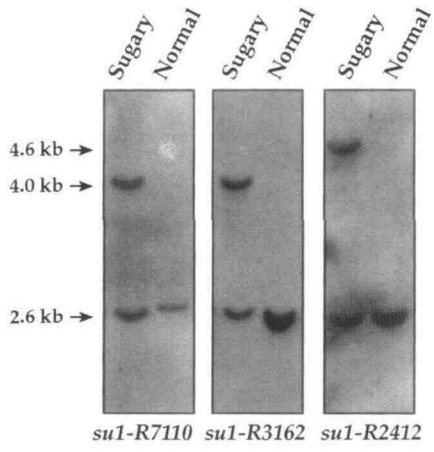

Figure 5. Genomic Insertions Are Linked to *su1-R2412*, *su1-R7110*, and *su1-R3162*.

Normal and sugary sibling kernels from populations segregating for the non-mutant allele *Su1* and either *su1-R7110*, *su1-R3162*, or *su1-R2412* were germinated (populations established as shown in Figure 1). Genomic DNA from the resulting seedlings was digested with EcoRI and probed on DNA gel blots with fragment BE1000 from the *su1-R4582*::*Mu1* genomic clone. The lengths of the fragments marked with arrows were estimated based on the mobility in the same agarose gel of standards of known nucleotide sequence.

examined. Because *su1-R7110* and *su1-R3162* arose in a *Mutator* background, it is likely that each resulted from the insertion of a 1.4-kb transposon, most likely *Mu1*, into the same 2.6-kb genomic interval that also was modified in plants containing *su1-R4582*::*Mu1*. The mutation *su1-R2412* is likely to have occurred via insertion of a 2.0-kb element into this same region. The broad band of ~2.6 kb observed in the *su1-R2412* and *su1-R3162* populations (Figure 5) was resolved in other gels into two fragments of ~2.6 and 2.7 kb (data not shown). Thus, in these backgrounds, the non-mutant allele *Su1* associates with an EcoRI fragment of 2.7 kb, in contrast with the 2.6-kb fragment observed for this allele in the families segregating for *su1-R4582*::*Mu1* (Figure 3C) or *su1-R7110* (Figure 5).

su1 Transcription and Analysis of a *su1* cDNA Clone

To characterize the product of the *su1* gene locus, SU1, a cDNA clone of the *su1* mRNA was obtained. The genomic fragment BE1000 (Figure 3B) was used as a probe in RNA gel blot analysis and detected a transcript of ~2.8 kb in total RNA isolated from wild-type kernels harvested 20 days after pollination (data not shown). This transcript was more abundant in the polyadenylated RNA fraction than in total RNA, suggesting BE1000 detected the *su1* mRNA. A cDNA library constructed from maize endosperm mRNA in a bacteriophage λ vector was screened for hybridization with probe BE1000; eight hybridizing clones were identified among ~200,000 recombinant phage examined. The longest cDNA insert in any of these clones was

~2.4 kb. Considering the 2.8-kb length estimated for the *su1* transcript, this cDNA clone most likely did not contain the entire mRNA sequence. After single-plaque purification, the longest cDNA insert was excised from the recombinant bacteriophage and subcloned in phagemid vectors. An additional 280 bp at the 5′ end of the cDNA were cloned by polymerase chain reaction amplification of the 5′ end of the *su1* mRNA (see Methods). Thus, the total length of the *su1* cDNA clone is ~2.7 kb, which, depending on the length of the poly(A) tail, comprises either the complete or nearly complete mRNA sequence.

The 2706-bp nucleotide sequence of the *su1* cDNA clone was determined (GenBank accession number U18908). A sequence of 14 consecutive A residues was located at one end of the clone, identifying the polyadenylation site and the 3′ end of the mRNA. A continuous open reading frame (ORF) of 742 codons could be discerned, beginning 223 nucleotides from the 5′ end of the cDNA clone and terminating 240 nucleotides prior to the poly(A) addition site. The extent to which this ORF continues at the 5′ end cannot be determined at present because of a short region where the nucleotide sequence could not be resolved on either strand and because the translational initiation codon is unknown. Currently, it is not known whether the cDNA clone contains the native 5′ terminus of the *su1* mRNA sequence. Thus, the deduced primary sequence of SU1 reported here could lack up to 100 residues at the N terminus of the native protein.

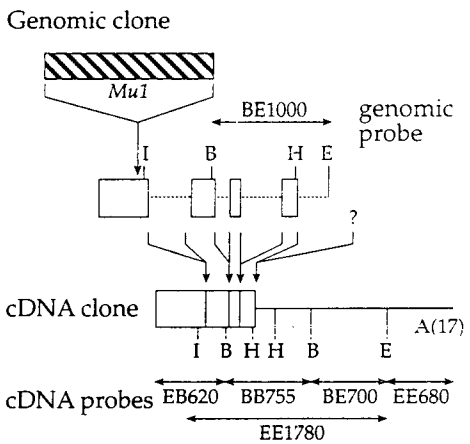

Figure 6. Partial Structure of the *su1* Gene Locus.

The nucleotide sequence of the *su1-R4582*::*Mu1* genomic clone was compared with that of the *su1* cDNA. White boxes represent regions of nucleotide sequence that are identical in the cDNA and genomic clones and thus are designated exons. Dashed lines indicate regions of genomic DNA not present in the cDNA sequence and thus are designated introns. The vertical arrows between the genomic and cDNA clones indicate splice junctions. The solid line indicates the region of the cDNA sequence derived from segments of the genome not included in the *su1-R4582*::*Mu1* clone. The hatched box indicates the position of the *Mu1* element in the genomic clone. The cDNA clone is 2.7 kb in length. The figure is drawn to scale. Restriction enzyme recognition sites are indicated for EcoRI (E), HincII (I), HindIII (H), and BamHI (B). The question mark implies that the extent of the indicated intron could not be determined because its 3′ end is not included in the genomic clone. Horizontal arrows indicate the positions of restriction fragments used as hybridization probes.

The sequence of a 1.6-kb region of the *su1-R4582*::*Mu1* genomic clone also was determined to ensure the cloned cDNA was derived from the putative *su1* gene locus and to reveal the presence of introns. Figure 6 shows that four exons and four introns were identified by comparing the cDNA and genomic sequences. The site of the *Mu1* insertion in *su1-R4582*::*Mu1* was identified in the cDNA clone (Figure 6), indicating the insertion occurred in an exon. As expected, the 9-bp sequence that is directly repeated at both ends of the *Mu1* insertion in the genomic clone was present only once in the cDNA. The available genomic sequence includes only 874 bp of the total 2706 bp in the cDNA and thus does not contain the entire *su1* gene locus. The genomic clone extends ~500 bp beyond the 5′ end of the cloned cDNA.

Transcription of the *su1* gene locus was examined in normal kernels and sugary kernels homozygous for various *su1* mutations, using as a probe a portion of the *su1* cDNA (Figure 6). Figure 7 shows that in RNA gel blot analysis the cDNA probe EE1780 detected a 2.8-kb mRNA in normal kernels harvested 20 days after pollination. As expected, this transcription pattern is identical to that detected by hybridization with the genomic probe BE1000. The 2.8-kb transcript was missing or severely reduced in concentration in kernels homozygous for *su1-R4582*::*Mu1*, *su1-R2412*, *su1-R7110*, or *su1-R3162*, whereas transcripts of approximately this length were present in kernels homozygous for *su1-R8064* or *su1-Ref* at seemingly normal levels (Figure 7).

Predicted Function of the *su1* Gene Product

The *su1* cDNA sequence allowed deduction of the great majority of the SU1 amino acid sequence. Amino acid sequence comparisons using the program BLAST (Altschul et al., 1990) revealed that regions of SU1 are similar in primary structure to specific portions of several types of enzyme known to hydrolyze glucose homopolymers. These include bacterial debranching enzymes such as isoamylases and pullulanases, enzymes that hydrolyze α-(1→4) glycosidic linkages such as α-amylases and cyclomaltodextrinase, and both prokaryotic and eukaryotic branching enzymes. Figure 8 shows pairwise comparisons of SU1 versus an isoamylase, an α-amylase, and a branching enzyme, all from prokaryotic organisms. The sequence identity between SU1 and *Pseudomonas* isoamylase extends over almost the entire 742 known residues of the maize protein, whereas only short regions of sequence identity were observed between SU1 and the branching enzyme or α-amylase. Thus, among known starch hydrolytic enzymes, SU1 is most closely related to those that hydrolyze α-(1→6) linkages. Among these, the relationship of SU1 to isoamylase is greater than to pullulanase, although the similarity with pullulanase is significantly more extensive than that with branching enzyme or α-amylase (data not shown). The most extensive amino

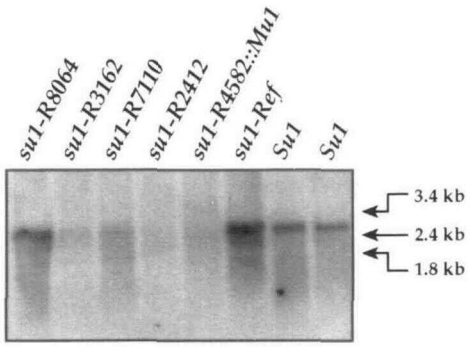

Figure 7. Detection of *su1* mRNA.

Total RNA isolated 20 days after pollination from kernels homozygous for the indicated mutation (10 μg per sample) was separated by electrophoresis and probed with fragment EE1780 from the *su1* cDNA. The *Su1/Su1* kernels are from two different standard lines, F_1 hybrid B77/B79 (right-most lane) and F_1 hybrid Q66/Q67 (second lane from the right). Arrows indicate the mobility in the same agarose gel of standards of known nucleotide sequence.

acid sequence similarity detected was with the deduced product of the *Escherichia coli* gene *glgX*, a gene of unknown function located in the operon that also codes for a glycogen branching enzyme (Romeo et al., 1988).

Figure 9 shows the deduced amino acid sequence of SU1 aligned with that of isoamylase from *P. amyloderamosa*. Of the 695 aligned residues, 32% are identical in the two polypeptides. Localized regions show an even higher degree of amino acid sequence identity. For example, of the 99 amino acids between positions 277 and 375 of SU1, 53% are identical to the residue found at that position in isoamylase. Other highly conserved regions of SU1 are residues 476 to 505, where 57% of the 30 amino acids are identical to those of isoamylase, and residues 180 to 222, where 53% of the 43 aligned amino acids are the same as in the bacterial protein. Two conserved sequence blocks observed previously in all known α-amylases, branching enzymes, and debranching enzymes (Svensson, 1988; MacGregor and Svensson, 1989; Jesperson et al., 1993) are found within SU1 (Figure 9). Two additional conserved sequences found specifically in the α-amylases, however, are lacking in SU1.

su1 Is a Member of a Multigene Family

DNA gel blot analysis using various segments of the *su1* cDNA as probes (see Figure 6) was utilized to examine whether *su1* is a unique sequence within the maize genome. Figure 10 shows that a single BamHI fragment hybridized strongly with cDNA probe EB620 or BB755. In contrast, probe EE680, located downstream of probe BB755 (Figure 6), hybridized with approximately equal efficiency with 14 different BamHI fragments. At most, two of these fragments could be explained by allelic diversity at the *su1* gene locus. Twelve other fragments, therefore, contain sequences that are sufficiently complementary in nucleotide sequence to probe EE680 to form heteroduplexes even under the high-stringency conditions used. Probe BE700, located immediately upstream of probe EE680 (Figure 6), also detected multiple fragments in the genome. These DNA gel blot data are in agreement with the single hybridization signal

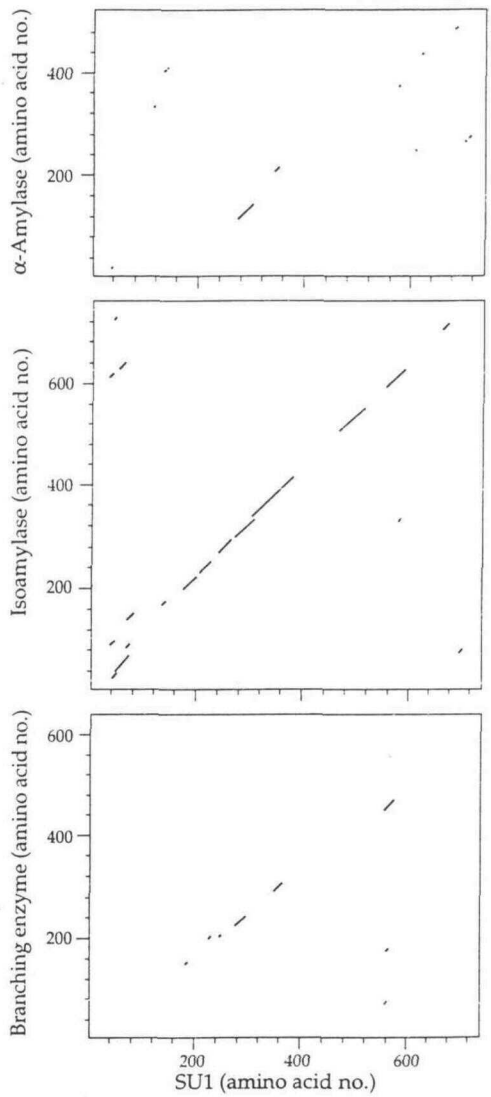

Figure 8. *su1* Codes for a Protein of the α-Amylase Superfamily.

The deduced amino acid sequence of SU1 was compared with that of isoamylase from *P. amyloderamosa* (GenBank accession number P10342), branching enzyme from *Bacillus stearothermophilus* (GenBank accession number P30538), or α-amylase from *B. megaterium* (GenBank accession number P20845). The program Compare was used with a window size of 30 and a stringency requirement of 16 identical residues. Thus, dots in the plot indicate that 16 of 30 residues are identical in the two proteins at the specified positions of their primary sequences.

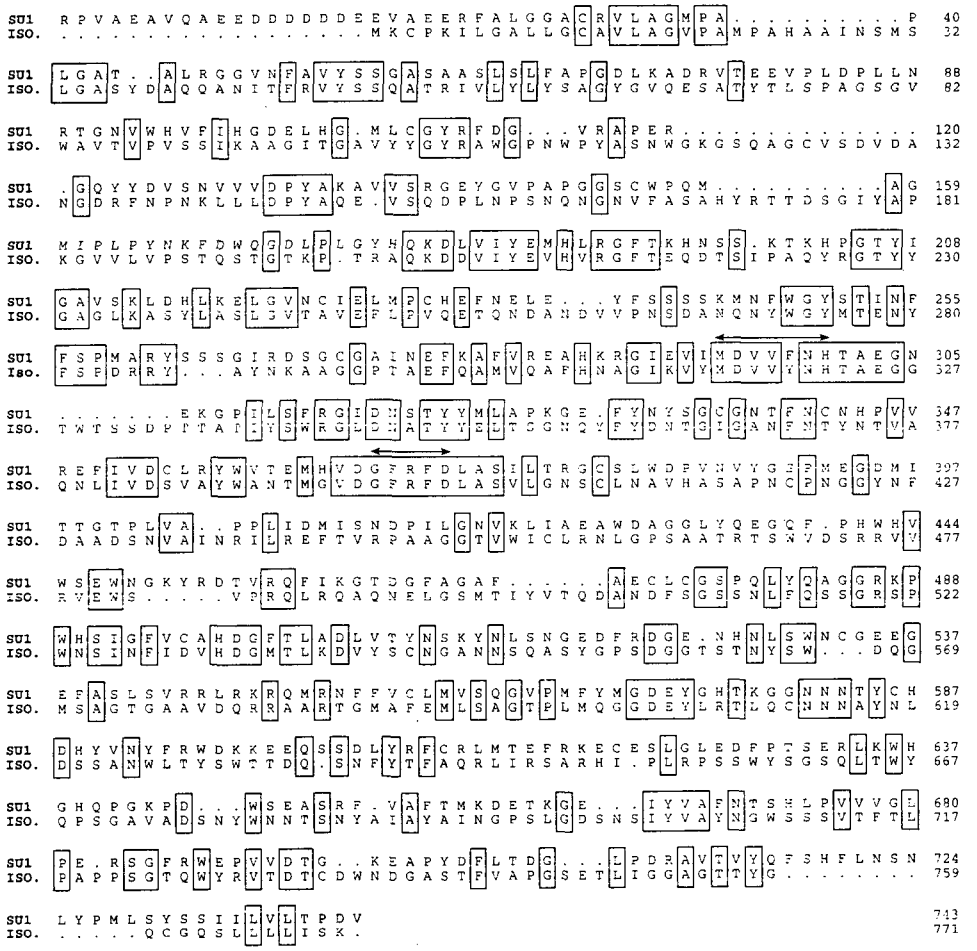

Figure 9. Amino Acid Sequence Comparison of SU1 with *Pseudomonas* Isoamylase.

The deduced primary sequences of SU1 and *Pseudomonas* isoamylase (ISO; GenBank accession number P10342) were aligned using the Gap program. Dots indicate the position of insertion/deletions in the two sequences. Identical residues are boxed. Residues in SU1 are numbered from the first discernible codon in the ORF; thus, residue 1 is not necessarily the N terminus of the native protein. Arrows indicate the position in SU1 of two conserved sequence blocks found in all known α-amylases, branching enzymes, and debranching enzymes.

obtained using genomic probe BE1000 (Figure 3C), because all the exons in this genomic interval are contained within cDNA probe BB755 (Figure 6).

DISCUSSION

The mutation *su1-Ref* is one of the earliest described and used mutations of maize (Correns, 1901; Vanderslice and Garwood, 1978). Stocks homozygous for this mutation have been widely cultivated as sweet corn varieties because of the accumulation of sugars in the endosperm and because phytoglycogen imparts a desirable texture to the kernels. Here, we report the isolation of the *su1* gene locus by transposon tagging and characterization of its transcript.

The transposon tagging strategy is based on the assumption that insertion of a transposable element in a target gene causes a mutation that can be identified by a specific phenotype. This approach by itself, however, cannot rule out the possibility that the mutation is independent of the insertion, even though the transposon and the mutant gene are tightly linked. In this study, two lines of evidence indicated that the cloned transposon-containing genomic DNA is within the *su1* locus. First, plants containing any of four independent *su1* mutations (*su1-R4582*::*Mu1*, *su1-R2412*, *su1-R7110*, and *su1-R3162*) displayed restriction fragment length polymorphisms in the same genomic interval, and these polymorphisms cosegregated with the sugary phenotype. The probability is very low that each of these rare genomic rearrangements would occur coincident with a *su1* mutation but not be causally related to *su1* function. Second, insertion of *Mu1* within the cloned region occurred de

novo within the same narrow developmental window in which *su1-R4582::Mu1* arose. Taken together, these data provide strong evidence that the cloned DNA described here is part of the *su1* gene locus.

The *su1* gene produces a mRNA transcript of ~2.8 kb in kernels. The nucleotide sequence of this transcript comprises a continuous ORF of at least 742 codons, which may include as many as 100 additional codons at the 5′ end. Observed amino acid sequence similarity established SU1 as a member of the α-amylase superfamily of starch hydrolytic enzymes; this family includes enzymes from bacteria, fungi, plants, and mammals (Svensson, 1988; MacGregor and Svensson, 1989). The sequence similarity between members of this superfamily is located primarily in two spatially conserved regions that are proposed to form catalytic and starch binding structures within the proteins (Matsuura et al., 1984; Nakajima et al., 1986; Jesperson et al., 1993). SU1 contains both of these conserved regions and, in addition, displays extensive primary sequence similarity with members of a particular subfamily of the α-amylase superfamily, namely, debranching enzymes. The characterized enzyme to which SU1 exhibits the highest degree of amino acid sequence identity, isoamylase from *Pseudomonas*, is known to completely hydrolyze the α-(1→6) linkages of amylopectin and glycogen (Yokobayashi et al., 1970; Amemura et al., 1988). Thus, the nucleotide sequence data reported here suggest that SU1 is a starch debranching enzyme active in maize endosperm. These data are consistent with the previous observation that mutations in the *su1* gene locus affect the activity of a starch debranching enzyme in developing maize endosperm tissue (Pan and Nelson, 1984).

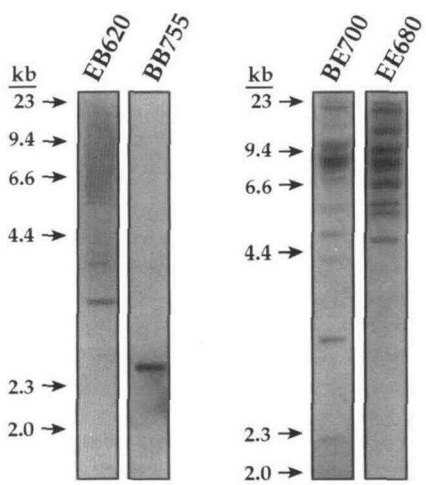

Figure 10. Hybridization of the *su1* cDNA to Maize Genomic DNA.

Restriction fragments from the *su1* cDNA were used as probes; the locations of these fragments are shown in Figure 6. Maize genomic DNA was digested with BamHI and hybridized with the probes in DNA gel blots under high-stringency conditions. Arrows indicate the mobility in the same agarose gels of standards of known nucleotide sequence.

The conclusion that *su1* mutations significantly alter storage carbohydrate composition in maize kernels by reducing the activity of an enzyme that hydrolyzes α-(1→6) linkages implies that debranching is an integral part of normal starch biosynthesis. The data suggest that a highly branched glucopolysaccharide is an intermediate in starch synthesis and that SU1 is required for modification of the intermediate during its conversion to amylopectin. Consistent with this notion is the finding that *ae1* mutations are epistatic to *su1* mutations, that is, eliminating this branching enzyme activity prevents accumulation of phytoglycogen in *ae1 su1* double mutants (Ayers and Creech, 1977). Two related models have been proposed to explain how such an intermediate might function in starch biosynthesis. Erlander (1958) suggested that phytoglycogen is formed by the action of starch synthases and branching enzymes and subsequently is converted to amylopectin by the action of a debranching enzyme. As opposed to this proposed linear progression from phytoglycogen to amylopectin, it has been suggested that an equilibrium exists between the activities of the branching and debranching enzymes (Pan and Nelson, 1984; Hannah et al., 1993). This equilibrium could result from a cycle in which kernels convert amylopectin into phytoglycogen by the action of branching enzyme, and then phytoglycogen is processed back into amylopectin by the action of SU1. Thus, *su1* mutations affecting the debranching enzyme would result in accumulation of phytoglycogen by shifting the proposed equilibrium toward production of the more highly branched polymer.

We propose an alternative, third model that does not require the production of a highly branched glucopolysaccharide as a normal intermediate. According to this model, individual starch molecules would be acted upon simultaneously as they form by the debranching enzyme SU1, branching enzyme(s), and possibly starch synthase(s). Thus, phytoglycogen would accumulate only when the debranching enzyme was missing and would not be produced in normal kernels, consistent with the finding that phytoglycogen can be detected only in very low amounts in normal maize endosperms (Creech, 1965; Black et al., 1966).

Indirect evidence supporting the hypothesis that SU1 acts on starch precursor molecules as they are being elongated is found in the observation that *su1* mutations resulted in noticeable loss of SSS activity in the endosperm (P. Keeling and G. Singletary, personal communication). We speculate that coordination of debranching, branching, and starch synthase activities during starch biosynthesis is accomplished by physical association of the enzymes in a complex within the amyloplast. Mutations at the *su1* gene locus could disrupt the complex through effects on the debranching enzyme, such that amylopectin production is decreased and the mutant enzyme complex produces phytoglycogen. Disruption of such a complex might also influence the activity of soluble starch synthase. In addition, evidence for a functional interaction between two of the starch biosynthetic enzymes was provided by the finding that the application of purified SBEI or SBEII

greatly stimulated the activity of endosperm SSSI (Boyer and Preiss, 1979).

The lack of a detectable transcript in RNA from kernels homozygous for *su1-R2412* seemingly is inconsistent with the relatively mild phenotype that results from the mutation. Typically, an absence of transcript implies a completely nonfunctional mutation. The fact that the kernels analyzed in this study are not in a homogeneous genetic background is one possible explanation for the less severe phenotype resulting from *su1-R2412* compared with *su1-Ref* or the other *su1* mutations. Another possible explanation is that the timing of *su1* expression is affected by *su1-R2412.* For example, a delay in transcription could result in SU1 deficiency in the more mature cells of the central region of the kernel crown and normal starch synthesis in the younger cells in the basal region of the kernel. This situation would result in the phenotype observed in *su1-R2412* mutant kernels in which glassiness and wrinkling occur only in the crown.

Availability of the *su1* cDNA facilitates direct examination of the putative maize endosperm debranching enzyme SU1 for the ability to hydrolyze α-(1→6) glycosidic linkages. The substrate specificities of bacterial isoamylases and pullulanases have been characterized (Gunja-Smith et al., 1970; Yokobayashi et al., 1970; Lee and Whelan, 1971; Harada et al., 1972; Kainuma et al., 1978), and this information suggests specific biochemical functions of SU1. Isoamylase is equally active toward amylopectin or glycogen, and the hydrolysis rate of α-(1→6) linkages in the polysaccharides is ~10-fold greater than in small oligosaccharides. Pullulanase is moderately active in the hydrolysis of amylopectin and is unable to hydrolyze glycogen; its preferred substrate is the ordered glucopolysaccharide pullulan. Neither debranching enzyme releases single glucosyl residues from α-(1→6) linkages. Pullulanase releases maltosyl groups, whereas isoamylase only releases maltotriosyl groups and larger oligosaccharides from branched molecules. Another activity specific to isoamylase is the ability to hydrolyze linkages between α-maltosaccharides and a tyrosine residue of the mammalian protein glycogenin, the putative primer for glycogen biosynthesis (Lomako et al., 1992). If this activity were conserved in SU1, the maize debranching enzyme could affect starch biosynthesis in the endosperm by regulating initiation of polysaccharide chain growth.

Observing hybridization of *su1* cDNA probes to multiple genomic DNA fragments suggested SU1 is a member of a gene family in maize. As many as 14 different regions of the genome formed heteroduplexes with a specific portion of the *su1* cDNA even under high-stringency conditions. Not all probe segments from the *su1* cDNA hybridized with high efficiency to multiple regions of the maize genome, suggesting that specific domains of SU1 are conserved in the putative gene family. The single transcript detected by hybridization to *su1* cDNA probes in maize kernels 20 days after pollination originates from *Su1* itself, and none of the putative *su1*-related genes is expressed in this tissue. This conclusion is based on the observations that (1) probe BB755 detected the kernel mRNA (data not shown), and this probe hybridized efficiently with only one genomic fragment (Figure 10); and (2) the transcript was not detected in kernels homozygous for *su1-R2412* or in *su1-R4582::Mu1.* The *su1*-related genes may code for enzymes present in germinating kernels or other plant tissues that are active in starch utilization; multiple α-(1→6) hydrolase activities have been characterized in these tissues (Manners and Rowe, 1969; Lee et al., 1971). The *su1* cDNA probes that formed heteroduplexes with multiple genomic segments comprise codons 380 to 743 of the discernible ORF. This region of SU1 is similar in amino acid sequence to *Pseudomonas* isoamylase (27% identity over 338 aligned residues) but not to as great an extent as are the other portions of the maize protein.

METHODS

Maize Stocks, Genetic Crosses, and Allele Nomenclature

Mutations at the *sugary1* (*su1*) gene locus were generated by crossing active *Mutator* (*Mu*) plants (Robertson, 1978) with standard lines as shown in Figure 1. Standard lines were the F_1 hybrids B77/B79 or Q66/Q67. These four inbred lines have no history of *Mutator* activity. Mutant alleles *su1-R4582::Mu1*, *su1-R2412*, *su1-R7110*, and *su1-R3162* were identified following the self-pollinations of the F_1 plants 82-83-4582-43, 87-2412-24, 79-7110-2, and 83-3162-15, respectively. Allele *su1-R8064* was identified following the open pollination of F_1 plant 88-8064-1, derived from the cross of a *Mutator* plant with a plant homozygous for *colored aleurone1 shrunken1 bronze1 waxy1.* These plant numbers are the laboratory designations used to identify each allele; inclusion of the letter *R* in the allele designations indicates the stocks are from the laboratory of Donald Robertson; inclusion of the term *Mu1* indicates the particular mutation is known to have resulted from insertion of a *Mu1* transposon. Stock No. 413B, from the Maize Genetics Cooperation Stock Center (Urbana, IL), was used for tests of allelism between each of the five new *su1* mutations and the reference allele *su1-Ref.*

DNA and RNA Gel Blot Analyses

Standard procedures were used for DNA and RNA gel blot analyses (Ausubel et al., 1989; Sambrook et al., 1989). Maize DNA was isolated from seedling tissue by the method of Dellaporta et al. (1983). Approximately 10 μg of genomic DNA was digested with 30 units of restriction enzyme, separated by electrophoresis in 0.8% agarose gels, and transferred to a nylon membrane (Magnacharge; Micron Separations Inc., Westboro, MA). Membranes were hybridized to DNA probes at 65°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% sarkosyl, 50 μg/mL denatured salmon sperm DNA (Sigma, St. Louis, MO); the probes were radioactively labeled by the random primer method. The membranes then were washed twice in 2 × SSC, 0.1% SDS, for 30 min at 65°C, twice in 0.2 × SSC, 0.1% SDS, for 20 min at 65°C, and once in 5 mM Tris-HCl, pH 8.0, for 5 to 10 min at 65°C, and they were exposed to x-ray film for 1 to 4 days.

Total RNA was isolated as described by Cone et al. (1986) from maize kernels harvested 20 days after pollination and stored at −80°C. During this procedure, water-soluble polysaccharide was removed from the nucleic acid fractions prepared from *su1* mutant kernels by centrifugation at 100,000*g* for 1 hr in a Beckman model TL-100 centrifuge.

Under these conditions, the water-soluble polysaccharide is separated as a gelatinous pellet from the aqueous phase that contains the RNA. Approximately 5 μg total RNA was denatured and separated by electrophoresis in the presence of formaldehyde and then transferred to nylon membranes (Ausubel et al., 1989). Membranes were hybridized at 42°C to radioactively labeled DNA probes in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, 50 μg/mL denatured salmon sperm DNA. After hybridization, the membranes were washed as described previously for DNA gel blot analysis. Radioactivity remaining on the membranes was detected using the Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Genomic and cDNA Cloning Procedures

A portion of the *su1* genomic locus was isolated as follows. Approximately 200 μg of genomic DNA isolated and pooled from three *su1-Ref/su1-R4582*::*Mu1* seedlings was digested with EcoRI and separated by electrophoresis. DNA was eluted and purified from gel fractions containing fragments ~3 to 5 kb in length and ligated to the bacteriophage λ vector NM1149 (Scalenghe et al., 1981). The genomic library was used to infect *Escherichia coli* C600*hfl*A cells (Sambrook et al., 1989), and ~200,000 plaques were screened with the *Mu1*-specific probe MM960. Seven hybridizing clones were single-plaque purified, and the genomic 4.0-kb EcoRI fragment present in one recombinant λ clone (H-4) was subcloned into the phagemid vector pBluescript KS+ (Stratagene), producing pMJ60.

A portion of the *su1* cDNA was isolated as follows. A maize endosperm cDNA library in the bacteriophage vector λgt11 was obtained from R. Schmidt, University of California, San Diego; EcoRI adapters were ligated to cDNA molecules during preparation of this library. Approximately 200,000 plaques from this library were screened with genomic probe BE1000. DNA from eight different hybridizing clones was digested with EcoRI; the largest cDNA insert present was 2.4 kb in length and comprised two EcoRI fragments of 1.8 and 0.6 kb. These two fragments were subcloned in pBluescript KS+ to produce pMJ67 and pMJ68, respectively.

Amplification of 5′ cDNA

To obtain the 5′ end of the *su1* cDNA, a modification of the rapid amplification of cDNA ends (RACE) protocol (Frohman et al., 1988) was used. Polyadenylated RNA from maize kernels (2 μg) was reverse transcribed using the *su1*-specific primer 5′-GTATGTACTAT TATCTATCCC-3′ (10 pmol) (nucleotides 1485 to 1465). Unincorporated nucleotides and excess primer were removed using Centricon 100 filters (Amicon, Beverly, MA). Poly(A)-tailed cDNA was amplified by polymerase chain reaction using the *su1*-specific primer 5′-GGGATCATACCAGCCATT-TGA-3′ (25 pmol) (nucleotides 707 to 687), RACE $(dT)_{17}$ adapter (10 pmol), and RACE amplification primer (25 pmol) (Frohman et al., 1988). The amplified products were digested with BamHI and EcoRI and cloned in phagemid vector pUC119. The resulting plasmid, pMJ125, was characterized by nucleotide sequence analysis and found to extend beyond the 5′ terminus of the cDNA clone present in pMJ67.

Nucleic Acid Hybridization Probes

The 960-bp MluI fragment MM960, contained within *Mu1*, was excised from plasmid pMJ9 (Barker et al., 1984) and used to detect genomic and cloned copies of this transposon. Probe BE1000, a 1.0-kb BamHI-EcoRI fragment comprising part of the *su1* gene locus, was excised from plasmid pMJ60. *su1* cDNA probes (Figure 6) were the 755-bp BamHI fragment BB755, the 700-bp BamHI-EcoRI fragment BE700, and the 1780-bp EcoRI fragment EE1780 (all excised from plasmid pMJ67), in addition to the 680-bp EcoRI fragment EE680 (excised from plasmid pMJ68) and the 620-bp EcoRI-BamHI fragment EB620 (excised from plasmid pMJ125).

Nucleotide Sequence Analysis

Nucleotide sequence was determined by the chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 (U.S. Biochemical Corp.). To determine the sequence of the maize genomic DNA adjacent to the *Mu1* termini in *su1-R4582*::*Mu1*, two EcoRI-NotI fragments from the insert in pMJ60 were subcloned in phagemid vectors; each of these two fragments contained one of the *Mu1* termini. Sequence was determined using oligonucleotide primers from within each terminus and extending in the 3′ direction toward the end of the transposon (5′-GGCTGTCGCGTGCGT-3′ and 5′-GCGTACGTCTCTAAA-3′). Portions of pMJ60 and all of pMJ67, pMJ68, and pMJ125 were analyzed using various oligonucleotide primers; the original plasmids and various subclones derived from them were used as templates. To sequence across the EcoRI site internal to the cDNA insert in phage λ clone H-4, the 2.9-kb KpnI fragment from this phage was subcloned in pBluescript KS+ to form pMJ99. This fragment extends from the KpnI site in the cDNA to the KpnI site of λgt11. Of the 2706-bp cDNA sequence reported here, ~2300 bp were analyzed on both strands, and all restriction sites used for subcloning were crossed.

The GCG sequence analysis software package (Genetics Computer Group, Madison, WI) was used for data base searches and amino acid sequence alignments. The GenBank accession number for the *su1* cDNA sequence is U18908.

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