Identification of cis-Acting Elements Important for Expression of the Starch-Branching Enzyme I Gene in Maize Endosperm

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The genes encoding the starch-branching enzymes (SBE) SBEI, SBEIIa, and SBEIIb in maize (Zea mays) are differentially regulated in tissue specificity and during kernel development. To gain insight into the regulatory mechanisms controlling their expression, we analyzed the 5'-flanking sequences of Sbe1 using a transient gene expression system. Although the 2.2-kb 5'-flanking sequence between −2,190 and +27 relative to the transcription initiation site was sufficient to promote transcription, the addition of the transcribed region between +28 and +228 containing the first exon and intron resulted in high-level expression in suspension-cultured maize endosperm cells. A series of 5' deletion and linker-substitution mutants identified two critical positive cis elements, −314 to −295 and −284 to −255. An electrophoretic mobility-shift assay showed that nuclear proteins prepared from maize kernels interact with the 60-bp fragment containing these two elements. Expression of the Sbe1 gene is regulated by sugar concentration in suspension-cultured maize endosperm cells, and the region −314 to −145 is essential for this effect. Interestingly, the expression of mEmBP-1, a bZIP transcription activator, in suspension-cultured maize endosperm cells resulted in a 5-fold decrease in Sbe1 promoter activity, suggesting a possible regulatory role of the G-box present in the Sbe1 promoter from −227 to −220.

Starch, the major form of carbon and energy reserve in plants, provides a major caloric source for the human population of the world and is also an important industrial commodity. Although the pathway of starch biosynthesis is not completely understood, there is no doubt that it involves at least four groups of committed enzymes: ADP-Glc pyrophosphorylase (EC 2.7.7.23), starch synthase (EC 2.4.1.21), starch-branching enzyme (SBE; EC 2.4.1.28) and starch-debranching enzyme (EC 2.4.1.41) (for review, see Preiss, 1991; Martin and Smith, 1995). Starch biosynthesis involves at least four groups of committed enzymes: ADP-Glc pyrophosphorylase (EC 2.7.7.23), starch synthase (EC 2.4.1.21), starch-branching enzyme (SBE; EC 2.4.1.28) and starch-debranching enzyme (EC 2.4.1.41) (for review, see Preiss, 1991; Martin and Smith, 1995). SBEs catalyze the formation of amylopectin by introducing α-1,6 branch points into the linear α-1,4-linked Glc chains. The introduction of branches not only changes many of chemical and physical properties of starch, but also facilitates starch synthesis by increasing the number of nonreducing ends, the site of Glc addition by starch synthases. Thus, SBEs are of crucial importance for the quantity and quality of starch synthesized in the plant (Edwards et al., 1988). In fact, mutations in Sbe genes of pea, maize (Zea mays), and rice severely decreases the total starch content and changes the ratio of amylose and amylopectin (Shannon and Garwood, 1984; Smith, 1988; Bhattacharyya et al., 1990; Mizuno et al., 1993).

Multiple forms of SBEs, differing in enzymatic and biochemical properties, have been identified and characterized in various plants, such as spinach (Hawker, 1974), pea (Matters and Boyer, 1981; Smith, 1988), potato (Griffin and Wu, 1968; Khoshnoodi et al., 1993), teosinte (Boyer and Fisher, 1984), rice (Mizuno et al., 1992; Nakamura et al., 1992; Yamanouchi and Nakamura, 1992), and maize (Hodges et al., 1969; Boyer and Preiss, 1978; Dang and Boyer, 1989). They were grouped into two distinct families based on their structural relatedness and named according to the prototypic family member from maize (Burton et al., 1995; Gao et al., 1996). The SBEI family consists of maize SBEI, rice SBEI, and pea SBEII; and the SBEII family encompasses maize SBEIIa and SBEIIb, rice SBEIII, and pea SBEI.

Significant differences in the enzymatic properties between the SBE families are well documented (for review, see Martin and Smith, 1995). SBEs belonging to the SBEI family have lower affinity for amylose than SBEI isoforms and prefer to use shorter glucan chains for further branch formation. Another noteworthy difference between the SBEI and SBEII families is that they are differentially regulated during seed development. The SBEII family genes are expressed earlier than the SBEI family members in developing seeds (Smith, 1988; Burton et al., 1995; Gao et al., 1996), which may result in changes in the SBEI to SBEII ratio. Since SBEI and SBEII have significantly different in vitro catalytic properties (as mentioned above), such changes in the SBEI to SBEII ratio may cause differences in the starch synthesized during kernel development. During pea embryo development, changes in the SBE isoform ratio was accompanied by transition in the branch lengths of amylopectin (Burton et al., 1995).

SBEII has been further resolved in maize endosperm by chromatography on 4-aminobutyl-Sepharose into two frac-
tions: SBEIIa and SBEIIb (Boyer and Preiss, 1978). Although these two isoforms are similar in molecular mass, amino acid composition, proteolytic digest map, and immunological reactivity, they do have distinct properties (for summary, see Fisher et al., 1996) and are encoded by different genes (Gao et al., 1997). For example, SBEIIb is more active than SBEIIa in the branching of amylopectin (Boyer and Preiss, 1978). Takeda et al. (1993) also showed that these two SBEII isoforms have different optimum temperature and specific activities in the branching linkage assay.

Isolation of the maize cDNAs encoding SBEI, SBEIIa, and SBEIIb has allowed us to investigate the Sbe genes at the molecular level (Fisher et al., 1993, 1995; Gao et al., 1996, 1997). Gao et al. (1996) showed that Sbe1 and Sbe2b are expressed in a coordinate fashion with the granule-bound starch synthase and ADP-Glc pyrophosphorylase, respectively, during maize endosperm development. The finding that many genes involved in starch biosynthesis are regulated by sugar availability (Muller-Rober et al., 1990; Koch et al., 1992; Giroux et al., 1994; Salehuzzaman et al., 1994; Fu et al., 1995b) suggests that they may share common regulatory mechanisms controlling their expression. Therefore, knowledge of the regulatory mechanisms for one of the starch biosynthetic genes aid in the understanding of how the other genes are controlled in plants. Unfortunately, however, little is known about promoter elements, transcription factors, or molecular mechanisms involved in the regulation of starch biosynthetic genes.

To begin to explore these questions, we have recently isolated and sequenced maize genomic DNA fragments containing the Sbe1 and Sbe2b genes (accession nos. AF072724 and AF072725, respectively), and established the complete genomic organization of the genes (Kim et al., 1998a, 1998b). We report in this study functional analysis of the Sbe1 promoter, which revealed DNA sequence elements important for the high-level, sugar-responsive expression of the Sbe1 gene in maize endosperm cells.

MATERIALS AND METHODS

Construction of Chimeric Plasmids

A transcriptional fusion of the Sbe1 promoter to a luciferase (LUC) reporter gene was made as follows. A BamHI restriction enzyme site was first created just before the translation initiation site of the Sbe1 gene by PCR: The DNA sequence between −253 and +27 of the Sbe1 gene was PCR-amplified with PI-1 and PI-2 primers (Table I). Four additional bases were included at 5′-end of the primers to provide for restriction enzyme sites at the ends of the PCR products for subsequent cloning. The bases were chosen randomly by considering their effect on Tm and on dimer and stem-loop formation of the primers. Pfu DNA polymerase (Stratagene), which has proofreading activity, was used to enhance the fidelity of PCR amplification. (Pfu DNA polymerase was used for all of the following PCRs.)

Since an Apal restriction enzyme site (GGGCCC) is located immediately downstream of the 5′ primer (PI-1) binding region of the Sbe1 promoter, −203 to −198, the PCR product was digested with Apal and BamHI. The resulting 236-bp fragment was then cloned into pBluescript SK− (Stratagene) and sequenced to verify that no misincorporation had occurred in the DNA sequence during the PCR amplification (all of the following PCR products were sequenced). Next, the 236-bp fragment was ligated to the 1,991-bp SacI-Apal Sbe1 promoter fragment and cloned into the promoterless LUC plasmid (pLN cut with SacI and BamHI) (promoterless LUC-NOS gene in pUC119) (Montgomery et al., 1993), thereby creating plasmid pKL101.

To construct a translational fusion of the Sbe1 promoter containing the first exon and intron to a LUC reporter plasmid, the DNA sequence between −253 and +228 was amplified with the PI-1 primer and a 3′ primer (PI-3) designed to anneal to the region just downstream of the first intron of the Sbe1 gene. The 493-bp PCR product was digested with Apal and BamHI, and the resulting 436-bp fragment was used to replace the Apal-BamHI fragment in pKL101. This construct was called pKLN101. To make pKL101, which contains the Sbe1 promoter with four exons and introns, the 236-bp Apal and BamHI fragment in pKL101 was replaced with the 1816-bp Sbe1 genomic DNA fragment.

The plasmid pKLNS101 was derived from pKLN101 by replacing the nopaline synthase (NOS) 3′ sequence with the native Sbe1 3′-flanking sequence. To accomplish this, two primers, PI-4 and PI-5, were designed to amplify Sbe1 DNA sequences containing the transcription stop signal and the polyadenylation site (from +5382 to +5780). A 419-bp PCR product was digested with SacI and EcoRI, and

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<tr>
<th>Primera</th>
<th>Sequencerb</th>
<th>Annealing Regionc</th>
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<tr>
<td>PI-1 U</td>
<td>CCAGCTCCAGGTGGACGTGTTTTTG</td>
<td>−253 to −232</td>
</tr>
<tr>
<td>PI-2 L</td>
<td>cgtctgactCGTCGAGCCCGGTGTTGAGTTCCC</td>
<td>+8 to +27</td>
</tr>
<tr>
<td>PI-3 L</td>
<td>agtccgatcTACGCAATGAGCCGGA</td>
<td>+209 to +228</td>
</tr>
<tr>
<td>PI-4 U</td>
<td>gactgtactATACAAATTGAACCGCAGG</td>
<td>+5382 to +5401</td>
</tr>
<tr>
<td>PI-5 L</td>
<td>actggaggctCGAACAGGAAACGGAAGAAA</td>
<td>+5761 to +5780</td>
</tr>
<tr>
<td>PI-6 U</td>
<td>gacgttagctACCGCAATGGACCAGGG</td>
<td>−254 to −235</td>
</tr>
<tr>
<td>PI-7 U</td>
<td>atcatctgACGTTCCAGGGCAAC</td>
<td>−196 to −177</td>
</tr>
<tr>
<td>PI-8 L</td>
<td>TGGCAGAGGGAGGGCCCA</td>
<td>−165 to −146</td>
</tr>
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a U and L indicate upper (sense) and lower (antisense) primers relative to Sbe1, respectively. b The lowercase letters designate restriction sites used for cloning. c Numbers represent distance relative to the transcription start site (+1) of Sbe1.
the resulting fragment was then used for substituting a 255-bp SucI-EcoRI NOS 3’ sequence in the pKLN101.

To create a series of 5’ deletions in the Sbe1 promoter, pKLN101 was first modified as follows: pKLN101 DNA was digested with HindIII and the resulting 7,190-bp fragment lacking the 452-bp HindIII fragment was gel purified. The fragment was blunt ended by Klenow fill-in DNA synthesis and ligated with SalI linkers. After complete digestion with SalI, the DNA fragment was partially digested with BamHI to isolate the 1,993-bp SalI-BamHI fragment, which was then gel purified and cloned into pLN cut with SalI and BamHI to produce pKLN101-1.

A series of 5’ deletion mutants were made from the plasmid pKLN101-1 using an S1-nuclease-based system (Erase-a-Base, Promega) to produce the 5’ deletion series plasmids pKLN102 to pKLN107. All constructs were sequenced with the pUC/M13 reverse primer to verify deletion end points. For the −254 and −196 deletion constructs, two regions of the Sbe1 promoter, −254 to −146 and −196 to −146, were PCR-amplified by primer PI-6 and PI-8, PI-7 and PI-8, respectively. Primers used in PCR to create the Sbe1-LUC constructs are shown in Table II. Since each 5’ primer, PI-7 and PI-8, contains a HindIII restriction enzyme site, and since a BstXI restriction enzyme site is located between −173 and −162, the PCR products were digested with HindIII and BstXI and the resulting fragments were used to replace the 2,047-bp HindIII-BstXI fragment of pKLN101.

Linker-Scanning Mutagenesis

A series of linker-scan mutations were introduced into the 60-bp DNA region from −314 to −255 as described by Kunkel et al. (1987). The HindIII-BamHI (−314 to +235) fragment from pKLN105, containing the DNA region to be altered, was subcloned into the corresponding sites of a M13 mp19 vector to produce a single-stranded template. To increase mutant recovery efficiencies, the template was prepared from an Escherichia coli datt+ wgg- strain (C232) that allowed the incorporation of uracil into the newly synthesized DNA. Next, a set of oligonucleotides with 10-bp mismatches (Table II) were annealed to the template and extended with T7 DNA polymerase. After the addition of T4 DNA ligase, the resulting heteroduplexes were introduced into a wild-type E. coli strain (MV1190) to generate mutated double-stranded DNAs. DNA sequencing was performed to verify that the desired mutations were correctly introduced and that no unintended mutations had occurred.

To create the mutated Sbe1 promoter-LUC constructs (pLS1-1 to pLS1-6), the HindIII-BamHI fragment in pKLN105 was replaced by each mutated DNA sequence.

**Particle Bombardment**

Suspension-cultured cells of maize (Zea mays) endosperm (inbred line A636), provided by J.L. Anthony (DEKALB Genetics Corporation, Mystic, CT), were grown in 250-mL large-mouth Erlenmeyer flasks containing 80 mL of Murashige and Skoog basal salt medium (Murashige and Skoog, 1962) supplemented with 0.4 mg/L of thiamine, 2 g/L of Asn, and 30 g/L of Suc (Shannon and Liu, 1977). The culture was maintained in the dark at 29°C on a rotary shaker (120 rpm) and subcultured every 7 d by transferring a portion of the cell suspension into fresh medium.

For particle bombardment, about 600 mg (fresh weight) of actively growing cells 3 d after subculture was evenly distributed over the surface of a piece of filter paper (Whatman no. 4, 55 mm in diameter) by vacuum filtration of 8 mL of suspension culture. The filter paper bearing the cells was then placed over three layers of filter paper (Whatman no. 4, 70 mm in diameter) moistened with 5 mL of the liquid medium containing 12% (w/v) Suc, and positioned in the middle of a 10-cm Petri dish.

Gold microcarriers (1.6-μm particle size, 60 mg) were washed three times with 1 mL of 100% (w/v) ethanol and twice with 1 mL of sterile de-ionized water, resuspended in 1 mL of sterile de-ionized water, and dispersed in 50-μL aliquots (3 mg/50 μL). The Sbe1 promoter-LUC constructs and a GUS reference plasmid (pBI221, Jefferson, 1987) were coprecipitated onto the gold particles as follows: under continuous vortexing, the following were added in order to each 50-μL aliquot of gold particles: 5 μL of DNA (8 μg of LUC reporter plasmid and 4 μg of GUS reference plasmid), 50 μL of 2.5 mM CaCl2, and 20 μL of 0.1 mM spermidine (free-base, tissue-culture grade). The gold particles coated with DNA were pelleted in an Eppendorf centrifuge at 10,000 rpm for 10 s, rinsed with 250 μL of 100% (w/v) ethanol, and resuspended in 60 μL of 100% (w/v) ethanol. Immediately after sonication, 8 μL of the DNA-coated gold particles was pipetted onto the center of macrocarriers (Bio-Rad) and dried in a low-humidity environment.

A He biolistic particle-delivery system (model PDS-1000, Bio-Rad) was used for particle bombardment. The bom-

### Table II. Oligonucleotides used in linker-scanning mutagenesis

| Constructs | Oligonucleotides
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<tbody>
<tr>
<td>pLS1-1</td>
<td>CCCGGTTTGGCTTATTCTTTCGACGTTGAGCTGGTgtcGactatcTTGGCCTTTGGGCTTGC</td>
</tr>
<tr>
<td>pLS1-2</td>
<td>TTGACAGCCTTCCCGGTTgatgcTggcgcATATTTATGTAAGCTTG</td>
</tr>
<tr>
<td>pLS1-3</td>
<td>GCCTTTTGGGCTTGCACGctgatgcTgctattgtTCGTTTTTTTTATTTTA</td>
</tr>
<tr>
<td>pLS1-4</td>
<td>GGGCCGATTGCGCCTTTGCATcgagcagCTTCGGCGTTGCGCTTTT</td>
</tr>
<tr>
<td>pLS1-5</td>
<td>AGCTGGTTTGGCGCGCAGcgacgatGGCTTGCAAGCTTCCCG</td>
</tr>
<tr>
<td>pLS1-6</td>
<td>ACAACCGTGAGCTTGTTacGcctttgTTGGCTTGGGCTTGC</td>
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</table>

*a The mutated bases are shown in lowercase letters, and restriction sites used for convenience of screening are underlined.
barronment parameters optimized included He pressure, gap distance (the distance from the power source to the microprojectile), and the target distance (the distance from microprojectile launch site to the sample target). After optimization, all bombardments were performed in a dimly lit room at 650 psi under a vacuum of 26 inches of Hg, with a distance of 10 cm between the cells and the barrel of the particle gun. Following the bombardments, the Petri dishes were sealed with laboratory film and incubated in the dark at 25°C for 24 h.

GUS and LUC Assays

The bombarded cells were harvested from the plates by vacuum filtration, frozen in liquid nitrogen, and ground with a mortar and pestle to a fine powder. The powder was then transferred into a microfuge tube and extracted with cell-culture lysis buffer containing 300 mM Tris-P, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetracetic acid, 10% (v/v) glycerol, and 1% (v/v) Triton X-100 (0.3 mL/g of tissue). Cell debris were pelleted in an Eppendorf centrifuge at 14,000 rpm for 10 min at 4°C, and the supernatant was split into two aliquots for assays of GUS and LUC activity.

For fluorometric GUS assays (Jefferson, 1987), 30 µL of the crude extract was incubated at 37°C with 2 mM 4-methylumbelliferyl glucuronide in 0.3 mL of GUS assay buffer (50 mM NaPO4, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [v/v] Sarkosyll, 10 mM ß-mercaptoethanol, and 20% [v/v] methanol). After 0, 1, and 2 h of incubation, 0.1-mL aliquots were removed and added to 0.9 mL of 0.2 M Na2CO3 to terminate the reaction. A fluorometer (model TKO 100, Hoeffer, San Francisco) calibrated by setting a 100 nM methylumbelliferone to 1,000 fluorescence units was used to measure fluorescence of the product, 4-methylumbelliferone.

The DNA-protein binding reaction was performed in 20 µL of solution containing 0.5 ng of labeled probe, 10 µg of nuclear protein, 1 µg of poly(dI-dC)poly(dI-dC), 12% (w/v) glycerol, 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-Cl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT. After a 20-min incubation at room temperature, the samples were loaded into a 4% (w/v) native polyacrylamide gel that had been prerun at 4°C for 1 h at 150 V and electrophoresed for 2.5 h at 150 V in Tris-Gly buffer at 4°C. Following electrophoresis, the gel was dried in a gel dryer (Bio-Rad) and exposed to Kodak x-ray film with two intensifying screens for 24 h.

Northern-Blot Analysis

Total RNA was isolated according to the method of Vries et al. (1988) from suspension-cultured maize endosperm cells that had been incubated for 24 h in the Murashige and Skoog basal salt medium supplemented with 0.4 mg/L of thiamine, 2 g/L of Asn, and various amounts of Suc from 0% to 15%. Northern-blot analysis was performed as described in Gao et al. (1996). Radioactivity was detected with a phosphor imager and quantified with the ImageQuant software program (both from Molecular Dynamics). To correct for minor loading errors between the lanes, the blot was washed at 95°C in a 0.1% (w/v) SDS solution to remove the 32P-labeled Sbe1 cDNA probe and rehybridized with a 32P-labeled tomato cDNA for 26S rRNA.

RESULTS

Transcribed Regions of the Sbe1 Gene Are Involved in Gene Expression

To determine whether the 5' flanking sequence of the Sbe1 gene has all of the DNA elements necessary to initiate transcription, a 2,217-bp fragment upstream of the translation start site (−2,191 to +27) was fused to the LUC re-
porter gene in pUC119 (pKL101) as shown in Figure 1A. The chimeric plasmid was then introduced into maize endosperm cells via particle bombardment along with a reference plasmid containing the CaMV 35S promoter linked to a GUS gene (pBI221, Jefferson, 1987) to correct for transfection efficiency. However, only very low levels of LUC activity were detected relative to the other promoters described above.

Since data have indicated that DNA sequences within transcribed regions such as exons, introns, and 3’ flanking regions are involved in the expression of genes in either a qualitative or a quantitative manner (Callis et al., 1987; Hamilton et al., 1992; Fu et al., 1995a; Ulmasov and Folk, 1995), three different types of translational fusion constructs were created to test the effect of downstream elements on Sbe1 gene expression. First, the 5’-flanking sequence, as well as the first exon and intron of the Sbe1 gene (–2,190 to +228), were fused in-frame to the LUC reporter gene to make pKLN101 (Fig. 1, A and B). Second, the NOS 3’ sequence in the pKLN101 was replaced with the Sbe1 3’ flanking sequence (399 bp in length), which contains the translation stop codon and polyadenylation signal to create pKLM101. Finally, to determine whether an increase in the number of exons/introns enhances gene expression, three more exons and introns from the Sbe1 gene were added to the pKLN101 to make pKLM101 (–2,190 to +1,617).

The results of transient expression assays using the chimeric constructs are shown in Figure 1C. Inclusion of the DNA sequence from +28 to +228 containing the first exon and intron increased the level of LUC expression by 14-fold, suggesting that the first exon and intron region is required for high-level expression of the Sbe1 gene in maize endosperm cells. Since pKLN101 produced a fusion protein, however, we cannot completely rule out the possibility that the increase may have been due to changes in enzyme activity and/or turnover rate caused by the added amino acid sequences. If the additional amino acids have a negative effect, the enhancement of LUC activity observed would be greater than 14-fold.

Replacement of the NOS 3’ end in pKLN101 with the Sbe1 3’ region did not have a significant effect on the level of LUC expression, implying that the Sbe1 3’ UTR does not have indispensable control elements. However, it is still possible that the region may be important for Sbe1 gene expression in other cell types or inductive conditions. Construct pKLM101 showed a slight reduction in LUC activity compared with pKLN101, indicating that additional exons and introns had an adverse effect on LUC expression in suspension-cultured maize endosperm cells. The adverse effect could be explained by inefficient splicing resulting from the introduction of multiple copies of the plasmid into a single cell, or by formation of fusion protein consisting of the 5’ end of SBEI and LUC, thus lowering LUC activity. Alternatively, it could be due to the presence of negative cis elements in this region.

**Figure 1.** Effect of Sbe1 gene exons/introns and 3’ end on the level of LUC expression driven by the Sbe1 promoter. A, Schematic diagram of chimeric Sbe1 promoter-LUC constructs. Numbers indicate the distance relative to the Sbe1 transcription start site. Translation initiation starts at position +28. The light-gray boxes indicate the Sbe1 promoter region. Angled lines indicate exons and introns in the Sbe1 gene. White and black boxes indicate LUC reporter gene and NOS 3’ end sequences, respectively. The striped box indicates the Sbe1 3’ flanking sequence. B, Junction sequences between the Sbe1 gene and LUC. The BamHI sites used to join the two genes are underlined. The translation start site of LUC is indicated by boldface letters. C, Expression levels of the construct shown in A. LUC to GUS ratios were calculated as described in “Materials and Methods.” Each value represents the average of four independent shootings. Error bars indicate st values.

**5’ Deletion Down to –314 Did Not Significantly Affect the Sbe1 Promoter Activity**

To identify promoter sequences critical for Sbe1 expression in maize endosperm cells, a series of 5’ deletion mutants were derived from pKLN101 as shown in Figure 2A. The activity of each 5’ deletion construct is presented in Figure 2B. Removal of the sequences to –1,332 caused a decrease in the level of LUC expression, while deletion of an additional 422 bp, to –910, resulted in an increase in the activity of the construct. This suggests that potential positive and negative distal cis regulatory elements may be located in the regions from –2,190 to –1,332 and from –1,332 to –910, respectively. Further deletions down to –315 did not significantly affect the promoter activity, but a severe reduction in activity was observed when an addi-
To further delimit sequences essential for high-level expression of the promoter, two additional 5′ deletions with about 60-bp intervals were created between −315 and −145. As shown in Figure 2, a deletion to −255 (pKLN108) severely reduced the expression of the LUC reporter gene, while a further deletion to −196 (pKLN109) did not further reduce promoter strength. This indicates that a strong positive regulatory element(s) is present in the 60-bp region between −315 and −255.

**A 60-bp Region Is Critical for the Promoter Activity**

Since the 5′ deletion analyses indicated that the region of the Sbe1 promoter from −314 to −255 is critical for promoter activity, the 60-bp DNA fragment was further dissected by oligonucleotide-directed in vitro mutagenesis, as described by Kunkel et al. (1987). A series of six different substitution mutants, designated pLS1 to pLS6, were created by altering the wild-type DNA sequence of the Sbe1 promoter at 10-bp intervals. The mutations were made by creation of transversion substitutions where possible, while at the same time introducing restriction enzyme sites for simplifying identification of the mutant forms.

The mutated constructs were tested for their promoter activity using the transient assay system, and the results of the experiments are shown in Figure 3. Mutations in the regions from −314 to −305 and from −304 to −295, corresponding to pLS-1 and pLS-2, caused a decrease in the Sbe1 promoter activity to 60% and 72% of wild-type (pKLN105) expression, respectively. The pLS3 construct showed almost the same level of the LUC expression as the wild-type promoter, suggesting that the nucleotides from −294 to −314 were important for promoter activity.

**Linker-Scan Analysis Reveals Two cis Elements within the 60-bp Region**

The 60-bp region in the Sbe1 promoter contains two cis elements, indicated by linker-scan analyses. These analyses revealed a decrease in promoter activity by 60% and 72% when the nucleotides from −314 to −305 and from −304 to −295 were altered, respectively. The pLS3 construct showed almost the same level of the LUC expression as the wild-type promoter, suggesting that the nucleotides from −294 to −314 are critical for promoter activity.

**Figure 2.** Effect of 5′ deletions on Sbe1 promoter activity. A, Schematic diagram of the 5′ deletion chimeric constructs. The thick black lines denote the Sbe1 promoter sequences. The numbers at left indicate deletion end points relative to the transcription initiation site (+1) of the Sbe1 gene. The light-gray boxes and the thin black angled line represent the first exon and intron in the Sbe1 gene, respectively. The white boxes indicate the LUC gene. The black boxes denote NOS 3′ end sequences. B, The relative activity levels of the constructs shown in A. The relative activity values are percentages of pKLN101 level. Each value represents the average of six to eight independent experiments. Error bars indicate ± values.

**Figure 3.** Linker-scan analyses of the 60-bp region in the Sbe1 promoter. A, Schematic diagram of the linker-scan constructs. DNA sequence of the 60-bp region in the Sbe1 promoter is shown to the right of the wild-type construct pKLN105. The mutated bases in the linker-scan constructs are shown in lowercase letters. Dashes represent the unaltered nucleotides. For an explanation of the other symbols, refer to the legend to Figure 2. B, Relative LUC activity levels of the constructs shown in A. The relative activity values are percentages of construct −314 level. Each value represents the average of four independent experiments. Error bars indicate ± values.
−285 are not important for the promoter activity in maize endosperm cells. However, a mutation in the pLS-4 region (−284 to −275) decreased promoter activity to 40% of the wild-type level. Also, other two mutants, pLS-5 and pLS-6, showed a reduction of promoter activity to 55% and 50% of the wild-type promoter, respectively.

The 60-bp Fragment Interacts with DNA-Binding Proteins

Electrophoretic mobility-shift assays were performed to investigate the possibility that a nuclear protein(s) might interact with the 60-bp Sbe1 promoter fragment from −314 to −255. The 60-bp fragment was 32P end-labeled with Klenow fill-in reaction and then incubated with nuclear extract prepared from 30 DAP maize kernels demonstrated to highly express the Sbe1 gene (Gao et al., 1996). As shown in Figure 4, two major shifted bands were observed in the lane containing nuclear extract (lane 2) compared with the control (lane 1). The bands were not detected after inclusion of proteinase K in the binding reaction (lane 7), indicating that the shifted bands represent DNA-protein complexes.

Competition assays were conducted to determine whether the complexes are due to the binding of sequence-specific proteins. Inclusion of 10- and 100-fold excess of the unlabeled 60-bp fragment in the binding reaction significantly reduced formation of the complexes (lanes 3 and 4), while the same amount of nonspecific competitor DNA failed to compete for binding (lanes 5 and 6). Thus, the complexes appear to be the result of sequence-specific interactions between a nuclear protein(s) and the DNA fragment, which is consistent with the functional identification of this region as an important regulatory element. Using six 60-bp fragments of linker-scan mutants (LS-1 to 6) as competitors, we found that LS-1 did not affect the intensity of the lower band, although the rest of the linker-scan mutants abolished its formation (data not shown). This suggests that the lower band may be the result of interactions between a trans-acting factor(s) and the sequence ACATAAAATA, which is located within LS-1. All of the linker-scan mutants reduced the intensity of the slower-migrating complex to varying degrees (data not shown). Since LS-4, LS-5, and LS-6 were less effective competitors, wild-type sequences spanning these regions (−284 to −255) may be involved in formation of this complex; however, binding may involve several overlapping regions in this fragment.

Expression of the Sbe1 Gene Is Sugar Regulated

The SBEs are expressed in a coordinate fashion with the granule-bound starch synthase and ADP-Glc pyrophosphorylase during maize endosperm development (Gao et al., 1996). The ADP-Glc pyrophosphorylase gene (AGPase S) from potato and the genes encoding granule-bound starch synthase and SBE in cassava plants have been shown to be induced by an exogenous supply of sugars (Muller-Rober et al., 1990; Giroux et al., 1994; Salehuzzaman et al., 1994). This led us to speculate that the Sbe1 gene in maize may also be regulated by the external sugar concentration.

To test this, suspension-cultured maize endosperm cells were incubated in Murashige and Skoog medium containing different concentrations of Suc, and their total endogenous RNAs were analyzed by northern-blot hybridization. Suc was used in preference to other metabolizable sugars, because it is known to be the major sugar unloading from the pedicel tissue of maize kernels (Porter et al., 1985). The results are shown in Figure 5. An increase in Suc concentration from 0% to 9% elevated the Sbe1 mRNA level 2-fold, and at higher concentrations the increase was reduced. Hexoses such as Glc, Fru, and myoinositol also increased the level of transcript in a similar fashion (data not shown). However, l-Glc and PEG 200 at concentrations calculated to have the same osmotic potential as a 9% Suc solution (263 mm) did not exhibit any effect, indicating that the response is not an osmotic but a sugar-specific phenomenon. These results suggest that, like other starch biosynthetic genes (Giroux et al., 1994), expression of the Sbe1 gene in maize endosperm cells is regulated by sugar availability. This metabolic feedback mechanism may serve as a system to fine-tune the expression levels of Sbe genes relative to the physiological status of a plant. Shannon et al. (1996) showed that nonallelic starch mutants of maize accumulating high levels of Suc in the endosperm contained increased SBE activities compared with the control.

Since we recently determined that two Sbe1 genes (Sbe1a and Sbe1b) with divergent 5′-flanking regions exist in the maize genome (Kim et al., 1998a), it was necessary to determine whether expression of the isolated Sbe1 gene (Sbe1a) promoter responds to external Suc concentrations. To test this, a gene not regulated by sugar concentration was required as an internal control for the transient assay system. Since a CaMV 35S promoter has been used as a control in other studies investigating the Suc responsive-
ness of plant genes, the effect of Suc on the expression of the CaMV 35S promoter-GUS chimeric gene (pBl221) in maize endosperm cells was first investigated.

The plasmid pBl221 was bombarded into suspension-cultured maize endosperm cells supplemented with 0% (w/v) Suc or 9% (w/v) Suc medium and incubated at 25°C in the dark. After 48 h of incubation, the GUS activity and protein concentration were measured from each sample to calculate specific GUS activity (data not shown). The results showed that specific GUS activities of 9% (w/v) Suc samples were almost 2.5-fold higher than those of 0% (w/v) Suc samples, which is consistent with other reports (Graham et al., 1994; Grierson et al., 1994). Since similar results were obtained from a ubiquitin promoter (pACH18) and a −64 CaMV 35S minimal promoter, which does not have an activation sequence (as)-1, a binding site for the transcription factor TGA-1a (Katagiri et al., 1989), it appeared that the elevated levels of expression by the CaMV 35S and ubiquitin promoters in 9% (w/v) Suc may have simply been a general phenomenon caused by an increase in energy source rather than a sugar-specific effect. Therefore, we reasoned that if the chimeric construct pKLN101 (the Sbe1 promoter-LUC) is sugar modulated, it will further enhance the level of LUC expression beyond the general increase at the higher Suc concentration.

As shown in Figure 6, after normalization to GUS activity driven by the CaMV 35S promoter, the plasmid pKLN101 still showed approximately 2-fold greater LUC activity in 9% (w/v) Suc medium than in 0% (w/v) Suc medium. This is consistent with the result of the endogenous RNA analysis indicating that the identified Sbe1 gene is regulated by sugar availability. It also suggests that the nucleotide sequence containing a 2.2-kb 5′-flanking region and the first exon/intron of the Sbe1 gene is sufficient to confer sugar responsiveness in maize endosperm cells.

To delimit a region(s) necessary for the response, two deletion constructs, pKLN105 and pKLN106, were also tested in the transient expression system (Fig. 6). Like pKLN101, pKLN105 (deletion end point −314) responded to a high Suc concentration (9%) by increasing LUC expression by approximately 2-fold. However, pKLN106 (deletion end point −145) showed similar levels of LUC expression in both low and high Suc conditions. These results suggest that the region between −314 and −145 contains a cis-regulatory element(s) necessary for the sugar response in maize endosperm cells. In addition, because the expression level was reduced in both Suc-treated and untreated cells, other regulatory elements may also reside in this region.

**Overexpression of mEmBP-1 Protein Represses the Sbe1 Gene Expression**

The canonical G-box sequence, CCACGTGG (Giuliano et al., 1988), was found in the 5′-flanking sequence of the maize Sbe1 (−228 to −221) as well as the rice Sbe1 gene (−170 to −163). This evolutionary conservation suggests a possible role of the G-box motif in the regulation of gene expression, although our 5′ deletion analysis did not show it as an important regulatory element. The G-box motif resides in the promoters of many plant genes, responding to a variety of different environmental and physiological stimuli, and is often associated with additional regions that act as coupling elements, determining signal response specificity (Menken et al., 1995).

Electrophoretic mobility shift analysis and DNase I footprint analyses were performed to determine whether the G-box in the maize Sbe1 promoter interacts with a G-box-binding protein in maize, mEmBP-1, which is a homolog of the wheat EmBP-1 (Guiltinan et al., 1990) and is expressed during endosperm development (Carlino et al., 1999). As expected, the analyses clearly showed that EmBP-1 inter-
acts with the G-box sequence in vitro (data not shown). Since EmBP-1, a basic Leu zipper (bZIP) transcription factor, is implicated in ABA-induced Em gene expression in wheat (Guitián et al., 1990), the data prompted us to ask two questions: First, is Sbe1 gene expression regulated by ABA concentration? Second, can the mEmBP-1 protein transactivate Sbe1 gene expression? Transient expression assays failed to show a relationship between the exogenous ABA concentration (1–100 μM) and Sbe1 promoter activity in suspension-cultured maize endosperm cells (data not shown), suggesting that the G-box in the Sbe1 promoter is not ABA responsive; however, we cannot rule out the possibility based on these data without further evaluation of ABA levels and responses of endogenous genes in our assay system.

To address the second question, a chimeric construct containing the CaMV 35S promoter fused to the full-length mEmBP-1 cDNA (35S-mEmBP-1) was created and co-introduced with the plasmid pKLN101 (a full-length Sbe1 promoter-LUC) into suspension-cultured maize endosperm cells. We predicted that overexpression of mEmBP-1 protein would enhance the LUC expression driven by the maize Sbe1 promoter greatly depends on the presence of the DNA region spanning the first exon and intron of the maize Sbe1. Addition of the DNA sequence (+28 to +228) containing the first exon and intron of the Sbe1 gene into the transcriptional chimeric construct (pKL101) increased reporter gene expression in suspension-cultured maize endosperm cells up to 14-fold. Since such DNA sequences containing transcriptional stimulating effects are useful in investigations of gene expression in plant cells and for plant genetic engineering, it will be necessary to determine whether the DNA sequence has the ability to increase gene expression under the control of other promoters.

There are several examples of plant genes that are regulated by DNA sequences within the transcribed region (Callis et al., 1987; Bruce et al., 1989; McElroy et al., 1990; Fu et al., 1995a). Among them, the first exon and intron sequences of the maize Sh1 gene are the best examples studied so far (Vasil et al., 1985; Maas et al., 1991; Clancy et al., 1994). The Sh1 exon appears to have two separate cis elements that act independently to increase gene expression via different mechanisms. One of the elements may contain a novel promoter element that has the ability to interact with transcription factors binding upstream. The other may act at the level of translation efficiency or mRNA stability. The enhancing effect of the Sh1 intron is likely the result of an increase in the level of mature cytoplasmic mRNA level, such as the maize Adh1 first intron (Callis et al., 1987).

5′-Deletion analysis of the maize Sbe1 promoter revealed several cis-regulatory elements affecting promoter activity in maize endosperm cells. Of special interest was the identification of the 60-bp positive element located in the region from −314 to −255 relative to the transcription initiation site. Further investigation of the region using linker-scan analysis identified at least two separate regions, −314 to −295 and −284 to −255, which are critical for gene expression in maize endosperm cells.

Interestingly, as shown in Figure 8, the −314/−295 region has striking similarity to the Suc-responsive element (SURE-1) of the potato patatin-1 promoter (Grierson et al., 1994), which has been shown to interact with a Suc-inducible nuclear protein(s). Grierson et al. (1994) demonstrated that a 100-bp patatin-1 promoter fragment encompassing SURE-1 is sufficient to confer Suc responsiveness. DNA sequences similar to the −314/−295 region are also

Figure 7. Effect of mEmBP-1 overexpression on Sbe1 promoter activity. Each reporter plasmid (4 μg of Sbe1 promoter-LUC, pKLN101, ubiquitin-LUC, or pACH18) and reference plasmid (CaMV 35S-GUS; pBI221) were coprecipitated onto gold particles with (hatched bars) or without (white bars) 4 μg of CaMV 35S-mEmBP-1. Suspension-cultured maize endosperm cells were bombarded with the gold particles and incubated at 25°C for 24 h in the dark. The relative activity values are percentages of the pKLN101 or pACH18 levels without mEmBP-1 overexpression. Each value represents the average of two independent shootings. Error bars indicate se values.
found in the promoter regions of other sugar-inducible genes, such as maize Suc synthase (Shaw et al., 1994), Arabidopsis β-amylose (Mita et al., 1995), and potato sporamin (Ohta et al., 1991) (Fig. 8). This finding, along with the sugar-enhanced expression of Sbe1 demonstrated by northern-blot analysis (Fig. 5) and transient expression assay (Fig. 6), strongly suggests that the conserved sequences may be implicated in mediating sugar responsiveness of the Sbe1 gene. This was further supported by our recent finding that the −314/−196 region of the Sbe1 promoter is sufficient to confer Suc responsiveness to the −64 CaMV 35S minimal promoter (data not shown). Since high-Suc media were used for the transient expression assays to maximize gene expression, it is understandable that mutation of this region would decrease the level of LUC expression. It remains to be determined whether other Sbe genes are also sugar modulated. To date, we have not detected sugar-dependent DNA-binding activity associated with the Sbe1 promoter.

In potato and cassava plants, sugars have been shown to regulate the expression of genes involved in starch biosynthesis (Muller-Rober et al., 1990; Salehuzzaman et al., 1994). Our results demonstrated that the maize Sbe1 is also modulated by sugar concentration. Such a sugar effect was not due to changes in the osmotic potential, because L-Glc and PEG, which are osmotically active, did not affect Sbe1 gene expression. Recently, Jang et al. (1997) provided evidence that hexokinase is involved in sensing sugar concentration in higher plants, and sugar signaling mediated through hexokinase is uncoupled from sugar metabolism.

Sequence comparison between the rice (Kawasaki et al., 1993) and maize Sbe1 genomic DNAs (Kim et al., 1998a) revealed that the 5′-flanking sequences proximal to the protein-coding regions are highly divergent except for the canonical G-box sequences (CCACTGG), which are located in similar positions relative to the corresponding transcription initiation sites. This evolutionary conservation between the species led us to postulate that the G-box may be involved in the regulation of the Sbe1 gene expression, possibly in response to one of the environmental or physiological stimuli, even though we failed to show the importance of the G-box in Sbe1 promoter activity using the 5′ deletion analysis. It is possible that a G-box-dependent mechanism controlling Sbe1 promoter activity could not be appraised in our suspension-cultured endosperm cells. This hypothesis is supported by the results showing interaction of the G-box with mEmBP-1 protein in vitro and repression of the Sbe1 promoter activity by overexpression of mEmBP-1 (Fig. 7).

Additionally, the finding that disruption of the G-box sequence (CCACGTGG) in pKLN105 did not cause a reduction in promoter activity (data not shown) led us to speculate that the G-box and its binding proteins are involved in down-regulation of the Sbe1 gene expression rather than up-regulation. Although a specific role for the G-box motif in Sbe1 gene expression has not been identified, there is a possibility that the G-box in the Sbe1 promoter may play a critical role under different environmental conditions or in different tissues.

It has been noted that mutations decreasing starch accumulation in maize endosperm also reduce storage protein synthesis, implying possible interactions between these pathways (Barbosa and Glover, 1978; Tsai et al., 1978). Giroux et al. (1994) showed that mutations affecting synthetic events in one biosynthetic pathway affect the expression of genes in both pathways, and demonstrated that the expression of genes involved in starch and storage protein synthesis of the maize endosperm are coordinately regulated. Elevation in sugar concentration or alteration of the osmotic potential of the endosperm was proposed to be a possible candidate for the primary signal triggering this coordinate expression. In this context, knowledge of the Sbe promoter elements and their associated regulatory proteins may eventually lead to a better understanding of the regulatory mechanisms controlling all of the starch biosynthetic genes and the genes encoding storage proteins in maize endosperm.

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