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Comparison of the cloned *H-2K^{bm1}* variant gene with the *H-2K^{b}* gene shows a cluster of seven nucleotide differences

(major histocompatibility complex/DNA sequence/structure–function/gene conversion/diversity)

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ABSTRACT **The gene for the H-2K class I antigen of the *bm1* variant was cloned and analyzed at the DNA level and compared with the previously cloned parent *B6/Kh* gene. Sequence determination and comparative restriction endonuclease studies indicate that *K^{bm1}* is derived from the *K^{b}* gene. Seven nucleotide changes within a 13-nucleotide stretch distinguish the mutant from the parent gene and result in amino acid differences at positions 152, 155, and 156 in the antigen. The data confirm previously reported changes at amino acid positions 155 and 156 (arginine to tyrosine and leucine to tyrosine, respectively) and extend the altered region to include two nucleotides encoding a glutamate to alanine substitution at amino acid 152, a change not detected by the protein studies because of limitations of the methods used. The DNA sequence encoding this region of the K^{bm1} glycoprotein is identical to the DNA sequence of at least one other known class I gene in the mouse, a finding consistent with the hypothesis that the mutation was not a random event but may be the result of a block transfer of information by a copy mechanism analogous to gene conversion. As the sequence analysis of the coding region for the first 273 amino acid residues shows identity between parent and mutant except for the seven nucleotide changes, all variant–parent functional differences must depend only on the cluster of three amino acid differences in the second domain of the K^{b} glycoprotein.**

Studies on the spontaneously occurring H-2 major histocompatibility complex (MHC) variants have permitted a unique approach to the analysis of structure–function relationships of class I genes. The variant mouse sublines were discovered and isolated by I. Egorov, D. Bailey, H. I. Kohn, and R. W. Melvold (for discussion, see ref. 1) and have altered reactivities not only in tissue graft rejection, by which they were identified, but also in cell-mediated lymphocytotoxicity, mixed lymphocyte reactivity, and H-2-associated recognition (1, 2). The genes determining the altered functional properties were mapped to discrete regions of the H-2 complex by classical approaches. Most of the variants occurred in the *B6/Kh* line and the histogenic reactivity was mapped to the *K* region. It was therefore postulated that they were mutants of the *H-2K^{b}* gene.

The H-2 class I histocompatibility molecules are dimeric structures consisting of a M_r 45,000 MHC-encoded polymorphic glycoprotein associated with a M_r 12,000 nonpolymorphic light chain (β_2-microglobulin) not encoded in the MHC. The first 280 amino acids of the class I glycoproteins are located external to the cell membrane. This region is divided into three domains of 90 residues each (3, 4). Earlier studies initiated to analyze the protein structure of the MHC products of *H-2K^{b}* variant mice using a combination of tryptic peptide mapping and amino acid sequence analysis suggested that only one or two amino acid differences distinguish the variant from the parental K^{b} molecule (5). The changes were localized to the first amino-terminal half (positions 1–180) of the molecule, indicating that the first two domains, N and C-1, are involved with cellular recognition of the K^{b} molecule (5). Studies on fragmentation of the K^{b} molecule have shown that alloantigenic determinants detected by monoclonal antibodies are also located in the N and C-1 domains (6). The third domain, residues 180–270, binds β_2-microglobulin (6).

In addition to structure–function correlations, study of the *K^{b}* mutant series is also relevant in defining the origin and mechanism of diversity and polymorphism in the MHC. For example, comparison of the structure of the K^{b} variant molecules with other class I sequences has suggested that the variants are generated by a block transfer of genetic information, possibly due to a copy mechanism analogous to gene conversion (7). Here, we report our molecular analysis of the cloned *K^{bm1}* gene and discuss the relevance of the findings to the questions of structure, function, and polymorphism.

MATERIALS AND METHODS

Isolation of the *K^{bm1}* Gene from a Charon 4 Library. High molecular weight genomic DNA was isolated from B6.C-H-2^{bm1} mouse spleen cells as described (8). The DNA was partially digested with the restriction endonuclease *Eco*RI and a library was made using λ phage Charon 4 (9). Recombinant clones were screened by hybridization to nitrocellulose filters (Schleicher & Schuell) containing replicas of phage plaques. The filters were prehybridized at 65°C for at least 4 hr with 0.75 M NaCl/0.075 M Na citrate/50 mM phosphate, pH 7.4/0.08% polyvinylpyrrolidone/0.08% Ficoll/0.08% bovine serum albumin containing denatured salmon sperm DNA (Worthington) at 50 μg/ml and then hybridized with the nick-translated probe pH202.5, a derivative of an H-2K^{b} cDNA clone (10), for 16 hr at 65°C in the same solution. The probe had a specific activity of 1×10^8 cpm/μg and was used at 1×10^5 cpm/ml.

The *H-2K* gene was identified among the members of the class I-positive clones by hybridization analysis using the specific synthetic nonadecanucleotide probes K^{b}-19 (C-C-A-G-A-G-A-T-C-A-C-C-T-G-A-A-T-A-G) and K^{bm1}-19 (A-G-G-C-C-C-T-G-T-A-A-T-A-C-T-C-T-G-C). The synthesis, purification, and labeling of oligonucleotide probes was carried out as described (11) except that the K^{bm1}-19 oligonucleotide was syn-

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Abbreviations: MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter; kb, kilobase(s); bp, base pair(s).

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thesized in a Systex Microsyn 1450 automated DNA synthesizer. For screening, nitrocellulose filters containing DNAs from various class I recombinant clones were hybridized in 0.9 M NaCl/0.18 M Tris·HCl, pH 8.0/12 mM EDTA/0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin/0.1% $NaDodSO_4$ containing the labeled oligonucleotide probe (specific activity, $>1 \times 10^9$ cpm/μg) at 10 μg/ml at 55°C for 2 hr. The filters were washed at room temperature for 15 min with three changes of 0.9 M NaCl/0.09 M sodium citrate, and again in the same solution at 55°C, for 1 min. The dried filters were exposed to x-ray film.

Fluorescence-Activated Cell Sorter (FACS) Analysis of Transfected Cells. Ltk^- cells were transfected with DNA from the K^b clone C1.4.1 or the K^{bm1} clone E107.1.1 by established procedures (12) and identified by using a direct binding rosette assay (13). Positive clones were analyzed on a FACS II (Becton Dickinson FACS Systems, Mountain View, CA) by indirect immunofluorescence after reaction with monoclonal antibodies B8-24-3 (14) and 5F1.2.14 (15) followed by staining with a fluorescein-conjugated goat anti-mouse IgG reagent.

Comparative Restriction Analysis. The 10-kilobase (kb) *Eco*RI fragments from clones C1.4.1 and E107.1.1 that contain the K^b and the K^{bm1} coding sequences, respectively, were subcloned into pBR322 according to methods described (16). The plasmids were isolated and digested with *Eco*RI. These DNAs were then separately digested with restriction endonucleases *Hin*fI, *Hpa* II, *Hae* III, and *Sau*3A (all from BRL) as specified by the supplier. A pairwise comparison between the subcloned K^b and K^{bm1} genes was achieved by resolving the fragments on a 5% bisacrylamide (29:1) gel (0.15 cm × 16 cm × 40 cm) run in Tris borate buffer at 100 V for 24 hr and then staining the DNA fragments with ethidium bromide.

DNA Sequence Analysis. The pBR322-subcloned 10-kb *Eco*RI fragments containing the coding portions of C1.4.1 (K^b) and E107.1.1 (K^{bm1}) were transformed into the DNA methylation-lacking host GM48 (17). The plasmid DNAs were digested with specific restriction endonucleases to yield fragments that could be readily labeled with reverse transcriptase or terminal deoxynucleotidyltransferase. The reverse transcriptase reaction (15 units of enzyme) was carried out in 50 mM Tris·HCl, pH 8.3/40 mM KCl/6 mM $MgCl_2$ at 37°C for 1 hr with a mixture of unlabeled nucleoside triphosphates (each at 10 mM) and one ^{32}P-labeled triphosphate (100 μCi; 1 Ci = 37 GBq). The strategy to obtain labeled fragments is shown in Fig. 1. The 340-base-pair (bp) *Sma* I fragment, including exon II (amino acids 1–91), was digested with either *Bst* I or *Ava* II and labeled with reverse transcriptase. When *Ava* II was used, fragments labeled at a single end were obtained from a strand-separating gel. The 1.8-kb *Xba* I fragment was digested with *Bcl* I and labeled with reverse transcriptase. The two labeled fragments could be recovered from an agarose gel for analysis of exon III (amino acids 91–182). To obtain labeled fragments to analyze exon IV (amino acids 183–273), a *Pst* I digest of subcloned DNA was labeled using cordycepin 5′-triphosphate and terminal deoxynucleotidyltransferase as described (18) and then digested with a second restriction enzyme, and the appropriate fragment was recovered from the gel. The nucleotide sequences of the appropriate labeled fragments were determined using the methods of Maxam and Gilbert (19) with minor modifications. The sequence for each portion of the gene was determined three times.

RESULTS

The $H\text{-}2K^{bm1}$ gene was isolated from a library of Charon 4 λ phage constructed from B6.C-H-2^{bm1} spleen DNA. A set of recombinant clones containing class I DNA sequences was detected by hybridization with the $H\text{-}2K^b$ cDNA clone pH202.5. Two 19-base synthetic oligomers were used to identify recombinant clones containing the K^{bm1} gene. The oligonucleotide probes were complementary to two regions of the $H\text{-}2K^{bm1}$ gene as follows: K^b-19 (C-C-A-G-A-G-A-T-C-A-C-C-T-G-A-A-T-A-G) is complementary to the DNA sequence encoding amino acids 94–100 and K^{bm1}-19 (A-G-G-C-C-C-T-G-T-A-A-T-A-C-T-C-T-G-C), to amino acids 153–159. The K^{bm1}-19 oligomer recognizes the region of the K^{bm1} gene known to contain two amino acid differences with respect to the K^b gene (arginine to tyrosine at position 155, leucine to tyrosine at position 156) as determined by earlier analyses at the protein level (20). The nucleotide order of oligomer K^b-19 was selected by reference to the sequence of the K^b cDNA clone (10) and the sequence of K^{bm1}-19 was suggested by reference to the sequence of the L^d gene (21, 22), which shares the Tyr-Tyr substitution at amino acid positions 155 and 156. Two recombinant clones, E88.2.1 and E107.1.1, hybridized to both probes and both contained the 10-kb *Eco*RI fragment characteristic of the $H\text{-}2K^b$ gene described earlier in the B6/Kh-derived clone C1.4.1 (23).

Clone E107.1.1 was selected for further study and was cotransformed into Ltk^- cells with herpesvirus thymidine kinase (tk) DNA. Cells surviving hypoxanthine/aminopterin/thymidine selection were screened with monoclonal antibody B8-24-3, which reacts with both parent $H\text{-}2K^b$ and mutant $H\text{-}2K^{bm1}$ gene products (24). Cotransformed cell lines that expressed $H\text{-}2K^b$-like specificity as determined by a direct binding rosette assay (13) were tested for the expression of $H\text{-}2K^{bm1}$ by using indirect immunofluorescence with a FACS. Two mono-

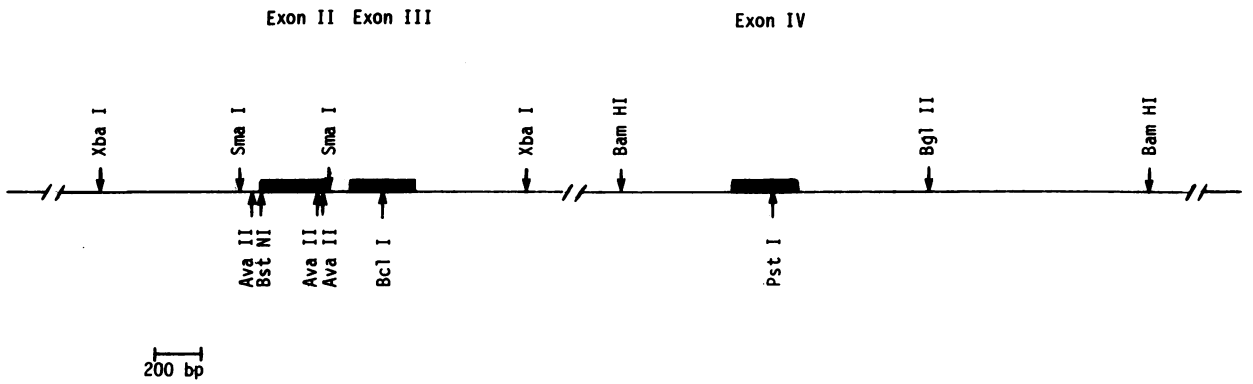

FIG. 1. Strategy for labeling fragments and analysis of the DNA sequences of the K^{bm1} gene encoding the extracellular region of the K^{bm1} antigen. The relevant portion of the 10-kb *Eco*RI fragment cloned in pBR322 and the positions of the restriction endonuclease sites are shown. The heavy line denotes the coding regions of the gene: exon II, amino acids 1–91; exon III, amino acids 92–183; exon IV, amino acids 183–273.

clonal antibodies were used to discriminate K^b from K^{bm1}. The first, B8-24-3, binds both the H-$2K^b$ and H-$2K^{bm1}$ products; the second 5F1.2.14, binds only the parent molecule (24). As shown in Fig. 2, E107.1.1 cotransformed L cells expressed a cell surface antigen having the specificity of the mutant glycoprotein H-$2K^{bm1}$, since the cells were bound by the B8-24-3 antibody but not by the 5F1.2.14 antibody, which binds only to the H-$2K^b$ product.

The 10-kb *Eco*RI fragments of both the *H-2K*^{b} clone C1.4.1 (23) from the parent mouse strain *B6/Kh* and the *H-2K*^{bm1} clone E107.1.1 were subcloned into pBR322 for comparative analyses of the two genes. To determine whether both genes were derived from the same stretch of chromosome or represented different members of a multigene family, the subclones were analyzed using four endonucleases (*Hin*fI, *Hpa* II, *Hae* III, *Sau*3A) that cleave frequently. As shown in Fig. 3, both subclones yielded virtually identical digestion patterns with the four enzymes, except for the differences in the *Hin*fI profile, where fragments of 140 and 225 bp are missing in the mutant E107.1.1 and a composite fragment of 365 bp is present. *Hin*fI recognizes the parent gene sequence at the site of mutation but does not recognize the mutant sequence. These comparative restriction analyses indicate that both the parent and variant *H-2* genes are derived from virtually identical regions of the genome.

The DNA sequence of the K^{bm1} gene encoding amino acids 1–273 was determined and compared with the sequence of a K^b cDNA (10) clone and a cloned K^b gene (23). The differences between the two genes were localized to the third exon, which encodes amino acids 91–182. The sequence of this region for both *H-2K*^{b} and *H-2K*^{bm1} is shown in Fig. 4. Seven base changes were detected in *H-2K*^{bm1}, accounting for the three amino acid substitutions, glutamate to alanine at position 152, arginine to tyrosine at position 155, and leucine to tyrosine at position 156.

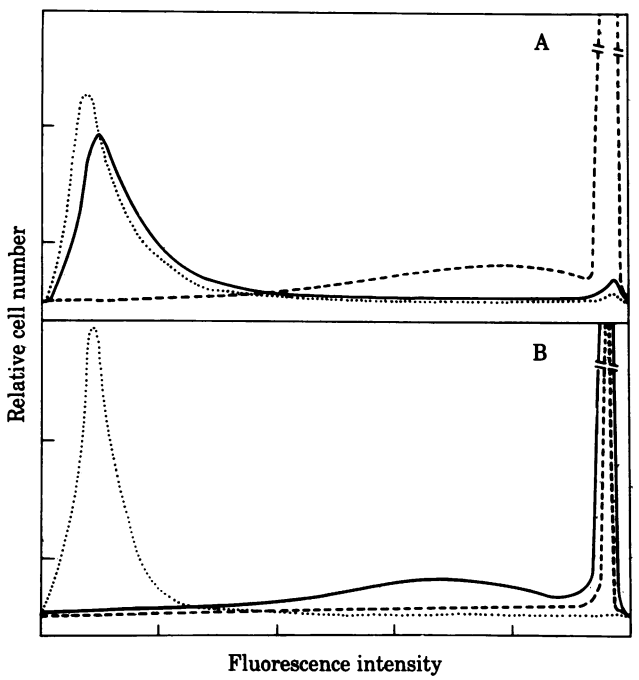

FIG. 2. Monoclonal antibody analysis of L cells transfected with K^b (C1.4.1) and K^{bm1} (E107.1.1) cloned genes and detected by indirect immunofluorescence using the FACS. Reactivity of 5F1.2.14 (anti-K^b) is shown in *A* and reactivity with B8-24-3 (anti-K^b/K^{bm1}) is shown in *B*. FACS patterns are as follows: ·····, negative control, untransfected L cells; ----, L cells transfected with K^b (C1.4.1); ——, L cells transfected with K^{bm1} (E107.1.1). Fluorescence intensity is in arbitrary units and increases from left to right.

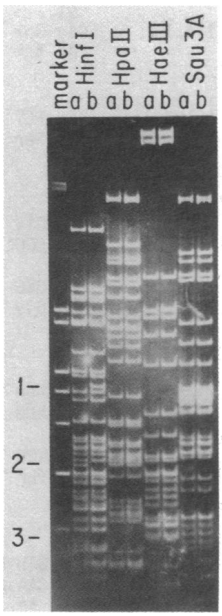

FIG. 3. Comparative restriction enzyme analysis of 10-kb *Eco*RI fragments containing the K^b and K^{bm1} genes. Homologous 10-kb *Eco*RI fragments, subcloned into pBR322, were digested with *Eco*RI and the indicated endonucleases. The resulting DNA fragments were resolved for pairwise comparison on a 5% bisacrylamide gel (29:1). Lanes: a, K^b; b, K^{bm1}. Molecular size markers are *Hin*fI fragments of pBR322. Differences between K^b and K^{bm1} in the *Hin*fI comparison are indicated: 1, 365-bp band present in the K^{bm1} digest; 2, 225-bp band present in the K^b digest; 3, 140-bp band present in the K^b digest.

DISCUSSION

The amino acid substitutions distinguishing the variant K^{bm1} glycoprotein from the standard K^b glycoprotein were established by determining the nucleotide sequences of the genes. Seven nucleotide differences were detected that result in three amino acid substitutions, alanine for glutamate at position 152, tyrosine for arginine at position 155, and tyrosine for leucine at position 156.

In a variety of assays, the *bm1* variant exhibits altered reactivity as compared with the parent. The disruption of serologic determinants is indicated by the failure of monoclonal antibody 5F1.2.14 to bind K^{bm1}. Differences in cellular recognition are shown by cell-mediated lymphocytotoxicity, mixed lymphocyte reactivity, and tissue grafting (25, 26). Changes in the ability to present antigen have also been detected in viral (27), chemical (28), and minor histocompatibility antigen assays (29). Thus, a variety of functions associated with class I molecules can be determined solely by a limited stretch of amino acids in the second domain of the amino terminus of the glycoprotein. The relationship between the amino acids at positions 152–156 and the remainder of the glycoprotein remains unclear, however, as substitutions in this region may alter function through steric modulation of target sites located elsewhere or may represent substitutions in the target site itself.

A number of models have been used to account for the high degree of polymorphism and diversity characteristic of major histocompatibility antigens. One suggests that the appearance of new antigens is due to inheritable regulatory changes in a battery of germ line-encoded genes present in the genomes of all individuals (30). Another hypothesis involves the classical concepts of sequence duplication followed by divergence due to point mutation (31). If the *H-2K*^{b} variants are the result

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                    91                                  100                                 110
Kb (C1.4.1)         Gly Ser His Thr Ile Gln Val Ile Ser Gly Cys Glu Val Gly Ser Asp Gly Arg Leu Leu
                    GC TCT CAC ACT ATT CAG GTG ATC TCT GGC TGT GAA GTG GGG TCC GAC GGG CGA CTC CTC

Kbm1 (E107.1.1)     GC TCT CAC ACT ATT CAG GTG ATC TCT GGC TGT GAA GTG GGG TCC GAC GGG CGA CTC CTC
                    Gly Ser His Thr Ile Gln Val Ile Ser Gly Cys Glu Val Gly Ser Asp Gly Arg Leu Leu

                                                120                                 130
Kb                  Arg Gly Tyr Gln Gln Tyr Arg Tyr Asp Gly Cys Asp Tyr Ile Ala Leu Asn Glu Asp Leu
                    CGC GGG TAC CAG CAG TAC GCC TAC GAC GGC TGC GAT TAC ATC GCC CTG AAC GAA GAC CTG

Kbm1                CGC GGG TAC CAG CAG TAC GCC TAC GAC GGC TGC GAT TAC ATC GCC CTG AAC GAA GAC CTG
                    Arg Gly Tyr Gln Gln Tyr Arg Tyr Asp Gly Cys Asp Tyr Ile Ala Leu Asn Glu Asp Leu

                                                140                                 150
Kb                  Lys Thr Trp Thr Ala Ala Asp Met Ala Ala Leu Ile Thr Lys His Lys Trp Glu Gln Ala
                    AAA ACG TGG ACG GCG GCG GAC ATG GCG GCG CTG ATC ACC AAA CAC AAG TGG GAG CAG GCT

Kbm1                AAA ACG TGG ACG GCG GCG GAC ATG GCG GCG CTG ATC ACC AAA CAC AAG TGG GAG CAG GCT
                    Lys Thr Trp Thr Ala Ala Asp Met Ala Ala Leu Ile Thr Lys His Lys Trp Glu Gln Ala

                                                160                                 170
Kb                  Gly Glu Ala Glu Arg Leu Arg Ala Tyr Leu Glu Gly Thr Cys Val Glu Trp Leu Arg Arg
                    GGT GAA GCA GAG AGA CTC AGG GCC TAC CTG GAG GGC ACG TGC GTG GAG TGG CTC CGC AGA
                        **      **  **
Kbm1                GGT GCT GCA GAG TAT TAC AGG GCC TAC CTG GAG GGC ACG TGC GTG GAG TGG CTC CGC AGA
                    Gly Ala Ala Glu Tyr Tyr Arg Ala Tyr Leu Glu Gly Thr Cys Val Glu Trp Leu Arg Arg

                                                180
Kb                  Tyr Leu Lys Asn Gly Asn Ala Thr Leu Leu Arg Thr
                    TAC CTG AAG AAC GGG AAC GCG ACG CTG CTG CGC ACAG

Kbm1                TAC CTG AAG AAC GGG AAC GCG ACG CTG CTG CGC ACAG
                    Tyr Leu Lys Asn Gly Asn Ala Thr Leu Leu Arg Thr
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FIG. 4. DNA sequence comparison of the exon encoding amino acids 91–182 from the K^b and K^{bm1} genes. DNAs and inferred protein sequences from both genes are shown. *, Difference between the sequences. The K^b DNA sequence is from refs. 10 and 23.

of a regulatory switch, both parent and variant genes must be present in the genome. This has been shown not to be the case for the *bm1* strain by hybridization analysis of *Pst* I-digested DNA, a procedure that can distinguish the K^{bm1} from the K^b gene because of an additional *Pst* I site in the K^{bm1} gene. This analysis showed the absence of the parental configuration in the DNA from the variant (7). Further, the variant gene appears to be derived from the parent gene because the K^{bm1} and K^b clones are not only identical in the coding regions determining the external domains (except for the mutation site in exon III coding for amino acids 152–156) but also extremely homologous over the entire 10-kb DNA segment (Fig. 3).

The concept that diversity is due to cumulative point mutations is unlikely for the K^b variant series because the amino acid changes in eight variants studied at the protein level could not have arisen by single point mutations and most of the variants contain complex substitutions requiring several clustered nucleotide base changes (5). Particularly relevant in this regard is the finding of seven nucleotide changes in the stretch of 13 nucleotides in the K^{bm1} variant gene.

We have recently proposed that a copy mechanism analogous to gene conversion in yeast may be operating in the MHC to generate the spontaneous variants seen in the H-2K^b series (7). Such a postulate has also been suggested for the MHC from other data (21, 32, 33). Several characteristics of the mutants support the copy mechanism hypothesis: (*i*) Variant amino acids in mutant *K* products are also present at homologous positions in other class I glycoproteins; (*ii*) the amino acid substitutions are multiple and clustered, requiring several nucleotide changes; (*iii*) the complex changes are arrayed in the same configuration as in other class I sequences; and (*iv*) the changes have been found repeatedly in independent isolates. The possibility of such findings being explained by single recombinations seems unlikely in view of the fact that each of the variant molecules has been found to be identical in structure to the parent K^b molecule amino and carboxyl terminal to the altered site.

These types of changes could result from a double recombination event occurring over short distances. However, we believe that it is more likely that a copy mechanism has introduced multiple base substitutions as a block into the K^b gene. This assumes that this same configuration of nucleotides is present elsewhere in the genome to serve as a donor, a distinct possibility in view of the multiple copies of class I genes (e.g., at least 36 genes in the $H\text{-}2^d$ haplotype) (34) known to exist in the genome. The stretch of 13 nucleotides containing the seven changes that we have identified in the K^{bm1} gene is identical to the homologous stretch in at least two other class I genes, L^d (21) and 27.5 (22), that have been identified in the $H\text{-}2^d$ haplotype. Therefore, these potential sequences exist in the mouse population.

The analysis at the DNA level extends our earlier biochemical studies on the *bm1* mutant (20). Whereas changes at amino acid positions 155 and 156 were detected in studies on the glycoproteins, and have been confirmed here, the alteration at amino acid position 152 was not detected because of technical limitations of the comparative peptide analysis and partial sequence approach (see discussion in ref. 5). The complete sequence of the DNA encoding the external region of K^{bm1} showed that only the three noted amino acid substitutions are present in the product, indicating the importance of the discrete changes in the amino acids in positions 152–156 in influencing the recognition of K^b by T cells.

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