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Molecular cloning and characterization of the *Amylose-Extender* gene encoding starch branching enzyme IIB in maize

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Abstract

The *amylose-extender* (*Ae*) gene encoding starch-branching enzyme IIb (SBEIIb) in maize is predominantly expressed in endosperm and embryos during kernel development. A maize genomic DNA fragment (−2964 to +20 485) containing the *Ae* gene was isolated and sequenced. The maize *Ae* mRNA is derived from 22 exons distributed over 16 914 bp. Twenty-one introns, differing in length from 76 bp to 4020 bp, all have conserved junction sequences (GT··AG). Sequence analysis of the 5′- and 3′-flanking regions revealed a consensus TATA-box sequence located 28 bp upstream of the transcription initiation site as determined by primer extension analysis, and a putative polyadenylation signal observed 29 bp upstream of the polyadenylation site based on cDNA sequence. Genomic Southern blot analysis suggests that a single *Ae* gene is present in the maize genome. Promoter activity was confirmed by testing a transcriptional fusion of the *Ae* 5′-flanking region between −2964 and +100 to a luciferase reporter gene in a transient expression assay using maize endosperm suspension cultured cells. 5′ deletion analysis revealed that the 111 bp region from −160 to −50 is essential for high-level promoter activity.

Introduction

Starch provides carbon and energy for vegetative and reproductive development of most higher plants. It is found as a water-insoluble granule which is mainly composed of two different polysaccharides, amylose and amylopectin [49]. Amylose is considered to consist of linear α-1,4-linked glucose chains about 1000 residues long. However, amylopectin is a more highly branched macromolecule consisting of linear α-1,4-glucose chains with α-1,6-glucosidic bonds at branch points.

It was generally thought that starch is synthesized in higher plants through the sequential action of three classes of enzymes, ADP-glucose pyrophosphorylase (EC 2.7.7.23), starch synthase (EC 2.4.1.21) and starch-branching enzyme (E. 2.4.1.28). However, recent analysis of the *sugary1* (*su1*) mutants of maize has demonstrated that starch-debranching enzyme (EC 3.2.1.41) is also involved in starch biosynthesis [25, 36]. It appears that the final branching structure of amylopectin is determined at least in part by a balance between the activities of debranching and branching enzymes.

Starch-branching enzymes (SBE) catalyze the formation of α-1,6 glucan linkages, thereby playing an important role in the synthesis of the amylopectin fraction of starch. This reaction involves two steps with the hydrolysis of an internal α-1,4 linkage and the reattachment of the released 1,4-glucan chain to the remaining or to another 1,4-glucan chain by a α-1,6 bond. Multiple forms of SBE differing in enzymatic and biochemical properties have been identified and characterized in various plants [30, 37]. They were grouped into two distinct families based on their structural relatedness and named according to the prototypic family member from maize, SBEI and SBEII [7, 18].

Three SBE isoforms, SBEI, SBEIIa and SBEIIb, have been resolved in maize endosperm by anion-exchange chromatography [3, 4]. Enzyme assays showed that SBEI has a lower K_m for amylose

and preferentially transfers longer glucan chains than SBEIIa and SBEIIb [22, 48]. The different catalytic properties of SBE isoforms led Guan and Preiss [22] to suggest that SBEIIa and SBEIIb use the slightly branched polysaccharides produced by SBEI as substrates for further branch formation. SBEI was also found to be distinct from SBE IIa and SBEIIb in immunological properties, molecular weight, amino acid composition, and proteolytic digestive maps. While SBEIIa and IIb did not exhibit significant differences in immunological and biochemical properties [3, 16, 46], they showed distinguishable enzyme activities in the branching linkage assay [48]. SBEIIa had more than 2-fold greater specific activity than SBEIIb, and the optimum temperatures are quite different, 25 °C and 15–20 °C, respectively.

Fisher *et al.* [14] isolated a full-length *Ae* cDNA by probing a maize endosperm library with the pea *rugosus* (*r*) gene which is known to be a structural gene for pea SBEI [1]. The *r* gene, which is one of the characters used by Mendel in his study on the laws of inheritance, determines the wrinkled versus round phenotype of mature seeds. In addition, Stinard *et al.* [47] cloned a dominant mutant allele of the amylose-extender (*ae*) locus through *Mutator1* (*Mu1*) transposon tagging. SBEIIb activity is absent from maize kernels containing the dominant *ae* mutant allele. Sequence comparison of the *ae* clone with *Sbe2b* cDNA showed near perfect identity, confirming that the SBEIIb is encoded by the *ae* gene. Later, a cDNA encoding maize SBEIIa was also isolated and shown to be distinct from *Sbe2b* (*Ae*) [19].

Recent studies have shown that the *Sbe* genes are differentially expressed in various tissues, suggesting that they may play distinct roles in starch biosynthesis [18, 19]. For example, while *Sbe1* and *Sbe2a* are expressed in vegetative tissues, *Ae* is not. Unlike *Sbe1* and *Sbe2b*, *Sbe2a* is more highly expressed in embryos than in endosperm. To study the regulatory mechanisms involved in expression of SBE isoforms during maize development, we have begun to characterize their genes. We previously reported the isolation and characterization of a full-length *Sbe1* genomic clone containing the entire coding region of *Sbe1* as well as 5′- and 3′-flanking sequences (Kim *et al.*, in press). We now describe the complete genomic organization of the *Ae* gene and the promoter regions critical for its expression in maize endosperm cells.

Materials and methods

Maize genomic library screening and DNA sequencing

Using ^{32}P-labeled full-length *Ae* cDNA [14] as probe, an EMBL-3 genomic library (Clonetech, Palo Alto, CA) prepared from maize seedlings (B73, 2-leaf stage) was screened essentially according to Sambrook *et al.* [39]. About 3×10^5 plaque-forming units were transferred onto nylon membranes (Hybond-N$^+$, Amersham, UK). Hybridization was performed at 55 °C for 20 h in a solution containing 0.5 M Na_2HPO_4 pH 7.2 and 7% SDS [10] with gentle agitation at 40 cycles per minute on a rotary shaker. After the hybridization, filters were washed twice in 5% SEN (5% w/v SDS, 1 mM EDTA, 0.04 M Na_2HPO_4 pH 7.2) and once in 1% SEN (1% SDS, 1 mM EDTA, 0.04 M Na_2HPO_4 pH 7.2) for 15 min at 65 °C. Plaques strongly hybridizing to the probe were selected and purified through three rounds of screening. Phage DNAs were isolated from the positive plaques according to Chisholm's method [9] and *Sal*I-digested inserts were subcloned into pBuescript SK$^-$. DNA sequences were determined by the dideoxynucleotide chain termination method [40] with Sequenase Version 2.0 (United States Biochemical, Cleveland, OH). Sequence analyses were performed using programs from DNASTAR (Madison, WI).

Primer extension analysis

To localize the transcription initiation site of the *Ae* gene, an oligonucleotide, 5′-GATCGGATCGAACTGATCAG-3′, which is complementary to the sense strand sequence of the *Ae* cDNA from −35 to −16 relative to the translation start site (ATG) was radiolabeled at its 5′ terminus with T4 polynucleotide kinase and γ-^{32}P-ATP [39]. About 10^5 cpm of the labeled primer was hybridized at 35 °C with 10 μg of total RNA, which was isolated from 30 DAP (days after pollination) maize kernels (B73) according to the protocol of Vries *et al.* [53]. After hybridization for 8 h, complementary DNA was synthesized from the annealed primer by the addition of reverse transcriptase and dNTP. After the addition of EDTA and RNase A into the reaction, the nucleic acid was precipitated with ethanol. The reaction products were resuspended in sequencing gel loading buffer, denatured at 95 °C, electrophoresed through a 5% w/v polyacrylamide sequencing gel, and visualized by autoradiography. In order to provide size markers, part of the *Ae* gene was

sequenced with the same primer used in the primer extension experiment.

Genomic Southern blot analysis

Maize genomic DNA was prepared from 7-day-old etiolated seedlings (inbred B73) according to the method described by Junghans *et al.* [28]. 10 μg of genomic DNA was digested with restriction enzymes, separated on 0.8% agarose gels, and transferred onto nylon membranes (Hybond-N, Amersham, UK) in 20× SSC containing 3 M NaCl and 0.3 M sodium citrate, pH 7.0, according to Sambrook *et al.* [39]. DNA was crosslinked to the membrane by 3.5 min of UV irradiation on a transilluminator (312 nm). Genomic blots were prehybridized at 65 °C for 1 h in 0.5 M Na_2HPO_4 pH 7.2, 7% SDS, and 100 μg/ml denatured salmon sperm DNA. 25 ng of a full-length *Ae* cDNA [14] was labeled with [α-^{32}P]-dCTP using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Labeled probe was added to the prehybridization solution and incubated at 65 °C for 18 h. Blots were washed twice in 5% SEN and once in 1% SEN for 15 min at 65 °C and were exposed to Kodak X-AR film at −80 °C for 48 h using two intensifying screens.

Construction of plasmids

For a transcriptional fusion of the *Ae* promoter to a luciferase (LUC) reporter gene, a *Bam*HI restriction enzyme site was created just before the translation initiation site (ATG) of the *Ae* gene as follows. The DNA sequence between −14 and +100 of the *Ae* gene was first amplified via polymerase chain reaction. The 5′ primer (PII-2), 5′-CCTAATTGTAGCCCTGCAGTCA-3′, is homologous to sequence of the *Ae* gene from −10 to +12. A *Pst*I site (CTGCAG) is located immediately downstream of the 5′ primer binding region of the *Ae* promoter, +4 to +9. The 3′ primer (PII-3), 5′-GACTGGATCCTCGCCTTCGCAGCCGGATCG-3′, consists of a DNA sequence complementary to that of the *Ae* gene from +80 to +100 and a *Bam*HI restriction enzyme site (GGATCC) flanked with four random nucleotides (underlined). The PCR product was digested with *Pst*I and *Bam*HI, and the resulting 100 bp fragment was ligated to the 2977 bp *Sal*I-*Pst*I *Ae* promoter fragment and cloned into plasmid pLN cut with *Sal*I and *Bam*HI (promoterless LUC-NOS gene in pUC119) [33], thereby creating plasmid pKL201. This construct as well as all the following constructs were verified by DNA sequencing.

To construct a translational fusion of the *Ae* promoter containing the first exon and intron to a LUC reporter plasmid, the *Ae* genomic clone, 3-2-1, was digested with *Xho*I and the resulting 866 bp fragment was gel-purified. The fragment was blunt ended by Klenow fill-in DNA synthesis and ligated with *Bam*HI linkers (CGGGATCCCG). After complete digestion with *Pst*I and *Bam*HI, the 325 bp DNA fragment was isolated and used to replace the 100 bp *Pst*I-*Bam*HI region in pKL201. This construct was designated pKLN201.

A series of 5′ deletion mutants were derived from pKL201 using available restriction enzyme sites and PCR techniques. To create pKL202, pKL203, pKL205 and pKL206, pKL201 was first digested with *Acc*I (−1719 to −1714), *Spe*I (−1128 to −1123), *Xho*I (−537 to −532) and *Apa*I (−348 to −343), respectively. Then, each linearlized plasmid was separately gel-purified and blunt-ended by Klenow fragment. After *Sal*I linker ligation, the modified plasmids were digested with *Sal*I, and the larger DNA fragments from each reaction were isolated and self-ligated to produce the relevant plasmids carrying different deletion end points.

For a construction of pKL204, the *Ae* promoter region between −755 and −500 was amplified with two primers. The 5′ primer containing a *Sal*I (GTCGAC) flanked with four extra nucleotides (underlined), 5′-GAAAGTCGACGAAGAGAGAATGAAAGCGAA-3′, and the 3′ primer, 5′-GCGCGGGTCCGTCGTGCCTTTT-3′, were designed to anneal to DNA sequences of the *Ae* gene from −755 to −736 and from −521 −500, respectively. The amplified 266 bp product was digested with *Sal*I and *Xho*I which was located 10 bp upstream of the 3′ primer binding region, and the resulting 230 bp fragment was used to substitute the 2.4 kb *Sal*I-*Xho*I fragment in pKL201.

To make pKL207, the *Ae* promoter region between −160 and +100 was first amplified with 5′ primer, 5′-GATAGTCGACCGACGCGCAACGGCCTGCCT-3′, and 3′ primer, 5′-GACTGGATCCTCGCCTTCGCAGCCGGATCG-3′, which contain *Sal*I and *Bam*HI sites, respectively, along with four arbitrary extra bases (underlined). Next, the PCR product was digested with *Sal*I and *Bam*HI and the resulting 267 bp DNA fragment was then used to replace the 3.1 kb *Sal*I-*Bam*HI fragment in pKL201. The same 3′ primer and method were used for a construction of pKL208 except for the 5′ primer, 5′-GA-

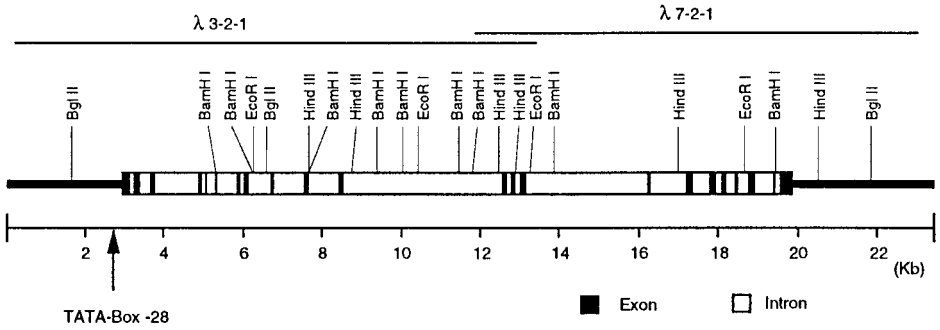

Figure 1. Genomic structure of the *Ae* gene. The complete structure of the *Ae* gene was constructed using two overlapping genomic clones: λ 3-2-1 and 7-2-1. The thick black lines indicate the 5′- or 3′-flanking sequences of the *Sbe1* gene. The solid black boxes indicate exons and the open boxes denote introns. The position of a putative TATA-box relative to the transcription initiation site (+1) is indicated.

TCGTCGACCGCTCGTCTCCGTCCTATAT-3′, homologous to the DNA sequence of the *Ae* gene from −49 to −32.

Particle bombardment

Suspension culture cells of maize (imbred A636) endosperm 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 [35] supplemented with 0.4 mg/l thiamine, 2 g/l asparagine and 30 g/l sucrose [44]. The culture was maintained in the dark at 29 °C on a rotary shaker (120 rpm) and was subcultured every 7 days by transferring a spoonful of the cell suspension into 80 ml of fresh medium.

For particle bombardment, about 600 mg (fresh weight) of actively growing cells 3 days after subculture was evenly distributed over the surface of filter paper (Whatmann #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 (Whatmann #4, 70 mm in diameter) moistened with 5 ml of the liquid medium containing 12% sucrose and positioned in the middle of a 10 cm Petri dish.

A 60 mg portion of gold microcarriers (1.6 μm particle size) was washed three times with 1 ml of 100% ethanol and twice with 1 ml of sterile deionized H_2O, resuspended in 1 ml of sterile deionized H_2O, and dispensed in 50 μl aliquots (3 mg/50 μl). The *Sbe1* promoter-LUC constructs and a GUS reference plasmid (pBI 221; Clontech, Palo Alto, CA) were co-precipitated onto 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, 4 μg of GUS reference plasmid), 50 μl of 2.5 M $CaCl_2$, and 20 μl of 0.1 M 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% ethanol, and resuspended in 60 μl of 100% ethanol. Immediately after sonication, 8 μl of the DNA-coated gold particles were pipetted onto the center of macrocarriers (BioRad, Hercules, CA) and dried in a low-humidity environment.

A BioRad PDS-1000/He Biolistic Particle Delivery system was used for particle bombardment. Bombardment parameters which were optimized include He pressure, gap distance (distance from power source to macroprojectile), and target distance (distance from microprojectile launch site to sample target). After optimization, all bombardments were performed in a dim room at 4.5 MPa under a vacuum of 88 kPa with a distance of 10 cm between the cells and the barrel of the particle gun. After the bombardments, the Petri dishes were sealed with Parafilm and then 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 pestle and mortal to a fine powder. The powder was then transferred into a microfuge tube and extracted with cell culture lysis buffer containing 300 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM, 1,2-diaminocyclohexane-N, N, N′, N′-tetraacetic acid, 10% glycerol and 1% Triton X-100 (0.3 ml/g of tissue). Cell debris was 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 [27], 30 μl of the crude extract was incubated at 37 °C with 2 mM 4-methylumbelliferyl glucuronide (MUG) in 0.3 ml of GUS assay buffer (50 mM $NaPO_4$ pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10 mM 2-mercaptoethanol, 20% 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 Na_2CO_3 to terminate the reaction. A TKO 100 fluorometer (Hoeffer, San Francisco, CA) calibrated by setting a 100 nM MU to 1000 fluorescence units was used to measure fluorescence of the product, 4-methylumbelliferone (4-MU). For each sample, results of GUS assay were plotted in a graph of OD_{405} (Y-axis) versus time in minutes and the GUS activity was expressed simply as the slope of the line. GUS activity from the maize endosperm suspension cells that had been bombarded with the naked gold particles (no DNA) was used as a control.

Using a luminometer (Monolight 1500; Analytical Luminescence Laboratory, San Diego, CA), luciferase activity was determined by measuring luminescence for 10 s after mixing 20 μl of cell extract with 100 μl of luciferase assay reagent containing 20 mM tricine pH 7.8, 1.07 mM $(MgCo_3)_4Mg(OH)_2{\cdot}5H_20$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP. LUC activity from the maize endosperm suspension cells that had been bombarded with the pLN (promoterless LUC plasmid) was used as a control. To correct differences in sample variability and transfection efficiency, the luciferase activity (in light units) was normalized with GUS activity, yielding the LUC/GUS ratio of each sample.

Results

Cloning and characterization of the Ae *gene*

After screening ca. 3×10^5 plaque-forming units of a genomic library prepared from maize seedlings (inbred B73), 12 lambda clones that strongly hybridized to the full-length *Ae* cDNA probe were isolated. These clones were hybridized to 5′ or 3′ cDNA fragment probes revealing that none of them contained both ends of the *Ae* gene. Based on restriction endonuclease maps, two clones, λ3-2-1, and λ7-2-1, were selected, subcloned, and sequenced. DNA sequences of the two clones revealed that the λ3-2-1 clone containing the 5′ end of the *Ae* gene had ca. 1.5 kb overlap with the λ7-2-1 clone containing the 3′ end of the gene. A complete restriction map of the *Ae* gene was constructed by combining the two overlapping genomic clones which encompasses the entire coding region of the gene as well as large regions of 5′- and 3′-flanking sequences (Figure 1).

Genomic organization of the Ae *gene*

Primer extension analysis was conducted to determine the transcription initiation site of the *Ae* gene (Figure 2). A single major reverse transcription product was observed which co-migrated with a G residue in the sequencing ladder, indicating the transcription initiates mainly at a position which is located 28 bp downstream from a putative TATA box. The transcription initiation site was numbered +1 in the sequence shown in Figure 3. In addition to the TATA box, a number of potential *cis*-regulatory elements were found in the 5′-flanking region of the *Ae* gene (Figure 3). The proximal promoter region from −300 to −87 contains two MRE boxes (TGCRCNC, R = purine, Y = pyrimidine), motifs essential for metal ion-dependent induction of both mouse and human metallothionein genes [50]. In addition, this region contains four GC boxes (CCGCCC), sequences recognized by the mammalian transcription factor Sp1 [32]. In a region further upstream, sequences identical to a Hex (ACGTCA), a conserved element found in plant histone gene promoters [31]; an I box (GATAGG), an element conserved in various *RbcS* genes encoding the small subunit of ribulose Rubisco [21]; and a RY repeat (ATGCCATG), a distal regulatory element which comprises a portion of the 28 bp legumin box [12], were present at positions −999, −647, and −1491, respectively. Interestingly, DNA sequences from −505 to +463 are extremely high in G+C content (66.5%) and CpG dinucleotide frequency (10.1 per 100 bp) compared to an average of 40% G+C and 3.2 CpG per 100 bp for the rest of the genomic fragment. This region includes the proximal promoter region, two exons, and one intron. These characteristics are typical of CpG islands found in the mammalian genome, which are usually unmethylated and flanked by methylated regions [2].

The genomic structure of the *Ae* gene was established by alignment with the published sequence of *Ae* cDNA [14]. The transcribed region of the gene consists of 22 exons and 21 introns distributed over 16 914 bp in length. Figure 1 summarizes the organization of the *Ae* gene. The published cDNA sequence is identical to the corresponding genomic sequence except for three nucleotides present in exon 4 and exon

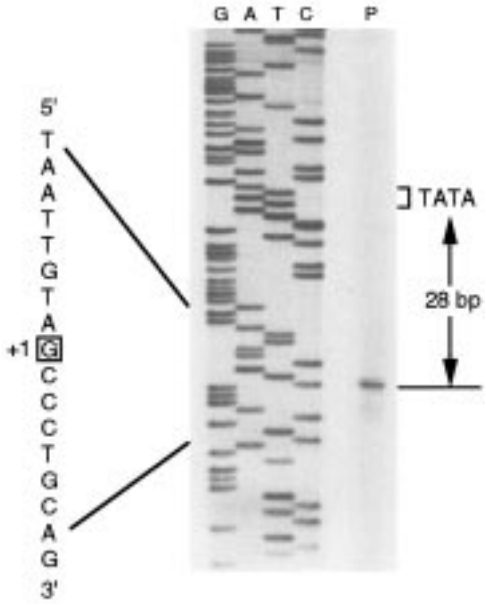

Figure 2. Primer extension analysis of the transcription initiation site of the *Ae* gene. A ^{32}P-end-labeled antisense primer was annealed to 10 μg of total RNA isolated from maize kernels (30 DAP) and extended using reverse transcriptase. The extended cDNA product was then analyzed on a 5% sequencing gel (lane P) along with a *Ae* 5′-flanking genomic DNA sequencing ladder (G, A, T, C) generated by the same primer. The number inside the vertical double-headed arrow on the right refers to distance from TATA box to the primer extension product indicated by the horizontal arrow. The sense sequence around the product is shown on the left and the transcription initiation site is boxed and assigned +1.

6. This may be due to the different genetic stocks used in the two studies. Table 1 shows the sequences around the exon/intron junctions and a list of putative branch point consensus sequences, which were derived as described by Brown [6]. The introns are relatively AT-rich (61%) compared to the exons (54%), and all have the conserved splice site sequences at their 5′ and 3′ ends, following the 'GT...AG' rule of plant introns [6]. The introns vary in length from 76 bp (intron 4) to 4020 bp (intron 11), and the exons vary in length from 43 bp to 303 bp. Exon 1 contains 100 bp of 5′-untranslated DNA sequence, and exon 22 contains the translation stop codon (TGA) and 3′-untranslated region. Although the canonical polyadenylation signal, AATAAA, was not found in the 3′ end of the gene, a similar sequence (AATTAAA) was observed 29 bp upstream of the polyadenylation site.

In addition, sequence analysis revealed that the 3′-flanking region of the *Ae* gene contains many direct repeat sequences and has a high degree of similarity to the pollen retroelement maize-2 (PREM-2), a copia-type retroelemt in maize which is expressed in a tissue-specific manner (Figure 3). A 13 bp polypurine tract (AAAAAGGGGGAGA) which is present in PREM-2 and necessary for retroelement replication was also found upstream of the region which is very similar to the 3′ long terminal repeat (LTR) of the PREM-2 [51]. Thus, the 3′-flanking region is likely to possess part of a PREM-2-type retroelement.

Genomic Southern blot analysis

Southern blot analyses were performed to determine the number of genes in the maize genome that are similar to *Ae*. When blots were probed with the full-length *Ae* cDNA [14] under high-stringency conditions, at least two strongly hybridizing bands were observed in each lane (Figure 4A). The band patterns agreed with the restriction map of the *Ae* genomic DNA, suggesting all the bands were derived from a single genetic locus. To confirm this, the blots were probed with a small fragment of the *Ae* genomic DNA which does not have any restriction enzyme sites for *Bam*HI, *Eco*RI, *Bgl*II and *Hin*dIII. As expected, only a single band was observed in every lane (Figure 4B), supporting the conclusion that a single copy of *Ae* is present in the maize genome.

Analysis of the 5′-flanking region of the Ae *gene*

To identify the 5′-flanking regions necessary for *Ae* gene expression, we utilized a transient expression assay system in maize endosperm suspension cells. Iodine staining and northern blot analysis showed that the maize endosperm suspension cells actually produce starch and the genes involved in starch biosynthesis are expressed (data not shown). In an initial experiment, a transcriptional chimeric construct containing the *Ae* gene fragment between −2964 to +100 linked to a luciferase (LUC) reporter gene in pUC119 was created and called pKL201. Since there are several examples of plant genes which are regulated by the first exon and/or intron sequences [8, 11, 17], a translational fusion construct (pKLN201) containing the corresponding region of the *Ae* gene was also created to test its effect on gene expression. pKLN201 was created by including an additional *Ae* DNA sequence from +101 to +329 in the plasmid pKL201. These plasmids were then tested by assaying LUC activity after introduction of DNA into maize endosperm suspension cells by particle bombardment. Plasmid pBI221 containing the CaMV 35S promoter linked to a GUS gene was used as an internal control to

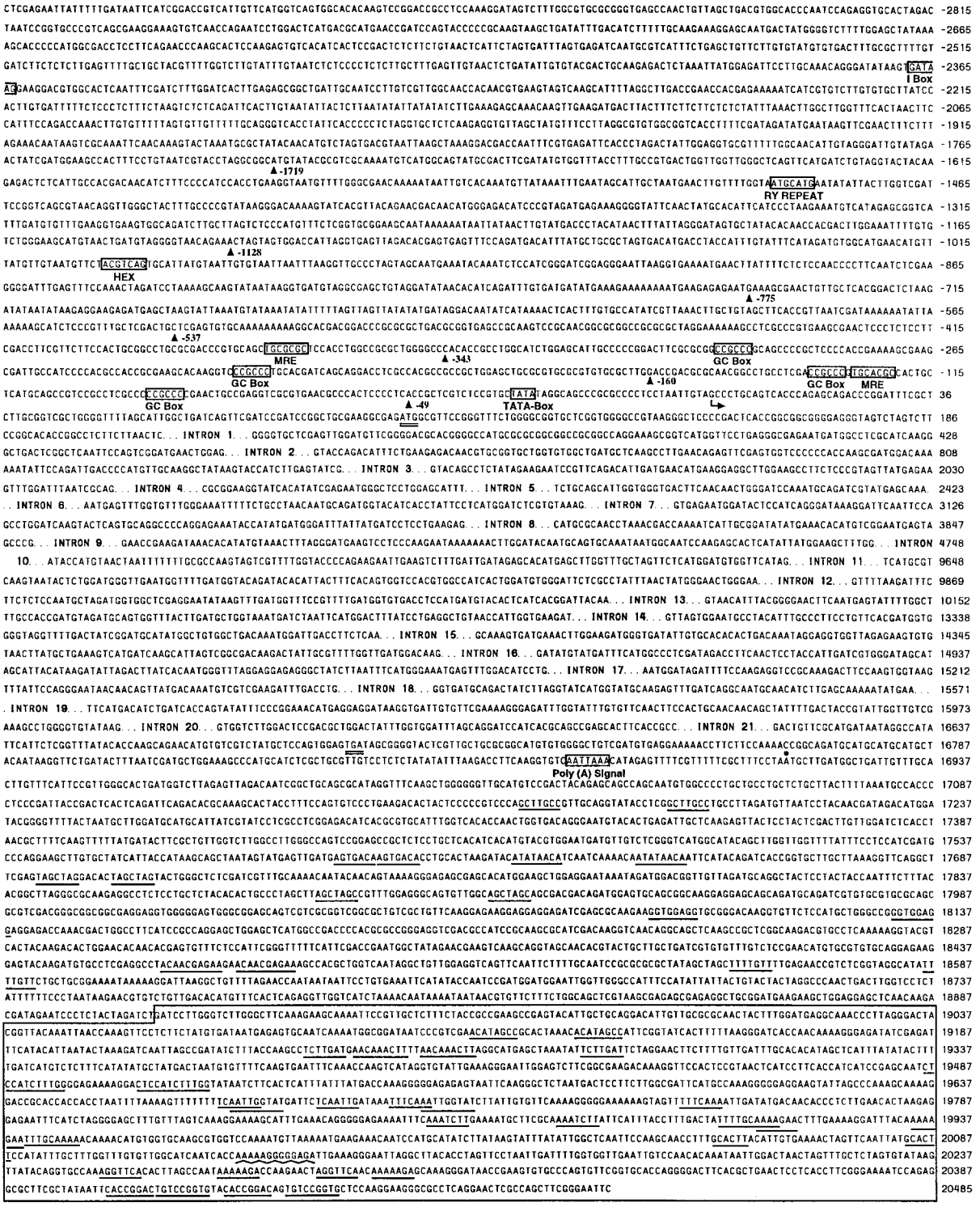

Figure 3. Nucleotide sequence of the *Ae* gene and 5′- and 3′-flanking regions. Numbers indicate the distance relative to the transcription start site (+1) which is indicated by the arrow. Intron sequences are omitted. Putative *cis* elements are indicated below the sequence and boxed. The end points of the 5′ deletion constructs were indicated by arrowheads below the first nucleotides of the deletions. Direct repeat sequences are underlined. The translation initiation codon (ATG) and the translation termination codon (TGA) are double-underlined. The dot indicates the polyadenylation site. The region exhibiting 80% sequence similarity with the PREM-2 internal and 3′ LTR region is boxed. The wavy line represents polypurine tract.

Table 1. List of introns and sequences of exon/intron borders in the *Ae* gene.

Intron number	Exon/	Putative intron branch point[a]	/Exon	Intron size (bp)	GC content (%)
1	ACTC	GTAA.....GTGAA..24[b].GCAG	GGGG	106	51.9
2	GGAG	GTTC.....CTGAA..29..CCAG	GTAC	244	41.4
3	ATCG	GTAT.....TTCAA..21..ACAG	GTAC	1086	43.9
4	GCAG	GTAT.....ATAAC..24..GTAG	CGCG	76	25.0
5	ATTT	GTAT.....TTCAG..25..TTAG	TCTG	196	31.1
6	CAAA	GTAT.....CTAAA..22..GCAG	AATG	499	33.5
7	AAAG	GTAG.....TTAAC..23..ATAG	GTGA	81	40.7
8	AAGA	GTCT.....TTAAG..21..GCAG	GTAA	567	30.3
9	CCCG	GTAT.....ATAAT..23..TTAG	GAAC	774	32.0
10	TTGG	GTAA.....TTAAT..21..GCAG	ATAC	751	33.2
11	ATAG	GTAA.....TTAAC..22..GCAG	TCAT	4020	44.0
12	GGAA	GTAC.....TTTAT..49..GCAG	GTTT	86	34.9
13	ACAA	GTAA.....CTAAA..19..TTAG	GTAA	148	31.1
14	GTAA	GTGC.....TTCAA..20..TCAG	GTTA	3051	41.8
15	TCAA	GTAA.....TTCAA..19..ACAG	GCAA	872	31.8
16	CAAG	GTTA.....ATGAG..25..GCAG	GATA	457	32.6
17	CCTG	GTGA.....GTCAT..21..GCAG	AATG	144	30.6
18	CCTG	GTAA.....ATTAT..28..TCAG	GGTG	226	30.1
19	TGAA	GTAT.....ATGAA..19..GCAG	TTCA	266	31.2
20	TAAG	GTAT.....CTGAC..21..CCAG	GTGG	448	40.0
21	CGCC	GTAA.....CTAAC..24..GCAG	GACT	96	45.8

[a] Consensus sequences between introns are underlined.
[b] Numbers indicate number of nucleotides between adjacent sequences.

correct for transfection efficiency [26]. The results showed that levels of LUC expression driven by the two constructs were almost the same (data not shown), suggesting that the first exon and intron region of *Ae* is not necessary for high-level gene expression in maize endosperm cells.

To define the promoter sequences important for *Ae* gene expression in maize endosperm cells via transient expression assays, a series of 5′ deletion mutants were derived from the plasmid pKL201 using available restriction enzyme sites and PCR techniques (Figure 5A, B). The activity of each 5′ deletion construct is presented in Figure 5C. Two consecutive deletions of the *Ae* 5′-flanking sequence down to −1128 decreased the level of the LUC expression to ca. 40% of the full-length promoter level. However, the removal of an additional 353 bp, to −775, restored LUC activity (Figure 5C). This suggests that potent positive and negative distal *cis*-regulatory elements may be located in the regions from −2964 to −1129 and from −1128 to −776, respectively. Although further deletions down to −160 did not significantly change the levels of LUC expression, a dramatic reduction in the promoter strength was observed when an additional 111 bp, to −49, was deleted. This indicates that very strong positive regulatory element(s) are located at the 111 bp region from −160 to −50. The presence of two GC boxes and one MRE motif in the region suggests the possibility that these conserved motifs may actually act as *cis*-regulatory elements essential for gene expression in maize endosperm cells.

Discussion

Analysis of the amylose-extender (*ae*) mutants in maize endosperm demonstrated the importance of SBEIIb activity in starch biosynthesis [20, 24, 34, 47]. The typical phenotype of *ae* maize mutants is a glassy, tarnished endosperm containing reduced amounts of starch with a higher proportion of amylose (up to 70%) [43]. In addition, *ae* mutants synthesize an amylopectin with a longer than average chain length (fewer branch points) in the endosperm. Endosperm extracts from the *ae* mutant contained no detectable amounts of SBEIIb, while SBEI and SBEIIa activities remained

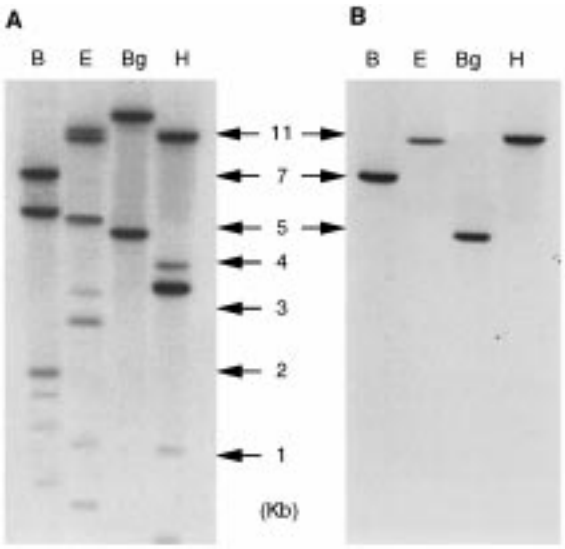

Figure 4. Southern blot analysis of maize genomic DNA. Each lane contains 10 μg of maize genomic DNA digested with the indicated restriction enzymes: B, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII. Genomic DNA was prepared from etiolated maize seedlings (inbred B73). Probes were prepared using the random primed DNA labeling kit (Boehringer Mannheim) and [α-^{32}P]dCTP. Hybridization and washes were performed at high-stringency conditions. Arrows indicate the position of the DNA size markers in kilobases. **A.** The full-length *Ae* cDNA was used as a probe. **B.** The 326 bp *Ae* genomic DNA fragment containing the first exon and intron region was used as a probe.

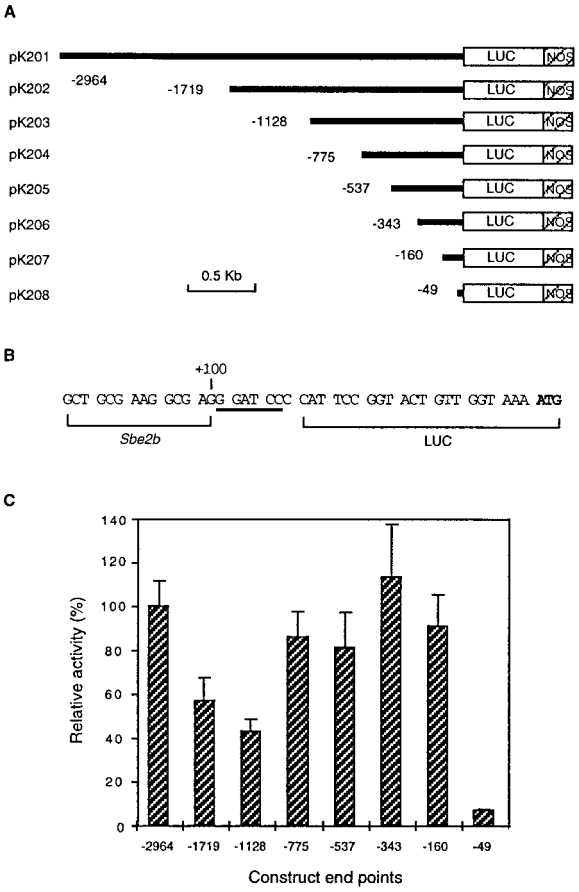

Figure 5. Schematic diagram of the 5′ deletion chimeric constructs. **A.** The thick black lines denote the *Ae* promoter sequences. The numbers at left indicate deletion-end points relative to the transcription initiation site (+1) of the *Ae* gene. The open and stripped boxes indicate luciferase gene and nopaline synthase 3′-end sequences, respectively. **B.** The junction sequences between the *Ae* gene and LUC. The *Bam*HI site used to join the two genes is underlined. The translation start site of *LUC* is indicated by boldface letters. **C.** Effect of 5′ deletions on *Ae* promoter activity. The relative activity values of the constructs are percentages of pKL201 level. Each value represents the average of three independent shootings. Error bars indicate standard errors of the means.

unaltered in the mutant [5]. Hedman and Boyer [23] also demonstrated that as the number of the functional *ae* alleles increases, SBEIIb activity increases almost linearly without affecting SBEI and SBEIIa activities. Despite these genetic data suggesting that SBEII and SBEIIb are encoded by two separate genes, it remained controversial as to whether they are the products of one or two genes.

According to the single-gene hypothesis which mainly relies on the similarities in immunological and biochemical properties between SBEIIa and IIb, the differences in the two isoforms are the result of post-transcriptional or post-translational modification of the *Sbe2a/2b* transcript or protein [37, 46]. Recently, however, two reports demonstrated that SBEIIa and SBEIIb are encoded by separate genes in maize endosperm. First, *ae*-B1 endosperm containing SBEIIa enzymatic activity was shown to have a virtually undetectable level of the SBEIIb transcript by northern blot analysis [15]. Second, a maize cDNA which has a DNA sequence distinct from *ae* cDNA was isolated and shown that the predicted amino acid sequence of the cDNA matches the N-terminal sequence of SBEIIa protein purified from maize endosperm [19]. Now, determination of the entire genomic sequence of the *Ae* gene in the present study allowed us to provide definitive evidence for independent genetic control of the SBEIIa and SBEIIb in maize endosperm cells. Comparison of the *Ae* genomic sequence with the recently cloned maize *Sbe2a* cDNA [19] indicated that the *Sbe2a* cDNA is not an alternatively spliced product of the *Ae* gene, supporting the conclusion that maize SBII and SBEIIb are the products of distinct genes.

We have established the complete genomic organization of the *Ae* gene, which has been previously mapped on the long arm of chromosome 5 [42]. Primer extension analysis demonstrated that transcription initiates predominantly at a nucleotide 100 bp upstream of the translation start site, 28 bp downstream from a consensus TATA-box. It is noteworthy that the 5′ end of the *Ae* gene from −505 to +463 contains a region resembling a mammalian CpG island in terms of G+C content, CpG frequency, position, and size. Typically, CpG islands are about 1 kb long and span from the proximal promoter region to the second exon [2].

Alignment of the genomic sequence with the *Ae* cDNA [14] revealed that poly(A) addition occurs at a position 219 bp downstream of the translation stop codon (TGA). The transcribed region of *Ae*, which consist of 22 exons and 21 introns, is 16 914 bp in length, almost three times longer than that of the *Sbe1* gene (Kim *et al.*, in press). Interestingly, part of the 3′-flanking region of the *Ae* gene is rich in repetitive DNAs and similar to the PREM-2 retroelement, suggesting the presence of a retroelement. At present, however, it is not yet clear whether the region is part of a larger moveable element or solo LTR like *Cin1* [45]. It has been known that most of the maize genes are flanked with retroelements which make up at least 50% of the nuclear DNA [41].

The coding regions of the genes *Sbe1* and *Ae* have ca. 60% similarity at the DNA level. Nevertheless, the genomic organization of the *Ae* gene was very different from that of the maize *Sbe1* gene (Kim *et al.*, in press), suggesting that the two genes are not the result of a recent gene duplication. The *Ae* gene is interrupted with almost twice as many introns as the *Sbe1* gene, and the total length of introns (14 194 bp) of the *Ae* gene is five times more than that of the *Sbe1* gene (2796 bp). Interestingly, this is contrasted by the interspecies conservation of the maize and rice *Sbe1* genes which have nearly identical genomic organization consisting of 14 exons and 13 introns, the positions of which are evolutionarily conserved. This together with other data also suggests that the SBEI and SBEII families evolved before monocots and dicots diverged.

Comparison of the maize *Sbe1* and *Ae* 5′-flanking sequences reveals little similarity, which possibly accounts for the different expression patterns of the two genes [18]. *Sbe1* is expressed in most maize tissues or organs including endosperm, embryos, leaf, stem, root and tassel, while *Ae* is expressed only in endosperm and embryos as well as young tassel. Additionally, the *Sbe1* and *Ae* genes are differentially expressed during kernel development. The timing of the highest transcript level of *Ae* comes prior to that of *Sbe1* in developing endosperm and embryos, which may result in changes in the SBEI/SBEIIb ratio. Since SBEI and SBEIIb have significantly different *in vitro* catalytic properties as mentioned before, such changes in the SBEI/SBEIIb ratio may cause differences in the starch synthesized during kernel development.

Transient expression assays of the *Ae*-LUC chimeric constructs showed that the 5′-flanking 3.0 kb region of the *Ae* gene is sufficient to initiate transcription in maize endosperm cells. Unlike the *Sbe1* gene, however, the DNA region containing the first exon and intron of the *Ae* gene does not have the ability to increase the level of gene expression. Among the possible *cis*-regulatory elements identified by 5′ deletion analysis, the 111 bp region between −160 and −50 relative to the transcription initiation site appears to be the most important. Removing this region almost abolished promoter activity. This is probably due to loss of two GC boxes and/or one MRE motif present in the region. In mammalian cells, these motifs were known to interact with transcription factors and mediate regulation of transcription [13, 29, 54].

Genomic Southern analysis indicated that a single *Ae* gene is present in the maize genome. This is probably one of the reasons why mutants devoid of SBEIIb activity have been identified. However, no mutants lacking either SBEI or SBEIIa activity have been reported so far. Possible explanations for this situation are that unlike *Ae*, more than one genes encoding each SBEI and SBEIIa may be present in the maize genome and/or their roles in starch biosynthesis can be compensated for by other SBE isoforms. Alternatively, such mutations may be lethal.

The complete genomic organization of the *Ae* gene established in this study broaden our understanding of the nature of *ae* mutations. In a study of the dominant *Ae-5180* mutant, Stinard *et al.* [47] isolated two *Hin*dIII fragments (12 bp and 12.5 kb) containing *Mu1* elements, demonstrating that the *Ae-5180* mutation is associated with two *Mu1* insertions flanked by complex rearrangements of *ae*-related sequences. The *Ae-5180* mutation is known to suppress *in trans* the accumulation of the *Ae* transcript from wild-type *ae* alleles. Comparison of our structural data of the *Ae* gene to the two HindIII fragments clearly reveals that both *Mu1* insertions occurred in the 5′-flanking sequences of the *Ae* gene, ca. 400 bp upstream of the transcription initiation site. Interestingly, the *Mu1* element in the 12 kb genomic fragment is flanked by inverted

repeats of *Ae* proximal promoter sequences which are sufficient to direct gene expression as demonstrated in transient expression assays of 5′ deletion mutants (Figure 5). Therefore, it is likely that all of the three promoter regions in *Ae-5180* may be functional *in vivo*. This finding prompted us to propose two molecular mechanisms which require promoter activity to explain a dominant action of the *Ae-5180* allele. First, since the two *Hin*dIII fragments were reported to be tightly linked genetically, there is a possibility that the *Ae-5180* allele actually produces antisense mRNA of the *Ae* gene if they are located next to each other. This also explains why wild-type revertants of the *Ae-5180* were accompanied with the loss of the inverted promoter regions in the 12 kb fragment. Second, if the *Ae-5180* mutation does not produce antisense RNA, a molecular mechanism underlying sense cosuppression may be involved in the dominant action of the *Ae-5180* allele. As suggested in Stinard *et al.*'s report [47], the fact that the *Ae-5180* allele completely suppresses the accumulation of the *Ae* transcript from wild-type *ae* alleles is similar to the phenomenon of cosuppression. Recent reports [38, 52] have shown that the silencing of gene expression is most effectively triggered by promoters which can produce transgene transcripts.

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