DNA Sequence Analyses Support the Role of Interrupted Gap Repair in the Origin of Internal Deletions of the Maize Transposon, *MuDR*

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ABSTRACT

Previous research has demonstrated that the autonomous *Cy* transposon can activate the excision of *Mu* transposons. To determine the relationship between *Cy* and the more recently described autonomous *Mu* transposon, *MuDR*, a *Cy* transposon inserted at the mutable *a1* allele, *a1-m5216*, was isolated and cloned. DNA sequence analyses established that this *Cy* insertion is identical to *MuDR* (*Mu9*, GenBank accession No.: m76978.gb_pl). Therefore, *Cy* will henceforth be termed *MuDR:Cy*. Defective derivatives of *MuDR:Cy* were isolated that had lost their capacity to activate their own excision or the excision of a *Mu7* transposon. Most of these derivatives are nonautonomous transposons because they can excise, but only in the presence of unlinked *MuDR:Cy* transposons. Physical mapping and DNA sequence analyses have established that six of these defective derivatives carry internal deletions. It has been proposed previously that such deletions arise via interrupted gap repair. The DNA sequences of the break points associated with all four sequenced deletions are consistent with this model. The finding that three of the excision-defective derivatives carry deletions that disrupt the coding region of the *mudrA* (but not the *mudrB*) transcript supports the view that *mudrA* plays a role in the excision of *Mu* transposons.

ABOUT a dozen families of transposons have been identified in maize since the discovery of the *Ac/Ds* family by McCLINTOCK during the 1940s (for a review, see PETERSON 1988). Transposons within a family share homologous inverted repeats and respond to the same transposase. A transposon that contains the internal sequence encoding the family-specific transposase is termed the autonomous (or regulatory) transposon of the family. Autonomous transposons can catalyze their own excision and activate, *in trans*, the excision of other nonautonomously transposing sequences termed receptors.

Originally identified by ROBERTSON in 1975, the *Mutator* transposon family of maize exhibits forward mutation rates 50-fold above the spontaneous rate (ROBERTSON 1978). This elevated mutation rate is termed "*Mutator* activity." Lines that exhibit *Mutator* activity and that are derived from ROBERTSON's stocks are termed *Mutator* stocks. The inheritance of *Mutator* activity is usually non-Mendelian; among the progeny of a cross between *Mutator* and non-*Mutator* stocks, ~90% of the progeny retain *Mutator* activity (ROBERTSON 1978). Many of the mutations recovered from *Mutator* stocks contain *Mu* transposon insertions. *Mu* transposons generally generate 9-bp direct target site duplications upon insertion. To date, eight classes of nonautonomous *Mu* transposons have been cloned either as insertions into previously cloned genes or by homology to the ~220-bp terminal inverted repeat sequences that are shared by all *Mu* transposons (for reviews, see WALBOT 1991; CHANDLER and HARDEMAN 1992). In most instances, members of different classes of *Mu* transposons do not exhibit internal sequence similarity.

The non-Mendelian inheritance of *Mutator* activity in *Mutator* stocks hampered the genetic identification of the autonomous transposon that regulates the transposition of Mu transposons. Working with the TEL (transposon element laden) population, which does not have recent common ancestors with *Mutator* stocks, SCHNABLE and PETERSON (1986) identified an autonomous transposon (*Cy*), which segregates in a near-Mendelian manner (SCHNABLE and PETERSON 1988) and that regulates the transposition of *Mu* transposons (SCHNABLE and PETERSON 1989). The *Cy* transposon was first identified by virtue of its ability to regulate the somatic instability of a nonautonomous allele (*bz1-rcy*) isolated from the TEL population. Cloning and sequencing of the *rcy:Mu7* transposon inserted at the *bz1-rcy* allele demonstrated that it had all the characteristics of a *Mu* transposon (SCHNABLE *et al.* 1989). Other tests established that: the *Cy* transposon is capable of regulating the excision of the *Mu1* transposon inserted at the *a1-mum2* allele (SCHNABLE and PETERSON 1989); strongly active *Cy* transposons are present only in the TEL population and *Mutator* stocks (SCHNABLE and PETERSON 1986); and in *Mutator* stocks, the presence of genetically active *Cy* transposons is correlated with *Mutator* activity (SCHNABLE and PETERSON 1989). In total, these data suggested that the *Cy* transposon is the regulatory transposon of *Mutator* family.

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More recently, a 4.9-kb transposon was cloned from *Mutator* stocks by several laboratories and termed variously *Mu9* (HERSHBERGER *et al.* 1991), *MuR* (CHOMET *et al.* 1991) and *MuA2* (QIN *et al.* 1991). Because *MuA2* and *Mu9* have only a single base pair difference (JAMES *et al.* 1993), it was agreed at the 1993 Maize Genetics Conference to rename this 4.9-kb transposon *MuDR* in recognition of DONALD ROBERTSON's contribution to the study of the *Mutator* family. Using a one-*MuDR* line carrying *a1-mum2* and related to a one-*MuDR* line developed by ROBERTSON and STINARD (1989), it was demonstrated that *MuDR* represents the autonomous transposon of the *Mutator* family (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991; QIN *et al.* 1991). Based upon this result, it became important to determine whether *Cy* and *MuDR* exhibit DNA sequence similarity.

Internal deletions arise within *MuDR* transposons at high rates (HARDEMAN and CHANDLER 1993; LISCH and FREELING 1994; LISCH *et al.* 1995). LISCH *et al.* (1995) proposed that these deletions may arise via interrupted gap repair, a model developed to explain the behavior of the *P* transposons of Drosophila (ENGELS *et al.* 1990). This model makes specific predictions regarding the characteristics of the sequences that define deletion end points. Hence, although sequences flanking *MuDR* deletions have not been reported, such sequences would represent a significant test of this model.

MuDR encodes two convergent transcripts (HERSHBERGER *et al.* 1991; JAMES *et al.* 1993), *mudrA* and *mudrB* (HERSHBERGER *et al.* 1995). Both transcripts are present in active *Mutator* stocks at all developmental stages in all tissue types tested, but not in inactive or non-*Mutator* lines (HERSHBERGER *et al.* 1995). Deletions that remove part of the *mudrA* coding region (as assayed via restriction mapping) generate defective *MuDR* transposons that appear incapable of conditioning excision of a nonautonomous *Mu1* transposon (LISCH and FREELING 1994). This suggests that *mudrA* plays a role in excision. Indeed, the protein predicted to be encoded by *mudrA* (*MURA*) shares a sequence motif with a group of bacterial insertion sequences suggesting that it encodes a transposase (EISEN *et al.* 1994). The biological role (if any) of *mudrB* is less clear. None of the *MuDR* deletions analyzed by LISCH and FREELING (1994) affected *mudrB* coding sequences exclusively (all their deletions included large portions of *mudrA* coding sequences).

In an effort to test the hypothesis that *Cy* and *MuDR* are one-in-the-same, we "trapped" a *Cy* transposon at the *a1* locus. In this report we substantiate the prediction that *Cy* is the autonomous transposon of the *Mutator* family (SCHNABLE *et al.* 1989) by demonstrating the absolute sequence identity between *Cy* and *MuDR*. Accordingly, *Cy* will henceforth be termed *MuDR:Cy*. We also describe the isolation and characterization of six nonautonomous deletion derivatives of *MuDR:Cy*. The DNA sequences flanking all four of the deletion junctions that were sequenced support the view that *MuDR* deletions arise via interrupted gap repair. The nature of these deletions also adds supports for a role of *mudrA* (and suggests a role for *mudrB*) in *Mu* transposon excision.

MATERIALS AND METHODS

Genetic stocks and gene symbols: According to the standard maize genetics nomenclature, loci and recessive alleles are designated by lowercase gene symbols, while dominant alleles are designated by uppercase symbols. The *a1* (*anthocyaninless1*) gene codes for dihydroflavonol reductase (REDDY *et al.* 1987) and is involved in the biosynthesis of anthocyanin throughout the plant, including the aleurone layer of the kernels. The *a1-m5216* allele contains a *MuDR:Cy* insertion (as reported in this manuscript). The *a1::rdt* allele has a *rdt* insertion at the fourth exon of *a1* and has been reviewed by XU *et al.* (1995). The stable, recessive *a1-dl* allele (previously termed a1-s by CIVARDI *et al.* 1994) contains a premature translation termination codon in the third exon (L. J. QIU and P. S. SCHNABLE, personal communication). This allele was obtained in coupling with *et1* in 1985 from P. A. PETERSON (Iowa State University) who had maintained it since 1955 (its pedigree prior to 1955 remains under investigation). The *et1* (*etched1*) gene product is thought to be involved in amylolytic enzyme activity (SANGEETHA and REDDY 1988) and is 12 cM centromere distal of *a1*. Homozygous recessive kernels have pitted endosperms (Figure 1C). Sweet Belle is an F_1 hybrid (from Asgrow) homozygous for *a1::rdt, sh2, Sh1* and *Bz1* (see CIVARDI *et al.* 1994, for details). The *shrunken2* (*sh2*) locus encodes ADP-glucose pyrophosphorylase, which is involved in starch biosynthesis (TSAI and NELSON 1966). The *bz1* (*bronze1*) locus encodes flavonol (O)3-glucosyl transferase (LARSON and COE 1968). Kernels homozygous for the recessive reference allele exhibit a pale to reddish brown (bronze) aleurone color. The *bz1-rcy* allele contains an *rcy:Mu7* insertion that can excise in the presence of *Cy* transposons (SCHNABLE and PETERSON 1986). The *bz1* tester stock carries the *bz1* reference allele in coupling with the closely linked (2 cM) marker *sh1* (*shrunken1*) and has the genotype *A1 Et1/A1 Et1 sh1 bz1/sh1 bz1*. The *sh1* locus encodes sucrose synthase (CHOUREY and NELSON 1976) and homozygous recessive kernels have smoothly indented endosperm upon drying. The *bz1-rcy* tester stock carries the *bz1-rcy* allele in coupling with *Sh1* and has the genotype *A1 Et1/A1 Et1 Sh1 bz1-rcy/sh1 bz1*. The *bz1* and *bz1-rcy* stocks do not harbor *Cy* activity. The *Cy* (*Cycler*) transposon controls the transposition of *rcy:Mu7* and *Mu1* (SCHNABLE and PETERSON 1986, 1988, 1989).

Genetic crosses:

Cross 1: Cy A1 Sh2 Et1/A1 Sh2 Et1 Sh1 bz1-rcy/(Sh1 bz1-rcy or *sh1 bz1)* × *a1::rdt sh2 Et1/a1::rdt sh2 Et1 Sh1 Bz1/Sh1 Bz1* (Sweet Belle).

Cross 2: a1-m5216 Sh2 Et1/a1::rdt sh2 Et1 Sh1 bz1-rcy/Sh1 Bz1 × *a1-dl Sh2 et1/a1-dl Sh2 et1 Sh1 Bz1/Sh1 Bz1.*

Cross 3: A1 Sh2 Et1/A1 Sh2 Et1 Sh1 bz1-rcy/sh1 bz1 × *a1-m5216 Sh2 Et1/a1-dl Sh2 Et1 Sh1 bz1-rcy/Sh1 Bz1.*

Cross 4: a1-m5216 Sh2 Et1/A1 Sh2 Et1 Sh1 bz1-rcy/(Sh1 bz1-rcy or *sh1 bz1)* × *a1-dl Sh2 et1/a1-dl Sh2 et1 Sh1 Bz1/Sh1 Bz1.*

Cross 5: A1 Sh2 Et1/A1 Sh2 Et1 Sh1 bz1-rcy/sh1 bz1 × *(a1-m5216 Sh2 Et1* or *A1 Sh2 Et1)/A1 Sh2 Et1 Sh1 bz1-rcy/(Sh1 bz1-rcy* or *sh1 bz1).*

Cross 6: a1-m5216 Sh2 Et1/a1-dl Sh2 et1 Sh1 Bz1/(Sh1 bz1-rcy or *Sh1 bz1)* × *a1-dl Sh2 et1/a1-dl Sh2 et1 Sh1 Bz1/Sh1 Bz1.*

Isolation of the *a1-m5216* allele: New mutations at the *a1* locus were recovered in exceptional kernels with the genotype *a1-m Sh2/a1::rdt sh2* (where *a1-m* is a newly arisen mutant allele). The analysis of one of these mutants (*a1-m5216*) is the subject of this report; two other confirmed *a1-m* mutants recovered from this screen remain under analysis (*a1-m5046* and *a1-m5259*). The kernel that carried *a1-m5216* exhibited

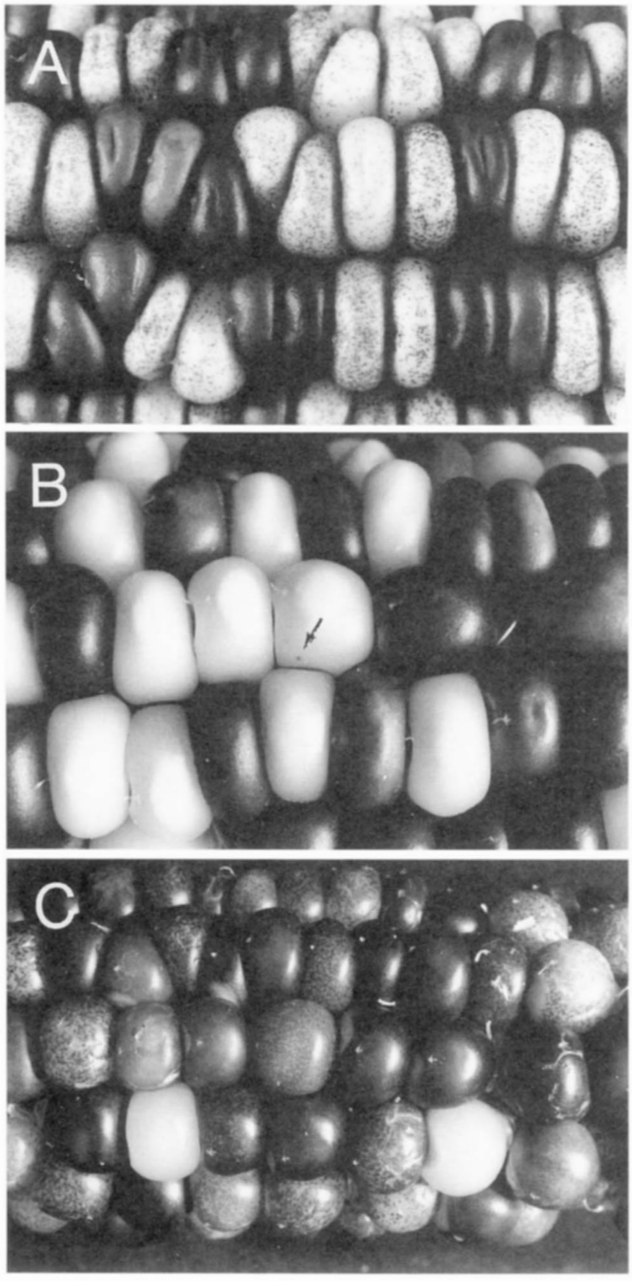

FIGURE 1.—Phenotypes of *a1-m5216* and its defective derivative alleles. (A) Standard a1-spotted kernels on an ear resulting from the cross: *A1/a1-m5216* × *a1-dl/a1-dl* (912908-1/2765). (B) Exceptional low a1-spotted phenotype found on an ear resulting from the cross: (*a1-m5216* /*A1* × *a1-dl/a1-dl* (912904-14/2711) where the female parent had been selected as having no *Cy* activity (a plant derived from a bronze kernel). This nonstandard a1-spotted phenotype is correlated with aberrant *Cy* activity (see footnotes *a*, *b*, and *f* in Table 3). Also note that there are fewer spotted kernels segregating than expected. (C) Exceptional colorless kernels on an ear resulting from the cross: *A1 Et1/a1-m5216 et1* × *a1-dl et1/a1-dl et1* (93g2007-1/2027). Such kernels potentially carry defective alleles of *a1-m5216*. The colorless kernel in the lower right-hand corner is etched. The other colorless kernel probably carries *Et1* as a result of a crossover.

the frequent, late somatic reversions typical of *Mu* transposon-induced mutants (Figure 1A). To conduct analyses on *a1-m5216*, it was necessary to distinguish it from *a1::rdt*. This could be done by virtue of the different phenotypes conditioned by the two alleles (high *vs.* very low spotting patterns) and the fact that *a1::rdt*, but not *a1-m5216*, was in coupling with the tightly linked (0.1 cM) *sh2* mutation. The ultimate success of this procedure was confirmed by comparing the DNA sequences of *a1::rdt* and the *A1* progenitor of *a1-m5216* (L. J. QIU and P. S. SCHNABLE, unpublished data) to *a1* sequences flanking the *MuDR* transposon insertion in an *a1-m5216* clone; these sequences are distinct from those of *a1::rdt* and identical to those of the *A1* progenitor of *a1-m5216* at all six DNA sequence polymorphisms in the ~200 bp 3′ of the *MuDR* insertion site in the *a1* locus (data not shown and L. J. QIU and P. S. SCHNABLE, personal communciation).

Estimating the number of *Cy* transposons segregating in a cross: The number of genetically active *Cy* transposons carried by the *a1-m5216* plant was estimated by the ratio of bz1-spotted (bz1-sp):bronze (bz) kernels among progeny from a *bz1-rcy* test cross: For example, the male parent in cross 3 will produce two classes of gametes of genotypes: *bz1-rcy* and *Bz1*. The female parent of cross 3 will also produce two classes of gametes with the genotypes: *bz1-rcy* and *bz1*. Therefore, the resulting progeny will be of three types in a 1:2:1 ratio *bz1-rcy/bz1-rcy*, (*bz1-rcy* or *bz1*)/*Bz1* and *bz1/bz1-rcy*. Kernels heterozygous for *Bz1* will be colored, and thus, will not be informative. Among the remainder of the kernels (which carry the *bz1-rcy* allele), the bz1-sp:bz ratio is expected to be 1:1 in the presence of one copy of an active *Cy* transposon. The expected ratios when two and three active *Cy* segregating are 3:1 and 7:1 respectively. This estimation method can be applied to other crosses. However, it is necessary to distinguish between the *bz1-rcy* and *bz1* alleles in some crosses (such as crosses 4 and 5). Stocks used in this study carry the *bz1-rcy* allele in coupling with a *Sh1* allele, and the *bz1* reference allele in coupling with a *sh1* allele. Hence, the *sh1* phenotype can be used to predict the *bz1* genotypes of progeny for the purpose of establishing expected segregation ratios in the presence of varying numbers of *Cy* (SCHNABLE and PETERSON 1986). The chi-square values are shown in parentheses, *i.e.*, ( ). For data that do not fit (at the 95% level of confidence) any of the three ratios tested (one, two and three copies of *Cy*), the number of *Cy* associated with the smallest chi-square value is shown in brackets, *i.e.*, [ ]. Some of these ratios may have arisen via Type I statistical errors (*i.e.*, statistical deviations); others may represent more complex biological processes (SCHNABLE and PETERSON 1988). Hence, *Cy* copy number estimates presented in brackets should be interpreted with caution. If the data fit more than one ratio, all numbers of *Cy* (between one and three) with nonsignificant chi-square values are shown. If the observed segregation ratio does not fit any of the three ratios and the chi-square values for the expected ratios of one *Cy* are larger than expected ratios for three *Cy*, the plant is estimated to carry more than three copies of *Cy*.

Southern blot analyses: Maize DNA samples used for Southern blot analyses were isolated from freeze-dried leaf samples by the method of SAGHAI-MAROOF *et al.* (1984) or from seedlings or immature ears according to the method of DELLAPORTA *et al.* (1983). DNA samples were digested for 3–4 hr using commercially available restriction enzymes according to manufacturers' specifications, electrophoresed on agarose gels and transferred to nylon membranes (Magnacharge, Micron Separations Inc., Westboro, MA) according to the method described by SAMBROOK *et al.* (1989). Probes used for hybridization were prepared by random hexamer priming (FEINBERG and VOGELSTEIN 1983) using ^{32}P-labeled dCTP. Membranes were hybridized, washed and exposed to X-ray film using standard procedures (SAMBROOK *et al.* 1989).

Genomic library preparation and screening: Three genomic libraries were prepared to isolate the overlapping clones

that comprise the *a1-m5216* sequence. In all instances, DNA was prepared from plants with the genotype *a1-m5216/a1-dl*. Plant DNA was isolated from immature ears by the method of DELLAPORTA *et al.* (1983), digested to completion with the appropriate enzymes according to manufacturers' specifications and electrophoresed on agarose gels (GTG grade, SeaKem agarose; FMC, Rockland, ME). DNA fragments of the desired sizes were recovered by electroelution (SAMBROOK *et al.* 1989) and extracted sequentially with phenol (pH 8.0), phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The *Eco*RI clone was obtained from a genomic library prepared with DNA from plant 91g7017 and the NM1149 lambda vector. Recovered maize DNA inserts were ligated into *Eco*RI-digested NM1149 arms overnight at 16°, packaged with commercial packaging extracts (Gigapack II, Stratagene, La Jolla, CA) and plated on the *Escherichia coli* host POP13. Plaques were screened using the 2.8-kb *Hin*dIII/*Bgl*II fragment from pALC2 (SCHWARZ-SOMMER *et al.* 1987, see DNA sequence analysis in MATERIALS AND METHODS) as a probe. The *Hin*dIII clone was obtained from a NM1149 genomic lambda library prepared as described above, except *Hin*dIII was used for restriction digests and maize DNA was obtained from plant 906828-6. The *Sal*I clone was obtained from a genomic library with maize inserts isolated from plant 92B403, cloned into Charon35 (Charon35 was a gift from F. R. BLATTNER, University of Wisconsin, Madison). Charon 35 arms were prepared by sucrose gradient centrifugation (SAMBROOK *et al.* 1989) to separate the stuffer fragment from the arms. The library was screened as described above on *E. coli* host NM538. A total of 12 independent clones from the *Sal*I library were analyzed and shown to be identical at the level afforded by restriction mapping.

PCR analysis: PCR reactions were performed using 200–400 ng of genomic DNA (SAMBROOK *et al.* 1989) and 0.5–1 μM of primers. The final concentration of reagents in the 50 μl reactions were: 200 mM dNTP/1.5 to 2.5 mM $MgCl_2$/50 mM KCl/10 mM Tris-HCL, pH 9.0/0.1% Triton X-100. The reactions were denatured at 94° for 0.5 to 1 min, annealed at 55–65° for 0.5–2 min, extended at 72° for 3–5 min (depending on the sizes of expected products), cycled 30–40 times and given a final 10-min extension at 72°. Reactions were electrophoresed on agarose gels using 5–10 μl of reactions, blotted and hybridized with the indicated probes. PCR products were purified by the GeneClean kit (BIO 101, Inc., Vista, CA) either directly (if a single product was obtained, as determined by gel electrophoresis) or after electrophoresis (if there were multiple products) and were subjected to restriction enzyme digestions followed by gel electrophoresis. Primers were prepared at the ISU Nucleic Acid Facility using a 394 DNA/RNA Synthesizer from Applied Biosystems (Foster City, CA). The primers, sp152, sp3-4, sp153 and sp7-2 were gifts from M. G. JAMES and M. J. SCANLON of the A. M. MYERS' Laboratory (Iowa State University). The positions (numbering according to zma1g.gb_pl and m76978.gb_pl) and sequences (5′ to 3′) of primers are listed as follows: A1.1 (GTCTTCATTGCACATGCACTGCAC, 2287–2301 of *a1*); A1.2 (GATTGTTGCTTAAGCGCCAATCGT, 3286–3263 of *a1*); A2667 (GGGTGGACATAAATAAAAGG, 2667–2648 of *a1*); Mu715 (ATCACAACTGGACTGGGA, 715–733 of *MuDR*); sp152 (TAGTGTGGACTCGAC, 1578–1592 of *MuDR*); Mu2270 (TGGCAGAGGTACGAGACAGC, 2270–2289 of *MuDR*); Mu2646 (GAAAACGAAAAAGCGACTCAAAAGG, 2646–2670 of *MuDR*); sp3-4 (GCAGAAAACAGAT, 3492–3504 of *MuDR*); Mu3960 (TCATCTACGGAAGGGTTGTC, 3960–3979 of *MuDR*); XX153 (CGCCTCCATTTCGTCGAATC, near-"universal" primer for *Mu* transposon inverted terminal repeats); sp153 (TACATGTGCTCTGAC, 1967–1981 of *MuDR*); Mu2117 (TCAGCCAAATCACACACAGGAAG, 2117–2095 of *MuDR*); Mu2183 (GAGCTCAGACAGATGGCAAAATAATAC, 2183–2162 of *MuDR* plus GAGCTC); sp7-2 (TCTGTCTGGGATATA, 3671–3657 of *MuDR*).

TABLE 1

Results of *a1-m5216* confirmation crosses

Plant no.	No. of kernels with indicated phenotypes: a1-spotted[a]	Colorless	χ^2 1:1[b]
A. Results from cross 2: *a1-m5216/a1::rdt* × *at-1dl/a1-dl*			
895216/5430	145	147	0.1[c]
895432/5216t[d]	114	140	2.48[c]

Plant no.	No. of kernels with indicated phenotypes: bz1-sp[e]	Bronze[f]	Colored	No. of *Cy*[g]
B. Results from cross 3: *A1/A1 bz1-rcy/bz1* × *a1-m5216/a1::rdt bz1-rcy/Bz1*				
895463/5216	210	50	208	[2](4.6)[h]

[a] a1-spotted indicates a phenotype of colored spots on a colorless background (Figure 1A).

[b] Chi-square value for a 1:1 ratio.

[c] No significant difference.

[d] t indicates tiller. This is a reciprocal cross of cross 2.

[e] bz1-sp (bz1-spotted) indicates a phenotype of colored spots on a bronze background.

[f] Bronze indicates a phenotype of bronze aleurone.

[g] Number of genetically active *Cy* elements carrying by the *a1-m5216* plant. See MATERIALS AND METHODS.

[h] According to the chi-square test results, the observed segregation ratio is significantly different than that expected for all possible numbers of *Cy*. The best fit is for two copies of *Cy*.

DNA sequencing and analyses: *a1-m5216* genomic clones were subcloned into the vectors pBluescript SK(+) or pBluescript KS(+) (Stratagene). PCR products were purified using a GeneClean kit (BIO 101, Inc.) directly or after gel electrophoresis and quantified for direct sequencing. DNA samples were sequenced at the ISU Nucleic Acid Facility using the double-stranded dye terminator technique on a ABI 373A Automated DNA Sequencer (Applied Biosystems). Sequence analyses were performed using the GCG program (Version 7, April 1991, Genetics Computer Group, Inc., Madison, Wisconsin) and comparison made to accessions m76978.gb_pl (*Mu9*) and zma1g.gb_pl (*a1*) from GenBank.

RESULTS

Isolation of the *a1-m5216* allele: As a first step in cloning a genetically active *Cy* transposon, cross 1 was used to "trap" a *Cy* transposon at the *a1* locus. The *a1* locus is one of several genes involved in the anthocyanin biosynthetic pathway (for a review, see DOONER and ROBBINS 1991). In the absence of mutations, all kernels on the ears derived from cross 1 would be expected to be colored because of the anthocyanin pigment in the aleurone layer. Thus, mutations at the *a1* gene can be recovered from cross 1 as rare kernels with colorless aleurone phenotypes. Several kernels with colored spots (somatic instability) on a colorless background (a1-spotted phenotype) were isolated from a population of 1.2 million progeny of cross 1.

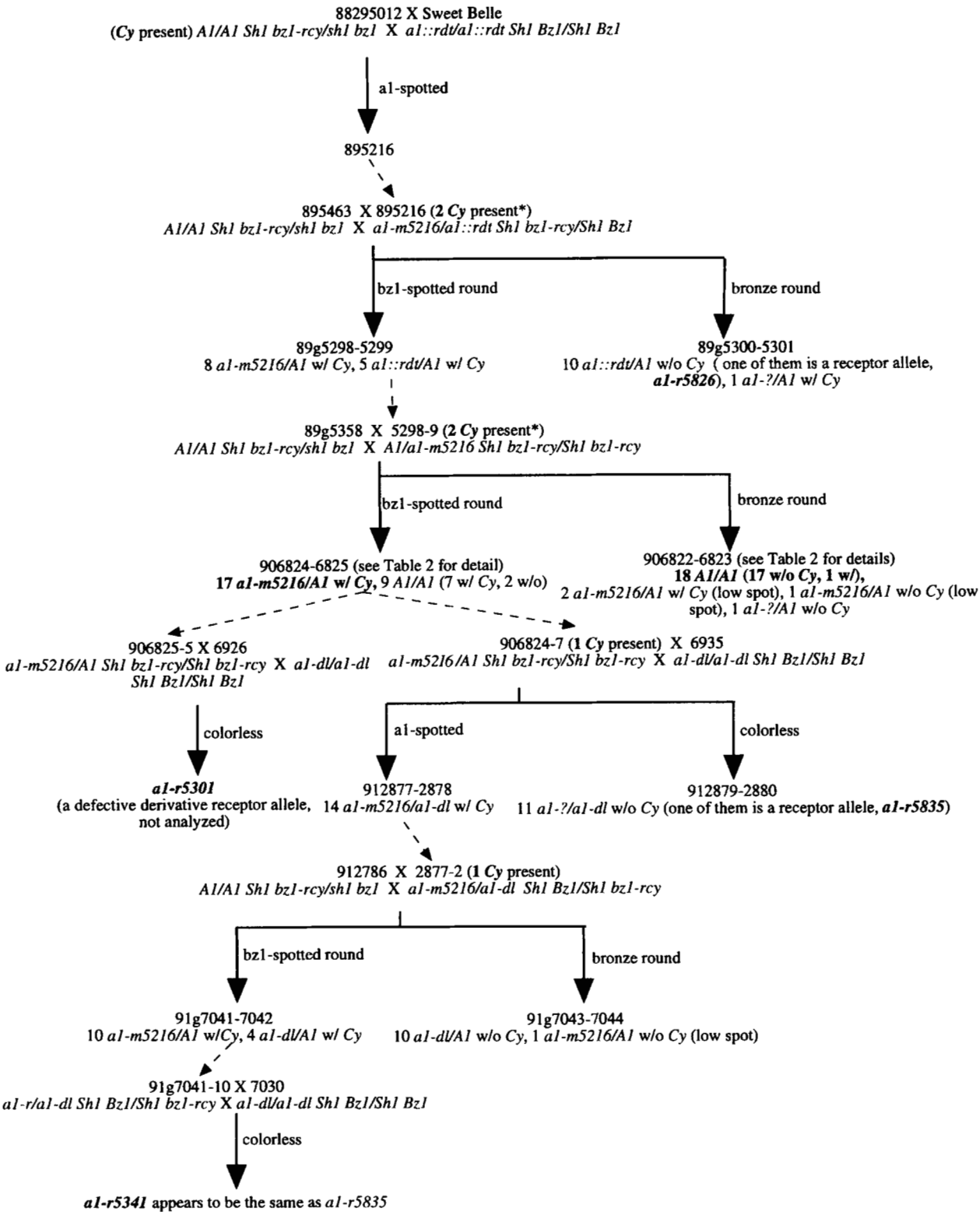

FIGURE 2.—Family 895298-9: an example of a pedigree carrying *a1-m5216* that was used to test the relationship between *Cy* activity and the a1-spotted phenotype. Phenotypes of selected kernels are indicated beside solid arrows. Above the solid arrows are the pedigree numbers and genotypes of the parent plants that gave rise to the ears from which seeds were selected. The row numbers of plants resulting from the selected seeds and the results of genetic tests on these plants are indicated below the solid arrow heads. *Cy* copy numbers were established *via* segregation ratios (see MATERIALS AND METHODS) and are indicated in parentheses. * indicates that the data of the *bz1-rcy* test cross of that plant does not fit the expected ratios for one, two or three copies of *Cy*. In such instances, the ratio with the smallest chi-square value was adopted for the estimation of the *Cy* copy number (such estimates must be interpreted with caution). Dashed arrows lead to the crosses that produced ears with seeds selected for further tests. Some data associated with this family are presented in Table 2.

TABLE 2

Analysis of progeny with and without *Cy* activity from family 89g5298-9

Results from cross 4: *a1-m5216/A1* × *a1-dl/a1-dl*				Results from cross 5: *A1/A1 bz1-rcy/bz1* × *a1-m5216/A1 bz1-rcyl*(bz1-rcy or bz1)			
	No. of kernels with indicated phenotypes			No. of kernels with indicated phenotypes		sh^g	
Plant no.	a1-sp[b]	cl[c]	Cl[d]	bz1-sp[e]	bz[f]	+/−	No. of *Cy*[h]
A. Progeny tests of bz1-spotted selections (with *Cy*) from 89g5358/5298-9[a]							
906824-3	26	1	18	415	23	−	>3
906824-7	206	46[i]	233	183	192	−	1 (0.25)
906824-8	207	1	174	369	83	−	>3
906824-9	177	0	193	179	132	−	[1] (7.3)
906824-10	74	1	74	78	67	+	>3
906824-11	142	2	196	313	20	−	>3
906824-16	222	0	223	260	200	+	>3
906825-3	185	14	171	172	66	−	2 (0.95)
906825-4	198	7	207	39	19	+	>3
906825-5	206	2	169	99	44	−	2 (2.54)
906825-7	152	2	133	241	16	−	>3
906825-8	153	16	189	285	46	−	3 (0.59)
906825-10	260	4	311	87	84	+	>3
906825-11	139	6	116	42	15	−	2 (0.05)
906825-12	203	2	242	30	14	−	1–2 (3.35, 0.09)
906825-13	173	23	189	190	38	−	3 (3.62)

Cross 1: Cy A1 Sh2/A1 Sh2 × *a1::rdt sh2/a1::rdt sh2*.*

The somatic instability of the a1-spotted kernels suggested that a transposon had inserted into the *a1* gene, thereby disrupting that gene's function, resulting in the colorless background phenotype. Somatic excisions of the transposon out of the mutant *a1* allele restore the gene function during development, resulting in somatic clonal sectors that express anthocyanin (colored spots).

One of the mutants derived from cross 1, *a1-m5216*, exhibited the frequent, late somatic reversions typical of *Mu* transposon-induced mutants (Figure 1A). Crosses to an *a1-dl et1* stock (cross 2) tested the inheritance of this mutable phenotype. The resulting segregation ratios are shown in Table 1A.

Cross 2: a1-m5216 Sh2/a1::rdt sh2 × *a1-dl Sh2/a1-dl Sh2.*

If the insertion at *a1-m5216* is a nonautonomous transposon and only one copy of an unlinked autonomous transposon is segregating among the progeny of cross 2, the ratio of a1-spotted to stable colorless would be 1:3. Instead, progeny from cross 2 exhibited a segregation ratio not significantly different than 1:1. This segregation ratio indicated that: the *a1-m5216* allele is under autonomous control, a nonautonomous transposon insertion at *a1-m5216* is responding to multiple *transacting* autonomous transposons present in the parents of crosses 2, or a nonautonomous transposon insertion at *a1-m5216* is responding to an autonomous transposon closely linked to the *a1* locus. Further tests described below will establish that *a1-m5216* arose via the insertion of an autonomous *Cy* transposon.

Mutability of *a1-m5216* is dependent upon *Cy* activity: To determine if somatic mutability of *a1-m5216* is conferred by a *Cy* transposon, *bz1-rcy* was used as a reporter allele in cross 3.

Cross 3: A1/A1 bz1-rcy/bz1 × *a1-m5216/a1-dl bz1-rcy/Bz1.*

In the absence of *Cy* transposons, *bz1-rcy* conditions a stable bronze phenotype. In the presence of a *Cy* transposon, however, the *rcy:Mu7* transposon insertion can excise from *bz1-rcy*, giving rise to somatic instability (bz1-spotted) (SCHNABLE and PETERSON 1989). The plant derived from the exceptional a1-spotted kernel isolated from cross 1 was crossed to *bz1-rcy* stocks (cross 3). The appearance of bz1-spotted kernels among the progeny of cross 3 demonstrated that this a1-spotted kernel carried *Cy* transposons (see Table 1B). The ratio of bz1-spotted to bronze kernels among the progeny of cross 3 suggests that two genetically active *Cy* transposons are present. However, the presence of *Cy* activity in this cross does not establish that *Cy* activity is associated with *a1-m5216* somatic instability.

To test the relationship between *a1-m5216* and *Cy* activity, progeny from crosses 2 and 3 (and related crosses) that would be expected to be segregating for *a1-m5216* and *Cy* were simultaneously tested for mutability at the *a1* locus (via crosses to the *a1-dl* stock) and *Cy* content (via crosses to the *bz1-rcy* stock). If somatic

* To avoid confusion, only the genes that are immediately relevant to the discussion at hand are indicated in each cross. The complete genotypes of each cross are listed in MATERIALS AND METHODS.

TABLE 2

Continued

Results from cross 4: (*a1-5216* or *A1*)/*A1* × *a1-dl*/*a1-dl*				Results from cross 5: *A1*/*A1 bz1-rcy*/*bz1* × (*a1-m5216* or *a1-dl*)/ *A1 bz1-rcy*/(*bz1-rcy* or *bz1*)			
	No. of kernels with indicated phenotypes			No. of kernels with indicated phenotypes		sh	
Plant no.	a1-sp	cl	Cl	bz1-sp	bz	+/−	No. of *Cy*
B. Progeny tests of bronze selections (witout *Cy* activity) from 89g5358/5298-9							
906822-1	0	0	all	0	all	ND[j]	0
906822-2	0	1/2	1/2	0	all	ND	0
906822-3	0	0	all	0	all	ND	0
906822-4	0	0	all	0	all	ND	0
906822-7	0	0	all	0	all	ND	0
906822-8	+[k](L)[l]	+	+	+(L)	+	ND	ND
906822-9	0	0	all	0	all	ND	0
906822-10	0	0	all	0	all	ND	0
906822-12	0	0	all	0	all	ND	0
906822-13	0	0	all	0	all	ND	0
906822-14[m]	18(L)	73	84	0	all	ND	0
906823-1	0	0	all	0	all	ND	0
906823-2	+	ND	+	+	+	ND	ND
906823-3	0	0	all	+	+	ND	ND
906823-4	0	0	all	0	all	ND	0
906823-5	0	0	all	0	all	ND	0
906823-6	0	0	all	0	all	ND	0
906823-7	0	0	all	0	all	ND	0
906823-8	0	0	all	0	all	ND	0
906823-9	0	0	all	0	all	ND	0
906823-10	0	0	all	0	all	ND	0
906823-12	0	0	all	0	all	ND	0

[a] Bronze, round, spotted kernels from ear 89g5358/5298-9 (that resulted from the cross: *A1*/*A1 Sh1 bz1-rcy*/*sh1 bz1* × *A1*/*a1-m5216 Sh1 bz1-rcy*/*sh1 bz1*) were planted in rows 906824-6825. The resulting plants were crossed by *a1-dl* (cross 4) and onto *bz1-rcy* testers (cross 5). The resulting ears were analyzed and counts of kernels with the indicated phenotypes are presented in section A. Data from the nine plants with *A1*/*A1* genotypes (seven with *Cy* activity and two without *Cy* activity) are not shown. Bronze, round kernels were selected and planted in rows 906822–6823 and crossed as described for rows 906824–6825. The resulting data are shown in section B.

[b] a1-sp (a1-spotted): kernels with colorless background and colored spots (Figure 1A).

[c] cl (colorless): colorless kernels.

[d] Cl (colored): colored kernels.

[e] bz1-sp (bz1-spotted): kernels with bronze background and colored spots.

[f] bz (bronze): bronze kernels.

[g] + indicates the presence of shrunken kernels; − indicates the absence of shrunken kernels.

[h] Estimated number of *Cy* elements in plants. see MATERIALS AND METHODS.

[i] Colorless kernels of ear 906824-7/6934 were further tested and shown to carry a deletion-derivative of *a1-m5216* (a receptor allele, *a1-r5835*).

[j] Not determined.

[k] Kernels with the indicated phenotypes were present but the numbers were not recorded.

[l] Low spotting pattern.

[m] Ears from the *a1* test cross contained a1-spotted kernels with the standard a1-spotted pattern. a1-spotted and colorless kernels were tested in the next generation by crossing to a *bz1-rcy* tester. The resulting ears from both selections had very few bz1-spotted kernels and the spotting patterns were low compared to standard bz1-spotted. This result indicated that the *Cy* element(s) carried by these plants had aberrant *Cy* activity. This is thought to explain the nonconcordant results (the presence of a1-mutability in the apparent absence of *Cy* activity) in the previous generation.

instability at *a1-m5216* is dependent upon *Cy* activity, then only progeny that carry *Cy* (as assayed via the *bz1-rcy* crosses) should exhibit the a1-spotted phenotype in crosses to *a1-dl* stocks, and none of the progeny that lack *Cy* should exhibit the a1-spotted phenotype. Data from one family tested in this manner are presented in Table 2 and Figure 2. More than 600 gametes from other families were subjected to similar tests over several generations. The results of these tests are summarized in Table 3. As indicated above, the critical result in these tests is whether the a1-spotted phenotype occurs in the absence of *Cy*. If such entries are not observed, then it can be concluded that *Cy* is responsible for mutability at *a1-m5216*. Among more than 600 tested ga-

TABLE 3

Summary of the association of *a1-m* mutability and *Cy* activity: numbers of individuals that on test crossing were shown to carry *a1-m*, *a1* or *A1*

Row no.	No. of gametes from the female parent with the indicated genotypes					
	With *Cy* activity			Without *Cy* activity		
	a1-m	*A1*	*a1*	*a1-m*	*A1*	*a1*
A. Progeny of cross: *a1-m5216/a1-dl Bz1-rcy/(bz1-rcy* or *bz1)* × *A1/A1 bz1-rcy/bz1*						
Selections: bz1-spotted and bronze						
89g5298-5301	8	0	6	0	0	10
912907-2908	8	0	1	0	0	3
91g7041-7044	10	0	0	5(L)[a]	0	10
912903-2904	7	0	11	1(VL)[b]	0	7
935930-5931	1	0	7	0	0	9
935932-5933	5	0	2	0	0	4
935934-5935	6	0	2	0	0	5
906844-6846	11	0	10	0	0	15
906847-6848	11	0	0	0	0	11
935925-5926	1	0	3	0	0	6
935928-5929	2	0	3	0	0	6
935936-5937	4	0	4	0	0	6
912893-2894	8	0	1	2(L)	0	2
Subtotal	82	0	50	8	0	94
B. Progeny of cross: *a1-m5216/A1 bz1-rcy/(bz1-rcy* or *bz1)* × *A1/A1 bz1-rcy/bz1*						
Selections: bz1-spotted and bronze						
906822-6825	20	8	0	1(L)	19	1
912901-2902	7	5	1(L)	0	9	0
906832-6838	18	12	0	1(L)	34	3(r)[c]
912905-2906	8	6	0	0	4	2
Subtotal	54	31	1	2	66	4
C. Progeny of cross: *a1-m5216/A1* × *a1/a1*						
Selections: a1-spotted and colorless						
912877-2880	14	ND[d]	0	0	0	11(r)[e]
923287u-v	3	ND	0	3(L)	0	0
923288u-v	1	ND	3	0	0	5
906829-6831	21	ND	2	0	0	1
912917-2918	2	ND	0	5(VL)	0	34
912915-2916	16	ND	1(L)	1(L)	0	11
912890, 2885	6	ND	0	0	0	2
912889, 2886	6	ND	1	1(VL)	0	3
912911-2914	27	ND	0	2(VL)	0	23
912909-2910	7	ND	0	0	0	5
912891-2892	2	ND	3	4(L)	0	5
Subtotal	105	0	10	16	0	100
Total	241	31	62	26[f]	66	198

[a] Low spotting pattern and very few spotted kernels.
[b] Very low spotting pattern and very few spotted kernels. See Figure 1B.
[c] Progeny from one of these ears was tested and shown to carry a responsive defective derivative allele, *a1-r182.*
[d] ND indicates no data; colored selection (*A1/_*) were not tested.
[e] Progeny from one of these ears was tested and shown to carry a responsive defective derivative allele, *a1-r5835.*
[f] All aberrant spotting patterns. See text for discussion.

metes, all of the 241 gametes that conditioned a standard, high excision rate from *a1-m5216* carried *Cy* activity as measured by mutability at *bz1-rcy*, and none of the 92 gametes that did not carry *Cy* conditioned this excision pattern. Hence, it can be concluded that *Cy* activity is necessary to achieve the standard, high rate of excision from *a1-m5216.* These extensive genetic tests therefore establish that mutability of *a1-m5216* (*i.e.*, ex-

cision of the resident transposon) is dependent upon the action of a *Cy* transposon.

Although *Cy* is clearly required for the standard high rate of excision at *a1-m5216*, 26 gametes were recovered from this experiment that conditioned nonstandard (low or very low) a1-spotting, even though they apparently lacked *Cy* activity when assayed with the *bz1-rcy* reporter allele (Table 3). Two of these exceptional discordant gametes were further tested and shown to in fact harbor aberrant *Cy* transposon(s). The analysis of one of those cases is described in footnote *m* in Table 2. In addition, the numbers of a1-spotted kernels resulting from the crosses between the discordant gametes and the *a1-dl* stock were, in 13 of these 22 instances, much less than expected, *e.g.*, one to 10 a1-spotted kernels out of several hundred. We therefore hypothesize that the rare appearance of the nonstandard a1-spotted phenotype in the apparent absence of *Cy* activity reflects a difference in the sensitivity of *a1-m5216* and *bz1-rcy* to the action of novel, aberrant *Cy* transposons that arose during these tests.

The genetic tests described above established that mutability at *a1-m5216* is dependent upon a *Cy* transposon. It remained to be established whether the transposon inserted at *a1-m5216* is itself a *Cy* transposon. If this transposon is a *Cy* transposon (or a *Cy* transposon is closely linked in coupling to *a1-m5216*), then selection for *Cy* activity should select for *a1-m5216 vs. A1* in families such as those presented in Table 3B. This tendency would be most pronounced in families segregating for few *Cy* transposons. Hence, within the families tested for the association between *Cy* activity and a1-mutability, progeny from ears that exhibited a one-*Cy* segregation pattern were preferentially selected and used for further crosses. However, the copy numbers of *Cy* transposons in the following generations failed to respond to this selection. This tendency is illustrated by an example presented in Table 2A. bz1-spotted kernels were selected from an ear with two *Cy* transposons (89g5358/5298-9) and were tested (906824-6825). Only five out of 16 analyzed progeny had one or two copies of *Cy* transposons; the remainder had three or more copies of *Cy*. However, even given the relatively high *Cy* copy number in many families, within some families (*e.g.*, 906822-6825, Table 3B), the pronounced association of *Cy* with *a1-m5216* gametes (20/28), suggests that *Cy* is either inserted at or closely linked to *a1* in *a1-m5216*.

Further evidence for this conclusion comes from the identification of plants that harbor only one or two *Cy* transposons (as assayed by segregation ratios in the *bz1-rcy* crosses) but that have ratios of greater than 3:1 a1-spotted:colorless on ears resulting from *a1-dl* testcrosses (*e.g.*, 906825-3, -5, -11, -12 in Table 2A). Given that the standard high rate of mutability at *a1-m5216* is dependent upon *Cy* activity (see above), this result could only occur if a *Cy* transposon is inserted at or closely linked to *a1-m5216*.

Molecular cloning and analyses of *a1-m5216*: The putative *Cy* insertion in the *a1-m5216* allele was isolated as several overlapping genomic clones using *a1* sequences as a probe (Figure 3). The *SalI*, *Hin*dIII and *Eco*RI *a1*-hybridizing clones were isolated from genomic lambda libraries prepared from *a1-m5216* DNA (see MATERIALS AND METHODS). With one exception, the restriction maps of the *Eco*RI, *Hin*dIII and *SalI* clones (which overlaps both the *Hin*dIII and *Eco*RI clones) are indistinguishable from that of *MuDR*. The single difference detected in the *SalI* clone resulted from an ~600-bp deletion (extending from position 1251 to 1854, numbering according to GenBank m76978.gb_pl) that removed the left-most *Hin*dIII site (position 1410). Because 12 out of 12 independent clones from the *SalI* library carry this deletion (data not shown), it appeared that the plant from which this library was prepared carried a deleted transposon at the *a1* locus. This hypothesis was confirmed by PCR amplification using primers A1.1 and Mu2183 directly from the genomic DNA that was used to prepare the *SalI* library (data not shown). Furthermore, the *Hin*dIII clone (isolated from DNA prepared from a different *a1-m5216*-containing plant) included the *Hin*dIII site missing in the *SalI* clones. PCR primers with homology to sites within the *MuDR* transposon (Mu2183) and *a1* sequences near the transposon insertion in the *a1* gene (A1.1) were used to amplify from genomic DNA containing the *a1-m5216* allele the 600-bp region that was deleted in the *SalI* clone. Restriction mapping, hybridization and sequence results confirmed that the resulting PCR product is from *a1-m5216* and includes the 600 bp present in *MuDR* that were deleted in the *SalI* clones. Hence, it can be concluded that the deletion present in the *SalI* clone does not reflect the structure of the intact *a1-m5216* allele. The complete sequence of the *a1-m5216* transposon (derived from the *Hin*dIII, *Eco*RI, and *SalI* clones and the A1.1/Mu2183 PCR product covering the deletion in the *SalI* clone) is 100% identical to *MuDR* (*Mu9*, GenBank accession m76978.gb_pl). Sequence analysis also demonstrated that the *a1-m5216* transposon generated a 9-bp target-site-duplication upon its insertion into the third exon of the *a1* locus between positions 2484 and 2485 (numbering according to zma1g.gb_pl in GenBank), characteristic of the *Mu* transposon family. These results demonstrate that the transposon cloned from *a1-m5216* is identical to *MuDR*.

Genetic data presented earlier established that a high rate of excision of the transposon inserted at *a1-m5216* is absolutely dependent upon *Cy* activity. Further tests established that *Cy* activity maps genetically to the vicinity of the *a1-m5216* allele. Hence, given that the transposon inserted at *a1-m5216* is a *MuDR* transposon, either *MuDR* is not autonomous and instead is dependent upon a closely linked *Cy* transposon for full excision activity or *MuDR* and *Cy* are one-in-the-same. Because *MuDR* is an autonomous transposon (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991; QIN *et al.*, 1991), we conclude that *MuDR* and *Cy* are identical. Therefore, *Cy* will henceforth be termed *MuDR:Cy*.

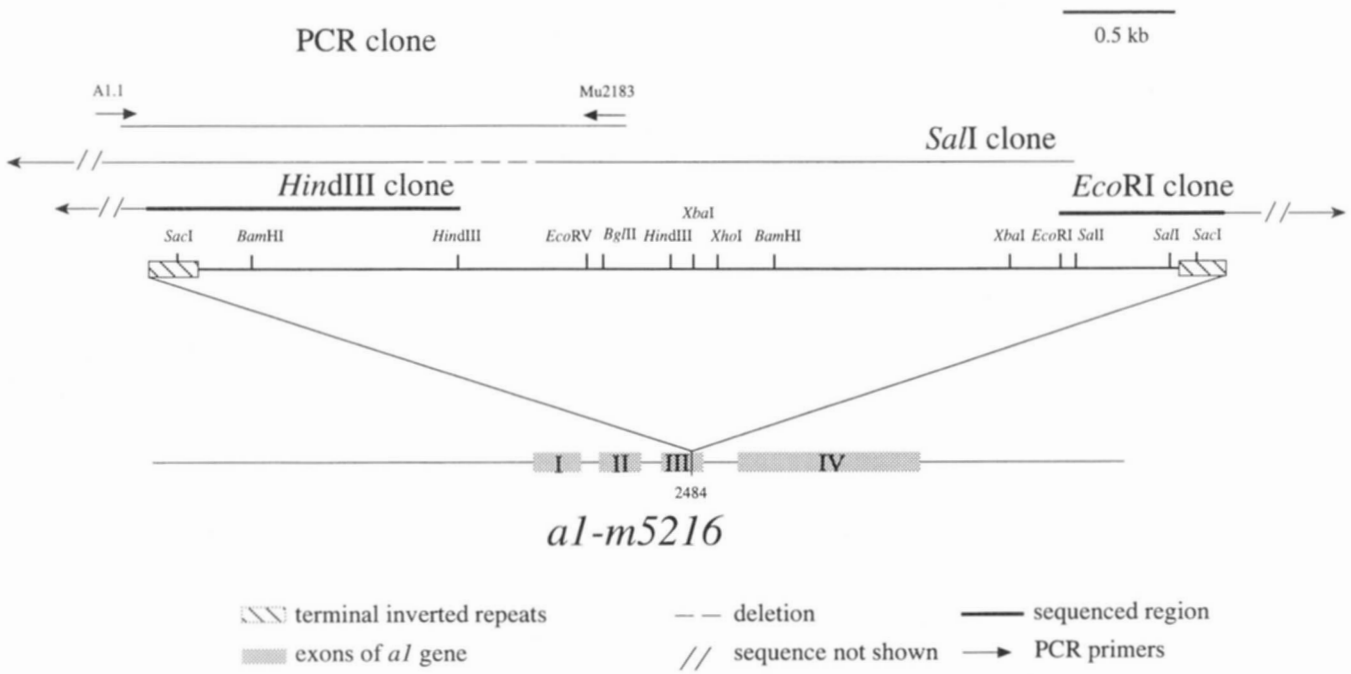

FIGURE 3.—Cloning of the *a1-m5216* allele. Four overlapping clones span the entire *a1-m5216* allele. The insertion site of *Cy* in the *a1* gene is indicated.

The isolation of defective derivatives of *MuDR*: It has been shown that deletion derivatives of *MuDR* arise at a high rate (HARDEMAN and CHANDLER 1993; LISCH and FREELING 1994; LISCH *et al.* 1995). An interrupted gap-repair model was proposed to be responsible for the creation of these deletion derivatives (LISCH *et al.* 1995). Although deletion derivatives of *MuDR* have been isolated and physically mapped (HARDEMAN and CHANDLER 1993; LISCH and FREELING 1994; LISCH *et al.* 1995), none have been sequenced. Because such sequences would represent an important test of this model, deletion derivatives of the *MuDR* transposon inserted at *a1-m5216* were isolated, analyzed and sequenced.

Defective derivative alleles of *a1-m5216* that condition a stable, nonspotted phenotype would be expected to fall into two classes. The first class would consist of those derivative alleles (*a1-r*) that can not undergo somatic reversion autonomously, but are responsive to *trans*-activation by active *MuDR:Cy* transposons. Alleles of the second class (*a1-nr*) would not exhibit somatic excision events even in the presence of active *MuDR:Cy* transposons.

Nine putative *a1-r* and three *a1-nr* alleles were isolated from crosses 4 and 6.

Cross 4: a1-m5216 et1/A1 Et1 × a1-dl et1/a1-dl et1.
Cross 6: a1-m5216 Et1/a1-dl et1 × a1-dl et1/a1-dl et1.

Progeny of cross 4 would be expected to segregate 1:1 for a1-spotted and colored kernels. Colorless nonspotted kernels (Figure 1C) from cross 4 were isolated as exceptions that potentially carried *a1-r* or *a1-nr* alleles (see Table 2A for examples) and analyzed. Progeny from cross 6 would be expected to segregate 1:1 for nonetched a1-spotted:etched, nonspotted, colorless kernels. Putative *a1-r* and *a1-nr* exceptions were selected as nonetched, colorless kernels in ear sectors.

Putative *a1-r* and *a1-nr* alleles derived from crosses 4 and 6 were subjected to three crosses: × *a1-dl/a1-dl* stocks to confirm the absence of a1-mutability; × *bz1-rcy* stocks to test for the presence of *Cy* activity; and to stocks carrying active *MuDR:Cy* transposons to test their ability to be reactivated. The classification of these exceptions as *a1-r* or *a1-nr* alleles is based upon the results of these genetic tests. Alleles that had lost a1-mutability but that are responsive to active *MuDR:Cy* transposons are classified as *a1-r* alleles. Alleles that lost a1-mutability but can not be activated by active *MuDR:Cy* transposon are classified as *a1-nr* alleles. As will be discussed below, sequence data from around the end points of *MuDR* deletion derivatives support the hypothesis that these deletion derivatives arise through interrupted gap repair.

Molecular analysis of *a1-r* and *a1-nr* alleles: Genomic mapping, PCR analyses and sequencing were used to identify the molecular lesions present in *a1-nr* and *a1-r* alleles relative to the intact *a1-m5216* allele. Initial characterizations were performed via genomic Southern blotting. Subsequently, PCR primers were designed to further dissect the lesions within each allele.

An example of one of these analyses is shown in Figure 4. Panel A shows the 2.4-kb PCR product expected to be amplified from *a1-m5216* using primers A1.1 and Mu2183. This product is observed in lane 2 of Panel B. In contrast, amplification of DNA from a plant carrying *a1-nr5940* yielded a novel 1.4-kb product (lane 3). Both of these PCR products hybridize to *MuDR* probes (lanes 2 and 3 in panel C, Figure 4). Each of the two PCR products was gel purified and double-digested with *Bam*HI and *Bgl*II. Digestion of

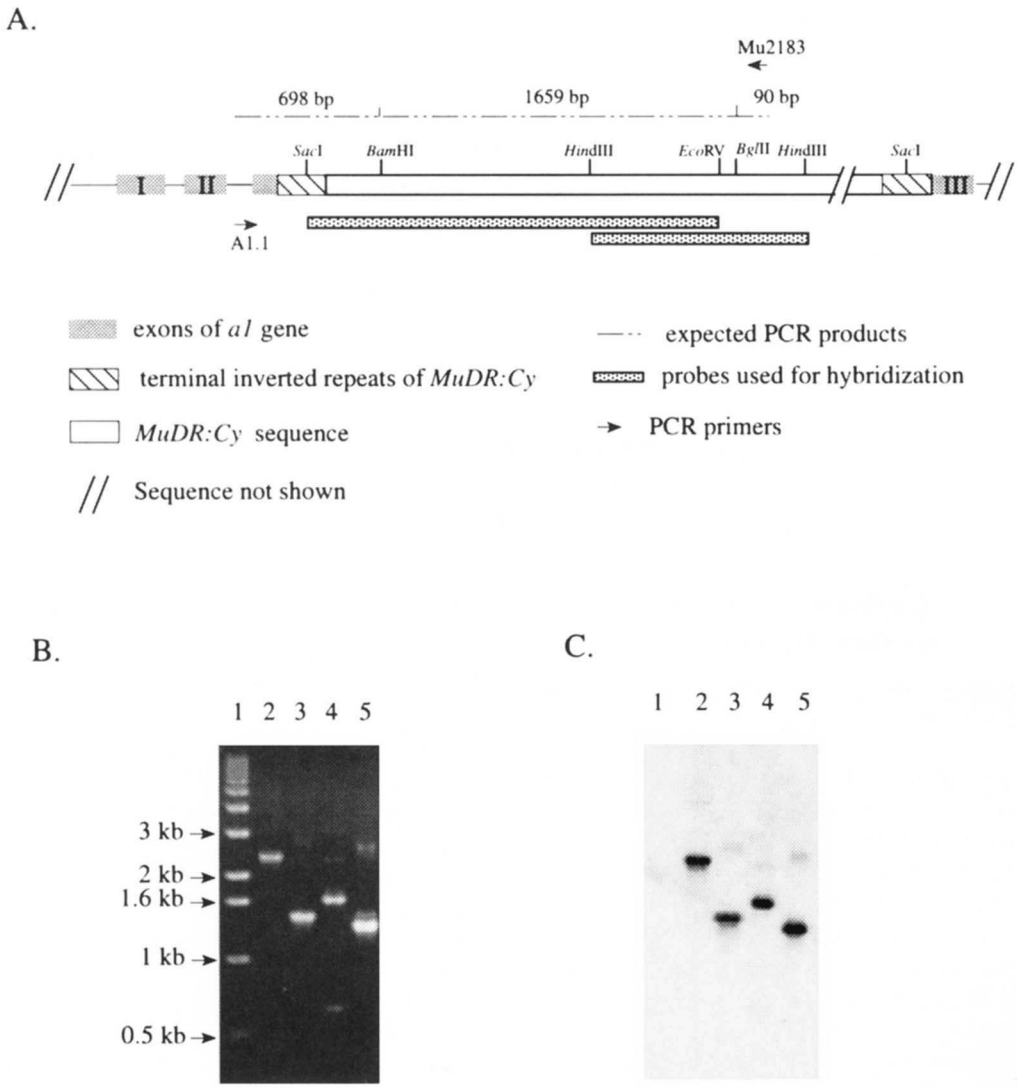

FIGURE 4.—PCR analysis of the defective derivative allele, *a1-nr5940*. (A) Partial restriction map of *a1-m5216*. The positions of the PCR primers used in this experiment (A1.1 and Mu2183) are indicated by arrows. The 2.4-kb PCR product expected from *a1-m5216* using these primers is shown. The expected sizes of the DNA fragments resulting from the digestion of this PCR product with *Bam*HI and *Bgl*II are indicated. (B) PCR products and digestion results. The PCR products obtained from *a1-m5216* and *a1-nr5940* using primers A1.1 and Mu2183 are shown in lanes two and three, respectively. These PCR products were gel purified and subject to *Bam*HI and *Bgl*II double digestion (lanes 4 and 5, respectively). The faint bands in lanes 3 and 5 are probably nonspecific products from the PCR reactions. The faint band in lane 4 is probably a partial digestion product. (C) The gel shown in Figure 4B was transferred to nylon membrane and hybridized with probes indicated in A. The 698-bp *Bam*HI fragment in lane four became visible following a longer exposure (data not shown).

the PCR product derived from the intact *MuDR* transposon revealed two of the expected three fragments (lane 4, Panel B). (The expected 0.1-kb fragment was too small to observe in this analysis.) In contrast, double-digestion of the PCR product derived from *a1-nr5940* released a 1.3-kb fragment (lane 5, Figure 4B). This result suggested that the *Bgl*II site, but not the *Bam*HI site, was retained in *a1-nr5940*. Further investigations, including direct sequencing of the PCR products (using the primers indicated in Figure 5), revealed that *a1-nr5940* contains a deletion from position 2468 in *a1* to position 949 in *MuDR* (Figure 5). The deletion removed part of exon 3 of *a1* and the promoter region and the 5′ end of the *mudrA* transcript.

In total, 15 defective derivative alleles of *a1-m5216* were analyzed in this fashion. Nine out of the 15 did not exhibit any changes at the resolution level of PCR analysis (*a1-r174, a1-r177, a1-r180, a1-r184, a1-r186, a1-r5826, a1-r5828, a1-nr176, a1-nr187*). Summaries of the results obtained from *a1-nr5940* and of 5 *a1-r* alleles that did exhibit sequence changes are shown in Figures 5 and 6. Two of these alleles were analyzed at the restriction mapping and hybridization level but not sequenced (*a1-r5306* and *a1-r5938*). Allele *a1-r5306* carries a 700-bp deletion between the *Bam*HI (at position 2865) and *Xba*I (at position 3945) sites that could affect either or both of the *mudrA* and *mudrB* transcripts (Figure 5). Allele *a1-r5938* has a 500-bp deletion between *Hin*dIII (at position 1410)

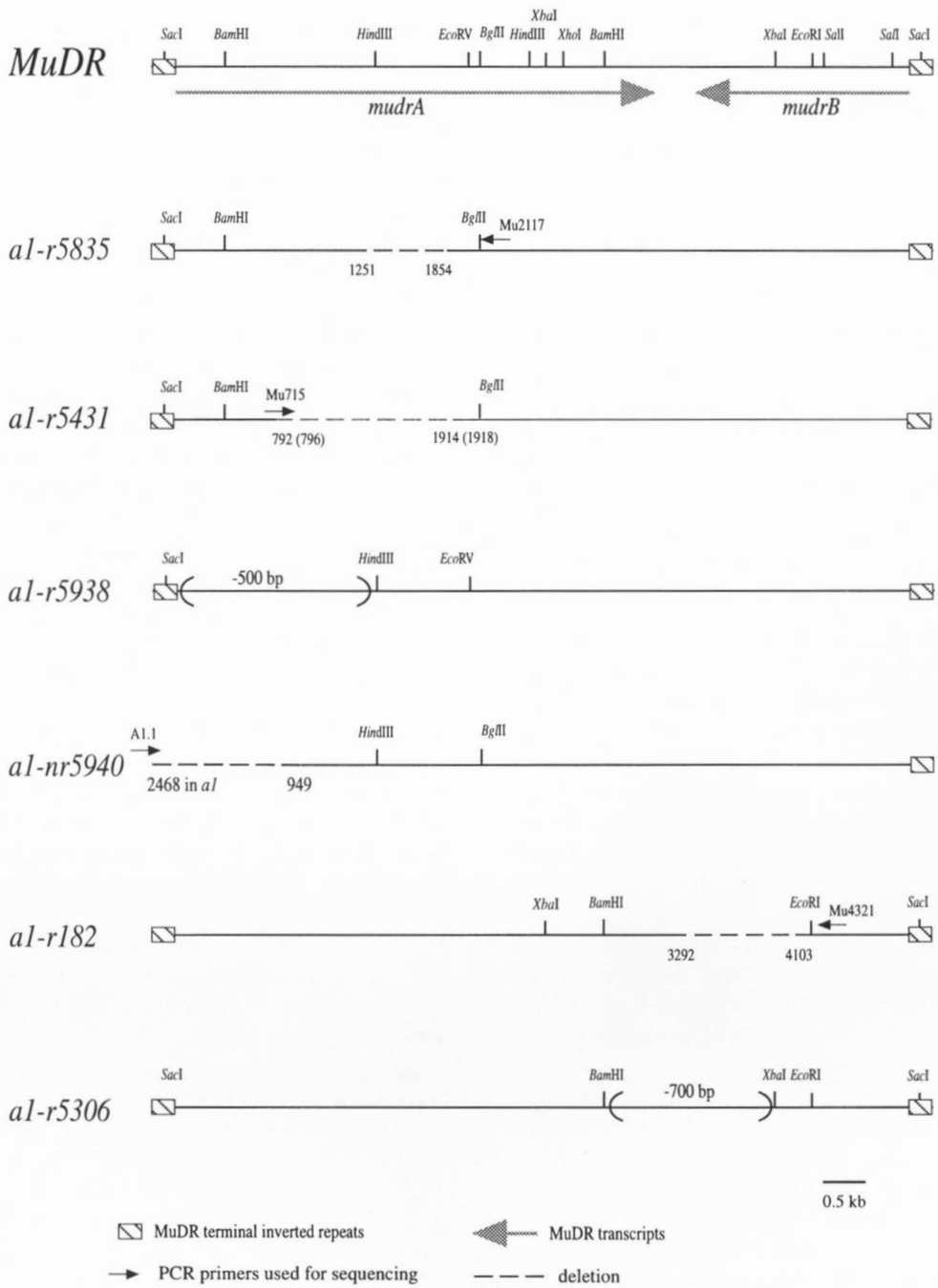

FIGURE 5.—Comparisons of six defective derivative alleles of *a1-m5216* to *MuDR*. Deleted regions are indicated by dashed lines. Deletion end points (as detected via sequencing) are listed under the restriction map. Deletions that were not sequenced were mapped between adjacent restriction enzyme site as indicated by parentheses. The sizes of parentheses are not proportional to the sizes of deletions. Restriction enzyme sites tested on the PCR products are indicated for each allele. The transcripts from *MuDR* are indicated by bold lines (*mudrA* and *mudrB*) under the partial restriction map of *MuDR*.

and *Sac*I (at position 127) in *MuDR* that will disrupt the *MudrA* transcript (Figure 5). Two of the *a1-r* alleles (*a1-r5835* and *a1-r5431*) that have been sequenced have deletions that will disrupt the *mudrA* coding region (1251–1854; 792–1914 or 796–1918, respectively) (Figures 5 and 6). In contrast, *a1-r182* harbors a deletion from position 3292 to 4103 in *MuDR* that disrupts the *mudrB* transcript coding region and the polyadenylation sites (HERSHBERGER *et al.* 1995) of *mudrA* (Figures 5 and 6).

DISCUSSION

***Cy* and *MuDR* are identical:** Previous studies (SCHNABLE and PETERSON 1986, 1989) suggested that the genetically defined *Cy* transposon is the autonomous transposon of the *Mutator* transposon system. More recently, the *MuDR* transposon has been cloned from *Mutator* stocks and shown to be the autonomous transposon of this transposon system. (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991; QIN *et al.* 1991). In this study, we

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a1-r5431   GAAAATGAGAtatattttaa--ctataggagaGATGTATTTG
               796                      1915
a1-nr5940  GAGGAACGGCAGAggcccgt--ccagatgaaaagaGGGTGGT
               2467 in a1                   950
a1-r5835   TCACATAGTTACCCAttaag--cgtggaatgcacccaACCAC
               1853                     1250
a1-r182    TGTTGTAAGACTgctcagtt--caccttgtacctCTGGAATA
               3292                     4103

Hershberger et al. 1995

           TCTTAGCGATACtaaaggct--gataaacaccacAAAAAATT
               2211                     2854
           ATTCTAGAAGggtggaacca--aaagactgagGATTAGTGCT
               2484                     3117
           GCTAGAAATAaaagtaccaa--attggttgtaAGACTGCTAA
               2627                     3254
           ACTCTACTGAaagattcaag--ctccaccaccGACAAGGAAA
               2457                     3069
```

FIGURE 6.—Sequences flanking *MuDR* deletion end points. The bases extending to each side of the deletions are shown in upper case. Deleted bases are shown in lower case. Deletion end points are numbered according to *Mu9* (accession No.: m76978.gb_pl). Direct repeats are underlined. Data are presented from this study and that of HERSHBERGER *et al.* 1995.

have reported the isolation of a mutable *a1* allele with a *Cy* insertion (*a1-m5216*). The sequence of the cloned transposon inserted at *a1-m5216* is identical to *MuDR*. Hence, these data establish that *Cy* is in fact the same as *MuDR*. The former will henceforth be termed *MuDR:Cy*. These data also establish that genetically active *MuDR* transposons are not confined to *Mutator* stocks because the TEL population from which *Cy* was isolated shares no recent pedigree with *Mutator* stocks (SCHNABLE and PETERSON 1986).

Functional analysis of deletions suggests roles for *MuDR* transcripts: *MuDR* codes for two convergent transcripts (HERSHBERGER *et al.*, 1991; JAMES *et al.* 1993), *mudrA* and *mudrB* (HERSHBERGER *et al.* 1995). The functions (if any) of these transcripts are not known because, although they cosegregate with *Mutator* activity, they are present in all organs and at all developmental stages tested in active *Mutator* stocks (HERSHBERGER *et al.* 1995) even though *Mu* transposon transposition occurs during limited developmental stages (reviewed by CHANDLER and HARDEMAN 1992). The deduced protein sequences of *mudrA* shares homology with the putative transposase of a group of bacterial transposons and thus is suggested to encode the transposase function of *MuDR* (EISEN *et al.* 1994). The function (if any) of *mudrB* is even less clear.

As a first step toward testing whether these transcripts are required for *MuDR* excision, defective derivative alleles of *a1-m5216* that are not capable of autonomous excision were isolated. Among the 15 alleles analyzed, only six exhibited lesions detectable at level of restriction mapping and PCR analysis. Among these six defective derivative alleles, five harbor deletions internal to *MuDR* and one, *a1-nr5940*, has a deletion spanning the junction of the *a1* gene and the 5′ end of the *MuDR* transposon. The five internal deletion derivatives can be transactivated to transpose by an intact *MuDR* elsewhere in the genome. The lesion of *a1-r5306* was only mapped to the resolution afforded by restriction enzyme digestion and may affect either one or both of the transcripts. However, three of the remaining internal deletions (a1-r5835, a1-r5431, a1-r5938) removed portions of the *mudrA* (but not *mudrB*) coding region. This result, in combination with *MuDR* deletions isolated by others (LISCH and FREELING 1994; LISCH *et al.* 1995) and characterized via restriction mapping, suggests that *mudrA* is indeed necessary for *MuDR* excision.

There is currently less support for a role of *mudrB* in *MuDR* excision. The isolation of *MuDR* deletions that are not excision autonomous and that have deletions that affect only *mudrB* would support such a role. However, none of the deletions analyzed by LISCH and FREELING (1994) and LISCH *et al.* (1995) affect *mudrB* exclusively.

DNA sequence analysis has demonstrated that only one of the deletions isolated in this study (*a1-r182*) unambiguously affects *mudrB*. This deletion spans more than half of the *mudrB* coding region and only the very 3′ end (32 bases) of *mudrA*, including the two putative poly(A) sites of *mudrA* at positions 3298 and 3324 (HERSHBERGER *et al.* 1995). It is possible that the *mudrA* transcript produced by *a1-r182* could still be polyadenylated even though it lacks both poly(A) sites (HUNT 1994). However, deletion of the intergenic region between *mudrA* and *mudrB* would be expected to remove the transcription termination sites of both transcripts. Such a loss could result in read-through of *mudrA* and/or *mudrB*. Such read-through transcripts would be antisense because *mudrA* and *mudrB* are transcribed in a convergent fashion (HERSHBERGER *et al.* 1995). However, because antisense *mudrA* and *mudrB* transcripts are present in active *Mutator* plants (HERSHBERGER *et al.* 1995), it is not clear whether additional antisense transcripts would affect *MuDR* excision. Unfortunately, because transcripts of different sizes are present in *Mutator* plants and transcripts of similar sizes are heterogenous at the sequence level (HERSHBERGER *et al.* 1995), northern analyses would probably not be useful in determining the kinds of transcripts produced by *a1-r182*. In the absence of such data, the role of *mudrB* in *Mu* transposon excision remains unresolved.

Break point sequences are consistent with *MuDR* deletions arising via interrupted gap repair: All of the *MuDR* mutations reported here with detectable sequence changes represented deletions. Because of the ease at which these events can be isolated (HARDEMAN and CHANDLER 1993; LISCH and FREELING 1994; LISCH *et al.* 1995), it must be assumed that *MuDR* deletions arise at rates far in excess of the spontaneous mutation rate in maize (10^{-5} to 10^{-6}, STADLER 1951). It has been proposed that such deletion derivatives may arise as the results of interrupted gap-repair processes (LISCH *et al.* 1995). The deletion break points of all four sequenced *MuDR:Cy* deletion derivatives occur adjacent to direct repeats (Figure 6). This finding is consistent with the interrupted gap-repair model proposed by ENGELS *et al.* (1990) as the mechanism for *P* element transposition.

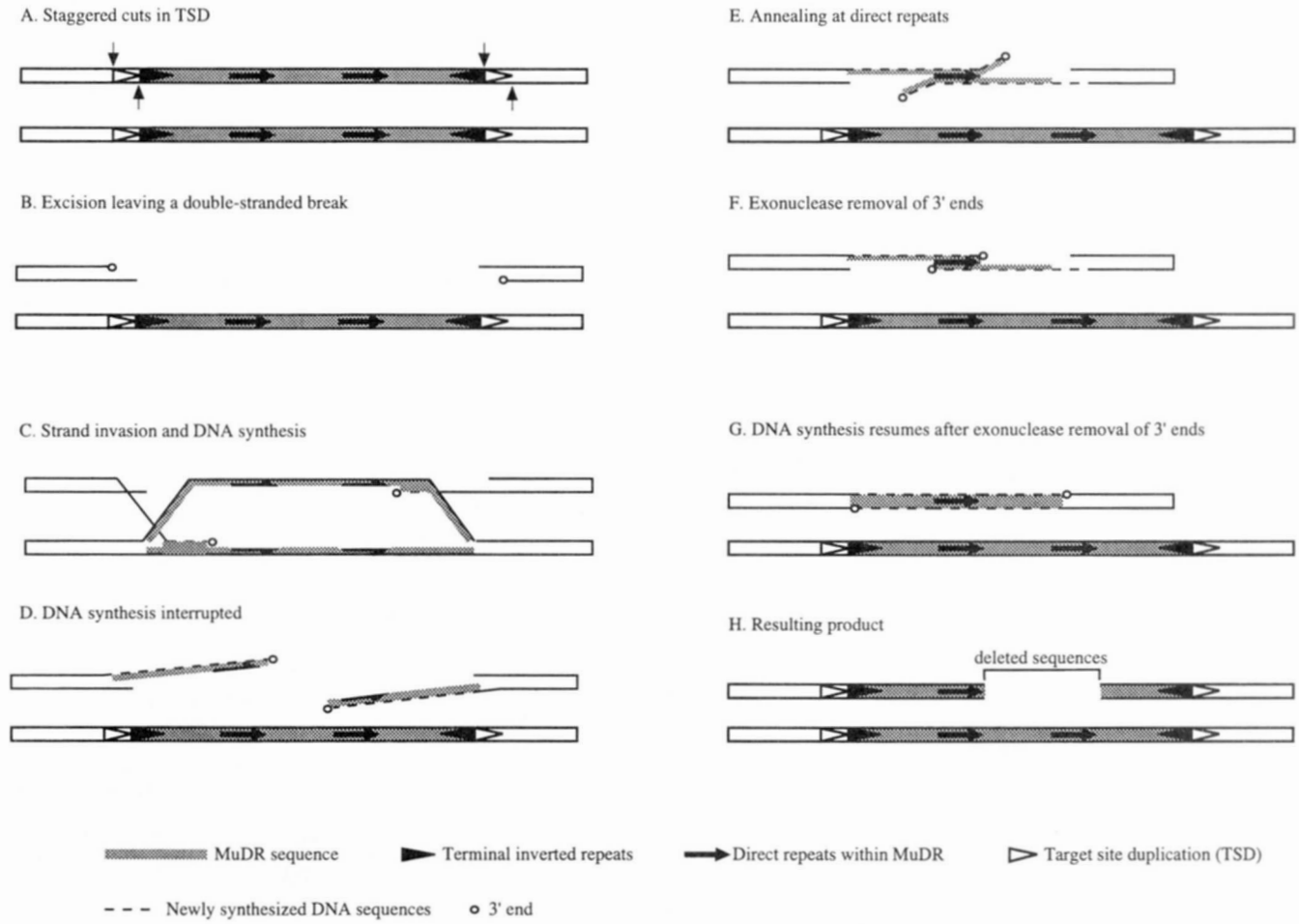

FIGURE 7.—Gap-repair model (adapted from KURKULOS *et al.* 1994). (A and B) *MuDR* excises from one sister chromatid and generates a double-stranded break. (C) After strand invasion, the free 3′ ends anneal with homologous sequences on the sister chromatid or elsewhere in the genome. DNA synthesis proceeds 5′ to 3′. (D and E) Repair is interrupted and the two partial strands anneal to each other at the site of a direct repeat. (F) 3′ overhangs are removed by exonuclease. (G) DNA synthesis resumes to fill the gaps. (H) The resulting molecule differs from the progenitor (see A) by the absence of one of the direct repeats and the entire sequence between the two direct repeats.

Support for this model has been obtained in Drosophila by the finding that deletions within *P* elements occur preferentially between direct repeats removing one copy of the repeats (KURKULOS *et al.* 1994).

According to this model (Figure 7), *MuDR* excision would result in a staggered double-stranded break with two free 3′ ends (which may be subject to exonuclease attack). These free 3′ ends can then anneal with homologous sequences that will serve as a template for DNA synthesis. This template could be the sister chromatid, the homologue, or sequences from elsewhere in the genome. Depending on the genotype of the template used, this repair process may result in precise excision (if the template does not contain a *MuDR* transposon) or the recovery of the *MuDR* transposon at the original site (if a template with the same allele, such as the sister chromatid, is used). Alternatively, if DNA synthesis is interrupted during gap repair, the two partial single-stranded overhangs may anneal with each other at sites with sequence homology (such as direct repeats) following which DNA synthesis can resume. At the conclusion of gap repair, the sequences between the two direct repeats plus one copy of the repeat will be lost.

Our data demonstrate that *a1-r5835, a1-nr5940, a1-r5431* and *a1-r182* have deletion break points of between two and five base pairs(Figure 6), consistent with their having arisen *via* interrupted gap repair. While this manuscript was under review, HERSHBERGER *et al.* (1995) published the sequences of four *MuDR* deletions obtained as random cDNA clones. Because the HERSHBERGER *et al.* deletions were obtained via reverse transcription of RNA, no functional analysis could be performed on them. However, three out of the four breakpoints associated with these deletions also occurred adjacent to direct repeats (Figure 6). This finding suggests that although interrupted gap repair may not account for all *MuDR* deletion events, many can be explained by the interrupted gap-repair model.

Because of the nature of our selection method and analyses, interrupted gap-repair events may have been preferentially recovered relative to more precise excision events. Other published sequences of somatic and

germinal *Mu* transposon excision products (SCHNABLE and PETERSON 1989; BRITT and WALBOT 1991; LEVY and WALBOT 1991) can be best explained by the SAEDLER and NEVERS (1985) model. This model proposes that the single-stranded target-site-duplication may act as a template for DNA synthesis following excision. Hence, the gap-repair and SAEDLER and NEVERS models may represent alternative processes to repair an excision site.

Our data suggest that events other than large deletions can affect *MuDR* function. The loss of *Mutator* activity has previously been associated with the hypermethylation of *Mu* transposons in several occasions (reviewed by CHANDLER and HARDEMAN 1992). Although the mechanism by which hypermethylation arises is not known, crossing inactive lines to active *Mutator* stocks sometimes restores *Mutator* activity coincident with the demethylation of the formerly hypermethylated transposons (reviewed by CHANDLER and HARDEMAN 1992). More recently, it has been shown that hypermethylation of *MuDR* transposons is correlated with loss of *MuDR* activity (GREENE *et al.* 1994; MARTIENSSEN and BARON 1994; LISCH *et al.* 1995). Hence, some of the nine defective alleles of *a1-m5216* that did not exhibit any detectable alterations at our level of analysis could have arisen via hypermethylation. Alternatively, they may represent small alterations in the sequence of *MuDR* that were not detected in our analysis.

Our selection scheme only allowed for the isolation of total loss of autonomous *MuDR* excision function (nonspotted kernels). Selection of altered (*e.g.*, lower) spotting patterns (changes of state) might give rise to a different spectrum of mutations in the *MuDR* transposon. Analysis of such events would be expected to help further dissect the functions of the two *MuDR* transcripts.

The genetic analyses of *MuDR:Cy* transposons conducted in the late 1980s were greatly facilitated by the near-Mendelian inheritance of *MuDR:Cy* (SCHNABLE and PETERSON 1988). In contrast, the inheritance of the autonomous transposons in *Mutator* stocks is typically extremely non-Mendelian (ROBERTSON 1978). It was this feature of *MuDR:Cy* that made it an excellent model for studying *Mutator* activity. However, efforts to isolate a line carrying *a1-m5216* (and no additional *MuDR:Cy* transposons) have proven fruitless (data not shown). In these experiments, *MuDR:Cy* copy number exhibited a strong tendency to increase from one generation to the next even in the presence of strong selection for low *MuDR:Cy* copy number (based on segregation ratios of spotted:nonspotted kernels). Those rare families within which it was possible to maintain low *MuDR:Cy* copy number invariably exhibited low excision rates as recorded at *a1-m5216* and *bz1-rcy*, thereby suggesting that the *a1-m5216* insertion had undergone mutation or modification. This difference in replication behavior between the original *MuDR:Cy* isolates and *a1-m5216* could be due to either compositional or positional differences among *MuDR:Cy* transposons.

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