Isolation and Characterization of a 1.7-kb Transposable Element from a Mutator Line of Maize

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ABSTRACT

We have cloned and sequenced a 1.7-kb *Mu* element from a Mutator line of maize and compared its structure to *Mu1*, a 1.4-kb element. With the exception of a 385-bp block of DNA present in the 1.7-kb element, these transposable elements are structurally similar, sharing terminally inverted and internal direct repeated sequences. Derivation of 1.4-kb elements from the 1.7-kb class via deletion of internal sequence is suggested by the finding that a portion of the extra DNA in *Mu1.7* is part of a truncated direct repeat sequence in the 1.4-kb element. An abundant poly(A)$^+$ RNA homologous to a portion of this extra DNA is present in several tissues of both Mutator and non-Mutator lines. Analysis of transcripts from an unstable mutant *bronze1* (*bz*) allele containing a *Mu1.7* element inserted in an exon of the gene detects three species of poly(A)$^+$ RNA that hybridize to a *Bz1* (*Bronze*) gene probe: the largest contains the entire *Mu1.7* element in the *Bz1* gene transcript; another appears to be a spliced, chimeric transcript; the smallest is normal size *Bz1* mRNA. The latter is most likely encoded by the normal-size alleles detected by Southern analysis of tissue expressing purple pigment, suggesting that normal gene function is restored by excision of the *Mu1.7* element.

ROBERTSON's Mutator stocks of maize are characterized by a 50-fold increase in the overall frequency of visible mutations. Genetic analysis of the Mutator trait by ROBERTSON (1978) showed that about one-third of the mutations are phenotypically unstable, suggesting the presence of insertion sequences. A new transposable element family was defined by analysis of an unstable *Alcohol dehydrogenase 1* (*Adh1*) allele arising in a Mutator line of maize (STROMMER *et al.* 1982). Sequence analysis showed that the *Mu1* element recovered from the mutant *Adh1-S3034* allele is 1376 bp in length and is flanked by 9-bp direct repeats of insertion site DNA; the element has terminal inverted repeats (TIR) of 215 and 213 bp and internal direct repeats of 104 bp (BARKER *et al.* 1984). Transcription studies of the *Adh1-S3034* allele show that the presence of the 1.4-kb element in the first intron affects the elongation of *Adh1* mRNA but that processing appears to be normal (ROWLAND and STROMMER 1985; VAYDA and FREELING 1986). The mutant produces normal sized *Adh1* mRNA at steady state levels about twofold lower than that produced by the nonmutant progenitor allele and contains no detectable *Mu1* sequences.

In other well-characterized transposable element systems an important regulator of transposition activity involves the presence of two distinct classes of transposable elements. Relying solely on genetic analysis, McClintock identified the two components of the *Ac-Ds* system almost 4 decades ago (MCCLINTOCK, 1949). Molecular and genetic analyses of the *Ac-Ds* system and other mobile element systems, *e.g.*, *Spm*(*En*) and the *P* elements of *Drosophila*, have identified nonautonomous members which are deletion derivatives of the corresponding autonomous elements (FEDOROFF, WESSLER and SHURE 1983; O'HARE and RUBIN 1983; PEREIRA *et al.* 1985). Analysis of these deletion derivatives has permitted identification of sequences crucial for activity of the autonomous transposable element (KARESS and RUBIN 1984; DOONER *et al.* 1986). While genetic evidence for autonomous and nonautonomous classes of *Mu* elements is not established, it is intriguing that Mutator stocks contain two major size classes of *Mu* elements: a larger element of approximately 1.7 kb (BARKER *et al.* 1984; WALBOT, CHANDLER and TAYLOR 1985; TAYLOR, CHANDLER and WALBOT 1986) in addition to the 1.4-kb size class typified by *Mu1*. This report describes the isolation of a representative of this larger size class, termed *Mu1.7* and comparison of its structure and sequence to *Mu1*. We show that insertion of *Mu1.7* into an exon of the *bronze1* gene creates an unstable allele. This *bz1-mu2* allele produces at least three species of mRNA, two of which contain *Mu1.7* sequences. We also show that an abundant transcript homologous to a portion of the *Mu1.7* element is present in both Mutator and non-Mutator lines.

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The sequence data presented in this article have been submitted to the EMBL/GenBank Data Libraries under the accession No. Y00603.

MATERIALS AND METHODS

Plant material: The RMGH1 and RMGH2 stocks correspond to D. ROBERTSON lines 71-9268-7^{n}/8269-1 and 71-9266-7^{n}/8266-9. They were the result of a cross involving 1966 and 1968 Mutator seed, respectively. The *bz2-mu1* lineage is described in WALBOT, BRIGGS and CHANDLER (1986). In this report individuals from the C230-8 family and subsequent generations were tested for the presence of *Mu1.7*. The derivation of the *bz1-mu2* and *bz2-mu1* alleles is described in TAYLOR, CHANDLER and WALBOT (1986) and in CHANDLER and WALBOT (1986), respectively. All alleles tested by Northern analysis were isolated from plants containing the *B* (*bz1-mu2*) or *B* and *Pl* (*bz1* and *Bz1*) genes. Non-Mutator lines were obtained from E. H. COE and M. G. NEUFFER (University of Missouri), the Maize Genetics Stock Center, and from M. FREELING (University of California, Berkeley).

DNA isolation, hybridization analysis and radiolabeling of probes: All methods are exactly as previously described (TAYLOR, CHANDLER and WALBOT 1986). Reconstructions are calculated on the basis of 6×10^9 bp per haploid genome and consist of the appropriate plasmid digest added to 5 μg of *Eco*RI-digested salmon sperm DNA as carrier.

RNA isolation and Northern blot analysis: Total RNA was extracted from maize tissues using guanidinium isothiocyanate according to SETZER *et al.* (1980). Poly(A)$^+$ RNA was isolated using oligo-dT-cellulose, separated on a 1.4% agarose gel containing 2.2 M formaldehyde (MANIATIS, FRITSCH and SAMBROOK 1983) and transferred to a nylon membrane (Nytran; Schleicher & Schuell). Hybridizations were performed sequentially with *Bz1* and *Mu* probes as described and washed at 60° (*Bz1*) or 65° (*Mu*) in 0.1× SSPE, 0.1% SDS. Probe removal was accomplished by agitating the membrane in 0.1× SSPE, 0.1% SDS at 95°, for three 15-min rinses.

Lanes 1 through 4 of Figure 6 are from an APT filter kindly provided by J. CALLIS. They contain total RNA from Efron S (1, 2) and Super Gold Pop (3, 4), maize lines described in WOODMAN and FREELING (1981). Lane 5 of Figure 6 contains total RNA from Black Mexican Sweet suspension culture cells described in FROMM, TAYLOR and WALBOT (1986).

Restriction mapping and DNA cloning: Restriction enzymes were obtained from Bethesda Research Laboratories and New England Biological Laboratories and used according to suppliers' instructions. Genomic cloning is described in TAYLOR and WALBOT (1985). The *Hin*fI fragment of *Mu1* was used as a probe to screen for *Mu*-containing phage. Subclones were prepared using directional cloning where possible into pBR322 or pUC8 (VIEIRA and MESSING 1982). When directional cloning was not possible, the fragment was isolated and the termini treated with T4 DNA polymerase in the presence of four deoxyribonucleotides (ENGLUND 1971) and blunt-end ligated into the *Sma*I site of pUC8. pMuLft (*Mlu*I to *Bst*EII sites in *Mu1*) and pSD2 (*Sma*I to *Dde*I sites in *Mu1.7*, Figure 2) were prepared in this manner. The *bronze1* probe pD3MS9 is described in SCHIEFELBEIN *et al.* (1985); pD3S3 consists of a *Sma*I fragment of a *Bz1* allele cloned into pUC8. *Bronze1* plasmid pMBzP17 is the source of fragments for the 3′ probe (see map, Figure 4A) and reconstructions. The location of this plasmid is described in TAYLOR, CHANDLER and WALBOT (1986). *Mu* probe pAB5 is described in CHANDLER and WALBOT (1986). The *Mu1*-containing plasmid pMJ9, described in BENNETZEN *et al.* (1984) was used as the sources of the full length *Mu1* element for restriction fragment comparisons.

DNA sequence analysis: DNA sequencing reactions were performed by the dideoxy chain termination protocol using M13 as vector (SANGER *et al.* 1980) or using double-stranded plasmid as the template (CHEN and SEEBERG 1985). DNA sequence analysis employed computer programs available from the BIONET National Computer Resource for Molecular Biology (RR-01685-03) or developed by RICHARD PATTIS.

RESULTS

Distribution of the two major size classes of Mutator elements: Restricting DNA from Mutator stocks with an enzyme which cuts external to the *Mu* elements and hybridizing the resulting Southern blot with a Mutator probe results in an array of fragments each representing an element located at a unique genomic location. Digesting genomic DNA with enzymes which cut within the terminal inverted repeat sequence, reduces all *Mu1*-size elements to a single band and consequently any heterogeneity in size classes of elements will be detected. *Hin*fI recognition sites are located ~35 bp from either end of the TIRs and digestion of the cloned representative of the 1.4-kb size class, *Mu1*, with this enzyme generates a 1.3-kb fragment. Active Mutator lines analyzed in this manner showed a second major size class of element approximately 1.7 kb in size (BARKER *et al.* 1984; TAYLOR, CHANDLER and WALBOT 1986).

To determine the range and ubiquity of the two major size classes of *Mu* elements, we performed *Hin*fI digestions and *Mu* hybridizations on active Mutator stocks as temporally and genetically separated as were available, in addition to analyzing closely related individuals. The earliest extant Mutator lines available are RMGH 1 and 2, which were produced from genetic crosses involving identified Mutator lines isolated in 1966 and 1968, respectively (gift of D. ROBERTSON, Iowa State University). *Hin*fI-digested DNA from plant RMGH 1 (Figure 1A, lane D) shows a characteristic pattern of two major fragments, about 1.3- and 1.7-kb in size, hybridizing to pAB5, a probe which contains the internal portion of the cloned *Mu1* element (Figure 2). As is the case in most Mutator lines examined to date (BENNETZEN *et al.* 1984; WALBOT, CHANDLER and TAYLOR 1985; CHANDLER and WALBOT 1986), the 1.3-kb fragment represented by the cloned *Mu1* element is the most prevalent. In addition to the 1.4- and 1.7-kb *Mu* size classes, *Hin*fI-digested DNA from plant RMGH 2 (lane C) shows larger fragments indicative of *Mu* elements in which one or both of the *Hin*fI restriction sites are modified (WALBOT, CHANDLER and TAYLOR 1985; CHANDLER and WALBOT 1986). Digestion of RMGH 2 DNA with *Tth*111-I, an enzyme which digests methylated DNA shows a pattern of two *Mu1* homologous bands at 1.0- and 1.4-kb which are the expected sizes for *Mu1* and

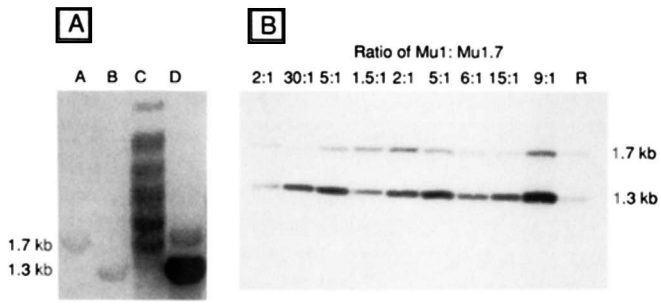

FIGURE 1.—Both major size classes of *Mu* elements are present in widely divergent lines but their absolute number and ratio vary, even between closely related plants. (A) Immature cob DNA (10 μg) from two of the earliest identified Mutator stocks available was digested with *Hin*fI, size-fractionated on a 1% agarose gel, blotted to APT paper (SEED 1982) and hybridized with the 1.3-kb *Hin*fI fragment from *Mu1*. Lanes C and D contain genomic DNA from RMGH2 and RMGH1, respectively. (See MATERIALS AND METHODS for ROBERTSON's original nomenclature for these stocks.) Lanes A and B are reconstructions of *Hin*fI digested plasmids containing *Mu1.7* and *Mu1*, respectively, at an equivalent of one copy per haploid genome. Fragment sizes are indicated on the left in kilobase pairs. (B) Nine siblings from a cross between an active Mutator plant (C230-8, used as male) containing the *bz2-mu1* allele and a non-Mutator *bz2* tester (K55/W23) were analyzed. An aliquot of 5 μg of total genomic seedling DNA was digested with *Hin*fI, a Southern blot prepared and hybridized to the Mutator probe, pAB5 (see Figure 2 for map of Mutator probes). The resulting hybridization pattern was scanned in a LKB densitometer, and the ratio of *Mu1.4*:*Mu1.7* was determined. These data are shown above each lane. The blot was washed free of Mutator probe and reprobed with a *Pst*I fragment from the coding region of the *Bronze1* gene of maize to normalize for variation in the amount of DNA per lane. These data were used to estimate the absolute copy number of each element when compared to a single genome equivalent reconstruction (lane R). The molecular mass in kilobases (kb) is on the right.

Mu1.7 elements (data not shown). This finding suggests that the potential for DNA modification and suppression of Mutator activity regulation is not a recently acquired property.

We have also examined the *Hin*fI pattern of individuals grown from 1983 stocks in which Mutator activity is monitored by observation of an unstable allele of *bronze2* (*bz2-mu1*) in the aleurone layer of the kernel. Molecular and genetic analysis over several generations of this lineage (family C230-8) shows that the Mutator system remains highly active as evidenced by a consistent pattern of uniform, small revertant sectors in the aleurone (WALBOT, BRIGGS and CHANDLER 1986; CHANDLER and WALBOT 1986). In addition we have surveyed several individuals from 4 generations of stocks carrying *Adh1-S* alleles containing *Mu1* elements (STROMMER *et al.* 1982); again, all of these lines show the presence of both size classes of elements (data not shown). Although the early Mutator lines, the *bz2-mu1* lineage and the *Adh1-S* stocks tested were descendants of the same original Mutator stock, they are as temporally separate as available, and have been maintained in different laboratories and out-crossed to widely different lines.

Ninety percent of the progeny of a cross between a Mutator and a non-Mutator plant can inherit the Mutator trait (ROBERTSON 1978). To explain this level of genetic transmission, multiple copies of autonomous elements must exist. If maize lines could be derived which segregate active and inactive Mutator progeny in the 1:1 ratio expected for the segregation of a single autonomous element, a genetic definition of the autonomous element would be possible. However, to date we have screened more than 300 Mutator individuals maintained for 5 or 6 generations of outcrossing to non-Mutator lines and have found that all lineages maintain multiple copies of both the *Mu1.4* and *Mu1.7* elements (L. P. TAYLOR, V. L. CHANDLER and V. WALBOT, unpublished data). A representative Southern blot of *Hin*fI digested DNA from nine sibling kernels from an active mutator stock containing the *bz2-mu1* allele was probed with the entire *Mu1.4* element (Figure 1B). Fragments of approximately 1.7- and 1.4-kb are present in all specimens; additionally the 1.7-kb fragment in all individuals hybridized to pSD2, a probe specific for the larger class of element (data not shown). Although they contain both major size classes of *Mu* elements, the ratio and copy number vary widely, even though these individuals are closely related. Densitometric scanning showed that in these nine plants the ratio of *Mu1.4*:*Mu1.7* varies over a 20-fold range from 30:1 to 1.5:1. By comparing the hybridization intensity of both sizes of *Mu* fragments in each individual with a 3 copy (*Mu1*) and a 1 copy (*Mu1.7*) reconstruction, and correcting for loading differences, the absolute number of *Mu1.7* elements was shown to vary from 10 copies (lane 9:1) to ~1 copy (lane 30:1) per haploid genome.

The presence of the *Mu1.7* element in early Mutator stocks and its persistence through repeated outcrossing, even though it is commonly found in lower copy number than the 1.4-kb size class, indicates that it may play an important role in the Mutator system. In an effort to more clearly define that role we undertook a structural analysis of a *Mu1.7* element.

Restriction map and DNA sequence of the 1.7-kb *Mu* element: A 1.7-kb *Mu* element was cloned from an active Mutator line containing the *Adh1-S3034a* allele (TAYLOR and WALBOT 1985). Thirty-five *Mu1*-homologous phage were recovered and screened by digesting the DNA with *Hin*fI. One clone contained a 13.5-kb insert which generated a 1.7-kb *Hin*fI fragment. A 2.5-kb *Bam*HI/*Hin*dIII fragment was subcloned into pUC8 and used in the subsequent restriction mapping and sequence analysis. Alignment of the restriction map of *Mu1.7* with *Mu1* shows that overall there is conservation of sequences between the two classes of elements. They share virtually identical terminal inverted repeats (TIR) and extensive internal homology. The difference in size between *Mu1.7* and

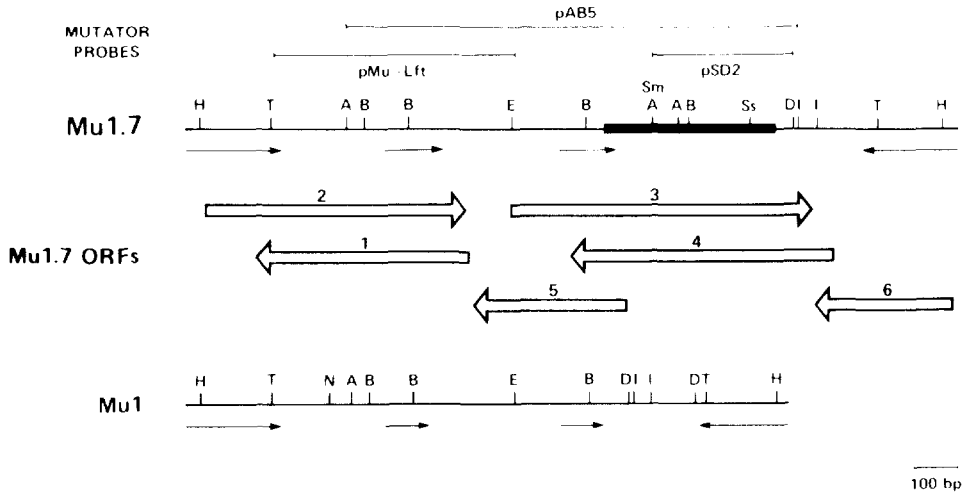

FIGURE 2.—A comparison of the restriction sites and repeat units in the *Mu1.7* and *Mu1* elements. The *black bar* in *Mu1.7* indicates the 385 bp of additional DNA not present in *Mu1*. *Arrows* indicate terminal inverted or direct repeat sequences. *Open arrows* labeled 1–6 are the open reading frames in *Mu1.7*. *Mu* probes used in this study are shown and their exact structure described in MATERIALS AND METHODS as are subcloning, restriction mapping, and hybridization conditions. Key to restriction enzymes sites: H = *Hin*fI, T = *Tth*111-I, N = *Not*I, B = *Bgl*I, E = *Bst*EII, D = *Dde*I, I = *Bst*NI, Sm = *Sma*I, Ss = *Sst*I, A = *Ava*I.

Mu1 is almost entirely the result of a block of DNA in the right half of the element as oriented in Figure 2. The *Mu1.7* element consists of 1745 bp and is flanked by a 9-bp direct repeat (Figure 3); this repeat presumably represents the host sequence duplication created at the insertion site. A comparison of the duplicated sequence flanking *Mu1.7* (CTCGTGCCT) with the 9-bp duplication flanking *Mu1* in the *Adh1-S3034* allele (TTTTGGGGA) (BENNETZEN *et al.* 1984) indicates no similarity in insertion sites. Within the approximately 1.35 kb shared between the two elements there are 37 single base changes. Transitions are more prevalent than transversions, representing 25 out of 37 (70%) of the altered bases; these are equally divided between A-G and C-T. These changes are scattered throughout the 1.7-kb element; one of these single base changes, a C to T transition, accounts for the lack of a *Not*I site in *Mu1.7* at position 321. In the left TIR 3 out of 215 bases are changed when compared to *Mu1*, while the right TIR is more diverged with 13 out of 213 bases changed. However comparison of the left with the right TIR of *Mu1.7* shows that they are 97% homologous.

In addition to the single base substitutions there are additions and deletions of sequence in *Mu1.7* as compared to *Mu1*. An 8-bp sequence (ACGGCAGC) near the internal junction of the left TIR (nucleotide 223) is not present in *Mu1.7*. At nucleotides 436 and 437 there are two additional bases (CG) present in *Mu1.7*. In the right portion of the *Mu1.7* element, there is an area containing two single base changes and two small insertions in *Mu1.7* relative to *Mu1*. One of the single base changes, an A to G transition creates a *Bst*NI site at position 1448, 55 bp away from a *Bst*NI site at position 1393 (Figure 3). Although there is only a single *Bst*NI site in the published sequence of *Mu1* at nucleotide 1027, the cloned *Mu1.7* and *Mu1* elements both generate an approximately 60-bp fragment upon digestion with this enzyme (data not shown), suggesting that the published sequence of *Mu1* in the region around nucleotide 1085 may be in error.

The most prominent difference between the two elements is a segment of DNA unique to *Mu1.7* in the right half of the element. This sequence contains single sites for several enzymes which do not restrict the *Mu1* element, including *Sma*I, *Sst*I, and *Sph*I. This novel DNA in *Mu1.7* is 385 bp long and is bounded by the trinucleotide GCC in direct orientation. Overall, the GC content (69%) of the DNA sequences unique to *Mu1.7* is comparable with that of the flanking DNA found in *Mu1*.

One of the most intriguing features of this segment of DNA unique to *Mu1.7* is its relationship to the 104-bp internal direct repeats which are found in both *Mu1.7* and *Mu1* (Figure 2). The extra DNA in *Mu1.7* is located immediately adjacent to the external terminus of the right direct repeat with the trinucleotide GCC which bounds the extra DNA forming the final 3 bases in the 104-bp direct repeat. The first 34 bp of the extra sequence in *Mu1.7* (position 960–994) are identical to the 34 bp adjacent to the internal terminus of the direct repeat in the left portion of both *Mu1.7* and of *Mu1* (Figure 3). The net result is that the direct repeats in *Mu1.7* are 138 bp long and 98% homologous instead of 104 bp and 96% homologous as in *Mu1*. Therefore only the left direct repeat of *Mu1* but not the right is 138 bp, identical to the direct repeats of *Mu1.7*. The length and orientation of the major open reading frames (ORFs) of *Mu1.7* are indicated in Figures 2 and 3. The numbering convention follows that of *Mu1* except for ORFs 5 and 6, two small reading frames not found in *Mu1*.

Analysis of a *Mu1.7*-induced mutation in *bronze1*: The physical properties of the cloned *Mu1.7* element, including the intact TIRs and the presence of 9-bp direct duplications flanking the element, suggest that this size class of element should be capable of responding to transposition signals. Evidence of transposition of the *Mu1.7* class of elements was provided by recov-

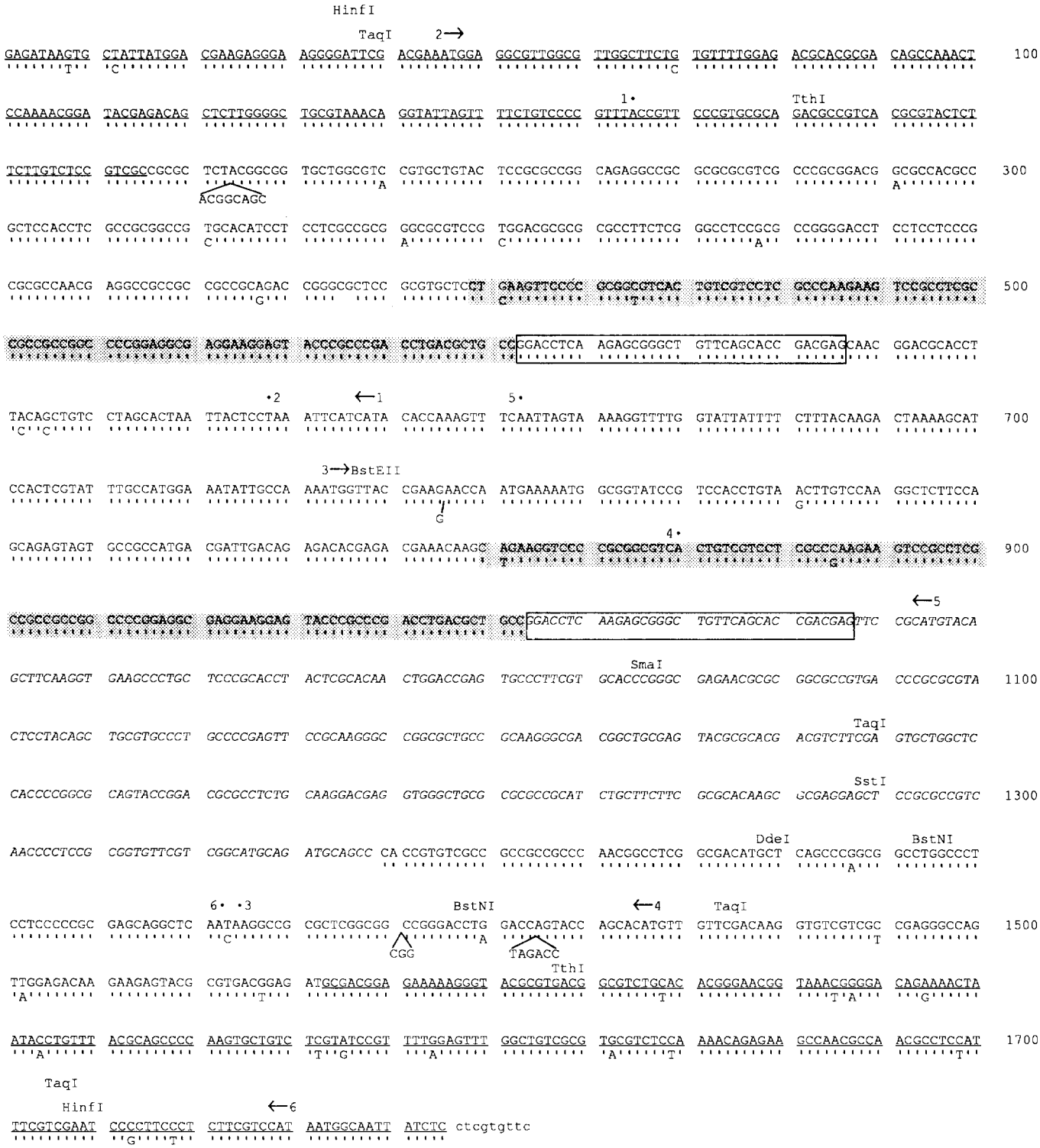

FIGURE 3.—Nucleotide sequence of *Mu1.7* and comparison to *Mu1*. The 1745 bp of *Mu1.7*, plus flanking sequences, was determined by the procedures described in MATERIALS AND METHODS. In the figure the sequences of *Mu1.7* (*top line*) is aligned to the relevant sequence of *Mu1* (*bottom line*) taken from BARKER *et al.* (1984). The large block of extra DNA present in *Mu1.7* is in *italics* and some relevant restriction sites are indicated. Single base substitutions, deletions and insertions of sequence between the two elements are indicated; nucleotide homology is indicated by a vertical bar (|). Terminal inverted repeats are *underlined* and the 104-bp internal direct repeats are *shaded* with the 34-bp extension boxed. The initiation codons and direction of ORFs 1–6 are indicated with an *arrow* and a (·) indicates the stop codons. ORFs 1 and 2 are about the same size in both *Mu1.7* and *Mu1* elements and have identical 5′ and 3′ ends. However, the deletion of 8 bp and the addition of 2 bp at positions 223 and 435, respectively, cause the ~200-bp segment of *Mu1.7* DNA between them to be in a different frame with respect to *Mu1*. The increase in size of reading frames 3 and 4 reflect the incorporation of all the *Mu1.7* novel DNA into both ORFs. The start codon for *Mu1.7* ORF3 is 14 bp to the right of that in *Mu1* but after 12 bp a 1-bp deletion causes a return to the same frame in both elements. In ORFs 1, 2 and 3 the direct repeats are in the same frame in *Mu1.7* as they are in *Mu1*.

ery of a mutable allele of *bronze1*, one of the genes required for anthocyanin biosynthesis in maize. This allele (*bz1-mu2*) contains a 1.7-kb *Mu1*-homologous insertion and displays somatic instability consisting of many fine revertant sectors, a pattern shared with many *Mu*-induced mutable alleles (WALBOT, BRIGGS and CHANDLER 1986). Preliminary genomic mapping of *bz1-mu2* using Southern analysis showed that a 1.7-kb *Mu1*-homologous sequence was inserted into a 650-bp *Bst*EII/*Sst*I fragment in the middle of a functional *Bz1* allele (TAYLOR, CHANDLER and WALBOT 1986). The sequence of cDNA clones of the *Bz1* transcript shows that a 100-bp intron lies within this fragment, the only intervening sequence in the gene (FURTEK 1986). To date all *Mu* element insertions associated with an unstable phenotype that have been analyzed at the molecular level have been located in either an intron or the 5′ untranslated region of the affected gene (BENNETZEN *et al.* 1984; ROWLAND and STROMMER 1985; FREELING and BENNETT 1985). In order to determine the orientation of *Mu1.7* within the 650 *Bst*EII/*Sst*I fragment and whether it has inserted within the *bronze1* intron, a detailed restriction map of the *bz1-mu2* mutant was undertaken using probes specific for *Mu1.7* (pSD2), *Bz1* (pD3MS9 and pD3S3), and the left half of *Mu* elements (pMuLft). The locations of the *Bz1* probes are indicated on the map of the *bz1-mu2* allele in Figure 4A and *Mu* probes are shown in Figure 2. *Mu1.7* elements have a single *Bst*EII site and a single *Sst*I site as shown in Figures 2 and 3, and no *Xho*II sites within the element. The 650-bp *Bst*EII/*Sst*I fragment of the *Bz1* gene has a *Xho*II site 175 bp from the *Sst*I site (Figure 4A). The placement and orientation of the *Mu* insert in *bz1-mu2* can therefore be determined by double digests using *Bst*EII/*Sst*I and *Bst*EII/*Xho*II. If the element has inserted to the left of the *Xho*II within the *Bst*EII/*Sst*I fragment (Figure 4A), then *Bst*EII/*Xho*II-digested *bz1-mu2* DNA should generate a fragment larger than the 500-bp wild-type fragment that will hybridize to *Bz1* probe pD3S3. If the insertion site is to the right (downstream) of the *Xho*II site, then the probe will hybridize to the wild type *Bst*EII/*Xho*II fragment of approximately 500 bp.

Figure 4B lane 1 shows a prominent band at 1.2 kb which hybridizes to pD3S3 indicating that the element is to the left or upstream of the *Xho*II site as oriented in Figure 4A. Referring to the orientation of *Mu1.7* shown in Figure 2 as A and the opposite orientation as B, a right and left half can be designated using the *Bst*EII site as the approximate center of the element. The direction of the *Bz1* transcript relative to the 650-bp *Bst*EII/*Sst*I fragment is indicated in Figure 4A (DOONER *et al.* 1985). In orientation A that half of *Mu1.7* containing pSD2 sequences would be nearer the *Sst*I site at the downstream end of the *Bz1* fragment while orientation B would be the reverse. Orientation A should generate a single 1.2-kb pD3S3

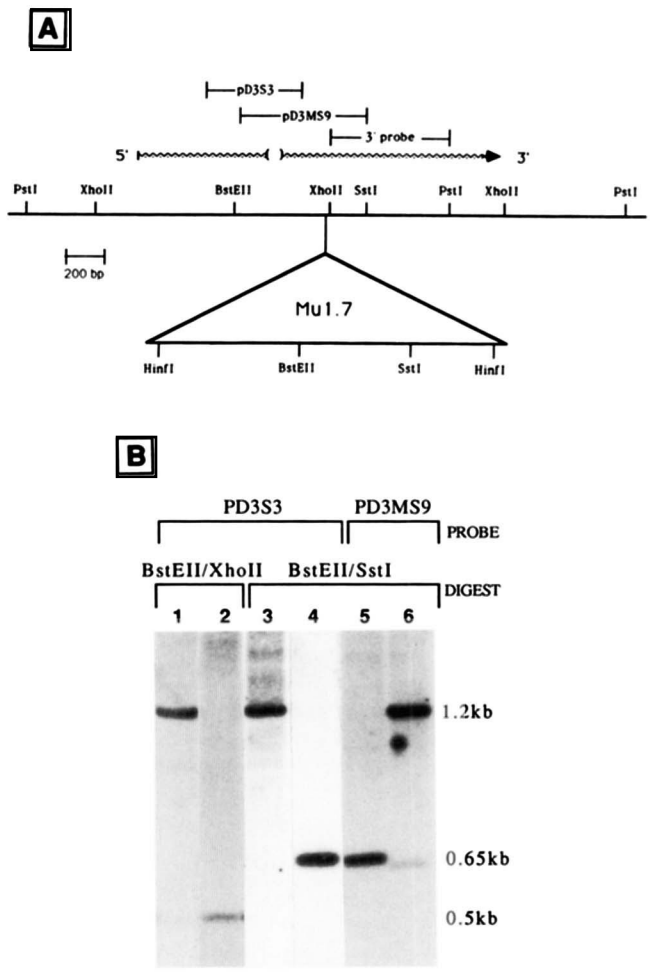

FIGURE 4.—Genomic restriction mapping localizes the insertion site of a *Mu1.7* element within *bronze1* sequences. (A) The restriction map of the *bz1-mu2* allele containing a *Mu1.7* element inserted adjacent to *Xho*II site. Information on the length and direction of a *Bz1* transcript, including a 100-bp intron (indicated by *brackets*) was supplied by FURTEK (1986) and H. DOONER (personal communication). The location of the *Bz1* probes used in this study are shown on the map. Mutator probes are shown in Figure 2. (B) Hybridization data used to confirm the location of the *Mu1.7* element. An aliquot of 5 μg of genomic DNA from *bz1-mu2* immature cobs was double digested with *Bst*EII plus *Sst*I or *Bst*EII plus *Xho*II, electrophoresed through a 1% agarose gel and transferred to a nylon membrane (Genetran, Plasco) by the method of SOUTHERN (1975). DNA from fully pigmented nonmutant sibling kernels, taken from the same ear on which the mutant kernel arose, were used as the source of the parental *Bz1* allele. *Bst*EII/*Xho*II digested DNA from *bz1-mu2* (lane 1) and one of its nonmutant siblings (lane 2) were probed with the *Bz1* probe pD3S3. *Bst*EII and *Sst*I digest of *bz1-mu2* (lane 3) DNA was probed with *Bz1* probe, pD3S3. After removing the previous hybridization signal, the blot was probed with the *Bz1* probe pD3MS9 to detect downstream sequences. Lane 6 is *bz1-mu2* and lane 5 is the nonmutant sibling. Lane 4 is a 5 copy reconstruction of a *Bst*EII/*Sst*I digest of pMBzP17 that contains the 2.2-kb *Pst*I fragment which encompasses most of the transcribed region of the *bronze1* gene (see Figure 4A). The size of fragments is given in right margin.

homologous fragment from *Bst*EII/*Sst*I digested *bz1-mu2* DNA while orientation B would generate a fragment of ~900 bp. In Figure 4B lane 3, a single 1.2-kb band hybridizes to pD3S3 indicating insertion of *Mu1.7* has occurred in orientation A. To confirm that the digestion and transfer were complete and that other fragments are present as predicted, the pD3S3 signal was removed and the lane probed with pD3MS9. As expected in addition to the 1.2-kb fragment, an ~630-bp fragment is detected by this probe (Figure 4, lane 6). This fragment is composed of *bronze1* sequences downstream of the insertion site and *Mu1.7* sequences rightward of the *Sst*I site within the element.

All blots were probed with *Mu* probes pMuLft and pSD2 and the resulting hybridization patterns confirmed that the expected size fragments were present and contained the appropriate sequences (data not shown). These fragment sizes and hybridization patterns are only consistent with the insertion of the 1.7-kb element within 50 bp upstream from the *Xho*II site in the 650-bp *Bst*EII/*Sst*I fragment of the *bronze1* gene (Figure 4A). This site is 217 bp downstream of the 3′ splice site of the intron. The orientation of the element relative to the direction of transcription of the *bronze1* gene is the same as that found for the *Mu1* element in the *Adh1-S3034* allele (Bennetzen *et al.* 1984) and in another *bronze1* mutant, *bz1-mu1* (Taylor, Chandler and Walbot 1986).

Analysis of transcripts of the *bz1-mu2* allele: In combination with the appropriate genetic factors (*B* or *B* plus *Pl*) anthocyanin pigmentation and high level expression of UDPglucose:flavonol 3-*O*-glucosyltransferase, the product of the *bronze1* locus, occur in maize leaf and stem tissue (Coe and Neuffer 1977). The husks of plants containing the *bz1-mu2* allele in a *B* background show a pattern of dark purple streaks on a light green background, indicating that some *Bz1* function is occurring in these plants. An analysis of the poly(A)$^+$ RNA produced in the husk tissue of plants containing the *bz1-mu2*, *bz1* recessive or *Bz1* wild-type alleles is shown in Figure 5A. The *Bz1* allele produces a single 1.9-kb RNA (Figure 5A, lane 3) while the recessive *bz1* allele has a single RNA (lane 2) about 350 bp smaller. This stable allele is associated with a 0.34-kb deletion within the coding region (Dooner *et al.* 1986). In contrast, the *bz1-mu2* allele produces 3 RNA species hybridizing to a *Bz1* gene probe (lane 1). A 3.6-kb transcript which hybridizes to both *Bz1* and the *Mu1.7*-specific probe pSD2 (Figure 5, lanes 1 and 4), is the major species. This size is the sum of the entire *Mu1.7* element plus the 1.9-kb mature *Bz1* message. Two less abundant species that hybridize to the *Bz1* probe (pD3MS9) are also present. The 1.9-kb class co-migrates with the nonmutant, parental *Bz1* message but does not hybridize to the

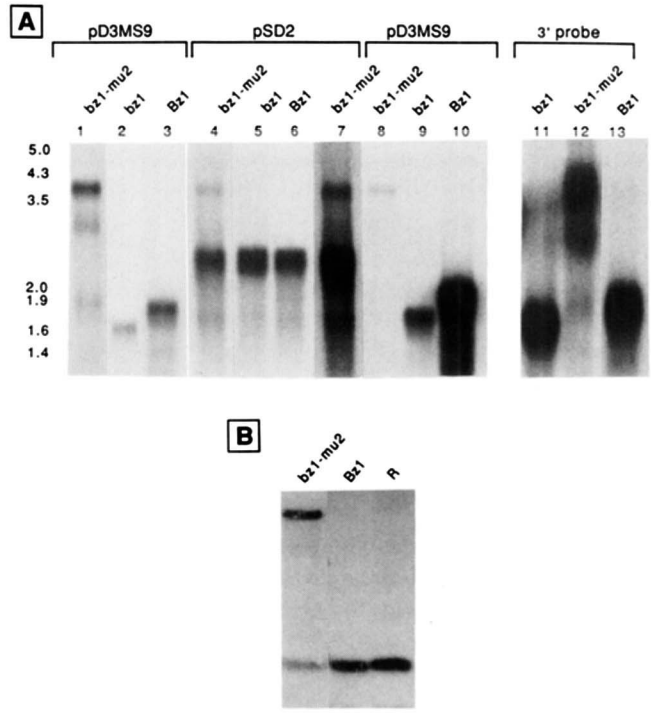

FIGURE 5.—Northern and Southern Blot analysis of nucleic acids from *bz1-mu2*, *bz1*, and *Bz1* husk tissue. (A) Three micrograms of poly(A)$^+$ RNA from husks of each genotype were electrophoresed through a 1.4% formaldehyde gel, blotted to nylon membrane and probed first with the *Bz1* probe pD3MS9 (*bz1-mu2*, *bz1*, and *Bz1* in lanes 1–3, respectively; lane 1 is a 72-hr exposure, lanes 2 and 3 are 3.6 hr). That signal was removed, and the blot rehybridized with the *Mu1.7* specific probe, pSD2 (lanes 4–7). Lane 4 shows the hybridization patterns using mRNA from the unstable mutant allele, *bz1-mu2*, and lane 7 is a 3 times longer exposure that lane. Lane 5 and 6 contain mRNA from the recessive *bz* tester allele and the parental *Bz1* allele respectively. Lanes 8–10 are a 24-hr exposure of the pD3MS9 hybridization pattern shown in lanes 1–3. Lanes 11–13 are from another Northern blot of mRNA from the three alleles hybridized with a DNA fragment from the 3′ portion of the *Bz1* transcript. See map Figure 4A for probe location. Denatured *Eco*RI/*Hin*dIII digested lambda DNA provided the size markers in the leftmost lane. (B) A Southern blot of *Bst*EII/*Sst*I-digested DNA isolated from identical husk tissue used for the RNA analysis in Panel A and probed with pD3S3, a *Bz1* probe. Lane 1, contains DNA from the mutant *bz1-mu2* and lane 2, DNA from wild type *Bz1* husk; Lane R is a 1 copy reconstruction from *Bst*EII/*Sst*I digested pMBzP17, a *bz1* plasmid containing the relevant sequences.

Mu1.7-specific probe (lane 4); this class of mRNA is probably produced in the purple sectors of husk tissue, as a result of the excision of the *Mu1.7* element from the gene. The third species is about 800 bases larger than the *Bz1* parental RNA but does not hybridize to the *Mu1.7*-specific probe (pSD2) (Figure 5A, lane 7, a longer exposure of lane 4). It does however hybridize to *Bronze1* sequences downstream of the insertion site of *Mu1.7*. An *Xho*II/*Pst*I fragment (see map, Figure 4A) representing the 3′-ward portion of the *Bronze1* mRNA was hybridized to another Northern blot of husk poly(A)$^+$ RNA from the three alleles. All three species of mRNA from the *bz1-mu2* allele show hybri-

dation to this probe including the 2.7-kb class (Figure 5A, lane 12). To further define the *Mu1.7* sequences present in this transcript, hybrid-selected *Bz1*-homologous RNA was electrophoresed through a 1.4% formaldehyde gel and probed with pAB5 (see map, Figure 2). Both the 3.6-kb and 2.7-kb species hybridize to this probe which is composed of sequences leftward of the unique region in *Mu1.7* (data not shown). These results would indicate that the 2.7-kb class of poly(A)$^+$ RNA contains most if not all of the *Bronze1* transcript but only sequences from the leftward portion of the *Mu1.7* element as oriented in Figure 2.

The mutation in *bz1-mu2* appears to reduce the level of the *Bz1* homologous RNA (Figure 5A, lanes 8–10). A densitometric scan of both the Northern blot containing poly(A)$^+$ RNA and a similar blot containing total RNA (data not shown) indicates that the relative abundance of *Bz1* homologous RNA in *bz1-mu2* is about 10% that of the wild type allele. Approximately one-half of the RNA present is the 3.6-kb species. In fact, this level of expression of *bz1-mu2* (in a *B* background) relative to the *Bz1* allele (in a *B* plus *Pl* background), agrees closely with the observation that *Bz1* activity and anthocyanin production in leaf sheaths of plants with only the *B* gene is about one-tenth that produced by plants having both *B* and *Pl* present (GERATS *et al.* 1984). To determine whether the mutation in *bz1-mu2* has any small effects on the amount of mRNA produced, it will be necessary to determine the expression from the allele in a *B* plus *Pl* background.

The conclusion that the normal size *Bz1* mRNA arises from somatic reversion in tissues of the unstable *bz1-mu2* stock is supported by the results of Southern analysis (Figure 5B). DNA isolated from the same plant material used for RNA analysis was doubly digested with *Bst*EII and *Sst*I, fractionated by gel electrophoresis, transferred to nylon membrane and probed with the *Bz1* probe pD3S3. Insertion of *Mu1.7* into the 650-bp *Bst*EII/*Sst*I *bronze1* fragment generates a unique 1.2-kb fragment when *bz1-mu2* DNA is digested with these enzymes (Figure 4B, lane 3). Reasonably precise excision of the element should restore the parental 650-bp fragment (Figure 4B, lane 5). Indeed, the *bz1-mu2* DNA extracted from the purple streaked husk tissue consists of two fragments (Figure 5B, lane 1), a major band of 1.2 kb and a weaker hybridizing fragment of 650 bp which co-migrates with the non-mutant *Bz1* parental allele shown in lane 2. This smaller fragment is presumably the result of excision of the element from the 650-bp *Bst*EII/*Sst*I *bronze1* fragment. These data indicate that the high frequency of somatic reversion seen in the *bz1-mu2* allele results from the perfect, or near perfect excision of the *Mu1.7* element, restoring approximately wild type size DNA fragments, normal mRNA size, and sufficient glucosyltransferase activity to create dark purple sectors in the husk tissue.

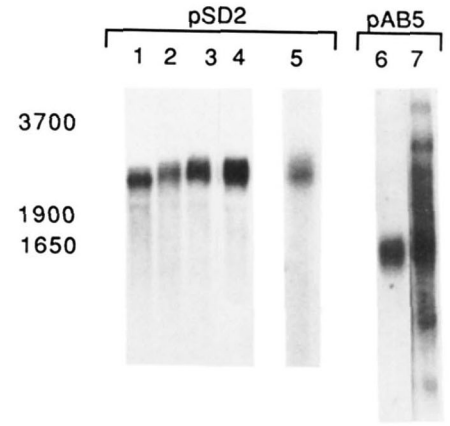

FIGURE 6.—*Mu1.7* homologous RNA is found in diverse maize tissues. Selected lanes from two Northern filters containing total RNA from immature embryos (lanes 1 and 4), roots (lanes 2 and 3) or suspension culture cells (lane 5) were probed with the *Mu1.7* unique region probe, pSD2. Lanes 1 through 4 contain 5 μg of RNA and lane 5 is the amount of RNA extracted from approximately 1 × 10^6 cells. See MATERIALS AND METHODS for the listing of these non-Mutator lines of maize. Lanes 11 and 12 of the Northern filter in Figure 5A were hybridized with pAB5 (see map, Figure 2) which detects *Mu* sequences surrounding the unique region of *Mu1.7*. The non-Mutator line containing the *bz1* allele is in lane 6 and lane 7 contains the Mutator line with the *bz1-mu2* allele. The size markers on the left represent the position of hybridization for the ribosomal DNA probe (3700 and 1900 bases) and *Adh1* (1650 bases).

An abundant transcript homologous to *Mu1.7* sequences: In addition to the 3.6-kb species, poly(A)$^+$ RNA from *bz1-mu2* contains a 2.2-kb transcript which hybridizes to the *Mu1.7* unique region probe (pSD2). Likewise the non-Mutator line carrying the *bz1* allele and the Mutator line carrying the *Bz1* allele have an identical size transcript at approximately the same abundance (Figure 5A, lanes 4–7).

The distribution of this transcript was tested by probing Northern blots of total RNA from developing embryos (20–30 days after pollination), roots and maize suspension culture cells. The tissue from these non-Mutator lines all have a single species of RNA approximately 2.2 kb that hybridizes to the *Mu1.7* unique probe (Figure 6, lanes 1–5). To determine whether other *Mu* sequences are represented in this transcript, the Northern blot in Figure 5A was probed with pAB5 which mainly detects sequences to the left of pSD2 in *Mu1.7* (see map, Figure 2). Lane 6 shows that the 2.2-kb transcript from the non-Mutator line containing the *bz1* allele, is the only species hybridizing to this probe. Poly(A)$^+$ RNA from the mutant *bz1-mu2* (lane 7) has a number of hybridizing bands, including one at about 2.2 kb. Presumably these extra bands reflect the presence of *Mu* sequences incorporated in read-through transcripts in this Mutator line.

The abundance and ubiquity of this transcript in

diverse tissues from both Mutator and non-Mutator lines is intriguing. It is not ribosomal RNA; in addition to being the wrong size it does not hybridize to an rDNA probe (data not shown).

It may derive from a sequence found in all maize lines that is closely homologous to the internal region of *Mu1.7*, including the pSD2 sequences, but with a large internal deletion and no *Mu* termini. This sequence has recently been cloned along with flanking sequences which show no homology to *Mu* sequences (L. TALBERT and V. CHANDLER, personal communication). We probed our Northern blots with these flanking sequences and find hybridization to the 2.2-kb species (data not shown).

DISCUSSION

Properties of *Mu1.7*: In an attempt to determine the factors required to produce and maintain an active Mutator system, we have cloned and analyzed a large *Mu* element found in Mutator lines. The *Mu1.7* element has the structural features of an active transposable element, including TIRs and host sequence duplication at its site of insertion; recovery of a *Mu1.7*-induced mutation in *bronze1* indicates that it can transpose. Structurally the two major elements in the Mutator stocks are nearly identical (97% homologous) in regions common to both elements. The major difference is a block of 385 bp of extra DNA in *Mu1.7* which could be the result of an insertion maize DNA into a *Mu1* element. In favor of an insertion model, the 385-bp block is bounded at both ends by the trinucleotide GCC in direct orientation. This trinucleotide is present at the same location in *Mu1* in a single copy; however, it is not clear that the second GCC in *Mu1.7* arose as a direct duplication from insertion of a transposable segment because the GCC motif is repeated >20 times in this region of *Mu1*. Another possibility for generating a *Mu1.7* element from a *Mu1* element would require at least two steps: duplication of the 34 bp present at the internal terminus of the left direct repeat and insertion of 351 bp of unique DNA.

Alternatively, comparison of the internal direct repeat sequences in both types of elements suggests a single step deletion relationship between *Mu1* and *Mu1.7*. The two copies of the 138-bp internal direct repeat of the larger element are not preserved in the smaller *Mu1* element. Only a single 138-bp copy is present in the left half of *Mu1* while in the right portion of the element the repeat is only 104 bp. We propose that deletion of 385 bp of DNA from the right half of the *Mu1.7* element, including 34 bp of the right direct repeat, generated a *Mu1* size class from a *Mu1.7* element (Figure 3). However, it is also possible that both *Mu1* and *Mu1.7* were derived independently from a larger precursor; such an element, if present in a low copy number, might not be detected until it causes a mutation in a cloned gene.

Somatic excision of *Mu1.7*: By SOUTHERN analysis (Figure 5B) it appears that ~10% of the *bz1-mu2* gene copies in husk tissue of a *bz1-mu2* homozygote have reverted during somatic growth to yield an approximately wild-type size *bronze1* gene. This level of reversion matches the phenotypic appearance of the husks, which contain numerous purple stripes on a green background. This is the first evidence that excision of a *Mu* element is associated with the presence of gene structure and function indistinguishable from wild type.

It was of interest, consequently, to determine the kinds of *Bz1* transcript in this tissue. Three major size classes of poly(A)$^+$ RNA were detected (Figure 5A). The largest size is the major species and is the size predicted if the entire 1.7-kb *Mu* element is included in the full length *Bz1* transcript; the fate of this RNA is unknown. Because there is purple tissue, some RNA encoding a functional gene product must also be present. Such an RNA could arise either from *Mu* excision in somatic revertants or by a splicing event removing the *Mu* element from the full length transcript of the *bz1-mu2* gene. Based on hybridization analyses (Figure 5A), the 1.9-kb RNA detected by *Bz1* but not by *Mu* probes is the same size as the wild-type mRNA and could result from either mechanism. However, because of the high level of somatic reversion detected by Southern analysis, we favor the hypothesis that this RNA is transcribed from revertant gene copies.

A third species of RNA of approximately 2.7 kb is 800 bases larger than the wild-type mRNA, however, it does not hybridize to pSD2, which is composed of sequences from the right portion of the *Mu1.7* element. It hybridizes to *Bronze1* probes 5′-ward (pD3MS9) and 3′-ward of the insertion site of *Mu1.7* and to a *Mu* probe (pAB5) which detects sequences in the left portion of *Mu1.7*. It does not hybridize to pSD2 which is composed of sequences in the right portion of the *Mu1.7* element.

Although sequencing of cDNA clones from the *bz1-mu2* allele will be required to determine the exact nature of all transcripts, based on the hybridization pattern we predict that this 2.7-kb mRNA results from a splicing event that begins within the *Mu1.7* sequence and terminates in the second exon of *Bz1*. A scan for potential splice sites in the *Mu1.7* element shows a sequence at nucleotide 1006 that contains 7 of 9 nucleotides of the consensus 5′ splice site, AAGGTGAGT (MOUNT 1982). The *Bz1* exon 2 contains two reasonable 3′ acceptor sites in the 100 bp surrounding the *Xho*II site; removal of this ~800-bp sequence from the 3.6-kb full length transcript would create an approximately 2.7-kb transcript.

The transcription termination effect of *Mu1* on the *Adh1-S3034* allele (ROWLAND and STROMMER 1985; VAYDA and FREELING 1986) is not seen in the *bz1-mu2* allele. Thus transcription termination is not a general effect of *Mu* element insertion in transcribed regions. The mechanism underlying the effect of *Mu* on transcription in the *Adh1-S3034* allele is unknown and could be specific to a particular site of element insertion within the locus. It should be noted that not only are the types of *Mu* elements and target genes different in these two examples but insertion in an exon (*Mu1.7* in *bz1-mu2*) may place far different constraints on gene expression than when the insertion occurs within an intron (*Mu1* in *Adh1-S3034*).

Nature of revertant alleles: Sequence analysis of excision products generated by transposable elements in plants has demonstrated a physical basis for the diversity noted in revertant alleles (SAEDLER and NEVERS 1985). It would be predicted that the frequency of recovery of phenotypically normal or near-normal revertants would depend on element type and insertion location, the propensity for precise excision events, and the impact of amino acid changes on the protein product. Studies of *Bz'* (purple seeds indicative of *bronze1* gene function) revertants recovered from other transposable element systems indicate that the *bronze1* gene product can be subsequentially altered and still produce phenotypically wild-type tissue. The same 650-bp *Bst*EII/*Sst*I fragment of the *bronze1* gene analyzed in this study is the site of insertion for two other transposable elements from which several stable *Bz'* derivatives have been recovered. Six of seven *Bz'* derivatives of *bz-m13* (*dSpm* inserted 33 bp downstream of the exon:intron junction (FEDOROFF *et al.* 1984; SCHIEFELBEIN *et al.* 1985; FURTEK 1986); had lower glucosyl transferase activity than the wild type progenitor allele, *Bz-McC* (NELSON and KLEIN 1984; V. RABOY and J. SCHIEFELBEIN, personal communication). DOONER and NELSON (1979) showed that in 15 stable *Bz'* derivatives of *bz-m2(DI)* [*Ds* element is inserted ~80 base pairs upstream of the *Xho*II site described in this study (DOONER *et al.* 1985; E. RALSTON and H. DOONER, personal communication)], two-thirds showed alterations in at least one of three criteria: enzyme specific activity, thermal stability or electrophoretic mobility. The other one-third were indistinguishable from the wild-type protein of the *Bz-McC* allele. The two classes may reflect alternative molecular events during excision from the *bronze1* locus. In this regard, precise excision of *Mu1.7* could generate a *bronze1* gene product containing three additional amino acids resulting from the 9-bp host sequence duplication. However, detailed analysis of cDNAs of mRNA from tissue containing the *bz1-mu2* allele will be required to determine the full spectrum of revertant events present after *Mu1.7* excision and to demonstrate whether an alternative splicing event removing part of the *Mu* element is responsible for an aberrant RNA product in some cases.

Role of *Mu1.7* in the mutator phenomenon: In the Mutator transposable element system up to 90% of the progeny of a Mutator parent can inherit the characteristic high level of mutation. This frequency dictates that multiple copies of autonomous elements must be transmitted to or be generated within each generation. We have maintained two independent lines of Mutator stocks that have been repeatedly outcrossed to non-Mutator lines. All of the >300 progeny we have tested for the presence of Mutator elements have contained both 1.4- and 1.7-kb size classes. Active Mutator lines originally containing a total of 10 copies or less of both size classes still maintain these elements even after 5 or 6 generations of outcrossing to non-Mutator lines. ALLEMAN and FREELING (1986) have reported an active Mutator line apparently devoid of *Mu1.7* elements; it will be informative to test these lines for pSD2 homologous sequences.

Elucidation of *Mu1.7* functions and interaction with other elements in the Mutator system is difficult by genetic techniques or molecular analysis. One way to test the activities of the various *Mu* elements that have been isolated is to introduce just one type of element into a non-Mutator line of maize. The introduction of DNA into maize protoplasts via electroporation (FROMM, TAYLOR and WALBOT 1985) and the subsequent regeneration of stably transformed callus (FROMM, TAYLOR and WALBOT 1986) offers a new approach to testing Mutator function. Experiments are in progress to follow the number and position of such introduced elements to document transposition. Once it has been established that a particular class of element is competent to transpose, mutated forms can be introduced to determine which nucleotide sequences are necessary for transposition. Assays to detect transcripts from the introduced copies or to recover excision products could then be performed.

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