THREE SPONTANEOUS *H-2D^b* MUTANTS ARE GENERATED BY GENETIC MICRO-RECOMBINATION (GENE CONVERSION) EVENTS

Impact on the H-2-restricted Immune Responsiveness

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The H-2 class I products encoded by the *K*, *D*, and *L* genes of the mouse MHC (1) play a crucial role as self-recognition elements in the immune response against tissue-associated foreign antigens (2). A hallmark of the H-2 system is the extraordinary degree of polymorphism; there are ~50-100 alleles identified for each genetic locus (3). Further, individual H-2 alleles of the same locus show remarkable diversity as they differ by 15-20% at the protein level (4, 5). The ability of mouse populations to respond to a variety of different foreign antigens in the context of class I molecules is thought to depend upon the polymorphism and structural diversity of H-2 molecules as well as the expression of multiple products (e.g., K, D, and L) on the cell surface. Consistent with this idea is the observation that different allelic products vary in their effectiveness. In many instances only one of the K, D, or L allelic products in an individual mouse functions as an antigen-presenting molecule for immune responsiveness against a particular viral antigen. For example, the CTL immune responses to Sendai virus (6) and Vesicular Stomatitis virus (7) are restricted to the K^b allele, whereas the D^b allele is utilized by the immune system for foreign antigen recognition of the Friend leukemia virus (8, 9), the Moloney murine leukemia virus (M-MuLV)[1] (9), and the influenza virus (10).

The study of mutant K^b mouse strains (11) has provided a model system to analyze MHC structure/function relationships as well as the mechanism for diversifying the H-2 gene family. The complex nature of the structural alterations found in the K^b genes from seven mutant mouse sublines suggests that a process of "micro-recombination" or "gene conversion" between class I genes generated the K^b mu-

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[1] *Abbreviation used in this paper:* M = MuLV, Moloney murine leukemia virus.

tants (12-16). This inference is based on the observation that the short sequences of genetic information found as changes in the mutant K^b genes are also found in other class I donor genes of the H-2^b haplotype. One implication of these data is that the reassortment of genetic information would play a major role in providing novel class I restriction elements in the population. To determine if such a recombination mechanism was generally responsible for diversification in class I genes other than K^b, we initiated a structural study of in vivo mutants occurring at the *H-2D* locus by analyzing a group of D^b mutants; D^{bm13}, D^{bm14} (17), and D^{bm24} (18).

The data obtained from the primary structural analysis of the D^b mutants can also be used to establish structure/function relationships by correlating sequence differences of the mutant products with the unique functional changes found in the D^b mutant lines. For instance, the cytotoxic T cells of C57BL/6 mice recognize M-MuLV antigens predominantly in association with the D^b molecule (9, 19, 20). These same M-MuLV-specific, D^b-restricted CTL can no longer recognize M-MuLV antigens in association with D^{bm13} or D^{bm14} (21). Similar functional alterations have been observed in the CTL response to the H-Y antigen (22). Thus, the pattern of D^b/viral antigen specificities recognized by the T cell are altered by the structural changes of the D^b mutant products.

Even more striking is the finding that the ability of bm13 and bm14 mice to mount an immune response against M-MuLV has been significantly altered. For example, bm13 mice exhibit a high CTL response to M-MuLV; however, the antiviral response by bm13 CTL is now restricted by both K^b and D^{bm13} gene products (21). More dramatically, bm14 mice are unable to generate a CTL response against M-MuLV.

The bm13 and bm14 mutations also have been shown to alter resistance to central nervous system demyelination after infection with Theiler's murine encephalomyelitis virus, a model system for human multiple sclerosis (23). Hybrids between DBA/2 and the parental B6 strain are completely resistant to such demyelination, while hybrids between DBA/2 and either bm13 or bm14 are highly susceptible. The virus establishes similar low level, chronic infections that persist for the lifetimes of both resistant and susceptible animals, so that the effects of the mutations appear not to be on an ability to actually clear the infectious agent, but rather with the ability to mount particular antiviral responses (probably delayed-type hypersensitivity) that also result in destruction of the axonal myelin sheaths.

In the present studies, characterization of the mutant D^b genes at the molecular level showed a unique alteration present for each mutant D^b gene. In two of the mutant genes (D^{bm13} and D^{bm24}), complex changes were found. In one, D^{bm14}, a single amino acid change was found. The existence of potential donor sequences in other class I genes supports the conclusion that much like the K^b mutant genes, these *D* region mutant genes appear to be generated by micro-recombination events with other class I genes. Thus the D^b mutants provide an example in which micro-recombinations have generated discrete, limited nucleotide changes that result in novel D^b gene products, which convey an altered immunological responsiveness by the mutant host animal.

Materials and Methods

Mice. B6.C-H-2^{bm13} (bm13), B6.C-H-2^{bm14} (bm14), and B10-H-2^{bm24} (bm24) were maintained in the laboratory of one of us, R. Melvold, Northwestern University Medical School, Chicago, IL. C57BL/6J (B6) were purchased from The Jackson Laboratory, Bar Harbor, ME.

The bm13, bm14 (17), and bm24 (18) strains were derived from mutant animals found to be incompatible with normal animals in the course of systematic screening for histocompatibility gene mutations. Skin grafts were reciprocally exchanged within groups of sex-matched syngeneic mice where all grafts should be accepted. Animals involved in occasional rejections were regrafted to confirm the original observations, and bred to establish the genetic transmission of the incompatibility. Backcrossing and intercrossing were carried out as necessary to establish congenic or coisogenic strains carrying the mutant genes. The mutated genes were mapped by conventional complementation testing with appropriate congenic, recombinant, and other defined mutant strains.

Synthetic Oligonucleotides. Oligonucleotides of varying length (15–21 nucleotides) were chemically synthesized by either the phosphotriester (Bachem Fine Chemicals, City of Hope, CA) or phosphoramidite (Applied Biosystems, Inc., Foster City, CA) solid-phase methods. Tables I and III contain a list of all oligonucleotides that were used for RNA/cDNA sequencing and Southern blot analysis.

DNA and RNA Preparation. High molecular weight genomic DNA from spleen cells was prepared as described previously (24). Whole cell RNA was prepared from liver, spleen, and kidney using the lithium chloride method described by Auffray and Rougeon (25). Polyadenylated RNA was purified by oligo-dT-cellulose chromatography (P-L Biochemicals, Milwaukee, WI) according to Aviv and Leder (26).

Analytical Southern Blots. The digestion of 30 μg of genomic DNA and 100 ng of cloned cosmid DNA by restriction enzymes was carried out under the conditions specified by the supplier (Bethesda Research Laboratories, Gaithersburg, MD). Digested DNA was size fractionated on 1.0% agarose gels, denatured, neutralized, transferred to GeneScreen (27), and crosslinked to the filter by exposure to shortwave UV light for 2 min (28).

Genomic DNA filters were prehybridized for at least 2 h in 5× SSC (1× SSC equals 150 mM NaCl, 15 mM sodium citrate), 20 mM sodium phosphate, pH 7.0, 10× Denhardts solution (1× Denhardts equals 0.02% Ficoll, 0.02% BSA, and 0.02% polyvinylpyrrolidone), 7% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. A 0.1-μg sample of oligonucleotide was incubated with 70 μCi (cosmid DNA blots) or 200 μCi (genomic DNA blots) (5,000 Ci/mmol) γ-[^{32}P]ATP (Amersham Corp., Arlington Heights, IL) and 7 U polynucleotide kinase (New England Biolabs, Beverly, MA) for 30 min at 37°C in the presence of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiotreitol, and 0.1 mM spermidine. The radiolabeled oligonucleotide was added to the prehybridization solution and filters were hybridized overnight. Hybridization was carried out 5°C lower than the calculated melting temperature, which was determined as described by Suggs et al. (29). Filters were washed three times for 30 min in 1× SSC and 1% SDS at hybridization temperatures.

C57BL/10 Cosmid Clones. Cosmid clones containing the class I genes of the C57BL/10 mouse strain were a generous gift from Dr.R. A. Flavell (30). Hybridization analysis of cosmid DNA blots was described elsewhere (14).

RNA/cDNA Sequencing Techniques. The RNA and cDNA sequencing techniques of class I histocompatibility antigens were developed in our laboratory by modifying previously described procedures (31, 32) and has been described elsewhere (15).

Results

RNA Sequence Analysis of D^b, D^{bm13}, D^{bm14}, and D^{bm24}. Nucleic acid sequence analysis was used to determine the nucleotide sequence of the D^b genes of the parental B6 mouse and the D^b genes of mouse strains bm13, bm14, and bm24. Seven oligonucleotides complementary to the D^b mRNA and five oligonucleotides complementary to D^b cDNA (Table I) were used as primers in the two sequencing methods described earlier (15). In the first procedure, ^{32}P-radiolabeled oligonucleotides, complementary to the mRNA, were used for the dideoxynucleotide sequencing of parental and mutant D^b RNA. The nucleotide sequence in the regions obscured by premature termination and band compression was determined by a second sequencing procedure, using uncloned D^b cDNA. In this technique, D^b mRNA was first cop-

TABLE I

Oligonucleotides Hybridizing to H-2D^b

Oligonucleotide	No.	Complementary to:	5′ - 3′ sequence	Calc T_d*
				°C
D^b	2	D^b (94–100)‡ s§	GCCAGACATCTGCTGGAGT	60
D^b	3	D^b (192–199) s	CTTCACCTTTAGATCTGGGGT	62
D^b	4	D^b (294–301) s	GCTCCAATGATGGCCATAGCT	64
D^b	5	D^b (150–157) s	TTGTAATGCTCTGCAGCACCA	62
D^b	6	D^b (254–260) s	CACGGCATGTGTAATTCTGCT	62
D^b	7	D^b (47–53) s	CCATCCACGGCGCCCGC	62
D^b	8	D^b (47–53) a	GCGGGCGCCGTGGATGG	62
D^b	9	D^b (3–10) s	TCTCGAAATACCGCATCGAGT	62
D^b	12	D^b (150–157) a	TGGTGCTGCAGAGCATTACAA	62
D^b	13	D^b (254–260) a	AGCAGAATTACACATGCCGTG	62
D^b	14	D^b (94–100) a	ACTCCAGCAGATGTCTGGC	60
D^b	21	D^b (3′ untransl) s	GACCTGAACACATCGTCTGTC	64
D^b	24	D^b (3–10) a	ACTCGATGCGGTATTTCGAGA	62

* According to Suggs et al. (29), melting temperature (T_d) is the temperature at which one-half of target segments are occupied by oligonucleotide probes.

‡ Numbers refer to amino acid positions.

§ s, sense strand; a, antisense strand.

ied into cDNA by reverse transcriptase using an unlabeled oligonucleotide primer complementary to the mRNA strand. A radiolabeled oligonucleotide, complementary to the cDNA strand, was then used as a primer for dideoxynucleotide sequencing of the parental and mutant D^b cDNA.

The 1,013 nucleotides corresponding to the exons 2-8 of D^b, D^{bm13}, D^{bm14}, and D^{bm24} were sequenced (Fig. 1). The parental D^b(B6) sequence is, with one exception, a silent base substitution in codon 120 (GGC instead of GGT), in full agreement with the published partial sequence of a D^b cDNA clone (33). Furthermore, the sequence corresponding to amino acids 1-81 is in complete agreement with the reported amino acid sequence of D^b (34). 59 of the 64 nucleotides in the leader exon (exon 1) were sequenced and no differences were found between the mutant and wild-type D^b genes (results not shown).

Distinct changes were found for each D^b mutant gene. The changes identified are the following (Fig. 1, Table II): (*a*) D^{bm13}, four altered nucleotides are clustered in a stretch of 17 nucleotides within the third exon, corresponding to the amino acid positions 114-119. The altered codons are at positions 114, 116, 118, and 119. The alteration at position 118 is silent. (*b*) D^{bm14}, a single nucleotide change is present at amino acid position 70. (*c*) D^{bm24}, eight nucleotide changes occur within the 51 nucleotides corresponding to amino acid positions 63-80. The altered nucleotides

FIGURE 1. Sequence comparison of exons 2–8 of D^b, D^{bm13}, D^{bm14}, D^{bm24}, and K^b. The nucleotide sequence of D^b is given. Numbers on top of the sequence refer to the position of the corresponding amino acid in the protein sequence. Dashes indicate sequence identity. The following nucleotide differences are found. A total of four in D^{bm13} at codons 114, 116, 118, and 119; one in D^{bm14}, a nucleotide change at the codon 70; and eight in D^{bm24} at the codons 63, 70, 73, 77, and 80. The most extended sequence of identity encompassing the mutation between D^{bm13}, D^{bm24}, and K^b, respectively, are boxed (K^b sequence, [36], partial D^b sequence, [33]). These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00806.

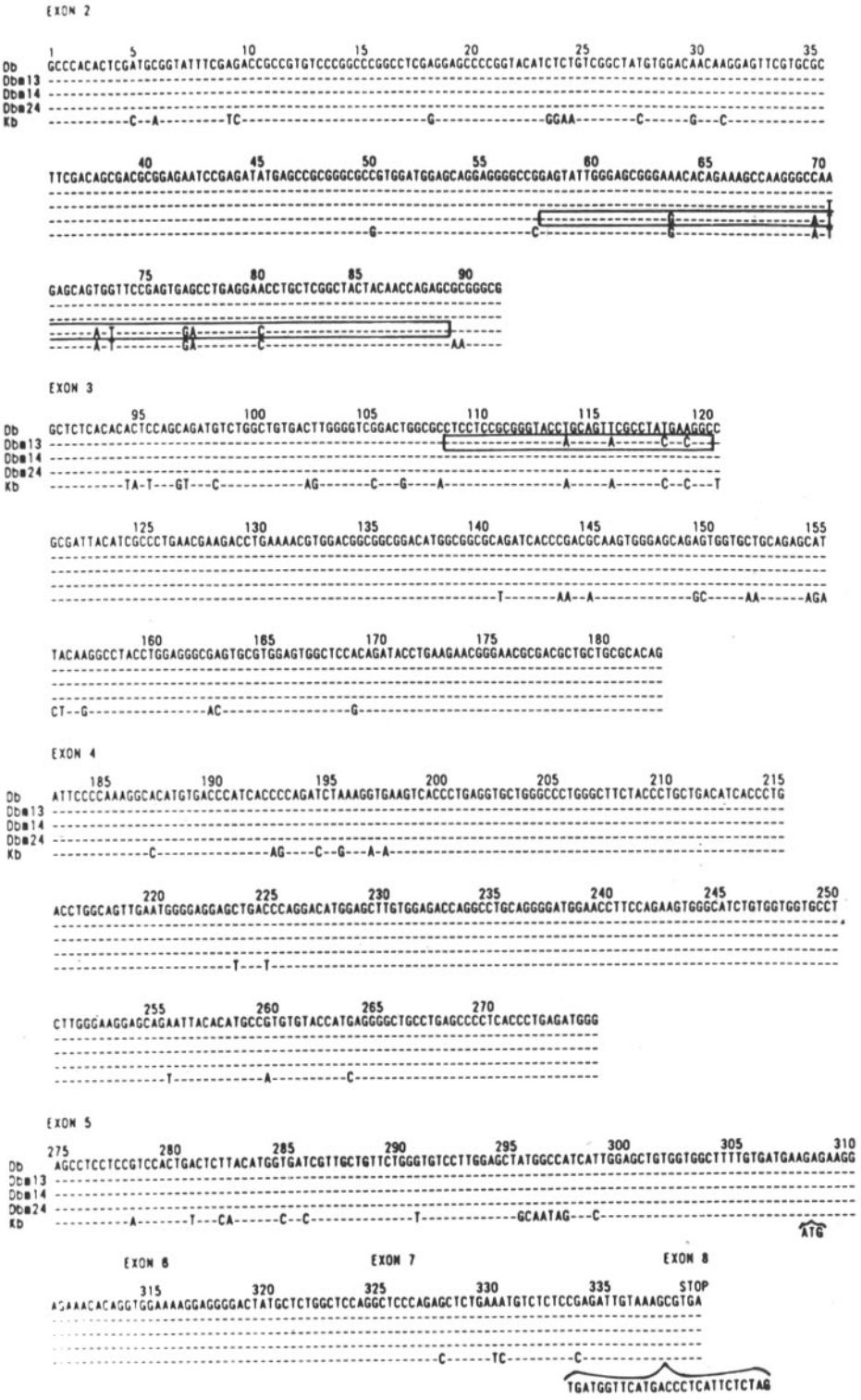
EXON 2
EXON 3
EXON 4
EXON 5
EXON 6
EXON 7
EXON 8
Db
Dbm13
Dbm14
Dbm24
Kb
ATG
STOP
TGATGGTTCATGACCCTCATTCTCTAG

TABLE II

Molecular Characteristics of D^b Mutations and Donor Genes

Mutant	Position of altered amino acids	Altered nucleotides/ consecutive nucleotides*	Donor gene‡	Length of identity encompassing mutation§
D^{bm13}	114 Leu Gln 116 Phe Tyr 118 Silent 119 Glu Asp	4:17	K^b	36
D^{bm14}	70 Gln His	1:1	K^b, *Q4, Q7, Q8, Q9, Q10, T3b, (T1b–T5b)*	
D^{bm24}	63 Silent 70 Gln Asn 73 Trp Ser 77 Ser Asp 80 Asn Thr	8:51	K^b	93

* The number of altered nucleotides/the number of consecutive nucleotides from the first to last alteration.

‡ Donor gene nucleotide sequences: K^b from Weiss et al. (36); *Q7, Q8, Q9* from Devlin et al. (39); *Q10* from Mellor et al. (35); T3b from Pontarotti et al. *(49)*.

§ The number of consecutive, identical nucleotides between the mutant *D* gene and the donor gene in the region of substitution.

are localized in codons for positions 63, 70, 73, 77, and 80. The alteration at position 63 is silent. The D^{bm24} gene contains the largest number of altered nucleotides ever found in a mutant *H-2* gene.

The K^b Gene Is the Donor Gene for D^{bm13} Mutation. Various MHC class I genes have been identified as donor genes in the generation of the K^b mutants analyzed thus far (15, 16, 35). To determine whether the D^{bm} alterations were generated by a recombination mechanism similar to that shown for the K^b mutants, we carried out a series of studies to identify potential donor sequences. A comparison of the nucleotide sequence of D^{bm13} with previously published murine class I gene sequences revealed that the altered stretch of D^{bm13} is identical to the homologous region in K^b (Fig. 1). To determine if the K^b gene is the only possible H-2^b-haplotype donor gene for the D^{bm13} changes, we synthesized a 21-base oligonucleotide ($D^{bm13mer}$) complementary to the altered stretch of the D^{bm13} sequence (Table III). This mutant-specific oligonucleotide was then hybridized to Bam HI-digested splenic DNA from B6 and bm13 mice (Fig. 2 *a*). The specificity of the oligonucleotide is shown by its

TABLE III

Oligonucleotides Binding to Mutant D^b Sequences

Oligonucleotide	Complementary to mRNA at codons	5′–3′ Sequence	Calculated T_d*
			°C
$D^{bm13mer}$	D^{bm13} (114–119)	G*TC*GTAGGCG*TACTGC*TGGTA	68
$D^{bm14mer}$	D^{bm14} (67–72)	CTGCTC*ATGGCCCTTGGC	60
$D^{bm24mer}$	D^{bm24} (73–79)	CCTCAG*G*TCCACTCGGAA*A*CT	66

* T_d, melting temperature.

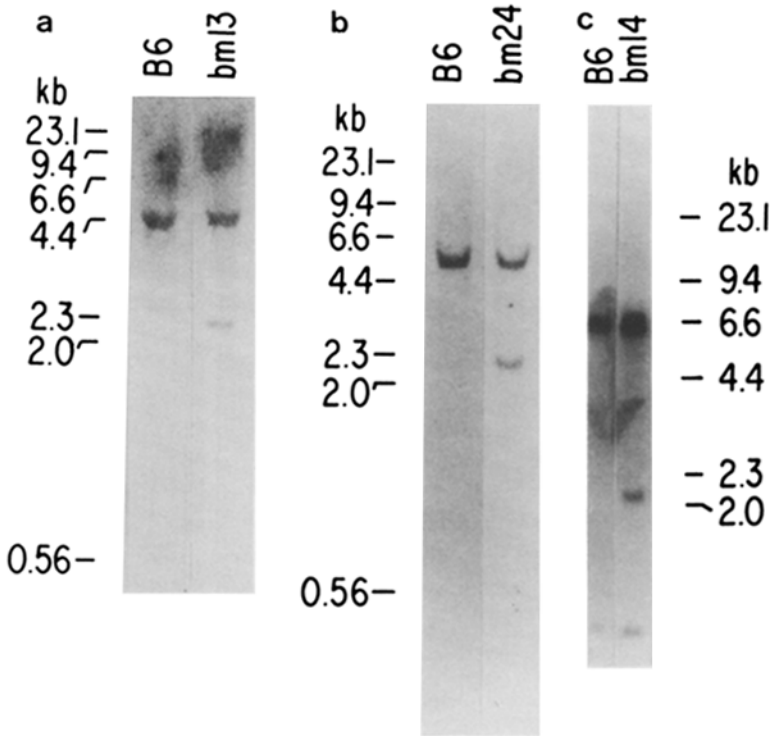

FIGURE 2. Hybridization of genomic DNA with mutant-specific oligonucleotide probes. DNA from parental and mutant mice were digested with Bam HI, size fractionated by agarose gel electrophoresis, transferred to GeneScreen, and hybridized with ^{32}P-labeled mutant-specific oligonucleotides for 16 h as previously described (14). Oligonucleotide probes are described in Table I. Hybridization and washing temperatures are (*a*) 60°C, (*b*) 55°C, (*c*) 60°C. After hybridization, filters were washed and exposed to Kodak XAR film as previously described (14). Numbers in kilobases are Hind III-digested λ DNA molecular size markers.

binding to the 2.1-kb Bam HI fragment corresponding to the D^{bm13} gene in bm13 DNA, but not to the corresponding D^b gene fragment in B6 DNA (Fig. 2 *a*). In addition, the oligonucleotide binds to another Bam HI restriction fragment of 4.8 kb found in the DNA from both mouse strains (Fig. 2 *a*). This band is identical in size to the Bam HI fragment containing the exons 1, 2, and 3 of the K^b gene (36).

To refine our search for potential donor genes, we also examined DNA prepared from cosmid clones containing all known class I genes of the H-2^b haplotype (30). This analysis revealed that the D^{bm13}-specific oligonucleotide hybridized only to the K^b gene. However, because bm13 arose in an (C57BL/6 × BALB/c) F_1 animal (17) we can not exclude the possibility that a mitotic recombination event in the zygote between an H-2^d haplotype gene and the D^b gene generated the D^{bm13} gene.

The K^b Gene Is the Donor Gene for D^{bm24}. A comparison of the altered nucleotide sequence of D^{bm24} with the class I sequences available in the literature reveals a sequence that is identical to the K^b gene from amino acid positions 63-80 (93 nucleotides) and includes the eight substituted nucleotides that differentiate D^{bm24} from D^b (Fig. 1). To determine if K^b was the only donor gene for D^{bm24}, a D^{bm24}-specific

oligonucleotide probe was synthesized ($D^{bm24mer}$) (Table III). The $D^{bm24mer}$ is complementary to codons 73-79 of D^{bm24} and contains the altered nucleotides at positions 73 and 77. The $D^{bm24mer}$ was radiolabeled and hybridized to Bam HI-digested bm24 DNA. The $D^{bm24mer}$ probe hybridizes to only one additional band in both lanes, a 4.8-kb Bam HI fragment of the K^b gene (Fig. 2 *b*). That this fragment is identical to the 4.8-kb Bam HI size fragment of the K^b gene was confirmed by hybridization analysis of the H-2^b class I cosmid clones (30) with the oligonucleotide $D^{bm24mer}$ (results not shown). The $D^{bm24mer}$ hybridized only to the K^b gene. Thus, from these hybridization data and the known sequence information on K^b, we conclude that the change in D^{bm24} was generated by a micro-recombination event between the D^b and the K^b genes.

D^{bm14} Contains a Single Nucleotide Substitution Possibly Generated by Micro-recombination. Since only one nucleotide change was found in D^{bm14} (Fig. 1), a point mutation as well as a micro-recombination event could account for this change. To ascertain whether this nucleotide was present in other class I genes at the homologous position, we prepared an 18-base oligonucleotide ($D^{bm14mer}$) complementary to the D^{bm14} sequence (Table III) to identify a potential donor gene. The particular length and sequence of the 18-base oligonucleotide was limited to the codons of the corresponding amino acid residues 67-72, because the flanking sequences of this stretch diverge in other H-2^b class I genes. DNA hybridization analysis of Bam HI-digested genomic DNA from B6 and bm14 showed that under stringent conditions the bm14 specific oligonucleotide bound to the 2.1-kb Bam HI D^{bm14} gene fragment in the bm14 DNA but not to D^b (Fig. 2 *c*). The oligonucleotide also hybridized to additional potential donor sequences located on Bam HI fragments of ~6 and ~0.8 kb. The higher intensity of autoradiographic band at 6 kb (relative to the intensity to the bands at 0.8 and 2.1 kb) suggests that there may be more than one hybridizing fragment migrating in this position. Hybridization analysis of cloned class I genes (30) reveals that the specific $D^{bm14mer}$ oligonucleotide hybridized to the ~6-kb fragments of the $Q7^b$ and $Q9^b$ genes and to a 0.8-kb Bam HI fragment from a cosmid clone containing the $T1^b$-$T5^b$ genes (results not shown). An examination of $Q7^b$ and $Q9^b$ sequences showed the presence of a nucleotide stretch identical to the sequence corresponding to the $D^{bm14mer}$. In addition, the K^b, *Q4*, *Q8*, *Q10*, and $T3^b$ genes contain the AAT codon at position 70 and share very short stretches of identity with D^{bm14} and, therefore, would not be detected by the $D^{bm14mer}$, but still could be donors of very short DNA stretches. Thus, genes from different regions of the MHC could have been the donors for a recombination event to generate the D^{bm14} gene.

Deduced Amino Acid Changes of the Mutant D^{bm13}, D^{bm14}, and D^{bm24} Molecules. The deduced amino acid changes in the protein products of the three D^b mutants (Fig. 3) are as follows. (*a*) The four nucleotide changes in D^{bm13} in the codons of amino acid positions 114 (CTG to CAG), 116 (TTC to TAC), 118 (TAT to TAC), and 119 (GAA to GAC) result in three amino acid changes in the α2 domains (Fig. 3), Leu to Gln at 114, Phe to Tyr at 116, and Glu to Asp at 119. The change in the codon for amino acid 118 is silent. The three amino acid substitutions are contained in a stretch of 13 amino acid residues identical in sequence to the K^b molecule (Fig. 3). Except for the amino acid difference between K^b and D^{bm13} at position 121 (Cys in place of Arg), this stretch of identity between D^{bm13} and K^b extends for a total

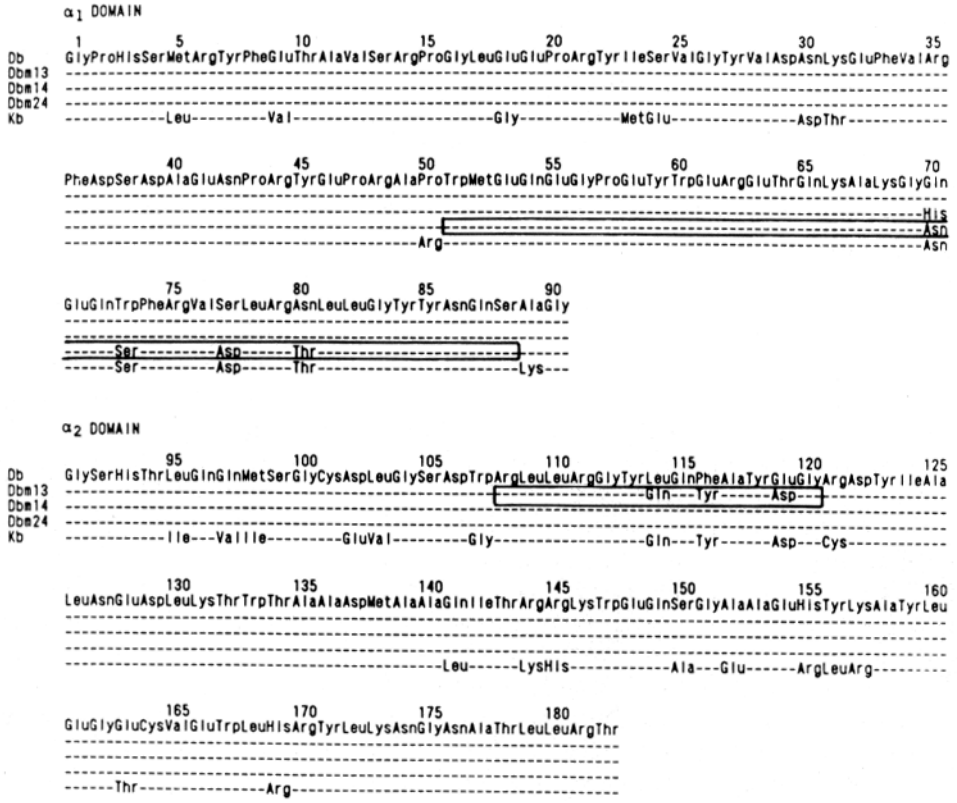

FIGURE 3. Amino acid sequence comparison of the α1 and α2 domains of D^b, D^{bm13}, D^{bm14}, D^{bm24}, and K^b. Differences of amino acids are found in D^{bm13} at position 114, 116, and 119; in D^{bm14} at position 70; and in D^{bm24} at position 70, 73, 77, and 80. Boxes show maximal length of amino acid identity between D^{bm13} and K^b, and D^{bm24} and K^b, respectively (K^b sequence, [36], D^b sequence, [33]). These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00806.

of 33 amino acid residues (108-141). (*b*) The single change found in D^{bm14} occurs in the codon of amino acid 70 (CAA to CAT) and replaces Gln by His. (*c*) The eight nucleotide changes found in D^{bm24} result in four amino acid replacements. The change in the codon for amino acid 63 (GAA to GAG) is silent. At amino acid position 70 a Gln is replaced by Asn (CAA to AAT). At position 73 a Trp is replaced by Ser (TGG to AGT). At position 77 a Ser is replaced by Asp (AGC to GAC). And finally, at position 80 an Asn is replaced by Thr (AAC to ACC). The amino acid identity between the K^b sequence and the altered D^{bm24} sequence (Fig. 3) extends to a stretch of 38 amino acids and, except for the difference at position 50 (Pro to Arg), the stretch of identity continues for another 20 amino acids. Thus, almost two thirds of the first domain of the D^{bm24} molecule is identical to K^b.

Discussion

Spontaneously arising variants expressing mutant H-2 molecules have been detected in inbred mouse strains at a relatively high frequency (11). We have estab-

lished the structural basis for the functional alterations observed in three spontaneously arising D^b mutants. The complete nucleotide sequences of the *D* genes from the bm13, bm14, and bm24 mutant mice (Fig. 1) establish that each variant has incurred unique structural mutations (Table II). D^{bm13} contains four altered nucleotides in the third exon (encoding the α2 domain) at the positions corresponding to amino acid residues 114, 116, 118, and 119. D^{bm14} contains a single nucleotide change in the codon for amino acid position 70 (α1 domain). D^{bm24} contains eight nucleotide changes in exon 2 (in the α1 domain) at positions corresponding to amino acid residues 63, 70, 73, 77, and 80. The changes in two of the three mutant D^b genes are multiple, clustered, and complex, and are therefore much like the changes found for the mutant K^b genes. These characteristics suggest that the alterations are not the result of random point mutation, but result from a recombination mechanism.

A significant conclusion from the studies of the K^b mutants (37) was that the altered K^b genes were generated by a micro-recombination process with other class I genes. Examination of a series of mutants in the *D* gene has permitted us to establish the generality at another MHC locus of the micro-recombination mechanism. The stretches of DNA containing the altered nucleotides in the D^{bm13}, D^{bm14}, and D^{bm24} genes are found in other class I genes of the H-2^b haplotype (Fig. 1, Table III). For example, the altered segment of D^{bm24} and D^{bm13} is identical to the homologous stretch in the K^b gene, suggesting that K^b could have been the donor gene for those mutants.

In contrast to the multiple changes found in D^{bm13} and D^{bm24}, a single nucleotide change was identified for D^{bm14}. We cannot, a priori, conclude whether a point mutation or a micro-recombination event could account for this alteration. However, when a single nucleotide change is flanked by a stretch of nucleotides with an identical counterpart in another class I gene, it is consistent with the idea that a recombination mechanism generated this alteration. A similar observation has been made for the K^{km1} mutant (38). Based on sequence analysis, a number of potential donor genes for D^{bm14} were identified. The $Q7^b$ and $Q9^b$ genes contain a stretch of 18 nucleotides identical in sequence and position to the D^{bm14} alteration (39). Hybridization analysis with a mutant-specific oligonucleotide indicated that these as well as another *T1a* gene could be potential donor genes.

Further in keeping with our contention that a micro-recombination generated the D^b mutants, is the finding of silent nucleotide alterations in the codons for amino acid positions 118 in D^{bm13} and 63 in D^{bm24} that are identical to those found in the K^b gene. Since silent or synonymous substitutions cannot be selected for at the product level, their presence in the mutant D^b genes cannot be explained by a point mutation/selection mechanism. Such data are most easily explained by the block transfer of genetic information (containing both synonymous and productive alterations) from the donor genes to D^b.

The direction of sequence transfer from donor to recipient in the micro-recombination process can proceed either telomerically or centromerically along the chromosome. For example, the D^b gene can act as a recipient of a stretch of sequence donated by the K^b gene to generate the mutant D^{bm24} and D^{bm13} genes. Conversely, D^b can also serve as a donor for the K^b gene (Fig. 4) as demonstrated by the K^{bm11} mutation (16).

Based on our interpretation that the mutants are generated by micro-recombination,

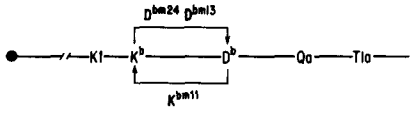

FIGURE 4. Schematic of D^b/K^b micro-recombinations. Solid line represents chromosome 17, with centromere at left. Capital letters represent the K, D, Qa, and Tla regions of the MHC. The centromeric *K1* gene is also included. Arrows indicate the transfer of genetic information from donor gene to recipient gene via the micro-recombination process. Mutant genes generated by micro-recombinations are indicated next to arrows. K^{bm11} data from Geliebter and Nathenson (16).

it is still an unexplained finding that more K^b mutant sublines (13 mutants) than D^b mutant sublines (three mutants) have been isolated. These apparent differences in mutation frequency could be the result of inherent properties of the DNA either within or surrounding the genes, or could be due to chromosomal location. For example, K^b is more centromerically located than D^b. Alternatively, relative to mutant K^b molecules, the mutant D^b molecules, due to specific antigenic features, may more often remain undetected by the isolation protocol of skin graft rejection.

It is important to consider other forces that mold the genetic features of the genes of the MHC. For example, the repeated expansion and contraction of the number of genes in the D region would lead to the homogenization of *D* gene sequences within the mouse population. In fact, the five genes in the D region of the $H\text{-}2^d$ haplotype may have resulted from the expansion of a D region containing a single gene, such as in $H\text{-}2^b$. Such an expansion has been postulated to have occurred by an unequal crossover between D and Qa region genes (40). Further, the spontaneous contraction of the $H\text{-}2^d$ D region to a single D^d gene (as in BALB/c-$H\text{-}2^{dm2}$) or a hybrid D^d/L^d gene (as in B10.D2-$H\text{-}2^{dm1}$) has been described (41, 42). However, despite the expansion and contraction of the D region, the extensive diversity and polymorphism of *D* and *L* genes in laboratory and wild mice are probably a result of the continuous micro-recombination process, coupled with natural selection. Thus, the evolution of the D region of the MHC reflects recombination events that diversify as well as homogenize its member genes.

Examination of the overall pattern of mutant/parent changes in the available mutants of the D^b and K^b genes reveals that the positions of the alterations at the protein level are clearly not random. This is evidenced from the examination of a diversity plot (43) of the altered amino residues of H-2 K^b mutants (bm1, bm8, bg series, bm3, bm11, bm23, bm4, and bm10) (Fig. 5 *b*) and the H-2D^b mutants (bm13, bm14, and bm24) (Fig. 5 *c*). The alterations of all three D^b molecules are found in the first or second domains at positions at which changes of the K^b mutants are also found. The sites of alteration of D^{bm14} and D^{bm24} are shared by three of the K^b mutants: K^{bm3} at amino acid positions 77 and 89 (16); K^{bm11} at positions 77 and 80 (16); and K^{bm23} at 75 and 77 (Ajitkumar, P., I. Egorov, and S. G. Nathenson, manuscript in preparation). The site of mutation of D^{bm13} is shared with five of the K^b mutants of the bg series; K^{bm5} and K^{bm16} have an alteration at position 116. K^{bm6}, K^{bm7}, and K^{bm9} have, in addition, an alteration at position 121.

The positions of the changes are of particular relevance when interpreted in relation to the recently determined three-dimensional structure of a class I molecule, HLA-A2 (44, 45). The α1 and α2 extracellular domains are organized into a super domain situated above the third domain, which is paired noncovalently with the

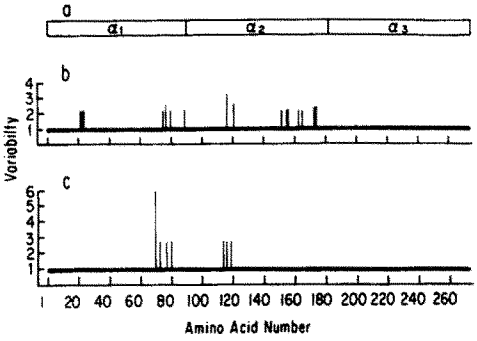

FIGURE 5. Diversity plot comparing K^b and D^b mutants. A class I molecule is schematically represented only on top (*a*). The three extracellular domains, α_1, α_2, and α_3, are compared. The variability was calculated according to formula used by Wu and Kabat (43): variability equals the number of different amino acids at a given position divided by the frequency of the most common amino acid at that position. Frequency equals the number of times the most common amino acid occurs divided by the total number of proteins examined. The amino acid residues from 12 K^b mutants (bm1, bm3, bm4, bm5, bm6, bm7, bm8, bm9, bm10, bm16, and bm23) and the wild-type K^b sequence were used for calculating the variability values of plot in *b*. Three regions contain multiple K^b mutations: ~70–90, 116–121, and 150–175. The variability values calculated for the amino acid residues from three D^b sequence are depicted in *c*. The changes of the D^b mutants fall into two of the K^b mutant regions.

β_2m subunit. The salient features of the structure of the α1 and α2 domains are two α helices, which are formed by the amino acid residues 50-84 of the α1 domain (α1 helix) and 138-181 of the α2 domain (α2 helix), and a deep groove (the putative antigen-binding site) between the α helices. The floor of the antigen-binding site is formed by strands of the antiparallel β-pleated sheet that run below the helices. It is possible to postulate that the K^b and D^b class I antigen-presenting molecules will have highly similar three-dimensional features in the α1 and α2 domains to HLA.A2 since these stretches are identical in length and are highly homologous in sequence. The amino acid residues of the α helices can be aligned without gaps or insertions, and there are identical regions of polymorphic and conserved residues (46). From such structural considerations using the α1 and α2 three-dimensional structure of an HLA-A2 molecule as a model, the positions of the allelic residues in the D^b mutants suggest a common theme. All stretches of changes include residues lining the putative antigen-binding groove. The D^{bm13} changes at 114, 116, and 119 are at the floor of the cleft formed by the β-pleated sheet. The D^{bm14} change at 70, and the D^{bm24} changes at 70, 73, and 77, are of residues that have side chains projecting into the antigen-binding cleft. Substitution of these residues might alter the structure of the antigen-binding site and the effect of such changes on the presentation of a peptide could be profound.

A major purpose of the present study was to identify the structural changes that result in alterations in the function of mutant D^b molecules. The influence of the mutant D^b changes has been established in studies for bm13 and bm14. Unfortunately, the bm24 strain was lost before extensive biological examination was possible. The D^{bm13} and D^{bm14} mutations influence the immunological response at two levels. In the CTL response against M-MuLV, neither of the two D^b mutant targets can be recognized by D^b-restricted CTL. Secondly, the mutations also profoundly alter the character of the immune response. For example, bm14 mice do not generate a CTL response against M-MuLV nor to H-Y (21, 22). Whereas M-MuLV antigens are usually predominantly presented by the D^b molecule, bm13 mice increase the K^b-associated component of the CTL response (21).

The D^{bm14} mutation at position 70 is a Gln to His. This residue on the α helix of the α1 domain in the HLA-A2 molecule has its side chain pointing into the antigen-binding groove. Since this one change abrogates the CTL response entirely, the nature of that side chain appears critical in controlling the capability for generating a response possibly due to an effect on the binding of immunodominant peptides of the foreign viral antigen. Such data agree with the importance of the homologous region of K molecules. For example, the bm3 (changes at 77 and 89) and bm11 (changes at 77 and 80) K^b mutants have become low responders for several K^b-restricted immune responses to viruses, such as Sendai, and vesicular stomatis virus (47, 48).

The modified immune status of the bm13 mouse compared with the wild-type B6 mouse is particularly intriguing because the CTL response to M-MuLV is not abrogated as for bm14, but rather altered to utilize the K^b product as the H-2 restriction element, as well as the D^{bm13} product (21). Our sequence results show that the stretch of amino acids from 108 to 120 contains 13 amino acids identical to K^b. The changes from the parental D^b sequences, Leu to Gln (position 114), a Phe to Tyr (position 116), and a Glu to Asp (position 119) occur on the β-pleated sheet that runs along the bottom of the antigen cleft. In the HLA-A2 molecule the side chains of 114 and 116 point up into the cleft and changes could alter binding of a foreign peptide. Interestingly, the gene conversion event results in remolding this stretch from 108 to 120 such that it is now identical to K^b. Since the K^b molecule is not used significantly by parental B6 mice as an antigen-presenting molecule, one hypothesis to explain the biological findings is that for the major CTL response against M-MuLV antigenic epitopes, the D^b molecule contains the proper configuration to present the major immunodominant peptide, a property for which K^b is inefficient. The D^{bm13} molecule thus still may retain the capability for presentation, but the MHC/peptide pattern important for CTL recognition is now shared by both K^b and D^{bm13} by their identity in the region 108-120.

The alteration found in D^{bm13} is the first example where a micro-recombination event has led to the appearance of a "new" class I molecule that at the same time alters the native immune responsiveness against a virus pathogen. In effect the bm13 mouse strain reflects a new haplotype in which K^b has now become an important restricting element for M-MuLV. Thus, this mutant could be considered an example of an intermediate stage in the process of evolution, by which genetic micro-recombination has provided the raw material for expanding, in the population, the number of possible restriction molecules that can present a particular antigen. The stability of the new MHC allele would then be decided by selective pressures at the population level.

Summary

Sequence analysis of the mutant D^{bm13}, D^{bm14}, and D^{bm24} genes indicate that they differ from the parental D^b gene by 4, 1, and 8 nucleotides, respectively. The mutant sequences substituted into D^{bm13} and D^{bm24} are identical to those found in the K^b gene, at the homologous positions. Thus, similar to the K^b gene, the D^b gene is able to undergo micro-recombination (gene conversion) events with other class I genes. Such data suggest that micro-recombination events could be an important mechanism for the diversification of all *H-2* genes. The D^b mutant products share a common theme: the alterations in all occur at amino acid residues whose side chains

in the homologous class I HLA-A2 molecule project into the postulated peptide antigen-binding cleft, and hence, would be expected to alter the binding of foreign or self peptides. Due to such changes, the bm14 mouse has become a nonresponder in the CTL response to Moloney murine leukemia virus (M-MuLV), as the alteration of one amino acid residue at position 70 (a Gln to His) is sufficient to entirely abrogate the cell-mediated response to the virus. On the other hand, the bm13 mouse has shifted the major part of its M-MuLV restriction to K^b, a profound alteration in CTL responsiveness due to the alteration of three amino acids (Leu to Gln at 114, Phe to Tyr at 116, and Glu to Asp at 119) in a peptide stretch of β-pleated sheet structure lining the bottom of the antigen-binding cleft. Thus, study of these mutants reveals that, in one step, micro-recombination at the genetic level has resulted at the protein level in profound changes in the immune response to viral infection. Such a mechanism operating at the population level can be a driving force during evolution for modulating the character of CTL immunity.

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