GENE CONVERSION BETWEEN MURINE CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX LOCI

Functional and Molecular Evidence from the bm12 Mutant

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The murine major histocompatibility complex (MHC)[1] is comprised of a cluster of closely linked genes that encode polymorphic cell-surface proteins important in mediating immune responses to a wide variety of immunological stimuli (1–3). The I region of the murine MHC encodes at least four polymorphic glycoproteins (Ia polypeptides) that are expressed primarily on the surfaces of antigen-presenting cells and B lymphocytes, and are involved in the control of immune responsiveness (4, 5). These associate noncovalently to form two functional heterodimers: I-A molecules ($A_\alpha A_\beta$) and I-E molecules ($E_\alpha E_\beta$) (6–8). The two subunits of the I-A molecule as well as the β-subunit of the I-E molecule are separately encoded within the I-A subregion of the MHC, while the α subunit of the I-E molecule is encoded within the I-E subregion (6) (Fig. 1).

Recent molecular data have shown that while both A_α and A_β exhibit substantial allelic protein polymorphism (10, 11), of the subunits comprising the I-E complex, only E_β displays significant allelic variation (12). Furthermore, A_α and E_β allelic polymorphism is clustered in short stretches of "hypervariability" within the first external protein domain (10, 12). Allelic variation in the A_β chain shows some clustering in the first domain, but polymorphism is seen in the second domain as well, although hypervariable regions are not as evident (11).

We have previously observed that regions of E_β allelic hypervariability (residues 1–13, 27–39, 68–75, and 87–93 of the mature E_β polypeptide) (12) in the first domain generally correspond to regions that show variability among A_β alleles, and that the stretches of sequence between these hypervariable regions are generally conserved both among alleles at a particular locus and between β loci (12). The pattern of E_β allelic polymorphism that has clusters of polymorphism separated by stretches of nucleotide sequence homology among E_β alleles and between class II β loci suggested the possibility that polymorphism in the β genes might be generated at least in part by a gene conversion–like mechanism.

Gene conversion and unequal crossing over were originally defined in fungi as two mechanisms that could act on the homologous members of multigene

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[1] *Abbreviations used in this paper:* bp, base pair; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; TdR, thymidine.

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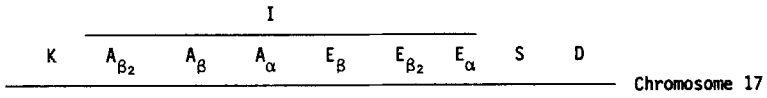

FIGURE 1. Genetic map of the I region and associated H-2 loci of the murine MHC on chromosome 17 (9).

families to maintain sequence homogeneity among closely related genes (13). Gene conversion has since been suggested to operate in several mammalian gene families, namely among the globin and immunoglobulin genes (14, 15), with a similar purpose. It has been speculated that the sequences of the multiple, homologous, tandemly arrayed genes of the mouse and human MHC might be generated and maintained by a mechanism such as gene conversion (16, 17). In the absence of nucleotide sequence data, however, these speculations could not be substantiated.

Recently, by nucleotide sequence comparison, it was suggested that the mutation in the bm1 mutant, a murine class I MHC K^b mutant, arose by gene conversion of a short stretch of nucleotide sequence from a homologous but nonallelic class I gene mapping to the Qa 2.3 locus (18). We now have evidence of a specific gene conversion event in the murine class II MHC genes.

The B6.C-H-2^{bm12} (bm12) mouse is a murine class II A_β^b mutant, derived by spontaneous mutation from a (BALB/c × B6)F_1 parent (19–21). The mutation was of a "gain and loss" type. The bm12 mutant and its B6 parent show reciprocal skin graft rejection and two-way mixed leukocyte reaction (MLR) (22), and the mutant exhibits differential responsiveness to some I-A–restricted antigens (23–25). Studies using the recombinant B10.MBR, and other genetic studies, mapped the bm12 mutation within the I-A^b subregion (26). Tryptic peptide comparison of Ia products from the bm12 strain and its B6 parent have shown the bm12 mutant to differ only in its I-A_β polypeptide (20, 27). Recent nucleotide sequence analysis of the A_β^{bm12} gene has shown it to differ by only three nucleotides from the parent A_β^b allele (28).

We now report the isolation of an alloreactive T cell clone that recognizes a determinant shared by E_β^b and A_β^{bm12}. To determine the extent of sequence homology between E_β^b and A_β^{bm12}, we have isolated and sequenced a cDNA clone of the murine class II E_β^b gene of the B10 mouse. Comparison of the nucleotide sequence of E_β^b with the sequences of A_β^b (11) and A_β^{bm12} (28) reveals that the bm12 mutation probably arose by gene conversion of a short stretch of nucleotide sequence from the E_β^b locus. This provides the first evidence of a gene conversion event involving two expressed class II genes. We provide further evidence that gene conversion–like events may play a prominent role in the generation of polymorphism in class II MHC β genes.

Materials and Methods

Mice. The adult inbred and F1 hybrid mice were bred in our animal facilities at the Department of Medicine, Stanford University, or purchased from The Jackson Laboratory, Bar Harbor, ME. Mice of either sex were used. Mouse strains used were C57BL/6 (B6), A/J (A), (C57BL/6 × A/J)F_1 (B6A), B10.A(5R), B6.C-H-2^{bm12} (bm12), and B10.

Monoclonal Antibodies. Anti-I-E^k monoclonal antibody 40K was generously provided

by Michelle Pierres, Marseilles, France, and has been described elsewhere (29). Monoclonal antibody BP107 (anti-I-A_β^b) has been previously demonstrated to react with the I-A_β^b chain (21, 30).

Long-term Cultured T Cell Clones. The establishment of alloreactive T cell clones has been previously reported (31).

Blocking Assay of Cloned T Cell Proliferation. The T cell clone proliferation assays and assays of monoclonal antibody blocking of T cell proliferation have been previously described (8). Briefly, 10^4 cloned T cells were cultured with 10^6 irradiated (3,300 rad) spleen cells from the strains indicated in the presence or absence of 1 μg purified anti-Ia antibody (29, 30). After a 2-d incubation, 1 μCi of [^{3}H]TdR was added per culture and TdR incorporation was measured by liquid scintillation counting as previously described (8). Counts represent the average of three replicate cultures. In most cases, the replicates were within 10% of the mean.

cDNA Cloning. Construction of a cDNA library from spleens of B10 mice has been described (10). The library was screened with a mouse E_β^k cDNA clone, pEB10 (12), by the method of Benton and Davis (32). Candidate clones were analyzed by restriction site mapping and Southern blotting (33).

DNA Sequence Determination. The nucleotide sequence of the pEBb1 insert was determined by the Sanger and Coulson dideoxynucleotide technique (34). Either short fragments were purified on agarose gels and cloned into the M13mp8 bacteriophage vector or the pEBb1 plasmid was digested with one or several restriction enzymes and cloned into M13mp8, and the resulting clones were screened with the appropriate EBb1 fragment as probe. In this way the sequence of the entire cDNA insert was determined on both strands (see Fig. 2).

Results

An Alloreactive T Cell Clone Recognizes $E_\alpha^k E_\beta^b$ and $A_\alpha^b A_\beta^{bm12}$. The initial suggestion that the A_β^{bm12} and E_β^b polypeptide chains might share a functionally important region of protein sequence not shared by the A_β^b polypeptide came from the observed reactivity pattern of an alloreactive murine T cell clone "4.1.4" (Table I). This clone was originally derived from a mixed lymphocyte culture in which strain A lymph node cells were repeatedly stimulated with irradiated spleen cells from B6A hybrids. The data presented in Table I suggest that clone 4.1.4 recognizes an allodeterminant on the $E_\alpha^k E_\beta^b$ molecule. This is demonstrated by the ability of clone 4.1.4 to be stimulated by B6A cells and

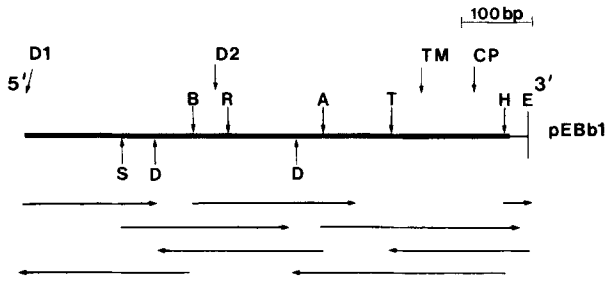

FIGURE 2. Partial restriction map of the pEBb1 insert. Heavy line represents coding sequence; lighter line represents 3′ untranslated sequence. 5′ and 3′ refer to the direction of transcription. 5′ start sites of first external protein domain (*D1*), second external protein domain (*D2*), transmembrane region (*TM*), and cytoplasmic tail region (*CP*) are shown. The original cloned gene contained a translocation of 300 bp of the 3′ untranslated region onto the 5′ end of the clone. This translocated segment has been deleted from the fragment shown. *S* = BstEII, *D* = HpaII, *B* = BglII, *R* = RsaI, *A* = AvaII, *T* = Tth111-I, *H* = HinfI, *E* = EcoRI. Horizontal arrows show direction and extent of nucleotide sequence determination.

TABLE I
Reactivity Pattern of Alloreactive T Cell Clones

Clone	A	B6	B6A	B10.A(5R)	bm12
4.1.4	336	647	9,025	8,213	7,603
S83.7	700	243	59,797	183	149
S83.4	422	22,024	22,160	20,712	14,657
I-A	$A_\alpha^k A_\beta^k$	$A_\alpha^b A_\beta^b$	$A_\alpha^b A_\beta^b$ $A_\alpha^k A_\beta^k$ $A_\alpha^b A_\beta^k$ $A_\alpha^k A_\beta^k$	$A_\alpha^b A_\beta^b$	$A_\alpha^b A_\beta^{bm12}$
I-E	$E_\alpha^k E_\beta^k$	None	$E_\alpha^k E_\beta^b$ $E_\alpha^k E_\beta^k$	$E_\alpha^k E_\beta^b$	None

The response, expressed as cpm of incorporated [^{3}H]TdR, of alloreactive T cell clones (10×10^3/well) to coculture with 1×10^6 irradiated (3,300 rad) spleen cells (1×10^6/well) in triplicate cultures for 72 h, including a terminal pulse with 1 μCi of [^{3}H]TdR. Culture conditions are as described (8). The I-A and I-E molecular complexes that exist on Ia-bearing cells of each strain are also shown.

B10.A(5R) cells, but not by B6 spleen cells. Unexpected was the observation that clone 4.1.4 recognizes spleen cells from strain bm12 equally well (Table I). For comparison, reactivity patterns of two additional alloreactive T cell clones are included which demonstrate recognition of either the $A_\alpha^b A_\beta^b$ molecule (clone S83.4) or a hybrid I-A molecule (clone S83.7).

Monoclonal Antibody-blocking Studies Show that Clone 4.1.4 Is Stimulated by I-E Region Products on B6A and B10.A(5R) Cells but by I-A Region Products on bm12 Cells. There are several possible interpretations of the reactivity pattern of clone 4.1.4. One possibility is that the bm12 strain in some manner uses the A_α^b chain in association with the E_β^b chain, and it is this complex that is recognized by clone 4.1.4. Recognition by clone 4.1.4 of a determinant on the E_β^b chain might then explain the similar reactivity patterns of strains bm12 and B10.A(5R) stimulator cells. This possibility seemed unlikely, since the association of A_α with E_β has not yet been demonstrated. However, to examine this possibility, and to better localize the determinant recognized by clone 4.1.4, clone 4.1.4 was stimulated by B6A, B10.A(5R), or bm12 cells in the presence of different monoclonal antibodies that have been previously characterized (21, 29).

Data presented in Table II demonstrate that clone 4.1.4 is stimulated by I-E region products on B10.A(5R) cells and B6A cells, but by I-A region products on bm12 cells. Antibody 40K is an anti-I-E–specific antibody (29). Antibody BP107 is an antibody to the A_β^b molecule (21). Using these two monoclonal antibodies, it was possible to demonstrate that antibody 40K blocked the determinant recognized by clone 4.1.4 on B10.A(5R) cells and B6A cells, but not on bm12 cells. This is consistent with the interpretation that the I-E region molecule is being recognized on the B10.A(5R) and B6A cells. Antibody BP107, which reacts with the A_β^b chain, blocks the ability of clone 4.1.4 to be stimulated by bm12 cells, but not by B10.A(5R) nor B6A stimulator cells. These data clearly demonstrate that clone 4.1.4 is stimulated by an I-E region product on B10.A(5R)

TABLE II
Monoclonal Anti-Ia Inhibition of Clone 4.1.4

	Stimulator strains				Antibody
	A	B6A	bm12	B10.A(5R)	
Clone 4.1.4	1,104	12,389	6,004	23,887	None
	NT	11,687	1,980	18,530	BP107
	NT	1,168	5,407	1,833	40K

Cloned T cells (10^4) were cocultured for 72 h with 1×10^6 irradiated spleen cells (3,300 rad) from the strains indicated in 200 μl MLR medium with or without the addition of 1 μg antibody (as indicated). Results are expressed as cpm of the mean of triplicate cultures following an overnight pulse with 1 μCi of [^{3}H]TdR before harvest and standard scintillation counting. Cultures inhibited by antibody are underlined for ease of presentation.

and B6A cells, yet is stimulated by an I-A region determinant on bm12 cells. The most likely hypothesis to correlate the reactivity pattern of clone 4.1.4 with the antibody blocking data is to suggest that one consequence of the bm12 mutation event is the sharing of a portion of protein sequence between E_β^b and the mutant A_β^{bm12} chain, resulting in the duplication of at least one functional determinant in both class II β chains. To determine the exact extent of nucleotide sequence homology between these two genes, and to elucidate the genetic mechanism for its generation, we undertook a molecular analysis of the E_β^b gene.

Isolation and Characterization of a cDNA Clone for the E_β^b Gene. Using a 1,050-basepair (bp) cDNA clone of the murine E_β^k gene (12) as a probe to screen a B10 (H-2^b) mouse cDNA library, we isolated multiple copies of E_β^b cDNA recombinants under highly stringent hybridization conditions. A partial restriction map of the longest of these clones, pEBb1, is shown in Fig. 2. We determined its nucleotide sequence by the M13-dideoxy method (34), using the strategy shown (Fig. 2). The 728-bp clone extends at the 5′ end from the codon for the fifth amino acid of the mature polypeptide through a 704-bp coding sequence and translational stop codon and terminates 24 bp into the 3′ untranslated region.

Identification of a Donor Gene for the bm12 Mutation Event. The nucleotide sequences of the bm12 mutant and the parent A_β^b allele differ by three nucleotides in a clustered region of 14 nucleotides between amino acid residues 67–71 of the mature polypeptide (28). The DNA sequence and predicted protein sequence of E_β^b is presented in Fig. 3, and is compared with the DNA sequences of A_β^b and A_β^{bm12} in the first domain region where the bm12 mutation has been localized. (Due to a first exon splicing difference between A_β and E_β, residue 1 of A_β corresponds to residue 2 of E_β when the sequences are aligned for maximum homology.) Comparison of sequences shows that the bm12 DNA sequence is identical to the E_β^b sequence in the region where it differs from A_β^b. Furthermore, this region is flanked by regions of exact homology between A_β^b and E_β^b which extend 20 nucleotides to the 5′ side and 9 nucleotides to the 3′ side of the bm12 mutation cluster. These flanking regions presumably provided stabilization of a heteroduplex formed between β genes, thereby allowing sequence transfer. Regions of inexact but significant homology extend even further in both direc-

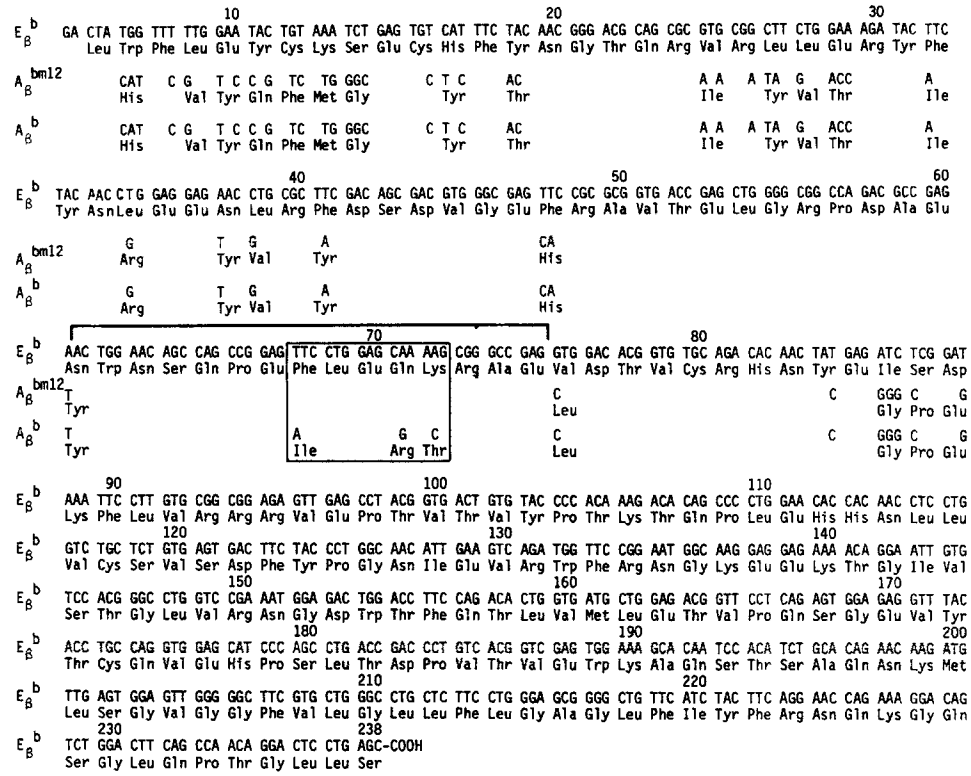

FIGURE 3. Comparison of the DNA and predicted amino acid sequences of the E_β^b donor gene (top line), the A_β^{bm12} mutant gene (center line, from reference 28), and the A_β^b gene (bottom line, from reference 11) in the first domain region where the bm12 mutation occurs, and the nucleotide and predicted amino acid sequence for the remainder of the E_β^b coding sequence. The bm12 mutation cluster is boxed. A horizontal bar above the sequences shows the predicted maximum extent of sequence transfer.

tions. We predict that the bm12 mutation arose by gene conversion of this region of the E_β^b gene into the corresponding region of the A_β^b gene. The maximum extent of sequence transfer between E_β^b and A_β^b is predicted to be 44 nucleotides, but could be as little as 14 nucleotides.

Evidence That Gene Conversion–like Events May Act More Generally among class II β Genes to Generate Sequence Diversity. The identification of a specific gene conversion event involving the two mouse class II β chain loci naturally leads to the question of the generality of such a mechanism. We have addressed this question by examining the potential for gene conversion by comparing nucleotide sequences of class II β genes. Fig. 4 compares the nucleotide sequences of $E_\beta^{k,d}$ (12, 35) with $A_\beta^{k,d}$ (11) and reveals that in roughly half of the amino acid positions where the E_β alleles are polymorphic, one of the substituted residues is the same as the residue at the corresponding position in one of the A_β alleles (boxed positions in Fig. 4). At each of the boxed positions in Fig. 4, all three nucleotide positions of the codon are conserved between β loci. This argues against selection at the protein level for certain residues at these positions, especially for residues such as arginine and leucine, which are each encoded by six codons, and show

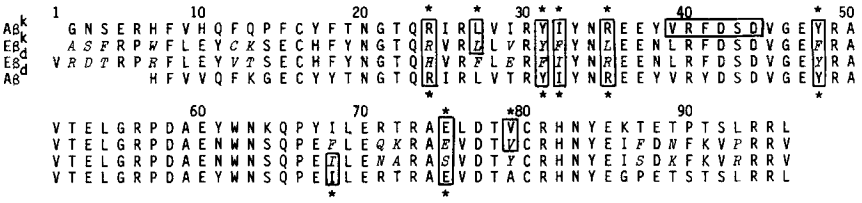

FIGURE 4. Comparison of the predicted protein sequences of the A_β^k gene (top line from reference 11), the E_β^k gene (second line, from reference 12), the E_β^d gene (third line, from reference 35), and the A_β^d gene (bottom line, from reference 11) in the first external protein domain. Letters in italics represent residues that are polymorphic between the two E_β alleles. Vertical boxes emphasize codons conserved between β loci (see text), and stars above or below certain A_β residues indicate that the extent of interlocus nucleotide sequence homology surrounding the starred residue is greater between the appropriate E_β allele and A_β^k (above) or A_β^d (below). The conserved hexapeptide from residue 39–45 (see text) is boxed.

no codon usage bias in the class II β genes. The same observation has been made for the human class II DR_β and DC_β genes (17). Furthermore, in the mouse, these identical codons are flanked by variable-length stretches of exact nucleotide sequence homology between β loci of between 15 and 57 nucleotides which could presumably mediate DNA sequence transfer between loci. The boxed hexapeptide in Fig. 4 is conserved at the nucleotide level, with the exception of one base change in E_β and one in A_β^d, in all human and mouse class I heavy chains and most class II light chains that have been examined (17). Its function is unknown; it may be important at the protein or nucleotide level, but certainly in the mouse class II β genes it could serve as part or all of the sequence that provides homology between β genes and allows intergenic exchange of sequences in the surrounding regions.

Discussion

We have reported the isolation of an alloreactive T cell clone 4.1.4 that is stimulated by B10.A(5R), B6A, and the A_β^b mutant line B6.C-H-2^{bm12} (bm12) spleen cells, but not by the bm12 parent B6 cells. Since clone 4.1.4 was derived from strain A anti-B6A, the stimulating Ia molecules on bm12 and B10.A(5R) cells must share an epitope recognized by this T cell clone. Correlation of the reactivity pattern of clone 4.1.4 with antibody-blocking data using monoclonal antibodies 40K (anti-I-E^k) and BP107 (anti-A_β^b) suggests that clone 4.1.4 recognizes an epitope determined by both the A_β^{bm12} and E_β^b chains, but not by the bm12 parent A_β^b allele. This T cell clone may recognize this shared β chain epitope in conjunction with a determinant on the associated α chain. Comparison of the protein sequences of A_α^b and E_α^k shows many stretches of sequence throughout the protein that are conserved between the chains (10, 36). The epitope recognized by clone 4.1.4 may be formed by shared A_β^{bm12} and E_β^b sequence and region(s) of E_α^k and A_α^b interlocus homology. Alternatively, clone 4.1.4 may recognize the shared β chain determinant alone.

That this shared A_β^{bm12} and E_β^b determinant might function effectively in association with either A_α^b or E_α^k is substantiated by recent data showing that an A_β^k gene, transfected into Ia^d-bearing B cells, was recognized by an antigen-dependent I-A^k–restricted T cell clone (37). Clearly, the antigen-reactive I-A^k–

restricted T cell clone was able to recognize the A_β^k gene product in the absence of an associated A_α^k chain. Presumably, the transfected A_β^k gene product associated at the cell surface with the endogenous A_β^d gene product to form a molecule capable of presenting antigen to the I-A^k-reactive clone (37). These data suggest that Ia β chains are capable of being appropriately recognized by some T cell clones independent of the genotype of the associated α chain. Regardless of the exact constitution of the epitope recognized by clone 4.1.4, at least a portion must be formed by sequence shared between A_β^{bm12} and E_β^b.

We have localized this shared sequence recognized by clone 4.1.4 and defined the molecular basis for the bm12 mutation by isolating a cDNA clone for the E_β^b gene and determining its nucleotide sequence. A_β^{bm12} differs from its A_β^b parent allele by only three nucleotides in a clustered region of 14 nucleotides between amino acid residues 67–71 of the mature A_β polypeptide (28). This clustering of mutations within a short stretch of nucleotide sequence suggested the possibility that the bm12 mutations were generated by a single mutational event. Isolation of the E_β^b gene revealed that in the bm12 mutation region, A_β^{bm12} is identical to E_β^b in the corresponding region. We therefore predict the bm12 mutation arose by gene conversion of this stretch of sequence from the E_β^b locus to the A_β^b locus.

Since the bm12 mutation arose in a (BALB/c × B6)F_1 (19), we examined the sequences of E_β^d and A_β^d for their ability to act as donor genes. In both cases, the nucleotide sequences in the presumed conversion region do not match the bm12 sequence in the same region. We therefore believe that the bm12 mutation arose by gene conversion from the E_β^b locus. This result extends the recent suggestion of a gene conversion event between class I MHC genes (18) to mouse class II β genes.

We have observed that allelic polymorphism in the E_β chains clusters in short stretches of hypervariability in the first domain, and have suggested that these regions form the active sites for interaction with foreign antigen or a T cell receptor (10, 12). The bm12 mutation cluster spans one of these hypervariable sequences (residues 68–75) (12). Recognition of this region by clone 4.1.4 provides the first direct evidence of the functional importance of these variable regions in T cell stimulation.

The intergenic transfer of a hypervariable region sequence in the generation of the bm12 mutation may be only one example of a more general mechanism, one that would implicate conversion events in conjunction with phenotypic selection in the evolution of the clustered pattern of polymorphisms observed among E_β alleles. Such a model is supported by our previous observation that the clustered regions of E_β allelic polymorphism are often flanked by regions of E_β vs. A_β homology that could presumably mediate sequence transfer (12). Indeed, there are regions in the E_β and A_β genes where DNA sequence differences between loci are no greater than the differences among alleles at either locus. Some stretches of E_β sequence are more homologous to A_β than to other E_β alleles (11, 12).

Gene conversion may act under different circumstances both to generate sequence diversity and maintain sequence homology in MHC genes, its phenotypic manifestation determined in part by the different selective pressures oper-

ating on different protein regions. It is highly likely that the extreme polymorphism among class II alleles arose to some degree by the intergenic exchange of sequences between class II loci, mediated by regions of interlocus homology. The identification of a conversion event involving the transfer of sequence corresponding to an allelic hypervariable region implicates conversion as a mechanism that acts on variable region sequences and, in combination with selection, generates polymorphism between class II β loci and perhaps also among alleles. (However, there is no evidence that gene conversion–like events are responsible for the generation of the clustered pattern of allelic polymorphism observed in the mouse A_α alleles (10)).

The extended regions of homology between class II loci in mouse and man (10, 12, 17, 36) might themselves be maintained by gene conversion events. In fact, by analogy to previously defined gene conversion events, conversion-like events in MHC genes might more generally act on homologous sequences in the first external protein domain or more highly conserved second domain to maintain sequence homogeneity in structurally important regions. However, in these cases, the identification of donor and recipient genes is made more difficult because of the scarcity of identifying allelic or interlocus polymorphism in these regions. Regions of homology are likely to be functionally important to these molecules, and conversion may serve to prevent their divergence, while random mutations in allelic hypervariable regions may be selected by the expanded ability they afford the organism to respond to foreign antigens; or conversion in combination with selection may act specifically on hypervariable regions, as in the case of bm12, to shift functionally important "mini-genes" between active sites to interact with antigen or T cell receptor.

Summary

The experiments presented in this study define the molecular basis of the bm12 mutation. Initial characterization of an alloreactive T cell clone, 4.1.4, showed this clone to recognize an allodeterminant present on the E_β^b and A_β^{bm12} chains, but not on the bm12 parent A_β^b chain. To define the extent of sequence shared between the I-E_β product and the mutant I-A_β product, we isolated a cDNA clone of the E_β^b gene and determined its nucleotide sequence. Comparison of the nucleotide sequences of E_β^b, A_β^b, and A_β^{bm12} shows the A_β^{bm12} gene to be identical to the E_β^b gene in the region where it differs from its A_β^b parent. We predict that the bm12 mutation arose by gene conversion of this region, which spans 14 nucleotides between amino acid residues 67–71 of the mature A_β chain, from the E_β^b locus to the corresponding position at the A_β^b locus. Recognition of this region, which spans one of the previously defined E_β allelic "hypervariable" regions, by an alloreactive T cell clone provides the first direct evidence of the functional importance of these hypervariable regions in T cell stimulation. The identification of a gene conversion event involving one of these allelic variable regions implicates conversion as a mechanism that acts on class II β genes to create sequence diversity in regions of Ia molecules that interact with foreign antigen or a T cell receptor, regions where protein sequence polymorphism would presumably be selected for by the expanded ability it affords the organism to mount effective immune responses against a wider variety of foreign antigens.

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