CONTROLLING-ELEMENT EVENTS AT THE SHRUNKEN LOCUS IN MAIZE

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Manuscript received November 19, 1980
Revised copy received March 16, 1981

ABSTRACT

We have examined insertions of the controlling element *Ds* at the Shrunken locus of maize. A cDNA probe complementary to a portion of the Shrunken locus mRNA was prepared. This probe recognizes a unique sequence in maize DNA. Using lines carrying derivatives of the same short arm of chromosome *9*, we have detected modifications at the nucleic acid level caused by *Ds*. The changes appear to be large insertions, one of which may be more than 20 kilobase pairs in length. These observations provide a basis for the isolation and molecular characterization of one of the maize controlling elements.

THE Shrunken (*Sh*) locus makes an abundant, well-characterized gene product, and controlling-element insertions at this locus have been described. As such, it is a logical place to begin a characterization of maize transposable elements. Known mutations at the *Sh* locus are recessive and are expressed as deeply dented, opaque kernels; they affect only the endosperm and do not reduce the viability of the plant. The locus was originally identified by a stable allele, *sh-R*, discovered in progeny of corn collected from a Nebraska Ponka Indian reservation (HUTCHISON 1921). SCHWARTZ (1960) showed that *sh* kernels (those homozygous for the reference allele or for either of two *Ds*-induced mutable *sh* alleles) lack a major soluble protein. Two chemically induced *sh* mutations make electrophoretic variants of the major soluble protein, indicating that the *Sh* locus is the structural gene encoding the protein (CHOUREY and SCHWARTZ 1971). Subsequently, CHOUREY and NELSON (1976) demonstrated that the *Sh* protein is sucrose synthetase (UDPglucose: D-fructose 2-glucosyltransferase). The enzyme catalyzes the reversible reaction: UDP + sucrose $\rightleftharpoons$ UDP-glucose + fructose. Sucrose is made in the leaves and is transported to the developing endosperm. In the endosperm, it is converted by the enzyme to a nucleoside diphosphate sugar that is eventually used as a glucosyl donor in starch synthesis. Mutant kernels make less starch and therefore collapse on drying. Sucrose synthetase has been purified to homogeneity from several plants (DELMER 1972; NOMURA and AKAZAWA 1973; SU and PREISS 1978). The purified enzyme from corn is reported to be a tetramer of identical subunits, $M_r = 88{,}000$ (SU and PREISS 1978).

The features of the two-element system, *Ac-Ds*, have been summarized (MCCLINTOCK 1956a). Here it is necessary to mention only a few of its character-

istics. *Ds* elements may be recognized when they insert into a locus and suppress or modulate gene action. These elements are stable in the absence of a *trans*-acting signal from *Ac*. When an active *Ac* is brought into the genome, *Ds* may transpose away from the locus, leaving a stably altered or restored allele. The chromosomal location of *Ds* also may be determined when it is not located at a defined locus by its ability to cause chromosome breaks. In response to *Ac*, some "states" of *Ds* can induce chromosome breaks where *Ds* is inserted (McCLINTOCK 1951). The resulting acentric fragment, carrying genes between *Ds* and the end of the chromosome, cannot migrate at anaphase and is lost. A dicentric chromosome carrying the markers proximal to *Ds* is formed in the daughter cell that receives the broken chromatid. This event initiates the breakage-fusion-bridge cycle (McCLINTOCK 1941). The dicentric chromosome breaks in the succeeding anaphases, leading to the random losses of proximal markers. The position of *Ds* therefore, can be deduced relative to other markers, because all markers distal to *Ds* are lost in the initial event, and proximal markers are lost in subsequent events (McCLINTOCK 1950).

McCLINTOCK (1951, 1952) obtained two independent transpositions of *Ds* from its standard position between *Wx* and the centromere on the short arm of chromosome *9* to a position just distal to *Sh* (Figure 1). A number of *sh* mutants were obtained from the two stocks carrying *Ds* adjacent to *Sh* (McCLINTOCK 1952, 1953). These mutants appeared only when *Ac* was present. The most interesting of these were mutable in the presence of *Ac*, and *Sh* revertants were obtained from these alleles, although at different frequencies. Two of the mutants we have analyzed, *sh-m5933* and *sh-m6233*, were in this group. All of the *sh*-mutables were transmitted normally by male and female gametes, and homozygous plants were viable. In addition to transposing, the *Ds* elements at *Sh* examined here also induce chromosome breaks in response to an active *Ac*. *Ds sh*-mutable alleles are exceptional in that when *Ds* occasionally transposes to give

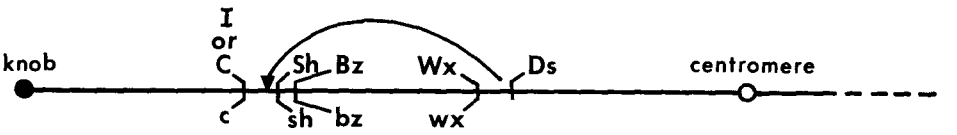

FIGURE 1.—Cytogenetic map of the short arm of chromosome *9* (redrawn from McCLINTOCK 1951). Relevant dominant markers (above the line) and their recessive alleles (below) are indicated. The arrow depicts the two transposition events that took *Ds* from its original location to positions just distal to *Sh*. *Ds* (*Dissociation*) is stable in the absence of a *trans*-acting signal from *Ac* (*Activator*). In the presence of an active *Ac*, it can cause adjacent deletions and chromosomal rearrangements; it can break chromosomes; it can transpose and modulate gene activity if inserted at a gene locus; or it can become inactive. *Wx* (Waxy) is the structural gene for starch-granule-bound ADP glucose glucosyl transferase. Mutants have reduced amylose; with iodine staining, the starch grains are red instead of blue. The gene is active in the endosperm and pollen. *Bz* (Bronze) is the structural gene for UDP glucose: flavonoid glucosyl transferase. Mutant kernels are bronze colored instead of deep purple. Plant color can also be affected. *Sh* (Shrunken) is the structural gene for sucrose synthetase. Wild-type kernels are full and vitreous; mutant kernels are deeply dented and chalky. *I* (Inhibitor) of *C*, one of eight factors that are required for aleurone color. Kernels with *I*, or that are homozygous for *c*, are colorless.

Sh revertants, they do not leave the immediate vicinity of the locus (McCLINTOCK 1953). This condition permitted McCLINTOCK (1956a) to continue the cycle and to isolate new mutable alleles from the *Sh* revertants; *sh-m6795*, analyzed in the present study, is an example of one of these secondarily derived mutants.

McCLINTOCK also obtained *sh* mutants that affected more than one locus, gave decreased recombination with adjacent loci or exhibited reduced male transmission. These are most easily explained as adjacent deletions extending distally or proximally from *Ds*. One of these, *sh bz-m4*, also analyzed in this paper, is a double mutation affecting both *Sh* and *Bz* (McCLINTOCK 1956b). In the absence of *Ac*, there is an altered pattern of synthesis of UDPglucose:flavanoid glucosyltransferase, the *Bz* enzyme (DOONER and NELSON 1977), and the *Sh* protein is not made (DOONER 1980). In the presence of *Ac*, sectors (or whole kernels) with the *Bz* phenotype appear; however, the kernels are stably *sh*. Furthermore, crossing over between *sh* and *bz-m* is reduced from about 4% to about 0.1% (DOONER 1980). Transmission of *sh bz-m4* is normal and homozygous plants are viable.

This work describes the preparation of a cDNA probe from wild-type sucrose synthetase mRNA and its use in examining the structure of wild-type and *sh* alleles. A preliminary account of some of these results has been presented (BURR and BURR 1980).

MATERIALS AND METHODS

Strains: All stocks are homozygous for markers on chromosome *9*. *Ds*, where present, responds to *Ac* when crossed to tester strains; however, none of the strains analyzed carry an active *Ac*.

(1) *Sh* = *C Sh Bz Wx Ds* or *I Sh Bz Wx Ds*, from McCLINTOCK.

(2) *sh-m5933* = *I Ds sh-m5933-1 Bz wx*, derived by McCLINTOCK from *Ds 5245* (*Ds* inserted just distal to *Sh*). Grown for 7 generations with *Ac* and for 3 generations without *Ac* before these analyses.

(3) *sh-m6233* = *C Ds sh-m6233A-2 Bz wx*, derived by McCLINTOCK from *Ds 4864A* (the other instance of *Ds* inserted just distal to *Sh*). Grown for 5 generations with *Ac* and for 2 generations without *Ac*.

(4) *sh-m6795* = *I Ds sh-m6795B-3 Bz Wx*, derived by McCLINTOCK indirectly from *Ds 5245*. A *Sh* revertant was selected from the original *Ds sh-m6258A* (not analyzed in this study), and the present *sh-m6795* was selected from progeny of this revertant as a new *sh*-mutable. It has been grown for 3 generations with *Ac* and for one generation without *Ac*.

(5) *sh bz-m4* = *C Ds sh bz-m4 Wx*, derived by McCLINTOCK from *Ds 4864A*. A simultaneous event gave rise to a *sh bz* kernel from which frequent dicentric events and less frequent sectors of *Bz* tissue occurred when *Ac* was present.

(6) *sh-R* = *C sh bz wx* from McCLINTOCK, *C sh bz Wx* (inbred W22) from O. E. NELSON and *c sh bz wx* from D. S. ROBERTSON; sh-R is the original stable allele described by HUTCHISON (1921).

Purification of sucrose synthetase and preparation of specific antiserum: Kernels of the hybrid WF9 × B37 were harvested 22 days after pollination, frozen in dry ice and stored at —20°. Five hundred grams of frozen kernels were homogenized in 500 ml ice-cold 0.1M potassium phosphate, pH 7.0, 1 mM EDTA and 1 mM DTT. The homogenate was filtered through two layers of cheesecloth and centrifuged at 16,000 × g for 20 min at 0°. The supernatant was decanted through glass wool, brought to 1% streptomycin sulfate and left at 4° for 30 min. The

precipitate was removed by centrifugation and the supernatant adjusted to 60% saturation with ammonium sulfate. The ammonium sulfate precipitate was dialyzed extensively against TAMD: 10 mM Tricine, pH 7.2, 50 mM potassium acetate, 0.5 mM magnesium titriplex (E. MERCK) and 1 mM DDT. The dialysate was centrifuged to remove undissolved material and then applied to a 2.5 × 25 cm DEAE cellulose (DE52, WHATMAN) column that had been equilibrated with TAMD. The column was washed with TAMD until no protein could be detected in the effluent and then developed with a one-liter linear gradient of 50 to 500 mM potassium acetate in TAMD. The gradient fractions were monitored by sucrose synthetase activity assayed in the direction of sucrose formation (NOMURA and AKAZAWA 1973). A broad peak of activity eluted in the middle of the gradient. The peak fractions were pooled and precipitated with 60% saturated ammonium sulfate. The precipitate was dissolved in a minimum volume of TAMD containing 0.5 mM DDT. This sample was applied to a 2.5 × 90 cm column of BioGel A-1.5m (BioRad) pre-equilibrated with the same buffer. The fractions were assayed by SDS-polyacrylamide gel electrophoresis and enzyme activity. Sucrose synthetase eluted shortly after the void volume and coincided with the major peak of absorbance. Occasionally, rechromotography on DEAE was necessary to remove a lower molecular weight contaminant.

Antibodies were raised to purified sucrose synthetase as described by BURR and NELSON (1975). Antisera were titered by double diffusion in agar, and specificity was assessed by immunoelectrophoresis of total soluble endosperm proteins.

Preparation of mRNA: Endosperms were dissected from kernels of the hybrid W64a × A297 twenty days after self-pollination, frozen in liquid nitrogen and stored at —85°. Twenty grams of frozen kernels were ground to a powder with dry ice in an electric coffee mill and stirred into an extraction buffer containing 7 M urea, 0.35 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 2% Sarkosyl and 5% phenol (FEDOROFF, WELLAUER and WALL 1977). After mixing on ice for 5 to 10 min, the mixture was phenol extracted. DNA was removed by spooling after the addition of one volume of 95% ethanol at —20°. A second volume of cold 95% ethanol was added, and the RNA was allowed to precipitate overnight at —20°. Poly(A)+ RNA was enriched by the method of DOTTIN, WEINER and LODISH (1976) and further purified on DMSO-sucrose gradients (BURR *et al.* 1978). Fractions from the DMSO-sucrose gradients were alcohol precipitated twice and portions were translated in the wheat germ cell-free system (BURR *et al.* 1978). Fractions containing mRNAs that gave translation products the size of sucrose synthetase were pooled and used for cDNA synthesis.

Immunoprecipitation of *in vitro*-synthesized polypeptides was performed by the method of KESSLER (1975).

Preparation of double-stranded cDNA clones: Complementary DNA was synthesized from size-fractionated poly(A)+ RNA, using the conditions of MYERS and SPIEGELMAN (1979). The RNA was hydrolyzed in 0.02 N NaOH for 8 hr at 37°. Second-strand synthesis and hairpin-loop cleavage were performed as described by EFSTRATIADIS *et al.* (1976). Overlapping ends of the double-stranded cDNA were evened with polymerase I, and synthetic *Eco*RI linkers (Collaborative Research) that had been phosphorylated were attached by blunt-end ligation (MANIATIS *et al.* 1978). The blunt-ended material was digested with *Eco*RI and the large fragments were recovered by gel filtration. These were ligated to plasmid pBR325 that had been digested with *EcoRI* (BOLIVAR 1978).

Transformation of *E. coli* LE392 (TIEMEIER, TILGHMAN and LEDER 1977) was done according to KUSHNER (1978), and bacterial growth was on L broth plates containing 10 μg/ml tetracycline. Colonies were picked onto duplicate tetracycline and chloramphenicol (25 μg/ml) plates. Bacteria that were *tet*^{r} *chl*^{s} were grown in one ml cultures of L broth. Rapid lysates were prepared and the DNA electrophoresed in 0.5% neutral agarose at 7 v/12 cm. Plasmids that were larger than the nonrecombinant pBR325, or its dimer, were chosen for further analysis. (It is estimated that plasmids with inserts of less than 100 nucleotides were not selected.) Plasmids were prepared (POTTER *et al.* 1979) from 500-ml cultures in L broth containing 40 μg/ml tetracycline, but not amplified with chloramphenicol.

Screening by hybridization-translation: Ten μg of plasmid DNA was nicked and denatured by boiling for 5 min in 0.2 N NaOH. The solution was neutralized and bound to a disc of DBM

paper, as described by STARK and WILLIAMS (1979). Hybridization of 10 μg endosperm poly(A)+ RNA and 110 μg total endosperm RNA was performed according to SMITH, SEARLE and WILLIAMS (1979) at 50° for 16 hr. The filters were washed (SMITH, SEARLE and WILLIAMS 1979) and the RNA removed by three elutions of 100 μl absolute formamide at 60°. The combined eluates were alcohol precipitated twice with one μg of wheat-germ tRNA as carrier. The wheat-germ tRNA had been prepared by the method of DUDOCK *et al.* (1969). The eluate was translated in the wheat-germ cell-free system in a final volume of 25 μl (BURR *et al.* 1978) and 10 μl of the reaction was electrophoresed on a 10% polyacrylamide-0.1% SDS gel (ANDERSON, BAUM and GESTELAND 1973). The gels were treated for fluorography as described by BONNER and LASKEY (1974).

Preparation of maize DNA and Southern transfers: Seeds were germinated on water-saturated germination paper in covered trays at 30° for 4 days in the dark. Shoots were frozen in liquid nitrogen as they were being harvested and stored at —85°. Frozen shoots were powdered in an electric coffee mill with dry ice. DNA was extracted by the same procedure described for the preparation of mRNA (see "Preparation of mRNA"). DNA was banded first in 10 mM Tris, pH 8, 1 mM EDTA, 1.25 g/ml $CsCl_2$ and one μg/ml ethidum bromide for 12 hr at 48,000 rpm in a Vi50Ti rotor and rebanded in 10 mM Tris, pH 8, one mM EDTA, 0.92 g/ml $CsCl_2$ and 0.6 mg/ml ethidium bromide. Ethidium bromide was removed by isoamyl alcohol and the DNA dialyzed extensively against 10 mM Tris, pH 8, one mM EDTA. Yields varied from 1–16 μg DNA/g fresh weight of shoots and appeared to be inversely related to the amount of polysaccharide that contaminated the spooled DNA.

Restriction endonucleases used were *Bam*HI, *Eco*RI, *Hind*III, and *Sst*I (BRL); *Bcl*I, *Bgl*I, and *Bgl*II (N.E. Biolabs); and *Bst*EII (Boehringer). Digestion conditions were as suggested by the suppliers. Electrophoresis was performed as described by MCDONELL, SIMON and STUDIER (1977) in 0.8 cm thick 0.4% neutral agarose gels in the presence of 0.5 mg/l ethidium bromide at 0.5 v/cm for 18 hr. Full-length and *Hae*II and *Pvu*II cut T7 DNA were used as molecular length markers (ROSENBERG *et al.* 1979). Gels were photographed on a short-wave UV light box for 6–8 min.

Transfers to nitrocellulose (SOUTHERN 1975) and hybridizations incorporated modifications of D. KLESSIG (personal communication). Hybridizations were carried out with 10^6 cpm/ml denatured probe, 100 μg/ml denatured salmon sperm DNA, one mM EDTA, 5 × Denhardt's solution (DENHARDT 1966), 6 × SSC and 0.5% SDS at 68° for 20 to 24 hr.

The cDNA insert in plasmid 1–333 was used as the probe. Plasmid 1–333 was digested with *Eco*RI and electrophoresed on a 1% neutral agarose gel (MCDONELL, SIMON and STUDIER 1977). The ethidium-bromide-stained gels were viewed with long wave UV, and the insert band was cut out of the gel. The fragment was recovered by solubilization of the agarose in 8 M sodium perchlorate and absorption of the DNA to glass powder (VOGELSTEIN and GILESPIE 1979). Labeling of the purified insert was by nick translation (RIGBY *et al.* 1977) in the presence of 200 pmole each of α-dATP^{32} and α-dCTP^{32} (New England Nuclear) to a specific activity of $0.5–1.0 \times 10^8$ cpm/μg.

Counts were removed from the filters, as described by SHANK *et al.* (1978), and the filters were rehybridized with ^{32}P-labeled maize rRNA. The maize rRNA had been labeled with γ-ATP^{32} and polynucleotide kinase after mild alkaline hydrolysis (MAIZELS 1976) to a specific activity of 1.5×10^7 cpm/μg. Hybridization was in 50% formamide, 3 × SSC, 1 × DENHARDT's solution, 0.1 M sodium phosphate, pH 7.2, 0.5% SDS and 0.5×10^6 cpm ^{32}P-labeled rRNA at 42° for 24 to 36 hr.

RESULTS AND DISCUSSION

Sucrose synthetase was purified to homogeneity by the criterion of SDS-polyacrylamide gel electrophoresis. We estimate it to have a subunit molecular weight of 89,000. The purified protein corresponds to the major soluble protein in developing endosperm when electrophoresed in nondenaturing gels (C. M.

WILSON, unpublished). Antiserum obtained from rabbits injected with the purified protein contained antibodies only to sucrose synthetase, as demonstrated by immunoelectrophoresis against total endosperm-soluble proteins.

Endosperm mRNA was obtained from kernels that were harvested slightly before the point of maximal enzyme activity (TSAI, SALAMINI and NELSON 1970). When the translation products of unfractionated or size-fractionated messages were examined on SDS-polyacrylamide gels, a band that co-migrated with the purified enzyme was noted. Specific antisucrose synthetase antiserum was used to absorb this band (Figure 2), demonstrating that sucrose synthetase is the only visible *in vitro* product of its size, and that the *in vitro* product was the same size as the mature enzyme subunit. Therefore, we felt confident in using the size of the translation product and its immunoprecipitability with specific immune serum to identify cDNA clones of the message.

Poly(A)+ RNA was enriched for sucrose synthetase mRNA by size fractionation of DMSO-sucrose gradients. When messenger RNA from each fraction was translated, the RNA between the two large rRNA peaks gave the bulk of the

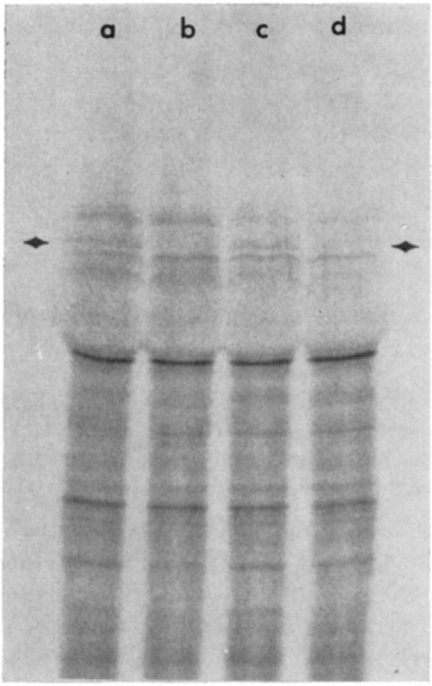

FIGURE 2.—Identification of sucrose synthetase among maize endosperm mRNA translation products. Total poly(A)+ RNA from developing endosperm, collected 20 days after pollination, was translated in the wheat-germ cell-free system. An equal volume of nonimmune serum (a and c) or serum containing antisucrose synthetase antibodies in excess (b and d) was added to the reactions after translation. Antigen-antibody complexes were removed by the addition of *Staphlococcus aureus* cell walls and centrifugation. The products in the supernatant were electrophoresed on a 10% polyacrylamide-SDS gel and visualized by fluorography. Purified sucrose synthetase (double-headed arrows) was electrophoresed in the adjacent channels and stained by Coomassie blue. (The bowed region is caused by the excess of serum protein.)

products the size of sucrose synthetase subunit. These fractions were pooled and used to synthesize double-stranded cDNA that was ligated to synthetic *Eco*RI restriction-site linkers. The double-stranded cDNA with *Eco*RI sticky ends was introduced into the unique *Eco*RI site in the plasmid pBR325 (BOLIVAR 1978). The *Eco*RI site is in a chloramphenicol-resistance marker, which permits rapid screening for plasmids with insertions in this site. The presence of inserts was verified by examining plasmid DNA prepared from rapid lysates and electrophoresed on agarose gels. Plasmid DNA was prepared from the clones carrying plasmids with detectable inserts. Eighty-four of these were screened by positive hybridization-translation, using total endosperm RNA. One of these, clone 1–333, hybridized a message that, when eluted and translated, gave a band the size of the sucrose synthetase subunit on SDS-polyacrylamide gels. The product could be specifically precipitated with sucrose synthetase antiserum (Figure 3). The insert is estimated to be 285 nucleotides in length. Using the insert from clone 1–333 as a hybridization probe, the other eighty-three clones were re-examined, and none could be shown to contain complementary sequences.

DNA prepared from clone 1–333 was digested with *EcoRI* and the insert

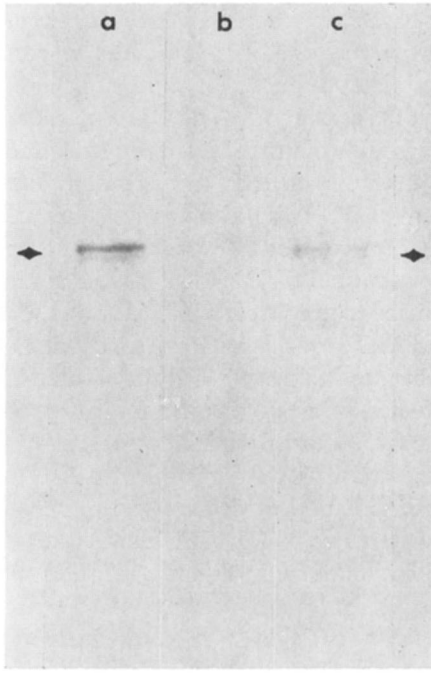

FIGURE 3.—Identification of a clone complementary to sucrose synthetase mRNA: Recombinant plasmids were screened by hybridization with endosperm mRNA and translation of the specifically eluted messages. Translation products were precipitated with antisucrose synthetase antiserum and *S. aureus* cell walls. (a) Products of total endosperm mRNA used in the hybridization assay, (b) clone 1–315, which bound no detectable mRNA, (c) clone 1–333, which gave a translation product the same size as sucrose synthetase mRNA. The material precipitated with specific antiserum migrates as a doublet. We have not investigated this phenomenon. (Note that this experiment examines products precipitated by immune serum; whereas, Figure 2 shows products remaining after immunoprecipitation.

fragment purified on agarose gels. Maize DNA was digested with restriction enzymes, electrophoresed on agarose gels and transferred to nitrocellulose filters. The filters were hybridized against the insert of clone 1–333 that had been radioactively labeled by nick translation. A single band was detected in all lines homozygous at the *Sh* locus. Similarly, GEISER *et al.* (1980) used an independently obtained cDNA probe to show that the *Sh* sequence was unique. During the course of these experiments, *Sh* sequences in a variety of genetic backgrounds were examined. With any given restriction enzyme, as many as four of the strains differed in the size of the fragment recognized. Because of the restriction polymorphism associated with the *Sh* locus, it was necessary to identify material that contained the progenitor *Sh* allele in MCCLINTOCK's stocks. Therefore, we used stocks homozygous for the short arm of chromosome *9*, with *Ds* in its standard position between *Wx* and the centromere. This is the same material MCCLINTOCK employed to obtain *Ds* transpositions adjacent to *Sh*.

Twenty-seven percent of the deoxycytosine of maize DNA is the 5-methyl derivative (SHAPIRO 1976). Just as with mammalian DNA, in our experiments, restriction enzymes that recognize the sequence -CpG- make few cuts in maize DNA. Within this limitation, we digested maize DNA with a number of the commercially available, six-base-pair recognition type II enzymes, and concentrated our efforts on those that gave large *Sh* fragments, as recognized by our probe. However, information is also presented for some of the enzymes that gave small fragment lengths. None of these enzymes cleaved our probe. Figure 4A shows the pattern of restriction fragments obtained from *Sh*, *sh-R* and the *sh-mutables* after *Eco*RI digestion. This enzyme clearly distinguishes *Sh* from *sh-m6233*, *sh-m6795* and *sh-R*. Also evident is the absence of a homologous fragment in *sh bz-m4* DNA. Figure 4B is a control to demonstrate complete cleavage and approximately equivalent amounts of DNA in all the samples: the radioactivity was melted off the filter used to prepare Figure 4A, and the filter was rehybridized with labeled maize rRNA. The 9-kb ribosomal repeat is seen in all channels. Other enzymes have been used to distinguish the *sh* alleles: *Bst*EII characterizes *Sh*, *sh-m6759* and *sh-R* (Figure 5), and *Bcl*I differentiates *Sh*, *sh-m593*, *sh-m6795* and *sh-R* (Figure 6). Not evident in these figures are small but consistent differences between *Sh* and *sh-m5933* digested with *EcoRI*, and between *Sh* and *sh-m6233* digested with *Bcl*I. In all three digests, no fragment complementary to the probe was detected in *sh bz-m4* DNA. This result is consistent with the genetic evidence for a deletion of *Sh* in this mutant (DOONER 1980).

The lengths of fragments complementary to our *Sh* probe produced by these and other enzymes are given in Table 1. The most interesting differences are the 21-kb difference between the *Sh* and *sh-m6795* *Bst*EII fragments, the approximately 12-Kb difference between the *Sh* and *sh-m5933* *Bcl*I fragments and the 11-kb difference between *Sh* and *sh-m6233* *Eco*RI fragments. These might be the result of large insertions into the *Sh* locus, but they could also be caused by a deletion of a restriction site or by an inversion. With our small probe, the only way to substantiate the idea that these are complete insertions would be to find two enzymes that give the same increase in size over *Sh* DNA for one of the

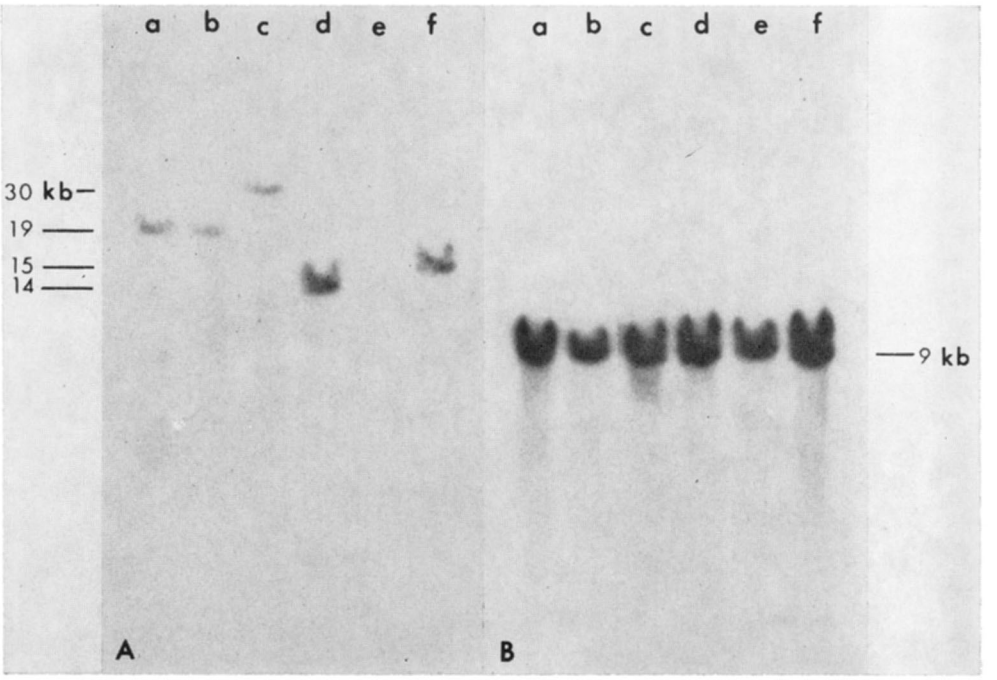

FIGURE 4.—Normal and mutant DNA digested with *EcoRI*, prepared for Southern transfer, and hybridized with a probe complementary to sucrose synthetase mRNA. (A) After autoradiography, the probe was melted off and the filter rehybridized with maise rRNA probe; (B) DNAs were prepared from (a) *Sh*, (b) *sh-m5933*, (c) *sh-m6233*, (d) *sh-m6795*, (e) *sh bz-m4*, (f) *sh-R*.

alleles. To date, this combination has not been found. This result could be explained if *Ds* were a large element carrying a number of internal restriction sites.

The existence of restriction polymorphism among *Sh* alleles of different maize stocks was mentioned previously. We have been asked whether the changes observed could be due to random mutations, rather than to specific *Ds*-induced events. It must be emphasized that inbred lines and laboratory stocks showing polymorphism at the *Sh* locus were independently derived from a very diverse germ plasm. In contrast, no polymorphism was detected in two of McCLINTOCK's stocks with *Sh* linked to *I* or *C*. The *sh-R* mutation has been propagated since 1914; we analyzed this mutation from three different collections and found no restriction polymorphism; *sh-m5933* and *sh-m6795* used in this study are, respectively, only ten and eight generations removed from *Ds5245*, their common *Sh* progenitor.

With most restriction enzymes, the stable *sh-R* allele gives fragment lengths that differ from our *Sh* DNA. While *sh-R* may eventually be found to differ from wild type by some chromosomal rearrangement—a deletion or insertion, for instance, rather than a point mutation—these differences probably reflect variations in the restriction polymorphism of its progenitor allele as compared to McCLINTOCK's *Sh* stock.

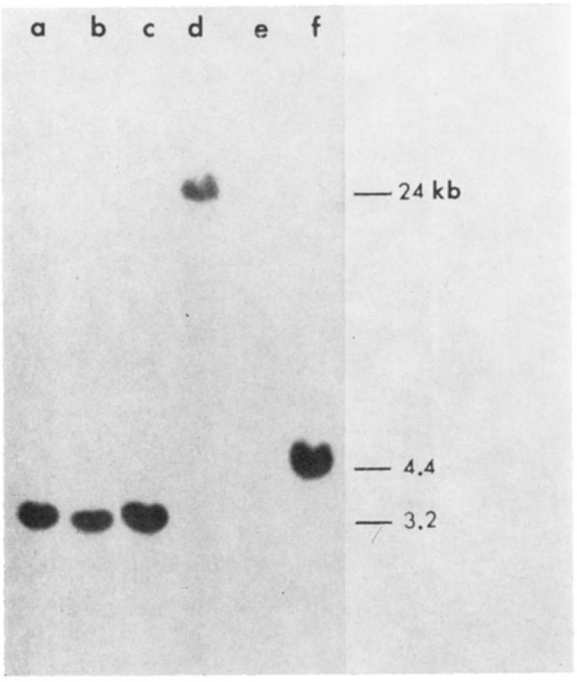

FIGURE 5.—Normal and mutant DNA digested with *Bst*EII and hybridized with sucrose synthetase probe. Source of DNAs and their arrangement are as in Figure 4.

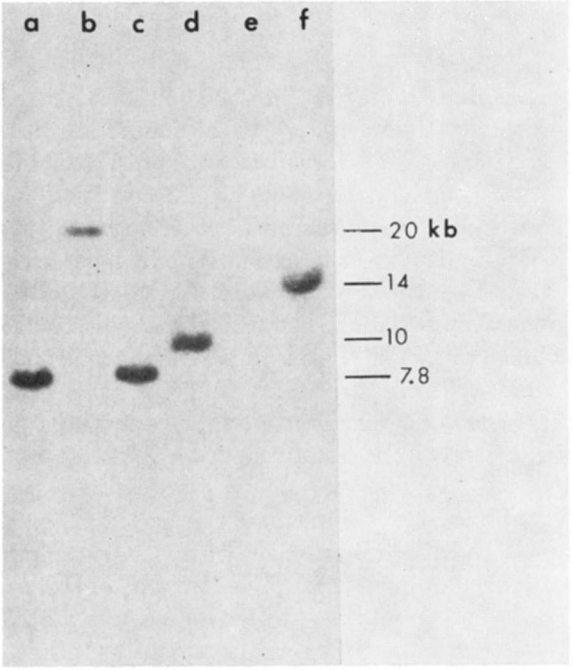

FIGURE 6.—Normal and mutant DNA digested with *Bcl*I and hybridized with sucrose synthetase probe. Source of DNAs and their arrangement are as in Figure 4.

TABLE 1

Lengths, in kilobase pairs, of restriction fragments recognized by the sucrose synthetase cDNA probe after digestion of normal and mutant DNA with restriction endonucleases and Southern hybridization

	*Bam*HI	*Bcl*I	*Bst*EII	*Eco*RI	*Sst*I	*Bgl*I	*Bgl*II	*Hind*III
Sh	15	7.8	3.2	19	2.2	12.4	6.1	0.7
sh-m5933	12	20	3.2	17	2.2	13.3	6.7	0.7
sh-m6233	16	8.1	3.2	30	2.2	16.3	6.5	0.7
sh-6795	10	10	24	14	5.5	16.6	13.2	0.7
sh bz-m4	—	—	—	—	—	n.e.	n.e.	n.e.
sh-R	17	14	4.4	15	2.2	14.0	6.7	0.7

n.e. = not examined.

Double digests of *Sh* DNA and hybridization with our cDNA probe allow the construction of a partial restriction map of the *Sh* locus. Figure 7 shows the restriction sites flanking the sequence complementary to our cloned cDNA probe. The *Ds* events within the locus can be assigned approximate positions relative to these restriction sites. For example, reference to Table 1 indicates that neither *Bst*EII nor *Sst*I detects modifications due to *sh-m5933* or *sh-m6233*, although all other enzymes used recognize these events. Since the *Bgl*I and *Bgl*II fragments, which extend only to the left of the *Bst*EII cuts, also detect these changes, we conclude that they must have occurred within an 800-bp region bounded by a *Bcl*I site on the left and the *Bst*EII site on the right. Likewise, as all enzymes used recognize the modification due to *sh-m6795*, this *Ds* event must have occurred in the 1400-bp overlap bounded by the *Sst*I site on the left and the *Bgl*II site on the right.

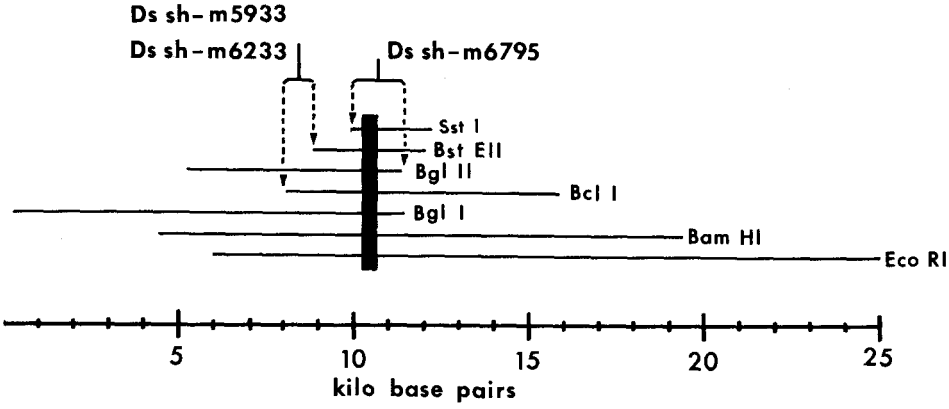

FIGURE 7.—Summary of Southern blotting analyses. Double digestion of *Sh* DNA with pairwise combinations of restriction endonucleases permits assignment of restriction sites flanking the sequence complementary to the cDNA probe. The horizontal lines represent the fragment lengths of *Sh* DNA that are produced with single restriction enzymes and that hybridize to the sucrose synthetase probe. Relative locations of *Ds* events were deduced by noting those enzymes that gave altered fragment sizes with *Ds sh*-mutable DNA compared with *Sh* DNA (see Table 1). The areas in which *Ds*-induced changes occurred are indicated by brackets. The heavy vertical bar represents the region complementary to the probe.

In making these conclusions, the *Ds*-induced mutations are assumed to be discrete events; that is, modifications of only a single site. This assumption is justifiable because the mutations were single events and are revertible. While most prokaryotic transposable elements show some site specificity of insertion, some have been shown to integrate into multiple sites within known genes (CALOS and MILLER 1980). It is not surprising, therefore, that *Ds* has modified different regions of the *Sh* locus. This observation is also consistent with NELSON's (1968) mapping of three *Ds* insertions at independent sites within the waxy locus of maize. It is not yet known how extensive the *Sh* locus is, or what regions are modified by the events. We hope that more precise physical mapping of these and other insertions will help define the gene.

These experiments were planned as the necessary prelude before attempting to isolate controlling elements. The evidence for alterations at the nucleic acid level brought about by *Ds* and the data on the restriction fragment sizes allow us to proceed with the molecular cloning experiments to obtain *Ds* from chromosomal DNA. It is hoped that the characterization of these sequences will answer such questions as how many copies there are in the genome, what is the relationship of *Ds* to *Ac* and how *Ds* and *Ac* transpose and regulate gene activity.

We would like to thank BARBARA MCCLINTOCK for her help and criticism, and for her continuous encouragement and interest. Skilled technical assistance was provided by MARJORY M. NEUBERGER and GREGORY P. BALLANTYNE. DAN KLESSIG provided invaluable instruction on Southern blotting and hybridization. This work was performed at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy, and was supported by the Science and Education Administration of the U.S. Department of Agriculture under Grant No. 5901-0410-8-0023-0 from the Competitive Research Grants Office.

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Corresponding editor: R. L. PHILLIPS