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Genetics

Spontaneous *H-2* mutants provide evidence that a copy mechanism analogous to gene conversion generates polymorphism in the major histocompatibility complex

(protein sequence/restriction endonuclease analysis/genetic hypothesis)

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ABSTRACT **The analysis of H-2K products from spontaneously generated major histocompatibility complex (MHC) mutants and of the primary structure of other class I antigens suggests the genetic hypothesis that diversity in the MHC results from a copy mechanism analogous to gene conversion. The hypothesis was tested by making precise structural predictions about three partially characterized MHC mutants (bm1, bm3, and bm8). The predictions were based on consensus sequences among class I genes that differ from *H-2K*^{b} in the same region of the molecule as do the *K*^{b} mutants. In two cases (bm3 and bm8) we successfully predicted the correct amino acid substitution at positions known to be altered but for which the specific nature of the substitution had not been determined. In two additional cases (bm1 and bm8) we predicted and found both new mutation sites and the specific amino acid substitutions. The positions and identifications of the variant amino acids were determined by radiolabeled amino acid sequence analysis and DNA restriction endonuclease analysis. The interaction of MHC genes through a copy mechanism to generate diversity permits the introduction of multiple nucleotide base substitutions into class I sequences by a single genetic event. Such a mechanism may account in part for the large structural divergence among alleles of MHC loci and the high degree of MHC polymorphism among wild mice.**

The class I transplantation antigens of the mouse, *Mus musculus*, are dimers made up of a 45,000 M_r polymorphic glycoprotein encoded in the major histocompatibility complex (MHC) and a noncovalently associated 12,000 M_r non-MHC-encoded protein (1). The extensive polymorphism among the products of the genes of the MHC is one of the mysteries of mammalian genetics. Over 50 alleles of the *H-2K* locus and 30 alleles of the *H-2D* locus have been found in laboratory and wild populations (2). These loci are among the most polymorphic genetic sequences known that produce functional protein products. Other mammalian species, including humans, exhibit a similar level of diversity among their MHC products (3, 4). Although other multigene families have been identified (5–7), none contain the high degree of polymorphism exhibited among alleles of the MHC genes.

Several models explain polymorphism in the MHC. One proposal, a regulatory hypothesis, suggests that all class I MHC genes, represented by a limited set of polymorphic variants, are present in the genomes of all individuals (8, 9). Diversity among cell surface antigens is achieved by a form of regulation that involves inheritable control of gene expression. A second hypothesis involves classic concepts of sequence duplication followed by divergence through point mutations (2). A modification of this hypothesis requires an extraordinarily high frequency of point mutation to account for the extensive diversity observed in this multigene family. Gene rearrangement similar to that observed during somatic differentiation of cells in the B cell lineage (10) has been ruled out by the absence of rearrangement among MHC sequences in differentiated cells (11). Both hypotheses are compatible with the large number of class I gene sequences identified by recombinant cosmids (12).

Our studies on the class I antigens of spontaneously generated MHC mutants are providing insight into the nature of a mechanism that generates change among class I MHC genes and that may contribute significantly to the extensive polymorphism seen in the system. The H-2K glycoproteins from 12 of the *H-2K*^{b} mutants have been analyzed biochemically (13–16), delineating limited structural differences between the parent and variant K^b molecules. Amino acid substitutions are confined to small regions of the glycoproteins. The variants did not arise by single point mutations because complex substitutions require several clustered nucleotide base changes. The finding that each of the variant molecules is identical in structure to the parent K^b molecule amino- and carboxy-terminal to the altered site is incompatible with a single recombination. However, a specialized double recombination event cannot be excluded.

Four characteristics of the *H-2K*^{b} mutants suggest that a copy mechanism analogous to gene conversion is operating to generate polymorphism in the MHC. First, variant amino acids in mutant H-2K glycoproteins are also present at homologous positions in other class I glycoproteins. Second, the amino acid substitutions in the mutants are clustered changes requiring multiple base substitutions. Third, the complex changes are arrayed in the same configuration in other class I sequences. Fourth, identical complex mutations have occurred repeatedly, because in a number of independently isolated mutants the same substitution patterns were found.

To test the hypothesis that polymorphism is generated by the interaction of existing gene sequences within the MHC, we made two types of predictions about the primary sequences of the *H-2K*^{b} mutants. The analyses presented below verified each prediction, supporting the hypothesis of gene interaction. Specifically, for the mutants bm3 and bm8, for which amino acid alterations had been previously localized but the precise nature not determined, the correct amino acid substitutions were predicted and found. In the H-2K molecules from the mutants bm1 and bm8, in which previously characterized substitutions were present, additional changes were predicted and found.

MATERIALS AND METHODS

Mice. All mice were maintained at Northwestern University Medical School (Chicago) by R. Melvold, and each mouse skin

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Abbreviations: MHC, major histocompatibility complex; kb, kilobase(s).

was grafted prior to use to establish the integrity of the mutation indicated by its pedigree (17).

Radiolabeling, Isolation, and Analysis of H-2K Glycoproteins and Glycopeptides. Spleen cells were labeled metabolically in culture and solubilized in Nonidet P-40 (18, 19). The glycoproteins were isolated, immunoprecipitated (20), and size fractionated on either a $NaDodSO_4$/Bio-Gel A-0.5m column (18) for tryptic digestion or on a Sepharose CL-6B column in 6 M guanidine·HCl for amino-terminal sequence analysis. The reduced and alkylated partially purified H-2K glycoproteins were digested with trypsin and applied to a cation-exchange column (18). The glycopeptides that elute in the void volume were isolated from each other by Sephadex G-50 column chromatography in 1 M HCOOH. Intact reduced and alkylated H-2K molecules were isolated by Sepharose CL-6B molecular sieve column chromatography in 6 M guanidine·HCl. The 44,000 M_r pool was desalted sequentially by Sephadex G-15 and Sephadex G-50 chromatography in 1 M HCOOH. The amino acid sequences of the intact molecules or peptides were determined as described (21, 22).

Analysis of DNA with Restriction Endonucleases. High molecular weight DNA was isolated from spleen cells as described (23). The DNA was digested to completion with the endonuclease *Pst* I (Bethesda Research Laboratories) under conditions recommended by the supplier and analyzed by hybridization to the MHC class I cDNA probe pH-2.III (11). The probe was nick-translated with DNA polymerase I (Boehringer Mannheim). Incorporation of [^{32}P]dCTP yielded a specific activity greater than 10^8 cpm/μg of DNA. Hybridization and washing conditions have been described (23).

RESULTS

Predictions. We have used the available structural data for MHC class I sequences (Fig. 1) to predict the location and nature of amino acid substitutions in three K^b mutants. Where peptide analysis or partial sequence analysis has localized the region of change in the mutant glycoproteins, specific predictions concerning the alterations can be derived from the panel of class I sequences by the assumption that the mutants arose from an interaction between the K^b gene and a similar set of genes. This hypothesis predicts specific changes in the K molecules of mutants bm3 (Lys to Ala at amino acid position 89), bm8 (Met to Ile and Glu to Ser at amino acid positions 23 and 24, respectively) and bm1 (Glu to Ala at amino acid position 152).

Ala at Amino Acid Position 89 in K^{bm3}. Previous structural studies on the mutant molecule K^{bm3} revealed that the parental H-2K^b Lys residue present at position 89 was replaced in the mutant molecule (14). The hypothesis that the changes resulted by a copy mechanism was used to predict that Ala was substituted for Lys at amino acid position 89 in the mutant bm3 (see *Discussion* and Fig. 1).

Spleen cells from bm3 were labeled with [^{3}H]alanine. The K^{bm3} molecules were immunoprecipitated, reduced and alkylated, and isolated by $NaDodSO_4$/Bio-Gel column chromatography. The 44,000 M_r glycoproteins were digested with trypsin and the resulting peptides were separated on a cation-exchange column, developed with a pyridine acetate gradient. There are two glycosylated peptides in the K^b and K^{bm3} molecules that elute in the first few fractions. In the K^b molecule they are of similar size and therefore difficult to resolve by molecular sieve chromatography. The substitution for Lys at position 89 in K^{bm3} eliminates a tryptic cleavage site and extends one peptide from 10 to 29 amino acids in length. The [^{3}H]alanine-bm3 glycopeptides were resolved by chromatography on a Sephadex G-50 column. The position of [^{3}H]alanine was determined for both peptides (peaks A and B) by automated Edman degradation (Fig. 2). Peak A contained [^{3}H]alanine at amino acid position 10 and peak B contained the labeled amino acid at position 4. In the parental K^b molecule, Ala was present in only one of the glycopeptides (amino acid positions 174–181, see Fig. 1). That peptide eluted at the same position as peak B in Fig. 2 and contained Ala at position 4. Because Lys at position 89 is the tenth amino acid in the glycopeptide in the K^b parent (which contains

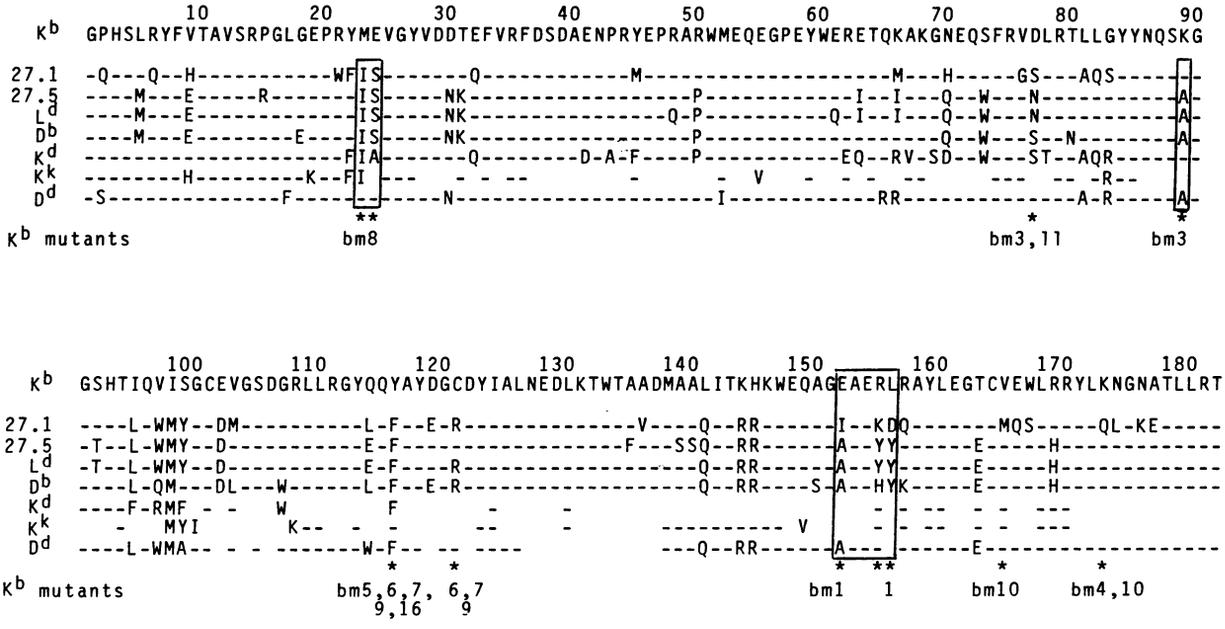

FIG. 1. Comparative sequences of class I molecules. The standard one-letter code for amino acids is used. Amino acids for positions 1 through 180 of eight class I sequences derived from protein and DNA sequence studies are presented. The sequence data were determined previously as follows: K^b (1); the *Qa* region pseudogene 27.1 (24); the L region genes from the *d* haplotype 27.5 (25) and L^d (26) and D^d (19, 27); K^d (28); D^b (29, 30); and K^k (31). Amino acid sequence identity with K^b is indicated by the hyphens. The positions of the K^b mutations are indicated (*), and the particular bm mutant is noted below the asterisk. The boxed sequences are those studied in the present paper.

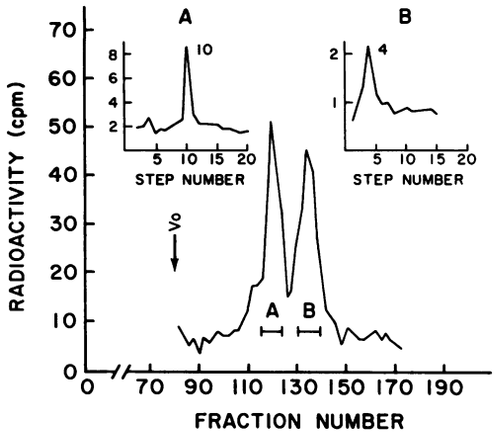

FIG. 2. Isolation of bm3 glycopeptides and sequence analysis. The tryptic glycopeptides from the [^{3}H]alanine-labeled K^b antigen were recovered from the breakthrough peak from a cation-exchange column and fractionated by Sephadex G-50 chromatography (220 × 1.0 cm) in 1 M HCOOH, eluted at 7 ml/hr. The glycopeptides (A and B) were pooled separately and the amino-terminal sequence was determined by automated Edman degradation for the position of [^{3}H]alanine (see *Insets*).

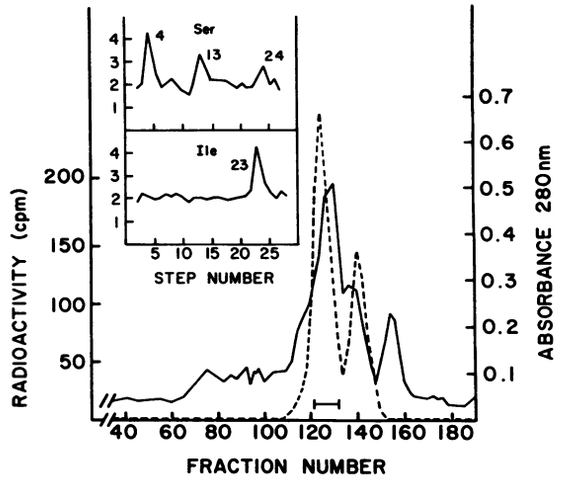

FIG. 3. Isolation and amino-terminal sequence analysis of K glycoproteins from bm8. The [^{3}H]isoleucine- and [^{3}H]serine-labeled K^{bm8} glycoproteins were reduced and alkylated and separated from high molecular weight viral contaminants and low molecular weight β_2-microglobulin by fractionation on a Sepharose CL-6B column (195 × 2.5 cm) equilibrated in 6 M guanidine·HCl, eluted at 7 ml/hr. The elution profile of the [^{3}H]serine fractionation is shown as a representative profile. ——, Radioactivity; – – –, A_{280}. The position of the radiolabeled amino acids relative to the amino terminus was determined by automated Edman degradation (see *Inset*).

no Ala), the presence of Ala at this position indicated a Lys to Ala substitution in the bm3 glycopeptide.

Ile and Ser at Amino Acid Positions 23 and 24, Respectively, in K^{bm8}. Previous structural studies on the K antigen from mutant bm8 identified the loss of Met at position 23 (unpublished data). By using the same logic as described for K^{bm3}, an Ile was predicted as the new amino acid at position 23 in K^{bm8}. The hypothesis predicted further a second undetected adjacent change at residue 24 where Ser is substituted for Glu. K^{bm8} immunoprecipitates were reduced and alkylated and fractionated on a Sepharose CL-6B column. The 44,000 M_r molecules were isolated and the positions containing [^{3}H]isoleucine and [^{3}H]serine were identified (Fig. 3) by sequence analysis from the amino terminus of the glycoprotein. Ile was present at residue 23, indicating a Met to Ile interchange in K^{bm8}. Ser was present at positions 4 and 13 in the parent K^b molecule but was detected at positions 4, 13, and 24 in K^{bm8}, indicating a Glu to Ser substitution at position 24 in the mutant molecule.

Ala at Amino Acid Position 152 in K^{bm1}. Previous structural studies on the K molecule of the mutant bm1 identified an Arg-Leu to Tyr-Tyr substitution at amino acid positions 155 and 156 (15). The Tyr-Tyr doublet is found at homologous positions in the L^d glycoprotein (32) but not in other known MHC sequences (Fig. 1). In two independent isolates of clones encoding L^d-like molecules from the *d* haplotype (25, 26), the sequence encoding Tyr-Tyr (155–156) is coupled to a sequence encoding Ala for amino acid position 152, whereas Glu is present at this position in K^b. We proposed that the genetic sequence in the bm1 haplotype might be similar to the L^d sequence, and that the copy mechanism could also have substituted Ala for Glu at position 152. Such a change would introduce a new *Pst* I endonuclease site into the *H-2K* gene of the bm1 mutant. In a hybridization analysis of *Pst* I-digested splenic DNA (Fig. 4), all of the class I gene sequences homologous to the cDNA probe pH-2.III (11) are identical except for the single difference of a band present in B6/Kh and bm6 DNA at 2.0 kilobases (kb) but missing from bm1 DNA.

In recent studies, the presence in the gene of the *Pst* I site at nucleotides corresponding to amino acid positions 152 through 154 has been confirmed in the analysis of genomic clones of the K^{bm1} gene (unpublished data). A unique smaller band of 1.6 kb is present in bm1 DNA. The analysis of the genomic clones verified the *H-2K*^{b} origin of the banding differences observed in the genomic digests. The presence of a new 1.6-kb band in the genomic bm1 digest (Fig. 4) is obscured by

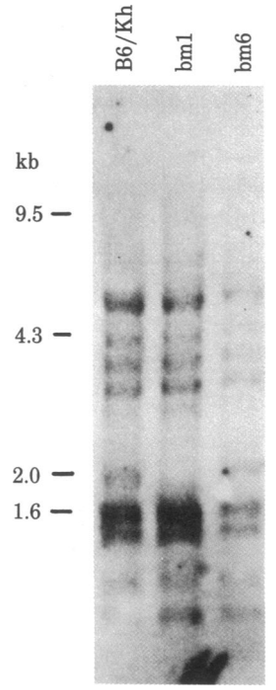

FIG. 4. *Pst* I restriction analysis of B6/Kh, bm1, and bm6 splenic DNA. Digested genomic DNAs were fractionated on a 1.2% agarose gel in 40 mM Tris acetate, pH 7.9/1 mM EDTA for 24 hr at 40 V/hr. The DNAs were transferred (23) to nitrocellulose paper and analyzed for class I sequences by hybridization to the nick-translated probe pH 2.III (11). The positions of selected DNA markers from λ phage DNA digested with *Hin*dIII and the plasmid pBR322 digested with *Hin*fI are shown.

other class I *Pst* I fragments of similar size.

The prediction of this new endonuclease site is tantamount to prediction of Ala at position 152 because the Glu in K^b is encoded by the codon GAA (33) and Ala in the L^d sequence is encoded by GCT. The second and third bases of the codon are the predicted base changes for the amino acid substitution Glu to Ala and both are specifically required as the first two bases in the *Pst* I recognition site. The remaining four bases (G-C-A-G) for the *Pst* I recognition site (C-T-G-C-A-G) are common to the K^b and L^d sequences and encode Ala at position 153 and part of the codon for Glu at position 154.

DISCUSSION

The pattern of substitutions detected in the spontaneous *H-2K*^{b} mutants relative to the primary amino acid sequences of eight class I molecules strongly suggests a hypothesis that not only accounts for the published data on the spontaneous mutants but also has permitted successful predictions of additional structural alterations. Four characteristics relate the K^b mutants to the class I sequences listed in Fig. 1. (*i*) In all cases in which an amino acid substitution has been characterized in a K^b mutant, the identical variant amino acid is present at a homologous position in another member of the class I family. The Tyr-Tyr substitution at positions 155 and 156 in K^{bm1} can be seen in the L^d molecules shown in Fig. 1. Similarly, the substitutions at position 116, Tyr to Phe in the K molecules of bm5, -6, -7, -9, and -16, and position 121, Cys to Arg in the K molecules of bm6, -7, and -9, members of the "bg series," are present in numerous other class I sequences. The Met substitution in K^{bm10} at position 165 occurs in the sequence of *Qa*-pseudogene clone 27.1. (*ii*) A majority of the mutants contain multiple substitutions. K^{bm1} has two adjacent changes at positions 155 and 156; K^{bm3}, two changes at positions 77 and 89; the K molecules from bm6, -7, and -9, two changes at positions 116 and 121; and K^{bm10}, two changes at positions 165 and 173. (*iii*) The multiple substitutions in the K^b mutants are found in the same array as they exist in the other class I sequences. The change to Tyr-Tyr at positions 155 and 156 in bm1 is found in both L^d sequences. Other class I molecules differ from K^b at positions 77 and 89, as found in bm3. The pseudogene 27.1, L^d, and D^b class I sequences contain Phe at 116 and Arg at 121 as found in the "bg series" sequences. The amino acid positions 165 and 173 in the pseudogene 27.1 match the position of changes in K^{bm10}. (*iv*) These mutants, although spontaneous and independent, are not random. Of the K molecules from 12 independently derived K^b mutants (13) 5 are known to contain identical substitutions at amino acid position 116. Three members of this group share multiple substitutions at positions 116 and 121 (13). Two other sets of mutants also share alterations at homologous amino acid positions, bm3 and bm11 (14) and bm4 and bm10 (ref. 16; unpublished data).

The studies described in the present report were designed to test the hypothesis that genetic sequences already present in the MHC are used by a mechanism analogous to gene conversion to introduce variation among class I genes. We have predicted changes in the K molecules of three mutants, bm3, bm8, and bm1, on the basis of a sample of class I gene sequences that are presumably representative of class I gene sequences in the *b* haplotype. Our previously published studies on K^{bm3} documented substitutions at amino acid residues 77 and 89, although the nature of the changes were not determined. As shown in Fig. 1, a majority of class I sequences differ from K^b at both of these residues. We predicted that Ala might be present at position 89 because all the MHC sequences that differ from K^b contain Ala at that position. A substitution of Ala for Lys would require a complex mutation of two base changes. As shown in Fig. 2, we identified Ala at position 89. The amino acid substitutions in the K^{bm3} must have resulted from at least three nucleotide base changes.

The known loss of Met at position 23 in K^{bm8} can be used to make a similar prediction. Six of the seven MHC sequences, excluding K^b (shown in Fig. 1), contain Ile at position 23, and, as shown in Fig. 3, we have found that Ile is also present in K^{bm8}. The previous protein analysis, which led to the identification of a change at amino acid position 23 in K^{bm8}, used a comparative approach that could uncover differences between parental and mutant peptides but could not determine the nature or extent of amino acid disparity (ref. 14, unpublished data). Single or multiple alterations would be indistinguishable. If a copy mechanism generated the Ile substitution at position 23, other tightly linked changes may have occurred in the same event. Most of the MHC molecules that contain Ile at position 23 also have Ser at position 24. A copy mechanism that substituted Ile for Met at position 23 in K^{bm8} might also have substituted the adjacent Ser for Glu at residue 24. We found that Ser is present at position 24 in K^{bm8} (Fig. 3). Thus, the hypothesis of a copy mechanism operating in the MHC allowed us to predict successfully in K^{bm8} not only the correct substitution at a site where a known change existed, but also the presence and nature of an additional substitution. These two amino acid substitutions would require a minimum of three base changes. Because three point mutations (one at amino acid position 23 and two at position 24) would not be consistent with known spontaneous mutation rates and the fact that bm8 occurred spontaneously in a single generation (17), this finding supports our hypothesis that a copy mechanism must be operating.

The most compelling argument for a copy mechanism stems from our analysis of the mutant bm1. For this case at least seven base changes must be invoked to explain the known and predicted substitutions. Protein analysis revealed a complex substitution at amino acid residues 155 and 156 (14). Five base changes are required to elicit the Arg-Leu to Tyr-Tyr substitution characterized in this mutant. The only class I molecules, aside from K^{bm1}, that are known to contain this substitution are the two L^d sequences determined by Moore *et al.* (25) and Evans *et al.* (26). Both molecules also contain a third tightly coupled difference from K^b at position 152 (Ala in L^d, Glu in K^b; see Fig. 1). The presence of Ala at position 152 in the context of the K^b genetic sequence (33) would create a new endonuclease site for the enzyme *Pst* I. We detected this change by hybridization analysis (Fig. 4), indicating that K^{bm1} contains an array of at least seven tightly linked base changes that closely resemble sequences in other class I genes. This finding also represents a second example of the prediction of a mutation site and the nature of the substitution according to the copy mechanism hypothesis.

The relationship of the spontaneous mutants detected in the laboratory to polymorphism characteristic of the genes of the MHC in wild populations has not been established clearly. The structural analysis of alleles of the *H-2* complex yielded two surprising results. First, alleles differ from each other by approximately 20% in amino acid sequences and, second, at least among alleles of the *K* and *D* loci, there is no apparent "*K*-ness" or "*D*-ness" (1). Alleles of *K* were as related to alleles of *D* as they were related among themselves. The hypothesis that a copy mechanism is operating in the MHC offers a way to generate both phenomena. The complex changes characteristic of the *H-2K*^{b} mutants provide an example of how multiple amino acid substitutions occur in a single event. This could be responsible for accelerating divergence among alleles, leading to differences as large as 20% (1). Because genetic sequences are

transferred among genes, the origin of point mutations (*K*-ness or *D*-ness) would be obscured. This could lead to the phenomenon of "homogenization" of homologous class I sequences among this gene family.

The hypothesis that conversion plays a role in the generation of polymorphism in the MHC is compatible with the principles underlying earlier attempts to explain this phenomenon. The major tenet of the duplication–divergence model is that the source of polymorphism is point mutation. Gene conversion, a mechanism for rapid rearrangement of genetic sequences, relies on duplication and divergence as the original source of variation. Diversity at the protein level could be achieved by a conversion event involving short stretches within genes, resulting in multiple, clustered substitutions, in an array already present elsewhere in the genome. A multigene family the size of the MHC (20–40 genes) would provide a repertoire of genetic sequences for such events. Models based on gene conversion or regulation rely on inheritable changes affecting the expression of MHC genetic sequences, and, in this sense, the conversion of a sequence in an active gene by an unexpressed pseudogene would be similar to germ-line regulation. However, fewer genes in the repertoire are necessary to generate the diversity characteristic of the MHC by gene conversion than by a germ-line repertoire containing each of the variant genes.

Gene conversion has yet to be conclusively documented in mammalian species, largely due to the absence of tetrads in mammalian reproductive cycles. Configurations among genes in various mammalian multigene families suggest that an event analogous to gene conversion in yeast takes place in mammals too (26, 34–38). The mutants of the MHC represent a unique example of these genetic events in that the lineage of the mice is known, permitting the resolution of the time frame governing the appearance of the variant (one generation), and in that the appropriate mouse lines are available and provide both the parental and mutant genetic configuration for tracing the origins of the conversion event.

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