Plant Physiol. (1996) 110: 611–619

Allelic Analysis of the Maize *amylose-extender* Locus Suggests That Independent Genes Encode Starch-Branching Enzymes IIa and IIb[1]

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Starch branching enzymes (SBE) catalyze the formation of α-1,6-glucan linkages in the biosynthesis of starch. Three distinct SBE isoforms have been identified in maize (*Zea mays* L.) endosperm, SBEI, IIa, and IIb. Independent genes have been identified that encode maize SBEI and IIb; however, it has remained controversial as to whether SBEIIa and IIb result from posttranscriptional processes acting on the product of a single gene or whether they are encoded by separate genes. To investigate this question, we analyzed 16 isogenic lines carrying independent alleles of the maize *amylose-extender* (*ae*) locus, the structural gene for SBEIIb. We show that 22 d after pollination *ae-B1* endosperm expressed little *Sbe2b* (*ae*)-hybridizing transcript, and as expected, *ae-B1* endosperm also lacked detectable SBEIIb enzymatic activity. Significantly, we show that *ae-B1* endosperm contained SBEIIa enzymatic activity, strongly supporting the hypothesis that endosperm SBEIIa and IIb are encoded by separate genes. Furthermore, we show that in addition to encoding the predominant *Sbe2b*-hybridizing message expressed in endosperm, the *ae* gene also encodes the major *Sbe2b*-like transcript expressed in developing embryos and tassels.

ADP-Glc pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), and SBE (EC 2.4.1.18) are three key enzymes involved in the biosynthesis of starch. SBEs (α-1,4-glucan:α-1,4-glucan-6-glycosyl transferase) catalyze the formation of α-1,6 linkages. The reaction involves hydrolysis of an α-1,4 linkage followed by the reattachment of the released 1,4-glucan chain to the remaining or to another 1,4-glucan chain by a 1,6 bond. This reaction creates branch points in the growing starch molecule and additional nonreducing ends for further synthesis of starch. Thus, SBEs play an important role in the synthesis of the amylopectin fraction of starch.

Multiple forms of SBE have been characterized biochemically from starch-producing tissues, including spinach leaf, pea embryo, potato tuber, maize (*Zea mays* L.) endosperm and leaf (reviewed by Preiss, 1991; Martin and Smith, 1995), and rice seed and other vegetative tissues (Mizuno et al., 1992; Nakamura et al., 1992; Yamanouchi and Nakamura, 1992). The starch-branching isoforms characterized and cDNAs cloned to date can be grouped into two distinct families, as initially defined by the Commission on Plant Gene Nomenclature (1994) and as suggested by Burton et al. (1995; class A and B). One family (*Sbe1* or class B) includes maize SBEI, rice SBEI, pea SBEII, and potato SBE. The second family (*Sbe2* or class A) includes maize SBEII, pea SBEI, and rice SBEIII. In maize, two members of the *Sbe2* family (SBEIIa and IIb) have been identified (Boyer and Preiss, 1978a, 1981).

The biochemical differences of the SBE isoforms in maize have been well characterized. SBEI is quite different from SBEIIa and IIb in molecular mass, chromatographic and enzyme kinetic properties, immunological reactivity, and amino acid composition (reviewed by Preiss, 1991; see below and Table I). Studies utilizing purified SBEs from maize endosperm have shown that the activities of SBEI and the SBEII isoforms are distinct (Table I). Guan and Preiss (1993) showed that SBEI has the highest rate in branching with amylose as substrate in vitro, and SBEIIa and IIb have higher rates than SBEI with amylopectin as substrate. Takeda et al. (1993) suggested that SBEI preferentially transfers longer glucan chains than either SBEIIa or SBEIIb. The model by Guan and Preiss (1993) suggests that SBEI produces slightly branched molecules, which would then serve as substrates for the action of SBEIIa and IIb. In this way, each SBE isoform may be responsible for a unique aspect of amylopectin biosynthesis and structure.

Maize endosperm SBEIIa and IIb can be separated by anion-exchange chromatography (Boyer and Preiss, 1978a) and have been shown to exhibit very similar but not identical biochemical properties (reviewed by Preiss, 1991). Estimates of molecular mass of the two proteins by gel filtration range from 70 to 92 kD (Boyer and Preiss, 1978a; Baba et al., 1982). Boyer and Preiss (1978a) estimated the size of both SBEIIa and IIb as 80 kD using disc-gel SDS-PAGE analysis. Recently, Guan and Preiss (1993) separated SBEIIa and IIb activity on DEAE-Sepharose columns and

[1] This work was supported by a grant from Pioneer Hi-Bred International Inc. (Johnston, IA) to M.J.G., C.D.B., and Jack Shannon and by the Pennsylvania State Agricultural Experiment Station (No. CRIS 3303).

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Abbreviations: *ae*, *amylose-extender*; DAP, days after pollination; EMS, ethyl methanesulfonate; *Mu1*, *Mutator1*; SBE, starch branching enzyme; *Sbe1*, gene encoding SBEI; *Sbe2b*, gene encoding SBEIIb; SBEI, protein encoded by *Sbe1*; SBEIIb, protein encoded by *Sbe2b*.

Table I. *Comparison of biochemical properties of maize endosperm SBE isoforms*

Data from biochemical analysis of purified maize endosperm SBE activity and cDNA cloning indicates the similarities and distinctions between SBEI, IIa, and IIb isoforms. Works cited in this summary are indicated below.

Method of Comparison	SBEI versus IIa/IIb	SBEIIa versus IIb
Immunological reaction[a,b]	Distinct	Similar
Amino acid composition[a]	Distinct	Similar
Proteolytic digest maps[a]	Distinct	Similar
Anion-exchange, hydrophobic columns[c,d]	Distinct	Distinct
Different reactivities in 3 SBE assays[c,d]	Distinct	Similar/ distinct
K_m for substrates (kinetic properties)[c,d]	Distinct	Similar
Molecular mass[c]	Distinct	Similar
Optimum temperature[e]	Distinct	Distinct
DNA and deduced amino acid sequence[f,g]	Distinct (I versus IIb)	No IIa data

[a] Singh and Preiss, 1985. [b] Fisher and Boyer, 1983. [c] Boyer and Preiss, 1978a. [d] Guan and Preiss, 1993. [e] Takeda et al., 1993. [f] Baba et al., 1991. [g] Fisher et al., 1993.

duplicated the results of Takeda et al. (1993), who showed that SBEIIa had more than twice the specific activity of SBEIIb in the branching linkage assay. Takeda et al. (1993) also showed that the optimum temperatures for SBEIIa and SBEIIb activity are different in the branching linkage assay. Clearly, research published to date describes maize endosperm SBEIIa and IIb as similar but distinguishable forms of SBE. A controversy central to the differentiation of the SBEIIa and IIb isoforms is whether they are the products of one or two genes.

To our knowledge, no maize mutants have been identified that are deficient in SBEI or IIa activities. However, analysis of the *ae* mutant has provided insight into the specific importance of SBEIIb to starch structure. The *ae* mutant, first observed by Vineyard and Bear (1952), affects SBEIIb activity in maize endosperm, resulting in an increase in the apparent amylose content of endosperm starch (Deatherage et al., 1954) in a gene dosage-dependent fashion (Fergason et al., 1966). Amylopectin in kernels from the *ae* mutant contains fewer branch points and longer chains than normal, contributing to higher apparent amylose (Boyer et al., 1980). Endosperm extracts homozygous for several different alleles of the *ae* mutation were shown to lack SBEIIb activity (Boyer and Preiss, 1978b; Baba et al., 1982; Hedman and Boyer, 1982). A linear dosage relationship of the functional *ae* allele and SBEIIb activity led to the suggestion that the *ae* locus encodes SBEIIb (Hedman and Boyer, 1982). In six *ae* alleles analyzed to date, normal levels of SBEI and IIa activity were found with absence of SBEIIb (Boyer and Preiss, 1978b; Baba et al., 1982; Hedman and Boyer, 1982). This suggested independent genetic control for SBEI, IIa, and IIb; however, the possibility of post-transcriptional modification of a single *Sbe2* gene product was not ruled out.

We recently isolated an *Sbe2b* cDNA from maize endosperm as verified by deduced amino acid sequence identity with other plant SBE genes and with the N-terminal sequence of purified maize SBEIIb protein (Fisher et al., 1993). A dominant mutant allele of the *ae* locus (*Ae*-5180) was identified in a maize *Mu1* population (Stinard et al., 1993). The genomic DNA sequence bordering the *Mu1* element shows 98% identity with a segment of the *Sbe2b* cDNA, the minor differences likely being due to the different genetic stocks used in the two studies (Stinard et al., 1993, standard B70 and Q60 hybrids; Fisher et al., 1993, hybrid of W64A and 182E). These results unambiguously demonstrate that the SBEIIb isoform is encoded by the *ae* gene.

To investigate the question of whether the *ae* gene also encodes SBEIIa, we utilized the cloned *Sbe2b* (*ae*) cDNA and an SBE assay to measure the levels of *Sbe2b* transcript and SBE activity levels in endosperm from isogenic lines containing 16 independent *ae* alleles. The results confirm that *ae* is the structural gene for SBEIIb. In one allele, endosperm cells with at least 100-fold reduced levels of *Sbe2b* mRNA show near-normal levels of SBEIIa enzymatic activity. In the same allele, there is also undetectable levels of *Sbe2b*-hybridizing transcript in developing embryos and tassels. These data indicate that a gene independent from *ae* encodes SBEIIa in maize endosperm and that the *ae* gene is the only *Sbe2b*-like gene expressed to appreciable levels in endosperm, embryo, and tassels.

MATERIALS AND METHODS

Nomenclature

The nomenclature used follows the recommendation of the Commission on Plant Gene Nomenclature (1994) and the method described by Shaw et al. (1994). The symbols *Sbe1* and *Sbe2b* refer to the structural genes for maize endosperm SBEI and SBEIIb, respectively. The symbol *ae* refers to the *amylose-extender* gene of maize, which is now synonymous with the *Sbe2b* gene. The *ae* gene was named by the kernel phenotype resulting from its loss of function. Recessive alleles of the *ae* gene are denoted *ae-* and a particular allelic designation follows the dash. The functional or wild-type form of the gene is referred to as *ae*. Dominant alleles of the gene are referred to as *Ae*. SBEI and SBEIIb refer to the proteins produced by the *Sbe1* and *Sbe2b* genes, respectively.

Plant Materials

Maize (*Zea mays* L.) inbred W64A and isogenic mutants of 16 independent *ae* alleles (*ae-B1, ae-B2, ae-B3, ae-B4, ae-B5, ae-M1, ae-M2, ae-i1, ae-i2, ae-EMS1, ae-EMS3, ae-MW1, ae-MW3, ae-MW5, ae-Z, and ae-Ref*) were field grown during the summer of 1994 at The Pennsylvania State University Horticultural Research Farm (Rock Springs). Alleles were obtained originally from R. Bear (*ae-B1, ae-B2, ae-B3, ae-B4, ae-B5*), M. Zuber (*ae-M1, ae-M2*), R. Creech (*ae-i1, ae-i2*), and R. Briggs (*ae-EMS1, ae-EMS3*). The entire collection of alleles was assembled by R. Creech at The Pennsylvania State University prior to 1970. Isogenic lines for each allele were constructed by at least nine backcrosses into inbred W64A. Each mutant has been tested for allelism with *ae-Ref* and

each contains the characteristic high amylose phenotype of the *ae* mutation (Garwood et al., 1976). The *ae-EMS1* and *ae-EMS3* alleles were identified after EMS mutagenesis, the *ae-i1* and *ae-i2* were from stocks containing *Ac-Ds* transposable elements, and all other alleles were from spontaneous mutational events. All plants were homozygous and were self- or sib-pollinated. Developing kernels of each genotype were harvested 22 DAP. Replicate ears from each genotype were used for both RNA analysis and SBE assays. All samples were immediately frozen in liquid nitrogen and stored at −70°C. For extracts prepared for chromatography, embryos were dissected from kernels prior to freezing. Immature tassels were harvested approximately 10 d prior to anthesis and frozen as above.

Reagents Used in Assays

Crystalline rabbit muscle phosphorylase a and Glc-1-P were purchased from Sigma. [α-^{32}P]dCTP was obtained from Amersham. Other reagents were purchased as described below or from Sigma.

Genomic Southern Analysis

Genomic DNA was isolated from B73 etiolated seedlings by the method of Dellaporta et al. (1983). After restriction enzyme digestion and electrophoresis, 20 μg of DNA was transferred to a nylon membrane (Hybond N+, Amersham) by capillary transfer. Filters were air dried for 1 h and fixed by a 30-s exposure under a UV lamp. The filters were prehybridized for 1 h at 65°C in 0.5 M $NaHPO_4$ (pH 7.2), 7% (w/v) SDS, and 80 μg/mL salmon sperm DNA. Hybridization was performed at 65°C in 0.5 M $NaHPO_4$ (pH 7.2), 7% (w/v) SDS, 40 μg/mL salmon sperm DNA, and 2.5×10^7 cpm of probe (specific activity of 10^9 cpm/μg) for 18 h. After hybridization, the membranes were washed twice at 65°C with 1% (w/v) SDS, 0.04 M $NaHPO_4$, 0.001 M EDTA for 20 min each and exposed to autoradiographic film.

Total RNA Extraction and Northern Analysis

Total RNA was separately extracted from independent ears of each genotype as described by McCarty (1986) and fractionated on a 2.2 M formaldehyde, 1.2% (w/v) agarose gel after denaturation (65°C for 15 min in 30 μL of the loading buffer containing 50% [v/v] formamide, 2.2 M formaldehyde, 20 mM Mops [pH 7.0], 5 mM sodium acetate, 1 mM EDTA, 4% [v/v] glycerol, 0.8% [w/v] bromphenol blue, and 1 μg of ethidium bromide). The denatured RNA samples were chilled on ice for 3 to 5 min, loaded, and allowed to rest in wells for 5 to 10 min before running at 2 to 3 V cm^{-1} for 4 to 5 h until bromphenol blue neared the bottom of the gel. Fractionated RNA was blotted on nylon membranes according to manufacturer's instruction (Hybond N, Amersham) and fixed by a 30-s exposure under a UV lamp. Membranes were prehybridized and hybridized at 65°C in 10 mL of hybridization buffer (6× SSC [0.9 M NaCl, 0.09 M sodium citrate, pH 7.0], 0.5% [w/v] SDS, 100 μg mL^{-1} salmon sperm DNA, and 5× Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) PVP, 0.1% (w/v) BSA]). The full-length maize *Sbe2b* (*ae*) cDNA (Fisher et al., 1993) and *Sbe1* cDNA (Fisher et al., 1995) were labeled with [^{32}P]dCTP using random-primed DNA labeling (Boehringer Mannheim, catalog No. 1004760) and purified with Sephadex G-50 spun columns (Sambrook et al., 1989). Membranes were washed with 2× SSC for 5 min, 2× SSC and 0.1% (w/v) SDS twice for 10 min each at room temperature, and 0.1 × SSC and 0.1% (w/v) SDS at 65°C for 15 min (high stringency). For low-stringency conditions, hybridization was carried out as described above except at 45°C, and the final low salt, 65°C wash was omitted. Total RNA concentrations were estimated based on spectrophotometric readings at 260 nm and then were calibrated a second time by northern hybridization with a tomato 26S rRNA cDNA probe, followed by quantification with a β-scope (Betagen, Mountain View, CA). The RNA concentrations were adjusted based on this calibration.

SBE Purification

SBEs were purified from isolated maize endosperm (*ae-B1* allele and wild type) and fractionated by chromatography on a DEAE-Sepharose FF anion-exchange column (Pharmacia Biotech, Piscataway, NJ) as previously described (Guan and Preiss, 1993).

Preswollen DEAE-Sepharose FF was equilibrated with 7 column volumes of running buffer (Guan and Preiss, 1993). Column chromatography was performed at 4°C using a 50-mL volume column (2.5 cm in diameter) and a Pharmacia Gradi-Frac chromatography apparatus. Dialyzed enzyme was loaded at 4 mg mL^{-1} resin bed volume. Fractions were collected at a flow rate of 1.2 mL min^{-1} at a volume of 7 mL per fraction. Two column volumes were collected as wash volume before application of a 0 to 0.4 M linear KCl gradient (500 mL volume). Fractions were collected and maintained at 0 to 4°C and assayed within 5 h.

SBE Activity Assay

SBE activity was assayed in each fraction based on the stimulation of α-D-glucan formation from Glc-1-P by crystalline rabbit muscle phosphorylase a, as described by Boyer and Preiss (1978a). Activity was measured by monitoring the amount of released Pi in the reaction stimulated by branching enzyme activity. Assay reactions were prepared as described by Boyer and Preiss (1978a), except that reaction volumes were 50 μL, containing up to 10 μL from each DEAE fraction. Reactions were incubated for 90 min at 30°C, prior to termination at 100°C for 1 min. Released Pi was then quantified using a colorimetric procedure (Lanzetta et al., 1979). SBE activity was expressed as micromoles of Pi released per 90-min reaction per milliliter of original DEAE fraction volume per milligram of total protein loaded onto the column.

SDS-PAGE and Western Analysis

DEAE-Sepharose FF fractions were concentrated using Centricon 30 tubes (Amicon, Beverly, MA). SDS-PAGE was performed according to the method of Sambrook et al. (1989) using 10% polyacrylamide resolving gels. Approxi-

mately 15 μg of total protein were loaded per lane from each concentrated fraction.

Immunoblotting was performed using Immobilon-P membranes (Millipore) by the method of Towbin et al. (1979). To detect SBEII proteins, a rabbit polyclonal antibody that recognizes maize endosperm SBEII was used (kindly provided by Bruce P. Wasserman of Rutgers University, Newark, NJ, and Peter L. Keeling and George W. Singletary of ExSeed Genetics, Ames, IA). These antibodies were raised against proteins extracted from starch granules as described by Mu et al. (1994). An 85-kD polypeptide was separated by SDS-PAGE, excised and electroeluted prior to injection into rabbits. The purity and identity of the purified SBEII protein were verified by protein microsequencing, which revealed identity to the maize *Sbe2b* cDNA (Fisher et al., 1993) deduced amino acid sequence (Bruce Wasserman, personal communication). This antibody was used as the primary antibody, and goat anti-rabbit alkaline phosphatase conjugate was used as the secondary antibody for indirect detection of SBE proteins, as described by Knecht and Dimond (1984).

RESULTS

Genomic Southern Analysis of the *Sbe2b* Gene Family

To evaluate the number of *Sbe2b*-like genes in the maize genome, we performed Southern analysis using the *Sbe2b* cDNA as a hybridization probe (Fig. 1). At moderately high stringency (65°C, 6× SSC), between two and five strongly hybridizing bands and two or three weakly hybridizing bands were detected, depending on the restriction enzymes used. At lower stringency (55°C) we obtained essentially identical results, and at low stringency (42°C) we obtained an autoradiogram with very high background and barely distinguishable bands (data not shown). From these data we estimate that there are two or three loci in maize that have strong sequence identity with the *Sbe2b* cDNA and that there are at least one or two additional loci of lower sequence relatedness, consistent with the multiple *Sbe2*-gene hypothesis.

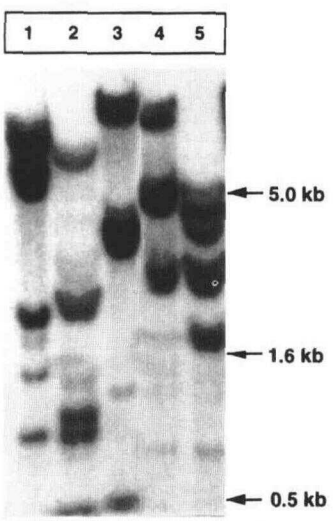

Figure 1. Southern blot of maize genomic DNA probed with the full-length *Sbe2b* probe (Fisher et al., 1993). Genomic DNA from maize inbred B73 was digested with the indicated restriction enzymes, fractionated, blotted, and probed under moderate-stringency conditions. Lane 1, *Bam*HI; lane 2, *Bam*HI/*Hin*dIII; lane 3, *Hin*dIII; lane 4, *Eco*RI; lane 5, *Eco*RI/*Bgl*I.

Analysis of *Sbe2b* (*ae*) Transcript Levels in Kernels of 16 *ae* Alleles

We previously described the cloning of the *Sbe2b* cDNA (Fisher et al., 1993), which was recently confirmed to be the product of the *ae* locus (Stinard et al., 1993). To study the effect of the *ae* mutation on *ae* mRNA accumulation, we used this full-length cDNA to perform northern analysis on kernels from 16 independent *ae* alleles. The *ae* alleles were collected from various donors (see "Materials and Methods") and each was backcrossed into W64A for at least nine generations. Northern blots of RNA extracted from 22-DAP kernels from each of the *ae* alleles were hybridized with the full-length *Sbe2b* (*ae*) cDNA probe (Fig. 2). RNA concentrations were calibrated by A_{260} and by hybridization with a 26S rRNA probe (see "Materials and Methods"). Nine of the 16 alleles showed approximately wild-type levels of the *Sbe2b* (*ae*) transcript (*ae-B5*, *ae-M1*, *ae-ref*, *ae-MW3*, *ae-i1*, *ae-B4*, *ae-Z*, *ae-MW5*, and *ae-B3*). Two showed obviously decreased levels of transcript (*ae-MW1* and *ae-EMS1*; Fig. 2, lanes 13 and 16), whereas two others showed a decreased level and altered size transcript (approximately 1.0 kb; Fig. 2, lanes 9 and 15; *ae-B2* and *ae-M2*). The remaining three alleles showed very low transcript levels (*ae-EMS3* and *ae-i2*; Fig. 2, lanes 3 and 14) and no detectable *Sbe2b* (*ae*) transcript (*ae-B1*; Fig. 2, lane 6). The same blots probed at low-stringency conditions showed similar results (data not shown). Northern blots of RNA from a replicate set of ears of each mutant were also essentially identical with those shown in Figure 2 (not shown). As a positive control, the same blots were stripped and reprobed with the *Sbe1* cDNA (Fig. 2). In the wild type and all of the *ae* alleles, an *Sbe1* transcript was strongly detected at nearly identical levels, verifying the equal loading and RNA integrity in each lane. This also demonstrated that there were no epistatic effects of *ae* mutations on *Sbe1* transcript accumulation in kernels.

In an attempt to detect low levels of *ae* transcript in *ae-B1* and/or transcripts from related genes, northern blots were made with increased amounts of total RNA from *ae-B1* with undetectable *Sbe2b* (*ae*) transcript and *ae-M2* with an altered *Sbe2b* (*ae*) transcript size. Figure 3 shows that little transcript could be detected in RNA from *ae-B1* kernels even with 25 and 50 μg of total RNA. This can be compared to the wild-type (W64A) lanes containing 1 μg of total RNA (Fig. 3), where *Sbe2b* (*ae*)-hybridizing transcript was easily detectable. Therefore, conservatively, at least 50-fold less *Sbe2b* (*ae*) transcript was present in *ae-B1*. β-scope readings showed that more than twice as much signal was detected in the wild-type lane containing 1 μg of RNA versus the 50-μg lane of *ae-B1*, indicating at least 100-fold less transcript in the *ae-B1* genotype.

Overloadings of the *ae-M2* allele also showed that some normal size transcript (2.7 kb) could be detected along with

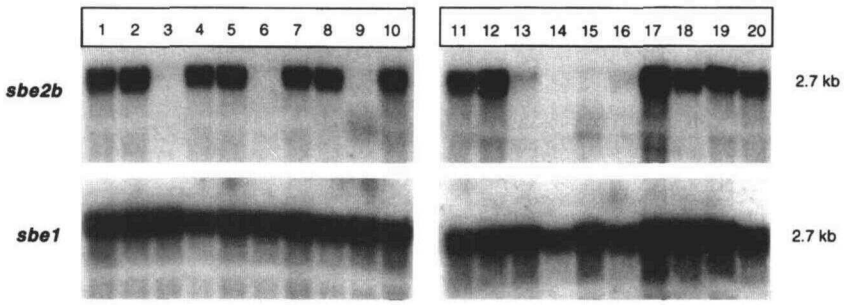

Figure 2. Northern analysis of 16 *ae* alleles. Total kernel RNA was fractionated, blotted, and probed with the full-length maize *Sbe2b* cDNA probe (top; Fisher et al., 1993) and stripped and reprobed with the full-length maize *Sbe1* cDNA (bottom; Fisher et al., 1995). Ten micrograms of RNA were loaded for each of 16 alleles and the wild type. Lane 1, W64A (wild type); lane 2, *ae-B5*; lane 3, *ae-EMS3*; lane 4, *ae-M1*; lane 5, *ae-Ref*; lane 6, *ae-B1*; lane 7, *ae-MW3*; lane 8, *ae-i1*; lane 9, *ae-B2*; lane 10, wild type. Lane 11, Wild type; lane 12, *ae-B4*; lane 13, *ae-MW1*; lane 14, *ae-i2*; lane 15, *ae-M2*; lane 16, *ae-EMS1*; lane 17, *ae-Z*; lane 18, *ae-MW5*; lane 19, *ae-B3*; lane 20, wild type. Transcript size is indicated in kb.

the aberrant transcript (approximately 1.0 kb) in 25 and 50 μg of total RNA. Similarly, 50 μg of *ae-M2* RNA yielded only the same or less normal *Sbe2b* (*ae*) transcript (2.7 kb) than 1 μg of wild-type RNA (W64A; Fig. 3). The same filter was stripped and reprobed with the *Sbe2b* cDNA under low-stringency hybridization conditions, yielding an identical result (Fig. 3). In the *ae-B1* and *-M2* kernels, the low levels of hybridizing 2.7-kb transcript may have been the products of a separate *Sbe2b*-like gene with some sequence similarity to the *Sbe2b* probe and/or with low transcript abundance, consistent with the existence of independent genes encoding SBEIIa and IIb. Perhaps the weak signal resulted from cross-hybridization of the *Sbe2b* probe with the presumptive *Sbe2a* transcript.

SBE Activity Analysis of Selected Alleles

We reasoned that, if SBEIIa and IIb are products of separate genes, alleles with little or no detectable *Sbe2b* transcript should still retain SBEIIa activity. To examine the levels of SBEIIa and IIb enzymatic activities, proteins isolated from *ae-B1* and wild-type endosperms were fractionated on a DEAE-Sepharose FF column to resolve the three SBE isoforms.

SBE activity in fractions from DEAE-Sepharose chromatographic elution profiles of endosperm extracts from the W64A wild-type sample are shown in Figure 4. SBEI activity passed through the flow-through and the SBEIIb and IIa activities eluted as a broad bimodal peak between 0.1 and 0.2 M KCl (fractions 42–65), in agreement with

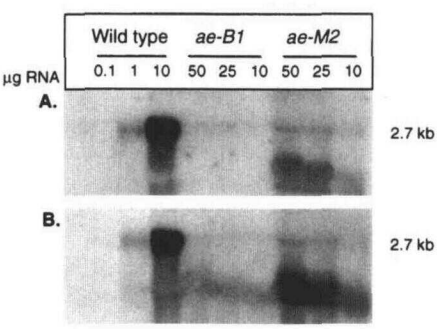

Figure 3. Northern analysis of selected *ae* alleles with increased loadings of RNA for detection of the *Sbe2b* (*ae*) transcript. A wild-type control is included for comparison. Total kernel RNA was fractionated, blotted, and probed with the full-length *Sbe2b* cDNA probe (Fisher et al., 1993) under high-stringency (A) and low-stringency (B) conditions (see "Materials and Methods"). From left to right: W64A (wild type) at 0.1, 1, and 10 μg of total RNA; *ae-B1* at 50, 25, and 10 μg of total RNA; *ae-M2* at 50, 25, and 10 μg of total RNA. Transcript size is indicated in kb.

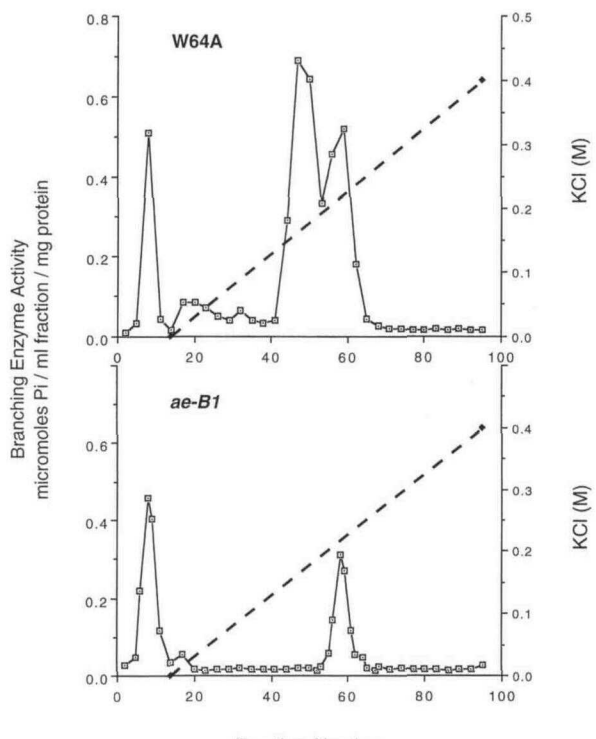

Figure 4. Elution profile from chromatography of maize endosperm SBE activity on DEAE-Sepharose FF. Wild type (W64A; top) and the *ae* allele *ae-B1* (bottom) are shown. SBE activity is defined as micromoles of Pi released in the assay reaction (phosphorylase a stimulation; 90 min.) per milliliter of column fraction per milligram of total protein. SBEI, fractions 2 to 12; SBEIIb, fractions 42 to 52; SBEIIa, fractions 53 to 65; the dashed line indicates the KCl gradient.

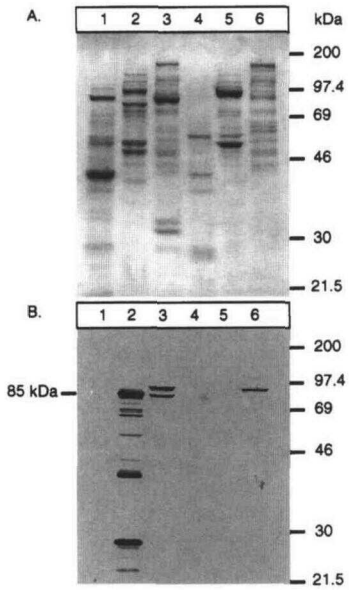

Figure 5. SDS-PAGE and western blot of DEAE-Sepharose FF fractions containing branching enzyme activity. Individual DEAE-Sepharose fractions containing the highest level of branching enzyme activity for each of the SBEI, SBEIIb, and SBEIIa peaks were concentrated and fractionated by SDS-PAGE. Duplicate gels were stained with Coomassie blue (A) or blotted onto polyvinylidene difluoride membrane for immunoreaction with a maize SBEII antibody (B). Lane 1, Wild type (W64A) SBEI fraction; lane 2, W64A SBEIIb fraction; lane 3, W64A SBEIIa fraction; lane 4, *ae-B1* SBEI fraction; lane 5, *ae-B1* SBEIIb fraction; lane 6, *ae-B1* SBEIIa fraction. Protein sizes are indicated in kD.

previous analysis (Boyer and Preiss, 1978a; Dang and Boyer, 1989). The SBEII bimodal peak consisted of SBEIIb (fractions 42–52) and SBEIIa (fractions 53–65) activities as previously defined (Boyer and Preiss, 1978a; Hedman and Boyer, 1983; Singh and Preiss, 1985) (Fig. 4).

SBEIIb enzymatic activity was not detectable in endosperm proteins from the *ae-B1* allele, as indicated by DEAE-Sepharose chromatography (Fig. 4). Endosperm cells from the *ae-B1* mutant lacked SBEIIb activity (fractions 42–52), but SBEIIa activity (fractions 53–65) remained at or near wild-type levels (Fig. 4), consistent with previous analyses of six different *ae* alleles (Boyer and Preiss, 1978b; Baba et al., 1982; Hedman and Boyer, 1982). As shown above, *ae-B1* kernels also had little or no transcript that hybridized to the *Sbe2b* (*ae*) probe, indicating that the observed SBEIIa activity must be encoded by a transcript that does not cross-hybridize significantly to the *Sbe2b* cDNA, or is of low abundance, or both. The similarity in SBEI activity levels among wild type and the *ae-B1* allele agrees with previous results showing that SBEI is unaffected by genotype at the *ae* locus and is consistent with our northern data, which also indicate no apparent pleiotropic effect on SBEI levels.

To verify the identity of the SBEII isoform peaks from the chromatographic separations, an antibody against purified maize endosperm SBEII was used to probe western blots of the fractionated proteins (Fig. 5). SBEIIb and IIa are very similar immunologically, and, as with previously described SBEII antibodies, the antibody used reacted to both SBEIIa and IIb (Fig. 5; Fisher and Boyer, 1983; Singh and Preiss, 1985). Chromatographic fractions representing the peaks containing maximal levels of SBEI (fraction 8), IIb (fraction 47), and IIa (fraction 59) activity were concentrated, separated by SDS-PAGE, and subjected to western analysis with the anti-maize SBEII antibody (Fig. 5). Coomassie staining of a similar gel indicated that, although different protein profiles are seen in each of the fractions as expected, approximately equal amounts of proteins were loaded in each of the lanes (lane 4 was slightly underloaded) (Fig. 5A).

Immunodetection with the SBEII antibody is depicted in Figure 5B. In the wild-type and *ae-B1* SBEI fractions, no SBEII immunoreactive proteins were detected as expected (lanes 1 and 4). In the wild-type samples, an 85-kD band was detected in the SBEIIb fraction along with a number of smaller bands that are likely degradative products of SBEIIb (lane 2). These smaller bands detected in the wild-type SBEIIb fraction (lane 2) were likely not nonspecific reaction to other proteins, since they were absent in the same fraction of *ae-B1* (lane 5). In the wild-type SBEIIa fraction, the 85-kD band and a second, larger band (89 kD) were strongly detected, and none of the smaller fragments were seen (lane 3). The presence of both proteins in the wild-type SBEIIa peak was due to the incomplete resolution of SBEIIb and IIa activity using this column. With the *ae-B1* extracts, fractions corresponding to SBEIIb in wild type showed no SBEII immunoreactive proteins, verifying the chromatography results above, which indicate that no SBEIIb activity was present in endosperm cells of this allele (lane 5). Significantly, in the SBEIIa fraction from the *ae-B1* allele, only the 89-kD SBEIIa band was detected (lane 6) at approximately wild-type levels. Together these data show that in the *ae-B1* endosperm cells, which showed at least 100-fold less *Sbe2b*-hybridizing transcript compared to wild type, near-normal levels of SBEIIa protein and enzymatic activity were present.

To investigate whether the *ae* gene or other related genes are also expressed in developing embryos and tassels, northern blot analysis was performed on RNA extracts made from dissected embryos and immature tassels from wild-type and *ae-B1* plants (Fig. 6). In wild-type tissues, as we have previously shown (Gas et al., 1996), strong hybridization to the *Sbe2b* probe was observed in both tissues, whereas in the *ae-B1* mutant, no *Sbe2b*-hybridizing transcript was detectable. This result demonstrates that the *ae* gene was also expressed strongly in developing embryos and tassel and that no other *Sbe2b*-like genes were expressed in these tissues to appreciable levels.

DISCUSSION

Data from several species (for review, see Preiss, 1991; Martin and Smith, 1995) indicate that SBEs exist as multiple isoforms in plant tissues. In maize other enzymes in the starch biosynthetic pathway, including ADP-Glc pyrophosphorylase (Prioul et al., 1994), starch synthase (for review, see Preiss, 1991), and Suc synthase (McCarty et al.,

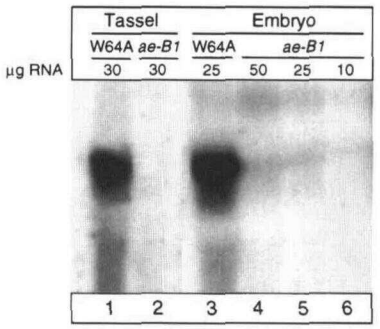

Figure 6. Northern analysis of tassel and embryo tissues. Total RNA from tassels 10 d prior to anthesis and 22-DAP embryos was fractionated, blotted, and probed with the full-length maize *Sbe2b* cDNA probe (Fisher et al., 1993). Genotypes and loading amounts are indicated. Transcript size detected is approximately 2.7 kb.

1986), also exist as related but distinct isoforms, which in some cases have been shown to be the products of a multigene family. To further our understanding of the role of SBE isoforms in plant development, it is essential to characterize their individual genetic loci.

As summarized in Table I, isoforms of SBEs in maize endosperm have been well characterized biochemically. SBEIIa and IIb are similar in many of their biochemical properties. Previous molecular mass estimates of the two proteins using gel filtration range from 70 to 92 kD (Boyer and Preiss, 1978a; Baba et al., 1982). The only previous SDS-PAGE estimate published for SBEIIa and IIb was 80 kD for both proteins (Boyer and Preiss, 1978a). However, these estimates were based on SDS-polyacrylamide disc-gel electrophoresis and were perhaps not able to resolve the differences between SBEIIa and SBEIIb, shown to be 89 and 85 kD, respectively, by our western analysis. Our 85-kD estimate for SBEIIb is in close agreement with the deduced amino acid sequence of the *Sbe2b* cDNA (Fisher et al., 1993; 85 kD).

Singh and Preiss (1985) suggested that SBEIIa and IIb are products of the same gene, citing their results showing similarities in reactions to antibodies and small differences in amino acid composition, proteolytic digestion maps, and K_m for substrates. However, this single-gene hypothesis does not agree with the results of Boyer and Preiss (1978b) and Hedman and Boyer (1982, 1983), which showed that alleles of the *ae* locus affect levels of SBEIIb but not SBEIIa in endosperm and that a linear relationship exists for increasing doses of the dominant *Ae* allele and SBEIIb activity (Hedman and Boyer, 1982). In addition, SBEIIa activity in maize leaves has been shown to be nearly identical with endosperm IIa, but SBEIIb activity is only found in endosperm (Dang and Boyer, 1988, 1989). These points argue for independent genetic control of SBEIIa and IIb and suggest that *ae* is the structural gene for SBEIIb alone. Formally, *ae* could be a mutation in a gene that is not the structural gene for SBEIIb, such as a regulatory or other locus that affects the stability or activity of SBEIIb. This possibility has been eliminated by cloning of the *Sbe2b* cDNA (Fisher et al., 1993), which has 98% DNA sequence identity with the corresponding *ae* locus (Stinard et al., 1993). However, the possibility that posttranscriptional modifications on the *ae* gene product result in both the SBEIIa and IIb isoforms had not been previously eliminated. Although it has been demonstrated that the *ae* locus encodes SBEIIb, the gene encoding SBEIIa is currently uncharacterized. Possible scenarios to explain the existence of two closely related forms of SBE, based on a one-gene and two-gene model, are described below.

Hypothesis I: SBEIIa and IIb Are Products of a Single (*ae*) Gene

Differences in the Two Forms Are the Result of Posttranslational Modification and/or Differential Intron Splicing

In this case the *ae* mutant is altered in some component of the modification or splicing mechanism that only affects SBEIIb activity. This hypothesis predicts that SBEIIb is a secondary product of the *Sbe2a/2b* transcript or protein. If this true, it would follow that any *ae* allele containing SBEIIa activity should still contain *ae* mRNA at normal levels for the synthesis of SBEIIa and that any alleles with no *Sbe2a/2b* transcript should have no SBEIIa activity. We have described *ae* alleles with at least 100-fold decreased levels of *Sbe2b* mRNA that still maintain approximately wild-type levels of SBEIIa activity. This is inconsistent with both of the above predictions and provides strong evidence against the single-gene hypothesis.

Differences in SBEIIa and IIb May Be the Result of Differential Interactions with Glucan (Starch) and/or Starch Synthase (Schiefer et al., 1973; Hawker et al., 1974)

Starch interactions have been proposed to explain the biochemical and chromatographic differences between SBEIIa and IIb (Preiss, 1988). Perhaps bound glucan or complexed starch synthase activity produce the two pools of activity from a single-gene product. In this case, the *ae* mutation is altered in some component that affects this SBE/starch or SBE/starch synthase complex, resulting in an absence of the SBEIIb form. Preiss (1988) also suggested that reduced SBE activity associated with the *ae* lesion may change starch structure such that it interacts with SBE differently, resulting in the loss of SBEIIb activity. Again, based on the same predictions discussed above, this scenario is not consistent with our result describing *ae* alleles with no detectable *Sbe2b* mRNA and near-normal SBEIIa activity.

Hypothesis II: SBEIIa and IIb Are Encoded by Independent Genes

Differences in the two forms may be the result of unique features of two independent genes and the proteins they encode. In this case, the *ae* locus would be the structural gene for SBEIIb alone. The presence of separate IIa and IIb genes would predict that *ae* alleles lacking SBEIIb activity and *ae* mRNA would still have normal levels of SBEIIa

activity. This hypothesis is supported by our analysis. Absence of *Sbe2b* transcript and SBEIIb enzymatic activity in the *ae-B1* allele further confirms that *ae* is the structural gene for SBEIIb. Significantly, the presence of SBEIIa enzymatic activity and yet little or no detectable *Sbe2b* mRNA in *ae-B1* is consistent with the presence of a second gene for SBEIIa. These data also indicate that the SBEIIa message does not significantly cross-hybridize with the *Sbe2b* cDNA, that it is present at very low abundance, or a combination of both of these possibilities.

We showed that the *Sbe2b* gene is also expressed in developing embryos and tassels. This is not the case for the large subunit of ADP-Glc pyrophosphorylase, which is encoded by an embryo-specific gene in addition to the gene expressed in maize endosperm (Giroux et al., 1995). The absence of expression of the *Sbe2b* gene in *ae-B1* tassel tissues is consistent with previous studies that showed that the *ae* locus also affected pollen phenotype (Moore and Creech, 1971). These studies mapped the genetic location of several *ae* alleles based on a pollen-staining technique and segregation analysis. Our data support the conclusion that this pollen phenotype is the result of an aberration in *Sbe2b* expression in these tissues.

We have shown that the *ae-B1* allele, which has little or no detectable transcript for the *Sbe2b* (*ae*) gene, maintains approximately normal levels of SBEIIa activity in endosperm. In the same allele, SBEIIb activity is undetectable. We conclude that endosperm SBEIIa is encoded by a gene distinct from *Sbe2b*. The isolation of the *Sbe2a* gene will further elucidate the distinct properties of the SBEIIa protein.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Jack C. Shannon for his insight and assistance in this project and Drs. Daniel J. Cosgrove, Jill Deikman, and Richard N. Arteca for reviewing the manuscript. The technical assistance of Chris Dervinis and Robert Oberheim is also greatly appreciated.

Received August 22, 1995; accepted November 10, 1995.
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