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Mu1-Related Transposable Elements of Maize Preferentially Insert into Low Copy Number DNA

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ABSTRACT

The *Mutator* transposable element system of maize was originally identified through its induction of mutations at an exceptionally high frequency and at a wide variety of loci. The *Mu1* subfamily of transposable elements within this system are responsible for the majority of *Mutator*-induced mutations. *Mu 1*-related elements were isolated from active *Mutator* plants and their flanking DNA was characterized. Sequence analyses revealed perfect nine base target duplications directly flanking the insert for 13 of the 14 elements studied. Hybridizational studies indicated that *Mu1*-like elements insert primarily into regions of the maize genome that are of low copy number. This preferential selection of low copy number DNA as targets for *Mu* element insertion was not directed by any specific secondary structure(s) that could be detected in this study, but the 9-bp target duplications exhibited a discernibly higher than random match with the consensus sequence 5′-G-T-T-G-G/C-A-G-G/A-G-3′.

THE *Mutator* transposable element system of maize is characterized by an exceptionally high rate of induced germinal mutations (ROBERTSON 1978) as well as by the unusually diverse nature of the mutants isolated (reviewed in BENNETZEN *et al.* 1993). Six different subfamilies of transposable elements have been identified in the *Mutator* system, all with similar termini but with apparently unrelated internal sequences (reviewed in WALBOT 1991; CHANDLER and HARDEMAN 1992; BENNETZEN *et al.* 1993). Most of the *Mutator*-induced mutations that have been characterized are due to the insertion of a member of the *Mu1* subfamily of elements (BENNETZEN *et al.* 1993).

Variable levels and degrees of insertion specificity have been observed in all of the transposable element systems, primarily in bacteria, where insertion preferences have been investigated. For instance, some bacterial elements seem to insert at or near a specific primary sequence, others seem to prefer a region with a particular base composition, while others show preferences for insertion into functionally significant regions, like gene promoters (reviewed in BERG and HOWE 1989). Relatively little is known about the insertion specificities of plant transposable elements. Genetic experiments indicated that *Ac* elements preferentially insert at sites linked to the initial (donor) element site in transpositions from the *bz1*, *p*, and *wx1* loci (VAN SCHAIK and BRINK 1959; GREENBLATT and BRINK 1962; DOONER and BELACHEW 1989; SCHWARTZ 1989). The asymmetric distribution of linked sites observed when *Ac* transposed from the *p* locus and an absense of *Ac* transposition events in a 4 map unit region close to the *p* locus indicated *Ac* insertion preferences within a given linked region (GREENBLATT 1984). In the *En/Spm* transposable element system of maize, transpositions of *En* from the *a1* locus also exhibited some preference for linkage to the donor element (PETERSON 1970; NOWICK and PETERSON 1981).

At the primary sequence level, short (6–14 nucleotide) direct repeats have been observed very close to the site of some *Ac*, *Ds* and *En/Spm* element insertions (DÖRING and STARLINGER 1984). Similarly, short homologous sequences have been found on either side of the *Tam3* elements in both the *Nivea* and *Palida* loci of *Antirrhinum majus* (COEN *et al.* 1986). *Tam1* elements of *A. majus* are found in both repetitive and low copy number sequences that are A+T rich (NACKEN *et al.* 1991), but specific target sequences have not been identified. Because these correlated sequence organizations of element insertion sites were not compared with the structure of randomly cloned maize or *A. majus* DNAs, it is not clear whether they have any functional significance. The *tourist* elements of maize show a strong preference for insertion at, and flanking duplication of, the sequence 5′-TAA-3′ (BUREAU and WESSLER 1992).

Information from early studies of *Mutator* indicated that the DNA near *Mu1*-related elements is not cytosine 5-methylated (BENNETZEN 1985; BENNETZEN *et al.* 1988, 1994). A similar specificity for insertion into unmethylated target sequences has also been observed for the *Ac* and *En/Spm* elements (CHEN *et al.* 1987b; CONE *et al.* 1988). In maize, nuclear DNAs that are 5′ cytosine

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unmethylated appear to be associated only with coding sequences (ANTEQUERA and BIRD 1988; WALBOT and WARREN 1990; BENNETZEN *et al.* 1994). The high mutagenic rate seen in some *Mutator* plants that contained relatively few *Mu1*-like elements suggested that *Mu1* might have a gene-specific insertion preference (BENNETZEN 1984; BENNETZEN *et al.* 1987). The uniquely broad variety of loci that have been associated with *Mutator*-induced mutation (reviewed in BENNETZEN *et al.* 1993) suggests that *Mu* elements can insert into essentially any gene, but mutations at some loci do occur up to 10 times more frequently than at others (ROBERTSON 1985), and different subclasses of *Mu* elements appear to prefer to insert into different genes (BROWN *et al.* 1989; BENNETZEN *et al.* 1993; HARDEMAN and CHANDLER 1993).

The few *Mu* elements that have been cloned did not exhibit any compelling homology at the nine base pair target duplications that are common to this system (reviewed in CHANDLER and HARDEMAN 1992). However, studies of *Mutator*-induced *bz1* mutations revealed that, although *Mu1* elements had inserted throughout *bz1*, one site was particularly preferred (BROWN *et al.* 1989; HARDEMAN and CHANDLER 1989). A study of dominant mutations associated with *Mu1* and *Mu8* insertions at *kn1* also revealed an apparent preferred region for insertions (GREENE *et al.* 1994). However, these three latter studies could have been biased by the nature of the mutant phenotypes selected.

In this paper, we present experiments that investigate the insertion specificity of *Mu1*-related transposable elements. We analyzed the insertion sites of many randomly selected *Mu1*-like elements. The evidence presented reveals preferential insertion of *Mu1* into low copy number sequences, but indicated only a weak preference for a specific target sequence consensus and no preference for any specific secondary structures at or near the insertion site.

MATERIALS AND METHODS

DNA isolation, genomic libraries and subcloning: Three active *Mutator* lines were separately used as sources of maize genomic DNA for library constructions: B1, D3 and D4. Genomic DNA was purified as previously described (BENNETZEN 1984; HULBERT *et al.* 1990). Genomic libraries were constructed in a Charon40 phage vector (DUNN and BLATTNER 1987) digested with *NaeI* and either *Eco*R1 or *Bam*HI. The maize DNAs for the B1 and D4 libraries were digested to completion with *Eco*R1. Maize DNA for the D3 library was partially digested with *Sau*3A and run over sucrose gradients to purify the 10- to 20-kb fraction. There are no sites for *Sau*3A or *Eco*R1 in *Mu1*-like elements. Ligations for all libraries were performed at 4° for 24 hr with 5 μg of Charon 40 DNA and 3 μg of insert DNA using 2 μl of T4 DNA ligase (400,000 units/ml, New England Biolabs). Ligated products were packaged with packaging extracts from *Escherichia coli* strains BHB 2688 and BHB 2690 (HOHN and MURRAY 1977). Libraries were plated onto *recA*$^-$ *E. coli* strain K802 at a density of ~5000 plaque forming units (pfu) per 100 mm plate or 25,000 pfu per 150-mm plate. Plaques were allowed to develop for 14 hr at 37°. Plaques were filter-replicated onto nylon membrane (Micro Separation Inc.) according to previously described methods (BENTON and DAVIS 1977).

The D4 library was screened with a 650-bp *Nco*I/*Hin*dIII fragment from *Mu1* (BARKER *et al.* 1984). The B1 and D3 libraries were screened with the internal, 1-kb *Tth*111I fragment of *Mu1* (BARKER *et al.* 1984). Approximately 5 × 10^6 cpm of probe were used per bag of 25 ml hybridization mix. DNA probes were radiolabeled with ^{32}P dCTP (Amersham) to a specific activity of at least 1 × 10^8 cpm/μg by random hexamer labeling (FEINBERG and VOGELSTEIN 1983). Hybridizations were performed at 65° in our standard hybridization buffer (BENNETZEN 1984). Positive plaques were identified and picked with the wide end of a pasteur pipet. Plugs were placed in microfuge tubes with 0.5 ml SM (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) and a drop of $CHCl_3$ and left at 4° overnight. Suspended phage were diluted and plated on K802 at a density of 50–200 pfu per plate. Resultant plaques were replicated onto nylon membranes and hybridized as described above. Single positive plaques were identified and picked from these plates.

Control recombinant clones were picked at random from each of the B1 and D3 libraries. The plugs were diluted as described above and plated onto *recA*– K802. X-gal (bromo-4-chloro-3 indolyl b-D-galactoside) was included in the top agarose for this plating to ensure identification of recombinant plaques. Positive phage clones were identified and plaque purified.

To facilitate DNA sequencing using primers with homology to the *Mu1* terminal inverted repeats (TIRs), individual *Mu1*-related elements were subcloned as two fragments employing their single internal *Nco*I site (BARKER *et al.* 1984). Single plaque isolates of each *Mu* element-containing phage were propagated and digested with *Sau*3A and *Nco*I for subcloning into pCEL80, a modified pUC vector (LEE *et al.* 1987). The plasmid vector was digested with *Bam*HI and *Nco*I. Linearized plasmid was purified over 1% agarose gels. Ligations were performed using a molar ratio (vector to insert) of at least 18:1. Ligation were introduced into competent DH5α cells, and white colonies were picked onto LB plates containing ampicillin. The resultant plates were replicated onto nylon membranes and screened with a *Mu1* internal probe.

DNA sequencing: The plasmid DNAs from positive clones were isolated according to BIRNBOIM and DOLY (1978) or HOLMES and QUIGLEY (1981). Double stranded plasmid DNA was alkali denatured and sequenced with Sequenase version 2.0 (United States Biochemical Corp., Inc.) and ^{35}S dATP (Amersham) according to the instructions in the Sequenase kit. The *Mu*-specific primer employed was 5′ CAGAGAAGCCAACGC 3′, which hybridizes ~50 bases from the external end of the *Mu1* TIR (BARKER *et al.* 1984). Denaturing electrophoresis was performed using 0.4 mm thick, 7% polyacrylamide gels in the presence of urea. Gels were fixed in 12% methanol and 10% acetic acid. After drying, gels were exposed to Kodak XAR-5 film for 24 hr at −70°.

Sequence analysis: Sequence information was analyzed using various programs from the University of Wisconsin GCG package on the Aids Center Laboratory for Computational Biochemistry vax cluster computers (DEVEREUX *et al.* 1984). The Compare program was used with a comparison (window) size of 9 and a stringency of 7. Other programs used were Repeat, Stemloop and Find. Fasta searches were performed using the Genbank and EMBL databases.

DNA gel blot hybridizations: The copy number classes of sequences flanking cloned *Mu* elements were determined using maize genomic DNA as a probe to *Mu* element-containing lambda clones. Restriction enzymes were identified that

would yield fragments between ~2 and 10 kb containing the *Mu1*-like elements plus flanking DNA. Control recombinant phage were also digested to yield maize DNA fragments of similar size to the *Mu*-flanking DNA alone. Digests were performed in 50 µl with spermidine added to a final concentration of 4 mM in otherwise standard digestion mixtures. Plasmid pB428 was digested with *Hin*dIII and *Bam*HI to yield three fragments that contained repetitive sequences from the *Adh1-S* allele (BENNETZEN *et al.* 1984, 1994) as repetitive DNA controls. The *Mu1*-containing plasmid, pMu100, was also included as a control. A *Sau*3A/*Hin*dIII fragment of pMJ9 (BENNETZEN 1984) was inserted into *Bam*HI/*Hin*dIII-digested pBR329 (COVARRUBIAS and BOLIVAR 1982) to create pMu100. Plasmid pMu100 was digested with *Hin*dIII and *Sal*I to yield a 1.8-kb fragment that contained the 1.4-kb *Mu1* element with some flanking sequence from within the *Adh1* gene (BARKER *et al.* 1984). Lambda DNA digested with *Hin*dIII and *Eco*RI was subjected to electrophoresis in parallel to provide molecular weight markers.

Digested DNAs were fractionated through 1.0% agarose gels and transferred to nylon membranes (Micron Separations Inc.) by capillary action (SOUTHERN 1975). Filters were baked for 2 hr at 80° under vacuum. Filters were hybridized sequentially to four different probes. The first probe was total DNA prepared from roots of 1-mo-old plants of maize line B73. The second probe was total DNA prepared from the leaves and stems of 2-week-old B73 plants. The third probe was a 1-kb *Tth*111I fragment internal to *Mu1* (BARKER *et al.* 1984). The fourth probe was a fragment internal to, and distinctive of, *Mu2*; this was a *Hin*dIII/*Eco*RI fragment of pSD2 (TAYLOR and WALBOT 1987), kindly provided by V. CHANDLER. Random labeling reactions were performed as described (FEINBERG and VOGELSTEIN 1983) using 100–150 ng of DNA. All hybridizations were performed in sealed plastic bags that were gently rocked at 65°, as previously described (BENNETZEN 1984). Washes for these hybridizations were as follows: first wash, 2× SSC, 0.1% SDS for ~15 min; second and third washes, 0.5× SSC, 0.2% SDS for 30–60 min; fourth wash, 1.5× SSC, 0.2% SDS for 30–60 min. All washes were performed at 65°. Filters were exposed to XAR-5 film for one to three days with one or two intensifying screens at −70°. Probes were stripped from filters by washing in 2 liters of 0.1× SSC, 0.1% SDS for 15 min at 95°.

RESULTS

Sequence analysis of DNA flanking *Mu1*-like elements: Twenty-five different *Mu1*-like elements were identified in genomic libraries from active *Mutator* plants. Gel blot hybridization with a fragment specific to *Mu2* (TAYLOR and WALBOT 1987) indicated that three of these clones (1, 14 and 17) carried *Mu2* elements and the remainder contained *Mu1* elements. The DNA immediately flanking each of 20 of these different elements was sequenced and analyzed in an attempt to identify a common sequence that served as a target for insertion. Sequencing data are shown in Figure 1. Because of the size of some insertions and the positions of the sequencing primers employed, we were only able to obtain full sequence information for the DNA near both ends for 14 of the *Mu* elements. Perfect nine base target repeats were observed flanking 13 of these 14 elements.

The sequences of the target repeats for these *Mu1*-like elements are all different. However, the 13 duplicated target sequences (1–13, Table 1) and the six sequences flanking one end of a *Mu* element (15–20, Table 1) could be aligned to give reasonable fit with the consensus sequence 5′-G-T-T-G-G/C-A-G-A/G-G-3′ (Table 2). Random sequence choice should give less than 3 matches with this sequence; all 13 target sites had three or more matches to the consensus, and most had five, six or seven matches. When oriented to yield the highest possible fit with this consensus sequence, our 19 individual target sites exhibited a Pu:Py ratio of 2.1 and a G:C ratio of 2.9.

The target sequences in this study, and the derived consensus, were compared to the flanking taget repeats from previously characterized *Mu* elements (BENNETZEN *et al.* 1984; O'REILLY *et al.* 1985; CHEN *et al.* 1987a; TAYLOR and WALBOT 1987; CHANDLER *et al.* 1988; ORTIZ *et al.* 1988; MCCARTY *et al.* 1989; SCHNABLE *et al.* 1989; TALBERT *et al.* 1989; NASH *et al.* 1990; BARKAN and MARTIENSSEN 1991; CHOMET *et al.* 1991; DOSEFF *et al.* 1991; HERSHBERGER *et al.* 1991; QIN *et al.* 1991). Although no exact sequence matches were observed, the consensus sequence derived from our data fit very well with the target sequences of the 23 previously characterized insertions sites reviewed by CHANDLER and HARDEMAN (1992). All but two of these previous 23 insertions sites matched our consensus sequence at three positions or more (data not shown). The two other target sites matched at only two bases, and these were associated with *Mu8* and *MuDR* elements. In this regard, the 13 *Mu1*-related elements previously analyzed (CHANDLER and HARDEMAN 1992) ranged from three to eight in fits with our consensus (averaging 5.2), while the other 10 elements from other *Mu* subfamilies matched much less well (ranging from two to six and averaging 4.0).

As a measure of the statistical significance of these results, 75 randomly chosen 9-bp sequences from each of 10 different 1000-bp sequences of DNA were screened for homology to the consensus. Five of these 1000-bp blocks were randomly generated using 25% for each base frequency (all random sequences were generated using the RANUNI subroutine in SAS v6.10). The other five were bases 3150–4149 (in intron number 3) and 4150–5149 (from exons 4 through 6) of the maize *y1* locus (B. BUCKNER, P. SANMIGUEL, and J. BENNETZEN, unpublished results), bases 200–1199 in the single long open reading frame (ORF) of a maize serine-threonine kinase gene that maps at the same position as npi422 on the short arm of chromosome *10* (P. SANMIGUEL, R. FREDERICK, K. HONG, S. HULBERT, and J. BENNETZEN, unpublished results), bases 4900–5899 just 3′ to the maize *adh1-S* gene (SACHS *et al.* 1986) and a 1000-base sequence just upstream of *mha1* (JIN and BENNETZEN 1994). The 75 randomly chosen 9-mers from these sequence blocks were scored for best fit to the consensus sequence in both orientations, and the higher homology was chosen. These 10 populations

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Mu Clone

1
1    GATCAGCGAG CTGGAGTCGG TGGCCGCGGG AGGTGTTCAC GAGTCTGAAG AGGTCCGGGT GGCGGGGGCA AAGCGTCGCG CGGAAGTCCA TGGCGAGCTC
101  GCGGGGATC
2
1    CATGTGTCGC CGGGACCGGA AGGCTGAAAC TGTCACAGTG ATGTAAAGTT TGGTTGGTGT TGGCGGCTCA CGAAAACCAG ACCTTTCCAA GTTTGAGGAC
101  ATATCTTGTG CACATAGAAG ATTGGTACAC ATGGT
3
1    GATCGACAAT ATATTCATTG GTACAAGATT AAATACGTGA ACATATTAAG TTACATCACA CGTGGGCAGC GTGTACATTT CTCCGGGAGA GCATTTAGCA
101  CACAAACATT ACTGCTGAAA GTTACATTGG ACAACATTTA AAAATAACTT ATTCTTGCTA CTATGGTATT GAACTTCTCG AGATC
4
1    GATCCAAATT GGGCTGAAGT TTTCAAAGCC CAGCTGAAAA CCCTGTTCCT TCCGACCTTG GCCACCTCGT ACGCATCGGC ATCGCGTCGC CCCGCCGCTC
101  TCCTCTCCTG CCGGCCTGTC CAACTTCAAA AGAGCACGCG CTTCCGCCCC CCGCCCTCCT GTACCGCTCT CCTTCCCCGA CGATGGATC
5
1    GATCGGCGCG TGGAGTGGAG AAGAAGAGAG CAGGATTCCA TGCGAGGTAA ATGGGGGTGG GCTGGGGAAC AGAGCAGCAG GATGCGAGGC GGCACCCACC
101  AACCCACCGC GGCAGGCAGG CAGGCGATTT CCCCCTTTCG TCCGTCCCCT CTCGGCTGCG CGGTGGCGTG ACGGTGGCGC CTGCTGTGAT GCGGCTTG
6
1    GATCCAGCCC CCCGACCCAC CCTGCCCCGT CTGCTCCCAG TCCCTTTCCG TCCCGCCCGA GTTAAACCCT AACTACAGTG CCGCCTCGCC TCCTCTCGCC
101  CATCCTCCCT AACCTTGTGA GACTTTACCG TCGTGTTGCG GCGGGGGGTG AGCGTGGTGG TGTTTAGCGT GTTTACTATC ATCACGGGGT CGGTGCGGAT
7
1    GATCATACTT GGCGACTTGC GGTGCTGCGG CGCCGAGATT TTGCGTGCGT TCTTGAGCGG TGTGAGGCGT TAATGGCGCA GCAGCCAGAC ACGCACCGGG
101  TTTTCTTGTA CCAGTACGTA CCCGTACGTG ATGTTACTGA TGGTGCTTTT GGCCGTAGTC TGCTGTGTCT GTCTTGGCTG CAGCCAGGGA GAGAAAGTTG
201  TTTAGCACCT GATC
8
1    GATCAAGTTC GGGTCCCCGA CCGCAAAAGC CGCTACAGAC ACAATCGGAA CCAGTTGAAG CGCAATAGCC GCGCGCAGCA GCATTGACGC ACGCAACGGC
101  ACGACAGGCA GAGGCAGGAC AGACAGCGAG AGAAGTGGTA GCTTACCCGC TTGGGCTCGC CGTTCTTCCC GCCGATGGTG GATGGAGATG ATATGGCCCG
201  TCACAGCGTT CCCTTCCCGT CACAGCGTCT
9
1    GATCCAACGG CGTCCCAGCA TTTTTAGTTC CACGTGTCTC CAATCCACCT GTCTCTTACT CTCCCACCCC AATCCTCCCA AATCCTCACC TTCCCCTTCC
101  CACTCGGCCA CTCCTCATCA GCCATCCCCA AAAGGCCAAA ACCCGCGCAC ACCGGCGACG CGGAGAACCT CCCGCCGGCC GCCGCCATGC CGCCGCCTCT
201  ACAGGCCCAC CGCCTACACA TCTCCACCGC CGCCTACCCC ACTCCAGTCT CG
10
1    GATCGCTCCA CGACAGGCTC CTTGTCCGCC ATAAGGTGGG TGATGCCGGC GCGATTCAGT TCGAAGAGGA CGCGACGGCG GGGAAGGGAT GAGAGACGAG
101  TAGGTGTTTT CCAGAAGCTT TGGGAATTGG AGCAGGGCTG GCTGCGCTGC GTGCTAGGGT TTTCGTGCGC GGAGAGGAGA CGTGACCAGC GAGGATAGGC
201  CCACTTTCAG AGAGTGGGAC CCGCGGCACG TGCCTCCTGT CAAACACGCG ACTCGAACTT CCGTTTGCAA AGCATCGTCG TCGGG
11
1    CCAATTTTTT TCTTAATTCT TTTTATCTAT TTTTTTGTTT CTAATCATTT GTGAGTGATT ATTAATTAGA CCCCTAAAAG TAAATAAATA AAGGAAAAAT
101  AATTGAAAAC ACATAGAGAG GGGTACAATA AACATTCTTT GGGGAAATCT TTGAACCCTT TCTTGCTCCT TTATTTAGCT TATCGTTCTA CATCTATAGA
201  CATATCTTAG GCTAGCAACT GTAACTAGTA CTTTGGATAT TCATTTAGCT TTGCTAATCA AATTTCGATT ATTGATAATC CATTGCTACT GTCAGCTATG
12
1    AAAAGAATAA AAAAAATCAT TTTGCTCTTT TGATTGATTG ATATGCTATA GCTGGAGTTC AAACCAACAG CGCACAGGCT ACAGGCCACA GCTAGCACAC
101  CCTTCTGCCA CGCTCTTGTC CGCACTCTAC TCGCCGAGAG TGTGAGTCGA GACGCCTCTC TCGCCCTCTC TTCTCCTCCC TCCACTCCGC CGCCGCGCCC
201  GAGAAAAGAG AGTTCCAATG CGCCTCCTCC GCTGCTCCTG CTCCTGTACC TCGCCGCTGT CGCGCGGCAC GGCAGCGACG CGACGCGCTG CTGGCCGCAA
301  GGCGCGCTCT CCGACCCCAC TGGGCGCTCG CGTCCTGGGA G
13
1    GAGGATGCGA AGGCTTTGGT TTGGTATGGC TCTCCGATTT GGCGTGTTCG TGGCGGTGTG GGGTGGTTGG TCTCCCGGGA TTTGACTCTA CCTAGCCTTT
101  TCGTCGCCGT GTTTCTTGTC CTTGGTTGGT CCGGAGGGGG GGAGGGAAGC CGAGAAGGTG GGGCCCGCCG TCCCAGCGAC TCCCGGCAGC TGCTCACCTC
201  ACGGATGACG GATGATACCC ACGTGCCAGG GATGAGGGCA GAAACAGGTA TGGACTTGGG CCGTGTACTG GTGGCCTCTA TGTGTAAATT GGTCTGGCTA
301  TGGCCCACGG ACATCCATAG TCATCTGCGT CGAGTCTATT TTTTAGCGTG CTAACCATGT ATCATGGCGA GCTGCCCGGG ATC
14
1    GTCCACACTG GCTGCACGAC GCTATCCTCA GTTTTGCTGC GCTAGTTGTC CCGCCTCGCT GTACTCGCAT CATCGCCTCT TACTCTTTAT ATAAGAG*CTT
101  AAAAGAGAAA GGAGAGCATC GCAGTGGCAC ACGCGCAATG GAAGGCTCGC GTGACGCGCC TCCCGGAGCT AACTCCCTCC CCGACGGCTT CGTTCCCGAC
15
1    CTTTTTAACA GGGAAGCGAA CAGGCTGAAC GGCCCTTGAT C
16
1    ACCGCCGCCG CAAGACGCAC GCTCTTCCGC TTTCTACGCC GCTTCGTCCT GCTGCT
17
1    CTGGGGGAGA TGTCCGCGAC CGCATCGGCA TCGCTGGTTT GGACAGGCAC GGACTCTGAT C
18
1    GTTGCGGTCA GTCTCAGGCC CCAACCCTTT TCCCACTTCC CGCCGGAGCG CTCTCCTCTT CGCCGCTCCA TCACCGCCTC CCAATCCCAT GGGCGCCGGG
101  CCGGATC
19
1    GCCTGTGTCA CCTGTGTCCG GGCGGCTCGC AGTCTTGGTT GGGCTCGGAT GGTCGGTGCC GGACGCCGGG CGCGGAACCC TAACCCTAGG GGCCGGCTAC
101  ATTAGATGCT GGGGGGCGG
20
1    GTAGCAAAGT GTTGAACAGT CAAATTCCTG CTTGAATTGC TCTCTTGCTT GGTCTGAAAG GCATACATTT TGTTGATGAA GATGGTAACG ATTTTTGTTT
101  GGTTTCAGTG GACGACGAGT ACATCATCAT TGGG
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FIGURE 1.—Sequences flanking *Mu1*-like elements. Reconstruction of the insertion sites of the *Mu1*-like elements in this study. For sequences 1–13, the underlined nucleotides represent the nine nucleotides that were directly duplicated upon *Mu* element insertion. In the sequence of clone 14, there is no nine base target repeat, the *Mu* element inserted between G at 97 and C at 98 as indicated by *. For sequences 15–20, where the sequencing was performed for only one side of *Mu1*-like elements, nucleotide No. 1 marks the start of flanking sequences.

gave average homologies of 3.35–3.63 hits for randomly generated sequences and of 3.25–3.71 hits for the actual maize sequences; no significant difference was found between any of these 10 populations, nor was there any significant bias toward the consensus sequence as determined by both REGWQ and Duncan's multiple range test at $\alpha = 0.05$ (SAS 1990). The mean number of 4.7 hits from the 23 duplicated target sequences previously listed in CHANDLER and HARDEMAN (1992) and the mean number of 5.5 hits from the se-

TABLE 1

Target duplications of randomly selected *Mu1*-related elements

Clone no.[a]	Target repeat	Matches with consensus
1	GCTGCCGGG	7
2	GATGCCGAT	6
3	GTTGAAGCG	7
4	CCCGGAGAA	5
5	GGATTTGGG	4
6	GCAGCAGGA	6
7	TAGGGAGGA	5
8	GTGCGTGTC	4
9	AGTCTGAAG	3
10	TTTGGTTGG	6
11	GCGAGAGAG	6
12	CATAGAGAG	6
13	ATGAGAGAC	5
14a[b]	ATATAAGAG	5
14b[b]	CTTAAAAGA	4
15	CTGGGGGAG	6
16	GGCGGCGGT	5
17	GTTAAAAAG	6
18	GACACAGGC	5
19	GTAGCAAAG	7
20	GTTGCGGTC	6

[a] Sequences are taken from the data presented in Figure 1; in 1–13, flanking DNA on both sides of the *Mu* insertion was sequenced and confirmed the target duplication. For 15–20, only one side of the *Mu* element was sequenced; hence the 9-bp duplication is inferred.

[b] Because the sequence duplicated, if any, could not be determined for this insertion, these data are not included in Table 2.

quences we determined both differed significantly from the mean number of hits measured from the 10 control populations (REGWQ and Duncan's multiple range test, $\alpha = 0.05$, SAS 1990).

The sequences surrounding the *Mu* element insertion site for each of these 20 *Mu1*-like elements were analyzed using the University of Wisconsin GCG program Compare. Significant homologies between these flanking sequences were not identified.

In some transposable element systems, there are often sequence similarities between the element and regions near the site of insertion. To determine whether this might be the case in this system, the flanking sequences from these *Mu1*-like elements were compared with the sequence of the *Mu1* element. As a control for this experiment, the flanking sequences were also compared to the first 1400 bp of the maize transposable elements *Ac* and *Spm*. Using the Compare program, homologies between the *Mu* element flanking sequences and the internal or terminal sequences of the *Mu1* element were no more commonly observed than were homologies between the *Mu* element flanking sequences and the first 1400 bp of *Ac* or *Spm*.

Each flanking sequence was analyzed for the presence of short inverted or direct repeats. Numerous direct and inverted repeats were observed in these *Mu* element flanking sequences. However, such sequences may be relatively common in plant DNA and, hence, the significance of such an association is not clear. As a test of the possible significance of *Mu* insertion site association with flanking repeats, we chose as a control to analyze the 3′ region of the *adh1*-S allele (DENNIS *et al.* 1984) for the presence of short direct and inverted repeats. While four *Mu1* elements and one *Mu3* element have been found in the 5′ region of *adh1*-S, no *Mu* element has yet been detected in the 3′ region (reviewed in BENNETZEN *et al.* 1993). We employed the Compare program with a 9-bp window, requiring seven matches, and analyzing bases 2200 (within intron III) to 5000 [600 bp after a poly(A) addition site] (DENNIS *et al.* 1984) in progressive units of 200 bp. Repeats were observed in the 3′ region of *adh1*-S, at a frequency and of types similar to those observed near *Mu1*-like element insertions (data not shown). Their presence indicates that the direct and inverted repeats observed in sequences flanking the *Mu1* and *Mu2* elements of this study are not unique to insertion sites for these elements.

The flanking sequences in this study were also scanned for the short direct repeat reported to be on one side of the *Mu1* element in *adh1*-S3034 (5′-TGAC/GTAATC/TTTGG-3′) (DÖRING and STARLINGER 1984). We also asked whether two other sequences present in the termini of *Mu1* (5′-CGGGAACGGTAAA-3′ and 5′-CGGCGTCT-3′) were found in these flanking sequences. These two sequences exhibit binding activity

TABLE 2

Bases at the target site duplications for 19 *Mu1*-related element insertions

Base	Position number									Total
	1	2	3	4	5	6	7	8	9	
T	2	8	8	1	2	3	1	2	2	29
G	12	3	5	11	9	3	15	7	10	75
C	3	4	3	2	6	3	0	1	4	26
A	2	4	3	5	2	10	3	9	3	41
Consensus	G	T	T	G	G/C	A	G	A/G	G	

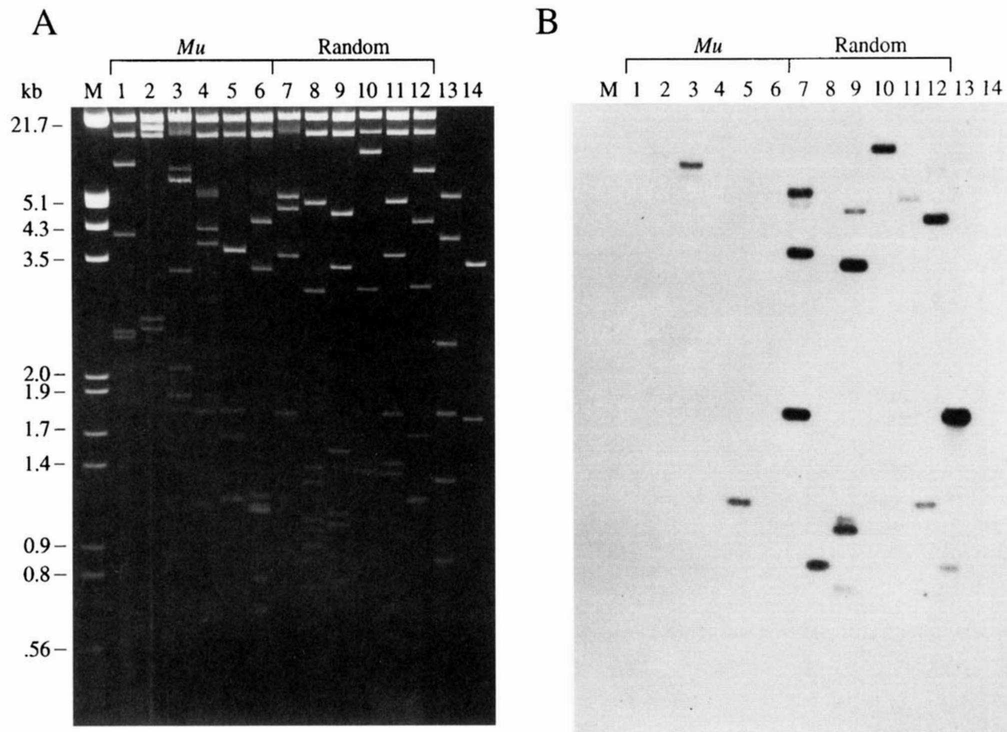

FIGURE 2.—Preferential insertion of *Mu1*-like elements into low copy number maize DNA. (A) Picture of an ethidium bromide stained 1.0% agarose gel containing 0.5 micrograms each of recombinant lambda phage DNA (lanes 1–12), 0.2 micrograms plasmid pB428, or 0.1 micrograms plasmid pMu100 digested with various restriction enzymes. (lanes 1–6) Clones that contained *Mu1*-like elements. (lanes 7–12) Randomly chosen recombinant maize/lambda clones. M, size standard of lambda DNA digested with *Hin*dIII and *Eco*RI. (lane 13) Plasmid pB428 digested with *Bam*HI and *Hin*dIII. (lane 14) Plasmid pMu100 digested with *Hin*dIII and *Sal*I. (B) Autoradiogram of a filter replica of the gel shown in A after hybridization to ^{32}P-labeled total maize DNA prepared from seedling leaves. Lanes 1–6 contain DNA from *Mu* clones 4 (digested with *Eco*RI), 8 (digested with *Hin*dIII), 17 (digested with *Sac*I), 23 (digested with *Xba*I), 3 (digested with *Hin*dIII/*Bam*HI), and 24 (digested with *Sac*I/*Xba*I), respectively. Lanes 7–12 contain DNA from control clones 56, 58, 59, 54, 53, and 57, respectively, digested with either *Sac*I (56) or *Hin*dIII (all others). At this short exposure time, only those fragments that were scored as very highly repetitive (strong bands) or highly repetitive (some weak or barely visible bands) yielded any detectable hybridization. For instance, the intense band in lane 13 has been previously shown to be very highly repetitive (>40,000 copies per haploid genome) while the weakly hybrdizing band in the same lane has been shown to be highly reiterated (~18,000 copies per haploid genome) (BENNETZEN *et al.* 1994).

to proteins present in active *Mutator* and *Mutator*-loss maize lines (ZHAO and SUNDARESAN 1991). No significant (>80%) homology to any of these three sequences was found adjacent to any of the *Mu* elements cloned and sequenced in this study.

We also performed Fasta searches through the Genbank and EMBL databases using each of the *Mu* element-flanking sequences presented here. No known sequences of genes were identified by these searches.

Identification of different repetition classes of maize DNA on clones that did and did not contain *Mu1*-related elements: Total DNA prepared from root tissue of maize line B73 was used as a probe to 17 appropriately digested lambda clones that each contained a *Mu* element and to 18 randomly chosen lambda clones from the same library. A large majority of the control clones (16/18) were found to carry repetitive DNA by this analysis, while less than half (7/17) of the *Mu* clones contained detected repetitive DNA (Figure 2). To determine whether any of the controls carried inserts from the maize chloroplast genome, filters were hybridized a second time to maize DNA from immature leaf and stem tissue (data not shown). Two control clones that exhibited more intense banding in the second hybridization experiment probably possessed chloroplast sequences and were not used for control fragment selection.

The sizes of bands carrying Mu elements were determined by hybridization to *Mu1* and *Mu2* probes. Control fragments that corresponded in size to the sequences flanking each *Mu1*-like element were chosen, before hybridization with total maize DNA, from randomly selected recombinant phage. Hence, control fragment sizes are ~1.4 kb smaller than each respective fragment that contains a *Mu1*-like element. An example of results from these experiments is shown in Figure 2 and the total data set is summarized in Table 3. Assignment of the copy numbers as very high, high, medium or low was by the purely operational criterion of hybridizational band intensity after a given period of filter exposure to X-ray film. Using the same assessment approach previously described, and by comparison to

TABLE 3

Summary of copy number determinations

Mu clone	Fragment size (kb)	Copy number	Control clone	Fragment size (kb)	Copy number
1[a]	3.6	Low	67	2.1	Very high
3	3.8	Low	—	—	—
4	4.0	Low	55	2.6	Very high
5	4.9	Medium	56	3.5	Very high
7	3.6	Low	62	2.3	High
8	10	Low	54	7.4	High
9	2.5	Low	—	—	—
11	4.9	Low	57	3.1	Low
13	2.2	Low	59	0.8	High
14[a]	3.5	Low	—	—	—
17[a]	3.4	Low	53	1.8	Medium
19	4.1	Low	73	2.6	High
20	4.1	Low	65	3.0	High
21	2.3	Low	71	0.9	Low
22	2.4	Low	61	0.9	Very high
23	3.0	Low	68	1.8	Very high
24	4.3	Low	58	3.0	Low

[a] Indicates a *Mu2*-size insertion; all other insertions are more closely related to *Mu1*.

many of these repeats as controls (BENNETZEN et al. 1994; SPRINGER *et al.* 1994), very high copy numbers represented ≥20,000 copies per haploid genomes, high copy numbers were between 1000 and 20,000 copies per haploid genomes, medium copy numbers were between 200 and 1000 copies per haploid genome and low copy numbers were <200 per haploid genome. The majority (11/14) of the control fragments were found to contain reiterated sequences, while only one of the 17 fragments carrying a *Mu1*-like element also contained detected repetitive DNA. The average length of flanking sequences in the *Mu*-element-containing fragments is 2.4 kb.

DISCUSSION

The *Mu* elements selected for this analysis were all of the *Mu1* subfamily but, unlike previous studies, were not selected for insertion into a specific gene or genes. These elements were cloned from active *Mutator* lines, which can generate new *Mu1* transpositions at rates averaging more than once per element per plant generation (ALLEMAN and FREELING 1986; BENNETZEN *et al.* 1987; HARDEMAN and CHANDLER 1989); hence most of the insertions are likely to have been relatively recent. It is formally possible that any preferences observed may be due not to insertion specificity alone but due to a combination of insertion and excision preferences. However, the germinal excision frequencies for *Mu* elements are usually two orders of magnitude or more lower than transposition rates (reviewed in BENNETZEN *et al.* 1993), so this is not likely to be a significant factor in this system.

A more significant limitation to this study is its exclusive focus on *Mu1*-related elements. Unique to the *Mutator* system, data are now accumulating that suggest that different subfamilies of elements can show differential preferences for insertion into different loci. For instance, the *bz1* locus of maize primarily acquires *Mu1*-related elements while the *sh1* locus (even in the same plant) seems to primarily acquire mutations due to elements of the *MuDR* (*Mu5/MuA/MuR/Mu9*) subfamily (BENNETZEN *et al.* 1993; HARDEMAN and CHANDLER 1994). Hence, any preferences observed, or not observed, for the *Mu1* subfamily may not hold true for other *Mutator* elements. This possibility is supported by our observation that members of the *Mu1/Mu2* subfamily have flanking duplications that average a closer fit to the consensus sequence that we have detected than do members of other *Mu* element subfamilies.

As in most previous experiments, we observed that *Mu1*-like elements usually generate a 9-bp target duplication. The only previous exception to this rule is a *MuA* element reported to have an 8-bp direct target duplication (QIN and ELLINGBOE 1990). The one exception observed here (clone 14) may be the result of a *Mutator*-induced rearrangement. Two deletions involving 75 and 77 nucleotides at the left terminal repeat of a *Mu1* element inserted into the *bz2* locus have been reported (LEVY and WALBOT 1991), and the deletion of DNA adjacent to one terminus of a *Mu1* element in the *Adh1* gene has also been observed (TAYLOR and WALBOT 1985). These rearrangements are postulated to result from aborted/aberrant transposition events.

This study detected a weak consensus sequence, 5′-G-T-T-G-G/C-A-G-G/A-G-3′, at the site of *Mu* element target duplication. This consensus, derived completely from a best fit to the data generated in our experiments,

is supported by our subsequent observation that the 23 *Mu* element target duplications summarized by CHANDLER and HARDEMAN (1992) also exhibited a better-than-random fit with this consensus sequence. Individual insertions average only about a five-out-of-nine fit with this consensus, where just over three out of nine would be random, so this may permit both the high frequency of mutation at most maize genes (reviewed in BENNETZEN *et al.* 1993) and the multiple insertions sites feasible in any single gene (BROWN *et al.* 1989). However, the preference for insertion into particular regions of genes, and different mutation rates at different loci in the same *Mutator* population (ROBERTSON 1985), may be partly accounted for by the availability of sequences with a near match to this consensus. Due to the limited area investigated by our sequencing analysis, the possibility also exists that a specific target sequence or structure might exist beyond the flanking regions investigated here.

The *Mu1*-related elements responsible for *Mutator*-induced mutations at the *bz1* locus are found in several locations, but the majority of insertions cluster 3′ to the first (and only) intron within this gene (TAYLOR and WALBOT 1987; BROWN *et al.* 1989; HARDEMAN and CHANDLER 1989). Both *Mu1* and *Mu8* elements have also been found to insert in a small region of *kn1* (GREENE *et al.* 1994). However, as we have previously discussed (BENNETZEN *et al.* 1993), this apparent insertion specificity may be due less to targetting of specific regions within a gene than it is due to the likelihood that a detectable mutation will only occur with insertions within a small region of these genes. This is particularly clear in the case of *kn1*, where dominant mutations due to ectopic expression were analyzed (GREENE *et al.* 1994).

If there were no insertion specificity in the *Mutator* system, we would have expected that ≥60% of the *Mu* element-containing fragments would carry high copy number DNA sequences. This is the percentage of randomly chosen fragments from randomly picked recombinant phage clones that were found to possess highly reiterated sequences. Given the striking difference between the copy number of the *Mu* element flanking sequences and the control fragments from the copy number determination experiments, we conclude that *Mu1*-like elements have a strong preference for insertion into sequences that have a low genomic copy number. The presence of many repetitive fragments within the control phage clones demonstrates that this preference of *Mu* elements for low or single copy number DNA is real and is not influenced by an inability to clone repetitive sequences from maize. Compared with the controls, our results indicate that *Mu1*-like elements exhibit an approximate 10-fold preference for insertion into the low copy number DNA component.

The copy number determination results presented here suggest that the length of single or low copy number sequences surrounding a *Mu1*-like element in the maize genome is usually ≥2.4 kb. This implies that sequences of low copy number extend on either side of a *Mu* element an average of ≥1200 bp. In our analysis of genome composition in a contiguous 280 kb region flanking the maize *Adh1* locus, we found long stretches (10 kb to >90 kb) of mixed highly repetitive and middle repetitive sequences, and only short segments of homogeneous low copy number DNA (SPRINGER *et al.* 1994). Our analyses of the total maize nuclear genome on pulsed field gels (BENNETZEN *et al.* 1994) has indicated the same general pattern; relatively short stretches of unmethylated single copy DNA interspersed among 20- to 200-kb regions of methylated repetitive DNAs. Because this indicates that any large restriction fragment containing low copy number DNA in maize is likely to also carry some flanking highly repeptitive DNA, we limited our analyses to short flanking regions. Moreover, the presence of middle repeptitive DNAs in both low copy number and repeated DNA blocks (HAKE and WALBOT 1980; BUREAU and WESSLER 1992; BENNETZEN *et al.* 1994) indicated that hybridization of clones to total maize DNA would be more informative than using selected fragments next to the *Mu1* elements for hybridization to gel blots of digested maize DNA. This was performed for several *Mu1*-flanking DNAs, however, to confirm their low copy number insertion preference (CRESSE 1992).

In preliminary RNA gel blot studies, we have observed that at least one of these elements (clone 9) is inserted into an endosperm-expressed gene and that relatively long stretches of GC-rich, ORF-containing DNA flank many insertion sites (CRESSE 1992). Because we have no appropriate controls, it is not possible for us to determine whether these results are statistically significant. Other groups analyzing the DNAs flanking maize transposable elements have also had this problem; particular sequences or repeats are found (DÖRING and STARLINGER 1984) but may not be anything more than an indication of the frequency of such sequences in a higher plant genome. We chose the 3′ end of the maize *adh1* gene, which has not been associated within any *Mu* element insertion, as a control for sequence comparisons. Tandem repeats of various types and homology with *Mu1* components were as frequently found in this region as they were in the regions flanking *Mu1*-like element insertion. Although we realize that this is far from a perfect control (which would be several independent stretches of randomly chosen and sequenced maize DNA), we feel that this comparison provides reasonable evidence that *Mu1*-related elements do not require any such sequences or structures at or near their site of insertion, beyond those which seem to be present in the DNA in and around most maize genes.

Because genes (the major known constituent of low copy number DNA in higher eukaryotes) are generally

not modified in plants (ANTEQUERA and BIRD 1988; WALBOT and WARREN 1990; BENNETZEN *et al.* 1994), *Mu1*-like element insertion into low copy number DNA is consistent with the observation that sequences flanking *Mu1* elements are not cytosine 5-methylated (BENNETZEN 1985; BENNETZEN *et al.* 1988, 1994). This preference for insertion into similarly unmethylated sequences has also been observed for the *Ac* and *Spm* systems of maize (CHEN *et al.* 1987b; CONE *et al.* 1988). This level of specificity may be a simple outcome of the openness, and general accessibility, of the presumably unmodified and gene-rich euchromatin compared with the compacted/modified/gene-poor heterochromatin. No matter what the cause, this preference for insertion into gene-like sequences is likely to be a major factor in the high mutation rates manifested by the *Mutator* system.

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