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Interaction Between K^b and *Q4* Gene Sequences Generates the K^{bm6} Mutation

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Genetic interaction as a mechanism for the generation of mutations is suggested by recurrent, multiple nucleotide substitutions that are identical to nucleotide sequences elsewhere in the genome. We have sequenced the mutant *K* gene from the bm6 mouse, which is one of a series of eight closely related, yet independently occuring mutants known collectively as the "bg series." Two changes from the K^b gene are found, positioned 15 nucleotides apart: an A-to-T change and a T-to-C change in the codons corresponding to amino acids 116 and 121, resulting in Tyr-to-Phe and Cys-to-Arg substitutions, respectively. Hybridization analysis with an oligonucleotide specific for the altered K^{bm6} sequence identifies one donor gene, *Q4*, located in the *Qa* region of the *H-2* complex. The two altered nucleotides that differentiate K^{bm6} and K^b are present in *Q4* in a region where K^b and *Q4* are otherwise identical for 95 nucleotides, delineating the maximum genetic transfer between the two genes. Because the K^{bm6} mutation arose in an homozygous mouse these data indicate that the *Q4* gene contains the only donor sequence and demonstrates that *Q*-region gene sequences can interact with the K^b gene to generate variant *K* molecules.

Class I genes of the murine major histocompatibility complex (*H-2*) constitute a multigene family consisting of genes for the classical transplantation antigens and the *Qa*- and *TL*-related molecules. With few exceptions, members of this family share amino acid and DNA sequence homology and similar intron-exon organization, and the gene products share the common biochemical properties of molecular weight, cell surface noncovalent association with β_2-microglobulin, and posttranslational glycosylation (28, 39). Classical transplantation antigens (*K*, *D*, *L*) are expressed on all somatic cells and are thought to play a role in the immunological recognition of self, nonself, and modified self (9, 49). *Qa* and *TL* antigens have a more limited tissue distribution and have as yet unknown functions (12, 39).

From serological studies of the *K* and *D* antigens in laboratory and wild mouse populations it has been estimated that greater than 50 alleles of each exist, attesting to the tremendous polymorphism of these loci (11). Sequence analyses of various alleles of the *K*, *D*, and *L* loci indicate that loci and alleles differ equally from each other by approximately 20% over the 200 amino-terminal amino acids and by 11% on the corresponding nucleotide level (8, 13, 28, 43). This large diversity among alleles is in sharp contrast to that observed at other genetic loci, where alleles usually differ from one another by only a few amino acids. This diversity is also remarkable with regard to the high conservation of *Qa*- and *TL*-region genes and their protein products, since limited tryptic peptide and DNA sequencing analyses indicate that alleles in these regions exhibit considerably less diversity and polymorphism than alleles in the *K* and *D* regions of the *H-2* complex (20, 24, 48).

The diversity noted among *K*, *D*, and *L* alleles is thought to play a role in the function of these glycoproteins as restricting elements in the murine immune system. However, the delineation of structure-function relationships of these molecules is hampered by the extraordinary diversity among alleles, since subtle, functionally crucial amino acid alterations would not be conspicuous against a background of 20% amino acid diversity. As a result, spontaneous in vivo mutations of the K^b gene have emerged as a powerful model system to study discrete amino acid changes associated with altered alloreactivity and associative recognition (10, 18, 23, 27). Mutant mice are detected by skin-graft incompatibility with individuals of the parental $H\text{-}2^b$ haplotype; with such tests, mutations of the K^b allele are observed at a frequency of approximately 2×10^{-4} per locus per gamete (10). These mutant alleles exhibit several unusual characteristics which argue against their being generated by point mutations: a number of mutant K^b molecules have multiple amino acid substitutions clustered over a small region, multiple nucleotide changes per codon would be required to effect many of the observed amino acid substitutions, and the same altered amino acid appears in several independently arising mutants (27, 31). Analysis of the available data showed that those amino acids substituted into the mutant K^b glycoproteins were also found at homologous positions in other class I molecules. This led to the speculation that a gene conversion-like mechanism may be responsible for the generation of diversity in the K^b mutants and other class I genes (6, 7, 10, 14, 31). Sequence analysis of the K^{bml} molecule and the identification of a potential donor gene further support this idea (21, 35, 44).

The "bg series" of mutants (bm5, bm6, bm7, bm9, bm16, bm17, bm18, bm20) are notable among the K^b variants because they represent a group of related but independently

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arising variants that are identical to each other with regard to all histocompatibility tests ("bg series" is an historical term for members of this group; they were originally designated bg1, bg2, bg3, etc., under the old nomenclature) (19, 22, 23). Peptide mapping and partial amino acid sequencing studies have indicated that the K^{bm5}, K^{bm6}, K^{bm7}, K^{bm9}, and K^{bm16} molecules share a Tyr-to-Phe substitution at amino acid position 116. In addition, K^{bm6}, K^{bm7}, and K^{bm9} molecules have a Cys-to-Arg alteration at position 121 (46, 47). The mutant K^{bm17}, K^{bm18}, and K^{bm20} molecules have yet to be characterized on the molecular level.

We have studied in detail one member of the "bg series" which arose in a C57BL/6Kh homozygous mouse and now report the cloning of the K^{bm6} gene. Sequence analysis of this mutant gene indicates that it differs from the K^b gene by only two nucleotides, positioned 15 nucleotides apart, resulting in amino acid substitutions at residues 116 and 121. Hybridization analysis with an oligonucleotide specific for the substituted region of the K^{bm6} gene indicates that only a single genetic sequence present in the C57BL/6Kh genome would be capable of interacting with the K^b gene to generate the K^{bm6} mutation. This sequence maps to the *Q4* gene in the *Qa* region of the *H-2* complex.

MATERIALS AND METHODS

Mice. C57BL/6Kh (B6) and B6-H-2^{bm6} (bm6) mice were obtained from the breeding and screening laboratory of Roger Melvold, Northwestern University Medical School, Chicago, Ill.

Preparation and screening of Charon 4 library. High-molecular-weight genomic DNA was prepared from spleen cells as previously described (30), digested to completion with *Eco*RI, and size fractionated by agarose gel electrophoresis (40 mM Tris acetate, 1 mM EDTA). The region of the gel corresponding to approximately 10-kilobase DNA fragments was excised, and the DNA was recovered by electroelution into dialysis tubing and cloned. The construction and screening of a Charon 4 library with pH202.5 for class I clones was previously described (35). Clones containing the K^{bm6} gene were identified by hybridization analysis of plaque replicas (GeneScreen; New England Nuclear Corp., Boston, Mass.) with ^{32}P-labeled K-mer (see below) as follows. Filters were prehybridized for 1 h at 55°C in 5 × SSPE (1× SSPE is 180 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM disodium EDTA [ph 7.4])–0.3% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml and hybridized for 2 h in 5× SSPE–0.3% SDS–100 μg of denatured salmon sperm DNA per ml with 2 × 10^5 cpm of ^{32}P-labeled K-mer per ml. Filters were washed twice for 30 min in 5× SSPE at room temperature and once for 1 min in 5× SSPE at 55°C.

Preparation and ^{32}P labeling of oligomers. The K-mer, a double stranded oligomer with the sequence 5′-CTGCAATAGTCACTGGAG-3′, was obtained by digestion of pH 202.10, a DNA subclone of the K^b gene containing the transmembrane exon (32) with *Bst*N-1, yielding a 275-base-pair fragment that was agarose gel purified, redigested with *Alu*I, and dephosphorylated with calf alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 2 h in 1× *Eco*RI buffer (see below). After labeling (see below), the K-mer was purified from free [γ-^{32}P]ATP and other labeled restriction fragments by electrophoresis through a 20% polyacrylamide (30:1 acrylamide-bisacrylamide)–8% urea gel (Tris borate buffer: 50 mM Tris borate, 1 mM EDTA), and elution of the oligomer from crushed gel slices in water.

The bm6-mer, K^b-TM, and oligonucleotides for dideoxy sequencing were synthesized by the phosphotriester solid-phase method as described in the manual for the Bachem oligonucleotide synthesizer. K^b-TM is a synthetic, single-stranded 21-base oligonucleotide covering the same region as the K-mer. A 0.1-μg sample of oligomer was labeled in the presence of 50 mM Tris hydrochloride (pH 7.6)–10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM spermidine–200 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and 7 U of polynucleotide kinase (New England Nuclear) at 37°C for 30 min.

Sequencing of the K^{bm6} and *Q4* genes. The 1.8-kilobase *Xba*I fragment containing the first three exons of K^{bm6} was subcloned in both orientations into M13mp10 (P-L Biochemicals, Milwaukee, Wis.). Exons 4, 5, and 6 are contained on a 1.5-kilobase *Bam*HI-*Sac*I restriction fragment and were also cloned into M13mp10. The subcloning of K^{bm6}, the preparation of single-stranded DNA, and the dideoxy sequencing procedure (34) were carried out as described in the instructions for the P-L Pharmaceutical DNA sequencing kit, with the exception that oligomers specific for the K^b gene were used as primers. Either [α-^{32}P]dATP or [α-^{35}S]dATP was used. Sequences were determined at least twice on exons 2, 3, and 4 and on both strands of exons 2 and 3.

The *Q4* gene is contained on the recombinant cosmid clone Bm1-2. A 3.1-kilobase *Bam*HI-*Hin*dIII fragment containing the 5′ half of *Q4* was subcloned, digested, end labeled, and sequenced by the method of Maxam and Gilbert (16) (see Results). The 3′ end labeling was done with an Amersham kit as described by the supplier.

Preparation of polyadenylated RNA. Whole cell RNA was prepared from fresh tissues as described by Auffray and Rougeon (1). The polyadenylated fraction of RNA was obtained by oligo(dT)-cellulose chromatography (P-L Biochemicals) (2).

Oligomer hybridization of genomic DNA and RNA. A 20-μg sample of genomic DNA was digested with the appropriate restriction enzyme, size fractionated on a 0.8% agarose gel (40 mM Tris acetate, 1 mM EDTA), denatured (0.5 N NaOH, 1.5 M NaCl, 45 min), neutralized (1 M Tris hydrochloride [pH 7.0], 2 M NaCl, 45 min), and transferred onto GeneScreen (38). DNA was fixed to the GeneScreen by a 2-min exposure to shortwave UV light (4). Filters were prehybridized for at least 2 h in 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate)–20 mM sodium phosphate (pH 7.0), 10× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone)–7% SDS–100 μg of denatured salmon sperm DNA per ml and hybridized overnight in prehybridization solution with 10% dextran sulfate and 50 ng of ^{32}P-labeled oligomer. Filters were washed twice at the hybridization temperature in 3× SSC–10 mM sodium phosphate (pH 7.0)–10× Denhardt solution–5% SDS for 1 min and then for 1 h and then once at the hybridization temperature for 1 h in 1× SSC–1% SDS. Hybridization temperatures were determined as described by Suggs et al. (41) and were 55°C for the K-mer, 60°C for K^b-TM, and 65°C for the K^{bm6}-mer.

Five micrograms of polyadenylated or nonpolyadenylated RNA were size fractionated on 1% agarose–formaldehyde gels (20 mM boric acid [pH 8.3], 1 mM EDTA, 3% formaldehyde), transferred to GeneScreen, and fixed by baking at 80°C for 2 h in vacuo. Filters were prehybridized, hybrid-

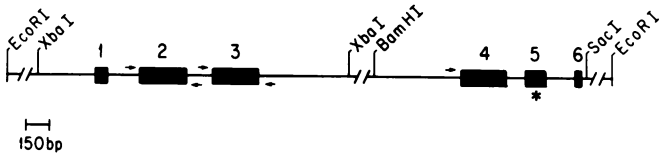

FIG. 1. Partial restriction map of the K^{bm6} gene. The 10-kilobase *Eco*RI fragment containing the K^{bm6} gene was obtained from recombinant clone λG298. Numbered, solid black boxes represent exons (exons 7 and 8 not shown). The black line represents introns and flanking DNA. The 1.8-kilobase *Xba*I and 1.5-kilobase *Bam*HI-*Sac*I restriction fragments were subcloned into M13mp10 and sequenced by the dideoxynucleotide method. Arrows represent oligonucleotide primers used for sequencing. The asterisk below exon 5 indicates the position of the K-mer, used to identify the K^{bm6} gene. For a more detailed restriction map of K^b, see references 35 and 43. bp, Base pairs.

ized, and washed as described above for filters containing genomic DNA.

Digestion of DNA with restriction enzymes. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim, or New England Biolabs (Beverly, Mass.) and used under the conditions recommended by the suppliers.

RESULTS

Cloning, identification, and sequence of the K^{bm6} gene. We have previously shown that the K^b gene is contained on an approximately 10-kilobase *Eco*RI restriction fragment of *B6* genomic DNA (36). Genomic DNA from bm6 mice was totally digested with *Eco*RI and size fractionated by agarose gel electrophoresis to enrich for 10-kilobase fragments. Size-selected DNA was ligated into Charon 4 arms and packaged in vitro. Approximately 200,000 unamplified recombinant phage were screened with pH202.5. Fifteen clones containing class I genes or gene fragments were detected. Two clones, λG286 and λG298, were determined to contain the K^{bm6} gene by hybridization to a *K* locus-specific oligomer (K-mer). Restriction enzyme analysis of the clone λG298 indicated that the K^{bm6} gene was located on an approximately 10-kilobase *Eco*RI fragment (Fig. 1). This fragment was subcloned into the *Eco*RI site of pBR322 and further subcloned into M13mp10 for dideoxynucleotide sequencing (Fig. 1). The exons corresponding to the three outer domains were sequenced. The nucleotide sequences of K^b and K^{bm6} are identical in the second and fourth domains

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          116          121
3'-TCAAGCGGATGCTGCCGGCGC-5'
    *            *
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FIG. 3. Nucleotide sequence of the bm6-mer. The nucleotide sequence of the bm6-mer is presented 3′ to 5′ to maintain the conventional amino acid orientation. Numbers above the sequence indicate the positions of the amino acids coded for by the bm6-mer. Asterisks indicate positions of nonidentity between the bm6-mer and the K^b sequence which are responsible for the hybridization specificity of the oligomer.

(data not shown). The nucleotide sequences of the third exon (α2 domain) of K^{bm6} and K^b are presented in a pairwise comparison in Fig. 2. Only two nucleotide changes are observed between the otherwise identical K^{bm6} and K^b: an A-to-T change and a T-to-C change at the positions corresponding to amino acids 116 and 121, respectively. These nucleotide alterations translate into the Tyr-to-Phe and Cys-to-Arg substitutions previously determined with amino acid sequencing techniques (47).

Identification of the donor sequence. A prediction of the model that genetic interaction is responsible for the generation of the K^b mutants is that a sequence identical to the altered K^{bm6} sequence is present elsewhere in the B6 genome (i.e., a donor sequence) (31). To examine this prediction, an oligomer of 21 nucleotides, complementary to the positive DNA strand of K^{bm6} corresponding to amino acids 115 through 122, was synthesized (bm6-mer) (Fig. 3). Genomic DNAs from both B6 and bm6 mice were digested with restriction enzymes, fractionated by agarose gel electrophoresis, transferred to GeneScreen, and hybridized to ^{32}P-labeled bm6-mer (Fig. 4). The specificity of the bm6-mer is demonstrated by its ability to hybridize to K^{bm6} sequences in bm6 DNA (Fig. 4a, 4.8-kilobase *Bam*HI fragment; Fig. 4b, 1.8-kilobase *Xba*I fragment) but not to the corresponding K^b fragments in B6 DNA. The K^{bm6}-mer also hybridized to a single donor sequence located on an 8.6-kilobase *Bam*HI fragment (Fig. 4a) or a 10-kilobase *Xba*I fragment (Fig. 4b) present in both the B6 and bm6 genomes. Similar results were obtained by hybridization analysis of *Kpn*I- and *Eco*RI-digested B6 and bm6 DNA (data not shown).

Genetic mapping of the donor gene. Hybridization analysis demonstrates that a single donor sequence for the K^{bm6} sequence is present in the B6 genome. To determine whether the donor sequence is contained in a class I gene, the bm6-mer was hybridized to a filter containing *Bam*HI-digested DNA of cosmid clones containing class I genes of

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                91          95          100         105         110         115         120
K^bm6           GCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGGGGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTTCGCCTACGACGGCCGCGATTACATC
K^b             ------------------------------------------------------------------------A-----------T-----------

                125         130         135         140         145         150         155
K^bm6           GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACACAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGG
K^b             ----------------------------------------------------------------------------------------------------

                    160         165         170         175         180
K^bm6           GCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAG
K^b             ------------------------------------------------------------------------
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FIG. 2. Pairwise sequence comparison of the third exon of K^{bm6} and K^b. The nucleotide sequence of the third exon, corresponding to the α2 domain, of K^{bm6} is given. The numbers on top of the sequence indicate the positions of the amino acids coded for by the corresponding codons. Dashes in the K^b sequence indicate identity to K^{bm6}. Two nucleotide differences exist between the two exons, at codons corresponding to amino acids 116 and 121. (K^b sequence from references 35 and 43.)

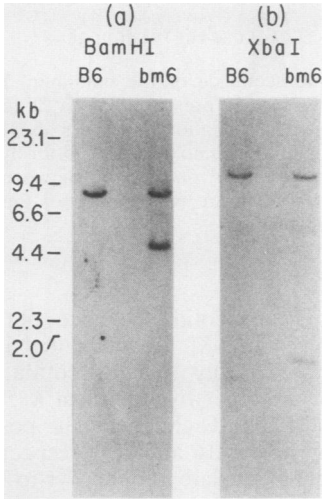

FIG. 4. Hybridization analysis of genomic DNA with bm6-mer. Splenic DNA from B6 and bm6 mice was digested with *Bam*HI (a) or *Xba*I (b), size fractionated by agarose gel electrophoresis, transferred to GeneScreen, and hybridized to ^{32}P-labeled bm6-mer (65°C). After hybridization, the filter was washed and exposed to Kodak XAR film for 4 days. Numbers, in kilobases, are *Hin*dIII-digested λ molecular weight markers.

the *H-2^b* haplotype (42) (Fig. 5). Of 24 clones comprising 26 genes, the bm6-mer hybridized only to clone B2.5, which contains the *Q4* and *Q5* genes located in the *Qa* region of the *H-2* complex. Subsequent restriction enzyme digestion and hybridization analysis indicated that the bm6-mer binds to an 8.6-kilobase *Bam*HI fragment of the *Q4* gene, identifying it as the same *Bam*HI fragment observed in *B6* genomic DNA (data not shown).

DNA sequence of the *Q4* gene. Previous results have shown that the *Q10* gene, the potential donor gene for the *K^{bm1}* mutation, shares a region of identity with the *K^b* gene that extends for 50 bases encompassing the seven substituted nucleotides (35, 44). To determine the extent of identity between *Q4* and *K^b*, the third exon of *Q4*, corresponding to the α2 domain, was sequenced (Fig. 6) and is presented in a pairwise comparison with the nucleotide sequence of the third exon of *K^b* (Fig. 7). *Q4* and *K^b* are nearly identical for the first 148 bases of exon 3. The two altered nucleotides in the codons for amino acids 116 and 121 that differentiate *K^{bm6}* from *K^b* are present in *Q4* at homologous positions. The substituted nucleotides are situated in a region of identity between *K^b* and *Q4* encompassing 95 nucleotides, corresponding to amino acids 109 through 140. Preceding a single-base, silent nucleotide difference in the codon for amino acid 108, *K^b* and *Q4* are identical for the remainder of the 5′ portion of the exon (52 nucleotides). *Q4* diverges from *K^b* by approximately 17% over the rest of exon 3, an amount comparable to other *Qa* region genes (12, 20, 40).

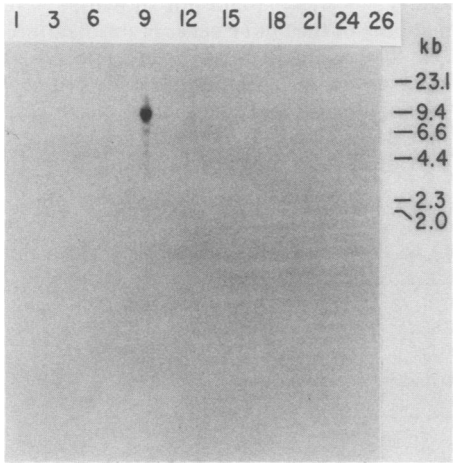

FIG. 5. Hybridization analysis of class I cosmid clones with bm6-mer. Twenty-four cosmid clones containing class I genes of the *H-2^b* haplotype were digested with *Bam*HI, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled bm6-mer (65°C). After hybridization the filter was washed and exposed to Kodak XAR film for 2 h. Numbers above gel indicate lanes of gel. Lanes 1 and 26 contain *Hin*dIII-digested λ markers.

Transcription analysis of the *Q4* gene. The *Q10* gene and its alleles are transcribed in the liver of several strains of mice, where it is processed as a soluble class I molecule (12, 20). The finding that *Q10* can be transcribed is consistent with the possibility that an RNA intermediate may participate in the genetic interactions thought to generate the *K^b* mutants (43). To determine whether the capability to be transcribed is a universal property of donor genes, polyadenylated RNA was prepared from various tissues from B6 and bm6 mice, size fractionated on formaldehyde-agarose gels, transferred to GeneScreen, and subjected to hybridization analysis with ^{32}P-labeled bm6-mer and K^b-TM. The bm6-mer detects both a major (17S) and a minor *K^{bm6}* mRNA species in bm6 spleen cells (Fig. 8a, lane 1). The bm6-mer, which does not hybridize to *K^b* mRNA, detects an intermediate size mRNA species, ostensibly a *Q4* transcript, in B6 spleen cells (Fig. 8a, lane 3). Although the *Q4* transcript is of a different size compared with the *K^{bm6}* transcripts, its presence is not detectable in RNA from bm6 spleen cells (Fig. 8a, lane 1), possibly due to the relatively low level of transcription of the *Q4* gene, which is overshadowed by the hybridization of the bm6-mer to *K^{bm6}* mRNA.

Hybridization analysis of liver cells with the bm6-mer, which detects the presence of *K^{bm6}* mRNA in bm6 cells (Fig. 8a, lane 2), indicates the absence of a *Q4* transcript in B6 cells (Fig. 8a, lane 4).

The filter in Fig. 8a was hybridized to ^{32}P-labeled K^b-TM, without prior removal of bound bm6-mer (Fig. 8b). Hybridization of K^b-TM to *K^b* mRNA in Fig. 8b, lanes 3 and 4,

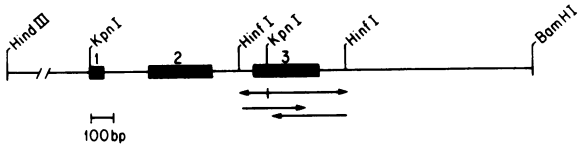

FIG. 6. 5′ portion of the *Q4* gene. The third exon of *Q4* was sequenced in the following manner. A 456-base-pair (bp) *Hin*fI fragment containing the third exon was purified from a 3.1-kilobase *Bam*HI-*Hin*dIII subclone of the *Q4* gene. End labeling of the *Hin*fI sites was accomplished by filling in with Klenow polymerase, followed by digestion with *Kpn*I and Maxam-Gilbert sequencing of each fragment. Alternatively, initial digestion of the *Bam*HI-*Hin*dIII subclone was carried out with *Kpn*I, followed by 3′ end labeling with [α-^{32}P]cordycepin triphosphate, digestion with *Hin*fI, and sequencing. Numbered, solid black boxes represent exons. The black line represents introns and flanking DNA. Arrows indicate positions and directions of sequencing.

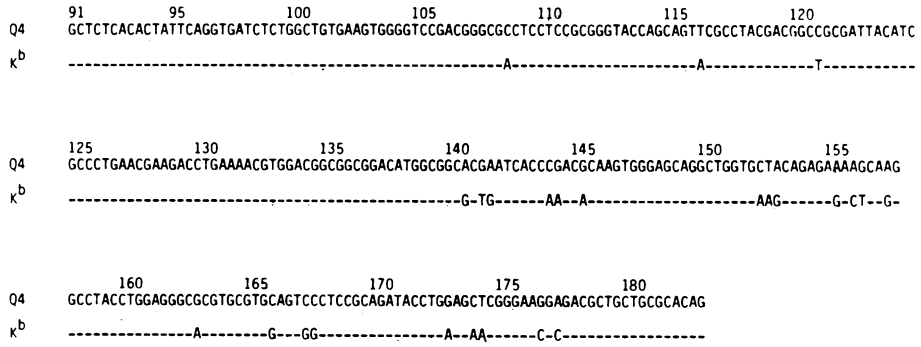

FIG. 7. Pairwise sequence comparison of the third exon of *Q4* and K^b. The nucleotide sequences of the third exons, corresponding to the α2 domains, of *Q4* and K^b are given. Numbers on top of the sequences indicate the positions of the amino acids coded for by the corresponding codons. Dashes in the K^b sequence indicate identity to *Q4*. (K^b sequence from references 35 and 43.)

attests to the integrity of the B6 RNA and demonstrates the relative level of transcription of K^b and *Q4* in B6 spleen cells (Fig. 8a and b, lanes 3). Therefore, the *Q4* gene appears to be transcriptionally active in B6 spleen cells, but not in B6 liver cells.

DISCUSSION

Although all class I major histocompatibility complex genes and products share substantial sequence homology and overall biochemical properties, a number of features differentiate members of this family (13, 28). Alleles of the *K*, *D*, and *L* genes, the products of which function as restriction-recognition molecules, are highly diverse (13, 28). Alleles of the *Qa* and *TL* genes exhibit much more conservation of sequence (20, 48). When compared with *Qa* and *TL* region loci on the population level, the *K*, *D*, and *L* loci are more polymorphic (11, 24). It seems reasonable that the polymorphism is a direct result of the mechanism underlying the generation of diversity of *K*, *D*, and *L* genes. These properties of the class I multigene family have made it a model for the study of the mechanisms involved in the generation of genetic diversity and the maintenance of polymorphism.

Spontaneous mutants of the K^b gene product have been instrumental in delineating structure-function relationships

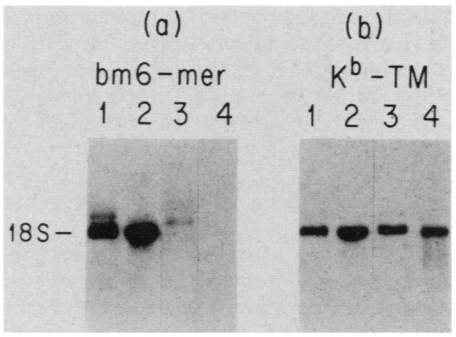

FIG. 8. Hybridization analysis of B6 and bm6 RNA. Whole cell RNA was prepared from B6 and bm6 livers and spleens. A 5-μg sample of polyadenylated RNA was fractionated by formaldehyde-agarose gel electrophoresis, transferred to GeneScreen, and hybridized to the bm6-mer (65°C) (a) or K^b-TM probe (60°C) (b). After hybridization the filters were washed and exposed to Kodak XAR film for 16 h. 18S ribosomal RNA represents a 1,950-base molecular weight marker present in the nonpolyadenylated fraction. Lanes: 1, bm6 spleen; 2, bm6 liver; 3, B6 spleen; 4, B6 liver.

involved in the recognition and effector response against allogeneic and altered-self histocompatibility antigens (10, 18, 23, 27). The "bg series" mutants (bm5, bm6, bm7, bm9, bm16, bm17, bm18, bm20) are of special interest because they constitute a group of related, repetitive, and yet independent events. Members of the "bg series" exhibit the longest mean survival time of reciprocal skin grafts with parental B6 mice of any of the K^b mutant strains (19, 27) and are histocompatible with each other (22). Amino acid sequencing studies have shown that the *K* molecules of bm5, bm6, bm7, bm9, and bm16 mice contain a Tyr-to-Phe substitution of amino acid position 116. In addition, K^{bm6}, K^{bm7}, and K^{bm9} molecules have a Cys-to-Arg substitution at position 121 (46, 47). We have now shown that the two amino acid substitutions in the K^{bm6} molecule are due to single nucleotide changes in each of the respective codons. Primary structural differences between the K^b and K^{bm6} molecules are limited to these two alterations, because the two alleles are otherwise identical over the first 274 amino acids.

It has previously been shown that the Cys at position 121 of the K^b molecule is not involved in disulfide bonding (15), so that a substitution at this position would not be expected to have a dramatic effect on the tertiary structure of the K^b molecule. It is surprising that the Cys-to-Arg alteration at position 121, which results in a net positive charge change, appears to be immunologically silent since it is present in the *K* genes of only some of the "bg series," which are all skin graft compatible. Consequently, the Tyr-to-Phe substitution at position 116, which does not result in a charge change and is present in the *K* genes of all members of the "bg series" tested, is apparently responsible for the antigenic phenotype of this group of K^b mutants. Experiments involving site-directed mutagenesis are presently under way to determine whether the presence of an Arg residue at position 121, in the absence of a Tyr-to-Phe substitution at position 116, is immunogenic. The present study demonstrates that the apparent nonimmunogenicity of the Cys-to-Arg substitution is not due to the presence of a third, compensatory change in the K^{bm6} molecule. It remains to be seen whether the K^{bm5} mutation contains a second compensatory change, but, as discussed below, this is unlikely.

The frequency and nature of the amino acid substitutions involved in the K^b mutants have led to the hypothesis that a gene conversion-like mechanism is involved in the mutation process (6, 31). The model of gene conversion between members of the class I multigene family has led to the successful prediction, in mutant mice, of additional amino acid substitutions not detected in conventional amino acid

sequencing procedures (31). A similar mechanism has also been proposed to generate diversity in class II molecules (17, 45).

The cloning and sequencing of the K^b (32, 36, 43) and K^{bm1} (35, 44) genes demonstrated that the two alleles differ from each other by seven nucleotides in the region corresponding to amino acids 152 to 156. A potential donor gene, *Q10*, was identified in an H-2^b cosmid library (20, 21). However, as the K^{bm1} mutation arose in a (C57BL/6 × BALB/c)F_1 ($H\text{-}2^b$ × $H\text{-}2^d$) mouse (3, 27), the nature of the donor gene is uncertain. If the conversion event occurred at the stage of the zygote, it is equally possible that the donor gene was of $H\text{-}2^b$ or $H\text{-}2^d$ origin (21, 44), since in addition to the *Q10* gene of the *b* haplotype, the L^d gene of the *d* haplotype has a sequence identical to the K^{bm1} gene at positions corresponding to amino acids 152 through 156 (6, 26). Furthermore, hybridization analysis of genomic digests with an oligomer specific for the altered K^{bm1} sequence has identified a *Q10*-like sequence in the *d* haplotype (24a). Therefore, the K^{bm1} mutation might have arisen as a result of the interaction of the K^b gene with one of the three potential donor genes.

In contrast, the K^{bm6} mutation arose in full sibling matings of C57BL/6Kh mice (23); therefore, the genetic interaction resulting in K^{bm6} had to involve exclusively genes of the *b* haplotype. Oligomer hybridization analysis of genomic DNA indicates that only one sequence in the C57BL/6 genome is identical to the substituted region of K^{bm6}. This sequence resides in an homologous position of the *Q4* gene, which is in the *Qa* region of the *H-2* complex. Restriction enzyme and hybridization analysis indicates that the sequences identified in genomic DNA and in cosmid cloned DNA are identical. Taken together, these data identify the existence of a single donor sequence in the C57BL/6 genome, located in the *Qa* region, and any genetic interactions with the K^b gene to generate the K^{bm6} mutation must have involved this sequence in the *Q4* gene. The identification of a single donor gene for the K^{bm6} mutation in the *Qa* region of the *H-2* complex, indicating the capability of *K* and *Q* genes to interact, strengthens, by analogy, the previous contention that the *Q10* gene is the donor gene of the *bm1* mutation (21). It is of interest that the two donor genes identified in the *b* haplotype reside in the *Qa* region. It is known from amino acid and nucleotide sequencing studies of other K^b mutants (27; Geliebter et al., unpublished observation) that *Q4* and *Q10* cannot comprise the complete reservoir of donor genes. Given the similarity of the *Q* genes to one another and to the *K* genes (12, 20, 40; Flavell et al., unpublished data), it would not be surprising to find other *Q* genes capable of interacting with and donating sequences to the *K* gene. In this regard it is interesting that *K*- and *Qa*-region genes have been found to share serological epitopes (5, 29). This may be explained by the transfer between these genes of stretches of DNA that code for the amino acids that comprise these antigenic sites.

The extent of identity between K^b and *Q4* around the substituted region of K^{bm6} is 95 bases, delineating the maximum amount of genetic material transferred between the two genes. Preceding a 1-base disparity in the codon for amino acid 108, *Q4* and K^b are identical for an additional 52 bases at the beginning of exon 3. Further continuous identity of 12 nucleotides exists in the 3′ region of the intron between exons 2 and 3. Given the long stretch of identity between *Q4* and K^b in the 5′ portion of exon 3, it would not be surprising to find the *K* genes of other members of the "bg series" carrying the silent change at amino acid position 108 in addition to the productive substitution at positions 116 and 121. Such a finding would further support the idea of a mechanism of genetic interaction generating diversity in class I genes. The extent of identity between the K^b and *Q4* genes around the region of substitution (95 nucleotides), and hence the maximum amount of sequence transferred between the two genes, is larger than that observed between the genes interacting to generate *bm1* mutation (50 nucleotides) or the class II *bm12* mutation (44 nucleotides) (17, 45). As discussed below, the relatively large region of identity between K^b and *Q4* may be responsible for the enhanced frequency of interaction resulting in the generation of the "bg series." Still, in comparison to studies on other mammalian systems which indicate genetic transfers of several hundred nucleotides (25, 33, 37), the amount of DNA transferred between *Q4* and K^b in the generation of the K^{bm6} gene is relatively small.

As indicated previously, amino acid sequence analysis has revealed a subgroup of the "bg series" (bm6, bm7, and bm9); although isolated independently, these mutants exhibit identical substitutions in their *K* molecules at positions 116 and 121 (47). Assuming that these mutants are also identical at the nucleotide level, it is surprising to find only one donor gene present in the B6 genome. The repetitiveness of such an event might have suggested that multiple donor genes be available, contributing to the frequency of this subset of mutations. This suggestion would be further strengthened if bm5 and bm16 mutants represent a partial interaction event with the *Q4* gene. Preliminary data indicate that the K^{bm5} gene has a substituted sequence which is shared with only the *Q4* gene (unpublished data). The singularity of the donor gene for the "bg series" would imply that something intrinsic to the *Q4* gene promotes its interaction with the K^b gene. Perhaps its large extent of identity to the K^b gene or its spatial orientation or location on the chromosome would increase its ability to form heteroduplexes with the K^b gene. Studies are presently under way to determine the nucleotide sequence of the K^b genes of other members of the "bg series."

The K^b gene is separated from the *Qa* region by more than 500 kilobases of DNA (42). This suggests that if interactions between the K^b and *Q* genes occur on the level of DNA, these regions may be spatially oriented on the chromosome much closer than their linear distance would imply. Alternatively, the genetic interactions leading to the generation of the K^b mutants could conceivably have occurred through a donor gene transcript or cDNA intermediate (21, 43). This would require that a donor gene be transcriptionally active and its RNA (or cDNA) be present at the time of genetic alteration. The finding that the *Q10* gene is transcribed in the liver (12, 20) indicates its potential to be transcribed in the germline. Likewise, that *Q4* is transcribed in spleen cells of B6 mice, albeit at a low level, indicates its potential to be active in the germ line. Studies are presently underway to determine the level of transcription, if any, of the *Q4* and *Q10* genes in germ cells.

The finding of a donor gene for the K^{bm6} mutation supports the contention that K^{bm6} did not result from two random point mutations. Furthermore, the possibility that two independent mutations in the K^b gene would have arisen in one generation is statistically small. It may be argued that the substitution at position 121, which may be immunologically silent, preexisted and was identified when the change at position 116 was introduced and detected by skin graft rejection. This is unlikely, because amino acid and DNA sequence analysis of over 50 parental type C57BL/6 mice failed to detect any substitutions at position 121 (unpublished data). Furthermore, the repetitiveness of the Tyr-to-

Phe substitution at position 116 in the "bg series" argues against point mutation and for genetic interaction as the mechanism generating this group of mutants.

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