MOLECULAR CHARACTERIZATION OF THE
C3HfB/HeN H-$2K^{km2}$ MUTATION

Implications for the Molecular Basis of Alloreactivity

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The class I histocompatibility (H-2) antigens, H-2K, H-2D, and H-2L, encoded by the murine MHC, are integral membrane surface glycoproteins that mediate the recognition and elimination of virally infected and foreign cells by the immune system (1, 2). H-2 antigens mediate two apparently different immune recognition phenomena. CTL recognition of cell-associated viral antigens in the context of particular self H-2 molecules is the basis for H-2 restriction (2), whereas recognition of nonself H-2 antigens alone appears responsible for the alloreactive response that results in tissue graft rejection between histoincompatible individuals. However, recent studies demonstrating that some H-2 restricted, virus-specific CTL can cross-react with nonself H-2 antigens (3), and also that CTL apparently recognize both viral and nonself H-2 antigens as processed peptides in the context of self H-2 (4–7), indicate that H-2 restriction and alloreactivity may result from a common mode of CTL recognition.

One of the most remarkable features of H-2 antigens is their extensive polymorphism. More than 100 serologically distinct H-2K and H-2D molecules have been identified among wild and inbred mouse populations (8), and molecular genetic analysis has revealed that such antigenic polymorphism derives largely from sequence variation among multiple H-2 genes (1, 9). Sharing an underlying 80–99% DNA sequence similarity, the H-2 genes, alleles as well as nonalleles, are highly diverse within an individual, as well as extremely polymorphic among members of a population (8). This degree of H-2 polymorphism is thought to result from selective pressure; since these antigens serve as foreign antigen presentation structures, the generation of new H-2 sequences would ensure a large and expandable repertoire of H-2 restriction elements that could present a virtually limitless number and variety of foreign antigens to the immune system, thereby assuring survival of the population. In contrast to the H-2 genes, other MHC class I genes, denoted Qa and Tla, are relatively nonpolymorphic, and their function is not yet known (10, 11).

Characterization of in vivo *H-2* mutants has provided evidence for molecular mechanisms by which new H-2 sequence diversity might be generated. In several spontaneous H-2K^b mutations, for example, a single cluster of multiple nucleotide changes results in a cluster of amino acid substitutions in the extracellular portion of the encoded molecule (12–16). In most cases, the substituted sequence of the mutant gene could be found intact at the same position in at least one other class I locus within the same strain (14, 16, 17), leading to the suggestion that such mutations arise from a nonreciprocal intergenic transfer of sequence information (13, 15, 16, 17). It is important to stress, however, that both the apparent lack of outside marker exchange and the inability to examine all products of a single meiosis in mammalian systems preclude use of a bona fide gene conversion mechanism to explain such *H-2* mutations. Aside from the *H-2K^b* mutants, only one other *H-2K* locus mutation has been characterized to date. This gene, CBA *H-2K^{km1}*, exhibits a single nucleotide difference relative to AKR *H-2K^k* (18, 19). Although a gene conversion mechanism was invoked, it seems just as likely that this mutation could have arisen by a point mutation not involving recombination.

To date, nearly all in vivo *H-2* mutants have been selected by skin graft incompatibility with the parental strain (14, 20, 21). An apparent spontaneous mutation at the *H-2K* locus in the C3HfB/HeN mouse, however, was identified by the growth behavior of syngeneic transplanted tumors. The C3HfB/HeN (C3Hf) and C3H/HeN (C3H) mouse strains, concurrently derived from the same C3H population and maintained separately by full sibling matings, were thought to be genetically identical except for the absence of mammary tumor virus (MTV)[1] in C3Hf (22). Transplantation studies with C3Hf-derived tumors (23), however, indicated that C3Hf had acquired a heritable alloantigenic alteration that mapped to the K end of the MHC (24, 25). Skin grafts between C3H and C3Hf mice were found to be reciprocally rejected (22), and both C3H anti-C3Hf and C3Hf anti-C3H CTL could be generated (26). Since this altered C3Hf H-2K^k phenotype appeared to have reverted back to that of the parental C3H strain in several C3Hf tumors, the C3Hf strain was thought to be unique among all other known *H-2* mutants, and for this reason, C3Hf was given the haplotype designation, H-2^{kv1} (variant 1) (27). Although peptide map analysis revealed limited structural alterations in the C3Hf H-2K^k polypeptide (28), the molecular basis for the observed phenotypic differences between C3Hf and C3H remained obscure.

Here, we report the molecular genetic analysis of the C3Hf *H-2K^{kv1}* gene, which we have redesignated *H-2K^{km2}* based upon our demonstration that C3Hf fulfills the requirements for a mutant haplotype (20). We demonstrate not only that the third exon of *H-2K^{km2}* bears a single clustered substitution of nucleotides not found in any other *H-2* mutant to date, but also that this mutant-specific sequence is present intact in the *H-2D^k* gene of both C3H and C3Hf, features that are consistent with a nonreciprocal sequence transfer mechanism. The *H-2K^{km2}* mutation encodes a cluster of three amino acid substitutions that map to the bottom of the peptide-binding cleft (29) of the H-2K^k molecule, in one of the most polymorphic β strand regions among H-2 antigens (30). The biological implications of the deduced structural changes in C3Hf H-2K^{km2} are discussed, particularly with respect to the possible molecular basis of H-2 alloantigenicity.

Materials and Methods

Mouse Strains. C3H/HeN (C3H) and pedigreed C3HfB/HeN (C3Hf) mice were obtained directly from Dr. W. Heston at the animal production unit, National Institutes of Health (NIH), Bethesda, MD. Different C3HfB/HeN colonies are not genetically identical[2]. Although all are derived from C3H and are foster nursed on MTV⁻ strains, only one C3Hf colony that we examined exhibited the H-2^{km2} mutant phenotype. At least one other colony that we examined carries the H-2^k phenotype of the parental C3H strain. The bona fide H-2^{km2} mutant mouse strain is now held by Dr. W. John Martin at the University of Southern California Medical School (Los Angeles, CA). This strain is maintained by full sibling matings and is checked periodically for histoincompatibility with C3H.

Biochemical Analysis of H-2K^k Molecules. Peptide maps and two-dimensional (2D) gels were generated as described previously (32, 33). Briefly, ∼5 × 10^7 C3H and C3Hf splenocytes were cultured separately in 0.5 mCi of [^{3}H]leucine (New England Nuclear, Boston, MA) for peptide mapping as well as for 2D gel analysis. Radiolabeled H-2K^k molecules were isolated by immunoprecipitation using the mAb H100-5.28 (34).

Library Construction and Screening. High molecular weight DNA from C3Hf H-2^{km2} spleen was prepared by standard methods. Poly(A)$^+$ RNA was isolated from C3Hf splenocytes (35) immediately after a 48-h treatment in culture with recombinant murine IFN-γ (Schering Corp.). Approximately 100 U of IFN-γ was added per 5 × 10^5 freshly removed spleen cells (100 U/ml), maintained in culture in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), antibiotics, and 2 mM glutamine before preparation of the RNA. The genomic and cDNA libraries were constructed by Stratagene Cloning Systems (Stratagene, San Diego, CA), using randomly sheared 7–10-kb genomic fragments or 0.5–3-kb cDNAs generated by a combination of random- and oligo-dT priming, respectively. Modified with Eco RI linkers, each was cloned into the Eco RI site of the λZAP vector (Stratagene).

Approximately 80 genomic and 280 cDNA λ clones were obtained from screening 2 × 10^6 and 10^6 recombinant phage, respectively. All clones containing class I sequences were isolated in the initial screen using the general class I probe, pK^{b}Δi3. The pK^{b}Δi3 clone contains the entire *H-2K^b* gene except for a deletion of intron 3, and was generously provided by Dr. R. Bruce Wallace (Beckman Research Institute of the City of Hope, Duarte, CA). Genomic and cDNA clones bearing *H-2K^{km2}* sequences were identified by their hybridization to the *H-2K^k* gene–specific oligonucleotide probe, K^k-TM (5'-GAGCTGCAATAGTCA-CTG-3'), which is homologous to a unique region of the *H-2K^k* transmembrane exon corresponding to amino acid codons 294–300 (19, 36).

Labeling Reactions and Southern Blot Analysis. All double-stranded DNA probes used for library screening and Southern analysis were generated by the random priming method (37). Single-stranded oligonucleotides were 5' end-labeled with γ-[^{32}P]ATP (Amersham Corp., Arlington Heights, IL) and polynucleotide kinase as described (38). Enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN), and were used under the conditions recommended by the suppliers. Restriction digests of genomic and cDNA clones were examined by Southern blot analysis on Zetaprobe nylon membranes (Bio-Rad Laboratories, Richmond, CA) using conditions described previously (39).

RNase A Protection Analysis. Uniformly labeled single-stranded RNA probes were synthesized from linearized DNA templates using the T7 polymerase reaction conditions recommended by the supplier (Promega Biotech, Madison, WI), except that 0.25 µM α-[^{32}P]UTP (400 Ci/mmole; Amersham Corp.) and 62 µM cold UTP were used to generate lower specific activity probes. The RNase A protection method of Myers et al. (40) for cleavage of single

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[2] We have found that the C3HfB/HeN colony maintained by the National Cancer Institute is histocompatible with C3H/HeN and does not exhibit any of the phenotypic differences relative to C3H, as previously observed for C3Hf of the original Heston (NIH) colony. We and others (31) have characterized the mice from the National Cancer Institute (NCI) colony, demonstrating that they carry the wild-type (C3H) *H-2K^k* allele in homozygous form, with no evidence of an *H-2K* coding region mutation (see Discussion).

base mismatches between RNA/DNA duplexes was used, and both sense and antisense RNA probes were tested against the same DNA templates.

DNA Sequencing. The C3Hf *H-2K^{km2}* gene from the genomic clone, pK33, was subcloned directionally as large fragments of 1–2 kb into M13mp18 and mp19. Single-stranded dideoxynucleotide sequencing with α-[^{35}S]dATP (Amersham Corp.), using a panel of internal oligonucleotide primers homologous to evenly spaced regions on both strands of the *H-2K^{k}* gene, has been described previously (41). C3H *H-2K^{k}* was sequenced in parallel with *H-2K^{km2}*, providing for an accurate, pairwise comparison of these two alleles. Except for a large part of intron 3, the entire *H-2K^{km2}* gene was sequenced at least once in each orientation, and exon 3 was sequenced two to three times in each direction. cDNA clones were sequenced by the alkaline denaturation method of double-stranded dideoxynucleotide sequencing (42).

Oligonucleotide Hybridization of Genomic Southern Blots. 5' end-labeled synthetic oligonucleotide probes were used for detection of single-copy genomic sequences (38). Essentially, 10-μg samples of genomic DNA, digested with Pst I and size fractionated on a 1% (wt/vol) agarose gel, were transferred to Genescreen (New England Nuclear) and fixed to the membrane by a 3-min exposure to short-wave UV light (254 nm, 1,200 μW/cm^2) at a distance of 35 cm, as described. Prehybridization for 1 h and hybridization for 16 h were performed at 45°C in 0.5 M NaH$_2$PO$_4$, pH 7.2, 1% (wt/vol) BSA, 7% (wt/vol) SDS. The final wash for each probe (1 × 30 min) was performed at 53°C in 3.2 M tetramethylammonium chloride, 1% SDS.

Results

Biochemical Characterization of the C3Hf H-2K^{km2} Molecule. Previously, peptide map analysis indicated that the C3Hf H-2K^{km2} (H-2K^{kv1}) molecule exhibits limited lysine peptide differences relative to C3H H-2K^{k} (28). To further characterize these structural alterations, we compared H-2K^{km2} with H 2K^{k} by 2D gel electrophoresis. C3HfB/HeN and C3H/HeN splenocytes were separately radiolabeled in culture with [^{3}H]leucine, and their respective H-2K antigens, immunoprecipitated with the H-2K^{k}-specific mAb, H100-5.28 (34), were analyzed on 2D polyacrylamide gels. The gel profiles of the two molecules were found to be virtually identical (data not shown), indicating that whatever structural alterations are borne by H-2K^{km2} do not change the apparent molecular weight or pI of the molecule.

For a more definitive characterization of the structural alteration in C3Hf H-2K^{km2}, additional peptide mapping was performed. Cultured splenocytes of both C3HfB/HeN and C3H/HeN mice again were radiolabeled with [^{3}H]leucine, an uncharged amino acid, and the H-2K^{k} H chains were purified after immunoprecipitation with H100-5.28. Comparison of the tryptic peptide profiles of H-2K^{k} and H-2K^{km2} revealed the gain of a single, distinct peptide in H-2K^{km2} relative to H-2K^{k} (Fig. 1), demonstrating that H-2K^{km2} appears to bear a labeled leucine residue within an analogous tryptic peptide that is unlabeled in H-2K^{k}. No other differences between the molecules were detected. Thus, peptide map analysis predicts a leucine amino acid substitution in the C3Hf H-2K^{km2} H chain.

Characterization of the C3Hf H-2K^{km2} Gene. Because a structural change in the H-2K^{km2} molecule could result either from a change in the coding sequence of the gene or from an alteration in splicing of the primary transcript (43), a genomic and a cDNA library were constructed with mRNA and DNA, respectively, from C3HfB/HeN mice whose sibs were shown to exhibit reciprocal skin graft rejection with C3H/HeN mice. Of the 80 genomic clones bearing class I sequences, one also hybridized strongly to the *H-2K^{k}* gene-specific transmembrane oligonucleotide probe, K^{k}-TM. This genomic clone, designated pK33, was shown to bear the complete *H-2K^{km2}* gene by gross restriction site identity with C3H *H-2K^{k}* (36) (Fig. 2
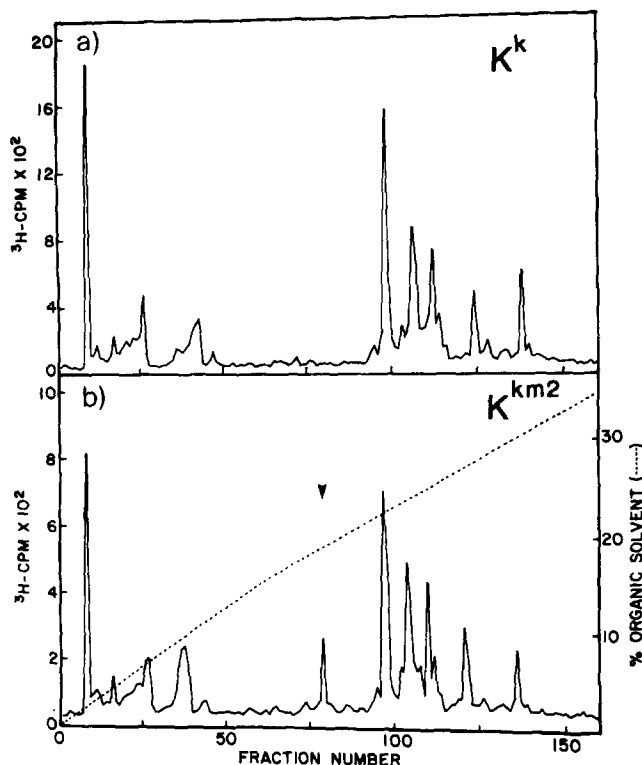
FIGURE 1. [^{3}H]leucine peptide map comparison of C3H H-2K^k (a) and C3Hf H-2K^{km2} (b). The gained peptide in H-2K^{km2} is indicated by an arrow. All other peptides are virtually identical between the two molecules.

a). Approximately 30 independent *H-2K^{km2}* cDNA clones were similarly identified by their hybridization to K^k-TM.

RNase A cleavage analysis with *H-2K^k*-derived probes was performed first to identify potential regions of mutation in *H-2K^{km2}*. Both sense and antisense RNA transcripts derived from pTK^{k}VSp, a subclone that contains all of the 5' exons and introns of *H-2K^k* (Fig. 2 *a*), were used for side-by-side analysis of the *H-2K^{km2}* and *H-2K^k* genomic clones. The DNA templates used were either intact or linearized at the Bss HII site in intron 1; cutting the DNA within the region of homology to the RNA probes allows precise localization of a detected mismatch relative to the known location of the restriction site. Fig. 3 *a* shows that uncut *H-2K^k* DNA (lane *1*) protects the full-length 1.05-kb RNA fragment (although this high molecular weight band is very faint and there are many smaller degradation products), and uncut *H-2K^{km2}* (lane *2*) protects two distinct fragments of 730 and 330 bp. Whereas the expected 235 and 820-bp RNA fragments were produced with Bss HII-linearized *H-2K^k* template (lane *3*), the 820-bp fragment is reduced to two fragments of 330 and 490 bp with Bss HII-cut H-2K^{km2} DNA (lane *4*). Represented schematically in Fig. 3 *b*, a single RNase A sensitive heterology in *H-2K^{km2}* relative to *H-2K^k*, thus, could be localized unambiguously to a point 330 bp from the 3' end of the RNA probe, mapping to within the first few codons of exon 3. No RNase A cleavable mismatches could be detected in the 3' end of *H-2K^{km2}* using probes derived from the 3' region of *H-2K^k* (data not shown). Since RNase A recognizes virtually all
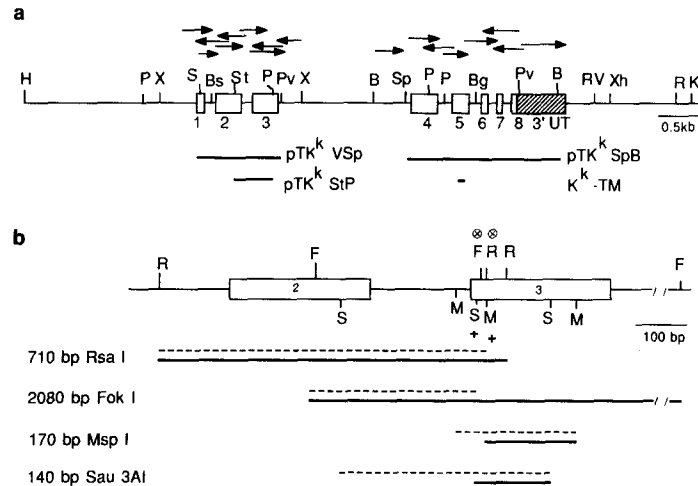
Figure 2. Structure of C3H *H-2K^k* and C3Hf *H-2K^{km2}*. (*a*) Gross restriction site identity between the two alleles. Horizontal arrows depict the strategy used for dideoxynucleotide sequencing of *H-2K^{km2}* with oligonucleotide primers homologous to evenly spaced regions within the gene. Exon numbers are indicated, and below the restriction map is shown the location of the *H-2K^k* subclones used as probes in these studies: pTK^kVSp contains a 1.05-kb Sph I–Pvu II fragment spanning all of the 5' exons and introns of *H-2K^k*; pTK^kStP bears a 0.5-kb Sty I–Pst I fragment spanning the middle of exon 2 through the end of exon 3; pTK^kSpB carries a 2.0-kb Spe I–Bam HI fragment encompassing the far 3' end of intron 3 through the end of the 3' untranslated region; and K^k-TM is an oligonucleotide homologous to part of the transmembrane exon. B, Bam HI; Bg, Bgl II; Bs, Bss HII; H, Hind III; K, Kpn I; P, Pst I; Pv, Pvu II; R, Eco RI; RV, Eco RV; S, Sph I; Sp, Spe I; St, Sty I; X, Xba I; Xh, Xho I. (*b*) Restriction site differences in exon 3 between *H-2K^k* and *H-2K^{km2}*. The Fok I (*F*) and Rsa I (*R*) sites absent from the *H-2K^{km2}* allele relative to *H-2K^k* are indicated (O), as well as the Msp I (*M*) and Sau 3AI (*S*) sites unique to *H-2K^{km2}* (+). The restriction fragments specific to *H-2K^k* (---) and to *H-2K^{km2}* (——) are shown, and the polymorphic restriction fragment sizes in *H-2K^{km2}* are given.

RNA/DNA mismatches of two or more nucleotide pairs, but only less than half of all possible single base pair mismatches (40), the efficient cleavage of both sense and antisense 5' probes when *H-2K^{km2}* DNA was the annealing partner suggested that a cluster of multiple nucleotide differences might be present at this single exon 3 region of *H-2K^{km2}*.

Examination of the published sequence of C3H *H-2K^k* (36) revealed several restriction enzyme sites at the 5'-end of exon 3 that could be polymorphic if *H-2K^{km2}* carried a mutation in this region. In fact, Southern blot analysis with seven different four-base recognition restriction enzymes using 5' and 3' *H-2K^k* probes (see Fig. 2 *a*) demonstrated four restriction enzyme site differences in *H-2K^{km2}*, all clustered within the same region at the 5' end of exon 3. A Fok I and a Rsa I site present in *H-2K^k* are absent in *H-2K^{km2}*, but new Msp I and Sau 3AI sites are present in the same region of the *H-2K^{km2}* gene (Fig. 2 *b*). No other restriction site polymorphisms in either the 5' or the 3' region of *H-2K^{km2}* could be discerned.

DNA sequence analysis was performed in order to define precisely the nucleotide sequence of the *H-2K^{m2}* exon 3 alteration and to verify that this gene bears no other sequence differences relative to *H-2K^k*. Using a series of oligonucleotide primers ho-
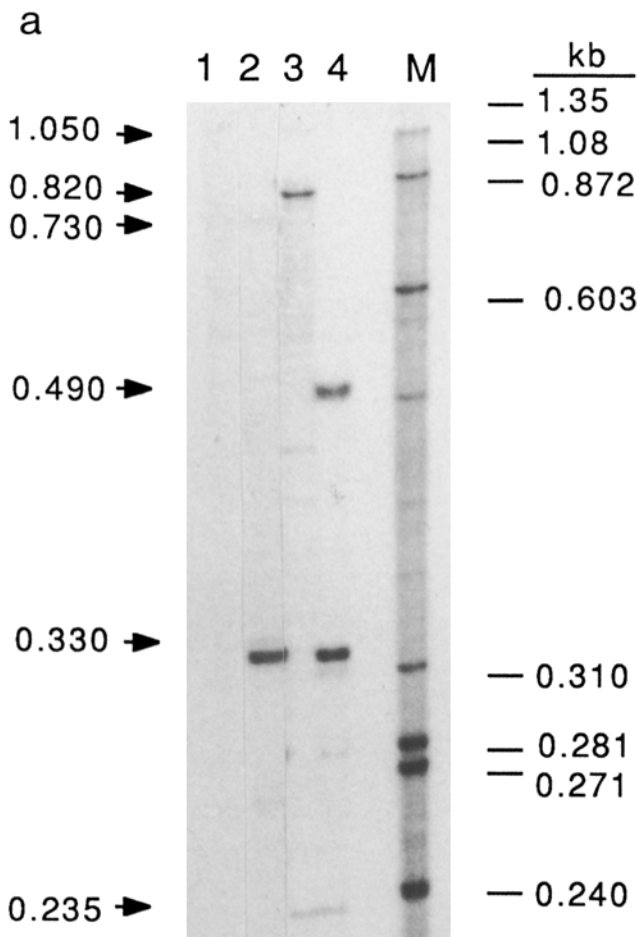
FIGURE 3. RNase A cleavage analysis of H-$2K^{km2}$. (a) An RNA probe transcribed in vitro from pΓK^{k}VSp (Figure 2 a) was used for concurrent analysis of the genomic H-$2K^k$ and H-$2K^{km2}$ clones. Lanes 1 and 3 show RNA fragments protected by H-$2K^k$. Lanes 2 and 4 show fragments protected by H-$2K^{km2}$. Protected RNA fragments of high molecular weight, particularly the 1.05-kb fragment in lane 1, appear subject to degradation, and therefore, this band is extremely faint. The DNA clones were either uncut (lanes 1 and 2) or linearized at the single Bss HII site in intron 1 (lanes 3 and 4) before annealing to the RNA probe. The fragments for each DNA template that are reproducibly protected are indicated. The faint, small molecular weight bands in all lanes represent probe degradation products. (b) A schematic representation of the results. The extent of the 1.05-kb pΓK^{k}VSp probe is shown. Exons are numbered and protected fragment sizes are indicated. Sp, Sph I; Bs, Bss HII; Pv, Pvu II.
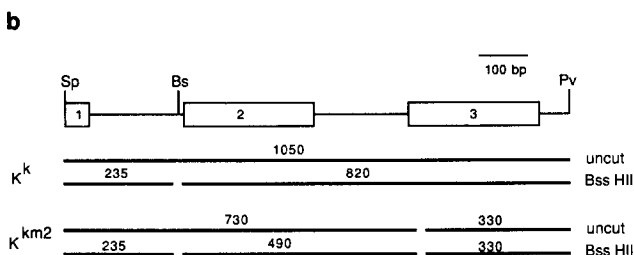
mologous to evenly spaced regions on both strands of the H-$2K^k$ gene, H-$2K^{km2}$ from pK33 was sequenced (see Fig. 2 a) concurrently with C3H H-$2K^k$, thus allowing a clear, pairwise comparison of the two alleles. The DNA sequences of H-$2K^{km2}$ and H-$2K^k$ were found to be identical, with the exception of a short region in exon 3, encoding amino acids 95–99 in the second external domain (α_2) of the molecule.

Within this region of difference, H-$2K^{km2}$ bears four base substitutions within a contiguous stretch of 14 nucleotides, three of which translate to produce the amino acid substitutions, Phe→Ile, Met→Leu, and Tyr→Ser at positions 95, 98, and 99, respectively in the H-2K^{km2} polypeptide (Fig. 4). The fourth nucleotide difference in H-$2K^{km2}$ does not alter the amino acid, Gln, at position 96.

The H-$2K^{km2}$ cDNA clones also were sequenced. Each of 10 clones containing exon 3 was found to bear the H-$2K^{km2}$ mutant-specific sequence, indicating that wild-type H-$2K^k$ transcripts are not likely to be present in C3Hf. In addition, consistent with the finding that all of the consensus splice donor and acceptor splice sites in the genomic clone are unaltered, all exons in every cDNA clone analyzed were spliced as shown previously for wild-type H-$2K^k$ (44). Taken together, analysis of both genomic and cDNA clones shows definitively that the structural difference between H-$2K^k$ and H-$2K^{km2}$ results from a coding region mutation that produces amino acid substitutions, rather than from a splice site mutation that could direct alternative splicing.

The DNA sequence of C3Hf H-$2K^{km2}$ is entirely consistent with the biochemical, the RNase A cleavage, and the restriction mapping results. First, as predicted by 2D gel analysis, the amino acid substitutions deduced from the DNA sequence are all conservative changes, conferring neither a charge nor a size difference on the H-2K^{km2} polypeptide. Moreover, the deduced Met→Leu substitution at codon position 98 corroborates the peptide mapping result that H-2K^{km2} has gained a single [^{3}H]leucine-labeled peptide, with no associated labeled peptide losses from H-2K^k. Finally, DNA sequencing not only confirmed the prediction from RNase A cleavage analysis that H-$2K^{km2}$ bears a sequence alteration only at the 5' end of exon 3, but also defined the four nucleotide substitutions that generate the four restriction enzyme site differences found between H-$2K^{km2}$ and H-$2K^k$ (Fig. 4).

Identification of a Potential Donor Gene in C3H.   To determine whether the H-$2K^{km2}$ mutation could have been generated by an intergenic nonreciprocal sequence transfer,
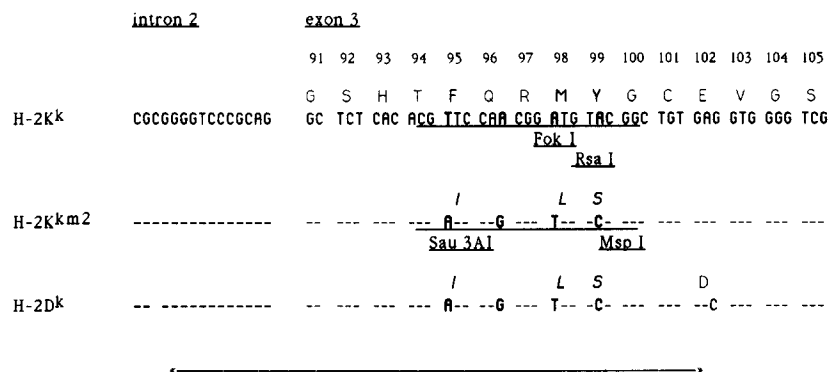

FIGURE 4.   DNA sequence comparison of H-$2K^k$ (19, 36), H-$2K^{km2}$, and H-$2D^k$ (36). Numbers depict the amino acid positions in the H-2 molecule. The exon 3 nucleotide and amino acid substitutions, as well as the polymorphic restriction enzyme sites in H-$2K^{km2}$ relative to H-$2K^k$, are indicated. The synthetic K^k and K^{km2} oligonucleotides used as probes in these studies are complementary to the underlined sequences in H-$2K^k$ and H-$2K^{km2}$, respectively. The arrow indicates the extent of contiguous homology between H-$2K^{km2}$ and H-$2D^k$.

as defined by analysis of the $H\text{-}2K^b$ mutants (14, 16, 17), we looked for a potential donor gene among C3H and C3Hf class I clones. All available C3H genomic clones, representing virtually the entire C3H class I family (36), as well as several C3Hf genomic and cDNA clones, were probed with the K^{km2} oligonucleotide, a 19-mer that corresponds to the substituted region of $H\text{-}2K^{km2}$ (see Fig. 4). This probe hybridized to only one gene, $H\text{-}2D^k$ (36), in the set of C3H clones. In contrast, it hybridized strongly to both $H\text{-}2D^k$ and $H\text{-}2K^{km2}$, and weakly to an unidentified genomic clone, designated pK73, in the C3Hf collection (data not shown).

DNA sequence analysis of the C3Hf $H\text{-}2D^k$ gene not only revealed its complete identity to the published C3H $H\text{-}2D^k$ sequence (36), but also demonstrated that both $H\text{-}2D^k$ genes are identical to $H\text{-}2K^{km2}$ in the region of the mutation in exon 3. The maximum length of contiguous sequence identity between $H\text{-}2D^k$ and $H\text{-}2K^{km2}$ encompassing the mutation is 45 bp, extending for 22 nucleotides upstream and for 9 nucleotides downstream of the 14-bp mutation site (Fig. 4). DNA sequence analysis of the pK73 gene, however, revealed that its exon 3 sequence differs by three nucleotides from the 14-bp core region of identity between $H\text{-}2K^{km2}$ and $H\text{-}2D^k$, and also that it differs from all other C3H H-2 and Qa genes characterized to date, including $H\text{-}2K1^k$, $Q1^k$, $Q4^k$, $Q5^k$, and $Q10^k$ (S. Watts, unpublished results). Therefore, despite its weak hybridization to the K^{km2} oligonucleotide probe, pK73, probably a Tla gene, is not likely to have been a donor for the $H\text{-}2K^{km2}$ mutation. Thus, clone analysis indicated that $H\text{-}2D^k$, unaltered in C3Hf, is the only possible donor gene for the C3Hf $H\text{-}2K^{km2}$ mutation.

Genomic Analysis of C3Hf. Although the results from tissue grafting (22) and cDNA clone analysis indicated that the parental $H\text{-}2K^k$ gene is not expressed at either the protein or the RNA level in C3Hf, presence of an unexpressed copy of $H\text{-}2K^k$ in the C3Hf genome could not be excluded. Therefore, C3H and C3Hf were examined by genomic Southern blot in order to verify that the $H\text{-}2K^{km2}$ allele is present in homozygous form in the C3Hf genome. First, C3H and C3Hf were shown to bear the identical Eco RI, Hind III, Kpn I, and Bam HI restriction sites (see Fig. 2 a) within and flanking their respective $H\text{-}2K$ loci (data not shown), indicating that there are no gross structural changes in either of the chromosomal copies of $H\text{-}2K^{km2}$ in C3Hf. Distinct Fok I and Rsa I restriction fragment differences between the two strains, however, were revealed by hybridization with a low-copy $H\text{-}2K^k$ 5' coding probe (Fig. 5). Each of the polymorphic Fok I and Rsa I fragments detected previously in the cloned $H\text{-}2K^k$ and $H\text{-}2K^{km2}$ alleles (see Fig. 2 b) are detected only in the respective C3H or C3Hf genome.

Analysis with the K^{km2} oligonucleotide probe, however, was required to demonstrate that the entire 14-bp $H\text{-}2K^{km2}$ mutation is intact and homozygous in the genome of C3Hf. The K^{km2} and the corresponding K^k probe (see Fig. 4) were hybridized to duplicate filters containing genomic Pst I digests. As shown in Fig. 6, the 3.5-kb band corresponding to the $H\text{-}2D^k$ gene hybridizes only to K^{km2} and is present at equal intensity in both C3H and C3Hf, providing final confirmation that $H\text{-}2D^k$ contains the mutant-specific sequence and that it is unaltered in the C3Hf genome. Moreover, detection of the $H\text{-}2K^k$ locus-specific 1.6-kb band only in C3Hf with the K^{km2} probe and only in C3H with the K^k probe confirms that the mutant or the parental allele-specific sequence, respectively, is intact and homozygous in the appropriate genome. This result also reveals that the $H\text{-}2K^k$ coding informa-
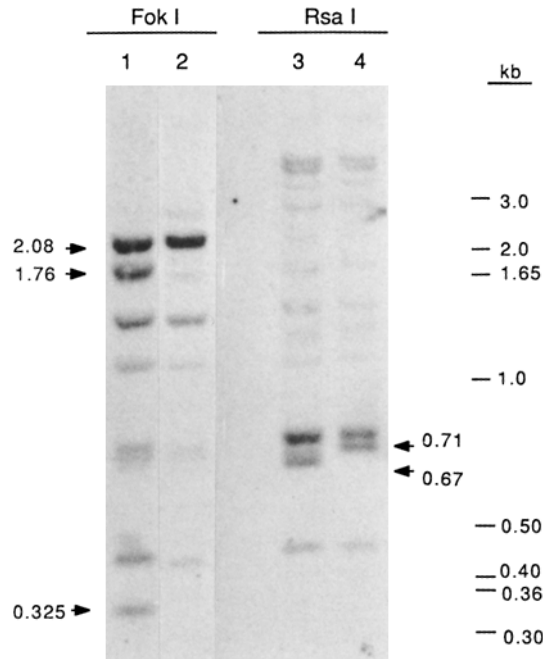
FIGURE 5. Genomic Southern blot analysis of polymorphic Fok I and Rsa I restriction sites. As indicated, the allele-specific restriction fragments identified in the cloned H-$2K^k$ and H-$2K^{km2}$ genes (Fig. 2 b) are detected uniquely in C3H (lanes 1 and 3) and C3Hf (lanes 2 and 4) genomic DNAs, respectively, with pTK^{k}StP probe (see Fig. 2 a). The 2.08-kb Fok I band corresponding to H-$2K^{km2}$ in C3Hf apparently overlaps another endogenous class I fragment hybridizing to the probe in both genomes.

tion corresponding to the K^k probe is completely absent from the C3Hf genome. The faint 0.9-kb band in both C3H and C3Hf hybridizing only to K^{km2} corresponds to the pK73 gene; the moderate stringency of the final wash apparently allowed its weak hybridization despite heterology to the probe. Taken together, all the results from molecular analysis of C3Hf H-$2K^{km2}$ provide convincing evidence for a homozygous alteration at the H-$2K$ locus of C3Hf, consistent with a nonreciprocal
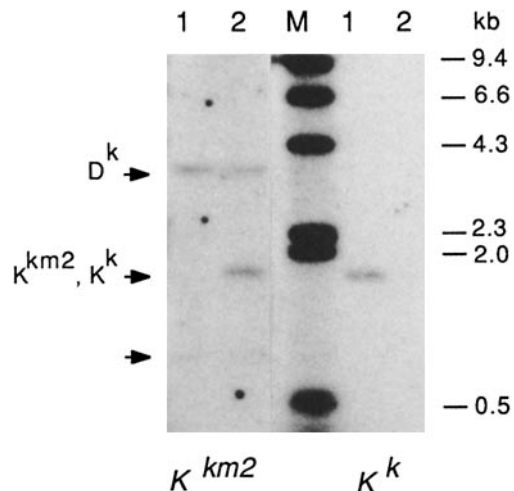

FIGURE 6. Genomic Southern blot analysis using K^{km2} and K^k oligonucleotide probes. Duplicate blots containing genomic Pst I digests of C3H (lane 1) and C3Hf (lane 2) DNA were hybridized with the indicated probes. The H-$2D^k$, H-$2K^k$, and H-$2K^{km2}$ gene-specific bands are indicated. The 0.9-kb band hybridizing faintly to the K^{km2} probe in both lanes represents the gene on pK73, which bears three base mismatches relative to the probe.

interaction between the parental H-$2K^k$ gene and the only possible donor gene in C3H, H-$2D^k$.

Predicted Structure of the H-2K^{km2} Antigen. The three dimensional structure of the class I antigen, deduced from crystallographic analysis of the human HLA-A2 antigen (29), reveals a prominent groove on the top surface of the molecule, bounded on two sides by α helices and on the bottom by the central strands of an antiparallel β pleated sheet. This cleft has been suggested to be the likely binding site for foreign antigen fragments (29, 30). Based on this structure, each of the three amino acid substitutions in H-2K^{km2}, at positions 95, 98, and 99, map to one of the two largest, most central β strands that form the bottom of this binding cleft (Fig. 7). It is interesting to note that this is one of the most polymorphic β strand regions among class I molecules. Although alterations at two of these three β strand positions, as well as substitutions at other β strand residues, are found in various H-2 mutants or HLA variants (30), the constellation of amino acid substituted residues in H-2K^{km2} is unique.

Discussion

Transplantation studies with syngeneic tumors indicated that C3Hf mice had acquired a genetic alteration, mapping to the K end of the MHC, some time after C3HfB/HeN mice were separated by Dr. W. Heston (NIH) from the C3H/He strain (22, 25). Although biochemical studies provided evidence for limited structural differences between the C3H and C3Hf H-2K^k molecules (28), the molecular basis for such an alteration remained obscure, and the C3Hf strain carried a variant haplotype designation, H-2^{kv1} (27). Recently, Minamide et al. (31) have shown that mice from the C3Hf colony currently maintained by the NCI carry an H-$2K$ gene that is virtually identical to wild-type H-$2K^k$. We also examined mice from this NCI colony, and have obtained identical results (J. Vogel, unpublished data). However, our molecular genetic analysis demonstrates that the NIH C3Hf mice from the colony maintained by Dr. W. Heston are homozygous for an H-$2K$ locus mutation, and
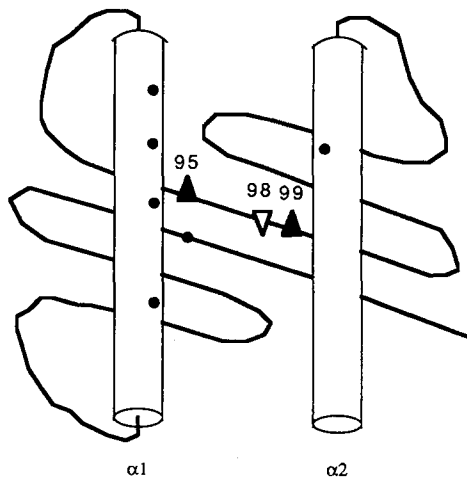

FIGURE 7. Schematic representation of the predicted structure of the H-2K^{km2} class I molecule. Based on the crystallographic structure of HLA-A2 (29, 30), the extracellular portion of H-2K^{km2} encoded by exons 2 and 3 is depicted. The molecule is viewed from the top, showing the antigen-binding cleft bounded by the two α helices (*cylinders*) and the antiparallel β strands (*thick lines*). The substituted amino acid residues at the bottom of the cleft in H-2K^{km2} are represented by large triangles. (▲) Residues 95 and 99, with side chains pointing up into the open space of the cleft; (▽) residue 98, with the side chain pointing down into the molecule. Residues oriented toward the antigen-binding cleft that remain polymorphic between H-2K^{km2} and H-2D^k are identified by small circles.

that these C3Hf mice meet the requirements (20) for the new mutant haplotype designation, H-2^{km2}. Although spontaneous reversion of an initial mutant *H-2K^{km2}* allele in the NCI mice could account for the presence of wild-type *H-2K^k*, there is no other gene in the C3Hf genome that could restore the wild-type sequence to the mutant gene (J. Vogel, unpublished data). Therefore, it seems most likely that the NCI C3Hf colony never carried the *H-2K^{km2}* gene when it was established.

Our results provide further insight into the genetic mechanisms by which *H-2* mutations may arise, and also reveal that clustered nucleotide substitution mutations potentially generated by nonreciprocal recombination mechanisms are not unique to the H-2^b haplotype. Although the C3Hf *H-2K^{km2}* mutation was neither selected for by standard methods of skin grafting nor identified within a single generation (20, 21), these C3Hf mice have all of the characteristic features of the *H-2K^b* mutants (14). First, similar to many of the mutant *H-2K^b* genes, *H-2K^{km2}* bears a single cluster of four nucleotide substitutions within a 14-bp stretch of exon 3, resulting in three clustered amino acid alterations located within the antigen binding cleft of the H-2K^{km2} molecule. Moreover, the DNA sequence of this substituted region is found intact at the homologous position in only one other gene, *H-2D^k*, of both the parental and mutant haplotypes, consistent with the suggestion that a nonreciprocal sequence transfer mechanism between nonallelic genes generates such clustered substitutions (13–17). Syngeneic tumor transplantation, in fact, has been used recently by others as a screening method for identifying several new *H-2* mutants that preexisted and were segregating within an inbred population (45). Taken together, all of the data on C3Hf are consistent with a spontaneous mutation in an *H-2K^k* gene having arisen within a closed, C3H-derived, *H-2K^k* homozygous stock, segregating in an early C3Hf population, and eventually becoming homozygous in at least one subsequent C3Hf colony (at the NIH) as a result of the strict brother-sister mating that was required to maintain this strain as MTV free. For these reasons, we propose that the haplotype of these C3HfB/HeN mice now be designated as H-2^{km2}, with the mutant allele denoted, *H-2K^{km2}*, following the standard *H-2* mutant nomenclature (20).

The relatively small 14–45-bp tract of sequence in *H-2K^{km2}* that could have been transferred from the *H-2D^k* potential donor gene is consistent with the 5–95-bp sequence tracts identified in the *H-2K^b* mutants (14, 16, 17). It is interesting to note that *H-2D^k* and *H-2K^{km2}* are identical for an additional 101 nucleotides beyond the single nucleotide difference that marks the 3' end of the shared 45-bp region. Although the 91.5% DNA sequence similarity between *H-2K^k* and *H-2D^k* in exon 2 is typical of most *H-2* gene pairs, the 96% exon 3 sequence similarity is remarkably high (see Table I). The mutation in *H-2K^{km2}* homogenizes these two *H-2* loci even more, increasing the exon 3 identity between *H-2K^{km2}* and *H-2D^k* to an exceptional 97.5%. It is tempting to speculate that the large region of near identity between *H-2K^k* and *H-2D^k* in exon 3 may have provided favorable conditions for initiation of the recombination event proposed to have generated the *H-2K^{km2}* mutation.

The clustered substitution of *H-2K^{km2}*, however, differs from the single nucleotide alteration of *H-2K^{km1}*, the only other *H-2K^k* mutation so far characterized (18). In fact, *H-2K^{km2}* provides the first clear example of a clustered substitution mutation within an *H-2* gene other than *H-2K^b* that is likely to have been generated by the proposed nonreciprocal recombination mechanism. *Qa* and *Tla* genes have been

TABLE I

The H-2K^{km2} Mutation Reduces the Variability between the H-2 Genes and Molecules in C3Hf

| H-2 pair | Number of differences/total (percent similarity) | | | | Number of polymorphic residues in binding cleft (residue positions) | |
| | Nucleotide | | Amino acid | | | |
	Exon 2	Exon 3	α₁ domain	α₂ domain	α helices	β sheet
K^k vs. D^k (C3H)	23/270 (91.5%)	11/276 (96.0%)	15/90 (83.3%)	8/92 (91.3%)	5 (63, 73, 77, 81, 152)	3 (9, 95, 99)
K^{km2} vs. D^k (C3Hf)	No change	7/276 (97.5%)	No change	5/92 (94.6%)	No change	1 (9)

H-2K^k and H-2D^k DNA and amino acid sequences are from Watts et al. (36). Amino acid residues whose side chains point toward the open space of the cleft, and therefore which are accessable for interaction with bound antigen, were deduced from the crystallographic structure of HLA-A2 (29, 30). Of these residues, only those that are polymorphic between the indicated molecules have been considered for this comparison. Categorized by their location in either the α helices or the β sheet, the total number of polymorphic residues, as well as their position numbers, are indicated.

identified as the potential donors for many of the H-2K^b mutations (see Table II), but a donor gene from the D end of the H-2 complex is not unprecedented. H-2D^b is the only possible donor gene for the H-2K^{bm11} mutation (14, 16); however, H-2D^b appears to have donated exon 2 sequences, whereas exon 3 sequences are apparently transferred from H-2D^k to H-2K^{km2}. It is also interesting to note that 7 of the 45 identical nucleotides between H-2K^{km2} and H-2D^k lie in intron 2 (Fig. 4), indicating that part of this intron might have been transferred along with the exon sequences; H-2K^{bm3} is the only other mutant H-2 gene for which part of an intron also could have been donated (14, 16). Finally, the equivalent 45-bp region of the parental H-2K^k allele contains four CpG dinucleotides, a feature that is consistent with a previously proposed model for H-2 sequence diversification involving the unequal repair of methylated CpG sequences (46). Of course, causal evidence for such non-reciprocal sequence transfers and for any active involvement of CpG sequences in the generation of these mutations awaits further experimentation.

All three of the substituted residues, representing relatively conservative changes in H-2K^{km2}, are buried within the molecule at the bottom of the peptide antigen-binding cleft (29, 30); and this constellation of amino acid substitutions is unique relative to all other known mutant H-2 or variant HLA molecules (14, 30). The two substitutions at positions 95 (F→I) and 99 (Y→S) are oriented toward the open space of the cleft (see Fig. 7), and most likely do not contact the TCR directly, but rather serve as ligands for the binding of processed antigenic peptides (30). In contrast, altered residue 98, also a conservative change (M→L), is oriented down into the molecule and could affect molecular conformation or stability. The specific contribution of each altered residue toward the biological properties of H-2K^{km2}, however, is not yet known.

Although the substitutions in H-2K^{km2} are located in one of the most polymorphic β strands among H-2 molecules, at residues thought to be important in determining the foreign antigen specificity of different H-2 molecules (30), the H-2K^{km2} mutation actually serves to reduce the polymorphism between H-2K^k and H-2D^k within the peptide antigen-binding site (Table I). Three of the eight polymorphic

TABLE II

The Molecular Characteristics of H-2K^{km2} Distinguish it from all other H-2 Mutants

Mutant	Altered amino acid positions	Location in H-2 molecule	Donor genes	Substituted region could:		
				Generate foreign peptides	Affect peptide antigen binding	Affect direct CTL interactions
H-2K^{km2}	95	β strand ●	D^k	±	+	−
	98	β strand				
	99	β strand ●				
H-2K^{bm1}	152	α helix ●	$Q10^b$	+	+	+
	155	α helix ●/↑				
	156	α helix ●				
H-2K^{bm3}	77	α helix ●	$Q8^b, K1^b, D^b$	+	+	±
	89	α/β loop				
H-2K^{bm4}	162	α helix ↑	$T5^b$	+	+	+
	163	α helix ●/↑				
	165	α helix				
	173	α helix				
	174	α helix				
H-2K^{bm5,16}	116	β strand ●	ND	+	+	−
H-2K^{bm6,7,9}	116	β strand ●	$Q4^b$	+	+	−
	121	β/β loop				
H-2K^{bm8}	22	β strand ●	ND	+	+	−
	23	β strand				
	24	β strand ●				
	30	β/β loop				
H-2K^{bm10}	163	α helix ●/↑	$K1^b$	+	+	+
	165	α helix				
	167	α helix ●				
	173	α helix				
	174	α helix				
H-2K^{bm11}	77	α helix ●	D^b	±	+	+
	80	α helix ●/↑				
H-2K^{bm23}	75	α helix	$Q10^b$	+	+	+
	77	α helix ●				
H-2K^{km1}	152	α helix ●	D^k + others	±	+	+

For each H-2 mutant, the substituted amino acid position numbers, the location of each substituted amino acid residue in the class I molecule, and the potential donor gene for each mutation are listed. Those residues likely to interact with bound antigen are indicated: ●, side chains point directly into the binding cleft: ↑, side chains point up and away from the cleft; ●/↑, side chains point up as well as into the binding cleft (29, 30). The information for the H-2K^b mutants is adapted from Nathenson et al. (14), and from Geliebter and Nathenson (16). The H-2K^{km1} information is taken from Martinko et al. (18) and from our own analysis of its potential donor genes. The identity of the respective donor gene and the deduced location of each amino acid substitution within the three-dimensional structure of the class I molecule were used to estimate the relative likelihood that each mutant molecule either could generate peptides that may be novel to the parental strain, could alter cellular and foreign peptide binding, or could affect direct contact with CTL. +, very likely; ±, may occur to a minor degree; −, not likely.

amino acid residues between H-2K^k and H-2D^k that could influence peptide anti-gen binding (30) are located in the β sheet at the bottom of the antigen-binding cleft. The H-2K^{km2} mutation results in the elimination of two of these differences, leaving the base of the H-2K^{km2} cleft virtually identical to that of H-2D^k (see Table I and Fig. 7). The remaining five polymorphic amino acid residues between H-2K^{km2} and H-2D^k that could affect antigen binding are located within the α helices and are oriented either toward the binding site or up away from the molecule. These residues may be critical for contact with foreign antigen or with the T cell, and to-gether with the one remaining β sheet difference, likely account for the continued immunological distinction between H-2K^{km2} and H-2D^k.

The predicted location of the amino acid substitutions in H-2K^{km2} is consistent with several previous observations. Since none of the alterations in H-2K^{km2} are ex-posed on the outer surface of the molecule and, therefore, would not be accessible to antibodies, it is now not surprising that serological differences between the C3H and C3Hf strains were never detected and that repeated attempts at generating a C3H anti-C3Hf antibody were not successful (23, 28). In addition, C3Hf cells have been found to exhibit altered viral restriction properties relative to C3H. For ex-ample, Martin and coworkers (unpublished results), have found that C3Hf is inefficient at presenting vaccinia virus antigen to C3H H-2K^k-restricted CTL. It seems rea-sonable to suggest, given their predicted location within the molecule, that the sub-stitutions in H-2K^{km2} affect viral restriction through impaired binding or presen-tation of viral antigen peptides (4), rather than by disrupting any direct interaction with the TCR. This is in contrast to many of the well-characterized *H-2K^b* mutants, for which at least one substituted residue is located on the top surface of the mole-cule, and therefore could make direct contacts with the T cell (see Table II). Con-ceivably, the ability of the modified H-2K^{km2} molecule to present other H-2K^k-restricted viral antigens to CTL is also affected, although verifying this prediction requires further analysis.

The alloreactive response, resulting in histoincompatibility between an *H-2* mu-tant and its parental strain, is thought to be a consequence of the viral restriction function of H-2 molecules (1, 2, 9). In support of this view, some virus-specific, self H-2–restricted CTL have been found to crossreact with nonself H-2 antigens (3). Given the premise that H-2 antigens are peptide receptors of highly degenerate specificity, presenting foreign antigens in the form of processed peptides for recogni-tion by CTL (4, 47, 48), two models have been proposed that attempt to explain nonself H-2 alloantigenicity in terms of foreign peptide antigen presentation. First, supported by the results of several independent studies (5–7), one model suggests that processed peptides derived from polymorphic regions of nonself H-2 or HLA molecules can be recognized as foreign in the context of intact self class I restriction elements. Thus, the alloreactive response may be self H-2 restricted, in which the nonself H-2 fragments effectively mimic a foreign viral antigen. The second model suggests that each H-2 antigen binds a distinct set of peptides, processed from both viral and normal cellular proteins, for presentation to CTL, but that only viral pep-tides normally are recognized by CTL as foreign due to tolerance developed toward the cellular peptides (47, 48). Therefore, alloreactivity would result from the ability of the nonself H-2 antigen to bind and present a novel set of cellular peptides, some

of which, identical in sequence or conformation to specific foreign antigen peptides, would be recognized as foreign by host CTL (47).

Structural information from a large number of alloantigenic mutant H-2 molecules, including H-2K^{km2}, provides an initial basis for evaluating the generality of these models. First, as summarized in Table II, many of the mutant H-2K^b substitutions appear to be derived from genes whose cell surface expression and tissue distribution are extremely limited, indicating that these mutant molecules may have the potential to serve as a source of peptides that could be seen as foreign by parental H-2-restricted CTL. It is also possible that most of the mutant H-2K^b as well as the H-2K^{km1} antigens themselves could bind and present cellular peptides differently to parental CTL, since at least one substituted residue in each case is oriented toward the open space of the peptide-binding cleft. Thus, the nature, location, and orientation of the substituted amino acids in most of these mutants are consistent with both models for nonself H-2 alloantigenicity, indicating that the two proposed molecular processes may not necessarily be mutually exclusive.

The features of the H-2K^{km2} substitution, however, distinguish this mutant from all of the others (see Table II). For example, the substituted residues in H-2K^{km2}, located at the bottom of the cleft, could readily alter the binding of cellular peptides, but are not likely to affect any of the direct molecular contacts between H-2 and the TCR. In addition, the substituted region of H-2K^{km2} is also represented in H-2D^k; therefore, virtually all processed peptides that could be derived from H-2K^{km2} would be expected to be identical to the repertoire of self H-2K^k or H-2D^k peptides already present in every C3H cell. Only the two H-2K^k/H-2D^k junction regions, both derived from a single β strand in H-2K^{km2}, could comprise peptides that might be recognized by C3H CTL as foreign. In view of the suggestion, however, that peptides bound to MHC antigens are in α helical form (49, 50), and that both primary sequence (51) and peptide conformation (49, 50) influence peptide binding, it is not known whether all possible β strand–derived peptides can bind to self H-2, or, if bound, whether they would be in the proper conformation to be seen as foreign by C3H CTL.

Therefore, the deduced structure of H-2K^{km2} is entirely consistent with its biological properties. It seems most likely that the altered H-2K^{km2} antigen would bind a set of cellular peptides, different from that bound by either H-2K^k or H-2D^k, and present a subset of these cellular peptides as foreign to CTL of the C3H parental strain. Since 10^2–10^3 times as many CTL appear to respond to a single nonself H-2 antigen as compared with an H-2-restricted viral antigen (1), the vigorous alloreactive response observed against H-2K^{km2} (22, 26) may simply result from recognition of a wide variety of bound self cellular peptides by a large number of different host CTL. Clearly, our prediction about the likely molecular basis for H-2K^{km2} alloantigenicity must be experimentally tested.

Summary

The C3HfB/HeN (C3Hf) mouse strain expresses an H-2K^k molecule, previously denoted H-2K^{kv1}, that is structurally and functionally distinct from H-2K^k of the parental C3H strain. By molecular genetic analysis, we demonstrate that the C3Hf *H-2K* gene carries a homozygous coding region mutation relative to the C3H allele, revealing that C3Hf meets the requirements for assignment of a mutant haplo-

type, H-2^{km2}. C3Hf H-$2K^{km2}$ bears a single clustered substitution of four nucleotides within 14 contiguous nucleotides in exon 3. Since this sequence also is present intact at the homologous position in H-$2D^k$ of both C3H and C3Hf, the origin of the H-$2K^{km2}$ mutation is consistent with a nonreciprocal sequence transfer from the H-$2D^k$ donor gene, analogous to the mechanism proposed for generation of the H-$2K^b$ mutations. The H-$2K^{km2}$ mutation encodes three clustered amino acid substitutions, at positions 95, 98, and 99, that map to one of the large β strands at the bottom of the peptide antigen binding cleft of the H-2K^{km2} molecule. The nature and location of these amino acid substitutions are unique relative to any other known H-2 mutant or HLA variant, and underscore the importance of the β-pleated sheet in influencing CTL recognition. These results indicate that H-2K^{km2} alloantigenicity may derive largely from altered presentation of self cellular peptides.

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