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The Molecular and Functional Characterization of a Dominant Minor H Antigen, H60[1,2]

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Minor histocompatibility (H) Ags elicit T cell responses and thereby cause chronic graft rejection and graft-vs-host disease among MHC identical individuals. Although numerous independent H loci exist in mice of a given MHC haplotype, certain H Ags dominate the immune response and are thus of considerable conceptual and therapeutic importance. To identify these H Ags and their genes, *lacZ*-inducible CD8$^+$ T cell hybrids were generated by immunizing C57BL/6 (B6) mice with MHC identical BALB.B spleen cells. The cDNA clones encoding the precursor for the antigenic peptide/K^b MHC class I complex were isolated by expression cloning using the BCZ39.84 T cell as a probe. The cDNAs defined a new *H* locus (termed *H60*), located on mouse chromosome 10, and encoded a novel protein that contains the naturally processed octapeptide LTFNYRNL (LYL8) presented by the K^b MHC molecule. Southern blot analysis revealed that the *H60* locus was polymorphic among the BALB and the B6 strains. However, none of the *H60* transcripts expressed in the donor BALB spleen were detected in the host B6 strain. The expression and immunogenicity of the LYL8/K^b complex in BALB.B and CXB recombinant inbred strains strongly suggested that the *H60* locus may account for one of the previously described antigenic activity among these strains. The results establish the source of an immunodominant autosomal minor H Ag that, by its differential transcription in the donor vs the host strains, provides a novel peptide/MHC target for host CD8$^+$ T cells. *The Journal of Immunology,* **1998, 161: 3501–3509.**

Despite sharing identical MHC, polymorphisms among the minor histocompatibility (H)[4] Ags cause T cell-mediated chronic graft rejection and graft-vs-host disease (1). During the past 80 yr, over 50 minor *H* loci have been described in recombinant and congenic mice. The vast majority of these loci are located on the autosomes, but a few also map to the mitochondrial and sex-chromosomes (2–4). The H Ags serve as the source of antigenic peptides presented by the MHC molecules that are detected by allogeneic T cells (5). The H Ag polymorphisms are therefore reflected in quantitative or qualitative differences in the processed peptide/MHC ligands that profoundly influence the T cell response.

The sources of H Ags and the mechanisms by which H Ag polymorphisms affect the T cell response have been debated for decades but are now beginning to be revealed. The mitochondrial ND1 protein was the first *H* gene product to be identified, from which a N-formylated peptide was presented by the nonclassical H2-M3 MHC class I^b molecule to CD8$^+$ T cells (6). Different alleles of the ND1 protein differed by a single amino acid substitution within this peptide sequence and were recognized bidirectionally by T cells in appropriate donor/host strain combinations. Since then, two other mitochondrial proteins, COI and ATPase 6, have been found to be sources of peptides presented by H2-M3 and rat RT1^a molecules, respectively (7, 8). By contrast, the unidirectional female anti-male CD8$^+$ T cell response (anti-*HY*) was recently shown to be specific for peptide/MHC complexes derived from the nuclear Y chromosome-encoded *Smcy* or the *Uty* genes (9–11). Again, multiple amino acid substitutions within the antigenic peptides in the corresponding female Smcx and Utx homologues presumably allowed the male peptides to be recognized as nonself. Furthermore, the *HY*-encoding genes are ubiquitously expressed, and their gene products are immunogenic in mouse, rat, and primates (12). Thus, identifying the precursor source proteins of H Ags in one species may also be relvant to H Ags in other species as well.

The relative simplicity of the mitochondrial and the Y chromosomes led to the identification of these *H* loci and their gene products. In contrast, the autosomal *H* loci have been rather difficult to identify by traditional positional cloning approaches due to the high gene density of the autosomal chromosomes. As a consequence, the role of polymorphic autosomal *H* loci in tissue rejection reactions has been difficult to elucidate. For example, several intriguing autosomal Ags have been detected in the MHC-matched C57BL/6 anti-BALB.B response, with hierarchical immunogenicity among themselves and relative to the sex-linked *HY* loci (13–17). Possible mechanisms that could account for their immunodominance include substitutions in antigenic peptide sequences and/or in the relative abundance of peptide/MHC expressed in the donor strain. Alternatively, homologous peptides in the host strain could cause differences in frequency of the responding T cell precursors by influencing the development of the T cell repertoire

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[2] The seqnence reported in this paper has been deposited in the GenBank database under accession No. AF084643.

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[4] Abbreviations used in this paper: H, minor histocompatibility; RI, recombinant inbred; B6, C57BL/6; CPRG, chlorophenol red β-galactopyranoside; SSCP, single strand conformational polymorphism; TFA, trifluoroacetic acid; NCBI, National Center for Biotechnology Information.

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(18–21). To test these hypotheses and to understand how genetic polymorphisms yield immunogenic peptide/MHC complexes, it is necessary to identify the H peptides, and their precursor proteins in the donor strain, and to determine their relationship to the corresponding host homologues.

We recently used a novel expression cloning strategy to define the autosomal *H13* locus at the molecular level (22–25). Here we apply this strategy to define a new autosomal *H* locus defined by C57BL/6 anti-BALB.B CTLs, designated *H60*.

Materials and Methods

Mice

All the indicated inbred mouse strains were obtained from or bred at The Jackson Laboratory (Bar Harbor, ME). The BALB.B and (BALB/c × BALB.B)F_1 mice were bred in the animal care facility at the University of California, Berkeley, CA, from The Jackson Laboratory stocks.

Cell lines

Cell lines were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (HyClone, Ogden, UT) or in DMEM supplemented as described (26). LMtk$^-$ (C3H, H-2^k) and its MHC-expressing transfectant derivatives have been described previously (27). EL4-B7 (H-2^b), RMA (H-2^b), and its TAP$^-$ derivative RMA/S cell lines were obtained from Drs. J Allison and D. Raulet (University of California, Berkeley, CA). To generate anti-BALB.B or anti-CXB-K-specific CTL, B6 mice were primed i.p. with 2×10^7 splenocytes from H2^b-matched BALB.B or CXB-K/By recombinant inbred (RI) mice. After 7 to 14 days, primed responder cells were harvested and restimulated in mixed lymphocyte culture (MLC) with 5×10^6 200-Gy-irradiated CXB-K cells in modified DMEM medium supplemented with 10 to 30 U/ml rIL-2, using established conditions (28). For restimulation in vitro, 4×10^6 cells from the mixed lymphocyte cultures were cocultured with 5×10^6 200-Gy-irradiated B6 spleen cells pulsed with 10 nM LYL8 peptide for 30 min at 37°C and then washed once in DMEM medium. All CTL lines were maintained by weekly stimulation with BALB.B spleen cells and 10 to 30 U/ml rIL-2, using established conditions (29). The *lacZ*-inducible T cell hybrid BCZ39.84 was generated by fusing the anti-BALB.B CTL line with the BWZ.36/CD8α fusion partner, as described (30).

T cell activation assays

T cell responses specific for peptide/MHC were measured by the production of β-galactosidase (lacZ) activity in the T cell hybrids (30, 31). T cell hybrids ($3–10 \times 10^4$) were cocultured overnight with APC ($2–5 \times 10^4$) either expressing the Ag endogenously, transfected with Ag cDNAs or with exogenous peptides in 96-well plates. The peptide/MHC-induced T cell response was assayed as lacZ activity using the substrate chlorophenol red β-galactoside (CPRG), as described (30). The conversion of CPRG to chlorophenol red was measured at 595 nm and 655 nm as a reference wavelength with a 96-well microplate reader (Bio-Rad, Richmond, CA). Data show the mean absorbance of replicate cultures and are representative of at least three independent experiments. For detection of cytolytic activity, 4-h ^{51}Cr release assays were performed. Peptides were tested using ^{51}Cr-labeled TAP-deficient RMA/S cells that were incubated with 10 nM LYL8 (H60) or with the VSV (RGYVYQGL) (32), at 30 min at 37°C, and washed twice to remove unbound peptide. Effector cells were then added to target cells in V-bottom plates at varied E:T cell ratios. Percent specific lysis, calculated from the amount of ^{51}Cr released into the culture supernatant, is shown as the mean of triplicate cultures.

cDNA library and expression screens

A unidirectional cDNA library was constructed (Superscript Choice System, Life Technologies) using poly(A)$^+$ mRNA from Con A-stimulated (BALB.B × BALB/c)F_1 spleen cells in the *Bst*XI/*Not*I sites of the mammalian expression vector pcDNA1 (Invitrogen, San Diego, CA) (23, 33). The cDNAs were screened by transforming competent bacteria with recombinant plasmids and culturing in pools of ~30 to 100 cfu in 96-well U-bottom plates. Aliquots of cDNA, prepared by the alkali lysis method directly in the 96-well plates (34), were transiently transfected into 3×10^4 LMtk$^-$ cells cotransfected with the relevant MHC Class I cDNA (10 ng/ml) and B7-2 cDNA (5 ng/ml). Two days later, 10×10^4 BCZ39.84 T cells were added per well and cocultured overnight. Positive pools were identified by adding CPRG and scoring pools with above background absorbance. The plasmid encoding the antigenic activity was identified by repeating the screen with individual colonies obtained from the positive cDNA pool. The *H60* cDNA sequence is available from the NCBI GenBank with the accession No. AF084643.

Expression constructs and peptides

Deletion constructs were generated by amplifying DNA fragments of the 28.64 plasmid with a vector-specific T7 forward primer (5′-AATACG ACTCACTATAG-3′) and three reverse primers R1 (5′-AGCAGTAGTGT GAAATCTCTTTAC), R2 (5′-GTATCGTAGATGTTTTATCCACTT), or R3 (5′-TTCATTGATGGATTCTGGGCCATC), using Pfu polymerase (Stratagene, La Jolla, CA). PCR fragments were digested with *Bam*HI in the 5′ flanking region of the vector and cloned into the *Bam*HI/*Eco*RV sites of pcDNA1. Minigene constructs MLYL8 (MLTFNYRNL), MIFL8 (MILSLFILL), and MFFI8 (MFIDGFWAI) shown in Figure 4 were prepared using complementary oligonucleotides corresponding to the indicated sequences. The synthetic peptides LTFNYRNL (LYL8), SIINFEKL (SL8) (35), RGYVYQGL (VSV) (32), ANYDFINV (AFNV8) (36), and SLVELTSL (SEL8) (33) were prepared using solid phase F-moc chemistry on the ABI Model 433 synthesizer (Perkin Elmer, Foster City, CA), purified by HPLC, and confirmed by mass spectrometry.

Genetic mapping and expression analysis

Southern and Northern blots were prepared according to established procedures (34). For Southern blots, genomic DNA from the indicated strains were either purchased from The Jackson Laboratories DNA resource or prepared from mouse liver. Genomic DNA was digested with *Bam*HI or *Xba*I before electrophoresis and transferred to nylon membranes (Zetabind, Bio-Rad). Following DNA cross-linking to the membranes by UV irradiation (Stratalinker; Stratagene), the blot was probed with ^{32}P-labeled DNA inserts of 28.64 or 22.26 plasmids containing the antigenic peptide, washed at 0.1× SSC at 60°C, and exposed for 24 to 48 h before autoradiography. For Northern blots, total RNA from resting or Con A + LPS-activated spleen cells was fractionated, transferred to nylon membranes, and probed with ^{32}P-labeled 22.26 cDNA fragment, washed, and autoradiographed, as above. For simple sequence length polymorphism (SSLP) analysis, splenic genomic DNA was amplified by PCR according to standard procedures (37), using the primer pairs for *D10Mit2* (5′-CTGCTCA CAACCCATTCCTT-3′) and (5′-GTTCATTTGAGGCACAAGCA-3′) purchased from Research Genetics (Huntsville, AL). PCR products were run on 3% agarose gels and visualized with a UV transilluminator, following ethidium bromide (EtBr) staining. For mapping of the *H60* gene, a single strand conformational polymorphism (SSCP) that distinguished parental B6 and SPRET/Ei strains was used to type The Jackson Laboratory BSB panel of (C57BL/6 × SPRET/Ei)F_1 × C57BL/6 DNAs (38). PCR primer pairs *H60*-F (5′-GTGTGATGACGATTTGTTGAG-3′) and H60-R (5′-ATTGATGGATTCTGGGCCATC-3′) amplified a 195-bp genomic DNA fragment from the 3′ region of the 28–64 cDNA. PCR amplification was performed in the presence of [α-^{32}P]dCTP (Amersham, Arlington Heights, IL), and the DNA samples were heat denatured and placed on ice before gel electrophoresis. MDE gels were run according to the manufacturer's (FMC Bioproducts, Natick, MA) recommended protocols, and the SSCP was visualized by autoradiography.

MHC binding assays

The synthetic LYL8 peptide was compared with AFNV8, SEL8, and SL8 peptides for its K^b-stabilization ability using RMA/S cells. RMA/S cells were first incubated overnight at 31°C to stabilize the "empty" K^b molecules and were added to varying concentration of the peptides in a 96-well U-bottom plate. The plate was incubated at 31°C for 30 min, allowing peptides to bind to previously stabilized K^b molecules. After this incubation period, unbound peptides were washed off. The cells were then incubated at 37°C for 4 to 5 h to cause dissociation of K^b molecules that remained empty or were weakly bound to the peptide. The residual K^b was measured by staining the cells with the anti-K^b mAbs Y3 or 5F1 and using flow cytometry. In the second assay, the ability of different peptides to compete with the SL8 peptide for binding to K^b MHC was measured using the recently described Ab, 25D1.16 (a kind gift of Drs. A. Porgador and R.N. Germain, National Institutes of Health, Bethesda, MD), which recognizes the SL8/K^b complex (39). To RMA/S cells, previously incubated at 31°C overnight, the SL8 peptide (50 nM) was added in the absence or presence of varying concentrations of each competitor peptide. Peptide-cell mixtures were incubated at 31°C for 4 to 5h, washed, and then stained with 25D1.16 ascites (1:500) to quantitate the relative levels of the same SL8/K^b complex. The decrease in expression of SL8/K^b complexes is a measure of K^b MHC binding by the competitor peptides.

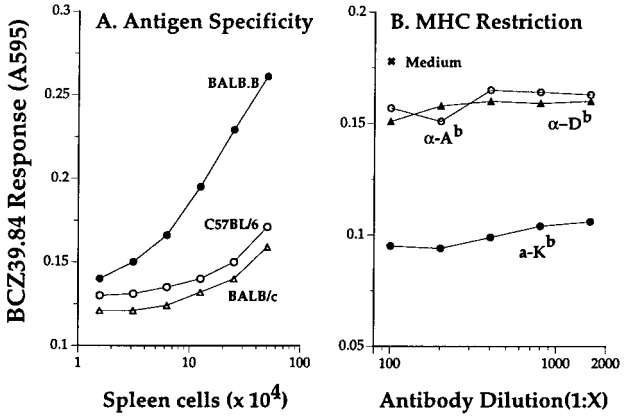


FIGURE 1. The BCZ39.84 T cell hybrid is specific for a polymorphic BALB Ag presented by the K^b MHC class I molecule. *A*, BCZ39.84 cells (10×10^4) were cocultured overnight with varying number of spleen cells from the indicated strains or (*B*) with 6×10^4 BALB.B cells and varying dilutions of anti-MHC mAbs. The T cell response was measured as the lacZ activity in culture lysates by the conversion of the substrate chlorophenol red β-galactopyranoside (CPRG) at 595 nm and 655 nm as reference.

Extraction and HPLC analysis of naturally processed peptides

Total acid soluble peptide pool from C3 cells was extracted as described (40, 41). Briefly, 1.5 to 3×10^8 C3 cells were washed with PBS and extracted with 1 ml of 1% trifluoroacetic acid (TFA) with boiling for 5 min. An irrelevant 17-mer peptide was added at 1 μM concentration to the extract as a carrier to prevent nonspecific losses. Cellular debris was removed by centrifugation, and the extract was fractionated by HPLC after filtration through a 10-kDa Millipore filter to remove large m.w. species. A narrow bore reverse phase C18 column (Vydac, 2.1×250 mm, 5 μm) was run in 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Flow rate was maintained at 0.35 ml/min and seven drop fractions were collected, dried in a vacuum centrifuge, and resuspended in 50 μl PBS + 10% DMSO. Serial dilutions of each fraction were assayed for stimulating BCZ39.84 T cells with K^b-L cells as APC, in a total volume of 200 μl, as described above. Mock injections with sample buffer alone were performed before each extract sample, using the same column and identical run conditions to demonstrate absence of cross-contamination between samples. The fractions from mock injections were assayed in the same experiment, using the same APC and T cells in parallel with fractions from the cell extracts and synthetic peptide standards. The minimal LYL8 peptide concentration required for BCZ 39.84 activation was typically 1 to 10 pM.

The abundance of naturally processed peptide was estimated by comparison with the synthetic LYL8 standard curve and taking procedural losses into account. Peptide loss during extraction was estimated by spiking the H60-negative EL-4 cells with known amounts of LYL8 and determining its recovery after TFA extraction and ultrafiltration by comparison with a synthetic LYL8 standard curve run in parallel (e.g., see Figure 4*D*). Likewise, known amounts of LYL8 peptide were injected into the HPLC, and peptide recovery in the active fractions was determined. In three independent measurements, the LYL8 activity recovered after extraction and ultrafiltration was 68.7 ± 8.8% (mean ± SD) and after HPLC fractionation, 67.8 ± 11%. The overall recovery was therefore judged to be 47% and was used to extrapolate the peptide activity in HPLC fractionated cell extracts to copies of LYL8 peptide/cell.

Results

Expression cloning the BCZ39.84 T cell defined BALB.B minor H cDNA

The $CD8^+$ BCZ39.84 T cell hybrid was obtained from B6 ($H\text{-}2^b$) mice immunized with spleen cells from the MHC identical BALB.B mice. The BCZ39.84 T cells responded to BALB.B spleen cells but not to the MHC congenic BALB/c ($H\text{-}2^d$) nor to the host B6 spleen cells (Fig. 1*A*). The Ag expression was restricted by the K^b MHC class I molecule because the BCZ39.84

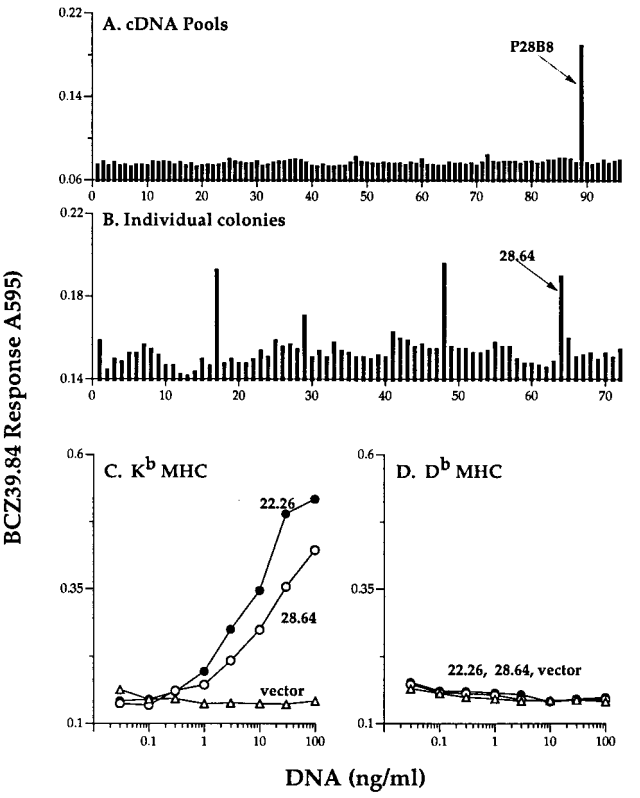


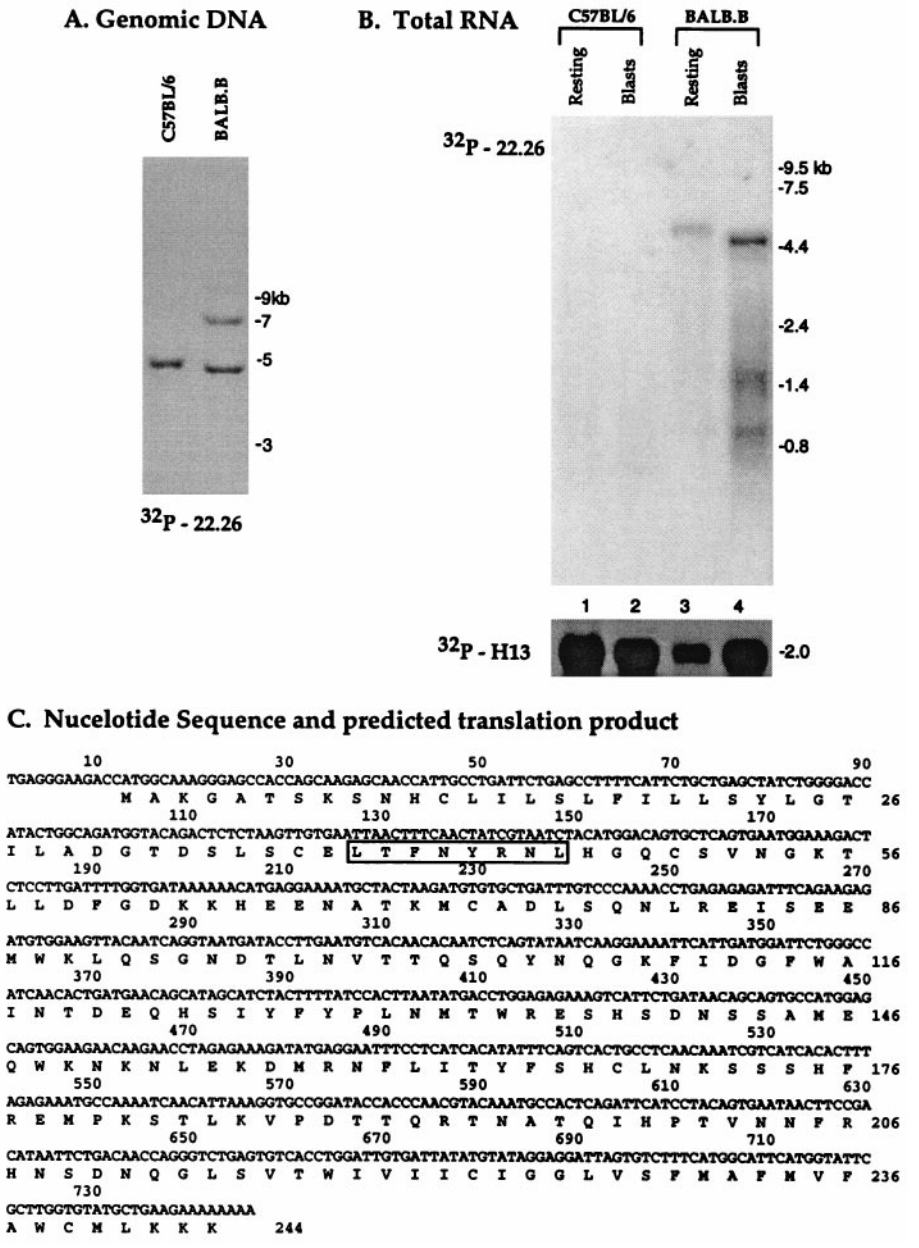
FIGURE 2. Isolation of the BALB cDNA clone that allows expression of the BCZ39.84 ligand in K^b-expressing recipient cells. Plasmid DNA from a Con A + LPS-stimulated (BALB.B × BALB/c)F_1 spleen cDNA library were transiently transfected into 3×10^4 $Lmtk^-$ cells as, (*A*) pools, or (*B*) individual colonies of the P28B8 positive pool. Recipient $Lmtk^-$ cells were transiently transfected with varying concentrations of the indicated plasmid DNA together with the cDNA encoding either (*C*) the K^b or (*D*) the D^b MHC molecules. Two days later, transfected cells were cocultured overnight with BCZ39.84 T cells, and their response was measured as in legend to Figure 1.

response was inhibited by anti-K^b (Y3), but not anti-D^b (B22.249.R1) MHC class I or by anti-A^b (28.16.8S) MHC class II Abs (Fig. 1*B*). The BCZ39.84 T cell thus defined a polymorphic BALB Ag presented by the K^b MHC molecule.

The BCZ39.84 T cell-stimulating Ag was isolated by expression cloning from a (BALB/c × BALB.B)F_1 cDNA library (22, 23, 25, 33). Representative data from one of the 96-well plates screened showed a cDNA pool, P28B8 with BCZ39.84-stimulating activity above the background (Fig. 2*A*). The screen was repeated with individual plasmids isolated from this cDNA pool, and one of the positive clones, termed 28.64, was selected for further analysis (Fig. 2*B*). The plasmid DNA from clone 28.64 and from another similarly isolated clone, 22.26, allowed generation of the BCZ39.84 ligand, in a dose-dependent manner, but only in recipient cells that expressed the K^b but not the D^b MHC molecule (Fig. 2, *C* and *D*). As shown below, both 28.64 and 22.26 clones encoded the same polypeptide sequence, which was thus a candidate precursor of the peptide/K^b complex recognized by the BCZ39.84 T cells.

The BCZ39.84 Ag cDNA defines a polymorphic locus encoding a previously unknown protein

To determine whether the BCZ39.84 T cells detected a polymorphic *H* locus represented by the isolated cDNAs, we compared the host B6 and donor BALB DNA in a Southern blot analysis. A

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      10                  30                  50                  70                  90
TGAGGGAAGACCATGGCAAAGGGAGCCACCAGCAAGAGCAACCATTGCCTGATTCTGAGCCTTTTCATTCTGCTGAGCTATCTGGGGACC
          M  A  K  G  A  T  S  K  S  N  H  C  L  I  L  S  L  F  I  L  L  S  Y  L  G  T   26
                110                 130                 150                 170
ATACTGGCAGATGGTACAGACTCTCTAAGTTGTGAATTAACTTTCAACTATCGTAATCTACATGGACAGTGCTCAGTGAATGGAAAGACT
I  L  A  D  G  T  D  S  L  S  C  E  L  T  F  N  Y  R  N  L  H  G  Q  C  S  V  N  G  K  T   56
      190                 210                 230                 250                 270
CTCCTTGATTTTGGTGATAAAAAACATGAGGAAAATGCTACTAAGATGTGTGCTGATTTGTCCCAAAACCTGAGAGAGATTTCAGAAGAG
L  L  D  F  G  D  K  K  H  E  E  N  A  T  K  M  C  A  D  L  S  Q  N  L  R  E  I  S  E  E   86
                290                 310                 330                 350
ATGTGGAAGTTACAATCAGGTAATGATACCTTGAATGTCACAACACAATCTCAGTATAATCAAGGAAAATTCATTGATGGATTCTGGGCC
M  W  K  L  Q  S  G  N  D  T  L  N  V  T  T  Q  S  Q  Y  N  Q  G  K  F  I  D  G  F  W  A  116
      370                 390                 410                 430                 450
ATCAACACTGATGAACAGCATAGCATCTACTTTTATCCACTTAATATGACCTGGAGAGAAAGTCATTCTGATAACAGCAGTGCCATGGAG
I  N  T  D  E  Q  H  S  I  Y  F  Y  P  L  N  M  T  W  R  E  S  H  S  D  N  S  S  A  M  E  146
                470                 490                 510                 530
CAGTGGAAGAACAAGAACCTAGAGAAAAGATATGAGGAATTTCCTCATCACATATTTCAGTCACTGCCTCAACAAATCGTCATCACACTTT
Q  W  K  N  K  N  L  E  K  D  M  R  N  F  L  I  T  Y  F  S  H  C  L  N  K  S  S  S  H  F  176
      550                 570                 590                 610                 630
AGAGAAATGCCAAAATCAACATTAAAGGTGCCGGATACCACCCAACGTACAAATGCCACTCAGATTCATCCTACAGTGAATAACTTCCGA
R  E  M  P  K  S  T  L  K  V  P  D  T  T  Q  R  T  N  A  T  Q  I  H  P  T  V  N  N  F  R  206
                650                 670                 690                 710
CATAATTCTGACAACCAGGGTCTGAGTGTCACCTGGATTGTGATTATATGTATAGGAGGATTAGTGTCTTTCATGGCATTCATGGTATTC
H  N  S  D  N  Q  G  L  S  V  T  W  I  V  I  I  C  I  G  G  L  V  S  F  M  A  F  M  V  F  236
      730
GCTTGGTGTATGCTGAAGAAAAAAAA
A  W  C  M  L  K  K  K      244
```

FIGURE 3. The 22.26 cDNA defines a polymorphic, differentially expressed novel gene. *A*, Southern blot of *Bam*HI-digested genomic DNA of the donor BALB.B or host C57BL/6 strains probed with the 22.26 probe. Numbers in the right margin indicate size in kb. *B*, Northern blot of 22.26 hybridizing transcripts in resting or Con A + LPS-stimulated spleen cell RNA. The autoradiogram below was obtained after the same blot was stripped and reprobed with the H13 cDNA (25). Numbers indicate sizes of RNA standards in kb. *C*, Nucleotide sequence and predicted translation product of 22.26 cDNA. The sequence of the antigenic LYL8 peptide is boxed. The sequence is available from the NCBI GenBank under the accession No. AF084643.

distinct set of bands was detected by the 22.26 cDNA probe in *Bam*HI-digested B6 and BALB.B DNAs (Fig. 3*A*). Neither the strong (4.7, 7.2 kb) nor weakly (~9 kb) hybridizing bands in the BALB.B DNA matched the single 5-kb band in the B6 DNA, demonstrating that the cDNA clone did detect a restriction enzyme site polymorphism among these two strains. The corresponding transcripts were then analyzed by Northern blot analysis of total splenic RNA (Fig. 3*B*). With the 22.26 probe, three different transcripts (approximately 1.0, 1.5, and 4.6 kb) were detected in the BALB.B splenic blasts, but only the single ~5-kb transcript was found in the resting BALB.B spleen. In contrast, transcription of this locus was undetectable in either resting or blasted B6 spleen cells. As a positive control, the same blot was stripped and rehybridized with the recently discovered *H13* cDNA probe (25). As expected for *H13*, both the B6 and the BALB spleen cells expressed these transcripts, demonstrating that the RNA samples were intact. We conclude that the 22.26 cDNA defined a polymorphic *H* locus that was differentially transcribed in the B6 and the BALB.B strains.

The characteristics of the antigenic precursor protein were inferred from the nucleotide sequence of the cDNA inserts (Fig. 3*C*). Comparison of the 28.64 and 22.26 sequences showed that both clones were identical in their coding sequence but differed in the length of their 5′ untranslated sequence. Because an in-frame termination codon was not found at the 3′ end, it is likely that these cDNA clones represent partial transcripts encoding 244 amino acids of a protein truncated at its C terminus. The relationship of the cDNAs and the putative polypeptides encoded by the three transcripts observed in the Northern blot is not yet known. Interestingly, a search of the nucleotide or the predicted protein sequence in the NCBI GenBank did not yield any significant matches in the nonredundant or the EST databases, demonstrating that these cDNAs represent a novel gene. However, a putative endoplasmic reticulum (ER) translocation signal sequence (aa 1–29), a transmembrane region (aa 213–241), and seven potential N-linked glycosylation sites were predicted by the Prosite program, indicating that this antigenic precursor polypeptide could be a transmembrane protein. We conclude that these

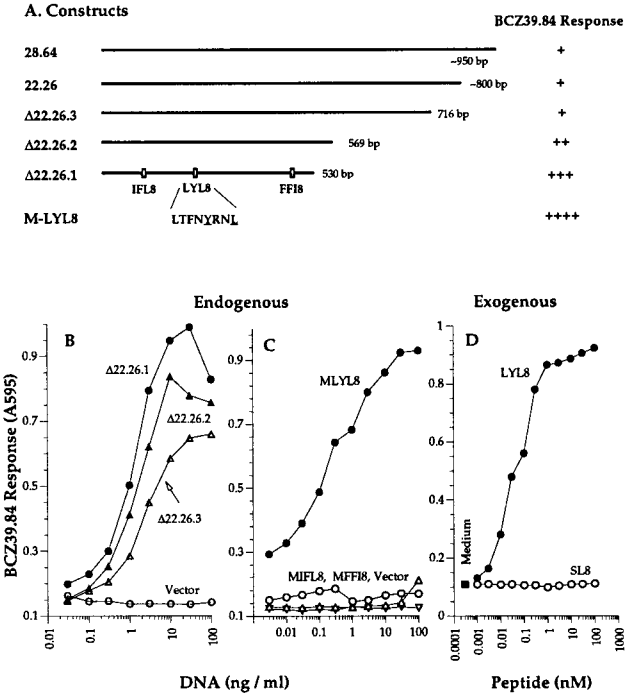

FIGURE 4. Identification of the antigenic peptide encoded by the 22.26 cDNA. *A*, Schematic representation of BCZ39.84 T cell response to cDNA deletion constructs generated as described in *Materials and Methods*. *B*, Plasmid DNAs of the indicated deletion constructs, or (*C*) the minigenes encoding the putative antigenic peptides indicated as boxes in (*A*), were transiently transfected into Lmtk$^-$ cells together with K^b MHC cDNA. The BCZ39.84 T cells were added after 48 h and assayed for lacZ activity the next day. *D*, The response of BCZ39.84 T cells after overnight culture with K^b-L cells and varying concentrations of either the synthetic LYL8 or the control SL8 (OVA 257–264) peptides.

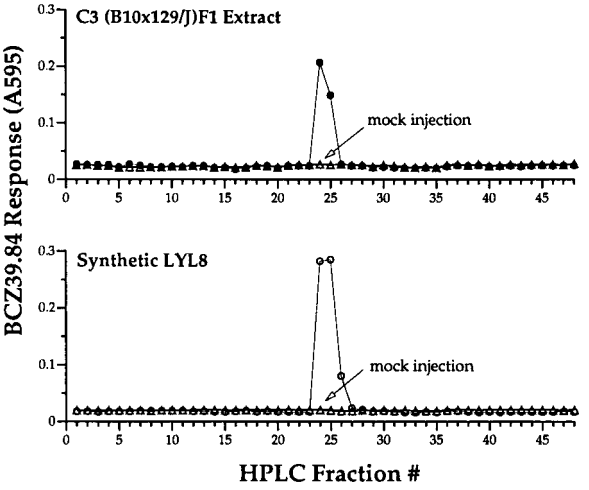

FIGURE 5. The naturally processed antigenic activity in cell extracts coelutes with the LYL8 synthetic peptide. A total TFA extract of C3, a pre-B line derived from the (B10 × 129/J)F$_1$ strain was fractionated by reverse phase HPLC, and the fractions were assayed for their ability to stimulate BCZ39.84 T cells with K^b-L cells as APC. Mock injection indicates fractions from runs collected and assayed before the sample in the same experiment to demonstrate absence of cross-contamination between runs.

cDNAs defined a polymorphic *H* locus, encoding a previously unknown, possibly transmembrane protein, whose first described function is to serve as a precursor for an antigenic peptide/K^b MHC complex.

Identification of the antigenic peptide within the cDNA

The minimal antigenic peptide within the cDNA clone was defined by deletional analysis. The vector encoded 5′ forward primer, and three reverse primers were used to generate 3′ deletions of the 22.26 cDNA by DNA amplification (Fig. 4*A*). All three 3′ deletion constructs were highly active in generating the BCZ39.84 ligand in transiently transfected cells (Fig. 4*B*). The antigenic activity was therefore located within the first 530 nt that encoded three peptides (see Figure 3*C*, ILSL**F**IL**L**, LTFN**Y**RN**L**, and FIDG**F**WA**I**) with the K^b binding motif, xxxx[F,Y]xx[I,V,L,M] (42). Minigene constructs with the oligonucleotide sequences encoding each of these octapeptides were transfected into K^b-expressing recipient cells and tested for stimulation of BCZ39.84 T cell. Among these, only one construct, encoding the peptide M-LTFN**Y**RN**L** (referred to as LYL8) was active (Fig. 4*C*). That the LYL8 coding sequence itself, without the translation initiator methionine, was sufficient to generate the BCZ39.84 stimulating activity was confirmed with the synthetic LYL8 peptide. In an exogenous assay with K^b-L cells as APC, the LYL8 peptide stimulated the BCZ39.84 T cells at <10 picomolar concentration (Fig. 4*D*). None of the other K^b-restricted T cells tested, e.g., OVA257–264 (SL8)-specific, B3Z, or the H4^b-specific T cells (not shown) were stimulated by the LYL8 peptide at even 10,000-fold higher concentration. We conclude that the antigenic ligand for the BCZ39.84 T cells was defined by the octapeptide LYL8/K^b complex.

Natural abundance and K^b binding activity of the LYL8 peptide

It has been suggested that the immunodominance of *H* peptides could be due to their high abundance in the donor tissues or due to their ability to form stable complexes with the MHC molecules relative to other peptides (13). To test these hypotheses, we analyzed the naturally processed LYL8 peptide in cell extracts. Despite being unrelated in their background genes, spleen cells from either BALB.B or 129/J strains stimulate BCZ39.84 T cells (Fig. 1, and data not shown). Attempts to extract the antigenic peptide from the BCZ39.84-stimulating spleen cells of BALB.B or 129/J strains were however unsuccessful, suggesting that the LYL8 peptide was expressed in spleen cells at a low level. But in the extract of the pre-B cell line, C3, derived from the (B10 × 129/J)F$_1$ mice, a single peak of BCZ39.84-stimulating activity was found after HPLC fractionation (Fig. 5*A*). This single peak of activity in the C3 extract precisely matched the retention time of the synthetic LYL8 peptide, strongly suggesting that the two were identical (Fig. 5*B*). Furthermore, by comparing the activity of the recovered natural peptide with synthetic LYL8 peptide, and taking procedural losses into account (see *Materials and Methods*), the natural abundance of the LYL8 peptide in three independent extracts of 263, 150, and 232 × 10^6 C3 cells was estimated to be 5.2, 6.8, and 14.6 copies/cell or an average of 9 copies/cell. We conclude that the synthetic LYL8 peptide coelutes with its naturally processed analogue and is expressed at the very low end of the abundance scale of peptide/MHC complexes.

The K^b MHC binding capacity of LYL8 vs other known K^b peptides was assessed using the TAP$^-$ RMA/S cells. The RMA/S cells were cultured at low temperature (31°C) overnight to obtain expression of "empty" peptide receptive MHC molecules on the cell surface. Further incubation of these cells at the normal 37°C temperature causes a loss of Ab epitopes on these MHC molecules unless peptide binding had occurred. In this assay, using the anti-

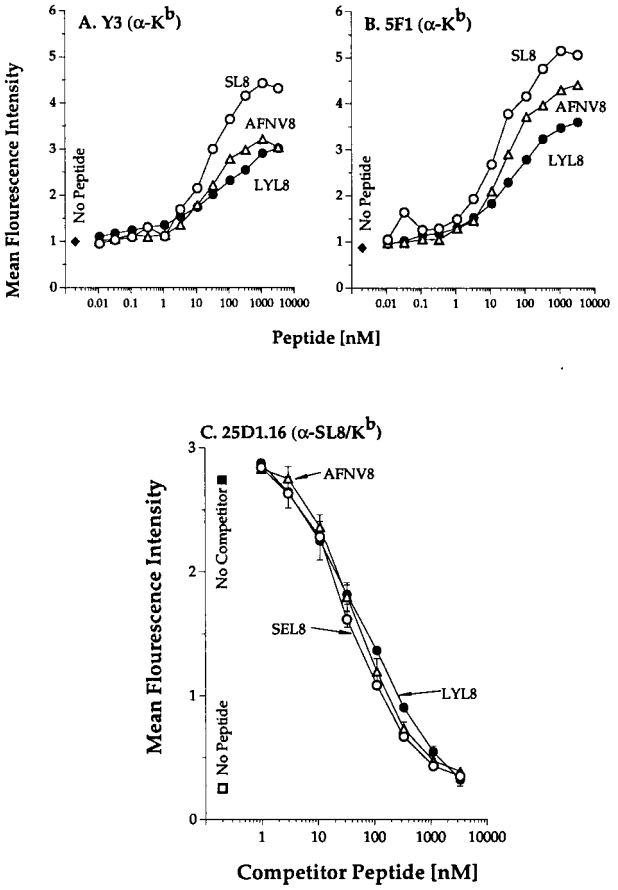

FIGURE 6. The LYL8 H peptide is comparable to other antigenic peptides in binding to the K^b MHC. *A* and *B*, The peptide-induced stabilization of K^b on surface of TAP^- RMA/S cells with varying concentrations of the indicated peptides was measured by flow cytometry of cells stained with anti-K^b mAbs Y3 or 5F1. *C*, Competition assay for inhibiting SL8 binding to K^b on RMA/S cells by varying concentration of indicated competitor peptides. The expression of SL8/K^b complex was measured by flow cytometry using the SL8/K^b-specific mAb 25D1.16 (39). The K^b binding peptides were SL8, SIINFEKL; AFNV8, ANYDFINV; and SEL8, SLVELTSL.

K^b mAB, Y3 or 5F1, the LYL8 peptide was found to be about 3- to 10-fold less effective in binding K^b than the SL8 (OVA 257–264) or the AFNV8 (MMTV) peptides (Fig. 6, *A* and *B*). However, this standard assay is sensitive to the influence of the peptide sequence on the conformational epitope recognized by the anti-MHC mAb (43, 44). To obviate this potential artifact, we also measured the ability of peptides to compete with SL8 for K^b binding as assessed by expression of the SL8/K^b epitope on the RMA/S cells using the recently described mAb 25D1.16 (39). As shown in Figure 6*C*, the expression of the SL8/K^b complex was specifically detected by the mAb 25D1.16 and was inversely proportional to the concentration of the competing peptides. Interestingly, superimposible inhibition curves were obtained with each of the three K^b binding peptides. This result indicates that, despite apparent differences in its ability to generate the Y3 or 5F1 epitopes, the LYL8 peptide was comparable to the SEL8 (33) and AFNV8 (36) peptides in its ability to compete with SL8 for binding K^b MHC. We therefore infer that, among the peptides tested, the K^b binding capacity of the LYL8 H peptide was indistinguishable from other foreign (AFNV8) or alloreactive (SEL8) peptides.

The antigen cDNA defines a new H *locus on chromosome 10*

To define the chromosomal location of this *H* locus, we used The Jackson Laboratory mapping panel, which consists of 94 (C57BL/6 × SPRET/Ei)F_1 × C57BL/6 backcross DNAs (38). Oligonucleotide PCR primers from the 3′ end of 28.64 cDNA were designed to amplify a 195-bp region, and sequence polymorphisms were detected by SSCP analysis (see *Materials and Methods*). The results summarized in Figure 7*A* showed that the 28.64 cDNA corresponds to a gene on mouse chromosome 10 that cosegregates with the *D10Mit2* marker located 6.4 cM proximal to *D10Hun15* and 7.5 cM distal to *D10Hun14*. None of the known minor H loci is consistent with this chromosomal position, with the possible exception of one linked to the male-sterile mutation (*mshi*) (45). The *mshi* locus can, however, be excluded because it was detected in H-2^d rather than the H-2^b mice analyzed here and because polymorphisms at this locus are detected only by skin grafts onto mutant mice as opposed to splenic expression of H60 shown here. Furthermore, none of B6.C bilineal congenic strains used to characterize unmapped minor H Ags are consistent with this map position (4, 46). The BCZ39.84 antigenic cDNA therefore defines a new *H* locus we termed *H60*.

The *H60* locus was further characterized by determining its allelic distribution among the CXB RI strains, CXB-E, CXB-G, CXB-I, CXB-J, and CXB-K. These RI lines have been extensively used by the Wettstein and Karre groups to define the immunodominant H Ags in the B6 anti-BALB.B cytotoxic T cell responses (13, 14). In Southern blots of *Xba*I-digested DNA, the presence of a unique 9.1-kb band and absence of a 3.7-kb band was observed in the BALB.B (allele c) and CXB-K strains but not in the B6 (allele b) or CXB-E, CXB-G, CXB-I, or CXB-J strains (Fig. 7*B*). This strain distribution pattern was also observed by simple sequence length polymorphism analysis of the RI strains using PCR primers that amplify the cosegregating chromosome 10 locus *D10Mit2* (data not shown). This analysis further confirms the genetic polymorphism of the *H60* locus and clearly showed that the CXB-K was the only RI strain among those tested that carried the BALB alleles at the *D10Mit2* as well as the *H60* loci.

The LYL8 epitope is dominant among B6 anti-BALB.B CTL

To determine the relative immunogenicity of the *H60* locus, and whether any of the previously described B6 anti-BALB.B CTL activities could be attributed to *H60*, bulk CTL lines were generated by priming B6 mice with BALB.B spleen cells. The CTL lines were tested for their lytic specificity using RMA/S cells loaded with the LYL8 peptide or with the K^b binding VSV peptide (32), as a negative control, as well as splenic blasts from B6, BALB.B, and the same panel of CXB RI strains as above (Fig. 7*C*). After the first wk of culture, high levels of anti-LYL8 (H60) peptide-specific lytic activity was detected, with the bulk CTLs using RMA/S cells as targets. Furthermore, these CTLs preferentially lysed the BALB.B, CXB-G, and CXB-K targets but lysed the CXB-I, CXB-J, and self-B6 weakly or not at all. Previous analysis of the B6 anti-BALB.B CTLs have shown that the CXB-K strain expresses the CTT-1 and CTT-5 Ags (13). To determine whether either of these two Ags corresponded to the H60 Ag, the LYL8-specific CTLs were further enriched by restimulating them for another 2 wk with LYL8-pulsed B6 cells. Again, the LYL8-pulsed RMA/S cells, as well as the BALB.B and CXB-K target cells, were specifically lysed, and no lysis of self B6 or the other four CXB RI cells was observed. That only the BALB.B and CXB-K cells expressed the H60 Ag was further confirmed by their ability to stimulate the monoclonal LYL8/K^b-specific BCZ39.84 T cell hybridoma (Fig. 7*C*). In an independent immunization of B6 mice with

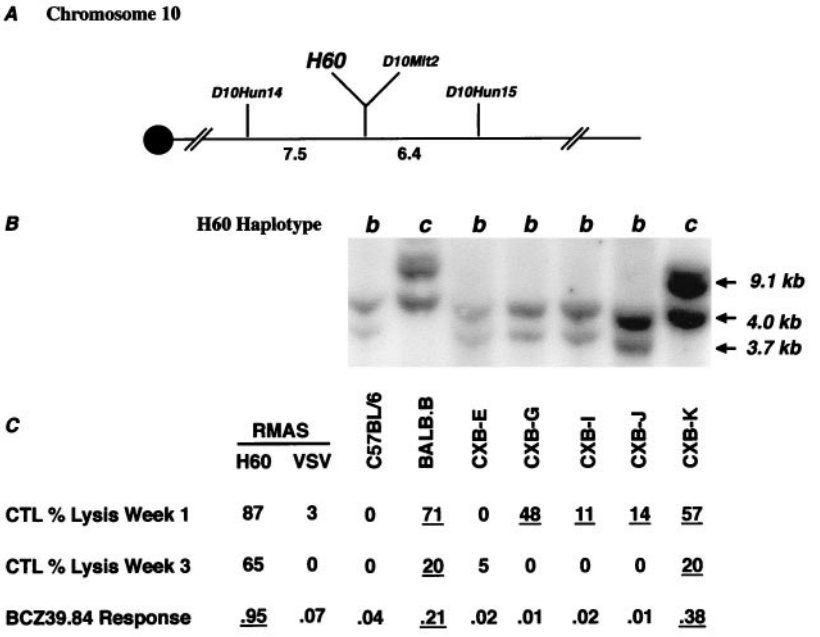

FIGURE 7. The 28.64 cDNA maps a new locus *H60*, on mouse chromosome 10, and its antigenic LYL8/K^b product is expressed only in BALB.B and the CXB-K RI strains. *A*, Positioning of *H60* relative to other loci on mouse chromosome 10 based on SSCP analysis of The Jackson Laboratory BSB interspecific backcross panel. Numbers indicate distance between loci in cM. *B*, Southern blot RFLP analysis of *Xba*I-digested DNA from CXB RI mouse strains. The 9.1-kb band correlates with BALB-derived DNA, and the 3.7-kb band correlates with B6-derived DNA. *C*, Effector cells were generated after priming of B6 mice with BALB.B splenocytes, followed by restimulation one or three times in vitro. Target cells were Con A-stimulated lymphoblasts from the indicated strains, or from RMA/S cells loaded with the 10-nM LYL8 peptide or a K^b-binding VSV peptides. Representative data of percent specific lysis at E:T cell ratios of 25:1 (Week 1) and 12.5:1 (Week 3) are shown for one of three independent CTL lines. The lacZ response of BCZ39.84 T cell hybrid is shown as the absorbance (A595) when incubated with spleen cells from the indicated strains as in legend to Figure 1.

BALB.B spleen cells, T cell hybrids were derived, and five of seven K^b-restricted hybrids were found to recognize the LYL8/K^b complex (data not shown). These results demonstrate that the anti-LYL8 specificity is predominant among primary B6 anti-BALB.B CTLs and that, among the RI strains tested, only the CXB-K strain expresses the LYL8/K^b complex. Taken together with the previous analysis of the same RI strains by Wettstein and colleagues, our data suggest that their CTT-5 Ag, which is also restricted by the K^b MHC, is in fact the LYL8 peptide derived from the *H60* locus described here.

Discussion

We describe here a novel murine minor H Ag locus, *H60*. The *H60* locus is located on chromosome 10, is differentially expressed in the MHC identical donor BALB.B vs the host C57BL/6 mice, and encodes a previously unknown protein that contains the rare, but immunodominant, naturally processed LYL8 (LTFNYRNL) peptide/K^b complex. These findings provide the molecular and functional characterization of a dominant minor H Ag and its precursor gene.

Sources of minor histocompatibility Ags

After decades of uncertainty, the identity of T cell-defined minor H loci is now being revealed. Among the available methods, genetic strategies, and in particular the expression-cloning strategies, are proving to be rather successful in providing not only the identity of the antigenic peptide but also, as an essential tool, the cDNA clone of the protein precursor as well (6–9, 11, 25). The availability of the precursor gene then allows a genetic analysis of the *H* locus, which provides the molecular explanation of their polymorphic behavior. By contrast, the sequence of the antigenic peptide, obtained after its biochemical purification, does not necessarily yield the identity of its donor protein if no match is found in the sequence data bases (47, 48).

As sources of H peptides, all four known sex-linked and autosomal *H* loci encode novel gene products. Both the Y chromosome-encoded *Smcy* and *Uty* genes, based upon their sequence motifs, are likely to be located in the nucleus. Sequence analysis of the two autosomal H Ags, *H13* and *H60*, suggests that these are likely to be membrane-bound proteins. These cellular locations are, however, also shared by other precursors of MHC class I binding peptides, such as the influenza nuclear protein or the surface hemagglutinin glycoprotein (49). Given that no intracellular compartment is excluded as a source of antigenic peptides, the finding that a variety of different proteins serve as sources of antigenic H peptides extends and reinforces this notion to minor histocompatibility Ags as well. The high likelihood that the yet unknown normal cellular functions of the H proteins will also vary suggests that H precursors will not share any functional characteristics either. However, whether the H proteins and their processed peptide/MHC complexes are distinguished by their distribution among different tissues, particularly the professional APCs and/or different target tissues, remains to be explored. Furthermore, the existence and immunogenicity of *H60* homologues among different mouse strains and other species remain to be determined.

The LYL8/K^b complex

The antigenic peptide derived from the H60 Ag precisely matched the K^b octapeptide consensus motif, xxxx[F,Y]xx[I,L,M,V] (42). Although exceptions to the MHC consensus motifs have been found among minor H as well as other antigenic peptides (25, 33, 50), knowledge of the K^b motif did narrow the search for the LYL8 octapeptide within the H60 coding sequence. The HPLC retention time of the synthetic LYL8 peptide also precisely matched the single peak of antigenic activity in the naturally processed peptide pool of the C3 (B10 $\times$ 129/J)F_1 cell line. Because the *H60* cDNA was isolated from the BALB background, it is likely that its homologue is also present in the distinct 129/J background. Remarkably, the amount of naturally processed LYL8 peptide was found to range between 5 and 15 copies/cell, which makes it a member of the rare rather than abundant set of peptides expressed on the cell surface (51). Furthermore, the MHC binding capacity of the LYL8 peptide was comparable to other peptides derived from foreign, viral, or allogeneic Ags. Thus, neither the MHC binding ability nor the natural abundance of the LYL8 peptide distinguish it from other known antigenic peptides. Among the other H peptides, the natural abundance of the mitochondrial ND1, COI, ATPase 6, or the HY-derived peptide/MHC complexes are yet unknown, but

our previous analysis of the H13^a-derived SVL9/D^b complexes had shown that it too was expressed at less than 50 copies/cell (25). Whether the expression of these antigenic H peptides in different cell types will reveal substantial differences in their natural abundance remains to be determined, but our findings strongly argue against the hypothesis that the potent immunogenicity of H Ags is due to their unusual MHC binding ability or due to their overall high abundance.

Polymorphisms of H *loci*

The ability of *H* loci to induce T cell immunity suggested that they were polymorphic between the donor and host strains. Comparison among the known *H* loci, however, reveal different mechanisms for their antigenic polymorphism. At one extreme are the male *HY*-encoding loci of the Y-chromosome, where their X-chromosome homologues in the female host contain amino acid substitutions within the relevant peptides presented by the MHC (9, 11). For the murine Smcy/K^k and Uty/D^b peptides, the substitutions in the *Smcx* and *Utx* homologues were drastic enough to prevent their ability to bind the relevant MHC, and they would therefore be absent from the repertoire of self peptides in the female host. The human Smcy/HLA-B7 and Smcy/HLA-A*0201 peptides may also be similar to their murine counterparts (10, 52). On the other extreme, polymorphic substitutions within the mitochondrial ND1 peptides presented by the nonclassical H2-M3 do not prevent their presentation, and T cell responses can be elicited in either direction (6)

The only two autosomal *H* loci known so far fall within these two extremes. We recently showed that a single conservative valine to isoleucine amino acid substitution within the murine autosomal H13 peptide SSV[I,V]GVWYL/D^b explained its bidirectional immunogenicity in strains expressing either the *H13^a* or the *H13^b* alleles (25). The *H13* locus is therefore similar to the mitochondrial *H* loci in this respect. The autosomal *H60* locus described here conceptually resembles the *HY*-encoding loci because its expression was not detected in the host B6 strain, making expression of any LYL8 homologue extremely unlikely. The reasons for this differential expression of the *H60* locus and its functional consequences, if any, are not yet known. Because transcription of *H60* was up-regulated in mitogen-stimulated spleen cells, it also suggests that *H60* may be an example of a transcriptionally regulated *H* gene in contrast to other identified *H* loci that appear to be constitutively expressed.

In conclusion, we have identified a new murine *H60* histocompatibility locus that encodes an immunodominant H Ag. Knowledge of this differentially expressed locus and its processed peptide now provides the genetic and biochemical tools to determine why, among thousands of estimated polymorphic loci (4, 53), only a small set is apparently immunogenic (13, 14).

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