Transcriptionally Active *MuDR*, the Regulatory Element of the Mutator Transposable Element Family of *Zea mays*, Is Present in Some Accessions of the Mexican land race Zapalote chico

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Manuscript received October 28, 1996
Accepted for publication January 2, 1998

ABSTRACT

To date, mobile *Mu* transposons and their autonomous regulator *MuDR* have been found only in the two known Mutator lines of maize and their immediate descendants. To gain insight into the origin, organization, and regulation of *Mutator* elements, we surveyed exotic maize and related species for cross-hybridization to *MuDR*. Some accessions of the mexican land race Zapalote chico contain one to several copies of full-length, unmethylated, and transcriptionally active *MuDR*-like elements plus non-autonomous *Mu* elements. The sequenced 5.0-kb *MuDR-Zc* element is 94.6% identical to *MuDR*, with only 20 amino acid changes in the 93-kD predicted protein encoded by *mudrA* and ten amino acid changes in the 23-kD predicted protein of *mudrB*. The terminal inverted repeat (TIR) A of *MuDR-Zc* is identical to standard *MuDR*; TIRB is 11.2% divergent from TIRA. In Zapalote chico, *mudrA* transcripts are very rare, while *mudrB* transcripts are as abundant as in Mutator lines with a few copies of *MuDR*. Zapalote chico lines with *MuDR*-like elements can *trans*-activate reporter alleles in inactive Mutator backgrounds; they match the characteristic increased forward mutation frequency of standard Mutator lines, but only after outcrossing to another line. Zapalote chico accessions that lack *MuDR*-like elements and the single copy *MuDR a1-mum2* line produce few mutations. New mutants recovered from Zapalote chico are somatically stable.

MOLECULAR, genetic, and anthropological data indicate that maize arose in what is now Mexico through domestication of teosinte, a grass closely related to present-day maize (Goodman and Brown 1988). Domestication is proposed to have occurred once, not more than 10,000 years ago (Doebley 1990). Because this is such a recent event on an evolutionary time scale, corn should have a very homogeneous genome. However, both the allelic diversity (Shattuck-Eidens *et al.* 1990) and the range of genome size (from 9.82 to 12.12 pg; Rayburn *et al.* 1985) are among the highest for any eukaryotic species. In the short time span since domestication, transposable elements and selection for growth in many habitats are proposed to have played important roles in first generating and then maintaining this diversity (Schwarz-Sommer *et al.* 1985; Kloeckener-Gruissem and Freeling 1995; Walbot 1996).

Characteristically, active transposable elements are found in a few populations within a species, and both the origin and maintenance of transposable elements remain enigmas (Capy *et al.* 1994). Within a lineage, transposons are transmitted in predictable, albeit non-Mendelian, patterns and often exhibit mechanisms to increase copy number. In addition, analysis of *P* and *Mariner* elements in *Drosophila* spp. and insertion sequences in bacteria implicate horizontal transmission as a possible explanation of the punctate distribution of active transposons, at least for some cases (Capy *et al.* 1994).

The Mutator transposons of maize are one of the most active transposable element families described in any organism. Standard Mutator activity is defined by a suite of characters: high forward mutation frequency (10^{-3} to 10^{-5} per gene per generation), frequent somatic excision late in development, and infrequent germinal excision ($<10^{-4}$ to 10^{-5} per gene per generation) (reviewed in Walbot 1992). These features have only been observed in plants derived from a single line of maize, first described as Mutator by Robertson (1978). These standard Mutator lines have multiple copies of a diverse family of transposable elements. *Mu* elements share ~210-bp Terminal Inverted Repeats (TIRs) and create 9-bp host sequence duplications at the site of insertion. The *Mu* transposon family is composed of at least six distinct subfamilies that are distinguished by unique internal sequences between the TIRs (reviewed in Bennetzen 1996). In standard Mutator lines, the 4942-bp *MuDR* regulatory elements are present in 5–50 copies (Hershberger *et al.* 1991), and non-autonomous *Mu* element copy numbers are even higher (Alleman and Freeling 1986; Taylor and Walbot 1987; reviewed in

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Walbot 1991). All of the *Mu* elements exhibit non-Mendelian inheritance, with copy number maintained on outcrossing by replicative transposition late in the sporophytic or during the gametophytic phase of the life cycle (reviewed in Walbot 1991; Bennetzen 1996). Collectively, the multi copy *Mu* elements increase the mutation frequency 20–100-fold or more above the spontaneous level (reviewed in Walbot 1992; Bennetzen *et al.* 1993). In these standard Mutator lines, loss of Mutator activity is an epigenetic phenomenon rather than the result of segregation of *MuDR*; loss of activity is correlated with increased methylation of the regulatory *MuDR* and non-autonomous *Mu* elements (reviewed in Chandler and Hardeman 1992; Bennetzen 1996).

A few exceptional Mutator lines contain only a single *MuDR* that segregates as a near-Mendelian factor; these lines were derived from standard, high copy number Robertson lines with the *a1-mum2* or *a1-mum3* reporter alleles (Robertson and Stinard 1989, 1992; Chomet *et al.* 1991; Lisch *et al.* 1995). In the most thoroughly analyzed example, the *MuDR* element is located on chromosome *2*L (Robertson and Stinard 1992; Lisch *et al.* 1995). At this location, it programs the standard pattern of high frequency somatic excision. However, few element insertions occur and both *MuDR* and *Mu* element copy numbers typically remain low (Lisch *et al.* 1995). Independently, Schnable and Peterson (1986, 1988, 1989a,b) described *Cy*/*r-cy*, a two-element transposable element system that has turned out to be a low activity Mutator line; *Cy* lines often contain a single, segregating regulatory element, now known to be a *MuDR* (Hsia and Schnable 1996). The sequence of *MuDR* (Hershberger *et al.* 1991) is identical to *Cy* (Hsia and Schnable 1996) and, with the exception of a single, inconsequential base change, identical to the single *MuDR* in the *a1-mum2* lines (James *et al.* 1993). Consequently, the differences between high- and low-activity Mutator lines cannot be explained by differences in the primary sequence of *MuDR*.

Based on Southern hybridization, all *Zea* species tested contain multiple, dispersed sequences homologous to segments of *Mu* elements; some of these widely shared sequences appear to be parts of genes that have become incorporated into a *Mu* element (Talbert *et al.* 1989). By Southern hybridization, TIR-homologous sequences are not found beyond the genus Zea and the maize X Tripsacum hybrid species *Tripsacum andersonii* (Talbert *et al.* 1990). However, genomic clones with sequences similar to *Mu* TIRs and limited regions of similarity to *MuDR* have been reported in rice (Eisen *et al.* 1994; Ishikawa *et al.* 1994). Within maize, *MuDR* is not widely distributed (Hershberger *et al.* 1991), nor is Mutator activity. The largest survey to date tested maize lines for a *Cy* capable of activating somatic instability of *bz1-rcy*. Schnable and Peterson (1986) found that active *Cy* elements were nearly restricted to the original *Cy* line and Robertson's Mutator lines. Weak *Cy* activity was found sporadically in a few plants in 7 of 47 other lines surveyed, but this rare occurrence could represent activation of cryptic regulatory elements from the *Cy* parent; this possibility could not be tested as *MuDR* had not yet been cloned. Because standard Mutator activity creates so many mutations, it is not surprising that *MuDR* is apparently missing from most strains of corn.

We are interested in whether *MuDR* can be maintained in the maize genome. To address this question, we surveyed for *MuDR* in American inbreds, exotic maize lines, and *Zea* spp. by Southern blot hybridization. While nearly all lines had cross-hybridizing fragments, only the Mexican land race Zapalote chico had a multicopy cross-hybridizing band of approximately the correct size. Analysis of different accessions of Zapalote chico demonstrated that only a subset of the population contains *MuDR*-like elements; only the Zapalote chico lines with *MuDR*-like elements exhibited a high forward frequency.

The sequenced example of a *MuDR*-like element of Zapalote chico (*MuDR-Zc*) is highly similar to *MuDR* and encodes similar transcripts. Unlike standard Mutator lines, which generate new mutants during selfing and outcrossing, Zapalote chico exhibits hybrid dysgenesis. Self-pollinated lines produce few mutations, but outcrosses to non-Mutator lines activate a high forward mutation frequency. In addition, new mutants are somatically stable, at least in seedlings. Zapalote chico is cultivated by the Zapotecs, a Native American people of Oaxaca, Mexico. This line is their economic staple and by their oral history has been cultivated for more than 5000 years, tracing to their cultural origin in the highlands of Central Oaxaca. We discuss the possibility that selection for a high-yielding stable crop has resulted in the novel properties of the Mutator system in Zapalote chico.

MATERIALS AND METHODS

Mutator terminology: *MuDR* is the regulatory Mutator element, and this name replaces prior nomenclature: *MuA2* (Qin *et al.* 1991), *MuR1* (Chomet *et al.* 1991), *Mu9* (Hershberger *et al.* 1991), and *Cy* (Hsia and Schnable 1996). *Mu* elements share ~200-bp TIRs with *MuDR* and require transcriptionally active *MuDR* to be mobilized.

Plant material: Active Mutator individuals with the *bz2-mu4::MuDR* allele (family M87) were used as the Mutator stock for DNA and RNA analysis; this is a multicopy, standard Mutator line with a *MuDR* transposon inserted in the second exon of the *bz2* gene (Hershberger *et al.* 1991). The *bz2* tester line in inbred W23 was used as the non-Mutator stock for most molecular analyses and for the mutagenesis tests; an *a2* tester constructed in W23 by this lab, and an *a2* tester obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL) in a mixed nuclear background, were used for the Southern blot survey. For the original survey, all of the exotic lines of maize and the *Zea* spp. (listed in 1982 inventory as *Zea mexicana* luxurians, mexicana, nobogame, parviglumis, and peruviensis, *Z. perennis*, and *Z. diploperennis*) were obtained from the Stock Center. The *Zea* spp. collections now reside at the USDA Plant Introduction Station (Ames, IA). For subse-

quent experiments, existing Zapalote chico accessions were obtained from Pioneer Hi-Bred (Johnston, IA) and an overlapping set from CIMMYT (International Maize and Wheat Improvement Center, Texcoco, Mexico). Both Ronald Phillips and Richard Kowles provided several generations of crosses between Zapalote chico (cytogenetically many knobs) and Wilbur's Knobless Flint. Thirty-five new accessions were collected as individual ears in Juchitán, (Oaxaca, Mexico) (16.15N, 95.00W) directly from Zapotec farmers; two Tuxpeño X Zapalote chico F_1 hybrids and the F_2 backcross ears were donated by M. C. Arredondo, a retired Mexican corn breeder living near Juchitán. Tuxpeño is a widely adapted inbred line developed in Mexico and used as the foundation for breeding experiments.

Forward mutation test: Individuals were self-pollinated to assess the phenotypes of any pre-existing mutations and crossed as pollen to the *bz2* tester. The outcross seed were planted, and the F_1 plants were self-pollinated, yielding F_2 ears. Thirty progeny kernels of the F_2 and selfed parental ears were planted side-by-side in the summer field; mutants were counted in the F_2 only if they were clearly distinguishable from any segregating phenotype in the parent. All novel phenotypes recorded appeared to be recessive, present in ~one-quarter of the progeny. In ambiguous cases, *i.e.*, in which there was low germination or only one or a few mutant plants were present, a second sample of 30 kernels was planted and evaluated. Somatic mutability was scored by eye and by observation through a stereozoom microscope (×20).

Ten kernels of each Zapalote chico accession were grown in summer, 1993 (M designations), at Stanford. Seed were planted in late June to promote flowering, because maturation of neotropical maize is inhibited by long days in the temperate zone. In most accessions, only a few individuals reached maturity within 75–90 days and could be both self-pollinated and crossed as pollen parent to *bz2* tester. In the 1994 winter nursery in Molokai, Hawaii, Zapalote chico lines matured within 50–55 days, and additional representatives of some lines were self-pollinated and crossed to *bz2* tester. Of all the Zapalote chico samples examined, one line M59 = N234 was the most consistent in flowering at Stanford, and additional individuals of the original accession were tested for forward mutations during 1994–1995. To assess spontaneous mutation frequency in a non-Mutator line, the crossing scheme was used with the *bz2* tester. Four standard Mutator lines were used for comparison; two lines were selfed and crossed to *bz2* in 1993 (M88, *bz2-mu2::Mu1* reporter allele; M121, *bz2-mu1::Mu1* reporter allele), and two lines in 1994 (N190, *bz1-mu1::Mu1* early somatic excision line; N285, Mutator with *Bz1*-revertant alleles from the early excision of the *bz1-mu1::Mu1* reporter allele). Two *a1-mum2::Mu1*, single *MuDR* lines were obtained from D. Robertson and evaluated in 1994 to compare the low *MuDR* copy number lines to Zapalote chico.

RNA blot analysis: Immature ears were collected from field-grown material of each line during the summer of 1994. Tissue was immediately frozen in liquid nitrogen and stored at −80° until RNA isolation. RNA was isolated by grinding the samples in liquid nitrogen, then extracting with Tri-Reagent (Molecular Research Center, Cincinnati, OH). Poly(A)$^+$ RNA was purified from total RNA using a Mini-oligo(dT) cellulose spin column kit (5 Prime→3 Prime, Boulder, CO).

For the RNA blots, 16–20 μg of poly(A)$^+$ RNA was electrophoresed through an agarose formaldehyde gel for 6 hr and transferred to Hybond-N (Amersham, Arlington Heights, IL) using standard techniques (Sambrook *et al.* 1989). Two probes were generated by PCR amplification from p*MuDR*. This plasmid was constructed and sequenced by R. J. Hershberger; it contains a full-length *MuDR* element, recovered from the *bz2-mu4::MuDR* allele (Hershberger *et al.* 1991), with a one base frameshift mutation in *mudrA* that allows maintenance in *Escherichia coli*. Probe PA contains 927 nucleotides of *mudrA* (positions 183–1100), and PB contains 978 nucleotides of *mudrB* (positions 3774–4752). A third probe, BX1.0 (Hershberger *et al.* 1995), was recovered from a *Bam*HI (nucleotide position 2865) to *Xba*I (nucleotide position 3945) digest of p*MuDR*; this probe recognizes both *mudrA* and *mudrB* (Figure 1). Probes were labeled by the random primer method, using the DECAprime II Kit from Ambion, Inc. (Austin, TX) (Feinberg and Vogelstein 1983) and purified on push columns (Stratagene, La Jolla, CA). Prehybridization and hybridization were performed according to the protocol published for GeneScreen (Du Pont, Wilmington, DE) using 10% dextran sulfate. Filters were washed once in 2× SSPE, 1% SDS at room temperature for 10 min, once in 1× SSPE, 1% SDS at 65° for 15 min, and once in 0.1× SSPE, 0.1% SDS at 65° for 15 min. Autoradiography was performed for 12–72 hr at −80° using two intensifying screens.

DNA blot analysis: Maize genomic DNA was prepared from immature ears of selfed Zapalote chico accessions grown in 1994 (N designations in Tables) and purified as described by Stapleton and Walbot (1994). For Southern analysis, three μg of DNA were digested with restriction enzymes (BRL, Gaithersburg, MD) according to the manufacturer's instructions, electrophoresed through agarose gels, and blotted onto Hybond-N (Amersham). Probes were prepared as described above. The blots were prehybridized, hybridized, and washed as recommended by the membrane manufacturer. To quantify *MuDR* copy number, a plasmid containing *MuDR* was digested with *Sst*I or *Sst*I/*Dra*I, diluted to the proper concentration equivalent to a specific copy number in the maize genome, and electrophoresed next to restriction digests of maize genomic DNA. Blots were probed with BX1.0. In some cases, stripped blots were reprobed with a 380-bp fragment of *Adh1* as a loading control. To check for the presence of *Mu1* and the related *Mu2* elements, probe pA/B5 was used (Taylor and Walbot 1987).

DNA amplification by polymerase chain reactions (PCR): DNA amplification reactions were performed in volumes of 25–100 μl overlaid with 50–100 μl paraffin oil. Each reaction contained 0.2 mm of each of the four deoxyribonucleotides, 100 ng of each oligonucleotide primer, a buffer (15 mm Tris pH 8.3, 50 mm KCl, 1% Gelatin, 1.8 mm $MgCl_2$), Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 50–100 ng of DNA. PCR reactions were carried out for 30 cycles of 1 min at 94°, followed by 1 min at 55°, and 1 min at 72°. The following DNA primers were used for *mudrA*: primer #183 5′-CGCCGT CTGGCAGGGCCTCTTGTCACCGTCTC-3′ with primer #1996 5′-GAATGTCATAGGTTGCATAG-3′ or primer #2017 5′-GATA CGTTGGATACTGTAAG-3′ with primer #2282 5′-TATGGAT GTAGAGACCTTAG-3′. For the *mudrA*/intergenic region primer #2281 5′-GATTCCAGAGATGTAGGTAT-3′ was used with primer #813 5′-CCAACCAAAGTAAGACCACA-3′. For the intergenic region to *mudrB* region, primer #2019 5′-GCCATTAGTTCTT ACAACCT-3′ was used with primer #2109 5′-ACAATACGCGT TAACCAAACA-3′. To amplify *mudrB* primer #3773 5′-CTTGT ACAGATCTTGTGACCAGTCGCA-3′ was used with primer #4752 5′-GTCCACAAATCGATGTTACGGTCGTT-3′. For TIRA primer #2466 5′-GCTGAGCCTCCTGCAGGGAGATAATTGCC-3′ was used with primer #2467 5′-CCATGGTACCAAAATCAG AG-3′. The resulting fragment contains all of TIRA, plus the region of the 5′ untranslated region that contains a transcriptional start site. To amplify TIRB, primer #2468 5′-TGAACGCCT CCTGCAGGAGAGATAATTGC-3′ was used with primer #2470 5′-CAATCGGTACCCACAGGAGCAAGAG-3′. The resulting fragment contains TIRB plus the 5′ untranslated region of *mudrB*.

Plasmids: Seven plasmids were constructed by amplifying genomic DNA of Zapalote chico line N215 by PCR with the

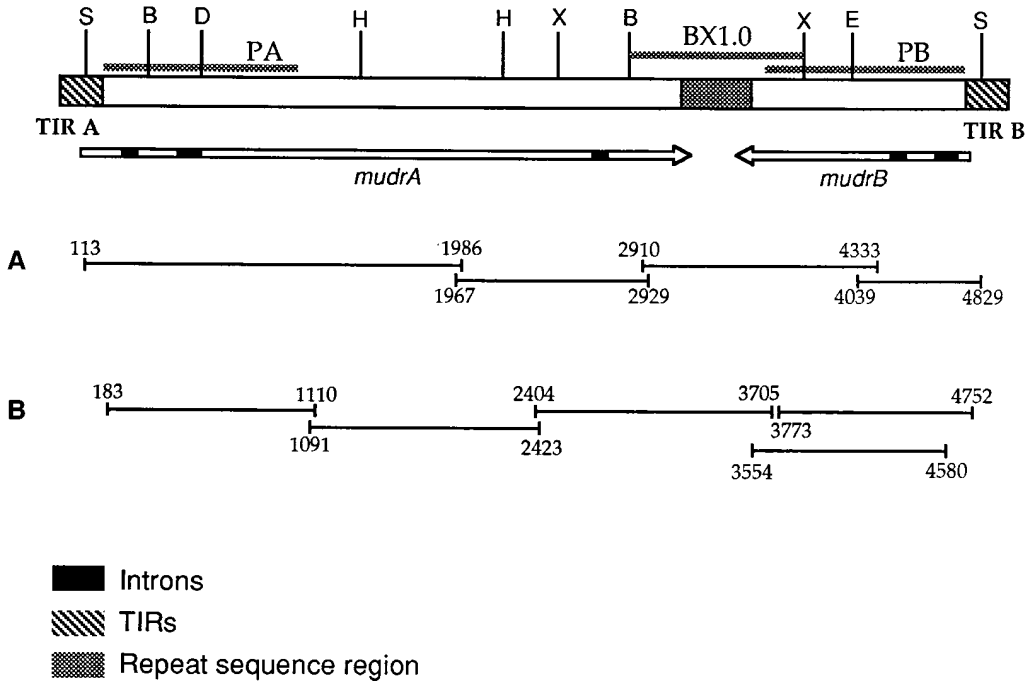

Figure 1.—Diagram of *MuDR*. DNA probes (PA, PB, BX1.0) used in Southern and Northern hybridizations are shown above the transposon structure. Below the transposon, the *mudrA* and *mudrB* convergent transcription units are illustrated; the first intron of each transcript is spliced ~100%, the second introns are spliced ~80%, as is the third intron of *mudrA*. A 120-bp in-frame intron of *mudrB* that is spliced in about 5% of transcripts of *MuDR* is not shown. (A) Regions of *MuDR* for which PCR amplification was attempted in various accessions of Zapalote chico. (B) PCR fragments generated in the cloning of the *MuDR*-like element. Nucleotide sequence numbering according to Hershberger *et al.* (1991). B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; X, *Xba*I.

primers listed above. Amplified fragments were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The *MuDR-zc* element was cloned in overlapping fragments, because the full-length *MuDR* is toxic to *E. coli*. Bracketed numbers to the right of each plasmid correspond to the base pairs of the standard *MuDR* sequence (numbering according to Hershberger *et al.* 1991). Plasmid pTIRAzc [1–455] has a 455-bp insert; pA1zch [183–1110] has a 927-bp insert; pA2zch [1091–2423] has a 1332-bp insert; pA3zch [2404–3705] has a 1301-bp insert; pA4zch [3554–4580] has a 1026-bp insert; pB1zch [3773–4752] has a 979-bp insert; and pTIRBzc [4476–4944] has a 468-bp insert.

DNA sequencing: *MuDR-Zc* regions were obtained as restriction fragments from the pAzch plasmid series and were subcloned into the M13mp19 vector for single-stranded sequencing (Norrander *et al.* 1983) with the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) or with the ABI 310 fluorometric automated sequencer. Both strands of all fragments were fully sequenced. To eliminate compression of bands that occurred when sequencing GC-rich regions, mixes containing deoxyinosine provided with the kit were used. Primers for sequencing were commercially available M13 primers; a few custom internal primers were used on long fragments. The *MuDR-Zc* sequence is registered in GenBank as accession number U75360.

RESULTS

Properties of *MuDR*: *MuDR* encodes two, convergently transcribed genes (Figure 1). The major transcription initiation sites are in the TIRs, and the most abundant transcripts are 2.8 kb (*mudrA*) and 1.0 kb (*mudrB*). Intron skipping, multiple polyadenylation sites, and a second transcription initiation site in the 5′ UTR of *mudrA* result in four distinct transcript types for each gene (Hershberger *et al.* 1995). Although the exact roles of the proteins encoded by *mudrA* and *mudrB* are unknown, *mudrA* encodes a polypeptide with homology, over an extended motif of ~150 amino acids, to a suite of bacterial transposons (Eisen *et al.* 1994). In addition, it has recently been demonstrated that *mudrA* encodes a DNA-binding protein which binds to specific sequences within the highly conserved *Mu* TIR, leading to the proposal that MURA is a transposase (Benito and Walbot 1997). Deletions within *mudrA* in lines with a single *MuDR* abolish somatic instability of the *a1-mum2* reporter allele; this evidence demonstrates that *mudrA* is essential for somatic excision (Hsia and Schnable 1996; Lisch and Freeling 1994).

Distribution of *MuDR* elements: Previously, we reported that the 4.7-kb *Sst*I fragment characteristic of an intact, unmethylated *MuDR* element (Figure 1) was multicopy in Mutator lines and was not present in standard inbreds of maize (Hershberger *et al.* 1991). Most non-Mutator lines did contain various sized fragments that hybridized to one or more internal *MuDR* probes, but there was no evidence for intact *MuDR* elements. To expand the analysis of distribution of *MuDR*-like elements, genomic

Southern blotting was used to screen additional inbreds, exotic lines, and *Zea* spp. for intact *MuDR* elements. Genomic DNA was digested with *Sst*I, which recognizes sites in unmethylated TIRs of *MuDR*, a Southern blot was prepared, and then hybridized with the BX1.0 fragment, which contains the 3′ portions of both *MudrA* and *MudrB* and the intergenic region (Figure 1).

Of the lines examined by this Southern blot survey, a Co-op accession of Zapalote chico had a fragment about the size expected for *MuDR* (~5.1 kb). This fragment was slightly larger than *MuDR* and was present in ~3–5 copies per genome (data not shown).

All of the other exotic lines examined, including Argentine popcorn, Tama flint, Strawberry popcorn, Papago flint, gourd seed, Northern flint, *Z. perennis*, *Z. diploperennis*, and five teosinte types (see materials and methods), hybridized weakly to the central *MuDR* probe. Similar cross-hybridization has been found in some (W23, K55, and A188) standard maize inbred lines (data not shown).

Screening for *MuDR* in Zapalote chico lines by PCR and Southern analysis: The first Zapalote chico sample examined was collected in the 1950s from Oaxaca Mexico; it has been maintained by the Maize Genetics Stock Center, by periodically growing and selfing the line. Using eight sets of PCR primers that spanned most of *MuDR*, we determined that all regions of this putative regulatory element in Zapalote chico could be amplified from an immature ear DNA sample of one individual. Furthermore, seven of the fragments were the expected size, and each of these contained one or two restriction sites at the same positions as in *MuDR*. There were no polymorphisms for the 12 enzyme sites examined (data not shown). With the primer pair that spanned the intergenic region, however, several size variants were detected, ranging from 100 to 300 bp larger than the comparable region of *MuDR* (data not shown). As the intergenic region is composed of a complex set of repetitive elements (Hershberger *et al.* 1995), we hypothesized that there had been an expansion of these motifs. Collectively, the results suggested that Zapalote chico contained elements that were very similar to *MuDR*.

To assess the distribution of *MuDR*-like elements in Zapalote chico populations, existing accessions were obtained from three other sources: seven examples of old accessions were obtained from CIMMYT, and a mostly overlapping set was obtained from Pioneer Hi-Bred. The CIMMYT materials were collected in the 1950s and 1960s, but then maintained under different growth conditions in central Mexico and in Iowa. Ronald Phillips and Richard Kowles contributed Zapalote chico X Wilbur's Knobless Flint hybrids, derived from a CIMMYT accession. Zapalote chico is classified as a land race, but it is also an economically important line. It is the only corn variety grown by the 300,000 Zapotecs living in southwestern Mexico. To obtain a current representation of Zapalote chico, 35 new accessions were collected in 1993 from farmers and from a corn-breeding program located in the main Zapotec population center, near Juchitán in the state of Oaxaca, Mexico. Most of these lines were successfully propagated at Stanford University in summer 1993. We conducted a more extensive investigation of the distribution of *MuDR* elements using Southern blot analysis and PCR experiments of genomic DNA samples from accessions that could be self-pollinated.

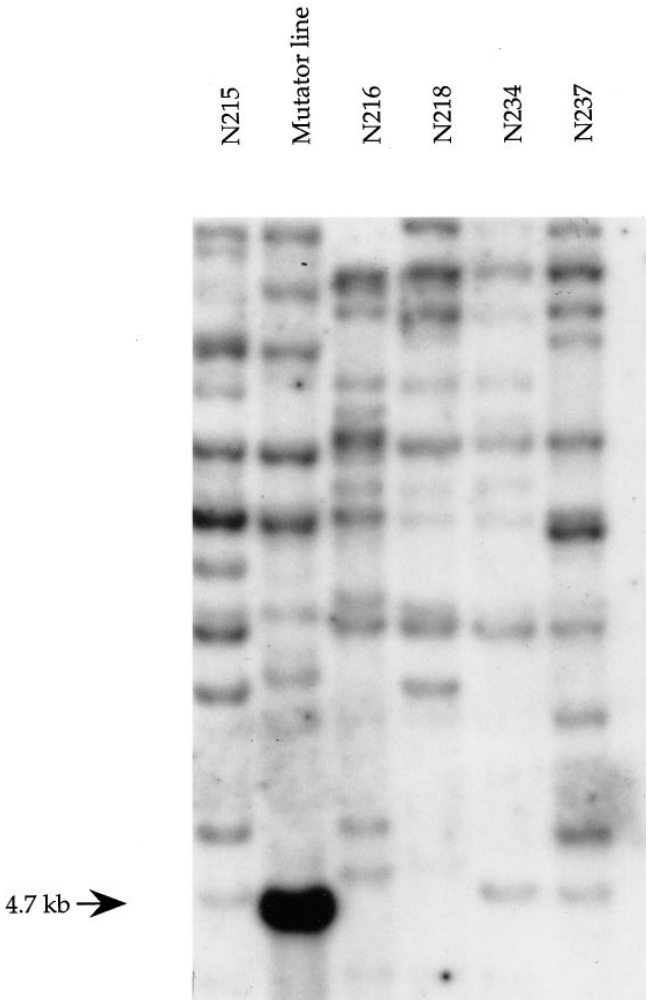

Figure 2.—Screening for *MuDR* in Zapalote chico lines by Southern analysis. 6–8 μg DNA samples were digested with *Sst*I and probed with BX1.0.

For Southern blot analysis, genomic DNA samples most likely to contain full-length elements (based on PCR screening; see Table 1 below) were digested with *Sst*I and probed with BX1.0. Figure 2 shows the ~4.7, kb *Sst*I fragment characteristic of an intact *MuDR* element was conserved in lines N215, N234, and N237, although the Zapalote chico hybridizing bands were always slightly larger (~50–100 bp) than *MuDR* from a standard Mutator line. Zapalote chico line N216 contained a fragment that is ~250 bp larger than *MuDR*; it may be similar to the larger *MuDR*-like element originally identified in the Maize Stock Center material.

TABLE 1

PCR survey for *MuDR* sequence in different accessions of *Zapalote chico*

	Primer sequence numbers			
Accessions	113–1986[a]	1967–2929	2910–4333	4039–4829
N200[c]	+[b]	−	−	+
Oax 50[c]	+	+	+	+
N201[c]	+	+	+	+
Oax 48[c]	+	+	−	+
N204[c]	−	−	−	+
N205[c]	−	−	−	−
N206[c]	−	−	−	−
Chis 224[c]	+	+	+	+
N207[c]	+	+	−	+
N211[d]	+	+	−	−
N213[d]	−	+	+	+
N214[d]	−	−	+	−
N215[d]	+	+	+	+
N216[d]	+	+	+	+
N217[d]	−	+	+	−
N219[d]	+	+	−	+
N220[d]	−	+	−	−
N221[d]	−	+	−	−
N222[d]	+	−	+	−
N226[d]	+	−	+	−
N230[d]	−	−	−	−
N234[d]	+	+	+	+
N236[e]	−	+	−	−
N237[e]	+	+	+	+
N240[f]	+	−	+	−
N241[g]	−	+	+	−
N249[g]	−	−	−	−
N252[h]	−	+	−	+
N255[d]	+	+	−	−
N257[d]	−	−	−	−
N264[c]	+	−	−	+
N267[c]	−	+	+	−
M4-1[c]	+	+	+	+

[a] Region amplified by PCR. Nucleotide numbering of *MuDR* according to Hershberger *et al.* (1991).
[b] Plus symbol indicates amplification of this region by PCR.
[c] Accession obtained from CYMMIT and grown at Stanford.
[d] Accession collected from farmers in Oaxaca, Mexico.
[e] Accession was a cross of Tuxpeño with Zapalote chico.
[f] Accession obtained from Maize Stock Center.
[g] Accession obtained from R. Phillips.
[h] Accession obtained from R. Kowles; F_1 hybrid of Zapalote chico and Wilbur's knobless flint.

To extend our analysis to additional lines, PCR experiments were carried out on samples from individual selfed progeny, using four sets of primer pairs that span *MuDR* (Figure 1). As shown in Table 1, eight samples (24%) yielded PCR products of the expected size with all four primer pairs; these samples represent seven distinct accessions, with the Oaxaca 50 accession represented from two distinct sources (N201 and Oaxaca 50 directly from CIMMYT). The majority (6/8) of the accessions positive for *MuDR*-like elements represented the most recently collected material, indicating that *MuDR*-like elements exist in the current Zapotec crops. Twenty accessions (61%) yielded products from a subset of the primer pairs, and five accessions (15%) did not seem to contain any amplifiable fragments. In this analysis, the particular individual sampled from the Maize Stock Center lineage (N240) gave a positive PCR result with only two primer pairs, suggesting that this individual did not contain an intact *MuDR* element. Also, two of the CIMMYT lines, Oaxaca 48 and Chiapas 224, yielded different PCR products in the two versions sampled. We conclude that there is heterogeneity within some accessions, reflecting either heterogeneity in the original material or changes during propagation at stock centers.

For a more detailed analysis of these *MuDR*-like elements, the PCR fragments generated from lines N234

and N215 were digested with enzymes for restriction sites present in the transcribed region of authentic *MuDR* elements. All ten of these enzymes produced fragments of identical size in digests from active Mutator lines and both N234 and N215 Zapalote chico accessions (data not shown). These data suggest that the differences in length between Zapalote chico *MuDR*-like elements and *MuDR* will be found in the TIRs and/or in the intergenic region.

Inheritance of the *MuDR*-like element: To examine the propagation of *MuDR*-like elements through outcrosses with non-Mutator lines, Southern blots were perfomed in two lineages: (N234 = P56) and CIMMYT accession Oaxaca 2 (N264 and N265). The founder individual (M59) of the N234 lineage appears to have one copy of a *MuDR*-like sequence per haploid genome; it is 100 bp larger than *MuDR*. The M59 founder was crossed to *bz2* tester, a non-Mutator source, and the progeny (individuals of family P56) contain ~1 copy of *MuDR* (Figure 3A, and as discussed below a PCR survey of 28 P56 individuals were all positive for *MuDR*). All P56 individuals could contain a single copy of *MuDR* if the founder had been homozygous for one *MuDR* locus. When individuals of P56 were outcrossed a second time to *bz2* tester, the copy number of ~1 is maintained (compare parent P56-12 and outcross progeny, lanes 1 and 3; parent P56-17 and outcross progeny, lanes 2 and 4; Figure 3A). Figure 3B provides more evidence for transmission of the element through two outcrosses. M3-4 (lane 1) contains the *MuDR*-like element, and this element is maintained when outcrossed to *bz2* tester (MH3, lane 2) and when MH3 was selfed to produce line N265 (lane 3). Siblings of line N265 are shown to contain the element (O70, O70-1, and O70-4, lanes 4–6), which is again maintained on selfing (OH59, P61, lanes 7 and 9), as well as after a second outcross (O70-4 × *bz2*, lane 8).

Figure 3C shows a lineage of progeny of M3-1 (a sibling of M3-4), which was outcrossed once, repeatedly selfed, and outcrossed once more. Selfed progeny of M3-1 do not show the *MuDR*-like element (lane 1). MH2 (lane 2), selfed F_1 progeny from an M3-1 outcross to *bz2* tester, do not show the element. Selfing of MH2 progeny produced line N264-1 (lane 3), which also lacks the *MuDR*-like element. However, after selfing of N264-1, bands of the correct *MuDR*-like element size appear in the siblings O69-5 and O59-9 (lanes 4 and 5) and persist in the F_1 of an outcross of O69-9 to *bz2* tester (lane 6). These results demonstrate that cryptic, presumably methylated copies of *MuDR*-like elements exist in Zapalote chico accessions, and that these elements can appear during a crossing program.

Larger hybridizing bands are present in all of the above Southerns, including Tuxpeño, a non-Mutator line. The relationship of these larger fragments to *MuDR* cannot be ascertained from the available data, although it is interesting that at least some part of *MuDR* is widely distributed in the genus. The fragments could represent disrupted copies of *MuDR*, sequence similarity to either *mudrA* or *mudrB*, or methylated intact *MuDR* element as we suggest for the M3-1 individual. *MuA*, for example, is a larger *MuDR*-like element recovered from a Mutator line; it is disrupted by several insertions (Qin and Ellingboe 1990). Internal deletions within *MuDR* that retain the TIRs produce *Sst*I fragments smaller than 4.7 kb (Hershberger *et al.* 1995), but deletions missing the *Sst*I site of one TIR could yield fragments larger than *MuDR*. As epigenetic loss of Mutator activity is correlated with DNA methylation, the larger fragments could also represent modified *MuDR*-like elements. The *Sst*I (=*Sac*I) sites (GAGCTC) in the TIRs are not followed by either G or NG, consequently, methylation of the "canonical" substrates CpG and CpNpG cannot explain the inability of these enzymes to digest methylated (epigenetic loss) *MuDR* elements (Martienssen and Baron 1994). Maize DNA can be methylated at other C residues (Wang *et al.* 1996), and it is possible that methylation at one or both of the internal C residues prevents digestion.

Distribution of *MuDR*-like elements in Zapalote chico families: Given the diversity between and within accessions of Zapalote chico, we wished to determine the inheritance of *MuDR*-like elements in individual lines in which a founding individual was demonstrated to contain one or a few copies of the *MuDR*-like element. Our strategy was to PCR amplify the *MuDR*-like element in two halves (positions 113–2423, yielding a 2310-bp fragment, and from positions 2404–4829, yielding a 2425-bp fragment) that cover nearly the entire element. PCR analysis of 28 individuals of line P56 (progeny of *bz2* × M59) indicated that all were positive for both halves of the *MuDR*-like element (data not shown); these data indicate either homozygosity of the M59 parent (although it was estimated to contain only a single *MuDR*-like element by Southern blotting) or copy number maintenance. Line P57 are progeny of the original Zapalote chico accession M62 (Tuxpeño X Zapalote chico) crossed to *bz2* tester. In the 28 second outcross progeny examined, all tested positive for both halves of *MuDR* (data not shown). As the original individual had only 1–2 copies of *MuDR*, it seems likely that copy number is maintained in the stock either by transposition or by recruitment of formerly cryptic elements.

We conclude from the combination of PCR analysis and Southern blot hybridization tests that the parental Zapalote chico lines, which had only 1–2 copies of the *MuDR*-like element, transmitted the element to all progeny examined. This is circumstantial evidence that replicative transposition of the *MuDR*-like element occurs in Zapalote chico as is proposed for all *Mu* elements in standard lines (Bennetzen 1996). The analysis is compromised, however, by possible recruitment of cryptic *MuDR*-like elements during the crossing scheme.

DNA sequence analysis: Because *MuDR* and the gene, cDNA, and exon3 of *mudrA* are unstable in *E. coli*, the

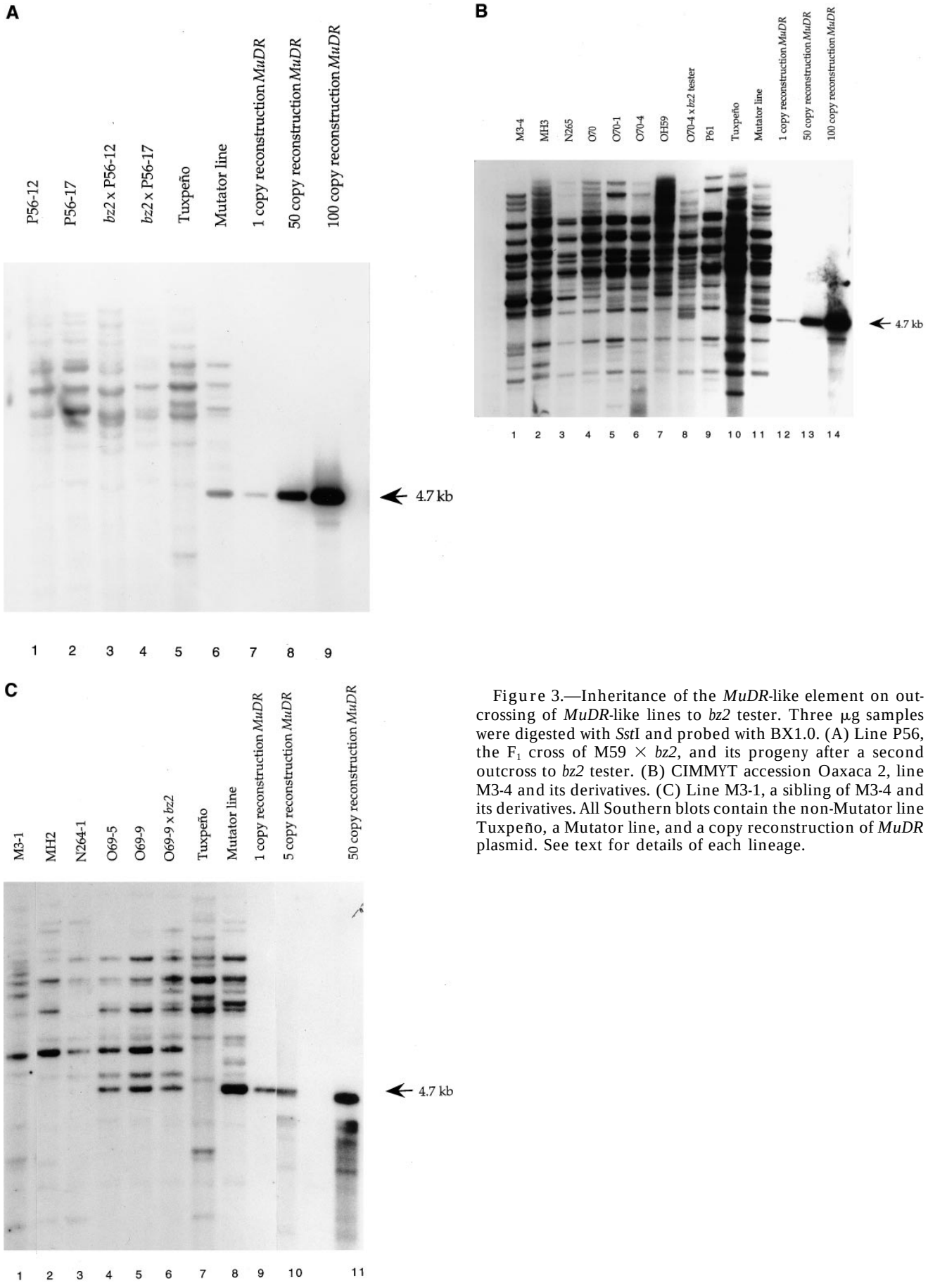

Figure 3.—Inheritance of the *MuDR*-like element on outcrossing of *MuDR*-like lines to *bz2* tester. Three μg samples were digested with *Sst*I and probed with BX1.0. (A) Line P56, the F_1 cross of M59 × *bz2*, and its progeny after a second outcross to *bz2* tester. (B) CIMMYT accession Oaxaca 2, line M3-4 and its derivatives. (C) Line M3-1, a sibling of M3-4 and its derivatives. All Southern blots contain the non-Mutator line Tuxpeño, a Mutator line, and a copy reconstruction of *MuDR* plasmid. See text for details of each lineage.

element and large subclones of it cannot be stably maintained on bacterial plasmids. Stable derivatives inevitably contain frameshift and deletion mutations that destroy the large open reading frame within exon3 (Hershberger *et al.* 1991, 1995). As we wished to obtain the sequence of the *MuDR*-like element of Zapalote chico without selecting for mutations during cloning, segments of the *MuDR*-like element(s) of line N215 were cloned in five overlapping fragments. The cloning strategy is shown in Figure 1. Given that Southern analysis indicated only that ~3 copies of a *MuDR*-like element are present in N215 (Figure 2) and that the 10-enzyme-restriction survey demonstrated that the reading frames had the expected enzyme sites, we reasoned that an element sequence assembled from these pieces would represent a single, full-length element. It is possible, however, that the individual pieces sequenced are from different, but very closely related, elements.

The complete sequence for the deduced element assembled from the overlapping clones is shown in Figure 4, and the sequence comparisions to *MuDR* are summarized in Table 2A. The *Sst*I/*Sst*I internal fragments of *MuDR* and the cloned *MuDR*-like element from line N215 share 94.6% DNA sequence identity. In comparison to the known sequence of *MuDR*, it is possible to identify two putative coding regions in the *MuDR*-like element which correspond to the *MudrA* and *MudrB* genes of *MuDR*. The greatest divergence between the two elements is found in the intergenic region and in the sequence of the first intron of the *MudrA* gene. The intergenic region of the *MuDR*-like element contains a number of nucleotide insertions, including an additional copy of a direct repeat sequence; these insertions likely account for the slightly higher apparent molecular weight of *MuDR*-like *Sst*I framents on genomic Southern blots (Figure 2). However, the putative coding regions and intron locations are highly conserved. As shown in Table 2B, there are only 10 nonsynonymous codon changes in MURB, and 20 nonsynonymous changes in the much larger MURA. Based on the high degree of conservation of the MURA and MURB proteins, we will designate the *MuDR*-like element of N215 Zapalote chico as *MuDR-Zc*.

Comparison of the TIR sequences of *MuDR* and N215 showed that TIRA of *MuDR-Zc* is 100% identical to TIRA of *MuDR*, whereas TIRB of *MuDR-Zc* is 91% identical to the TIRB of *MuDR*. The two TIRs of *MuDR-Zc* are only 88.1% identical to each other. Although the left and right TIRs of other *Mu* elements are rarely identical, the extent of divergence between the TIRs of *MuDR-Zc* is much higher than in *Mu1–Mu8* (Walbot 1991). In *MuDR*, there are only two base changes in the first 180 bp of the TIRs, and overall the 215-bp TIRs are 96% identical (Hershberger *et al.* 1991). These nearly identical TIRs of *MuDR* contain the promoter regions and major transcription start sites for the two genes (Benito and Walbot 1994; Hershberger *et al.* 1995).

Transcription of *MuDR-Zc*: The biological significance of *MuDR-Zc* is best addressed by determining whether *MuDR-Zc* is an active element. Active and inactive Mutator lines can be distinguished by the presence or absence, respectively, of *MuDR*-hybridizing transcripts. The expression of the *MuDR-Zc* was examined by Northern blot hybridization. Figure 5 shows the analysis of a standard Mutator line and several Zapalote chico lines that yielded PCR (N201), or both PCR and Southern hybridization results (N215, N234, and N237), consistent with full-length *MuDR*-like elements. In poly(A)$^+$ RNA samples, the active Mutator plants of standard lines have abundant transcripts for both *mudrA* (Figure 5A, lane 5) and *mudrB* (Figure 5B, lane 6). The *MuDR* transcripts are relatively abundant as they are readily observed in total RNA (data not shown; see Hershberger *et al.* 1995). In the Zapalote chico samples, however, it was technically difficult to visualize *mudrA* transcripts using total RNA. With poly(A)$^+$ RNA, very low levels of *mudrA* could be detected as a faint band of ~2.8 kb (Figure 5A, lanes 1–3). These transcripts are similar in size to those produced by the standard Mutator plants. The Zapalote chico *mudrB* transcripts were easily detected with poly(A)$^+$ RNA (Figure 5B, lanes 1–4). Surprisingly, the *mudrB* probe identified two different sized transcripts, one slightly larger (1.05 kb) and one slightly smaller (0.95 kb) than the 1.0-kb transcript from the standard Mutator line.

Unexpectedly, we also observed novel-sized RNAs in the non-Mutator *bz2* tester line that hybridized with the *MuDR* probe (Figure 5A, lane 4, and Figure 5B, lane 5). Similar size transcripts are also present at very low, comparable levels in the standard Mutator sample and in some of the Zapalote chico samples. The *mudrA* and *mudrB* gene probes may fortuitously recognize ubiquitous maize transcripts. In standard Mutator lines, transcripts as long as the entire element (4.9 kb) and truncated transcripts from internally deleted *MuDR* elements have been reported (Hershberger *et al.* 1995). However, the cross-hybridizing material in the poly(A)+ sample from *bz2* tester is the first report of any cross-hybridization with a non-Mutator line.

Non-autonomous *Mu* elements in Zapalote chico accessions: One hallmark of active Mutator lines shared by both standard and low copy number lines is the presence of unmethylated *Mu* elements. For the *Mu1* and related *Mu2* elements, methylation status is conveniently assessed by Southern blot analysis after digestion with *Hin*fI. There is a recognition site for this enzyme near the end of each TIR of the 1.4-kb *Mu1* and 1.7-kb *Mu2* elements. As shown in Figure 6, derivatives of accessions N201, N215, N234, and N237, four accessions with full-length *MuDR*-like elements, yield both the 1.3- and 1.6-kb expected fragments that hybridize to a probe that can detect both *Mu1* and *Mu2*. Considering the *Mu1* and *Mu2* elements together, it appears that the Zapalote chico accessions examined contain ~3–10 cop-

GAGATAATTGCCATTATAGACGAAGAGCGGAAGGGATTCGACGAAATGGAGGCCATGGCGTTGGCTTCTATGATCTGGAG 80
ACGCAGAGGACAGCCAATCGCCAAAACAGAAAGGTGACAGCGCTTGGAGCTCCTTAAACAGGTATTACTCTCCTGTCGGC 160
*GTTTACCGTTCGCCCGCGCACACGCCGTCTGGCATACTCCTCTTGTCACC*GTCTCTCCTCTAAATGCTCTCTGGTTCGGC 240
CTGCTCGCGGCAGCTGGCGTACTCCTCTCCTCGCCGAATTGGACTGCTCTCGGGAGCTGGCGTCTTCCTACTGCGGCTGC 320
TTCCGGTTTCCTGTTCGTGAGTTCTCCTGCTATCCTCTGTCTCCCATGGCTATCTTATGTGAACCATGGCTATCGTGTTC 400
CCCTCACCGAACCCGGTTGTGAACTTAGGTTTTCTCTGATTTTGGATCC**ATG**GACTTCACGCCCAGTTGCAATTCGCCAG 480
ACTCCAACGACATTCCCAACTCCCCCGATGTAGATCCGGCATTGGGCGAAACAGGTGGCAGTGAGGTGAGTTCAATTTAG 560
ATACATTGTCTTCAATTTCTAAAATAGTGTTGGATGGTCTCGTCCTGCATGGTCTTCAATTTTTAAAACAGTCTCGCTTA 640
TGCTTGGAGGCTCCTACATTGTCTGTAATATAGGGACTTCAGAAGATTGATGGGGAATCACAACTGGACTGGGATTCGAT 720
TATAGTTTCAGATGTATTGGATGATGAAGGCAGAGTACAAGTACCATGCGAAAATGAGATATATTTTAATCTTGGACTCA 800
ATAAAGAGGATGAGGCTGCCAATAATAGGTTTTCTGGCAGTGGTACAAATTGTCATGCACAAGGAAGTTTGGATACGGAC 880
AACGAAGACCACCATGCTGATCAGCCTTGTCAAGACTACATTCCAGACGAAAAGAGGGTGGTGTATAATAGGATGAATCC 960
TTCTATGCAGCCAGGTTGTTTGTTTCCTAACATGAAAGAATTTAGGATTGCTATGCGACAGTATGCAATAAAACATGAGT 1040
TCGAGCTTGGAATTGAAGTTACTTCGACAACAAGATACGTTGGATACTGTAAGGGTGGTGATTGCCCTTGGAGGATATAT 1120
GCACGTGAAGAGAAGAAAGGATTGCCTACTATTGTGGTAGCTGTACTAGATGATGTTCACACTTGCACATCTAGTGGAAG 1200
GAGGGGGACTACTAGGCCAACTTGTGGTTGGGTCGCATTCCACGCTAAACCCTTGCTCATGAAGAAACCACAAATGGGTG 1280
GTAAAGAGTTACAACAAACACTACAGACAACTCATAACGTCACTATTGGGTATGATACAGTTTGGAAGGGGAAAGAGAAG 1360
GCTTTGAGAGAGTTGTATGGATCTTGGGAGGAAAGCTTCCAGCTCTTGTACTCTTGGAAGGAGGCTGTAATTGCAGTGAT 1440
GCCCGATAGTGTGATTGAGATTGATGTTATTTTGGAAGATGGGAAGTACTATTTTAGTCGATTCTTTTGTGCCTTTGGTC 1520
CATGCATATCTGGGTTCCGAGATGGGTGCAGACCTTATCTTAGTGTGGACTCGACAGCATTGAACGGTAGATGGAACGAA 1600
CATCTTGCATCTGCTACTGGTGTAGATGGCCACAATTGGATGTACCCAGTATGTTTTGGCTTTTTCCAAGCTGAGACGGT 1680
TGACAATTGGATTTGGTTCATGAAACAGCTGAAAAAGGTTGTGGGTGATATGACACTTCTAGCTATATGTTCAGATGCAC 1760
AAAAAGGTCTGATGCATGCTGTAAATGAGGTATTTCCTTATGCTGAGAGAAGAGAATGCTTCAGACACTTAATGGGAAAC 1840
TATGTGAAACACCATGCTGGTTCAGAGCACATGTATCCAGCAGCAAGGGCCTATAGGAGAGATGTATTTGAACACCATGT 1920
TACCAAGGTCAGAAATGTTCACAAGATTGCTGAGTACTTAGACCAACACCATAAATTCCTTTGGTACAGGAGTGGTTTCA 2000
ACAAAGATATCAAATGTGATTACATCACAAATAACATGGCTGAGGTTTATAATAACTGGGTTAAAGACCACAAAGACCTT 2080
CCTGTGTGTGATTTGGCTGAGAAAATTAGGGAGATGACAATGGGACTGTTTCATCGTAGGCGAAGGATTGGTCATAAGCT 2160
TCATGGTATTATTTTGCCATCTGCTTTAGCGATACTAAAGGCTCGCACTAGAGGGTTGGGCCACTTGTCCATTGTAAAAT 2240
GTGACAACTACATGGCAGAGGTACGAGACAGCACTAATTGTATGACTAAACATGTCGTGAATGCAGAACTGAAACAGTGT 2320
TCTTGTGAGGAATGGCAACACACTGGGAAACCGTGTCAACATGGTCTAGCCCTAATAATAGCCCAAGATTCCAGAGATGT 2400
AGGTATGGAAAATTTTGTTGACGATTATTACTCTACTGAAAGATTCAAGATAGCATATTCTAGAAGGGTGGAACCAATTG 2480
GTGATCGTTCGTTTTGGCCATCCGTTGATTTCGCCAGTGGAGTGTTTGCACCAATAGCTAGAAGAGGTCTTGGAAGACAA 2560
CGAAAAAATAGAATTAAAAGCTGTCTCGAGGGTAGGAGTGCTAGAAAAAAAAGTACCAACGAAAATGAGAAAACGAAAAA 2640
GCGACTCAAAAGGCAATACACTTGTCCTAATTGTGGTGAATTGGGACACCGCCAATCTAGCTACAAGTGCCCTTTGAATG 2760
GGACAAAAAAAAGGCTAGTTCTCAACTTACTTCTATATGTTCAATTTATATTAGTACTCGTGAACTAAATGTTTGAGTAT 2800
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ATGTACCAGAACAGCCAGACGTAGCAGAGGAAATCACTGAACAAGAGCTAGAAGATCCACAGCCAGAGACAGAACAATTG 2960
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TTCTCCACCACCGACAAGGAAATGGCTAGTGAAGAAAATCACCCCCAAGAAAAGACTGAGGATTAGTGCTCAGAAGAAGC 3120
AGTAT**TAA**CTGCTAAGAACAACCACCGTGCTCAGAAGAAACACAGTATTTGTTGTAAGACAACCACTGTATTTGTTGTAA 3200
GACTGTTATGTAAGACTGCTACGAACAAACACTATGTAACCTCCACCTGTATTGGTTGTAAGACTGCTAAGAACAAGCCC 3280
AGTGTATTTGTTGTAAGACTGTTCAGTTTTAGTTGCCAGTTCGGTGCTTCCCAGGTTCAGTTTTATTTCAGTTCCCAGTT 3360
CGGTGCTTCCCAGTTTTCCCAGTTCGGTCTTGCAATTGTTGCTGCTTGCAATTGACGCCCAGTTCGGTCACTTGCAATTG 3440
GGTCTTGCAATTGTTGCTGCTTGCCCAGTTTCAGAGAAACAGATTGCTTGCAATTGTCGCCCAGTTCAGAGAAACAGATT 3520
GCTTGCCCAGTTTCAGAGAAATAGAGAGCAGAAAACAGATAAAATATTACACAAAACAGATGACATATGACACACATGAA 3600
TAACAGTGAGCCATTAGTTCTTACAACCTCATCTCCACAACACAGAAAACAGACAACACTAATGTTCTTACAAACGCCAT 3680
TCATCAGGCCTTAACACGACAACAAACACTAGGGTTCTTACATCAGATAATAGGTCATACAACAAATATCAGTTGCGTCC 3760
TTCCAAAATATATCCCAGACAGACCAAAATGACACCAGAATGAAACCAACCCACCAAAGGCAACCTCAAGTCCACAA**CTA** 3840
CATGTTACGGTCGTTTATCTCTTCGAACCTGTAGTTTATGACACGATAGTGCTCTTCGAATGAGACATTAGCTTTAATCT 3920
CTTCCCACTTGAAGTCTTCAAAAACCATTTCCCATAGCTCTGGATCTTCTGTACTGTACCCATCACCAAGTTCATCATCA 4000
TCTAGAGGATGTTCATCATCTACGGAAGGGTTGTCGTAAAGATCCCAAGTTGGATTCTTTGTTTCTAAATCTTCTTCACA 4080
GACAATTGAAGTTTTGTCTAGCTCCTTGCACACATGTTCCAGATGCCCGAGAACCTTACCTATCTTGCCACCTTGTACCT 4160
CTGGAATAGTCGAACACAATTTTTGAACCGAATGCAACAGTTTAGTGTGCCTCCTCATTTCTTGAATAGCATATCTCAAC 4240
GAATTCTTTATGTTCACATGATACTTGAAGTCGACAAGATCCTGTGCAACAACCAGATGACAATCCATTTCTATCAACAA 4320
AAAAAATGAGTGTATGAGTTTGATAATCCATACCACATTCGATGAGGCCTTAACTGCTTCAACCTTGTCCACGGCAATGG 4400
CGGCGAACTGAACCGGAGCCTTGGCCTCCTTCTCAGCAACAAATACCGCGCATCTCGCCTCCGAGGCCGCAACGGCAGCA 4480
GCACGAGCAGCTTCCACAGCATCTGCAACCACTTTGCTCGGTGGACAATC**CAT**CCGCACAGGAGCAGAGAAATTACGGCT 4560
AGGGTTTCTGGATTCAGGATGGGGGCGGCATGAACAAGGAACGACGGCTAGGGTTCGGCAACGCGTTAACCATACAAGGC 4640
AGAGATGGGAATCGGTGCAGCATTGTCGGAGCAGGAGAACTCACCGCTGAGAATAGGAGACCGCAAGCAGCCGCAAGAGG 4720
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GTCACCTTTCCAATTTGGCGATTGGTTGTCGTCTGCGTCTCCAGAACAGAGAAACCAACGCCATGGCCTCCATTTCGTCG 4960
AATCCCGTCCGCTCTTCGTCTACAATGGCAATTATCTC 4998

Figure 4.—Complete nucleotide sequence of the 4998-bp *MuDR-zc* element of accession N215. The TIRs are in italics. Bold bases are the ATG of *mudrA* (position 450), stop codon of *mudrA* (position 3126), start codon of *mudrB* (position 4531, in antisense orientation), and the stop codon of *mudrB* (position 3838; in antisense orientation).

TABLE 2

Summary of differences between *MuDR* and *MuDR-Zc* sequences

Region	% Identity
A. Comparison of DNA sequences	
TIRA: *MuDR* to *MuDR-Zc*	100
mudrA: MuDR to *MuDR-Zc*	97.6
mudrB: MuDR to *MuDR-Zc*	95.2
TIRB: *MuDR* to *MuDR-Zc*	91.0
TIRA to TIRB of *MuDR-Zc*	88.1

Type of Change	Number in MURA	Number in MURB
B. Comparison of MURA and MURB predicted proteins[a]		
Synonymous codons	26	1
Conservative changes	9	7
Charged to neutral	3	1
Neutral to charged	8	0

[a] Based on the fully spliced MURA of 823 amino acids and the 207 amino acid MURB with intron 3 retained.

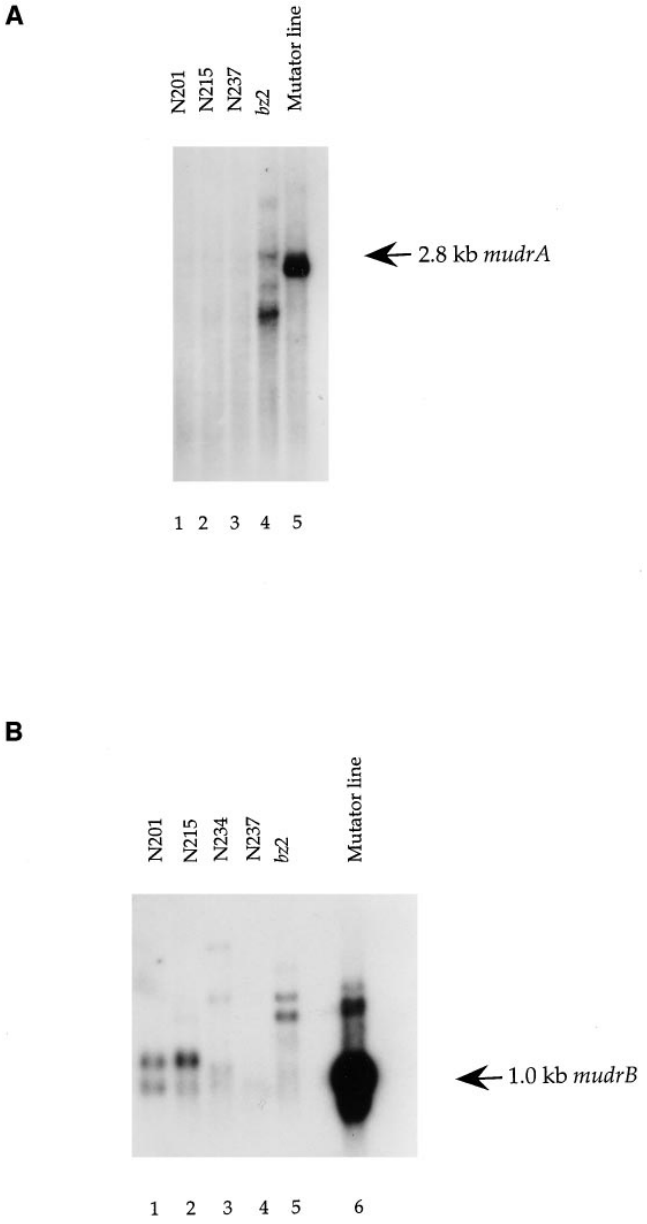

Figure 5.—Northern hybridization analysis of Mutator lines and Zapalote chico. Each sample contains 16–20 μg of poly (A)+ RNA. (A) The probe is PA corresponding to internal *mudrA* sequence. (B) The probe is PB corresponding to internal *mudrB* sequence.

ies of these non-autonomous elements in an unmethylated form. Although *Mu1* elements typically predominate in standard Mutator individuals (Taylor and Walbot 1987), the *Mu2* elements are more abundant in the Zapalote chico accessions examined. In addition, the probe detects additional size classes that may represent one of the common deleted forms of these *Mu* elements (reviewed in Walbot 1991), novel types of *Mu1*-derivatives or methylated copies of *Mu1* or *Mu2*. Other known or novel *Mu* elements may also be present.

Non-Mutator lines contain from zero to three *Mu1* and *Mu2* elements (Bennetzen 1984; Chandler *et al.* 1986; Chandler and Walbot, 1986). These elements are completely stable in position and copy number, and they remain methylated in a non-Mutator line. On crossing with a standard, active Mutator line, the *Hin*fI sites in the termini of the *Mu1* element in inbred line B37 lost methylation and could be digested with methylation-sensitive enzymes, such as *Hin*fI (Chandler *et al.* 1988). Thus, the moderate copy number and presence of unmethylated non-autonomous *Mu* elements suggest that these accessions of Zapalote chico are active Mutator lines.

Elevated forward mutation frequency in some Zapalote chico accessions: The multiple copies of unmethylated *Mu* regulatory and non-autonomous elements in some accessions of Zapalote chico are similar to what is found in standard, active Mutator lines. On the other hand, the low abundance of *MuDR*-related transcripts is more similar to the single copy *MuDR a1-mum2* lines (Qin and Ellingboe 1990) in which *MuDR* transcripts are only reliably detected with poly(A)+ RNA. The standard and single-copy *MuDR* lines both program the same pattern of high frequency somatic excision of *Mu* elements from reporter alleles, but the lines differ in the frequency of new mutants recovered (Robertson and Stinard 1992). Given that the Zapalote chico lines share specific properties with each of the two characterized types of Mutator lines, we were interested in defining the forward mutation frequency.

We used the test devised by Robertson (1978). Each individual is self-pollinated to score pre-existing recessive mutants; each individual is also crossed to a non-Mutator line, and multiple F_1 plants are grown and self-pollinated to score for new mutants generated in the gametes of the presumptive Mutator parent. As *Mu* insertions occur late in development, new mutants are

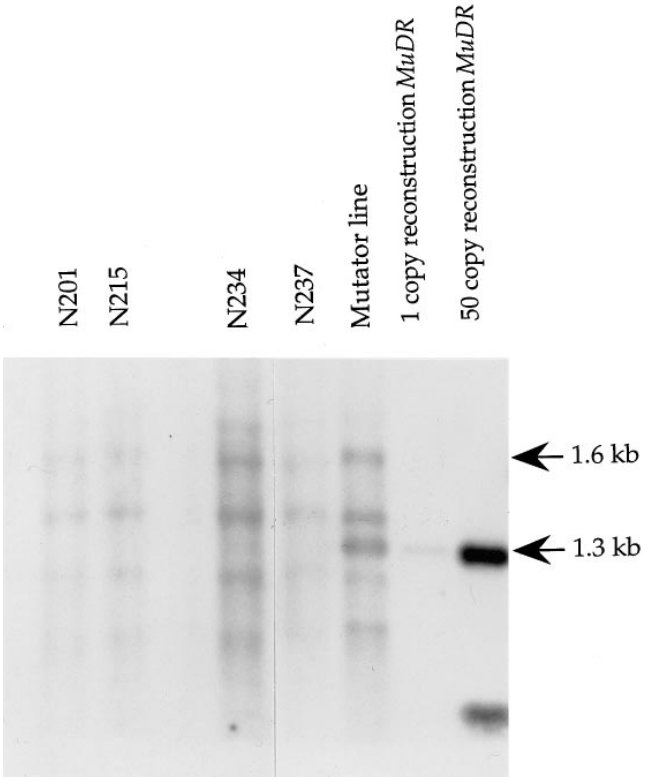

Figure 6.—Southern blot analysis of non-autonomous *Mu* elements in four accessions with full-length *MuDR-Zc* elements. DNA was digested with *Hinf* I and probed with pA/B5, which hybridizes to both *Mu1* and *Mu2*.

almost always recovered in only a single gamete (Robertson 1981; reviewed in Walbot 1991). One or a few individuals in most accessions of Zapalote chico were self-pollinated and crossed as pollen to *bz2* tester. Mutants recovered in the parental selfed ear and the F_2 selfed ear were scored as visible seedling traits 10 and 28 days after germination in the summer field. For comparison, we also assessed forward mutation frequency with several standard Mutator lines, two single-copy *MuDR* lines with the *a1-mum2* reporter allele, and the *bz2* tester in the W23 inbred line.

As expected, the four standard Mutator lines generated many mutants (Table 3D). On selfing, nearly half (28/62) of the parental plants segregated 3:1 wild-type:mutant for a pre-existing, visible seedling mutation. Common recessive phenotypes included albinos, zebra-striped leaves, pale green, pale yellow, and developmental mutants with twisted, shredded or midrib-only leaves. In the outcross to *bz2* tester, followed by selfing, 240 new mutants were observed in 835 families (29% mutation frequency) for the four standard Mutator lines. In the control for spontaneous mutation, no seedling mutants were observed in the selfed *bz2* tester (Table 3F). We can estimate spontaneous mutation frequency in the *bz2* tester if we also consider *defective kernel* (*dek*) mutations; one new *dek* mutation was recovered from the *bz2* tester (1/120 = 0.8%), a value similar to the spontaneous mutation frequency found in other non-Mutator lines examined in this test (Robertson 1981). The *dek* phenotype is among the most common recessive class in standard Mutator lines, representing failure of the embryo, endosperm or both (reviewed in Walbot 1991). There were 131 new *dek* mutations (131/835 = 16%) in the standard Mutator sample, ~20-fold more than in *bz2*.

In contrast to standard Mutator lines, the single *MuDR a1-mum2* line had a low forward mutation frequency (one mutant/344 families, Table 3E). Thus, the forward mutation frequency characteristic of standard Mutator may require multiple copies of *MuDR* and, most likely, a large population of non-autonomous elements.

The forward mutation test was completed before we classified the Zapalote chico accessions for *MuDR*-like elements and was therefore unbiased in selecting individuals for analysis. For simplicity, however, Table 3 groups lines on the basis of their *MuDR* phenotype. The four Zapalote chico accessions shown by PCR to contain all four segments of a *MuDR*-like element (Table 1) generated many new mutants after outcrossing as pollen parent to *bz2*; we observed 79 new mutations in 187 families (42%). The frequency of new mutations is equal to the most active standard Mutator line (M121), which gave 106 new mutants in 255 families (42%). In contrast, for the 11 Zapalote chico accessions in which PCR failed to detect all four segments of *MuDR*-like elements, the forward mutation frequency was low (five mutants/392 families = 1.2%, Table 3B); this is within the range of the spontaneous mutation frequency in other non-Mutator lines analyzed by this test (Robertson 1978), and similar to our results with *bz2* and with the two single-copy *MuDR* lines. A third group of 10 Zapalote chico accessions was analyzed for mutation frequency but not tested by PCR (Table 3C). These lines yield an intermediate value, with 31 new mutants in 217 families (14.2%). This group is clearly heterogeneous, with some accessions generating multiple mutants and some none.

Because both plastids and mitochondria are maternally transmitted in maize, reciprocal crosses between disparate lines often lead to defective kernels as a result of nuclear-cytoplasmic incompatibility. We observed many *dek* mutants in the self-pollinated F_2 ears of the Zapalote chico outcrosses, particularly among accessions that also gave rise to seedling mutants (data not shown). This class was excluded from analysis, however, because it is unknown whether mutations or incompatibility are responsible for the small or defective kernel phenotype (Allen *et al.* 1989).

Unusual features of mutant induction in Zapalote chico: It seems likely that both Mutator activity and *MuDR*-like elements are unevenly distributed in Zapalote chico populations. Only a subset of the Zapalote chico accessions qualify as Mutator lines by the forward mutation assay. This assay does not pinpoint what types

TABLE 3

Forward mutation frequency

Line	Self-pollinated parent: No. of Individuals	Self-pollinated parent: No. of Mutants	Outcrossed to *bz2*: *No. of* F_1 Families	Outcrossed to *bz2*: No. of F_2 Families	N
A. Zapalote chico accessions with *MuDR*-like elements					
M2	2	0	33	11	N201
M25	1	0	6	3	N215
M26	1	0	19	11	N216
M59	11	1	83	47	N234
M62	4	1	41	13	N237
Total	19	2	182	85	
B. Zapalote chico accessions without intact elements					
M1	1	0	14	0	N200
M5	4	1	41	1	N204
M6	1	0	11	1	N205
M7	2	1	40	0	N206
M8	1	0	75	0	N207
M24	2	1	44	0	N214
M29	3	2	42	0	N219
M30	3	1	47	2	N220
M32	2	0	25	1	N222
M36	2	0	32	0	N226
M41	2	0	21	0	N230
M58	1	1	16	1	N233
Total	24	7	408	6	
C. Zapalote chico accessions not classified by PCR					
M3	2	1	28	2	N202
M4	1	1	16	4	N203
M17	1	0	17	3	N208
M18	1	0	16	4	N209
M33	2	0	26	0	N223
M34	2	1	37	0	N224
M35	3	1	29	4	N225
M37	1	0	17	3	N227
M38	1	0	9	0	N228
M40	2	0	22	11	N229
Total	16	4	217	31	
D. Standard Mutator lines					
M88	2	1	32	6	n.a.
M121	20	11	255	106	n.a.
N285	20	9	309	57	n.a.
N190	20	7	239	71	n.a.
Total	62	28	835	240	
E. Single *MuDR a1-mum2* line					
N55	10	0	197	0	n.a.
N56	10	0	147	1	n.a.
Total	20	0	344	1	
F. *bz2* tester					
M72	10	0	120	0	

of elements cause mutations. It is possible that some Zapalote chico lines contain several types of transposable elements.

One curious feature of the analysis is that Zapalote chico lines yielded few mutants on selfing. In the subset of lines with *MuDR*-like elements, we identified only two visible seedling mutations among the 19 parents (Table 3A). In subsequent years, continuous selfing of these lines, and tests with more individuals from the original accessions, have produced few new mutants (data not

TABLE 4

Reactivation test with cryptic *bronze2* reporter alleles

Reporter allele[b]	No. tested	Number of ears with spotted kernels[a] after the indicated cross			
		(X)	to *bz2*	Zapalote chico[c]	Standard Mutator
bz2::MuDR	25	0	0	12	8
bz2::Mu1-mu1	17	0	0	0	1
bz2::Mu1-mu2	13	0	0	3	11

[a] Ears were scored as positive if at least 5% of the progeny kernels exhibited the frequent, fine spotting phenotype.

[b] The *bz2::MuDR* allele (formerly called *bz2-mu4*) has a full-length *MuDR* element inserted in the second exon (Hershberger *et al.* 1991); the other alleles have *Mu1* insertions in the first (*-mu2*) and second (*-mu1*) exons of *Bz2*. For the test, unspotted kernels were chosen from lines that were fully inactivated (*bz2::Mu1-mu1*), scored as no somatic mutability over several generations or from lines that were just inactivating; in the latter lines, selfed ears had just a few very lightly spotted kernels while progeny ears on *bz2* tester had no somatically unstable kernels.

[c] Results are pooled for the N237 and N264-derived *bz2* Zapalote chico lines.

shown). The number of visible mutants was similar to what we found in the "no *MuDR* group" (seven visible mutations in 24 parents) and the unclassified group (four mutants in 16 parents). The Zapalote chico accessions contain more "mutants" than *bz2*, but one plausible explanation is that temperature-sensitive alleles were recognized as mutant at Stanford that have no mutant phenotype in the much warmer conditions of Oaxaca.

The low incidence of visible mutants in Zapalote chico lines containing *MuDR* (2/19) is particularly striking considering the incidence of such pre-existing mutants in standard Mutator lines (28/62). In contrast, the F_2 ears from the outcross part of the forward mutation test exhibit similar frequencies of newly induced mutants. In its native habitat, only selfing or crosses within Zapalote chico germplasm occur, because Zapotec farmers grow only this type of corn. The activation of a high forward mutation frequency on crossing with a heterologous line suggests that hybrid dysgenesis occurs. We completed too few exact reciprocal crosses between Zapalote chico and *bz2* to determine whether the elevated mutation frequency results when an active Zapalote chico individual is the female parent as well as the pollen donor.

A second curious feature of the many new seedling mutants produced by the various Zapalote chico accessions is that none displayed somatic variegation. Typically, small wild-type sectors indicative of late somatic excision are visible in at least half of all new mutants produced by a standard Mutator line (Robertson 1981; reviewed in Walbot 1991). In the collection of mutants produced for this study, we also found that about half of the new albino, pale green and yellow mutants recovered from standard Mutator lines had visible dots of green on the first leaf (data not shown). The absence of somatic reversion is a novel property of new mutants produced in Zapalote chico.

Zapalote chico lines with *MuDR-Zc* can restore somatic mutability to cryptic *bz2* mutable reporter alleles: The lack of somatic instability of newly induced mutations in unknown genes made it difficult to analyze whether *Mu* elements were involved. To gain more direct evidence that *MuDR-Zc* elements were genetically active, we used a *trans*-activation test for Mutator activity. Lines derived from N237 and N264 with full-length *MuDR-Zc* (based on Southern blot hybridization) were crossed twice with *bz2*, in effect creating *bz2* tester lines after selection for individuals without the dominant *C-I* allele. This allele prevents anthocyanin accumulation and was present in most Zapalote chico accessions. For the activation test, inactive Mutator lines homozygous for one of three well-characterized *bronze2* alleles with precisely mapped *Mu* element insertions were selected from our collection; these lines contain multiple, methylated copies of *MuDR* and somatically stable *Mu* elements. As shown in Table 4, each inactive individual was self-pollinated and crossed to *bz2* tester to score spontaneous reactivation of somatic mutability at the cryptic reporter allele; no instance of spontaneous reactivation was observed in the 55 individuals tested. On crossing to Zapalote chico or standard Mutator *bz2* lines, fine purple spotting indicative of late, frequent somatic excision was restored in from zero to 85% of the test crosses. Such wide variation in reactivation is typical of Mutator reactivation tests (Walbot 1986).

DISCUSSION

A high forward mutation frequency is a defining characteristic of standard Mutator lines; mutation frequency is elevated 20–100-fold above spontaneous or above what is observed in active *Ac* or *Spm* lines (reviewed in Walbot 1992). Mutations in Mutator lines are caused by a diverse family of *Mu* elements, which share ~200-bp TIRs. Germinal insertion and somatic excision activities are controlled by the regulatory element *MuDR*. To date, *MuDR* has been found only in standard Mutator

lines, in their immediate derivatives, and in the *Cy* germplasm (Bennetzen 1996). In the standard U.S. germplasm, land races, and *Zea* spp. we have examined, we find evidence for unmethylated *MuDR*-like elements and Mutator activity in Zapalote chico. Even within this land race, only a subset of accessions appear to contain full-length elements.

For a neotropical maize, Zapalote chico is relatively tolerant of long daylength. It can be grown to maturity in the temperate zone and crossed with U.S. germplasm. Because it is so adaptable and contains many traits of potential agronomic importance, Zapalote chico has been used in breeding for disease, insect, and wind-damage resistance (Muñoz *et al.* 1992). Zapalote chico contains large numbers of prominent heterochromatic knobs, and this line has been used in maize cytogenetic research (Goodman and Brown 1988).

Several lines of evidence indicate that some accessions of Zapalote chico qualify as Mutator lines. First, they exhibit a high forward mutation frequency, similar to standard Mutator lines. Second, they contain multiple, unmethylated copies of non-autonomous *Mu* elements. *Mu* elements are methylated in inactive or non-Mutator lines (Chandler *et al.* 1988). Third, they contain multicopy unmethylated and transcriptionally active *MuDR*-like elements, which to date have been found only in standard Mutator lines (Bennetzen 1996). Fourth, *MuDR*-like element copy number is maintained through several outcrosses to non-Mutator lines. Approximately one-fourth of the Zapalote chico accessions examined appear to have Mutator activity by one or more of these criteria.

Molecular analysis of *Mu* elements in Zapalote chico accessions: The three sequenced examples of *MuDR* are nearly identical, and it was expected that a search for additional Mutator sources would identify only this element. We have cloned the *MuDR-Zc* element in several fragments from one accession of Zapalote chico (N215) that contains several copies of the putative regulatory transposon. The *MuDR-Zc* sequence assembled from the fragments is highly similar, but clearly diverged, from the *MuDR* present in standard and the derived low-copy *MuDR* Mutator lines. *MuDR-Zc* is 4998 bp, 56 bp larger than the 4942-bp *MuDR*. Overall, *MuDR* and *MuDR-Zc* exhibit 94.6% DNA sequence identity. Identity is highest in TIRA and in the coding regions, with the intergenic region being the most divergent part of the element. At the amino acid level, the *mudrA*-like gene (*mudrAzc*) is more similar to that of *MuDR*, 97%, than the *mudrB*-like gene (*mudrBzc*), 95.2%. A portion of *MuDR-Zc* was also cloned and sequenced from N234; in the region 4398–4524, this sequence is identical to *MuDR-Zc* of line N215.

Southern blot analysis clearly demonstrates the presence of intact ~5.0-kb *MuDR-Zc* elements in N215. Because *MuDR-Zc* was cloned in fragments by PCR amplification, however, we do not have proof that all of the polymorphisms exist in the same element. It is also possible that a few of the nucleotide polymorphisms are from PCR mutation. Because *MuDR* is toxic to *E. coli*, point mutations are common during attempts to clone the intact element; for this reason we cloned *MuDR-Zc* in pieces that appear to be tolerated in *E. coli*. However, we were also able to amplify the fragment in two, large overlapping PCR fragments (position 113–2423 yielded a 2310-bp fragment; positions 2404–4829 yielded a 2425-bp fragment). Future recovery of overlapping genomic clones of *MuDR-Zc* and cDNA clones will confirm the distribution of sequence differences within individual *MuDR-Zc* elements in line N215. To gain a better understanding of the diversity of *MuDR*-like elements, full sequencing of elements from additional Zapalote chico accessions could be informative as well.

Evidence for Mutator activity in some Zapalote chico accessions: Several approaches were taken to demonstrate that some Zapalote chico lines not only carry intact *MuDR*-like elements but may also have an actively transposing population of *Mu* elements. The first measure of Mutator activity was by Northern analysis, because it has been shown that only active Mutator lines express *MuDR* transcripts (Hershberger *et al.* 1995). Second, we examined the methylation status and copy number of *Mu* elements and the transmission of *MuDR*-like elements to progeny. The third measure was a forward mutation test to determine if any Zapalote chico accessions had an elevated mutation frequency, and whether mutation frequency correlated with *MuDR*-like elements. Fourth, we examined the ability of Zapalote chico to activate somatic instability in inactive Mutator lines.

Northern analysis demonstrated that *MuDR-Zc* is actively transcribed; however, the levels and patterns of expression are different from standard Mutator lines (Hershberger *et al.* 1995). *mudrA* and *mudrB* transcripts are easily detected in total RNA of standard Mutator lines and are approximately equally abundant, although in immature (prefertilization) ears there is an ~1:4 ratio of *mudrA*:*mudrB* transcripts (Hershberger *et al.* 1995). Low transcript abundance is characteristic of the single-copy *MuDR* lines, but these lines have approximately equal amounts of transcript from genes A and B (Qin *et al.* 1991; James *et al.* 1993). In Zapalote chico, however, *mudrAzc* transcript levels are extremely low, while those of *mudrBzc* are relatively more abundant. Both transcripts are only readily detected from poly(A)$^+$ RNA. As the *mudrAzc* and *mudrBzc* transcripts are approximately the size of standard Mutator transcripts, we infer that the TIRs also act as the promoter elements in Zapalote chico, as well as constituting part of the 5′ UTR of each transcript type. In the sequenced example of *MuDR-Zc*, TIRA is identical to TIRA of *MuDR* but TIRB is only 91% identical. The differences in TIRB may allow a higher level of *mudrBzc* transcription or increased transcript stability. It is possible that *mudrA*

and *mudrB* differ in transcript abundance because there is Zapalote chico-specific host regulation or new forms of autoregulation by the *MuDR*-like elements.

It is not clear why there are two *mudrBzc* transcripts in the Zapalote chico accessions examined. It is possible that these transcripts are produced by two different, but related, *MuDR-Zc* elements. It is also possible that they are produced by alternative transcription start sites, differential splicing or different polyadenylation events from a single transcription unit. As mentioned earlier, both alternative splicing and multiple polyadenylation sites exist in *mudrB* transcripts in standard Mutator lines (Hershberger *et al.* 1995), and such post-transcriptional events may explain the two transcripts found in Zapalote chico. We also observed novel-sized RNAs in the *bz2* tester line that hybridized with the *MuDR* probe. These cross-hybridizing RNAs may result from fortuitous similarity or regions of similarity to *MuDR* in this non-Mutator line.

Methylation has previously been shown to be correlated with the loss of activity of *MuDR* (Martienssen and Baron 1994). In inactive Mutator lines with methylated *Mu* elements, *Mu* copy number decreases by approximately half with each successive outcross to a non-Mutator line (Walbot and Warren 1988). Neither *MuDR-Zc* elements nor *Mu1* and *Mu2* are methylated at the enzyme sites examined. Furthermore, the *MuDR-Zc* copy number is maintained on outcrossing: parents with just 1–3 elements transmit them to all progeny and through at least two outcrosses. Maintenance of *Mu*-element copy number is a key property of active Mutator lines (Alleman and Freeling 1986; Walbot and Warren 1988), although we are uncertain whether transposition or demethylation of cryptic elements is responsible for copy number maintenance in Zapalote chico.

In the test for forward mutation frequency, we established that standard Mutator lines have a high forward mutation frequency (29% of families contain a new visible seedling mutation) compared to the low frequency of a standard inbred line of maize (*bz2* tester in W23 background) or a single *MuDR* line (*a1-mum2*). In Zapalote chico, an elevated mutation frequency correlates with the presence of *MuDR*-like elements, but transposable elements of additional families and *Mu* elements of several types may contribute to the observed mutation frequency. The accessions for which there is molecular evidence of regulatory elements had a 42% forward mutation frequency, matching the level of the most active standard Mutator line. All of the new mutations recovered appear to be recessive, based on segregation data (data not shown).

Several properties of Mutator activity in Zapalote chico are distinct from both standard and single-copy *MuDR* Mutator lines. In contrast to standard Mutator lines, we found few mutations segregating in the original Zapalote chico parents. New mutants occur after outcrossing Zapalote chico as pollen donor onto a non-Mutator line. Consequently, new mutations occur as a result of hybrid dysgenesis and must be induced during or after fertilization. In standard Mutator lines, many new mutations are recovered as single-kernel events, indicative of *Mu* insertions that affect single gametophytes (reviewed in Walbot 1991; Chandler and Hardeman 1992). When an active Mutator plant is used as a pollen donor, nonconcordant embryo and endosperm mutations occur in ~20% of the new mutants selected at *Y1* (Robertson and Stinard 1993). The lack of correspondence between the embryo and endosperm genotypes indicates that *Mu* insertions can occur after the mitosis that separates the two sperm in each pollen grain (Robertson and Stinard 1993). Our data provide evidence that mutations in Zapalote chico sperm can be induced even later, after fertilization, provided the sperm interact with a non-Mutator egg.

A second unusual feature of new mutations induced in Zapalote chico is that they are not somatically mutable. Frequent late somatic excision is characteristic of both standard and low-copy Mutator lines (reviewed in Walbot 1991; Lisch *et al.* 1995). If the Zapalote chico mutations are caused by *Mu* insertions, then the lack of somatic instability suggests that there is novel developmental regulation of element excision behavior. In Mutator lines losing activity, often assessed by a loss of somatic excision at a reporter allele, the levels of *MuDR* transcripts decline precipitously (Joanin *et al.* 1996). The low abundance of *MuDR-Zc* transcripts may similarly be below the threshold required to program somatic excision.

A mutation screen is currently in progress to isolate mutations in anthocyanin reporter genes, using Zapalote chico accessions with *MuDR-Zc* elements. The isolation of a *Mu* element inserted into a known gene will provide the opportunity to analyze the type of insert and its excision behavior more precisely. It is possible that mutations in Zapalote chico lineages result from more than one type of transposon.

Implications of hybrid dysgenesis: The seed accessions used in this study were gathered from different sources, and at different times. Only a subset of the Zapalote chico lines contains *MuDR-Zc* and exhibits an elevated mutation frequency. In the past, Zapalote chico has been included in a variety of corn-breeding programs. In crosses with other lines, however, hybrids are often abandoned because of high sterility (Muñoz *et al.* 1992) or poor vigor (W. Tracy, personal communication). Yet this land race is a commercial crop when grown and maintained by inbreeding by the Zapotec farmers in Oaxaca, México. Zapalote chico is the staple of the human and animal diet of the Zapotec people. Zapotecs prize this variety of corn for preparation of totopos, a baked corn cracker that is the main starchy food in their diet. We hypothesize that the Zapotec farmers have selected for the alterations in Mutator activity that

we observe as a low abundance of transcripts and unremarkable mutation frequency in inbred Zapalote chico.

The apparent restriction of a high forward mutation frequency to outcrosses involving Zapalote chico may be the explanation for the stability of this line in crop fields. Our molecular and genetic observations confirm a Zapotec myth that their corn will kill other lines of maize if interbred. This myth is one reason Zapotecs grow only Zapalote chico to ensure a reasonable yield. The basis of this myth may be hybrid dysgenesis. This phenomenon was first described by analysis of the repression and activation of *P* elements in *Drosophila melanogaster* in crosses that involved wild-caught and laboratory flies (reviewed by Engles 1989). With the appropriate combination of breeding scheme and *P*-element types, this transposable element family is quiescent, effectively tamed.

Similarly, we found a low-mutation frequency after selfing Zapalote chico lines with transcriptionally active *MuDR*-like elements. This contrasted with the high-mutation frequency observed in the progeny of these same plants crossed as pollen donor to inbred W23 and in the derivatives of the Tuxpeño X Zapalote chico lines crossed to W23. The difference in mutation frequencies suggests that Zapalote chico germplasm could contain a novel factor that suppresses Mutator activities or has lost a host factor required for activation. When Zapalote chico is crossed as pollen donor to other lines, the "repressor" of Mutator activity is missing or ineffective. The somatic stability of new mutants in the dysgenic crosses is also striking, and again suggests that the *MuDR-Zc* or the Zapalote chico background confers novel and stabilizing properties on the Mutator transposons. Further genetic and molecular analysis will be required to identify the proposed repressor, if it exists, and to probe the interactions of standard *MuDR* and *MuDR-Zc*.

We thank Stewart Gillmor, María-Inés Benito and Manish Raizada for their comments on a draft of the manuscript, and M.-I.B. for much helpful advice and support. We thank Joseph Sarsero for computer assistance. M.G.-N. was supported by the Fondación UNAM during this work. Support for the collecting trip to Juchitán, Oaxaca, was provided by the Eppley Foundation; other research support was provided by National Institutes of Health grant GM-49681 to V.W.

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Communicating editor: J. A. Birchler