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Recognition of the Product of a Novel MHC TL Region Gene (27^b) by a Mouse $\gamma\delta$ T Cell Receptor

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Summary

The $\gamma\delta$ T cell receptor (TCR) derived from the mouse KN6 T cell hybridoma recognizes an autologous determinant encoded by a broadly expressed gene mapping in the TL region of the major histocompatibility complex (MHC). We have cloned the gene and demonstrated that it is a novel class I gene (designated 27^b) belonging to a hitherto undescribed TL region gene cluster in strain C57BL/6. The BALB/c allele of 27^b, gene $T17^c$, is defective because it lacks an appropriate splice acceptor site, which explains the lack of recognition of BALB/c stimulator cells by the KN6 cells. We propose that $\gamma\delta$ TCR and nonclassical MHC and MHC-related class I molecules have coevolved to recognize a conserved set of endogenous and foreign determinants.

Introduction

In the vertebrate immune system, the recognition of antigen by lymphocytes is carried out by two sets of glycoproteins, immunoglobulins and T cell receptors (TCRs). Much of the diversity in these molecules is generated by somatic DNA rearrangement (Tonegawa, 1983; Davis and Bjorkman, 1988). Until recently, all TCRs were thought to be composed of heterodimers consisting of α and β subunits. However, during the search for the genes encoding these subunits, we found a third gene, TCR γ, that also rearranges in some T cells and shares a number of structural properties with the TCR α and β genes (Saito et al., 1984; Kranz et al., 1985). Further studies on the expression of the γ gene led to the discovery of a second T cell receptor, the $\gamma\delta$ TCR (Brenner et al., 1986; Lew et al., 1986). A given T cell expresses either $\alpha\beta$ TCRs or $\gamma\delta$ TCRs: no T cells, mature or immature, that coexpress both types of TCR have been detected (Chen et al., 1988; Itohara et al., 1989).

$\gamma\delta$ T cells represent a relatively minor T cell subpopulation in the lymphoid organs of the mouse (Bluestone et al., 1987; Itohara et al., 1989) and human (Borst et al., 1988), and are selectively localized in the mouse epidermis (Koning et al., 1987; Kuziel et al., 1987), in the intestinal epithelial layers of several species (Bonneville et al., 1988; Goodman and Lefrancois, 1988), and in several other epithelial tissues that form the outer or inner surfaces of the body (Augustin et al., 1989; Itohara et al., 1990). $\gamma\delta$ T cells that reside in different peripheral sites represent distinct subsets differing in the utilization of γ and δ gene segments and in the degree of diversity. Particularly intriguing are the observations that the $\gamma\delta$ T cells associated with the epidermis (dendritic epidermal cells or DECs) and with the epithelia of vagina, uterus, and tongue (vagina-, uterus-, and tongue-intraepithelial lymphocytes or vut-IELs) express distinct and homogeneous TCRs (Asarnow et al., 1988; Itohara et al., 1990). Both these $\gamma\delta$ T cell subsets appear to be positively selected in the fetal thymus (Lafaille et al., 1989). The other $\gamma\delta$ T cell subsets, which are generated later in ontogeny and localize to other peripheral sites, express more diverse TCRs (Cron et al., 1988; Lafaille et al., 1989; Takagaki et al., 1989).

To elucidate the biological role of $\gamma\delta$ T cells, it is essential to obtain information on the ligands that they recognize. Three pieces of information are available to date in this regard. First, a subset of mouse and human $\gamma\delta$ T cells and their clones are reactive with the mycobacterial heat shock protein hsp65 (O'Brien et al., 1989; Holoshitz et al., 1989). Although classical class I MHC molecules (H-2K, H-2D, and H-2L) do not seem to serve as restriction elements in this recognition, it is unclear whether other MHC products are involved. Second, a mouse $\gamma\delta$ T cell clone (Bluestone et al., 1988) and a $\gamma\delta$ T cell hybridoma (KN6) (Bonneville et al., 1989) were identified that recognize determinants encoded by an MHC-linked gene mapping distal to the Q region. Whether these determinants reside on class I molecules or other molecules encoded by genes in this chromosomal segment is unknown. Third, some human $\gamma\delta$ T cell clones recognize CD1 molecules, a set of glycoproteins evolutionarily related to MHC class I molecules but encoded by genes unlinked to the MHC (Porcelli et al., 1989).

In this study, we demonstrate that the determinant recognized by the KN6 $\gamma\delta$ TCR is encoded by a novel MHC class I gene located in a hitherto undescribed TL region gene cluster in C57BL/6 mice (for review of the TL region, see Flaherty et al., 1990). This observation has important implications for both $\gamma\delta$ T cell function and the function of nonclassical class I molecules.

Results

The KN6 $\gamma\delta$ TCR Recognizes a Broadly Expressed TL Region-Controlled Ligand

Utilizing a growth inhibition assay, we have previously shown that the KN6 T cell hybridoma recognizes a deter-

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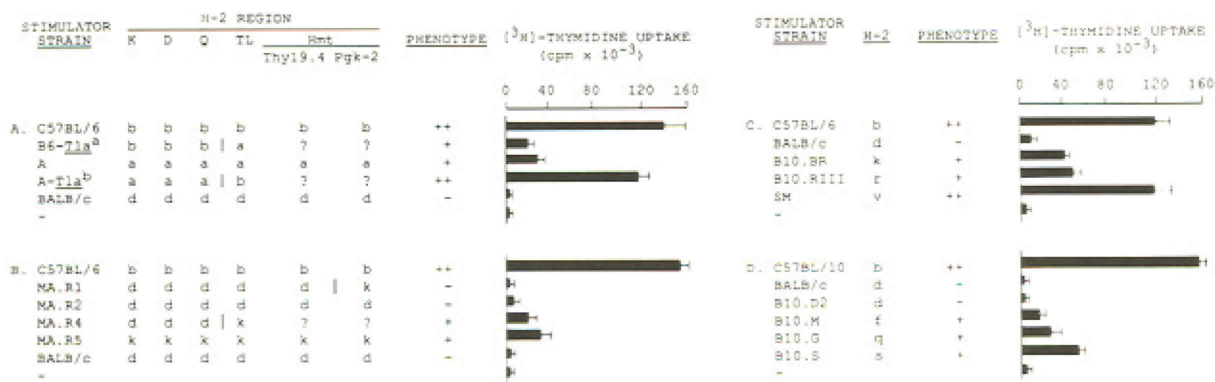


Figure 1. Proliferative Response of KN6 γδ T Cells to Peritoneal Exudate Cells from Various Strains of Mice

Approximately 10^4 CD4⁻8⁻ splenic T cells (>95% KN6 γδ T cells and no detectable αβ cells) from KN6 transgenic mice were cultured with 2.5×10^4 irradiated (1500 rads) peritoneal cells from the indicated strains of mice. The data are expressed as mean cpm and standard deviations of triplicate samples. Responses are classified into three types: ++, strong response; +, intermediate response; and −, no response. The crossover positions in the recombinant strains are indicated by vertical bars. Other strains tested: C57BL/10 (B10), C3H.SW, B6.AC1, B6.KB1, B10.A(R149)-*Tla*^{b}, B10.A(R410)-*Tla*^{b}, 129 (strong response); A.CA, BALB.S, B6-*H-2*^{k}, B10.PL(73NS), CBA, C3H, NZB, P, B6.AC3, B6.KB2, B10.A, B10(R297)-*Tla*^{a}, B10(R310)-*Tla*^{a} (intermediate response); BALB.S(R3) (no response).

minant controlled by a gene mapping distal to the Q region of the MHC (Bonneville et al., 1989). In the present study, cells from transgenic mice that express the productively rearranged KN6 γ and δ TCR genes were analyzed in mixed lymphocyte culture for their proliferative response. Three stimulator phenotypes were observed: strong stimulation, e.g., strain C57BL/6 (B6); intermediate stimulation, e.g., strain A; and no stimulation, e.g., strain BALB/c (Figure 1A). Analysis of recombinant strains confirmed our previous study (Bonneville et al., 1989) showing that a gene mapping distal to the Q region controls the ligand recognized by the KN6 γδ TCR (Figure 1A), and showed further that this gene maps proximal to the Hmt region gene *Pgk-2* (data with recombinant strain MA.R1, Figure 1B). Molecular mapping data shown in the next section formally prove that the gene encoding the KN6 ligand is situated between the Q and Hmt regions, in the TL region. Also consistent with our earlier study is the observation that strains that derived their TL region from BALB/c or B10.D2 mice failed to stimulate a response. However, in contrast to our previous findings, all other strains tested clearly stimulated a response (Figures 1A, 1C, and 1D). Since these latter strains all gave intermediate levels of stimulation in the proliferative assay, this could have been missed by the less sensitive growth-inhibition assay employed in our previous study. The TL region–controlled ligand recognized by the KN6 γδ TCR is thus expressed in numerous mouse strains.

The KN6 Ligand Is Encoded by a TL Region Class I Gene

To characterize the KN6 ligand further, we searched for cell lines that stimulate KN6 proliferation. As shown in Figure 2, KN6 cells responded to syngeneic (B6) thymomas EL4 and 2052C but not to allogeneic (BALB/c) thymomas WEHI7.1 and S49. In addition, strain 129/Sv embryonal carcinoma (EC) cell line PCC3 but not F9 or 402AX stimulated KN6 cell proliferation. PCC3 cells were selected for further study because we assumed that the TL region gene encoding the KN6 ligand is a class I gene, and because PCC3 is known to express TL region class I determinants but not classical H-2K, H-2D, or H-2L class I products (Stern et al., 1986). A cDNA library was prepared from PCC3 and screened with the pan-H-2 class I probe pH2-IIa, and eight cDNA clones were isolated and sequenced. Four clones were identified as Q region genes. Three clones exhibited the same sequence as Q7^b, except for one nucleotide, namely, A instead of G in position 2857 of the genomic sequence. This difference most likely reflects a strain-dependent polymorphism between strain 129/Sv and B10. The sequence of the fourth cDNA clone was identical to that of another Q region gene, Q8^b (data not shown).

The remaining four cDNA clones contained sequences that are clearly distinct from previously published class I gene sequences. Clone PCC3-10 exhibited high (87%–91%) sequence homology with Q region genes and therefore most probably belongs to the Qa gene family (data not shown). The nucleotide sequences of clones PCC3-4 and PCC3-27 were identical in the protein coding region, although the latter had a 109 bp deletion in the 5′ untranslated region (Figure 3). Hereafter, we use the designation PCC3-4/27 to refer to either PCC3-4 or PCC3-27. While being highly homologous (>88% homology) in exon IV, elsewhere the PCC3-4/27 sequence differed substantially from all known class I gene sequences (Table 1). Since this pattern of sequence homology is typical for TL region genes (Flaherty et al., 1990), we suspected that these cDNAs are derived from a hitherto unsequenced TL region gene. The eighth cDNA clone, PCC3-24, exhibited a high degree (96%) of sequence homology with PCC3-4/27 (Figure 3), and its pattern of sequence homology with other class I genes was similar to that of PCC3-4/27 (data not shown). It is therefore also likely to represent a TL gene. It should, however, be pointed out that this cDNA sequence has a 36 bp deletion encompassing the translation termination

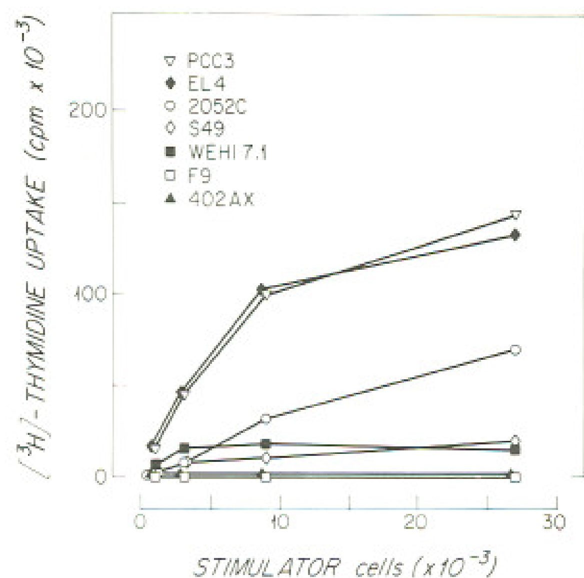


Figure 2. Proliferative Responses of KN6 γδ T Cells to Various Cultured Cell Lines

Approximately 10^4 $CD4^-8^-$ splenic T cells (>95% KN6 γδ T cells) from KN6 transgenic mice (H-2^d) were cultured with the various irradiated (3000 rads) stimulator cell lines. The data are expressed as mean cpm of triplicate samples.

codon of PCC3-4/27, and consequently its protein product would contain a prolonged carboxyl-terminal region (Figure 3).

Evidence that the PCC3-4/27 gene encodes the KN6 ligand comes from DNA-mediated gene transfer studies. Mouse L cells were transfected with a plasmid containing PCC3-4/27 cDNA under the control of the metallothionein promoter, and stable transfectants were isolated and tested for their KN6-stimulatory capacity. L cells transfected with PCC3-4 or PCC3-27 clearly stimulated the proliferation of KN6 cells, while nontransfected cells or vector-transfected cells did not (Figure 4A). L cells transfected with PCC3-24 were not recognized by KN6 cells (Figure 4A), although they efficiently transcribed the introduced DNA (data not shown). Recognition of L cells transfected with PCC3-4 or PCC3-27 could be partially blocked by preincubation of the responder KN6 cells with the anti-KN6 γδ clonotypic monoclonal antibody (MAb) 5C10, but not with the anti-H-$2K^b/D^b$ MAb 28-8-6, indicating that the stimulation is mediated by the γδ TCR (Figure 4B). Similar results were obtained with the KN6 ligand–negative BALB/c lymphomas WEHI7.1 and S49 that had been transiently transfected with PCC3-4, PCC3-27, or PCC3-24 cDNA subcloned into an SV40 expression vector, pSVL (data not shown).

Formal proof that the PCC3-4/27 gene maps in the TL region comes from Southern blotting analysis of genomic DNA from several recombinant strains (Figure 5). Restriction fragment length polymorphism (RFLP) analysis was conducted using the 5′ and 3′ subfragments of PCC3-4, pTL4-1 and pTL4-2, respectively as hybridization probes (Figure 5A). As shown in Figure 5B, a BamHI digest of

Table 1. Percentage of Nucleotide Sequence Homology of the cDNA Clones PCC3-4/27 with Known Class I Genes

Region	Gene	Exons I	II	III	IV	V	VI
K	K^d	55	70	62	92	49	52
D	D^k	53	69	62	95	50	55
Q	$Q1^k$	47	70	62	96	52	42
	$Q10^b$	52	72	60	95	36	49
TL	$T1^c$	55	60	50	88	50	46
	$T3^b$	48	66	56	92	41	46
	37^c	52	71	65	95	39	39
Hmt	Thy19.4	37	63	60	88	42	–
	Mb1	42	58	50	75	44	–

The exon–exon boundaries of PCC3-4/27 were determined by sequencing 27^b, the corresponding gene in C57BL/6. Only the protein coding region was compared. Sequences other than that of PCC3-4/27 were obtained from the GenBank data base.

C57BL/6 genomic DNA generated bands of 3.0 kb and 2.5 kb when probed with pTL4-2, while a single band of 2.7 kb was obtained with strain A/Boy. Since recombinant strains A-*Tla*^{b}/Boy and B6-*Tla*^{a}/Boy gave the C57BL/6- and A/Boy-type patterns of hybridization, respectively, the PCC3-4/27 gene maps distal to the Q region, thus confirming the functional mapping data (Figure 1). Data with the recombinant strain B6.CAS3(R4) also show that the PCC3-4/27 gene does not map in the Hmt region (Figure 5C). Since the *Pgk-2* gene maps within the Hmt region, and functional study above has already shown that the PCC3-4/27 gene maps proximal to *Pgk-2* (Figure 1C), the PCC3-4/27 gene maps between the Q and Hmt regions, in the TL region.

The Gene (27^b) Encoding the KN6 Ligand Is a Novel TL^b Region Gene

The TL region of strain 129/Sv, from which PCC3 was derived, has not been characterized at the molecular level. Although this strain is usually included with other H-2^b strains, differences in expression of TL determinants have been reported relative to strains C57BL/6 and C57BL/10 (Shen et al., 1982). To determine the TL^b counterpart of PCC3-4/27, we screened a cDNA library prepared from the thymocytes of newborn C57BL/6 mice using the 3′ PCC3-4 probe, pTL4-2 (Figure 5A), which hybridized only with PCC3-4/27 and PCC3-24 among all class I cDNA clones isolated from PCC3 cells (data not shown). From a total of eight cDNA clones isolated, only five hybridized also with an oligonucleotide probe, pTL4-3 (Figure 5A), that is specific for PCC3-4/27. We sequenced one of these five cDNA clones (clone B6-6) and one of the remaining three cDNA clones (clone B6-8). In the protein coding region, B6-6 differed from PCC3-4/27 only by a single nucleotide (position 915 in B6-6) that caused no amino acid replacement (Figure 3 and Table 2). Thus, the TL-encoded KN6 ligand is identical at the amino acid sequence level in strains C57BL/6 and 129/Sv. In addition, the sequence relationship summarized in Table 2 suggests that clone B6-8 is the C57BL/6 counterpart of PCC3-24. This cDNA contained an 11 bp duplication in exon III

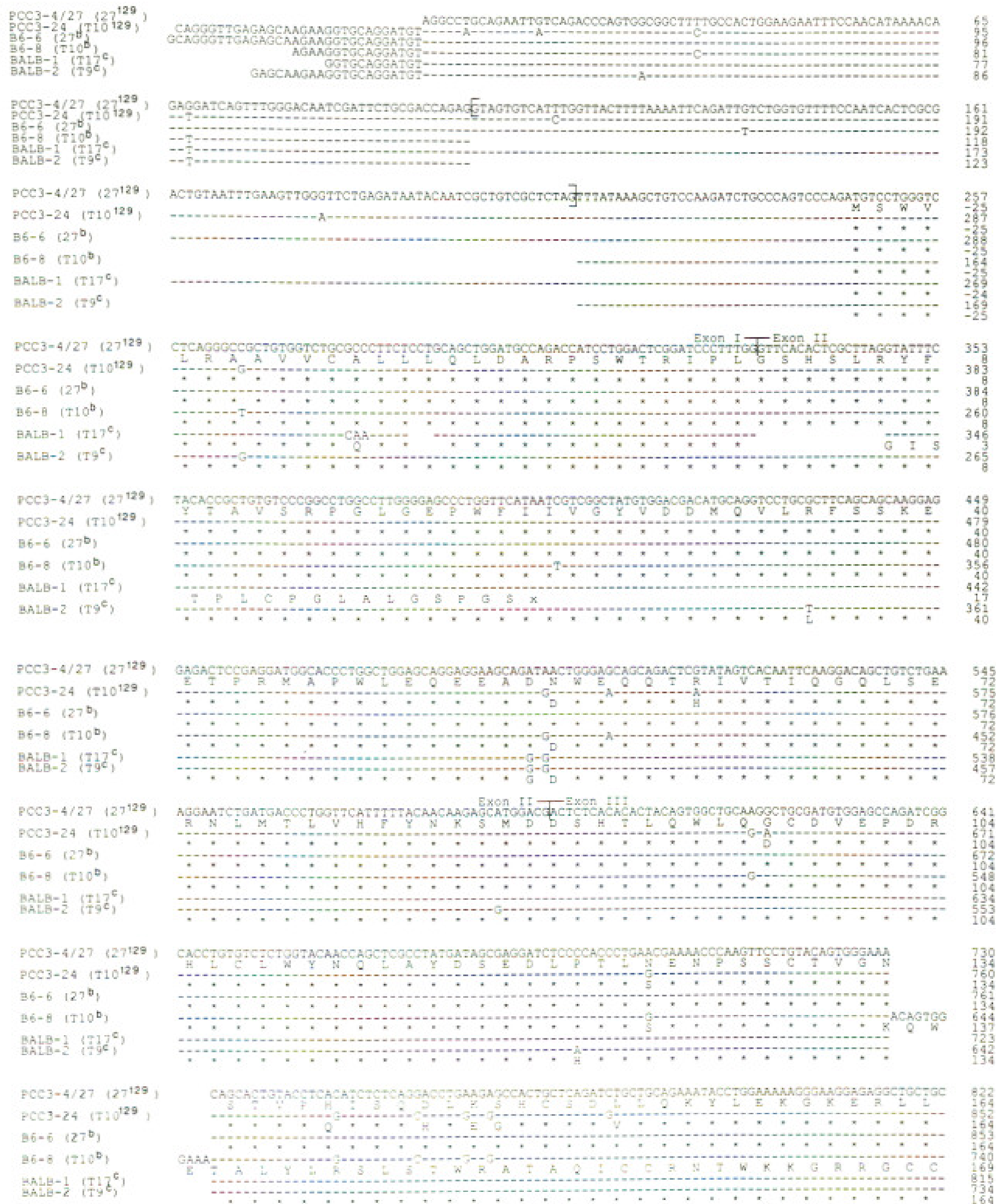
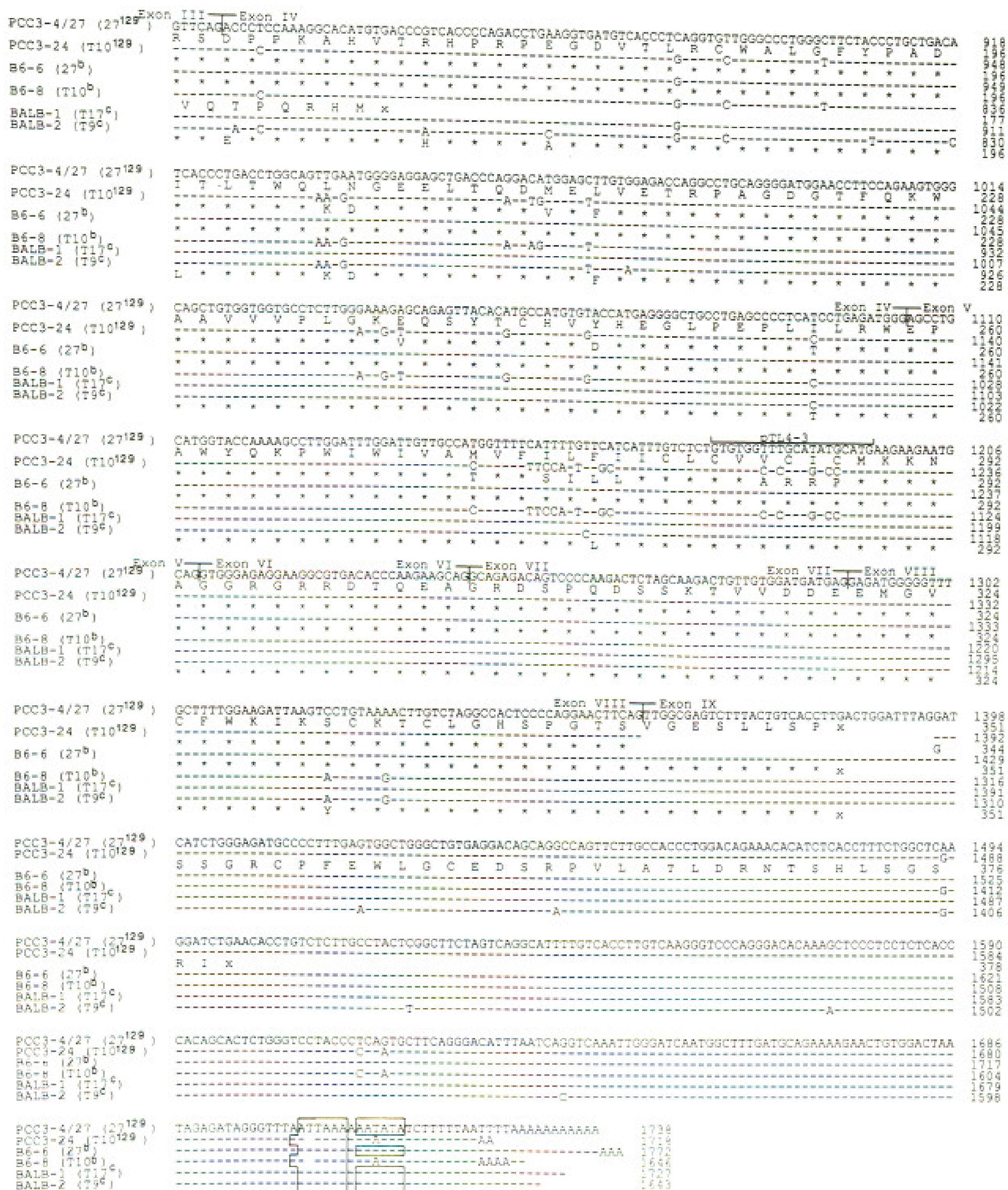


Figure 3. Nucleotide Sequences of PCC3-4-Related cDNA Clones and Their Predicted Amino Acid Sequences

The nucleotide sequences of cDNA clones PCC3-4 and PCC3-27 are identical except for the 109 bp deletion (indicated by brackets) in the 5′ untranslated region of the latter. A dash indicates that a related cDNA shares an identical nucleotide with PCC3-4/27. Nucleotides are numbered beginning with the 5′-most nucleotide of each cDNA. The exon boundaries of clones B6-6 and B6-8 were determined by sequencing the corresponding genes



27^b and $T10^b$, respectively (data not shown). Exon boundaries of the other cDNAs were inferred. Putative polyadenylation signals are boxed. The location of the oligonucleotide probe pTL4-3, which is specific for the KN6 ligand gene, is indicated. Deduced amino acid sequences are indicated by the one-letter code below each nucleotide sequence. Asterisks indicate amino acid identity with the PCC3-4/27 sequence, while "x" indicates a termination codon. The designation of each cDNA clone is followed by the designation of the corresponding gene.

causing a frameshift and premature termination of the putative polypeptide product, and therefore it cannot encode a complete class I molecule (Figure 3).

To determine whether B6-6 (PCC3-4/27 counterpart) and B6-8 (PCC3-24 counterpart) correspond to previously identified class I genes, hybridization analysis was conducted with cosmids containing these genes (Flavell et al., 1986). In the past, 15 TL region genes ($T1^b$–$T15^b$) have

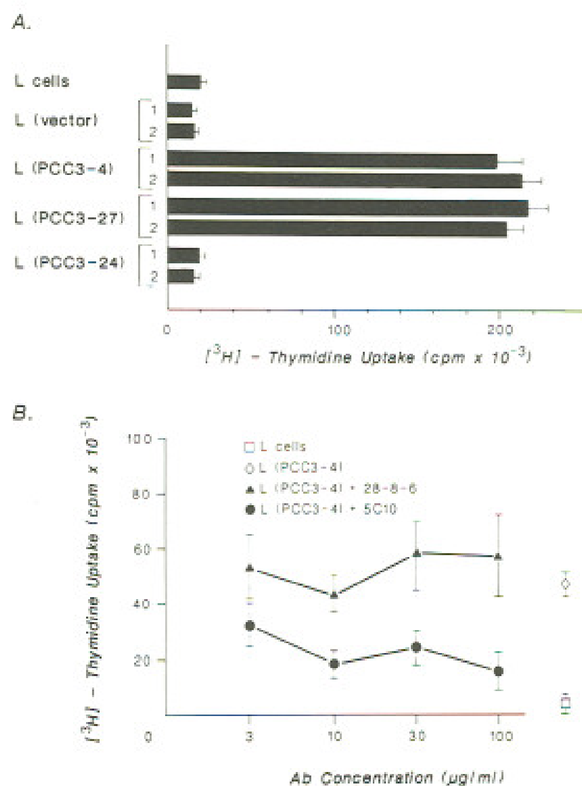


Figure 4. Proliferative Response of KN6 $\gamma\delta$ T Cells to L Cells Transfected with PCC3-4 and PCC3-27 cDNA Clones

Data are expressed as mean cpm and standard deviations of triplicate samples. (A) Approximately 10^4 responder cells from the short-term KN6 $\gamma\delta$ T cell lines (>99% KN6 $\gamma\delta$ T cells) were cultured with 2.5×10^4 irradiated (3000 rads) L cells or L cell transfectants. Two independently transfected clones were used in each case. (B) Approximately 10^4 $CD4^-8^-$ splenic T cells (>95% KN6 $\gamma\delta$ T cells) from KN6 transgenic mice were preincubated for 30 min with varying concentrations of the MAb 5C10 (anti-KN6 clonotypic) or MAb 28-8-6 (anti-H-$2K^b/D^b$) and cocultured with 2.5×10^4 preirradiated (3000 rads) L cells transfected with PCC3-4 in the presence of the respective MAb. As a control, proliferation was also measured using untransfected L cells or L cells transfected with PCC3-4 in the absence of MAb.

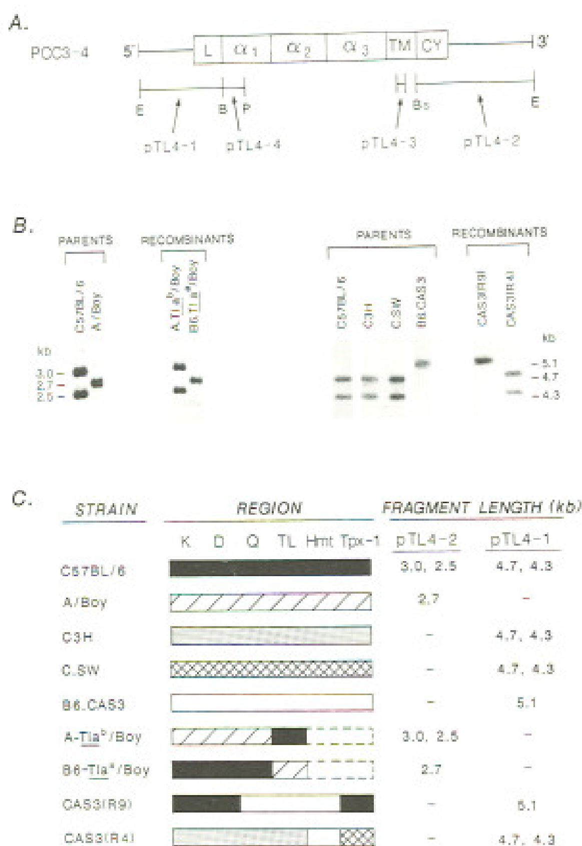


Figure 5. RFLP Analysis Maps Gene 27^b in the H-2 TL Region

(A) Schematic illustration of hybridization probes pTL4-1 and pTL4-2 used for the RFLP mapping and probes pTL4-3 and pTL4-4 used elsewhere in this study. L, leader peptide; α_1, α_2, and α_3, three extracellular domains; TM, transmembrane domain; CY, cytoplasmic domain. Restriction sites: B, BamHI; Bs, BspMI; E, EcoRI; P, PvuII.
(B) Southern blot analysis of genomic DNA from parental and H-2 recombinant strains. BamHI-digested genomic DNA isolated from the thymus of the indicated strains of mice was analyzed with the pTL4-2 (left panel) or pTL4-1 (right panel) probe. The length (kb) of the detected DNA fragments is indicated. (C) The origin of the H-2 region carried by recombinant and parental strains is schematically indicated along with a summary of the detected DNA fragments.

been cloned from the genome of C57BL/10 or C57BL/6 mice, which are thought to have identical or very similar TL regions (Flaherty et al., 1990) (Figure 6A). Among the 13 TL genes derived from C57BL/10 ($T1^b$ to $T13^b$), none hybridized with the KN6 ligand gene–specific pTL4-3 probe (data not shown). However, $T10^b$ hybridized with the pTL4-1 and pTL4-2 probes, which are known to detect both the KN6 ligand gene (B6-6) and its closest relative (B6-8). The identity of $T10^b$ with the defective B6-8 gene (PCC3-24 counterpart) was confirmed by sequencing the former (data not shown). Hereafter, we will refer to this defective gene as $T10^b$. The C57BL/6-derived TL genes $T14^b$ and $T15^b$ were not available for a similar analysis. However, it is unlikely that either encodes the KN6 ligand because the $T14^b$ gene is known to be defective (disrupted by an inserted retroviral sequence) and a $T15^b$-derived probe did not hybridize with $T10^b$ (Brown et al., 1988), which is highly homologous (96% homology) to B6-6 (Table 2).

Isolation of the gene encoding the KN6 ligand in the H-2^b haplotype was achieved by preparing a new cosmid library of C57BL/6 DNA, and screening the library with the pTL4-3 probe. We isolated one cosmid clone, B6.1, mapped the gene controlling the ligand within the cosmid, and determined the nucleotide sequence. As expected, this turned out to be identical to the sequence of B6-6 in the exons (data not shown). Thus, the KN6 ligand is encoded by a new TL^b region class I gene, which we provisionally designate gene 27^b. The PCC3-4/27 counterpart in strain 129 is hereafter referred to as gene 27^{129}.

Gene 27^b Belongs to a Novel TL^b Gene Cluster Related to the TL^d-B Cluster in BALB/c Mice

In BALB/c mice, two clusters of TL genes, TL^d-A (genes $T1^c$ to $T10^c$) and TL^d-B (genes $T11^c$ to $T17^c$ and gene 37^c) (Fisher et al., 1985; Transy et al., 1987), have been identi-

Table 2. Number of Nucleotide Differences among PCC3-4/27-Related cDNA Clones

	PCC3-4/27 (27^{129})	PCC3-24 ($T10^{129}$)	B6-6 (27^b)	B6-8 ($T10^b$)	BALB-1 ($T17^c$)	BALB-2 ($T9^c$)
PCC3-4/27 (27^{129})	0					
PCC3-24 ($T10^{129}$)	43	0				
B6-6 (27^b)	1	42	0			
B6-8 ($T10^b$)	43	8	42	0		
BALB-1 ($T17^c$)	6	45	5	45	0	
BALB-2 ($T9^c$)	23	46	22	43	23	0

Only the protein coding region was considered. Deletions and duplications (see Figure 3) were not counted as nucleotide differences. The genes corresponding to the cDNAs are indicated in parentheses.

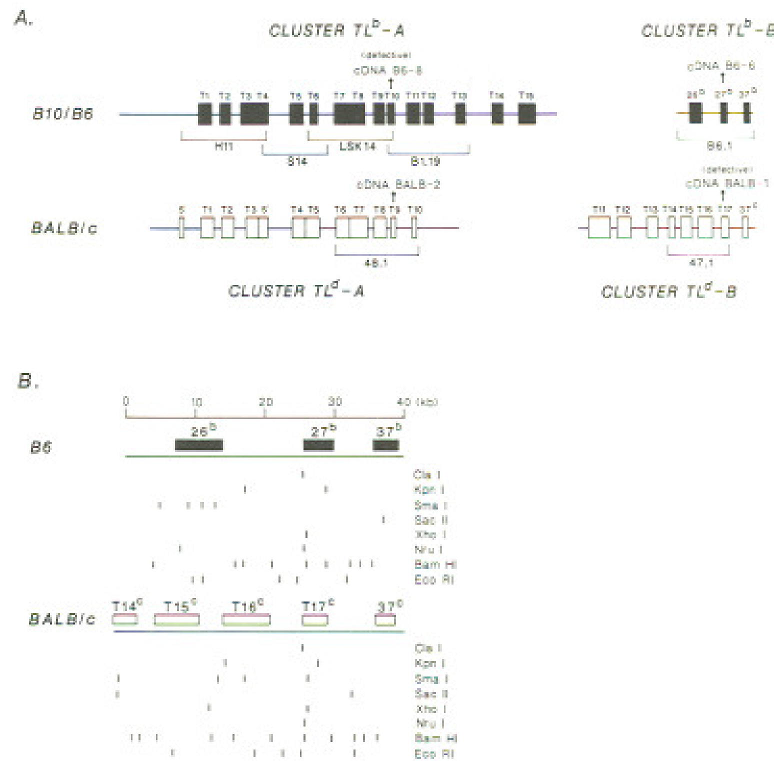


Figure 6. TL Region Genes in C57BL/10(6) and BALB/c Mice

(A) The new TL region gene cluster of strain C57BL/6, cluster TL^b-B, is indicated together with previously identified TL region gene clusters, TL^b-A (C57BL/10(6)), TL^d-A (BALB/c), and TL^d-B (BALB/c). The name and appropriate regions of the genomic DNA covered by the cosmid clones used in this study are indicated below the gene maps. Clone B6.1 was isolated in this study; clones H11, S14, LSK14, and B1.19 were a gift from R. A. Flavell (Yale University School of Medicine, New Haven, CT); clones 48.1 and 47.1 were kindly provided by M. Steinmetz (F. Hoffmann-La Roche, Basel, Switzerland). The cDNA clones described in this study are also indicated.

(B) Comparison of the restriction map of the cosmid B6.1 constructed in this study with the published (Transy et al., 1987) restriction map of the right half of the TL^d-B cluster of BALB/c.

fied which are related by duplication (Figure 6A). In contrast, the 15 TL genes (genes $T1^b$ to $T15^b$) of C57BL/10 or C57BL/6 mice comprise a single cluster that is more related to TL^d-A than to TL^d-B. $T10^b$ appears to be allelic to $T9^c$ (Brown et al., 1988), and $T9^c$ and $T17^c$ are believed to be derived from the duplication of a common ancestral gene (Fisher et al., 1985).

Since 27^b is closely related to $T10^b$, we suspected that $T17^c$ is the BALB/c allele of gene 27^b. To prove this, we isolated cDNA clones (represented by clone BALB-1) from a BALB/c cDNA library using the 27^b/$T10^b$-specific probe pTL4-2 (Figure 5A) and determined the sequence of clone BALB-1 (Figure 3) as well as the sequence of gene $T17^c$ (data not shown). The complete identity of the two sequences in the exons confirmed that $T17^c$ is the BALB/c allele of gene 27^b. In the protein coding region, the BALB-1 clone differed by only 5 nucleotides from B6-6 (27^b) and by only 6 nucleotides from PCC3-4/27 (27^{129}) (Table 2). Screening of the BALB/c cDNA library with the pTL4-2 probe also allowed the isolation of a second type of cDNA clone, represented by clone BALB-2. We determined the nucleotide sequence of this cDNA clone (Figure 3) as well as that of the gene $T9^c$ (data not shown) and demonstrated that the two sequences are identical in the exons. In agreement with these findings, the exon V nucleotide sequences of $T9^c$ and $T17^c$ have recently been described and are identical to the exon V sequences of BALB-2 and BALB-1, respectively (Brorson et al., 1989). Thus 27^{129} (PCC3-4/27), 27^b (B6-6), and $T17^c$ (BALB-1) are allelic, as are $T10^{129}$ (PCC3-24), $T10^b$ (B6-8), and $T9^c$ (BALB-2) (Table 3). Atypically, the overall homology of gene $T9^c$ with gene $T17^c$ is greater than with its alleles, $T10^{129}$ and $T10^b$ (Table 2). This is apparently due to extensive gene conversion in exons IV and V in which $T17^c$ served as the sequence donor to $T10^c$ (Figure 3).

The allelic relationship between 27^b and $T17^c$ implies that the former belongs to a novel TL^b gene cluster related to the TL^d-B cluster. Three lines of evidence sup-

Table 3. 27^b-Related Genes and Their cDNA Clones

	TL-A Cluster		TL-B Cluster	
Strain	Gene	cDNA	Gene	cDNA
129/Sv	$T10^{129}$	PCC3-24	27^{129}	PCC3-4 PCC3-27
C57BL/6 (C57BL/10)	$T10^b$	B6-8	27^b	B6-6
BALB/c	$T9^c$	BALB-2	$T17^c$	BALB-1

port this hypothesis. First, 7 kb downstream of the 27^b gene on the cosmid clone B6.1 is another class I gene (37^b) that is detectable not only with the pan-class I probe, pH2-IIa, but also with a probe specific for the gene 37^c (data not shown; but see Figure 6B). Gene 37^c is known to lie at about the same distance away from gene $T17^c$ (Transy et al., 1987). Second, an additional gene (26^b) detectable with the pH2-IIa probe on cosmid B6.1 is upstream of gene 27^b (Figure 6B), indicating that this is a cluster of genes. Finally, the restriction enzyme map of the downstream half of cosmid B6.1 matches well with that of the downstream end of the TL^d-B cluster (Figure 6B).

Molecular Basis for the Inability of TL^d Cells to Stimulate KN6 Proliferation

The failure of TL^d strains (BALB/c, B10.D2) to stimulate KN6 cell proliferation (Bonneville et al., 1989; Figure 1) is readily apparent upon examination of the BALB-1 ($T17^c$) sequence. Because of a 16 nucleotide deletion present at the start of exon II, which leads to a frameshift and premature termination of translation (Figure 3), the BALB-1 mRNA cannot encode a complete class I protein. This deletion is apparently due to a defect in the splice acceptor site in the intron I–exon II boundary of the $T17^c$ gene (Figure 7). In contrast, no structural defect was detected in the BALB-2 ($T9^c$) gene (Figure 3).

Tissue and Cell Expression of 27^b and $T10^b$

Since some TL determinants are known to be expressed in a tissue-specific manner (Boyse et al., 1965), we investigated the transcription levels of genes 27^b and $T10^b$ in various tissues and cells by RNAase protection assays. The 27^b gene was widely transcribed, although the levels of expression varied among tissues and cells (Figure 8). A high level was detected in spleen; intermediate levels were detected in thymus, liver, kidney, and lung; and low levels were detected in heart, muscle, brain, intestine, blood (Figure 8A), and skin (Figure 8C). With purified cell populations (Figure 8B), the RNA levels were greatest in $Mac\text{-}1^+$ peritoneal exudate cells and $Lyt\text{-}1^+$ peritoneal exudate cells, lower in splenic B cells ($B220^+$) and thymocytes, and lower still in splenic T cells ($Thy\text{-}1^+$). In addition, the purified intestinal epithelium and epidermis gave low levels of the 27^b RNA (Figure 8C). In contrast to the widely distributed 27^b RNA, $T10^b$ RNA is restricted to spleen, thymus, and peritoneal exudate cells and its level was about one-fifth the level of the 27^b RNA in the corresponding tissue.

Discussion

A Novel TL Region Gene Product As a Ligand for $\gamma\delta$ TCR

Data presented in this study show that a novel TL region gene (27^b) in strain C57BL/6 encodes the ligand recognized by the KN6 $\gamma\delta$ TCR. This role for a TL region gene was formally demonstrated by DNA-mediated gene transfer analysis and by Southern blotting analysis of genomic DNA from MHC-recombinant strains that positioned the gene between the Q and Hmt regions. In the transfection experiments, we cannot rule out the possibility that the introduced cDNA, instead of directly encoding the KN6 ligand, regulates expression of a host gene that encodes the ligand. This is highly unlikely, however, since no such regulatory function of MHC class I genes has been reported.

Based on RNA analysis, the 27^b gene is expressed in numerous tissues and cells, while the $T10^b$ gene is restricted in expression primarily to spleen, thymus, and peritoneal exudate cells (Figure 8). It thus appears that some TL genes, including 27^b and 37^c (Transy et al., 1987), are widely expressed while others, such as $T10^b$ and $T13^c$, which controls Tla thymic antigens (Boyse et al., 1965; Flaherty et al., 1990), are restricted in their expression.

To the extent examined, the 27^b gene displays limited polymorphism. Based on functional study, all strains tested, except BALB/c and B10.D2, appear to express this gene. Interestingly, cells from many of these strains stimulated KN6 cell proliferation at intermediate levels. Whether this is due to differences in allelic TL region gene sequences or levels of expression, or to differences in a putative endogenous peptide that might be presented by the class I product or by closely related products, is currently being investigated. Structural polymorphism was observed in the three strains analyzed. In the protein cod-

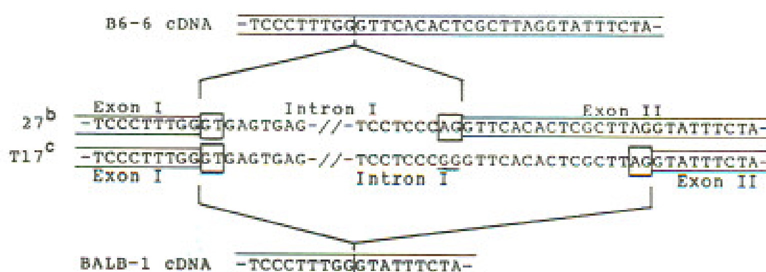


Figure 7. A Defective Splice Acceptor Site in Gene $T17^c$

The exon I–intron I and intron I–exon II junctions of genes 27^b and $T17^c$ are shown and compared with the corresponding sequences of cDNA clones B6-6 and BALB-1. The splice donor and acceptor consensus elements, the dinucleotides GT and AG, are boxed. The dinucleotide GG in the defective splice acceptor site in gene $T17^c$ is underlined.

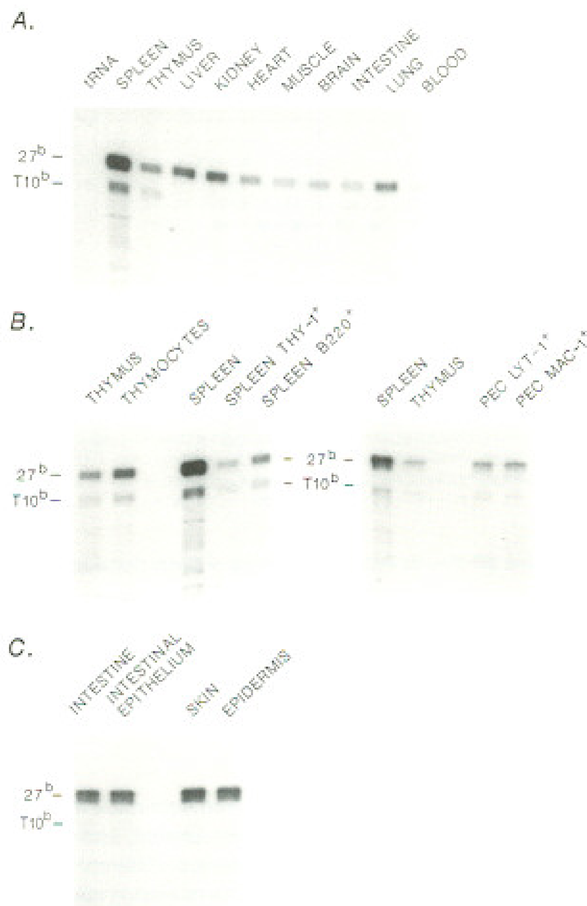


Figure 8. RNAase Protection Assay of 27^b and $T10^b$ RNA from Various Tissues and Purified Cells

An autoradiograph of the protected RNA fragments (27^b and $T10^b$ RNAs are 217 and 190 bases long, respectively) is shown. Band intensity is comparable only within each experiment. (A) Total cytoplasmic RNA from the various tissues and purified yeast tRNA (5 μg each) as a negative control was analyzed. (B) Total cytoplasmic RNA from various tissues and purified cell subpopulations thereof (1 μg) was analyzed. Thy-1+ and B220+ spleen cells and Lyt-1+ and Mac-1+ peritoneal exudate cells (PEC) were purified using a fluorescence-activated cell sorter. (C) Ten micrograms of each RNA was used. Intestinal epithelial cells were purified by Percoll gradient centrifugation as described (Bonneville et al., 1988). For the purification of epidermal cells, skin pieces from newborn mice were treated with dispase (3,000 U/ml in PBS) for 1 hr at 4°C. The epidermis was separated from the dermis and treated with a trypsin–EDTA solution (0.125% trypsin and 0.02% EDTA in PBS) for 5 min. The enzyme was inactivated by calf serum, and epidermal cells were purified by Percoll gradient centrifugation.

ing region, strains C57BL/6 and 129/Sv differed by a single nucleotide that caused no amino acid replacement. In contrast, BALB/c has a structurally defective RNA splice site, which explains why BALB/c cells fail to stimulate KN6 proliferation. Apart from this defect, the BALB/c allele is very similar to the C57BL/6 and 129 alleles, and their protein products would differ by only 3 amino acids. In contrast, $H\text{-}2K^d$ and $H\text{-}2K^b$ differ by 58 amino acids, and $H\text{-}2D^d$ and $H\text{-}2D^b$ by 56 amino acids (Watts et al., 1989). The relatively small differences among the gene 27 alleles is consistent with previous studies suggesting that TL region genes are much less polymorphic than classical H-2 genes (Boyse et al., 1965; Transy et al., 1987; Flaherty et al., 1990).

The BALB/c allele of gene 27^b turned out to be the previously cloned but unsequenced gene $T17^c$. Coupled with the observation that other class I genes, including 37^b, are closely linked to 27^b, these data provide evidence for a second TL^b region gene cluster related to the TL^d-B cluster. A summary of the relationships between different TL region genes and alleles is included in Figure 6 and Table 3. Future study is required to determine which genes in this cluster are expressed at the protein level, and to elucidate their function.

Structural Features of the Gene 27^b Product

The α_1 and α_2 domains of MHC class I molecule determine the antigen binding and T cell recognition specificity of these molecules (Reiss et al., 1983). Since the three-dimensional structure of a human class I molecule, HLA-A2, is known (Bjorkman et al., 1987a, 1987b), it would be interesting to compare the amino acid sequences of the 27^b molecule and HLA-A2 in these domains. The sequence homology between these two class I molecules is 54% in the α_1 domain and 39% in the α_2 domain (Figure 9). Secondary structure analysis by the Chou and Fasman (1978) algorithm suggests that the α_1 and α_2 domains of the 27^b protein fold into a β sheet and α helices structure not grossly different from the antigen combining site of HLA-A2.

Several differences between the 27^b and HLA-A2 structures are, however, worth noting. First, there is a 13 amino acid deletion in the middle of the 27^b α_2 domain (Figure 9). However, the two ends remaining after the deletion (positions 134 and 148) may be near each other should the 27^b protein adopt the HLA-A2 structure (see Bjorkman et al., 1987a, 1987b for the HLA-A2 structure). Thus, the deletion may not disrupt the remainder of the site, but only one end. Second, there is another deletion of 3 amino acids in the α_1 domain (Figure 9), which might disrupt the 45 pocket at the left end of the site (Garrett et al., 1989). Third, there are two new N-linked oligosaccharide sites, at residues 129 (NPS) and 150 (NST) (Figure 9). The former would probably direct the oligosaccharide away from the site, and the latter could direct an oligosaccharide into the region of the deletion covering the binding groove at the right end (D. Wiley, personal communication). Finally, the 12 residues after the 13 residue deletion are very different from most class I sequences, and the Chou and Fasman analysis does not give a high α helix propensity in this region. This could mean that the lower α helix is partially disrupted at the right end. Despite these differences, 27^b could still function by presenting antigens.

Structural Features of the Products of the 27^b-Related TL Genes

Among the 27^b-related TL genes studied (Table 3), genes $T10^{129}$ and $T9^c$ (129/Sv and BALB/c alleles, respectively, of $T10^b$) do not show any obvious structural defect (Figure 3), and yet their products do not seem to be recognized by the KN6 γδ TCR (Figure 1 and Figure 4A). The pre-

α1 domain

```
                     1   5    10   15   20   25   30   35   40   45    50   55   60   65   70   75   80   85   90
                     |   |    |    |    |    |    |    |    |    |     |    |    |    |    |    |    |    |    |
B6-6 (27b)           GSHSLRYFYTAVSRPGLGEPWFIIVGYVDDMQVLRFSSKEETPRM   APWLEQEEADNWEQQTRIVTIQGQLSERNLMTLVHFYNKSMD
PCC3-24 (T10129)     ---------------------------------------------   ----------D-----H-------------------------
BALB-2 (T9c)         ----------------------------------L----------   ---------GD-------------------------------
H-2Kd                -P------V-----------R--A------T-FV--D-DADN--FEPR---M---GPEY--E--QRAKSDE-WFRVS-R-AQRY--Q-KG
HLA-A2               ----M---F-S-----R---R--A------T-FV--D-DAASQ--EPR---I---GPEY-DGE--K-KAHS-THRVD-G--RGY--Q-EA
HLA-Aw68             ----M-----S-----R---R--A------T-FV--D-DAASQ--EPR---I---GPEY-DRN--N-KA-S-TDRVD-G--RGY--Q-EA
                         * * *              * * *                              ●|* ●|*●|**●|*●|**●|*   **|  *
```

α2 domain

```
                      95   100  105  110  115  120  125  130  135  140  145  150  155  160  165  170  175  180
                      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
B6-6 (27b)           DSHTLQWLQGCDVEPDRHLCLWYNQLAYDSEDLPTLNENPSSCT                 VGNSTVPHISQDLKSHCSDLLQKYLEKGKERLLRS
PCC3-24 (T10129)     ---------D------------------------S-------                 --------Q---H-EG---V---------------
BALB-2 (T9c)         --------------------------------H----------                 -----------------------------------
H-2Kd                G---F-RMF----GS-WR-LRG-H-F---GR-YIA---DLKTW-AADTAALITRRKWEQAGDAEYYRAY-EGE-VEW-RR---L-N-T---T
HLA-A2               G---V-RMY----GS-WRFLRG-H-Y---GK-YIA-K-DLR-W-AADMAAQTTKHKWEAAHVAEQLRAY-EGT-VEW-RR---N---T-Q-T
HLA-Aw68             G---I-MMY----GS-GRFLRG-R-D---GK-YIA-K-DLR-W-AADMAAQTTKHKWEAAHVAEQWRAY-EGT-VEW-RR---N---T-Q-T
                         * * *           * *                           * ||* |||* ||* |* ●||  |* ● *
                                                                         *         *        *
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Figure 9. Comparison of the α_1 and α_2 Domain Amino Acid Sequences of the 27^b and 27^b-Related Proteins with Those of Some Classical Mouse and Human MHC Class I Molecules

Amino acid sequences of the B6-6 (27^b), PCC3-24 ($T10^{129}$), and BALB-2 ($T9^c$) proteins are aligned with those of H-$2K^d$, HLA-A2, and HLA-Aw68. Asparagine (N) residues of potential N-linked glycosylation sites (NXS or NXT) and the conserved cysteines (C) in the α_2 domains are boxed. The symbols under the amino acid sequences designate HLA-A2 residues that have been judged by X-ray crystallography (Bjorkman et al., 1987b) to be in the vicinity of the recognition site for processed foreign antigen: asterisks, residues pointing toward the site; vertical lines, residues on an α helix pointing up away from the membrane-proximal immunoglobulin-like domain; filled circles, residues on an α helix pointing away from the site. The amino acid sequences of H-$2K^d$, HLA-A2, and HLA-Aw68 were obtained from the GenBank data base.

dicted amino acid sequences of $T10^{129}$ and $T9^c$ would differ from that of 27^b by 9 and 4 residues, respectively, in the combined α_1 and α_2 region (Figure 9). Seven of the 9 variant residues of $T10^{129}$ and 2 of the 4 variant residues of $T9^c$ occupy the positions at which HLA-A2 contains amino acids that could potentially interact with a bound peptide or a TCR (Figure 9) (Bjorkman et al., 1987b). Thus, the apparent lack of KN6 reactivity may be attributed to some of these amino acid substitutions. However, in the case of the $T9^c$ gene, we cannot exclude the possibility that its product is recognizable by the KN6 γδ TCR but was not expressed on the surface of the stimulator cells tested.

Implications and Speculation

The observation that a TL region class I gene product is a ligand for the γδ TCR has important implications for MHC function as well as γδ T cell function. The role of MHC products as the ligand for the TCRs has now been extended to a class I molecule encodes in the TL region. This raises the obvious possibility that other TL region–encoded class I molecules may also serve as the ligand for the γδ TCR. Data in several systems support the notion that γδ T cells recognize nonclassical class I molecules. Thus, a T cell clone derived from a BALB/c nu/nu mouse responded to an allogeneic determinant controlled by a gene mapping distal to the Q region in H-2^b mice (Bluestone et al., 1988). A T helper hybridoma specific for a GT copolymer apparently presented by the Qa-1 molecule expresses a γδ TCR (Vidović et al., 1989). In the human system, γδ T cells reactive to non-MHC-linked class I molecules, CD1, have been identified (Porcelli et al., 1989). γδ T cell clones reactive with allogeneic H-2 class I and class II products have also been reported (Bluestone et al., 1988). These clones were obtained from nude mice after strong selection by allogeneic stimulation, and exhibit a broad cross-reactivity for the products of different alleles of class I and class II MHC genes. These clones may have been derived from rare cells that are specific for nonclassical MHC proteins but were selected by in vivo and/or repeated in vitro stimulation on the basis of their cross-reactivity with classical MHC proteins. Finally, except for one case (Haregewoin et al., 1989), murine and human γδ T cells reactive with Mycobacterium bovis hsp65 do not seem to be restricted by the classical polymorphic MHC molecules (Holoshitz et al., 1989; O'Brien et al., 1989). Since these mouse γδ T cells are also reactive with purified protein derivative (a source of hsp65 that is denatured and probably degraded), it was suggested that the recognition involves nonclassical peptide-presenting molecules (O'Brien et al., 1989).

The 27^b product may be recognized by the KN6 γδ TCR as such or as an antigen-presenting molecule. We favor the latter hypothesis on the basis of the general structural similarities between the TL region–encoded and the classical (i.e., H-2K, H-2D, and H-2L) class I products and between the αβ TCR and γδ TCR. If this hypothesis is correct, the putative antigen recognized by the KN6 γδ TCR is probably a ubiquitously expressed self molecule (Tonegawa et al., 1989). The third possibility, that a 27^b-derived peptide is presented by a classical H-2 molecule to the KN6 γδ TCR, is unlikely since PCC3 stimulates KN6 cells and does not express any surface classical H-2 molecules, and because the functional assay did not reveal any influence of these polymorphic molecules on the response (Figure 1).

It was previously speculated that γδ T cells recognize

nonclassical class I molecules induced by infection and transformation (Janeway et al., 1988). In view of the more recent data, including those presented in this paper, it is tempting to speculate that nonclassical class I restriction elements and γδ TCRs have coevolved for the recognition of certain endogenous and common microbial antigens. While the classical restriction elements for αβ T cells show extensive allotypic polymorphism (Watts et al., 1989) to ensure the recognition of any peptide by the αβ T cells of a species, the nonclassical restriction elements for γδ T cells show mainly isotypic polymorphism, perhaps to ensure the recognition of evolutionarily conserved antigens by each member of a species. Interestingly, in the membrane-distal domains the various TL molecules have diverged from each other much more than the classical MHC molecules have (Table 1 and Flaherty et al., 1990), and these domains of CD1 molecules are quite distinct from those of TL molecules (Bradbury et al., 1988). Since these domains are likely to contain the binding sites not only for antigens but also for the TCR, the diverse α_1 and α_2 domains of TL and CD1 proteins might be specialized for the presentation of different sets of antigens and for the recognition by different γδ TCR subsets. In this regard, it is intriguing that the two γδ T cell subsets with homogeneous TCRs, namely, DECs and vut-IELs, while sharing the same δ gene, utilize two distinct Vγ gene segments with a relatively low sequence homology (35% at the amino acid sequence level [Garman et al., 1986]). According to the above model, this may coincide with the expression not only of particular endogenous antigens but also of distinct nonclassical class I molecules in the epidermis and the relevant epithelia. A similar tissue-specific expression of a particular nonclassical class I molecule may also occur in the intestinal epithelium because the associated γδ T cells, i-IELs, preferentially utilize yet another distinct Vγ gene segment, Vγ7 (Bonneville et al., 1988). On the other hand, the TCRs expressed by γδ T cells circulating in the peripheral lymphoid organs are more diverse (Cron et al., 1988) and are encoded by multiple combinations of Vγ and Vδ gene segments, although some of them (e.g., Vγ4 and Vδ5) are utilized more frequently than others (S. Itohara, personal communication). This subset of γδ TCRs to which the KN6 TCR belongs may recognize more diverse foreign and endogenous antigens that are presented by multiple nonclassical MHC molecules in a variety of tissues.

Experimental Procedures

Mice, Cells, and Antibodies

Mice were from our breeding colony or were purchased from Jackson Laboratory, except for the following. Many of the Q and TL region recombinant strains were generously provided by L. Flaherty (The Wadsworth Center, Albany, NY). The MA series of recombinants was provided by H. Passmore (Rutgers University, Piscataway, NJ). The B6.CAS3 series of recombinants were provided by K. Fischer Lindahl (University of Texas Southwestern Medical Center, Dallas). The KN6 γδ transgenic mice (H-2^d homozygous) have been described (Bonneville et al., 1989). The Abelson murine leukemia virus–transformed thymoma line 2052C (strain C57BL/6) was obtained from R. Risser (University of Wisconsin, Madison). Embryonic carcinoma lines F9, PCC3, and 402AX (all from strain 129/Sv) were obtained from P. Sharp at the Center for Cancer Research (MIT) or from S. Ostrand-Rosenberg (University of Maryland, Baltimore). T cell lymphomas S49 and WEHI7.1 (strain BALB/c) and EL4 (strain C57BL/6) were from H. N. Eisen of the Center for Cancer Research (MIT). L cells were from the American Type Culture Collection (Rockville, MD). MAbs 5C10 (anti-KN6 γδ TCR clonotypic), H57-597 (anti-pan-αβ TCR), and 2C11 (anti-CD3) were previously described (Itohara et al., 1989). MAbs 30-H12 (anti-Thy-1), 53.7.3 (anti-Lyt-1), RAS.3A1 (anti B220), and M1/70 (anti-Mac-1) were obtained from Becton Dickinson (Mountain View, CA) or A. Coutinho (Institut Pasteur, Paris).

DNA Probes

The pH2-IIa probe (Steinmetz et al., 1981) hybridizes with the exon IV sequences of all known H-2 class I genes (Weiss et al., 1984). Probe pTL4-1 is a 326 bp EcoRI–BamHI fragment from the 5′ region of cDNA clone PCC3-4 (Figure 5A). Probe pTL4-2 is a 543 bp BspMI–EcoRI fragment from the 3′ region of cDNA clone PCC3-4 (Figure 5A). Probe pTL4-3 is an oligonucleotide, 5′-CATGCATATGCAAACCACAC-3′, that constitutes a part of the exon V sequence of clone PCC3-4 (Figures 3 and 5A) and is specific for the KN6 ligand gene 27^b and its strain 129/Sv and BALB/c counterparts. See Figure 5A for the locations of the various pTL probe sequences in the cDNA clone PCC3-4.

cDNA and Genomic DNA Libraries

For preparation of the PCC3 cDNA library, cDNA was ligated to a synthetic EcoRI adaptor, size fractionated by Bio-Gel A50m gel filtration, ligated to EcoRI-cleaved λgt10 vector arms, and packaged using commercially available packaging extracts (Stratagene, La Jolla, CA). The C57BL/6 newborn thymus cDNA library, constructed in the eukaryotic expression vector CDM8, was kindly provided by B. Seed (Massachusetts General Hospital, Boston). The BALB/c cDNA library was constructed in the vector λZAP (Stratagene). The C57BL/6 genomic library was constructed in the cosmid vector pWE15 (Stratagene).

Proliferation Assay

CD4$^-$8$^-$ splenic T cells were prepared from transgenic mice that express productively rearranged KN6 γ and δ TCR genes (KN6 transgenic mice), as described previously (Maeda et al., 1987). Flow cytometric analysis indicated that over 95% of these cells express the KN6 γδ TCR and none expressed the αβ TCR. For the experiments described in Figures 1, 2, and 4B, CD4$^-$8$^-$ splenic T cells were used as responder cells, while the data in Figure 4A were obtained using short-term KN6 cell lines, over 99% of which consisted of T cells expressing the KN6 γδ TCR (KN6 γδ T cells). For the proliferation assay, 10^4 responder cells were cultured with various stimulator cells for 72 hr as described previously (Bonneville et al., 1989), and the proliferating cells were labeled with [^{3}H]thymidine (1 μCi per well) for 6 hr. Flow cytometric analysis of the cultured cells indicated that at least 95% of CD3$^+$ blast cells also expressed the KN6 γδ TCR. Specific proliferation of KN6 γδ T cells in the cultures was also confirmed by demonstrating the ability of the clonotypic KN6 γδ TCR MAb 5C10 to inhibit the proliferation.

Transfection

Mouse L cells were transfected using the calcium phosphate method with the PCC3-4, PCC3-27, and PCC3-24 cDNAs inserted into the bovine papilloma virus–derived expression vector BMGNeo (Karasuyama and Melchers, 1988). Stably transfected cells were isolated by G418 selection (600 μg/ml). WEHI7.1 and S49 cells were transiently transfected by the DEAE-dextran method with the PCC3-4, PCC3-27, and PCC3-24 cDNAs inserted into the SV40-derived expression vector pSVL (Templeton and Eckhart, 1984). Sixty hours after transfection, the cells were analyzed for their ability to stimulate KN6 proliferation.

Nucleotide Sequencing

Nucleotide sequences were determined by the dideoxy chain termination method using Sequenase DNA polymerase (United States Biochemical Corp., Cleveland, OH).

RNAase Protection Assay

RNAase mapping analyses were performed according to Melton et al. (1984). To make any antisense RNA probe, pTL4-4, a 217 bp BamHI–PvuII fragment from the exon I and II region of cDNA clone PCC3-4

(Figure 5A) was cloned into the pBluescript vector (Stratagene). The [α-^{32}P]CTP- or [α-^{32}P]UTP-labeled RNA probe was synthesized using the linearized plasmid as the template and bacteriophage T3 RNA polymerase (Promega, Madison, WI). One to ten micrograms of cytoplasmic RNA, prepared from C57BL/6-derived tissues and purified cells by the guanidinium thiocyanate–CsCl gradient method, was hybridized overnight with the probe at 45°C. Protected RNA fragments were analyzed by electrophoresis on 5% acrylamide–urea gels.

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GenBank Accession Numbers

The accession numbers for the cDNA sequences reported in this paper are M35243 (PCC3-4), M35244 (PCC3-24), M35245 (B6-6), M35246 (B6-8), M35247 (BALB-1), and M35248 (BALB-2).

Note Added in Proof

Under the newly revised mouse MHC nomenclature, gene 27^b will be designated gene $T22^b$ (Klein et al., Immunogenetics, in press, 1990).