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# Recombination within the myelin basic protein gene created the dysmyelinating shiverer mouse mutation

(neurogenetics/gene deletion/Z-DNA/staggered nucleotide rearrangement)

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ABSTRACT **Shiverer (*shi*) is an autosomal recessive mutation in the mouse characterized by an almost total lack of central nervous system myelin. While small amounts of other myelin components are present in the brain of the shi mouse, the four forms of myelin basic protein (MBP) are not detectable. Previous investigations by us and others indicate that the MBP gene has undergone a major rearrangement in the *shi* mutant. Herein, we report in detail the nature and extent of the rearrangement: a 20-kilobase region within the MBP gene is missing in the mutant. We map the 5′ breakpoint of the deletion to the second intron and the 3′ breakpoint to a site 2 kilobases beyond the last MBP exon. The junction of the upstream and downstream portions of the gene contains only one nucleotide not accounted for by the wild-type MBP gene sequence. The 3′ side of the deletion occurs in the 3rd of 11 tandem repeats of a 31-base-pair sequence. This region is rich in alternating purine and pyrimidine stretches, sequences that have been associated with both Z-DNA structures and gene rearrangements. The recombination junction shares several features with the junctions characterized by Anderson *et al.* [Anderson, R., Kato, S. & Camerini-Otero, D. (1984) *Proc. Natl. Acad. Sci. USA* 81, 206–210] in mouse L cells and is consistent with their model for a partially homologous recombination event. The structure of the shi recombination junction suggests that the donor DNA molecules were aligned in a partially homologous region before staggered cutting and joining occurred.**

Mammalian oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) produce a highly specialized multilamellar myelin membrane that surrounds the neuronal axons and greatly facilitates conduction of electrical impulses (reviewed in ref. 1). The myelin of the CNS has a relatively simple composition in which a family of closely related myelin basic proteins (MBPs) accounts for 30–40% of the total protein in the sheath (reviewed in ref. 2). Peripheral nervous system myelin contains a smaller and dispensable amount of the same MBPs (3, 4). Recent work by us (5) and others (6) has shown that a single gene encodes the family of MBPs and that the various forms of the MBPs are generated by a mechanism of alternative splicing of the mRNA.

A number of mutations have been identified in mice that affect oligodendrocyte function and/or Schwann cell function and, consequently, lead to dysmyelination in the CNS and/or the PNS (reviewed in ref. 7). The shiverer (*shi*) mutation is autosomal recessive and is characterized by the onset of tremors at about the 12th day of life, seizures at later times, and a progressive deterioration ending in an early death (8, 9). The mutation principally affects oligodendrocyte functions and the CNS of the shi mice is almost entirely devoid of myelin membrane (10). The normal amount of myelin is present in the PNS of these animals, but differences in its structure have been noted (4, 11, 12). The primary defect in shi mice appears to be the absence of MBP (13). By radioimmune assay, MBP levels in shi mice are <0.1% that of wild type (14).

The *shi* allele has been mapped to chromosome 18 by Sidman *et al.* (15) using classic genetic techniques. The gene encoding the MBP family has also been mapped to chromosome 18 by *in situ* hybridization and Southern blot analysis of DNA isolated from mouse–hamster somatic cell hybrids (16). Recent work by Roach *et al.* (17, 18) and by us (5) demonstrates that the MBP gene is structurally altered in the shi mouse and has led to the conclusion that the *shi* mutation is an allele of the MBP gene.

To further characterize the genetic defect in the MBP gene of the shi mouse, we have isolated and analyzed genomic clones spanning the remnants of the MBP gene in shi DNA. We have determined the 5′ and 3′ end points of the deletion and have determined the nucleotide sequence surrounding these points in normal and shi DNA. shi DNA is deleted for ≈20 kilobases (kb) from the middle of the second intron to a point 2 kb beyond the final exon of the MBP gene. Our evidence suggests that a single breakage and reunion event led to the formation of the *shi* allele.

## MATERIALS AND METHODS

**Animals.** The *shi* mutation was maintained on a C57BL/65 × C3H/HeJ $F_1$ hybrid background. Animals were originally obtained from M. K. Wolf (Anatomy Dept., Univ. of Massachusetts) and bred according to his protocols (19). C57BL and C3H mice were used as wild-type controls.

**Construction of Genomic Libraries.** High molecular weight DNA from a homozygous shi mouse was partially digested with *Mbo* I and fractionated on a 10–40% sucrose gradient as described by Maniatis *et al.* (20). Fractions containing DNA in the 15- to 20-kb size range were pooled and used to construct a genomic library. shi DNA (0.25 μg) was ligated to 0.5 μg of EMBL 3 arms digested with *Bam*HI (Vector Cloning Systems, San Diego, CA) and packaged in an *in vitro* packaging reaction (Gigapack, Vector Cloning Systems). After plating on NM539 cells (21), the plaques were screened directly without amplification. A library of 800,000 clones was obtained in this manner.

**Southern Transfers.** Blotting was carried out essentially as described by Southern (22) except that DNA was transferred to nylon membranes. For preparation of probe, fragments of MBP DNA were isolated on agarose gels and phenol-

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Abbreviations: CNS, central nervous system; PNS, peripheral nervous system; MBP, myelin basic protein; *shi*, shiverer gene; kb, kilobase(s); bp, base pair(s).

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extracted or electroeluted prior to nick-translation. The nylon transfers were prehybridized in 5× SSPE (1× SSPE = 180 mM NaCl/10 mM $NaPO_4$, pH 7.7/1 mM EDTA)/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/50% formamide/sonicated salmon sperm DNA (100 μg/ml)/1% $NaDodSO_4$. Hybridization was carried out at 42°C for 48 hr in a fresh portion of the same solution, but containing 10–20 × $10^6$ cpm of denatured probe DNA. Transfers were washed in 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.2% $NaDodSO_4$ at 55°C and exposed to x-ray film for 1–3 days.

**DNA Sequencing.** Fragments of DNA to be sequenced were subcloned into pUC8 or M13 vectors and sequenced by either the dideoxy-chain termination method (23) or the chemical modification and cleavage method (24).

## RESULTS

**Localization of the 5′ Boundary of the *shi* Deletion in the MBP Gene.** Analysis of *shi* mutant mouse DNA with probes from wild-type MBP genes has revealed that the MBP gene is partially deleted and several exons from the 3′ portion of the gene are missing (5, 17, 18, 25). We have extended the mapping studies in an effort to precisely localize the 5′ boundary of the deletion. The organization of the MBP gene has been determined by restriction endonuclease mapping, Southern transfer analyses, and, ultimately, sequencing all exons and flanking regions (5, 18). The gene is composed of seven exons spanning 32 kb of DNA. The information encoded in exons 2 and/or 6 are specifically deleted from some forms of mature MBP mRNAs—a phenomenon that accounts for the four forms of MBP found in the rodent.

We have examined the remnant of the MBP gene found in the *shi* mutant and compared it to the wild-type gene by Southern transfer analyses using a mixed cDNA probe that hybridizes to all seven exons (Fig. 1). DNA from the *shi* mutant exhibited only two of the four *Bam*HI fragments found in the wild-type DNA. The 15- and 7.5-kb fragments containing the first and second exon of the gene are present, but the 4.4-kb fragment containing the third exon and the 20-kb fragment containing exons 4, 5, 6, and 7 are absent in the shi DNA. Similar conclusions are drawn from the pattern obtained after digestion with *Eco*RI: only the 6.5- and 3.5-kb fragments containing the first two exons can be identified in the shi DNA. The deletion boundary, therefore, is located in the 11-kb intron 2, and since the 7.5-kb *Bam*HI fragment containing exon 2 is intact in shi DNA, the deletion must start beyond the *Bam*HI site defining the end of that fragment. We then used intron probes to precisely map the 5′ boundary of the deletion. When a 3.5-kb *Bam*HI intron fragment (B7 in Fig. 2) was used as a probe, a 7-kb *Bam*HI fragment was detected in shi DNA, indicating that the deletion site was within this region.

A λ phage library prepared from shi DNA was screened with the 3.5-kb *Bam*HI fragment (B7) as a probe. Restriction enzyme mapping and analyses indicate that one of the two positive clones, clone 25, contains a 22-kb insert that extends from a site downstream of the second exon to a site 15 kb beyond the deletion site (Fig. 2) and contains the 7-kb *Bam*HI fragment.

**Identification of the 3′ Boundary of the Deletion.** Evidence presented in Fig. 1 and in the previous section indicates that the deletion in the *shi* mutant joined sequences near the second exon of MBP to sequences that lie beyond the end of the MBP gene. Clone 25 of shi DNA contains the sequences downstream from the deletion and could be used as a probe to identify them in wild-type genomic clones. We subcloned a 1.9-kb *Kpn* I/*Sac* I fragment that contained the sequences flanking the deletion site and used it to probe a set of overlapping λ clones that contained information beyond the MBP gene; we were fortunate in finding that L2, a previously characterized MBP clone, contained sequences from the 3′ side of the *shi* deletion. Southern transfer experiments demonstrated that a 1.5-kb *Sac* I fragment adjacent to the 3.2-kb *Sac* I fragment containing exon 7 was responsible for the positive hybridization signal (data not shown). In addition, the restriction enzyme map of the L2 clone aligns perfectly with the map of clone 25 (Fig. 2). Taken together, these data indicate that the 3′ boundary for the *shi* deletion was located within a few hundred bases of the 5′-most *Sac* I site of the 1.5-kb fragment.

**Nucleotide Sequence of the DNA Surrounding the Breakage and Reunion Sites.** We sought to better define the boundaries of the *shi* deletion and to examine the nucleotide sequence of the relevant regions of DNA in the hope that these data might reveal features of the recombinational events generating the mutation. To that end, the DNA in the region of the deletion site in the *shi* chromosome and the corresponding regions in the wild-type MBP gene were sequenced. Fig. 3 shows the restriction maps of these regions and the sequencing strategy used. A comparison of the sequences of the wild-type and shi DNA shown in Fig. 4 reveals that the shi and wild-type sequences are perfectly homologous on either side of the deletion site with the exception of a single cytosine residue inserted at the recombination site. Such additions of one or

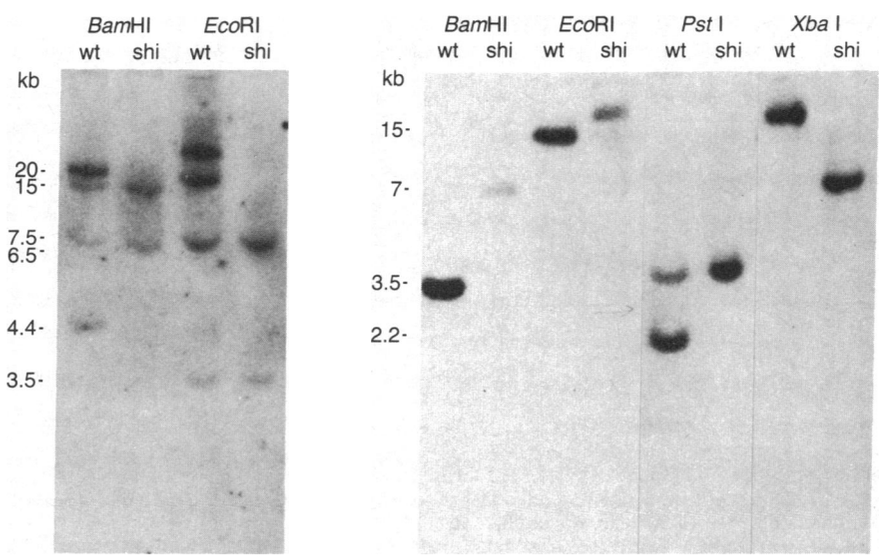


FIG. 1. Southern transfer analysis of the shi and wild-type (wt) mouse DNA. Ten-microgram samples of DNAs from shi and normal mice were cleaved with the indicated restriction endonuclease, and the resultant fragments were separated by electrophoresis in agarose gels and transferred to nylon membranes. (*Left*) Results were obtained with a cDNA probe, pdF191, which recognizes all seven MBP exons; (*Right*) results were obtained with an intron-specific probe, the *Bam*HI fragment B-7 shown in Fig. 2.

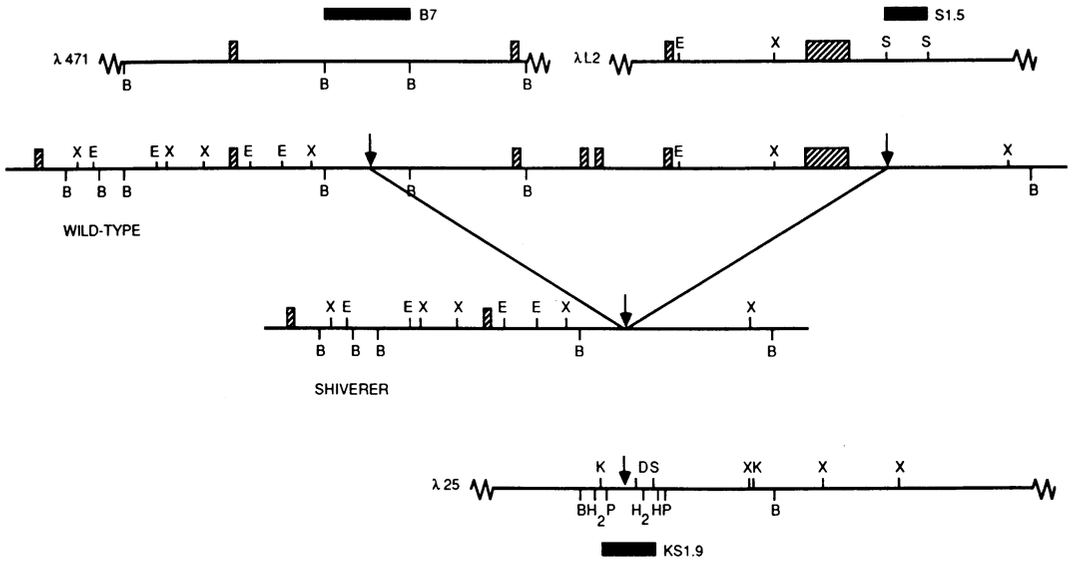


FIG. 2. Restriction map of wild-type and shi mouse DNA in the vicinity of the MBP gene. The restriction maps of the relevant λ phage clones of wild-type and shi DNA. Hatched boxes represent the exons of the MBP gene. B7, S1.5, and KS1.9 represent 3.5-kb *Bam*HI intron fragment, 1.5-kb *Sac* I fragment, and 1.9-kb *Kpn* I/*Sac* I fragment, respectively. E, *Eco*RI; B, *Bam*HI; X, *Xba* I; S, *Sac* I; K, *Kpn* I; $H_2$, *Hin*cII; P, *Pst* I. Arrows indicate the breakage and reunion points involved in the *shi* deletion.

more nucleotides, usually pyrimidines, have been found in other DNA rearrangements and have been postulated to be a tailing activity of one of the enzymes involved in recombination (26). The inclusion of only a single extra nucleotide suggests that a single recombinational event may have generated the deletion. The 3′ side of the recombination site, which is located beyond the last MBP exon, occurs in the middle of a region of DNA that contains 11 tandem imperfect repeats of a 31-base-pair (bp) sequence. This tandem repeat is largely composed of alternating purine and pyrimidine residues and is preceded by a stretch of simple sequence $(dG\text{-}dT)_{17}$. Stretches of alternating purine–pyrimidine residues have been associated with Z-DNA and, in some cases, recombinational events. The relationship of the tandem repeat, the 34-bp dG-dT stretch, and the breakage point in the DNA is illustrated in Figs. 3 and 4. A frequently observed characteristic of DNA that has undergone illegitimate recombination is a short stretch of homologous sequence in both recombining DNA. We have searched the sequences near the 5′ and 3′ breakage points for stretches of homology and have identified one area in which 15 of 20 bases (75%) are homologous (Fig. 5).

## DISCUSSION

Mutations of mice that specifically affect myelin synthesis or maintenance fall into three categories: those like *shi* and jimpy (*jp*), which affect primarily oligodendrocyte function; those like trembler (*Tr*), which affect Schwann cell function; and, finally, those like quaking (*qk*) and twitcher (*twi*), which affect both Schwann cells and oligodendrocytes and, consequently, both PNS and CNS myelination (reviewed in ref. 7). All three categories of mutations exert pleiotropic effects, making it difficult to correlate the mutation with a specific primary deficiency. The *shi* mutation, for example, results in a dysmyelination characterized by reduced levels of galactolipids, cholesterol, proteolipid protein, and the four forms of MBP (8, 13, 27). Despite this pleiotropy, many investigators have suggested that the *shi* mutation primarily affects MBP gene expression and only indirectly affects the other characteristics because the levels of the MBP are most

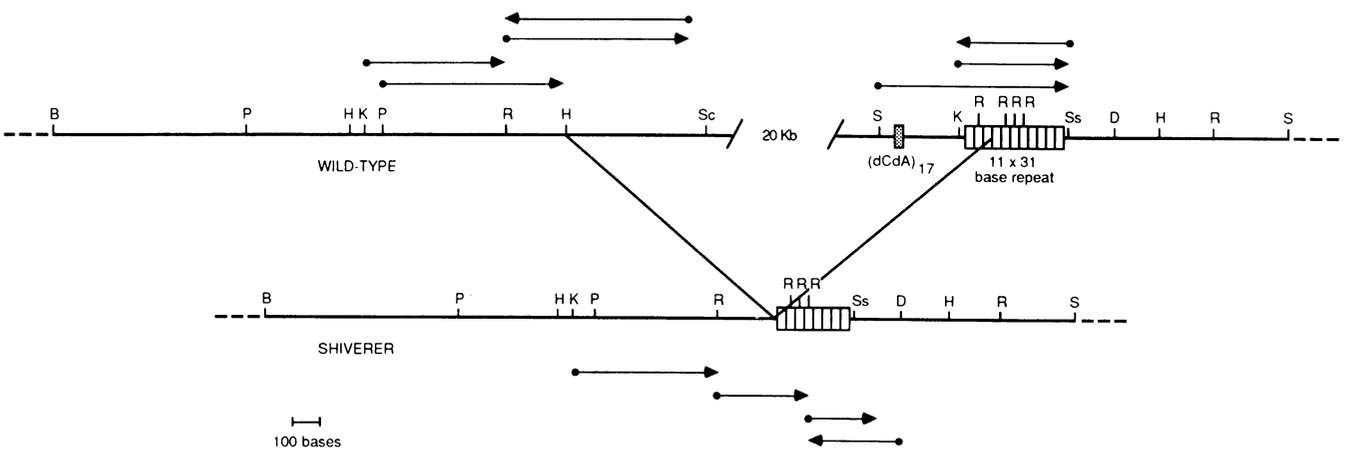


FIG. 3. Sequencing strategy and structural elements of the mouse DNA in the vicinity of the 3′ breakpoint. DNA was sequenced by the dideoxy-chain termination method. Arrows indicate direction and extent of individual sequencing experiments. Stippled box indicates position of $(dC\text{-}dA)_{17}$ sequence; open boxes indicate position of the 11 tandem repeats. Ss indicates *Ssp* I restriction site, other letters are as defined in legend of Fig. 2.

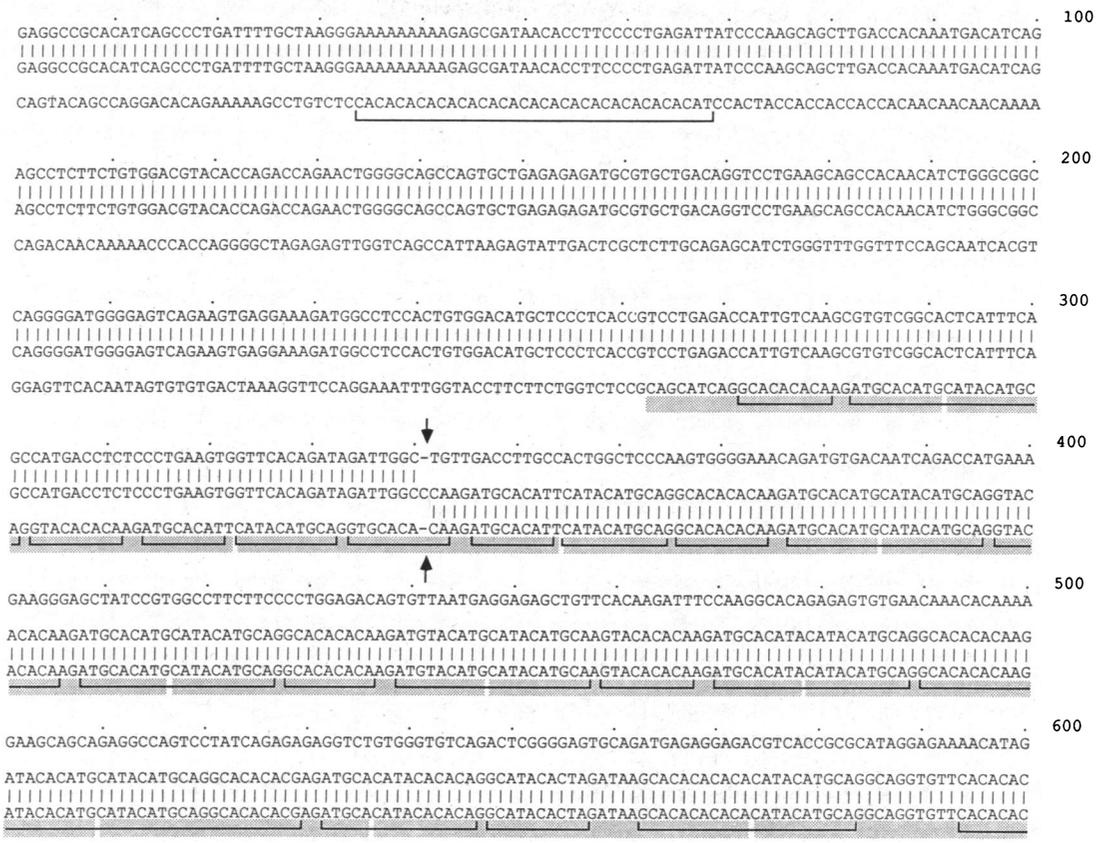


FIG. 4. Sequence of wild-type and shi DNA in the vicinity of the deletion site. Top line is the wild-type DNA sequence in the vicinity of the 5′ breakpoint. Middle line is the sequence of the shi DNA across the recombination site. Bottom line is the wild-type sequence at the 3′ breakpoint. Arrows indicate the recombination site. Alternating purine–pyrimidine sequences are underlined. Sequences highlighted with stippled bars are the 11 tandem repeats.

severely reduced (14, 28). The demonstration by Roach *et al.* (17, 18), Kimura *et al.* (25), and us (5) that the shi mice have an altered MBP gene strongly supports this interpretation. The association between the *shi* mutation and the MBP gene is also strengthened by the demonstration that both loci map to chromosome 18 (15, 16, 18).

Even though all available evidence indicates that the *shi* mutation is a deletion of the MBP gene, the details of the rearrangement had not been explored heretofore. In this communication, we precisely map the 5′ and 3′ termini of the deletion, establishing that it is a 20-kb deletion that removes exons 3, 4, 5, 6, and 7 of the MBP gene. By locating the 3′ end of the deletion to a point 2 kb downstream of the last MBP exon, we demonstrate that the *shi* mutation is confined to a single gene. Thus, the changes in the levels of other constituents of myelin are likely to be secondary effects of the lesion in the MBP gene.

We have studied the deleted sequences as well as the sequences flanking the deletion site for elements that might shed light on the events leading to the formation of the deletion. Three organizational elements of the DNA were identified and bear further comment: an extensive stretch of alternating purine and pyrimidine residues, a tandem array of 11 repeats of a 31-bp sequence also containing an alternating

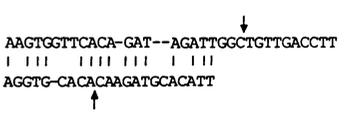


FIG. 5. Sequence homologies near the termini of the deleted segment of MBP DNA. Upper line, sequence near 5′ breakpoint; lower line, sequence near 3′ breakpoint; arrows indicate breakpoints.

pyrimidine–purine sequence, and a short sequence homology between opposite ends of the deletion.

The 3′ region of the deleted DNA sequence contains two stretches of alternating purine and pyrimidine residues. Within 250 bases of the distal breakpoint is the sequence $(dG\text{-}dT)_{17}$. This alternating pyrimidine–purine sequence has been identified in eukaryotic genomes and shown to be present $\approx 10^5$ times or, on the average, once every $3 \times 10^4$ kb in the mouse (29, 30). Judging from the few examples of this simple DNA that have been sequenced, they frequently contain arrays of 15–30 dG-dT repeats (30, 31). Both regions of alternating purine–pyrimidines near the distal breakpoint are of potential interest because DNA of this type has been shown to assume a Z or left-handed helical conformation under mild conditions such as those imposed by the torsional stress of superhelicity (32–34). Furthermore, the $(dG\text{-}dT)_n$ sequence or Z-DNA has been identified near the sites of both homologous and nonhomologous recombination (31, 35, 36).

The second feature of the recombination site is a 31-base repeated sequence. The sequence data of Fig. 2 and the schematic diagram of Fig. 4 show that the 3′ side of the deletion occurs in the 3rd of the 11 tandem repeats. This repeated array is notable for several reasons, including the abundance of alternating purines and pyrimidines discussed above. The number of tandem copies is low when compared to the highly reiterated sequences of satellite DNA (37, 38). It is not related to the known interspersed (SINE or LINE) repeat sequences (38–40). In the case of the immunoglobulin genes, nonreiterated tandemly repeated DNA has been implicated in recombinational events. The $S_\mu$, $S_{\gamma 1}$, $S_{\gamma 2B}$, $S_{\gamma 3}$, and $S_\alpha$ switch regions of the immunoglobulin genes contain unique tandemly arrayed elements ranging in size from 5 to 80 bp (41, 42). In addition to their normal functions in

promoting rearrangements that bring the variable–diversity–joining region (VDJ) fusion of the immunoglobulin heavy chain into close proximity of the constant region, these sequences have been implicated in the aberrant chromosomal translocations involving the c-*myc* gene in mouse plasmacytoma and human Burkitt lymphoma lines (26). However, the known involvement of tandem short repeats in recombination is limited to differentiating B cells, and there is no direct indication that the tandem sequences of the MBP gene were functionally involved in generating the deletion. Nonetheless, their presence at the deletion site invites speculation.

Short stretches of homology between sequences at or near the ends of the deleted segment of DNA have been reported by several investigators. The number of examples is sufficiently large that several models defining a functional role for these homologous sequences have been proposed to explain the generation of deletions. It was of interest, therefore, to search the sequences at the ends of the deleted segments of the MBP gene and those flanking the deletion site for short homologous sequences similar to those reported for other deletions. The sequences containing 5′ and 3′ ends of the deleted segment exhibit 75% homology (15/20) as shown in Fig. 5. Sequences of similar size and similar positions relative to deletion sites have been proposed to play a role in misaligning of chromosomes during meiosis, leading to unequal interchromosomal exchange (43), or in a "slippage" of the replicative enzyme complex, which generates deletions by an intrachromosomal mechanism (44). However, the partially homologous sequences we find in the mouse MBP gene seem to best satisfy the model proposed by Anderson *et al.* (45) for certain recombinational events. These authors examined a number of recombinants between pBR322 and herpesvirus thymidine kinase gene DNAs and found that the recombinant DNAs had homologous sequences near the recombination site. If the parental DNA sequences were aligned by the partial homology, then one donor molecule was always cut in the region of homology while the other was cut at a small multiple of 13.5 nucleotides away. The size and the extent of the homologies we find in the MBP gene (Fig. 5) is within the range found by Anderson *et al.* (45). Furthermore, the alignment of the parental sequences places the breakage and reunion sites of the MBP sequences 14 nucleotides apart (Fig. 5). Thus, the sequences surrounding the MBP deletion best conform to the model proposed by Anderson *et al.* that involves a staggered cutting and joining of the DNA.

Since the portion of the MBP gene retained in the *shi* genome contains the promoter and first two exons, one might expect the remnant gene to be transcriptionally active. It is somewhat surprising that transcripts from the gene are present at only 1/16 the levels found in normal mice (18). It is not possible to decide on the basis of these studies whether the reduction in the level of MBP-specific RNA is due to a decrease in the rate of transcription or an increase in degradation of aberrant transcripts. Although there is presently no indication that the *shi* mutation altered the promoter on any upstream sequences, it may have removed important regulatory sequences at the 3′ region of the MBP gene, the absence of which restricts transcription. On the other hand, the shi MBP transcripts, which are abnormally spliced and polyadenylylated (18), may exhibit a decreased stability and a low steady-state level. It is clear that further transcriptional studies on the *shi* gene are needed to understand the control mechanisms involved in the complex splicing pattern of MBP transcripts.

The thorough characterization of the *shi* mutation and the cDNA clones encoding the individual forms of MBP (5) should open the way for interesting transgenic studies directed at understanding the role of the individual forms of the MBP and the origin of the pleiotropy exhibited by the *shi* deletion.


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