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Characterization of a Meiotic Crossover in Maize Identified by a Restriction Fragment Length Polymorphism-Based Method

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ABSTRACT

Genetic map lengths do not correlate directly with genome size, suggesting that meiotic recombination is not uniform throughout the genome. Further, the abundance of repeated sequences in plant genomes requires that crossing over is restricted to particular genomic regions. We used a physical mapping approach to identify these regions without the bias introduced by phenotypic selection. This approach is based on the detection of nonparental polymorphisms formed by recombination between polymorphic alleles. In an F_2 population of 48 maize plants, we identified a crossover at two of the seven restriction fragment length polymorphism loci tested. Characterization of one recombination event revealed that the crossover mapped within a 534-bp region of perfect homology between the parental alleles embedded in a 2773-bp unique sequence. No transcripts from this region could be detected. Sequences immediately surrounding the crossover site were not detectably methylated, except for an *SstI* site probably methylated via non-CpG or CpXpG cytosine methylation. Parental methylation patterns at this *SstI* site and at the flanking repetitive sequences were faithfully inherited by the recombinant allele. Our observations suggest that meiotic recombination in maize occurs between perfectly homologous sequences, within unmethylated, nonrepetitive regions of the genome.

RECOMBINATION is an important aspect of meiosis in most eukaryotic organisms. It ensures proper chromosome disjunction during the reductional division of meiosis I by providing a physical connection between homologues. Meiotic recombination also plays an essential role in generating genetic diversity and thus contributes to genome evolution. In plants, where reproductive tissue is often derived from somatic lineages late in development, mitotic recombination can also contribute to genome diversity (DAS *et al.* 1990b).

Higher plant genomes differ in size over three orders of magnitude, largely due to variation in repetitive DNA sequences such as multigene families and repetitive transposable elements (FLAVELL 1985). Repeated sequences can potentially affect genome plasticity by their effects on homologous recombination. Unequal crossing over between repeats can give rise to deletions and amplifications, and other recombinational processes can give rise to inversions and translocations. To maintain genome stability in plants, homologous recombination via crossing over needs to be tightly controlled and restricted to particular genomic segments, whether in somatic or meiotic tissue.

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The current understanding of recombination mechanisms stems largely from genetic and molecular studies in bacteria and fungi (PETES *et al.* 1991). Little is known about the recombination process in plants. Several studies have utilized transgenic approaches to determine mitotic recombination frequencies between repeated sequences in plants (PETERHANS *et al.* 1990; GAL *et al.* 1991; ASSAAD and SIGNER 1992; TOVAR and LICHTENSTEIN 1992; SWOBODA *et al.* 1993, 1994). The frequency of such events varies between 10^{-4} and 10^{-7} per cell and appears to be affected by repeat length (PETERHANS *et al.* 1990), degree of homology (GAL *et al.* 1991), and position in the genome (ASSAAD and SINGER 1992; SWOBODA *et al.* 1993). Similar to mitotic recombination in yeast, gene conversion-like exchange seems to occur more frequently than crossing over, since many of the recombinational events are not associated with exchange of flanking markers (ASSAAD and SIGNER 1992; TOVAR and LICHTENSTEIN 1992).

Mitotic recombination between tandemly duplicated sequences at the *p* locus (ATHMA and PETERSON 1991) and the *kn1* locus (LOWE *et al.* 1992) in maize was found to be strongly enhanced (between several hundred- to several thousand-fold) by the presence of an active transposable element between the repeats. Molecular analysis of *Mu1* excision footprints at the *bz1* locus also suggested the occurrence of recombinational events upon transposon excision (DOSEFF *et al.* 1991). It is likely that transposase-induced double-strand breaks near the ends of the *Mu* element stimulate recombina-

tion, as demonstrated for the *P* element in Drosophila (Gloor *et al.* 1991).

Meiotic recombination has been studied in maize by selecting for wild-type intragenic recombinants between combinations of mutant heteroalleles of phenotypic marker loci. Such analysis has led to an understanding of the effect of particular heteroallelic combinations on the frequency of recombination and the type of genetic exchange (*i.e.*, crossing over or gene conversion-like exchange). For example, the presence of a single *Ds* insertion within a gene suppresses intragenic recombination and favors crossing over (Dooner 1986), whereas the presence of two different *Ds* insertions seems to favor conversion-like exchanges (Dooner and Kermicle 1986). This type of analysis also revealed that the maximal frequencies of intragenic recombination at the maize loci *a1* (Brown and Sundaresan 1991; Civardi *et al.* 1994), *adh1* (Freeling 1978), *b* (Patterson *et al.* 1995), *bz1* (Dooner 1986; Dooner and Kermicle 1986), *gl1* (Salamini and Lorenzoni 1970), *r* (Dooner and Kermicle 1986), and *wx* (Nelson 1968) were approximately comparable, ranging from 0.9 to 1.3×10^{-3}. Recombination frequencies at these loci were about two orders of magnitude higher than the average rate of recombination per kb for the whole maize genome, indicating that genes may be more susceptible to recombination compared to repetitive sequences that make up the majority of the genome.

Based on these and similar observations in other organisms, it has been proposed that recombination might be preferentially initiated at regions in the genome containing functional genes (Thuriaux 1977). This view is supported by the fact that genetic map lengths of most organisms are comparable despite large differences in genome size. The validity of this model in plants is not easily determined, because the genetic approaches used to study both meiotic and mitotic recombination have relied on phenotypic selection to identify recombinants within known genes. However, one way to address if recombination occurs preferentially within functional genes is to use physical mapping to identify unselected recombinants.

Here, we describe such a method based on the detection of nonparental polymorphisms formed by recombination between polymorphic restriction fragment length polymorphism (RFLP) alleles. Using this assay, two recombination events were identified in a population of 48 F_2 plants that were screened for recombination at seven RFLP loci. Both recombinational events were associated with flanking marker exchange, consistent with crossing over. The origin of one recombinant allele was characterized in detail at the molecular level, revealing that sequence homology, level of methylation, and possibly chromatin structure may specify sites for meiotic crossing over.

MATERIALS AND METHODS

Plant materials: Maize inbred lines A188, W64A and W22 were derived from stocks kindly provided by R. L. Phillips (University of Minnesota, St. Paul, MN).

DNA gel blot analysis: Genomic DNA from leaf samples were isolated as described (Das *et al.* 1990a). DNA samples used for copy number estimations of repeated genomic sequences were further purified on CsCl density gradients. Southern blots onto Nytran membrane were prepared by standard protocols and hybridized at 42° in 50% formamide, 5× SSC, 1% SDS, 5× Denhardt's solution, 0.05% sodium pyrophosphate (pH 8.0) and 50 μg/ml salmon sperm DNA. Membranes were washed three times with 1× SSC, 0.1% SDS at room temperature and twice with 0.1 or 0.2× SSC, 0.5% SDS at 65° for 20 min each. RFLP and other probes were isolated with the Gene-Clean kit (Bio 101) and labeled with α^{32}P-dCTP by random priming. Total maize genomic DNA (50–100 ng) was labeled using the same random priming method and used in hybridizations under conditions described above.

Construction and screening of subgenomic libraries: Approximately 75 μg genomic DNA from the inbred lines A188 and W64A, and a plant homozygous for the recombinant allele of *umc116* was digested with *Eco*RI and separated by agarose gel electrophoresis. DNA in the range of 6–10 kb was transferred onto NA-45 membrane (Schleicher and Schuell). The membrane was divided into five segments, and DNA was eluted from each segment to further fractionate the DNA fragments. An approximately 300-ng insert DNA from the appropriate fractions was ligated to 500 ng *Eco*RI-digested EMBL4 DNA and packaged using the Gigapack Gold kit (Stratagene). The libraries were screened with probe *umc116* or bs450 and positive plaques were plaque-purified twice. The *Eco*RI inserts from two positive lambda clones of each library were subcloned into pUC119. The resulting plasmid clones were used for all further analysis. PCR amplifications to confirm the contiguity of the A188 lambda clones were carried out on 10 ng genomic DNA from inbred line A188 under the following conditions: 4 min at 94°, followed by 30 cycles of 1 min at 65°, 2 min at 72°, 1 min at 94°, ending at 1 min at 72°.

DNA sequence analysis: The 2.4-kb region encompassing the crossover site was subcloned as ~1-kb *Bgl*II-*Sst*I, ~0.9-kb *Sst*I-*Bam*HI, and ~450- or ~600-bp *Bam*HI-*Sst*I fragments into pUC119. These subclones were sequenced at both strands using the Sequenase v2.0 DNA Sequencing kit (USB). Sequence data were compiled and analyzed using the GCG sequence analysis software package (University of Wisconsin, Madison). The ends of the insertion elements present in the A188 allele and their flanking regions were sequenced from subclones that spanned a 2.3-kb *Pst*I-*Sal*I region near the 5′ end of the allele and a 1.3-kb *Kpn*I-*Bgl*II region near the 3′ end of the large insertion element. Sequence information obtained from these subclones was compared to sequence data from the 450-bp *Pst*I fragment (black box Figure 5) of the W64A allele using the GCG package.

RNA analysis: Total RNA was extracted from immature and mature maize tissues from the inbred line W64A according to Das *et al.* (1990a). Poly-A$^+$ RNA was isolated using the PolyATtract mRNA isolation system (Promega). RNA samples were fractionated on formaldehyde-agarose gels and blotted to Nytran membranes. Northern blots were hybridized and washed under conditions described above. First-strand cDNA synthesis for PCR was carried out using the cDNA Cycle kit (Invitrogen) according to the manufacturer's protocol. Approximately 0.5 μg poly-A$^+$ RNA from embryo and endosperm [18 days after pollination (dap)], seedling leaves, and immature ears was treated with DNAse I and subsequently con-

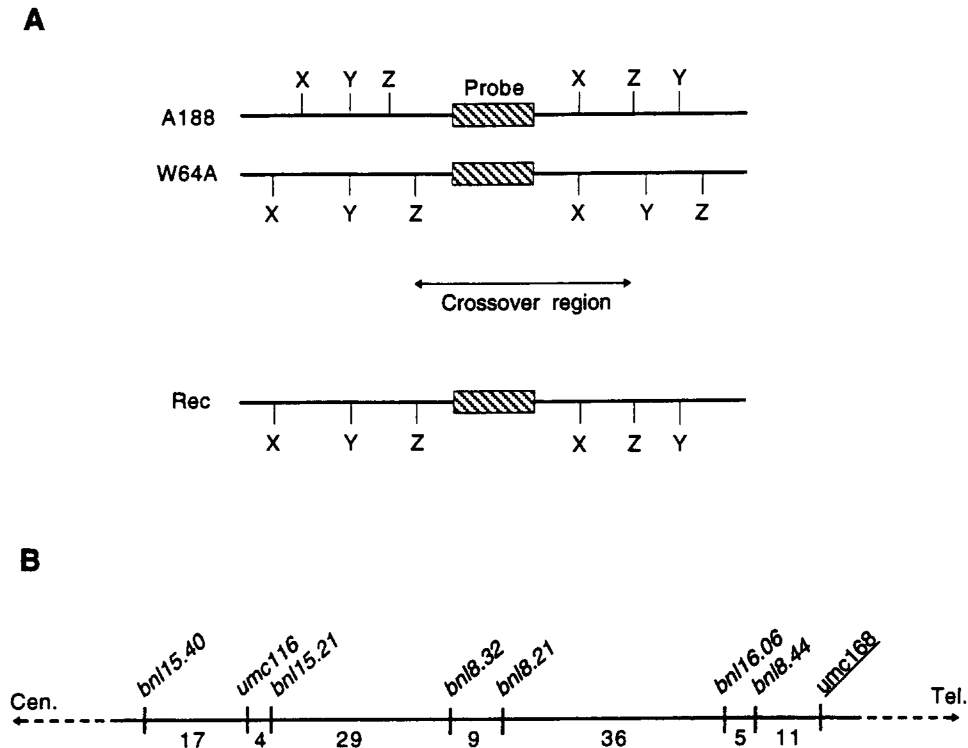

FIGURE 1.—Physical mapping as a means to identify unselected crossovers. (A) The diagram represents schematically the identification of recombinant alleles using RFLP analysis, using the two inbred lines A188 and W64A as examples. ▨, region of homology to the probe. Flanking restriction sites are indicated arbitrarily as X, Y, and Z. Recombination within the region indicated would generate two new alleles with altered distribution of restriction sites. One possible recombinant allele is shown, which resembles the W64A allele with enzyme X, but resembles the A188 allele with enzyme Y, and produces a nonparental restriction fragment with enzyme Z. (B) Linkage map of chromosome 7L. The RFLP loci used to identify unselected crossovers are shown, and the map distances (in cM) between each pair of RFLP loci are also indicated. *umc168* (underlined) was not used to identify crossovers but was used to establish the mechanism of recombination at the *bnl8.44* locus.

verted to first-strand cDNA using an oligo-dT primer. One-tenth of the products were added directly as template DNA in the PCR reaction. PCR reactions with 0.05 μg poly-A$^+$ RNA were included to control erroneous amplification from remaining genomic DNA. PCR amplifications were carried out under the following conditions: 1 min at 94°, 1 min at 60°, 2 min at 72°, and 40 cycles. PCR primers specific for the *hcf106* (R. MARTIENSSEN, unpublished data) and *dzs10* (KIRIHARA *et al.* 1988) cDNAs were used as positive controls and produced specific PCR products with seedling, and embryo and endosperm mRNA, respectively.

RESULTS

Identification of unselected recombinant alleles: Restriction analysis using Southern blots has been proposed as a method for the identification of recombinant alleles without selecting for restored gene function (MEAGHER *et al.* 1988). A schematic representation of the basis of this method, as used in the experiments reported here, is given in Figure 1A. Recombinant alleles resulting from a crossover event in the vicinity of a unique probe can be identified by comparing RFLP patterns between parents and progeny, using multiple restriction enzymes for each probe. In the absence of recombination, a given progeny plant should reveal the same genotype at a particular locus regardless of the restriction enzyme used. However, if recombination occurs between polymorphic restriction sites at a locus, two types of aberrant results can be observed. With restriction enzymes that detect polymorphisms on both sides of the crossover site (enzyme Z in Figure 1A), a recombinant allele will produce nonparental restriction fragments. With restriction enzymes that detect polymorphisms on only one side of the crossover site (enzymes X and Y Figure 1A), a recombinant allele will generate either one or the other of the parental restriction fragments, and the genotype of the progeny plant that inherited the recombinant allele will be scored differently depending on the enzyme used. Therefore, Southern blot analysis of genomic DNA from a segregating population using several different enzymes and the same probe can identify putative recombinants in the progeny by nonparental hybridizing bands or genotypes that are scored differently depending on the restriction enzyme used.

Such an analysis was conducted for genomic DNA from 48 self-pollinated F_2 progeny of A188 × W64A hybrids. DNA was digested with eight different restriction enzymes, and Southern blots were hybridized with

TABLE 1

Restriction site polymorphisms at RFLP loci of chromosome 7L

Restriction Enzymes	Probes						
	bnl15.40	*umc116*	*bnl15.21*	*bnl8.32*	*bnl8.21*	*bnl16.06*	*bnl8.44*
*Eco*RI	Yes	Yes	Yes	Yes	No	Yes	Yes
*Hind*III	Yes	No	Yes	Yes	No	Yes	Yes
*Eco*RV	Yes	Yes	No	No	No	Yes	Yes
*Xba*I	No	Yes[a]	Yes	Yes[a]	Yes	Yes	Yes
*Bam*HI	Yes	Yes	Yes	Yes[a]	Yes	Yes[a]	Yes
*Kpn*I	Yes	Yes	No	Yes[a]	Yes	Yes[a]	Yes[a]
*Sst*I	Yes[a]	Yes[a]	Yes[a]	Yes[a]	Yes[a]	No	Yes[a]
*Apa*I	Yes[a]	Yes	No	Yes[a]	No	No	Yes[a]

A total of 48 F_2 progeny from A188 × W64A hybrids were analyzed, in groups of 28 and 20.

[a] Cases where only the first group of 28 was tested. The presence or absence of polymorphisms for the relevant combination are indicated by yes or no, respectively.

10 RFLP probes from chromosome arm 7L. The RFLP probes *umc45, umc91,* and *bnl4.24* either did not reveal many polymorphisms between the A188 and W64A alleles, or yielded poorly resolved hybridization signals. However, seven RFLP probes did reveal extensive polymorphisms between the parental alleles. The relative map positions of these seven loci are shown in Figure 1B, and the presence or absence of polymorphisms for each enzyme and probe combination is summarized in Table 1.

By the criteria described above, two potential recombinants were identified at the loci *umc116* (Figure 2) and *bnl8.44* (Figure 3). Figure 2A shows the Southern blot data obtained after probing with *umc116* for 10 of the 48 F_2 plants tested. The genotype of the plant represented in lane 7 appears to be homozygous for the W64A allele upon digestion with *Bam*HI, but heterozygous for the A188 and W64A alleles following digestion with *Kpn*I. A novel restriction fragment was observed with *Eco*RI, which is slightly smaller than the band of the W64A allele. Similar results were obtained for other restriction enzymes, as summarized in Figure 2B (asterisks indicate a nonparental band). Based on the above model, the plant represented in lane 7 should be heterozygous for the W64A allele and a recombinant allele.

Similar observations were made for another plant in Southern blot analysis using *bnl8.44* as a probe. Figure 3A shows Southern blot hybridization data for a different set of 10 plants. The scored genotype of the plant represented in lane 4 depends on the restriction enzyme used. This plant appears to have inherited one

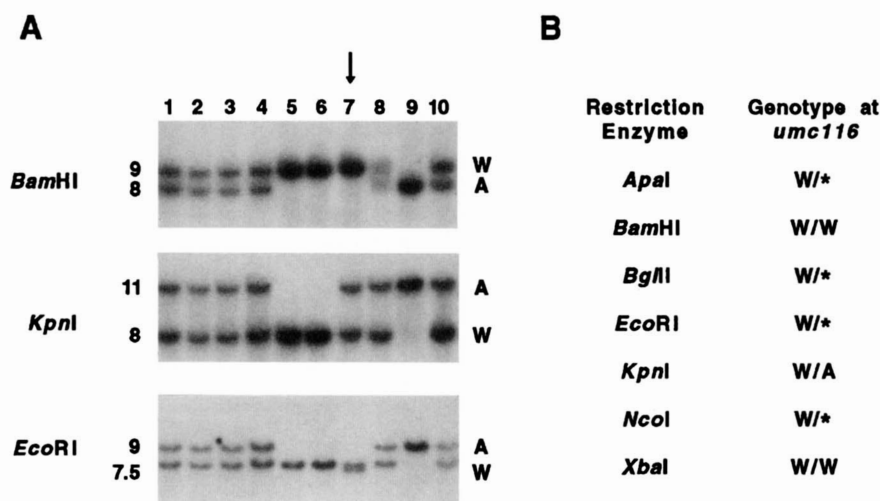

FIGURE 2.—Identification of the recombinant allele at the *umc116* locus. DNA prepared from 48 self-pollinated F_2 progeny of A188 × W64A hybrids was digested with eight different restriction enzymes, and the resulting genomic blots were probed with the RFLP marker *umc116*. (A) Southern blot data for 10 F_2 progeny. The restriction enzyme used and the restriction fragment sizes of the parental alleles are indicated. The plant represented in lane 7 appears to be homozygous for the W64A allele with *Bam*HI, heterozygous for the A188 and W64A alleles with *Kpn*I, and reveals a novel restriction fragment with *Eco*RI. (B) The observed genotype of the plant represented in lane 7 for each restriction enzyme used in the Southern blot analysis. A, A188 allele; W, W64A allele; *, recombinant allele.

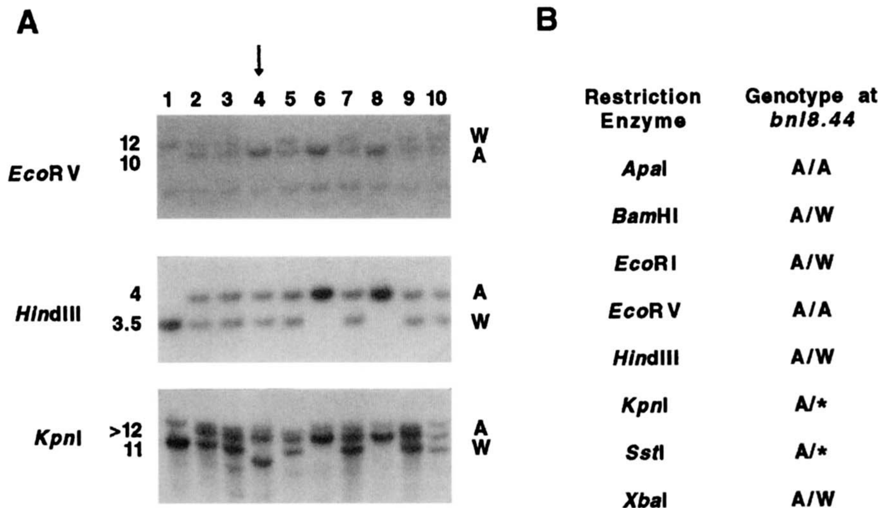

FIGURE 3.—Identification of the recombinant allele at the *bnl8.44* locus. Similar analysis as described for Figure 2. The plant represented in lane 4 appears to be homozygous for the A188 allele with *Eco*RV, heterozygous for the A188 and W64A alleles with *Hin*dIII, and reveals a novel restriction fragment with *Kpn*I. (B) The observed genotype of plant 4 for each restriction enzyme used in the Southern blot analysis. A, A188 allele; W, W64A allele; *, recombinant allele.

parental A188 allele and a potential recombinant allele. The new allele resembles the A188 allele upon digestion with *Eco*RV, the W64A allele with several restriction enzymes (*e.g.*, *Hin*dIII), and produces nonparental fragments with *Kpn*I and *Sac*I. Note that the other nine samples in Figure 2A and 3A show proper correspondence in genotype for all three restriction enzymes. This was the typical result for all other samples and loci tested.

Segregation analysis of the putative recombinant alleles: Segregation of the potential new alleles was analyzed in outcrosses to the inbred line W22 to confirm that the novel restriction patterns resulted from intralocus recombination and not from artifacts in the Southern blot analysis. Both plants carrying a putative recombinant allele were crossed to W22, and the genotype of several progeny was determined by Southern blot hybridization. Both new alleles segregated as expected, as shown in Figure 4A for the *umc116* allele and Figure 4B for the *bnl8.44* allele. Outcross progeny 1, 3, 4, 6, and 7 inherited the W64A allele of *umc116,* whereas the progeny 2, 5, and 8 appear to have inherited the recombinant allele (Figure 4A). Digestion of genomic DNA from plants 2, 5, and 8 with *Xba*I suggests inheritance of the W64A allele, whereas *Kpn*I digests suggest inheritance of the A188 allele. A nonparental restriction fragment was observed with *Eco*RI, which migrates as expected, slightly faster than the band of the W64A allele. The restriction pattern of the allele inherited by these three plants appeared identical to that of the putative recombinant allele in the F_2 parent for all enzymes listed in Figure 2B (data not shown).

The putative recombinant allele at *bnl8.44* also segregated as expected (Figure 4B). Plants 1, 2, and 5 appear to have inherited the recombinant allele, whereas the remaining progeny inherited the A188 allele. Again, the restriction pattern of the recombinant allele was identical to that of the parental F_2 plant (Figure 3A, lane 4) using additional enzymes. The two recombinant alleles also showed expected segregation ratios in a subsequent generation. Plants heterozygous for either one of the rearranged alleles were self-pollinated and three out of 17, and four out of 17 progeny were found to be homozygous for the new *umc116* allele and *bnl8.44* allele, respectively.

Flanking marker assortment in the recombinant chromosomes: Recombinational events giving rise to novel restriction patterns may occur either via crossing over or via a gene conversion-like mechanism. Crossing over is associated with exchange of flanking markers, whereas the arrangement of such markers remains parental in recombinants derived by a gene conversion-like mechanism. The type of recombinational exchange that occurred at the *umc116* and *bnl8.44* loci was established by determining the genotype at flanking marker loci in the original F_2 plants. For the F_2 plant carrying the recombinant allele of *umc116,* the genotype at the flanking RFLP loci *bnl15.40* and *bnl15.21* was already evident from the initial Southern blot analysis. However, because *bnl8.44* was the most distal marker on chromosome 7L used in our analysis, genomic DNA from the F_2 plant carrying the recombinant allele of *bnl8.44* was probed with the RFLP probe *umc168,* which maps 11 cM distal to *bnl8.44* (Figure 1B). The F_2 plant carrying the recombinant allele at *umc116* was heterozygous for the A188 and W64A alleles at *bnl15.40* and homozygous for the W64A allele at *bnl15.21.* Similarly, the F_2 plant carrying the *bnl8.44* recombinant allele was heterozygous at *bnl16.06* and homozygous for the A188 allele at *umc168* (data not shown). These observations are consistent with both recombination events being associated with exchange of flanking markers, but because we used a self-pollinated F_2 population rather than a backcross, we cannot be certain of the flanking marker assortment in the recombinant chromosomes. However, to explain the observed genotypes without

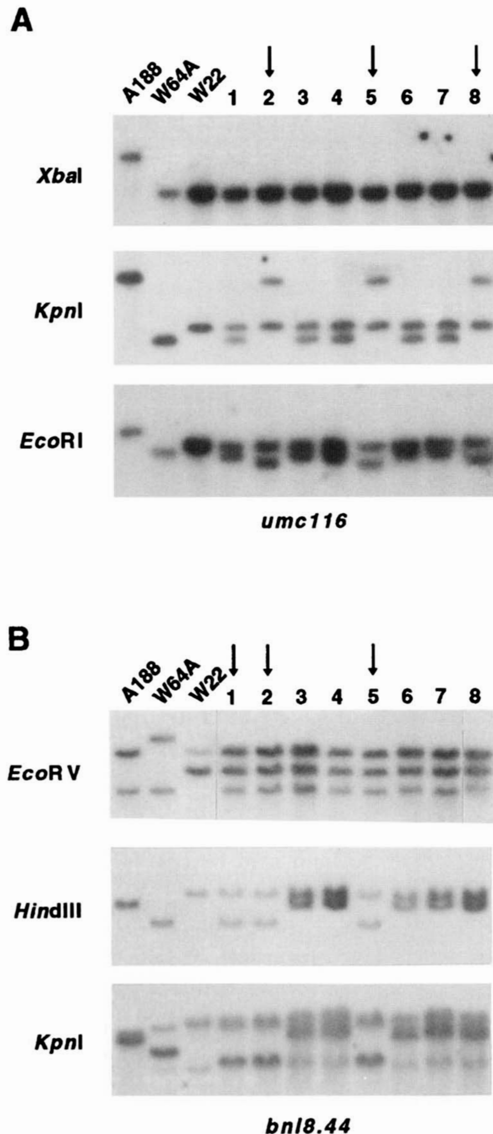

FIGURE 4.—Segregation analysis of the recombinant *umc116* and *bnl8.44* alleles. Plants carrying the recombinant allele at the *umc116* and *bnl8.44* loci were outcrossed to the inbred line W22. DNA from individual outcross progeny was digested with various enzymes and probed with *umc116* and *bnl8.44*, respectively. DNA from the inbred lines A188, W64A, and W22 was also included in the analysis. (A) Segregation of the recombinant allele at the *umc116* locus. Progeny 1, 3, 4, 6, and 7 are heterozygous for the W64A and W22 alleles with all three restriction enzymes. Progeny 2, 5, and 8 appear to be heterozygous for the W64A and W22 alleles with *Xba*I, heterozygous for the A188 and W22 alleles with *Kpn*I, and heterozygous for a new allele (a novel restriction fragment) and the W22 allele with *Eco*RI. This restriction pattern is expected following inheritance of the recombinant allele. (B) Segregation analysis of the recombinant allele at the *bnl8.44* locus. Progeny 3, 4, 6, 7, and 8 are heterozygous for the A188 and W22 alleles. Progeny 1, 2, and 5 reveal a hybridization pattern predicted for plants heterozygous for the recombinant allele and the W22 allele. The recombinant allele resembles the A188 allele with *Eco*RV, the W64A allele with *Hin*dIII, and produces a novel restriction fragment with *Kpn*I.

exchange of flanking markers, multiple recombination events at and flanking the *umc116* or *bnl8.44* loci need to be invoked.

Molecular analysis of the crossover at the *umc116* locus: Both parental alleles and the recombinant allele of the *umc116* locus were characterized molecularly to obtain information about the kind of sequences involved in this unselected meiotic crossover. Size-selected *Eco*RI libraries from the inbred lines A188 and W64A, and from a plant homozygous for the recombinant allele were screened with *umc116*. Positive clones were ~9, 7.5, and 7 kb in size, respectively, consistent with previous Southern blot data (Figures 2 and 4A). However, restriction mapping and cross-hybridization between the subclones indicated no homology between the A188- and W64A-derived clones outside a 1-kb fragment including *umc116*. A second size-selected *Eco*RI library from A188 was, therefore, constructed and screened with the probe bs450 (Figure 5). This fragment is unique in the genome and is homologous but polymorphic between the W64A and recombinant alleles. Moreover, the hybridization pattern of bs450 to genomic DNA from a plant homozygous for the recombinant allele resembled that of A188 (see below). A positive clone of 7 kb was isolated from an A188 library using probe bs450.

The contiguity of the A188-derived clones identified with the *umc116* and bs450 probes was confirmed by PCR analysis of genomic DNA from A188. The appropriate ends of the two lambda clones were sequenced and PCR primers were designed accordingly. A PCR product of the expected size was obtained from A188 genomic DNA. This PCR product was sequenced directly, and a sequence identical to the ends of the lambda clones was obtained (data not shown). However, because the region amplified is part of a repetitive element (see below), it is likely that the amplified and sequenced product was not derived specifically from the cloned region but rather from different copies present in the genome. Nonetheless, the observed sequence identity between the amplified fragment and the respective ends of the two lambda clones and the presence of an *Eco*RI site at the correct location in the amplified fragment suggests the contiguity of the two A188-derived lambda clones. If the two clones were not contiguous, one has to assume that the repetitive element near the *umc116* locus differs from the amplified copies precisely by the insertion of an unknown fragment flanked by *Eco*RI sites. Such a possibility is unlikely.

Restriction maps of all three *umc116* alleles are shown in Figure 5. The restriction map of the recombinant allele is identical to W64A around *umc116*, but identical to A188 around bs450. Assuming a simple crossover event as the mechanism of recombination, we can ascribe a proximal and distal side to the locus based on

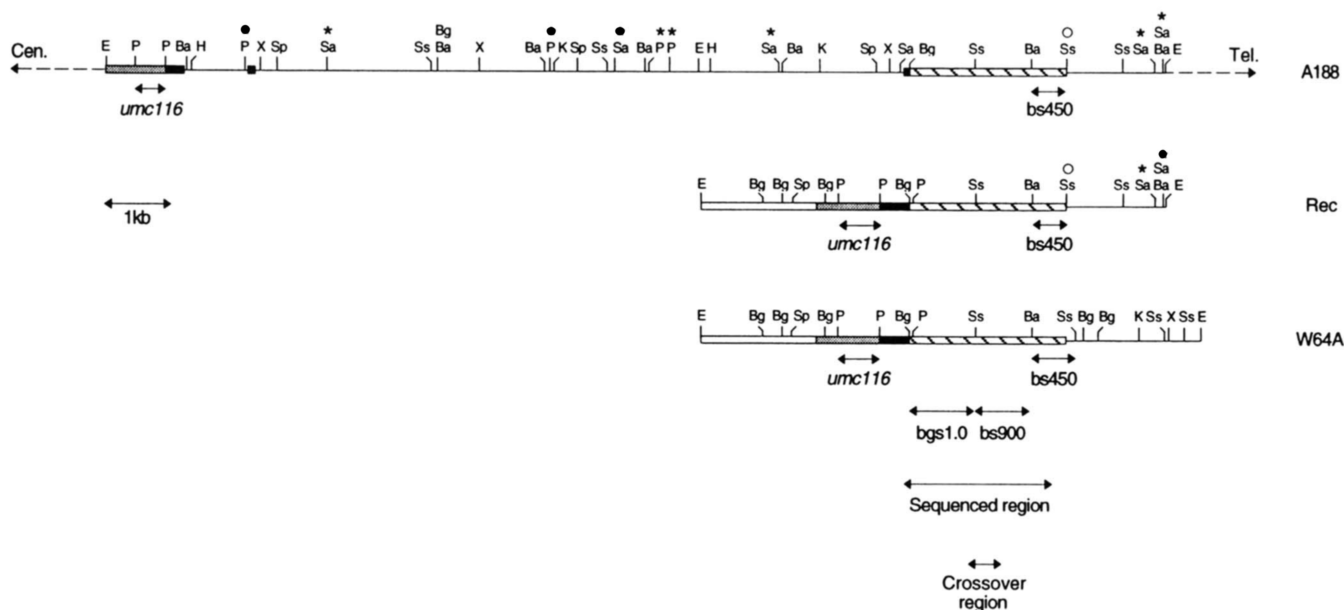

FIGURE 5.—Restriction maps of the A188, W64A, and the recombinant allele of the *umc116* locus. Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I. The centromeric and telomeric ends of the alleles are indicated. Single lines represent restriction fragments hybridizing to a repetitive genomic element, whereas boxes represent regions of the *umc116* locus hybridizing to unique sequences in the genome. Regions of homology between the alleles are indicated by identical highlighting. *Pst*I and *Sal*I sites marked with an asterisk were found to be methylated in genomic DNA prepared from leaf tissue. The *Sst*I site marked with ○ is subjected to *de novo* methylation. The restriction fragments hybridizing to the *umc116*, bgs1.0, bs900, and bs450 probes are indicated. The region of each allele that was sequenced to determine the crossover site is indicated, as well as the crossover region.

these restriction maps and the flanking marker assortment mentioned above (Figure 5). Regions of homology between the three clones were determined by cross-hybridization with various fragments derived from each clone. These regions are indicated by differently highlighted boxes in Figure 5. The proximal ends of the parental alleles were not homologous. In addition, the A188 allele has two insertions of approximately 10 kb and 860 bp relative to the W64A allele. Southern hybridization and sequence analysis revealed that the 10-kb insertion is a long terminal repeat (LTR)-retrotransposon found at ~1500 copies per haploid genome. The 860-bp insertion appears to be a solo LTR with a copy number of ~500 copies per haploid genome. Neither of these insertions is present in the recovered recombinant allele due to the position of the crossover site. The presence of different repetitive elements could explain the lack of homology and the unusually high degree of restriction site polymorphism between these alleles.

Both the restriction maps and the homology between the clones placed the crossover site within a ~2.4-kb *Bgl*II-*Sst*I fragment. This region was sequenced for all three genomic clones, to locate the crossover site more precisely. Figure 6 shows a schematic representation of the sequence comparisons of this region between the different alleles. Vertical lines indicate sequence polymorphisms. The width of each line correlates with the extent of the polymorphic region, but no distinction between insertions, deletions, or substitutions is made. The W64A and A188 alleles share a 534-bp fragment with perfect homology. The flanking regions are each about 900 bp in size and are only 93% (near the *Bgl*II site) and 95% homologous. Sequence comparisons with the recombinant allele located the crossover site within the 534-bp fragment. No polymorphisms unique to the recombinant allele were observed, suggesting that the crossover was conservative. Results from two studies using transgenic plants suggest that mitotic intrachromosomal recombination occurs also with high fidelity (PETERHANS *et al.* 1990; GAL *et al.* 1991).

Repetitive DNA sequences in each genomic clone were identified by Southern hybridization of the lambda clones with total maize DNA as a probe (data not shown). Individual restriction fragments derived from regions of the *umc116* locus that hybridized to genomic maize DNA in this analysis were subsequently used as probes on genomic blots to confirm that they contained repetitive DNA sequences. However, at this level of analysis, the presence of unique sequences embedded in these repetitive elements cannot be ruled out. Restriction fragments that did not hybridize to genomic maize DNA were also used as probes on genomic blots to confirm that they indeed corresponded to single copy regions in the genome. Interestingly, the homologous regions between the A188 and W64A alleles were found to correspond to unique sequences in the

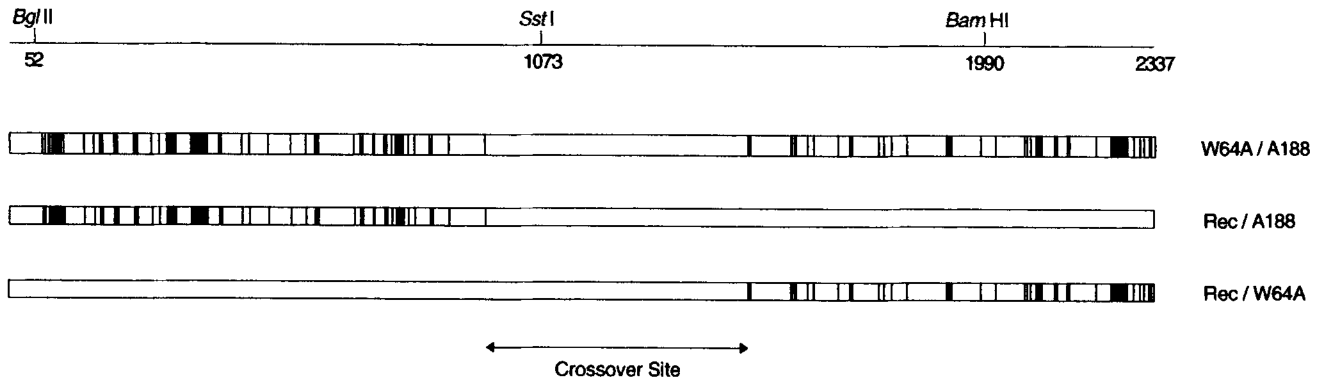

FIGURE 6.—Pairwise sequence comparisons between the recombinant, A188, and W64A alleles. A schematic representation of the sequence analysis of the crossover region is shown. The vertical lines represent sequence polymorphisms between the compared alleles. The width of the lines reflects the extent of the polymorphic region, but no distinction is made between nucleotide deletions, insertions, or substitutions. The 534-bp fragment encompassing the crossover site is indicated by the arrow.

maize genome (indicated as boxes in Figure 5), whereas nonhomologous regions hybridized to repetitive genomic sequences (indicated by single lines in Figure 5).

The meiotic crossover occurred within a perfectly homologous region of a single copy sequence. This observation is probably not due to the fact that single copy RFLP probes were used to identify potential recombinant alleles. The identification of crossovers depends on the presence of polymorphic restriction sites, which can be several kb outside the probed region. The *Eco*RI fragments hybridizing to the *umc116* probe included several repeat sequences. Moreover, the crossover site in the A188 allele is >10 kb removed from the *umc116* sequence and separated by repetitive elements.

Gene conversions and crossovers have been proposed to reflect the search for homology necessary to allow pairing between homologous chromosomes (see PETES *et al.* 1991). If crossovers indeed result from the homology search during chromosome pairing, then the previous information suggests an organization as shown in Figure 7 for the alignment of the parental alleles during meiosis I. Allelic pairing between short regions of single copy sequences with high homology is interrupted by the presence of nonhomologous repetitive elements.

Transcription of the crossover region: The high frequency of intragenic recombination in maize suggests that genes could be the preferred sites for meiotic recombination. To determine if the crossover region is part of a transcription unit, various probes encompassing all unique sequences of the W64A genomic clone were hybridized to RNA isolated from several immature and mature tissues of the inbred line W64A. No transcripts were detected using Northern blots containing 1 μg of poly-A$^+$ RNA from roots, seedling leaves, mature leaves, apices, immature ears, embryos 18 and 30 dap, and endosperms 18 and 30 dap (data not shown). However, due to the limited sensitivity of this assay, it cannot be ruled out that the crossover site is part of a transcription unit.

We used the "FEXA" computer program (SOLOVYEV *et al.* 1994) from the "BCM Gene Finder" to locate possible exons within the ~2.4-kb sequenced region, the 450-bp *Pst*I fragment from W64A (black box in Figure 5), and the corresponding regions from A188. The FEXA program predicts internal exons based on the presence of potential splice donor and acceptor sites, and open reading frames. The parameters of this exon prediction program are optimized for plant sequences. The FEXA program, however, did not recognize any potential exons in the A188 and W64A sequences. In addition, we used the program "Grail" (UBERBACHER and MURAL 1991), which is designed to recognize promoter sequences, open reading frames, splice donor and acceptor sites, and poly-adenylation signals and

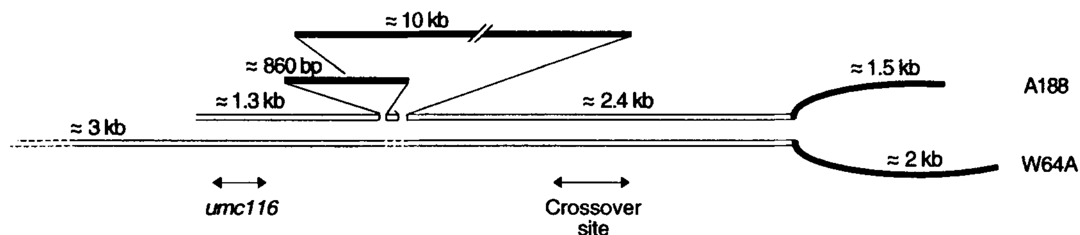

FIGURE 7.—Representation of the crossover region. Repetitive and methylated genomic sequences are shown as filled lines and unique, unmethylated genomic sequences are indicated by open lines. Regions of homology between the A188 and W64A alleles are aligned. The crossover region and the region hybridizing to the *umc116* probe are also indicated.

thus predicts the position of introns and exons. Since this program is optimized for the recognition of exons in human sequences and because transcription recognition signals in plants can be quite diverged from mammalian consensus sequences, the program might not recognize all possible exons. Four exons were identified in the sequence of the W64A allele, but only two were found for the A188 allele. This apparent discrepancy results from the presence of the two insertion elements in the A188 allele. Based on the computer analysis, both insertions occurred within an exon. The two exons conserved between the A188 and W64A alleles were located within the perfectly homologous region encompassing the crossover site. The protein sequences of the predicted exons were not homologous to any known protein sequence in the database. Also the DNA sequence of the ~2.4-kb sequenced regions from A188 and W64A showed no homology to any known sequence in the database.

Primers were designed for the potential exons conserved between the A188 and W64A alleles, and an RT-PCR experiment using poly-A$^+$ RNA from immature ears, embryo and endosperm 18 dap, and seedling leaves was performed to test for splicing, indicative of transcription. No specific RT-PCR product was obtained from these tissue samples, but products of the expected size were obtained using genomic DNA as a PCR template (data not shown). Specific PCR products were obtained in control experiments using *hcf106*-derived primers (R. MARTIENSSEN, unpublished data) together with mRNA isolated from seedling leaf tissue, and *dzs10*-derived primers (KIRIHARA *et al.* 1988) in combination with mRNA from embryo and endosperm tissue. Therefore, we have not found any evidence so far that the crossover region is part of a transcription unit. Moreover, since the insertion elements present in the A188 allele are located in the other two exons identified with the Grail program, it seems unlikely that the crossover region in the A188 allele is part of a functional transcription unit.

Methylation pattern of the crossover region: DNA methylation has been shown to suppress recombination in mammals (HSIEH and LIEBER 1992; GOODHART *et al.* 1993). We, therefore, compared DNA methylation patterns between the parental and recombinant alleles using methylation-sensitive restriction enzymes. In addition, we wished to determine whether methylation determinants were maintained after recombination. Leaf DNA from A188, W64A, and a plant homozygous for the recombinant allele was digested with *Sal*I and *Pst*I in combination with several methylation-insensitive enzymes and hybridized to multiple probes derived from the locus (data not shown). The results from these analyses are summarized in Figure 5. Methylated restriction sites are indicated by asterisks. In general, all *Sal*I and *Pst*I sites located within repetitive sequences were found to be methylated in mature leaves, whereas those restriction sites present in unique sequences were not. The only exception is the unmethylated *Sal*I site immediately preceding the 2.4-kb unique sequence encompassing the crossover site in A188.

The methylation pattern of the recombinant allele reflected the parental A188 origin for sites to the right of the crossover site. To examine potential W64A methylation sites to the left and to characterize the overall methylation of the locus, the unique regions immediately surrounding the crossover site were analyzed in more detail for the presence of methylated sequences. Leaf DNA was digested with *Hpa*II, *Msp*I, *Hae*III, *Hha*I, and *Pvu*II, in combination with methylation-insensitive enzymes *Bgl*II and *Bam*HI (the *Bam*HI site is not methylated in its sequence context) and hybridized to both the *Bgl*II-*Sst*I and *Sst*I-*Bam*HI fragments (bgs1.0 and bs900 in Figure 5). The hybridization patterns obtained indicated that the 35 restriction sites tested were unmethylated for all three alleles (data not shown).

Interestingly, the *Sst*I site of the bs450 fragment was found to be methylated for the A188 and recombinant allele but not for the W64A allele (marked with an open circle in Figure 5). The restriction maps derived from the lambda clones predict a 1.4- and 1.6-kb *Sst*I fragment upon hybridization with the bs450 probe for A188 and W64A genomic DNA, respectively. We observed the expected 1.6-kb fragment for W64A but obtained a 2.1-kb fragment for both the A188 and recombinant alleles (Figure 8). The restriction enzyme *Sst*I is not inhibited by CpG or CpXpG cytosine methylation but is affected by methylation at the internal C in its recognition sequence GAGCTC. Non-CpG or CpXpG cytosine methylation has been observed for several maize transposable elements, where it has been correlated with inactivation of the element (MCCARTY *et al.* 1989; SCHWARTZ 1989; MARTIENSSEN and BARON 1994). This type of cytosine methylation cannot be maintained by known maintenance methyltransferases but may need to be introduced *de novo* after each round of replication. Therefore, *cis* sequences containing the required information for the *de novo* methylation are inherited by the recombinant allele. Together, these data are consistent with the possibility that methylation determinants of individual chromosomes are maintained through the recombination process, including the nonclassical determinant mentioned above.

DISCUSSION

We describe a method to identify meiotic recombination events without selecting for restoration of gene function. This Southern blot assay is based on the occurrence of novel restriction patterns upon recombination between polymorphic alleles. Since it allows the identification of unselected recombination events, this

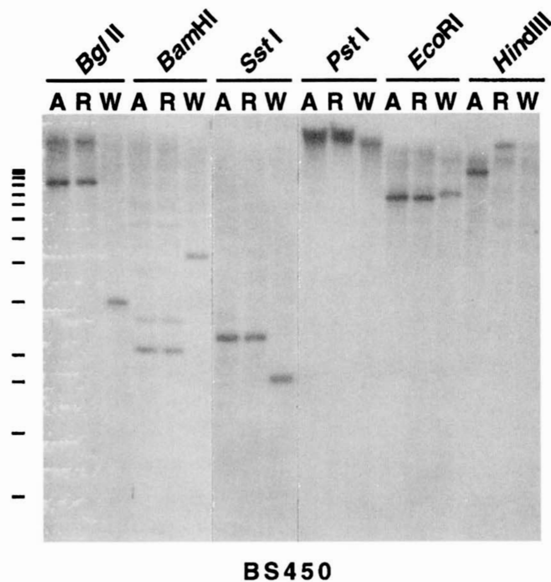

FIGURE 8.—*De novo* methylation at the *umc116* locus in the A188 and recombinant alleles. Genomic DNA was prepared from leaf tissue of plants homozygous for either the A188, W64A, and recombinant allele of the *umc116* locus. The DNA was digested with several enzymes and subjected to Southern blot analysis using the bs450 fragment as a probe. A 2.1-kb restriction fragment was observed after digestion with *SstI* for the A188 and recombinant allele, instead of the expected 1.6-kb fragment. The molecular weight marker is the 1-kb ladder (BRL). A, A188; R, recombinant; W, W64A.

method is particularly suited to test whether crossing over is confined to particular genomic segments. The assay depends on the presence of polymorphic restriction sites around a probed region. Since the incidence of polymorphism differs greatly among plant species, the usefulness of this assay might vary between plants. However, maize exhibits a relatively high frequency of polymorphism (SHATTUCK-EIDENS *et al.* 1990), and our assay could be particularly useful in this model system.

The high degree of polymorphism in maize is also apparent from our analysis. The W64A and A188 inbred lines showed polymorphism with multiple restriction enzymes at seven of the 10 RFLP loci tested (Table 1). At the *umc116* locus, the high frequency of restriction site polymorphism can be largely ascribed to the presence of different repeat sequences in the A188 and W64A alleles. This type of polymorphism has also been observed at the *adh1* (JOHNS *et al.* 1983) and *bz1* (FURTEK *et al.* 1988; RALSTON *et al.* 1988) loci and is likely very common in maize. In addition, the *umc116* locus of A188 and W64A contained ~5% sequence polymorphism within the conserved region (Figure 6). This type of polymorphism is also common in maize and has been observed for each locus where sequence information from multiple alleles was compared (*adh1*, SACHS *et al.* 1986; *bz1*, FURTEK *et al.* 1988; RALSTON *et al.* 1988; *sh1*, WERR *et al.* 1985; ZACK *et al.* 1986; 27 kDa zein gene, DAS *et al.* 1991b).

Meiotic intragenic recombination frequencies have been previously determined for the maize genes *a1* (BROWN and SUNDARESAN 1991; CIVARDI *et al.* 1994), *adh1* (FREELING 1978), *b* (PATTERSON *et al.* 1995), *bz1* (DOONER 1986; DOONER and KERMICLE 1986), *gl1* (SALAMINI and LORENZONI 1970), *r* (DOONER and KERMICLE 1986), and *wx* (NELSON 1968), and the maximum frequency for each of these genes varied between 0.9–1.3×10^{-3}. Because we have sampled 672 potential events (seven loci × 96 chromosomes) and observed two recombination events, the frequency we observed was 3×10^{-3}. However, the two situations are not strictly comparable. First, our method should detect crossovers that occur anywhere between polymorphic restriction sites that can extend several kb into neighboring repetitive sequences. In contrast, the phenotypic selection method detects crossovers between two mutations within the gene (and often within the coding sequence) separated by a fixed distance that is usually smaller. Second, our method is theoretically independent of the fidelity of recombination since no selection is imposed. Phenotypic selection for restoration of gene function from mutant heteroalleles requires that the recombination event occurs without introduction of any new mutations within coding regions or otherwise critical sequences. These two reasons would indicate that the RFLP method could detect higher recombination frequencies compared to the phenotypic selection approach. Furthermore, the F_2 population analyzed here may carry heritable activities that increase recombination frequencies along chromosome 7 by approximately twofold (M. TIMMERMANS, O. P. DAS, J. BRADEEN and J. MESSING, unpublished results).

The detailed characterization of one of the identified unselected recombinants revealed several interesting aspects about the site at which this crossover occurred. The crossover site mapped within a perfectly homologous region of a single copy sequence that was unmethylated. A combination of these properties could be important in targeting crossovers to particular allelic sequences and preventing nonallelic repetitive elements from crossing over. Ectopic recombination between repeats can result in large genomic rearrangements, such as inversions, deletions and translocations. Ectopic recombination frequencies between naturally occurring repeat sequences in yeast are suppressed in meiosis (KUPIEC and PETES 1988a,b). In addition, the observed recombination events were rarely associated with crossing over. The high frequency of intragenic recombination relative to the average recombination rate for the whole genome also suggests the existence of mechanisms involved in suppressing recombination frequencies between repetitive elements in maize.

Fungi have developed several mechanisms to suppress recombination between repeats. For instance, reduced recombination between Ty elements in *Saccharo-*

myces cerevisiae is partly mediated by the *trans*-acting *EDR1* system (ROTHSTEIN 1984; WALLIS *et al.* 1989). In *Neurospora crassa*, repeat-induced point mutations (RIP) occur premeiotically between repeated sequences (SELKER 1990). This reduces the homology between the repeats and thus inhibits recombination. In *Ascobolus immersus*, repeated sequences become methylated premeiotically (MIP), which likely reduces their recombination frequency (ROSSIGNOL and FAUGERON 1995). Although the cosuppression phenomena (NAPOLI *et al.* 1990; JORGENSEN 1995) suggest that plants can recognize ectopic sequence homology, there is no evidence yet for the existence of an active mechanism that suppresses recombination or crossing over between repeats. However, the degree of homology and the methylation status of particular sequences are likely to play a role.

Characterization of the *umc116* crossover showed that the crossover junction mapped to the largest stretch of perfect homology between the two parental sequences. This stretch is 534 bp long, within a total homologous region of at least 2337 bp. A reduced frequency of recombination resulting from mismatches in the recombining DNA molecules has been documented for bacteria (SHEN and HUANG 1986), yeast (BORTS and HABER 1987; HAYDEN and BYERS 1992; SELVA *et al.* 1995), Drosophila (CARPENTER 1982), and mammalian cells (WALDMAN and LISKAY 1988). Recombination frequency appears to be determined by a minimal length of perfect uninterrupted homology, rather than the overall percentage of sequence homology. For example, the minimal length of homology required for crossing over in yeast (HAYDEN and BYERS 1992) and mammalian cells (WALDMAN and LISKAY 1988) was found to be about 200–250 nucleotides. The presence of mismatches in the heteroduplex affects branch migration (SELVA *et al.* 1995) and possibly the resolution of recombination intermediates, such that recombination events associated with short stretches of homologous heteroduplex are less likely to be resolved as crossovers (ROEDER 1990; PETES *et al.* 1991; HAYDEN and BYERS 1992).

In plants, few recombination events have been characterized in detail and consequently the minimal homology required for recombination is unknown. At the 27-kDa zein locus in maize, two recombinant alleles derived from recombination between the tandemly duplicated zein genes have previously been identified. One recombinant allele most likely arose from meiotic unequal crossing over between the duplications (DAS *et al.* 1991a). In this case also, the crossover site mapped to an 1168-bp region of perfect homology, within a 10-kb region of lesser homology. The other recombinant allele appears to have arisen via a mitotic gene conversion-like rearrangement (W. HU, M. TIMMERMANS and J. MESSING, in preparation). Interestingly, the crossover site in this case was not mapped to a region of high homology but instead to a region with many sequence differences (DAS *et al.* 1990b, 1991b).

Most transgenic studies in tobacco, Brassica, and Arabidopsis used perfect repeats of 350–600 bp as recombination substrates. However, GAL *et al.* (1991) determined crossover sites resulting from mitotic intrachromosomal "pop-out" recombination between imperfect repeat sequences and observed that most crossovers occurred within the longest stretch of perfect homology, a 122-bp fragment. In another study, a 53-bp repeat sequence provided enough homology to facilitate mitotic recombination, but differences in the experimental set up did not allow direct comparisons of recombination frequencies and no distinction between gene conversion and crossing over was possible (PETERHANS *et al.* 1990). The homology requirement for gene conversion in yeast is only 25–60 bp (HAYDEN and BYERS 1992).

Based on our results and the previous observations at the 27-kDa zein gene locus, we suggest that meiotic crossing over in maize preferentially occurs between sequence domains with perfect homology. Depending on the divergence between repeats, this homology requirement could reduce their frequency of crossing over, as in *N. crassa* (SELKER 1990). In addition, the distribution of repetitive elements varies between inbred lines, as seen here at the *umc116* locus. This could further reduce the frequency of crossing over between repeats since only ectopic recombination is possible. Consistent with this is the observation that crossover frequencies between linked T-DNAs were several fold higher when measured in petunia inbreds than in hybrids (ROBBINS *et al.* 1995).

Methylation has been postulated to affect recombination (HOLLIDAY 1987), and site-specific recombination of methylated immunoglobulin genes is much reduced in mammalian cells (HSIEH and LIEBER 1992; GOODHART *et al.* 1993). Several observations from the *r* locus in maize suggest that recombination frequencies at this locus are also inversely correlated with DNA methylation levels (EGGLESTON *et al.* 1995). Most of the unequal recombination events at the *R-r:standard* or *R-stippled* complexes occurred between the 3′ ends of the *r* genes, which are less methylated in comparison to the 5′ ends of the genes. Furthermore, early studies found that recombination frequencies within *R-r:standard* and its derivatives are reduced following paramutation (BRAY and BRINK 1966). These reduced recombination frequencies have recently been correlated with increased levels of methylation at the *r* locus (see EGGLESTON *et al.* 1995). Consistent with these observations, the crossover site at the *umc116* locus mapped to unmethylated sequences. However, the methylation pattern observed in the leaf might not accurately reflect that present during sporogenesis.

The parental methylation pattern was faithfully inherited by the recombinant allele, even after several generations. This includes the *SstI* site that is methylated *de novo* in the A188 and recombinant alleles. Faithful maintenance of methylation through the recombination process may be a significant factor in generating allelic diversity and could be important for the maintenance of specific epigenetic states (HOLLIDAY 1987). Moreover, recombination events like the one described here could possibly impose a different epigenetic regulation on a gene.

The high frequency of intragenic recombination in maize and the frequent occurrence of recombination hotspots near genes in other species could reflect the postulated enhancing effect of transcription on recombination. Using Northern analysis and RT-PCR analysis on mRNA isolated from several immature and mature maize tissues, we were unable to detect any transcripts from the region encompassing the crossover site. However, since most genes are not expressed during sporogenesis, it is more likely that the increased recombination frequency near genes results from an indirect effect of a specific chromatin structure near transcribed regions in the genome. Alterations in chromatin organization do play a role in determining where meiotic recombination events are initiated in yeast (OHTA *et al.* 1994; WU and LICHTEN 1994). The observed position effect on recombination frequencies seen in experiments using transgenic plants (ASSAAD and SIGNER 1992; SWOBODA *et al.* 1993) and the reduced frequency of intragenic recombination at the *wx* locus observed for *wx*-translocation stocks in maize (YU and PETERSON 1973) could also be a consequence of altered chromatin structure of the recombination substrate at different genomic positions.

The unselected crossover identified by the physical mapping approach described here occurred within a single copy sequence of perfect homology between the parental chromosomes, that is unmethylated and probably not transcribed. Although the significance of these observations needs to be confirmed with additional crossovers, the results are consistent with the previous genetic observation that intralocus recombination frequencies are several fold higher than the recombination frequency between loci.

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