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Microrecombinations Generate Sequence Diversity in the Murine Major Histocompatibility Complex: Analysis of the K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11} Mutants

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The mechanism that generates spontaneous mutants of the K^b histocompatibility gene was analyzed. Nucleotide sequence analysis of four mutant genes (K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11}) revealed that each mutant *K* gene contains clustered, multiple nucleotide substitutions. Hybridization analyses of parental B6 genomic DNA and cloned class I genes with mutant-specific oligonucleotide probes, followed by sequence analyses, have identified major histocompatibility complex class I genes in the *K*, *D*, and *Tla* regions (*K1*, D^b, and *T5*, respectively) that contain the exact sequences as substituted into mutant K^b genes. These data provide evidence for the hypothesis that the mutant K^b genes are generated by a microrecombination (gene conversion) mechanism that results in the transfer of small DNA segments from class I genes of all four regions of the major histocompatibility complex (*K*, *D*, *Qa*, and *Tla*) to K^b. Many of the nucleotides substituted into the mutant K^b genes were identical to those found in other naturally occurring *K* alleles such as K^d. Thus, we propose that the accumulation of microrecombination products within the *K* genes of a mouse population is responsible for the high sequence diversity among *H-2* alleles.

Class I genes of the murine major histocompatibility complex (MHC) are tandemly arrayed in four regions (*K*, *D*, *Qa*, and *Tla*) along chromosome 17. Members of this multigene family share sequence homology and similar intron-exon organization, and their products share similar biochemical characteristics (8, 12, 26, 35). The *K* and *D* regions contain the genes encoding the classical H-2 transplantation antigens (K, D, L) which are found on all somatic cells and which function as restricting elements in the presentation of foreign, cell-associated antigens to T cells. Alleles of each *H-2* locus exhibit approximately 10 to 15% nucleotide sequence diversity (diversity refers to differences in primary sequence) and are very polymorphic at the population level (12, 14, 15, 35). The diversity and polymorphism of *H-2* gene products are thought to be important in their function as antigen-presenting molecules. Products of the *Qa* and *Tla* region genes have a more restricted tissue distribution and an, as yet, unknown function. Alleles of *Qa* and *Tla* loci display limited sequence diversity (8, 23, 30). The particular assortment of alleles of the various class I genes defines the haplotype of the mouse strain (e.g., C57BL/6, $H\text{-}2^b$; BALB/c, $H\text{-}2^d$).

H-2 molecules are integral membrane glycoproteins whose three extracellular domains (α1, α2, and α3) are noncovalently associated with β_2 microglobulin. This multimeric complex interacts with the T-cell receptor. Sequence analysis of *H-2* genes and their products indicates that sequence diversity among *H-2* alleles is not randomly distributed throughout the molecule but is rather concentrated in variable regions which are primarily in the α1 and α2 domains (exons 2 and 3) (2, 12). Interestingly, there is greater homology between class I variable-region sequences of nonallelic genes than between those of alleles of the same locus. The sharing of variable regions among nonallelic genes results in *H-2* alleles that resemble combinatorial assortments of various class I gene segments. These findings suggested that the sequence diversity of *H-2* genes is the result of recombination (e.g., crossing over or gene conversion) among the various class I genes (6, 11, 18, 28, 39). However, the level of sequence variability among *H-2* alleles is so high that it is very difficult to determine evolutionary relationships between *H-2* genes and to provide evidence that *H-2* diversity is generated by a recombination mechanism.

Spontaneous, in vivo mutants of the $H\text{-}2K^b$ gene have been detected in the C57BL/6 mouse strain by skin graft analysis at a frequency of 2×10^{-4} per gamete (13). (Mutant mice are denoted bm1, bm2, etc.; mutant K^b genes are denoted K^{bm1}, K^{bm2}, etc.) In contrast to the analysis of naturally occurring, diverse *H-2* alleles, the analysis of mutant K^b genes provides a model system for the study of the individual steps that lead to the diversification of *H-2* genes. Partial amino acid sequence analysis of several K^b mutant molecules indicate that they differ from the parental K^b molecule by clustered, multiple amino acid substitutions (25). It has also been observed that the amino acids substituted into the mutant K^b glycoproteins are also present, at homologous positions, in other class I molecules of various H-2 haplotypes (6, 29). These findings suggest that a recombination mechanism between class I genes, such as gene conversion, may be responsible for the generation of the K^b mutants (6, 29). Nucleic acid analysis of the K^{bm1}, K^{bm6}, and K^{bm9} mutants indicates that they each contain a single cluster of altered nucleotides (K^{bm6} and K^{bm9} arose independently but contain the same mutations) (9, 10, 34, 40). Two *Qa* region genes of the $H\text{-}2^b$ haplotype, *Q10* and *Q4*, contain sequences identical to those substituted into K^{bm1} and K^{bm6}/K^{bm9}, respectively (9, 10, 21). These data support a recombination hypothesis which suggests that these two donor

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genes recombined with the K^b gene to generate their respective mutant K^b genes.

Since only two different K^b mutants have been analyzed, it was important to study more mutant genes to expand our understanding of the mechanism of mutant generation. This analysis would determine whether recombination is a major factor in the generation of K^b mutants and, by implication, in the diversification of *H-2* genes. Also, the identification of all possible donor genes from all regions of the MHC would expand our understanding of the concerted evolution of the class I multigene family.

In this paper, we present the nucleotide sequences of the K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11} mutants. Each mutant gene contains multiple nucleotide substitutions localized to a single cluster of 11, 35, 37, or 38 nucleotides. The short segments containing the mutant sequences are also present in other class I donor genes from the *K*, *D*, and *Tla* regions. These data provide overwhelming evidence that mutant K^b genes are generated by a microrecombination process that results in the transfer of short segments of DNA from donor genes to K^b. The data also indicate that genes from all regions of the MHC can engage in microrecombination events with the K^b gene. The finding that the positions of the altered nucleotides in the mutant K^b genes coincide with variable regions of *H-2* alleles suggests that microrecombinations among class I genes can provide the raw materials for the diversification of *H-2* genes. The accumulation of many microrecombination products would result in the mosaic structure that is characteristic of these genes. Thus, these data provide evidence that the concerted evolution of multigene families can include recombination events which diversify, as well as homogenize, the member genes.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), B6-H-2^{bm3}, B6.C-H-2^{bm4}, B6.C-H-2^{bm10}, and B6.C-H-2^{bm11} mice were obtained from the breeding and screening laboratories of R. Melvold (Northwestern University Medical School, Chicago, Ill.), C. David (Mayo Clinic, Rochester, Minn.), and The Jackson Laboratory. C57B/6Y (B6/Y) mice were obtained from the Academy of Medical Sciences of the USSR, Moscow.

Preparation of DNA, RNA, and synthetic oligonucleotides. The preparation of genomic DNA, polyadenylated liver RNA, and synthetic oligonucleotides and procedures for hybridization analysis of DNA and RNA with ^{32}P-labeled oligonucleotides have been described elsewhere (9, 10).

RNA and uncloned-cDNA sequence analysis. The RNA and uncloned-cDNA dideoxynucleotide-sequencing techniques utilizing K^b-specific, ^{32}P-labeled oligonucleotide primers have been described elsewhere (9). A total of 849 nucleotides of each of the transcripts for the K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11} genes were sequenced. The 849 nucleotides correspond to the codons for the amino-terminal 283 amino acids of the mutant K^b molecules and include the three external domains, α1, α2, and α3 (exons 2, 3, and 4), and nine amino acids encoded for by the transmembrane exon.

Molecular genetic mapping of donor genes. Cosmid clones containing class I genes of the $H\text{-}2^b$ haplotype were obtained from Richard A. Flavell (38). DNA from 15 clones were digested with *Bam*HI, size fractionated on agarose gels, and transferred to GeneScreen. The filter was prehybridized for 2 h (5× SSPE, 0.3% sodium dodecyl sulfate, 200 μg of denatured salmon sperm DNA per ml), hybridized for 2 to 16 h in fresh prehybridization solution containing 100 ng of ^{32}P-labeled, mutant-specific oligonucleotide probe. (See Fig. 3 for hybridization temperatures.) Following hybridization, the filter was twice washed in 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM disodium EDTA [pH 7.4]) at room temperature for 30 min each and once at the hybridization temperature for 10 min. After being washed, filters were exposed to Kodak XAR film. Bound oligonucleotide probes were removed from the filter by washing in 5× SSPE at 80°C for 5 min.

DNA sequencing of donor genes. Following the identification of a potential donor gene in a cosmid clone, the exon containing the donor sequence was subcloned into M13mp10 and M13mp11 vectors and sequenced with the Pharmacia (Piscataway, N.J.) dideoxynucleotide-sequencing kit. Universal M13-sequencing primers and mutant-specific primers were used, and [α^{35}S]dATP (Amersham Corp., Arlington Heights, Ill.) was incorporated as label.

RESULTS

K^{bm4}. Sequence analysis of the K^{bm4} transcript indicated that it differs from the parental K^b transcript by six nucleotides in the codons for amino acids 162, 163, 165, 173, and 174 (Fig. 1a). K^b and K^{bm4} were otherwise identical over the 849 nucleotides sequenced, which included the exons for the three external domains (domains α1, α2, and α3; exons 2, 3, and 4) and part of the transmembrane exon. All of the nucleotide substitutions occurred in base positions 1 and 2 of their respective codons and resulted in amino acid replacements.

To confirm the sequence data generated by RNA and uncloned-cDNA sequencing, mutant nucleotides were analyzed to determine whether they generated or destroyed restriction enzyme sites. The mutant sequence at positions 173 and 174 (GAGCTC) generated a *Sac*I restriction enzyme site in K^{bm4} that is absent in K^b (Fig. 1a). This sequence was confirmed by restriction enzyme digestion and hybridization analysis. Hybridization analysis of *Sac*I-digested B6 and bm4 DNA with an *H-2K*-specific oligonucleotide to the transmembrane region demonstrated that the K^{bm4} gene contained the predicted additional *Sac*I site (Fig. 2a). This finding is confirmed by the fact that the oligonucleotide probe hybridized to the smaller, 2.6-kilobase K^{bm4} gene fragment in bm4 DNA, compared with the larger, 6.3-kilobase K^b fragment in parental B6 DNA (Fig. 2a). We know that the new 2.6-kilobase K^{bm4} *Sac*I fragment has the predicted size because both the K^b and K^{bm4} genes contained a *Sac*I site in intron 6, which was approximately 2.6 kilobases downstream from positions 173 and 174.

The nucleotide alterations in positions 162 and 163 of K^{bm4} generated an *Ava*II restriction enzyme site (GGACC) not present in the K^b gene (Fig. 1a). The parental K^b gene contains *Ava*II sites at codon 104 and 363 nucleotides into the large intron between exons 3 and 4 (domains 2 and 3). This 600-nucleotide restriction fragment was detectable in genomic DNA by an oligonucleotide probe to codons 151 through 157 (Fig. 2b). The presence of a new *Ava*II restriction site in K^{bm4} was confirmed by the reduction of the parental 600-nucleotide *Ava*II fragment to approximately 172 nucleotides, the linear distance between codons 104 and 162 (Fig. 2b). The G → C alteration in codon 165 of the K^{bm4} gene generated a *Bst*NI restriction enzyme site. However, this sequence is particularly difficult to demonstrate by restriction enzyme digestion and hybridization analysis because the alteration in codon 173 generated another *Bst*NI site and would require the resolution and detection of a 21-nucleotide genomic DNA fragment.

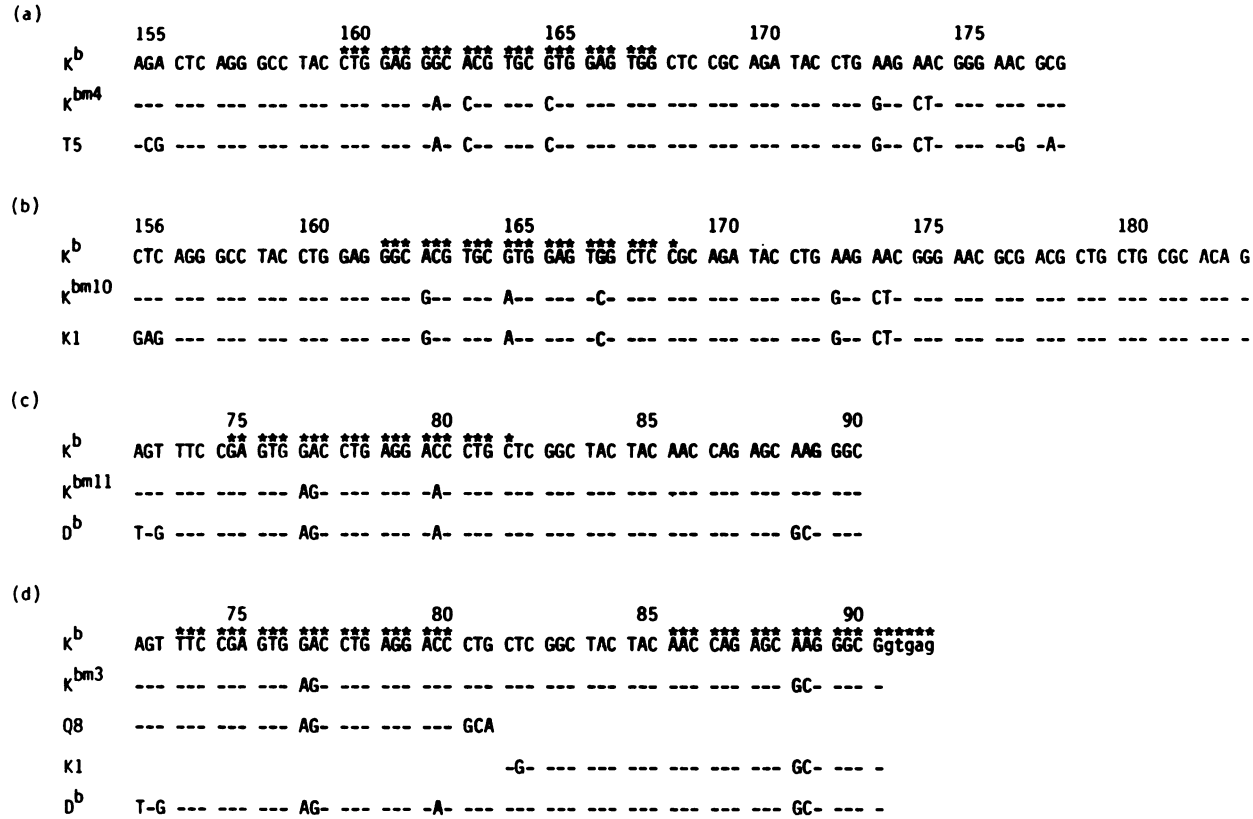

FIG. 1. Nucleotide sequence comparisons of K^b, mutant K^b, and donor genes. The nucleotide sequence of K^b is given in comparison with those of mutants K^{bm4} (a), K^{bm10} (b), K^{bm11} (c), and K^{bm3} (d) and the respective donor genes. The numbers on top of the K^b sequence indicate the amino acid position coded for by the codon. Dashes in mutant K^b and donor gene sequences indicate identity to K^b. Asterisks indicate the positions where mutant-specific oligonucleotides would hybridize to mutant K^b and donor gene DNA and RNA. Lower-case letters indicate intron sequences. The K^b and *K1* (gene A) sequences are according to Weiss et al. (39); the D^b sequence is taken from Hemmi et al. (S. Hemmi, J. Geliebter, R. W. Melvold, and S. G. Nathenson, J. Exp. Med., in press). The *Q8* sequence is from Devlin et al. (5). The K^b sequence in (d) is from both the B6/J and B6/Y strains.

If the six nucleotide substitutions in K^{bm4} are the result of a recombination event, an identical sequence should be present elsewhere in the B6 genome. An oligonucleotide probe specific for the K^{bm4} mutations should hybridize to the K^{bm4} gene and the donor sequence. To determine whether the six nucleotide substitutions in K^{bm4} are the result of recombination between the K^b gene and a donor sequence, a 24-base oligonucleotide complementary to the mutant sequence for codons 160 through 167 (encompassing the mutations at 162, 163, and 165) was synthesized (Fig. 1a). This oligonucleotide (the bm4-mer) was radiolabeled and hybridized to *Bam*HI-digested B6, bm4, and BALB/c mouse genomic DNA (Fig. 3a). The hybridization of the bm4-mer to the 4.8-kilobase BamHI fragment in bm4 DNA that contains exons 1, 2, and 3 of the K^{bm4} gene confirmed the mutant sequence. The specificity of the bm4-mer was demonstrated by the fact that it did not hybridize to the 4.8-kilobase *Bam*HI fragment of K^b in B6 DNA. The bm4-mer also hybridized to a 9-kilobase donor sequence in both B6 and bm4 DNA. A potential donor sequence was also present in BALB/c mouse genomic DNA (the original bm4 mouse was a B6 × BALB/c F_1; see Discussion). To determine whether the donor sequence is present in a class I gene, radiolabeled bm4-mer was hybridized to a filter containing *Bam*HI-digested DNA from 15 class I cosmid clones, corresponding to all of the class I genes of the $H\text{-}2^b$ haplotype. The bm4-mer hybridized to a single class I gene, *T5*, located in the *Tla* region of the murine MHC (data not shown).

To determine whether the *T5* gene contains the same sequence as that substituted into K^{bm4}, a restriction enzyme map of the *T5* gene was constructed, and an approximately 525-nucleotide *Bam*HI-*Pst*I fragment containing exon 3 (corresponding to the α2 domain) was subcloned into M13mp11 and M13mp10 and sequenced. The *T5* gene sequence was identical to that of K^{bm4} at the altered positions 162, 163, 165, 173, and 174 (Fig. 1a). This result provides very strong evidence that *T5* is the donor gene for K^{bm4} mutations. The 37 nucleotides between the base substitutions at positions 162 and 174 represent the minimum size of the recombination event between K^b and *T5* that is necessary to generate the K^{bm4} gene. The sequences of K^{bm4} and *T5* are identical from the codon for amino acid 156 until position 3 of codon 176. Thus, 62 nucleotides represent the maximum recombination between K^b and *T5*. Otherwise, K^{bm4} would have contained additional nucleotide substitutions at positions 155 and 176. Comparative sequence analysis of exons 3 from K^b and *T5* indicate that they are 86.6% homologous (Fig. 4).

K^{bm10}. Dideoxynucleotide sequencing of K^{bm10} mRNA revealed that it contains six nucleotide alterations in the codons for amino acids 163, 165, 167, 173, and 174 (Fig. 1b). All six nucleotide substitutions were in codon positions 1 and 2 and resulted in amino acid replacements. At positions

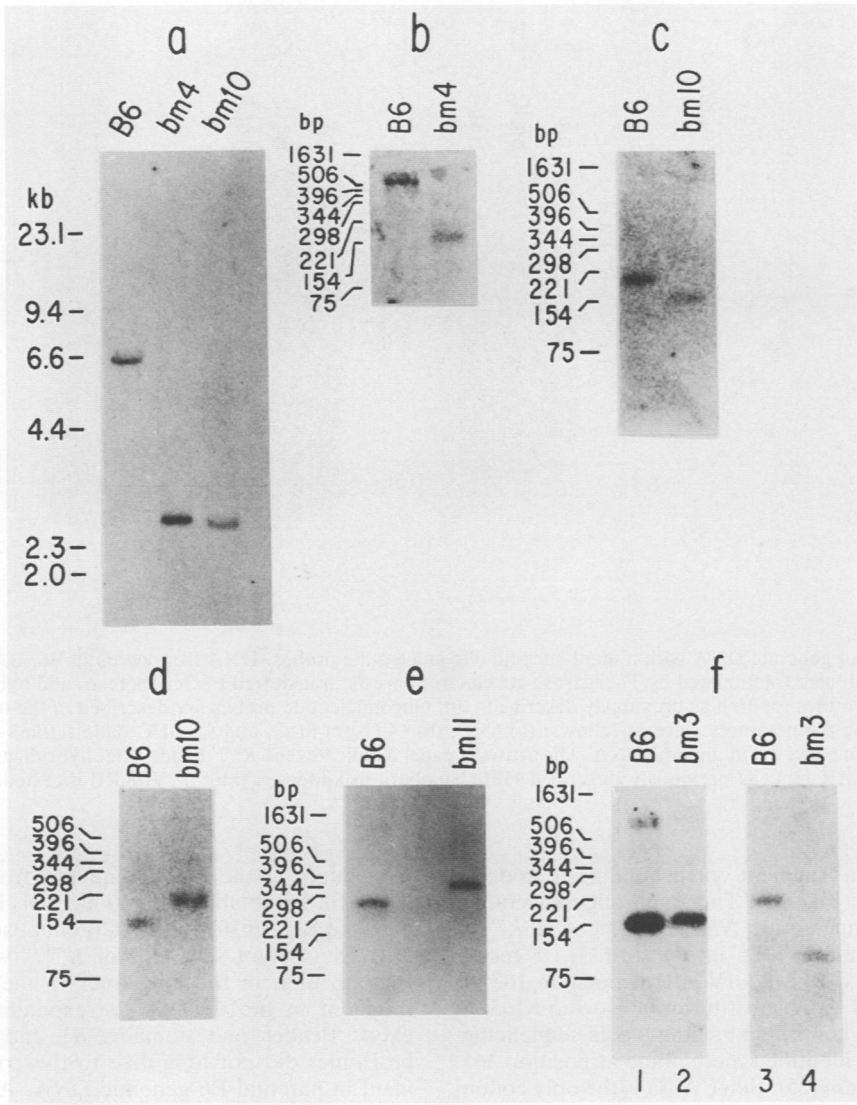

FIG. 2. Confirmation of mutant sequence by restriction enzyme digestion and hybridization analysis. A 50-μg sample of genomic DNA from parental B6 and various mutant mice was digested with various restriction enzymes, size fractionated by agarose gel electrophoresis, transferred to GeneScreen, and hybridized with ^{32}P-labeled, oligonucleotide probes for 16 h as previously described (10). The enzymes used for digestion, percent agarose gel used for fractionation, and hybridization and washing temperatures were as follows: (a) *Sac*I, 0.8%, 59°C; (b) *Ava*II, 2.25%, 55°C; (c) *Tha*I, 3%, 55°C; (d) *Nla*IV, 3%, 55°C; (e) *Ava*II, 3%, 60°C; and (f) *Rsa*I-*Ava*II, 3%, 60°C (lanes 1 and 2) and *Tha*I, 3%, 60°C (lanes 3 and 4). After hybridization, filters were washed and exposed to Kodak XAR film, as previously described (10). Numbers in kilobases (kb) are either *Hin*dIII-digested λ DNA, or *Hin*fI-digested pBR322 DNA molecular size markers. Restriction enzyme sites in K^b are from Weiss et al. (39). bp, Base pair.

173 and 174, K^{bm10} contained substitutions identical to those of K^{bm4}. The nucleotide changes at positions 163 and 165 were different from the alterations in the same codons of K^{bm4}.

As described above, the mutant sequence at positions 173 and 174 (GAGCTC) created a new *Sac*I restriction enzyme site in K^{bm10} (and K^{bm4}) that is not present in the K^b gene (Fig. 1b). Hybridization analysis of *Sac*I-digested B6 and bm10 genomic DNA with an *H-2K*-specific probe confirmed the presence of an additional *Sac*I site in the K^{bm10} gene (Fig. 2a). The smaller, 2.6-kilobase K^{bm10} fragment generated by *Sac*I had the predicted size of a K^b gene with an additional *Sac*I recognition site at positions 173 and 174.

The A → C substitution in codon 163 of K^{bm10} generated the new *Tha*I restriction enzyme site (CGCG) (Fig. 1b). The parental K^b gene contains *Tha*I sites at codons 111 and 176, which yielded a 197-nucleotide fragment detectable in genomic B6 DNA with an oligonucleotide probe to codons 151 through 157 (Fig. 2c). The presence of a new *Tha*I site in codon 163 of K^{bm10} was demonstrated by the hybridization of the probe to a smaller, 155-nucleotide fragment in bm10 DNA (Fig. 2c). A 155-nucleotide fragment corresponded to the size of a restriction fragment from codons 111 through 163. The G → C alteration at position 167 of K^{bm10} destroyed an *Nla*IV restriction enzyme site (GGNNCC) present in K^b (Fig. 1b). The K^b gene contains *Nla*IV sites at the codons for amino acid positions 112 and 167 and six nucleotides into the intron between exons 3 and 4 (domains 2 and 3). Hybridization analysis with an oligonucleotide complementary to positions 151 through 157 detected the 165-nucleotide *Nla*IV

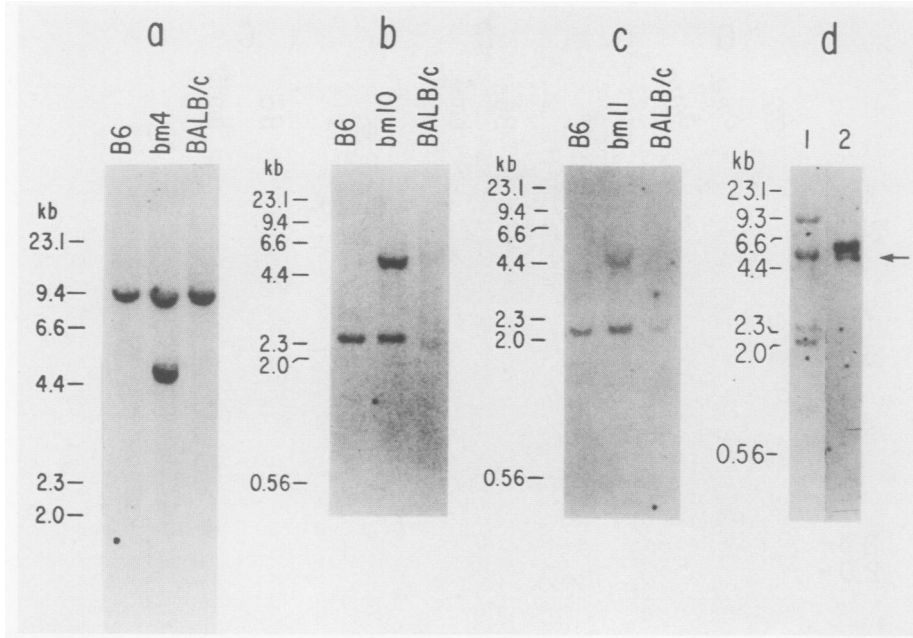

FIG. 3. Hybridization of genomic DNA with mutant-specific oligonucleotide probes. DNA from parental B6, BALB/c, and mutant mice were digested with *Bam*HI, size fractionated by 1% agarose gel electrophoresis, transferred to GeneScreen, and hybridized with ^{32}P-labeled mutant-specific oligonucleotides for 16 h as previously described (10). Oligonucleotide probes are described in the text and shown in Fig. 1. Hybridization and washing temperatures were as follows: (a) 65°C, (b) 64°C, (c) 61°C, and (d) 61°C (lane 1 [bm3-mer1]) and 65°C (lane 2 [bm3-mer-2]). Both lanes in panel d contain bm3 DNA. The arrow in panel d indicates the K^{bm3} band. After hybridization, filters were washed and exposed to Kodak XAR film, as previously described (10). Numbers in kilobases (kb) are *Hin*dIII-digested λ DNA molecular size markers.

fragment in B6 DNA, a fragment corresponding to codons 112 through 167 of K^b (Fig. 2d). The same oligonucleotide hybridized to the 220-nucleotide *Nla*IV fragment of K^{bm10} (position 112 to six nucleotides into intron 3), a result demonstrating the loss of the *Nla*IV site at position 167 of K^{bm10} (Fig. 2d). The G → A substitution at position 165 in K^{bm10} has already been confirmed by amino acid sequencing techniques which have identified methionine at position 165 of the mutant glycoprotein (25). Since ATG is the only codon coding for methionine, the G → A substitution can be inferred by the genetic code.

To ascertain whether recombination was responsible for generating the six nucleotide substitutions in K^{bm10}, a mutant-specific oligonucleotide was synthesized to identify a donor sequence. The mutant-specific oligonucleotide (the bm10-mer) was composed of 22 nucleotides and was complementary to the K^{bm10} sequence from codons 162 to 169 (Fig. 1b). Radiolabeled bm10-mer was hybridized to *Bam*HI-digested B6, bm10, and BALB/c mouse genomic DNA (Fig. 3b). The mutant sequence of K^{bm10} was confirmed by the hybridization of the bm10-mer to the 4.8-kilobase *Bam*HI fragment in bm10 DNA corresponding to the first three exons (leader and domains α1 and α2) of K^{bm10}. The bm10-mer did not hybridize to the corresponding K^b fragment in parental B6 genomic DNA. A 2.3-kilobase *Bam*HI fragment in both B6 and bm10 DNA hybridized to the bm10-mer, representing the donor sequence. A potential donor sequence was not present in BALB/c mouse DNA (the original bm10 mouse was a B6 × BALB/c F_1; see Discussion). When hybridized to a filter containing class I cosmid clones, the bm10-mer hybridized to a single gene, *K1* (data not shown). The *K1* gene is approximately 15 kilobases

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     91                        100                       110
T5   GCTCTCACACATTTCAGAGGATGTACGGCTGTGACCTGGGGTCGGACGGGCGCCTCCTCCGCGGGTACT
Kb   ----------TA-----GT---C-CT--------AG-------C--------A----------------C

                  120                       130
T5   GGCAGTTCGCTTACGACGGCAGCGATTACATCGCCCTGAACCAAGACCTGAAAACGTGGACGGCGGCGG
Kb   A-----A---C---------T--------------------G--------------------------

          140                       150                       1
T5   ACTTGGCGGCGCAGATCACTCGACGCAGGTGGGAGCAGGGTGGTGTTGCAGAGACGCTCAGGGCCTACC
Kb   --A---------T------CAA--A--A-----------C-----AA-------GA-------------

     60                        170                       180
T5   TGGAGGACCCGTGCCTGGAGTGGCTCCGCAGATACCTGGAGCTCGGGAAGGAGACGCTGCTGCGCACAG
Kb   ------G-A-----G----------------------A--AA------C-C----------------
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FIG. 4. Sequence comparison of exons 3 (α2 domains) of *T5* and K^b. Numbers indicate the amino acid positions coded for by the corresponding codons. Dashes in K^b indicate identity to *T5*. The K^b sequence is from Weiss et al. (39).

centromeric to the K^b gene on chromosome 17 (38). Exon 3 (domain α2) of the *K1* gene is present on a 2.3-kilobase *Bam*HI fragment identifying *K1* as the gene identified in genomic DNA (data not shown). A restriction enzyme profile of the *K1* gene was constructed, and a 600-nucleotide *Xma*I-*Pst*I fragment that contained exon 3 of *K1* was subcloned into M13mp10 and M13mp11 and sequenced. The sequence of the *K1* gene at codons 163, 165, 167, 173, and 174 is identical to the sequence substituted into the mutant K^{bm10} gene (Fig. 1b), an observation confirming that *K1* can be the donor gene for the K^{bm10} mutations. The 35 nucleotides between codons 163 and 174 represent the minimum recombination between K^b and *K1* to generate the K^{bm10} gene. The K^{bm10} and *K1* genes are identical in at least 79 nucleotides, beginning with codon 157 and extending into intron 3. Because the K^{bm10} sequence was determined by mRNA sequencing, the intron sequence was not obtainable, and the maximum recombination between K^b and *K1* is not known.

K^{bm11}. The nucleotide sequence of K^{bm11} differs from that of K^b by three nucleotides at codons 77 and 80 (Fig. 1c). The double nucleotide substitution at position 77 and the single alteration at position 80, were changes in base position 1 or 2 and resulted in amino acid replacements.

The nucleotide substitutions in codons 77 and 80 destroyed two *Ava*II restriction enzyme sites (GGACC) (Fig. 1c). This fact was confirmed by restriction enzyme digestion and hybridization analysis. In addition to positions 77 and 80, the parental K^b gene contains *Ava*II sites at 30 nucleotides 5′ and 60 nucleotides 3′ to exon 2. An oligonucleotide probe to positions 53 to 59 hybridized to a 256-nucleotide *Ava*II restriction fragment of K^b (Fig. 2e). The loss of two *Ava*II sites in K^{bm11} was demonstrated by the hybridization of the probe to the approximately 364-nucleotide *Ava*II restriction fragment of K^{bm11} (Fig. 2e). Thus, restriction enzyme and hybridization analysis confirmed the loss of the parental sequence at positions 77 and 80 of K^{bm11}.

A K^{bm11}-specific oligonucleotide (bm11-mer) complementary to the mutant sequences at positions 77 and 80 was synthesized (Fig. 1c). The bm11-mer was radiolabeled and hybridized to digested genomic DNA to detect the presence of a donor sequence (Fig. 3c). The specificity of the bm11-mer was demonstrated by the fact that it hybridized to the 4.8-kilobase *Bam*HI gene fragment of K^{bm11} DNA (thus confirming the mutant sequence) but not to the corresponding K^b fragment in B6 DNA. A 2.1-kilobase potential donor sequence was present in both the parental B6 and mutant bm11 genomic DNA, as well as in BALB/c mouse DNA (the strain of the father of the original bm11 mutant; see Discussion). Hybridization analysis of radiolabeled bm11-mer on cloned class I genes revealed that the D^b gene is the only class I gene that hybridizes to the bm11-mer (data not shown). The D^b gene fragment that hybridized to the bm11-mer was 2.1 kilobases long, a result confirming the genomic DNA data. An inspection of the nucleotide sequence of D^b (Hemmi et al., in press) indicates that the D^b and K^{bm11} sequences are identical at positions 77 and 80 (Fig. 1c). Thus, the D^b gene is the donor gene for the K^{bm11} mutations. The 11 nucleotides that encompass the three nucleotide substitutions define the minimum recombination length between K^b and D^b to generate the K^{bm11} mutant. The 45 nucleotides that are identical in D^b and K^{bm11} delineate the maximum recombination between K^b and D^b to produce the K^{bm11} mutations. A longer recombination event would have resulted in additional nucleotide alterations at positions 73 and 89.

K^{bm3}. Nucleotide sequence analysis of K^{bm3} transcripts indicated that K^{bm3} differs from the parental K^b sequence of B6/J and B6/Y mice by four nucleotides, two each in the codons for amino acids 77 and 89 (Fig. 1d). The nucleotide substitutions in codon 77 were identical to those substituted into the K^{bm11} gene. All four nucleotide alterations were changes in codon position 1 or 2 and resulted in amino acid replacements.

All the nucleotide replacements in K^{bm3} were confirmed by restriction enzyme and hybridization analysis. As with the K^{bm11} gene, the alteration at position 77 of K^{bm3} disrupted an *Ava*II site (GGACC) (Fig. 1d). However, because both K^b and K^{bm3} contain *Ava*II restriction sites at codon 80, digestion with *Ava*II will yield a mutant K^{bm3} fragment that is only eight nucleotides longer than the parental K^b fragment. To enhance the detection of this eight-nucleotide difference, B6 and bm3 genomic DNA were restricted with both *Rsa*I and *Ava*II. Since *Rsa*I digests both the parental and mutant K^b genes at codon 22, the double digestion should yield a parental fragment of 164 nucleotides (codons 22 through 77) and a mutant fragment of 172 nucleotides (codons 22 through 80). Hybridization analysis with a radiolabeled oligonucleotide probe to codons 53 to 59 confirmed the loss of an *Ava*II site in K^{bm3} by showing that hybridization was to a slightly larger K^{bm3} fragment (Fig. 2f, lanes 1 and 2).

The AA → GC substitution at position 89 in K^{bm3} created a new *Tha*I restriction site (Fig. 1d). The K^b gene contains *Tha*I restriction sites at codon 49 and 133 nucleotides into intron 2 (the intron between exons 2 and 3). Hybridization analysis with an oligonucleotide probe to positions 53 to 59 indicated that the 258-nucleotide parental K^b fragment was reduced to 118 nucleotides in K^{bm3} (Fig. 2f, lanes 3 and 4). This reduction corresponded to a *Tha*I restriction fragment extending from codons 49 to 89. Thus, both altered codons in K^{bm3} were confirmed by restriction enzyme digestion and hybridization analysis.

Two K^{bm3}-specific oligonucleotide probes were constructed, one probe spanning the K^{bm3} alteration at position 77 (bm3-mer-1) and the other spanning the alteration at position 89 (bm3-mer-2) (Fig. 1d). Hybridization analysis of bm3 genomic DNA with these oligonucleotides indicated that each probe hybridized to the K^{bm3} gene (see arrow in Fig. 3d), as well as to several potential donor sequences (Fig. 3d). However, the two K^{bm3} probes did not bind to the same potential donor fragment, a result indicating that whereas the B6 genome contains several sequences with the AGC codon at position 77 (e.g., *Q8* and D^b) and several sequences with the GCG codon at position 89 (e.g., *K1* and D^b), no single donor gene was identical to K^{bm3} at positions 77 through 89 (Fig. 1d). The absence of a single donor gene identical to K^{bm3} at positions 77 through 89 was also indicated when these two oligonucleotides (and other, smaller K^{bm3}-specific probes) were hybridized to $H\text{-}2^b$ class I cosmid clones (data not shown). Interestingly, the D^b gene was identical to K^{bm3} at position 77 and 89 but contained a mismatch at position 80 (Fig. 1d).

Transcription analysis of donor genes. The *Q4* and *Q10* donor genes are transcribed in various tissues (4, 10). This fact is consistent with the possibility of microrecombination via an mRNA or cDNA intermediate (21, 39), as well as the observation of a linkage between transcription and recombination (37). To determine the transcriptional activity of the donor genes identified in this study, 10 μg of liver poly(A)$^+$ RNA from parental B6 and mutant bm4, bm10, and bm11 mice were size fractionated on agarose-formaldehyde gels,

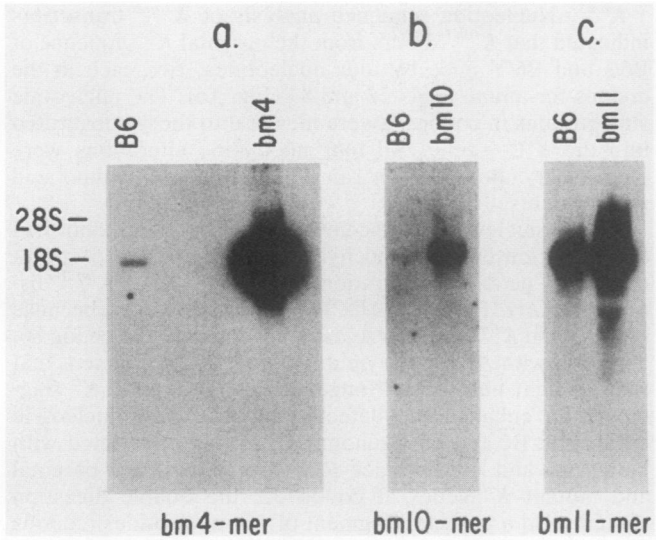

FIG. 5. Hybridization analysis of B6 and mutant mouse RNA. Whole-cell liver RNA was prepared from parental B6 mice and various mutant mice. A 10-μg sample of poly(A)$^+$ RNA was fractionated by formaldehyde-agarose gel electrophoresis, transferred to GeneScreen, and hybridized with ^{32}P-labeled mutant-specific oligonucleotides for 16 h as previously described (10). Oligonucleotide probes are described in the text and shown in Fig. 1a to c. Hybridization and washing temperatures were as follows: (a) 65°C, (b) 64°C, and (c) 61°C. After hybridization, filters were washed and exposed to Kodak XAR film, as previously described (10). The 28S and 18S rRNAs are 5,000- and 1,950-base molecular size markers present in the poly(A)$^-$ fraction.

transferred to GeneScreen, and hybridized with mutant-specific oligonucleotide probes. (The D^b donor gene is expressed in all tissues, as are all classical transplantation molecules, and the analysis of its transcriptional activity is included for completeness.) Hybridization analysis indicated that the *K1*, *T5*, and D^b donor genes were all transcribed in the parental B6 liver (Fig. 5). (The faint darkenings below 17S in B6 RNA (Fig. 5b) were not confined to the lane. The approximately 12S band in bm11 RNA (Fig. 5c) was artifactual.)

Sequence comparison of spontaneous K^b mutants and naturally occurring *K* alleles. The identification of donor sequences for all the mutations of the K^b mutants sequenced to date establishes the microrecombination process as the mechanism generating spontaneous *H-2* mutants. If microrecombinations are a continuously occurring, natural phenomenon, natural *K* alleles would be expected to contain some of the same donor gene sequences as those found in the mutant K^b genes. Figure 6a indicates that the K^d allele of the DBA/2J mouse contains many nucleotides that are identical to those substituted into K^b mutant genes. This observation implicates the microrecombination process in the sequence diversification of *H-2* genes and reassortment of class I sequences in nature. It is possible that the progenitor of the K^d allele may have undergone several microrecombinations with donor genes to attain its present mosaic structure of D^b-, *Q4*-, *Q10*-, *T5*-, and *K1*-like sequences. Conversely, the mutant K^b microrecombinations can be considered as individual events that, if allowed to accumulate, would diversify the K^b gene such that it would resemble K^d. It should also be noted that the K^k and K^{w28} alleles also contain codons that are identical to those substituted into the various K^b mutants (2, 24), a finding that supports the hypothesis that microrecombinations are an ongoing process in nature.

That the microrecombination process plays a major role in the diversification process of *H-2* genes is also supported by the finding that the most variable codons (here termed hypervariable codons) in exons 2 and 3 among the *K* alleles are also those that are altered in the spontaneous K^b mutants. Figure 6b illustrates that of the 12 codons that contain three or more different triplets in the four *K* alleles sequenced, 7 positions were also altered in the K^b mutants (positions 24, 77, 116, 152, 156, 163, and 173). Further, as discussed above, in many instances, the triplet of the naturally occurring *K* allele was identical to that of the mutant K^{bm} gene. These data suggest that the hypervariability of certain codons of various *K* alleles may be the result of the microrecombination process that operates in the different mouse strains and leads to sequence diversity among *H-2* alleles.

DISCUSSION

H-2 genes, members of the class I MHC multigene family, exhibit extensive allelic sequence diversity and are very polymorphic at the population level (12, 14, 15, 35). In contrast, the *Qa* and *Tla* class I MHC genes display little sequence diversity among the few alleles detected (8, 14). The mosaic structure of *H-2* genes hint that a recombination mechanism such as gene conversion between class I genes could promote the diversification of *H-2* genes (6, 11, 18, 28, 39). The analysis of spontaneous *H-2* mutants that represent single-generation events (13, 22) has provided a system of studying the role of recombination in the concerted evolution of class I genes (9, 10, 21, 25, 34, 40).

The K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11} mutants contained four, six, six, and three nucleotide substitutions, respectively (Fig. 1a to d) and were otherwise identical to K^b over the 849 nucleotides sequenced. Within each mutant gene, the nucleotide substitutions were clustered into segments of 38 or fewer nucleotides. The clustering of multiple alterations and the finding that identical nucleotide substitutions occur in independently arising mutants (positions 77, 173, and 174) are consistent with the mutants being generated by a nonrandom, complex mechanism such as recombination. Critical to a recombination hypothesis is a donor sequence in the parental genome that would contain the exact sequence substituted into the mutant K^b gene. All nucleotide substitutions in K^{bm3}, K^{bm4}, K^{bm10}, and K^{bm11} had donor counterparts in the B6 genome (Fig. 1a to d). The K^{bm4} gene resulted from the interaction of the *T5* gene with K^b. The *K1* gene was the donor gene for the K^{bm10} mutations. Recombination between the K^b and D^b genes generated the K^{bm11} mutant. Although a single donor gene for the K^{bm3} mutant has not been definitively established, potential donor sequences for each of its substitutions have been identified.

Nucleotide sequence comparisons of the K^b gene, mutant K^b genes, and donor genes defined the minimum and maximum extents of recombination between K^b and the donor gene to generate a particular mutant. For example, the K^{bm4} mutant resulted from the transfer of at least 37 but fewer than 62 nucleotides from *T5* to K^b (Fig. 1a). The analysis of other mutant K^b genes in this and other studies indicates that the extent of recombination between K^b and donor genes to generate each of the K^b mutants has also been less than 100 nucleotides (9, 21). Such recombinations can be called microrecombinations, a term reflecting the transfer of very small segments of DNA (tens of nucleotides). A similar

(a)

	77	116	121	155	156	173	174
K^b	GAC	TAC	TGC	AGA	CTC	AAG	AAC
$K^{bm3,11}$	AG-	---	---	---	---	---	---
$K^{bm6,9}$	---	-T-	C--	---	---	---	---
K^{bm1}	---	---	---	TAT	TA-	---	---
$K^{bm4,10}$	---	---	---	---	---	G--	CT-
K^d	AG-	-T-	C--	TAT	TA-	G--	CT-

(b)

	*24	63	66	69	73	*77	99	*116	*152	*156	*163	*173
K^b	GAA	GAG	AAA	GGC	AGT	GAC	TCT	TAC	GAA	CTC	ACG	AAG
K^d	-CT	C--	-G-	A--	T--	AG-	-TC	-T-	--T	TA-	GA-	G--
K^k	TCT	A-C	-TC	---	-T-	A--	-AC	---	--T	GA-	---	C--
K^{w28}	TCT	A-C	-TC	-A-	---	---	-AC	GT-	-CT	-GT	G--	---

FIG. 6. Sequence comparisons of codons from *K* alleles and mutant K^b genes. (a) The nucleotide sequence of K^b is given in comparison with mutant K^b and K^d sequences. The numbers above the K^b sequence indicate the amino acid position coded for by the codon. Dashes in mutant K^b and K^d sequences indicate identity to K^b. The K^b sequence is from Weiss et al. (39); the K^d sequence is from Kvist et al. (17). K^{bm6} and K^{bm9} sequences are from Geliebter et al. (9), and the K^{bm1} sequence is from Weiss et al. (40) and Schulze et al. (34). (b) The nucleotide sequences of hypervariable codons of K^b, K^d, K^k, and K^{w28} are given. The numbers above the K^b sequence indicate the amino acid position coded by the hypervariable codon. Dashes in the K^d, K^k, and K^{w28} sequences indicate identity to K^b. Asterisks above numbers indicate hypervariable codons altered in the various K^b mutants. The K^k sequence is from Arnold et al. (2); the K^{w28} sequence is from Morita et al. (24).

microrecombination has been described in the generation of the class II $I\text{-}A^{bm12}$ mutant, in which fewer than 44 nucleotides were recombined (20, 41). Microrecombinations may not be a phenomenon restricted to MHC genes or even to mammals, since the somatic diversification of the chicken immunoglobulin light chains may proceed by a similar mechanism (31).

The identification of *K1*, D^b, and *T5* as donor genes for the K^b mutants firmly establishes and broadens our understanding of the microrecombination process in the concerted evolution of class I genes. Previously, only the *Qa* region genes *Q4* and *Q10* were identified as donors, capable of interaction with the K^b gene (9, 10, 21). The lack of a known function for the *Qa* genes fueled speculation that the approximately 25 non-*H-2* class I genes (*Qa* and *Tla*) function to provide a reservoir of alternative sequences to diversify *H-2* genes. The finding that the D^b gene, whose product is a functional antigen-presenting molecule, is also a donor gene, suggests that donation of genetic information to *H-2* genes is not the sole function of non-*H-2* class I genes. That *Q4* and *Q10* reside in the telomeric portion of the MHC also suggested that microrecombination processes were directional, proceeding from telomeric donor genes to the centromeric K^b gene. The fact that *K1* is approximately 15 kilobases centromeric to K^b (38) and is the donor gene for the K^{bm10} mutant indicates that microrecombination can occur in a telomeric direction. Thus, there does not appear to be a directionality factor in the recombination among class I genes along chromosome 17.

The finding that the *T5* gene is a donor gene is significant because comparative hybridization and sequence analyses have indicated that those *Tla* region genes analyzed to date are very divergent from *H-2* and *Qa* genes (7, 27, 30, 32). In particular, exons 3 of *Tla* region genes are approximately 25% divergent from exons 3 of *H-2* and *Qa* genes (7, 27, 30, 32). These findings suggested that recombination between *H-2* and *Tla* genes occur infrequently, if at all. The *T5* gene is the donor gene for the K^{bm4} mutant, a finding that demonstrates that microrecombination between K^b and a *Tla* gene can occur. Further, the extent of homology between exons 3 of *T5* and K^b (86.6%) is comparable with the level of homology among *H-2* and *Qa* region genes (Fig. 4) (2, 30). This comparability suggests that some *Tla* region genes may be more closely related to *H-2* genes than previously thought.

The K^{bm3} allele was segregating in the C57BL/6Y (Yurlovo, USSR) mouse colony when it was isolated during routine skin graft testing for homozygosity (16). The B6/Y mouse subline had been separated from its parent line B6/J for less than 5 years when the bm3 mutant was detected. The K^{bm3} gene contains double nucleotide substitutions in each of codons 77 and 89. Although there are several potential donor genes for the alterations at position 77 (i.e., D^b and *Q8*) or 89 (i.e., D^b and *K1*) there is no one potential donor gene that is identical to K^{bm3} from positions 77 through 89 (Fig. 1d). Several explanations may account for this finding. One possibility is that the K^{bm3} gene may have been generated in a single generation by recombination with the D^b gene (Fig. 1d). This recombination could involve heteroduplex formation between K^b and D^b with mismatch repair at position 80 to the K^b sequence, followed by resolution of the heteroduplex in favor of the D^b sequence at positions 77 and 89. The patchwork resolution of artificially created heteroduplexes has been described (1). A second possibility is that the K^{bm3} gene was generated by the recombination of the K^b gene with two donor genes. Whatever the nature of the recombination events that generated the K^{bm3} mutant, the identification of potential donor genes for each of its double nucleotide substitutions in the B6 genome is consistent with the recombination hypothesis.

The microrecombinations described for the K^b mutants occur in the germ line (mitotically or meiotically) or in the

zygote. This latter possibility is of particular importance because the bm4, bm10, and bm11 mutants were detected in the F_1 generation of B6 × BALB/c mouse matings (16), and donor genes could conceivably come from the genome of either parent during zygotic recombination. Hybridization analysis with mutant-specific oligonucleotides for K^{bm4} and K^{bm11} (but not K^{bm10}) detected potential donor sequences in both the B6 (*T5* and D^b) and BALB/c mouse genomes. However, because donor sequences within the *H-2*^{b} MHC have been identified for all of the K^b mutants analyzed and because several microrecombinations must have occurred during the mitotic amplification of the germ line (bm6, bm9, and bm23; 9), it is probable that microrecombinations occur in the germ line and zygotic events are of little or no consequence.

The microrecombination process may be regulated by several factors including sex, mouse strain, and the donor and recipient genes involved. For example, most microrecombinations can be traced to the maternal chromosome. Of the 11 independently arising spontaneous class I *H-2* mutants detected in the F_1 generation of B6 × BALB/c mouse matings, all have occurred in the maternal *H-2*^{b} chromosome, 10 occurred in the K^b gene, and 1 occurred in the D^b gene (25). It has been suggested that the preponderance of mutants in the maternal *H-2*^{b} haplotype is caused by the preferential occurrence of recombination (gene conversion) in the female (B6 in these matings) (19), perhaps because of the longer duration of meiosis in females compared with males (42). This hypothesis is not totally correct because at least some, if not all, of the K^b mutants were generated during the mitotic amplification of the germ line and not during meiosis, and thus, the duration of meiosis would not be a factor (9). Alternatively, because the mother of all of the above-described F_1 mutants was B6, the preponderance of *H-2*^{b} mutants may be due to strain differences, which are either MHC linked or background. A possible example of strain-dependent recombination is that whereas *H-2*^{b} mutants are of the microrecombination type, *H-2*^{d} mutants involve single crossovers (33, 36). Additional parameters must also control microrecombinations, because the differential in the number of K^b versus D^b mutants (10:1) cannot be explained by strain or sex differences. Structural or mechanistic factors, such as the location or orientation of the K^b and D^b genes relative to each other and to other class I genes on chromosome 17 and the presence or absence of recombinogenic sequences, may be important in the microrecombination process.

The product of K^{bm} microrecombination, a K^b gene containing a small segment of nonallelic class I DNA, may be the result of a double, unequal crossover or a gene conversion-like event. Because we cannot recover all of the products of a mammalian germ cell recombination, we cannot distinguish between these two processes. We also cannot determine whether microrecombination events involve nonallelic genes on the same chromatid, sister chromatids, or homologous chromosomes. We can, however, conclude that single crossovers are not involved in the generation of the K^b mutants. Such events would yield hybrid products, genes that are one-half K^b and one-half donor gene, similar to that described for the D^{dm1} mutant (36). This possibility can be ruled out because all K^b mutants analyzed were entirely K^b with a very small substitution of donor gene sequences. Single crossovers between K^b and other class I genes may occur, but their products may be eliminated by selection. Depending on the relative 5′ → 3′ orientation of the two genes, single crossovers may result in large inversions or accentric and dicentric chromosomes.

The observation that intron sequences of allelic *H-2* genes are more conserved than exon sequences suggested that diversification by microrecombination events may be mediated by an RNA or cDNA intermediate (21, 39). This hypothesis is consistent with the finding that all donor genes analyzed to date are transcriptionally active in various tissues, a fact indicating their potential to also be active in germ cells where microrecombinations must occur. The transcriptional activity of the *T5*, *K1*, and D^b donor genes (Fig. 5) is also consistent with the proposed linkage between transcription and recombination (37).

A comparison of *H-2K* locus alleles indicates that they are composed of regions of conserved and variable primary sequences (2, 12). When the *K* alleles, K^b, K^d, K^k, and K^{w28} were compared, several hypervariable codons were apparent within the variable regions (Fig. 6b). At these positions, the hypervariable codons code for at least three different amino acid residues among the four *K* alleles. Most striking is the fact that of the 12 hypervariable codons 7 were in codons altered in the K^b mutant series. This finding supports the hypothesis that *K* gene sequence variability (diversity) and hypervariability are a direct result of the microrecombination process; i.e., the regions of the *K* gene that are diverse are those that have been involved in microrecombinations. Therefore, on an evolutionary level, microrecombination between class I genes would provide the raw materials for the diversification of the *H-2* genes. The accumulation of various mutant sequences within a single K^b gene via several, sequential microrecombinations with different donor genes will result in a chimeric *K* gene that is diverse from K^b. This result is indeed the observation with naturally occurring *K* alleles; they appear as mosaics containing blocks of sequences also present in other class I genes (39). Further support for the contention that *H-2* allelic diversity is a result of the microrecombination process is the fact that many of the nucleotides substituted into the various K^b mutants are identical to those at the homologous positions of the naturally occurring K^d allele (Fig. 6a). Thus, it appears that the mutant K^b genes are at a transient first step to becoming divergent, naturally occurring *K* alleles.

The microrecombination-diversification scheme for the dynamic evolution of *H-2* genes has significant implications on the rate at which *H-2* alleles will diverge. *H-2* genes, and particularly the K^b gene, mutate at a relatively high frequency (13). The ultimate source of almost all de novo genomic sequence variation is point mutation. However, *H-2* genes are part of a much larger multigene family of approximately 30 genes, which would collectively accumulate point mutations at a rate approximately 30 times faster than a single gene does. When the point mutations of the entire multigene family are coupled with a microrecombination mechanism that can reassort sequence segments between *H-2* genes and other class I genes, *H-2* evolution and diversification can proceed at a rate many times faster than that of a single gene. The finding that several mutant K^b molecules cannot present certain viral antigens to the T-cell receptor (3) suggests that the maintenance of microrecombination products in the population probably depends on natural selection. Thus, the demand for antigen-presenting molecules of many different specificities may be the driving force that utilizes the microrecombination process for *H-2* sequence diversification.

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