MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 1865–1872
0270-7306/87/051865-08$02.00/0

Vol. 7, No. 5

Molecular Analysis of Antigen Recognition by Insulin-Specific T-Cell Hybridomas from B6 Wild-Type and bm12 Mutant Mice

DAVID H. SHERMAN,[1,2] PAULA S. HOCHMAN,[3] ROBERT DICK,[3] RICHARD TIZARD,[1] KUZHALMANNAM L. RAMACHANDRAN,[1] RICHARD A. FLAVELL,[1]* AND BRIGITTE T. HUBER[1,3]

Biogen Research Corporation, Cambridge, Massachusetts 02142[1]; Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[2]; and Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111[3]

Received 24 November 1986/Accepted 28 January 1987

Molecular analysis of the heterodimeric T-cell antigen receptor of insulin-specific class II-restricted T-cell hybridomas (THys) derived from C57BL/6 (B6) wild-type and B6.C-*H-2*^{bm12} (bm12) mutant mice revealed that such T cells use a diverse V gene repertoire. Analysis of three THys that use related V genes, however, showed a number of novel features. (i) Two THys that share major histocompatibility complex restriction use V_α genes that are 98.6% homologous. (ii) Two THys sharing the same antigen fine specificity use a particular germ line $V_\beta D_\beta J_\beta$ combination. (iii) A 21-base-pair deletion in the 5′ segment of the J_β gene occurs in one THy, suggesting a novel mechanism for generating diversity in T-cell antigen receptor β genes. (iv) The first amino acid encoded by N sequences at the V-D junction is conserved in a pair of T cells which recognize identical antigenic epitopes. The implications of these findings for the structural mechanisms underlying major histocompatibility complex-restricted antigen-specific T-cell recognition are discussed.

In contrast to the B-cell antigen receptor, immunoglobulin, the T-cell receptor (Tcr) does not react with soluble antigen, but recognizes an as yet undefined cell surface molecular complex. This complex is apparently formed by the association of an antigen fragment with major histocompatibility complex (MHC) class I or class II proteins. The Tcr is thought to interact with a specific site on the MHC molecule, the histotope, while another site of the Tcr is believed to interact with the antigen epitope (for a review, see reference 53). A rigorous test of this hypothesis has awaited the identification of the Tcr proteins and the genes which encode them. Initial biochemical analysis of the Tcr was made possible by the production of monoclonal anti-idiotypic antibodies which allowed for the isolation of the Tcr protein. This protein was shown to be a disulfide-linked heterodimer made up of an acidic α chain and a more basic β chain each with an apparent molecular mass of 40 to 50 kilodaltons (1–3, 16, 23, 24, 37, 38, 42, 46, 47). The identification of the genes encoding the β chain (12, 19, 26–28, 39, 40, 49, 51, 55, 56, 60), and the α chain (11, 50) has shown a striking organizational and structural similarity to the immunoglobulin genes (33); both consist of variable- and constant-region segments. Three gene segments, variable (V), diversity (D), and joining (J), make up the V segment of immunoglobulin heavy chain and Tcr β, while the immunoglobulin light-chain and Tcr α segments consist of V and J elements only. All V genes are rearranged in somatic cells that transcribe the genes. Despite these recent biochemical and genetic analyses, the mechanism by which the Tcr gene products mediate MHC-restricted antigen recognition remains unclear. Transfection experiments by Steinmetz and his collaborators (15) have clearly demonstrated that the α and β genes are sufficient to encode for MHC-restricted T-cell antigen recognition.

To study the mechanism(s) of T-cell recognition, we developed a cellular system in which both the antigenic specificity and the MHC restriction can be precisely defined. We compared the Tcr structure of insulin-specific helper T cells that were derived from C57BL/6 (B6) wild-type (Ia^b) and mutant B6.C-*H-2*^{bm12} (bm12) (Ia^{bm12}) mice. Insulin is a chemically well-defined peptide hormone (molecular weight, ~ 5,000), which consists of an A and a B chain that are linked by two disulfide bridges; an intrachain disulfide bond creates a loop in the A chain which contains the amino acids at positions A7, A8, A9, and A10. Using the various species of insulin which differ at single amino acids, we were able to determine the fine specificity of our T cells (30, 31, 34).

To define the MHC restriction of our insulin-reactive T helper hybridoma (THy) lines precisely, we made use of the class II mutant bm12 mouse and the B6 wild type. The I-A_β gene of the bm12 mouse differs from that of the wild type at three nucleotide positions, which give rise to three amino acid changes clustered within the first extracellular domain of the A_β molecule (43). Both structural and functional analyses suggest that this mutation is the product of a gene conversion-like event in which a segment of 14 to 42 nucleotides was exchanged between I-A^b_β and I-E^b_β (30, 32, 44, 45, 58). This mutation creates a strong histocompatibility barrier such that the majority of B6-derived helper T cells, regardless of their antigen specificity, are restricted to recognize antigen solely in the context of Ia^b, but not Ia^{bm12}, and vice versa (31, 36). In this paper we report the results of our initial analysis of the structural mechanism of MHC-restricted, antigen-specific T-cell recognition using 12 B6-derived THys (2 insulin A-chain specific, 9 insulin B-chain specific, and 1 autoreactive) and 9 bm12-derived THys (2 insulin A-chain specific, 5 insulin B-chain specific, 1 autoreactive, and 1 reactive to an Ia determinant shared between B6 and bm12).

MATERIALS AND METHODS

THy production and specificity testing. THys were produced as described previously (31). Briefly, 7 to 10 days after in vivo priming with beef insulin (Sigma Chemical Co., St. Louis, Mo.) (one injection of a B6 mouse for 52H10F11, one injection of a bm12 mouse for 42H11, and two injections of

* Corresponding author.

a bm12 mouse for 5.3.18), popliteal, inguinal, and periaortic lymph node cells were fused at a ratio of 1:1 to the hypoxanthine-aminopterin-thymidine-sensitive AKR thymoma BW5147, using 50% polyethylene glycol 1540 (J. T. Baker Chemical Co., Phillipsburg, N.J.) for 8 min. Then 2×10^5 cells were plated in hypoxanthine-aminopterin-thymidine medium in 96-well plates containing 2×10^3 thymocyte feeder cells. Fourteen days after fusion, hybrids were transferred to 24-well plates, expanded, and tested for their ability to produce interleukin-2 (IL-2) when challenged with the species variants of insulin (100 μg/ml) in the presence of antigen-presenting cells (spleen cells) syngeneic to the original T-cell donor. Antigen-specific hybrids were cloned at 0.25 cell per well.

cDNA cloning. Cytoplasmic poly(A)$^+$ RNA was prepared by the method of Maniatis et al. (41) from 10^8 THys that had been tested for function at the time of lysis. cDNA plasmid libraries of about 5×10^5 independent colonies were constructed by the method of Okayama and Berg (48), using the commerically prepared dT-tailed vector (pSV7186) and dG-tailed linker piece (pSV1932) (PL Biochemicals, Inc., Milwaukee, Wis.). The 52H10F11 cDNA library was constructed in the pSV7186 vector, but instead of a linker piece, double-stranded cDNA was blunt-end ligated to the vector by the method of C. Gerald, G. Freeman, and H. Cantor (personal communication). cDNAs specific for the α and β chains of the Tcr were selected by subtracting the V_α- and V_β-positive clones specific for the fusion partner BW5147 from all the clones that were positive with constant-region C_α and C_β probes. V_β BW5147 was kindly provided by S. Hedrick. The proportions of V_α BW5147/V_α T cell- and V_β BW5147/V_β T cell-positive clones varied significantly from THy to THy. It is therefore difficult to estimate the exact frequency of T-cell α- and β-chain gene message, but it is approximately 0.01%.

DNA sequencing. A novel procedure for rapidly sequencing Tcr α-chain and β-chain cDNA clones was developed, based on the procedure of Church and Gilbert (13), as modified by R. Tizard and H. Nick (unpublished data). Plasmid DNA was cut to completion (α chain cut with *Nco*I, β chain cut with *Stu*I) and subjected to base-specific chemical cleavage reactions, and the fragments were resolved on a sequencing gel. After transfer to a nylon membrane, the filter was probed with a ^{32}P-labeled oligonucleotide probe (24mer that hybridizes to the α-chain C region just upstream of the *Nco*I site, at base pair [bp] 545 in Fig. 3; 24mer that hybridizes to the β-chain C region upstream of the *Stu*I site, at bp 640 in Fig. 4A). The filter was washed by the method of Church and Gilbert (13), except that the sodium ion concentration was increased by 1 M for the hybridization and 0.2 M for the washes by adding sodium chloride. The hybridization and washes were performed at 50°C. All sequences were determined from sense and antisense strands.

Genomic Southern analysis. DNA was prepared from AKR liver, B6 liver, and different THys by the method of Maniatis et al. (41), digested with *Eco*RI, electrophoresed in agarose gels, blotted onto a nylon membrane (Biotrans; ICN Biomedicals, Inc., Irvine, Calif.), and UV irradiated (13). Filters hybridized with the nick-translated probe were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C. Hybridization of the nylon filters with oligonucleotide probes was performed at 45°C in plaque screen buffer (50 mM Tris hydrochloride [pH 7.5], 1 M NaCl, 0.2% polyvinylpyrrolidone, 0.2% Ficoll-400, 0.2% bovine serum albumin, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate) containing 100 μg of tRNA per ml. The filters were washed three times with plaque screen buffer at 45°C and then twice with 3.2 M tetramethylammonium chloride (59) and 1% sodium dodecyl sulfate at 58°C.

TABLE 1. Reactivity profile of insulin- and autoreactive THy lines

T-cell line	Ia restriction	Reactivity
BW5147		
42G8	bm12	B6-auto
42F7	bm12	bm12-auto
10.10.58	bm12	B chain
5.3.18	bm12	B chain
41C8	bm12	A chain
21.2-B8	bm12	B chain
42H11	bm12	A chain
84E1G12	B6	A chain
52H10F11	B6	A chain
83H10F7	B6	B6-auto
84C6H5	B6	B chain
51A4C10	B6	B chain
84C6G10	B6	B chain
82B8E8	B6	B chain
84E1G11	B6	B chain
88E3	bm12	B chain
84E1A3	B6	B chain
82B8F5	B6	B chain
88G11	bm12	B chain
84E7G3	B6	B chain
84C6A7	B6	B chain

RESULTS

Analysis of Ia^b- and Ia^{bm12}-restricted THys. The 21 THy clones used in this analysis were produced in six independent fusions of BW5147 cells with lymph node T cells from insulin-primed B6 and bm12 mice (Table 1). Of the 12 B6-derived clones, 2 were specific for the insulin A chain, 9 were specific for the insulin B chain, and 1 was autoreactive as assessed in IL-2 production assays and B-cell proliferation and differentiation assays (30, 31). Of the nine bm12-derived clones, five were insulin B-chain reactive, two were insulin A-chain reactive, one was autoreactive, and one recognized an Ia determinant shared by B6 and bm12 mice. Of these 21 hybridomas, 3 were utilized for extensive analysis of their Tcr genes. The fine specificities of these clones, 52H10F11, 5.3.18, and 42H11, for the different insulins are shown in Table 2. The B6-derived hybridoma 52H10F11 is restricted to recognize insulin in the context of Ia^b, while 5.3.18 and 42H11 recognize insulin presented only by Ia^{bm12} (31). 52H10F11 and 42H11 recognize an A-chain insulin determinant shared by beef and sheep, but not pork, insulin. 5.3.18 is a B-chain-reactive hybridoma capable of recognizing beef, sheep, and pork insulin (which have identical B chains distinct from mouse insulin B chain) as well as the isolated oxidized B chain of pork insulin. In addition to recognizing insulin A chain in the context of Ia^{bm12}, 42H11 is also alloreactive, recognizing both Ia^b (31) and Ia^d (7; M. Kelliher and B. Huber, unpublished observations). The structure of the Tcr genes isolated from these three THys and the published sequence of the Tcr β gene of the LB2 cell line, which is an Ia^b-restricted, chicken erythrocyte-specific clone (49), are analyzed (for summary see Fig. 1).

Comparison of related Tcr V_α gene segments. Our analysis began with the isolation and sequencing of the α-chain cDNA from the 42H11 THy clone. To address the question

TABLE 2. Profile of reactivities of THys used in these studies

Antigen-presenting cells	Insulin	Proliferation of CTLL-2 cells[a]		
		B6 hybridoma 52H10F11	bm12 hybridomas	
			42H11	5.3.18
−	None	2,304	813	1,514
+	None	1,939	1,590	1,703
+	Beef	39,277	5,184	5,665
+	Sheep	17,095	11,930	9,502
+	Pork	2,194	1,570	5,588
+	B chain	1,503	1,516	2,488

[a] Counts per minute of tritiated thymidine incorporated by CTLL-2 cells. Mixtures of 5×10^4 THys, 5×10^5 irradiated syngeneic antigen-presenting spleen cells, and 100 μg of insulin per ml were cultured for 24 to 48 h in 96-well microtiter plates. Then 75 μl of supernatant was harvested from each well and individually assayed for IL-2 content by measuring the ability to support the growth of IL-2-dependent CTLL-2 cells in 24-h cultures.

of whether this same V_α segment is shared by other THy clones of similar specificities, we used a 460-bp *Bgl*II-*Hin*cII fragment derived from the 42H11 V_α segment to probe Northern blots (data not shown). The results of these blots showed that a second THy, 5.3.18, uses a similar V_α gene segment as suggested by its strong cross-hybridization to the 42H11 probe. Southern analysis of genomic DNA with the 42H11 V_α probe showed that this gene family consists of five members (Fig. 2A, lane 5). From the intensity of the bands, it is likely that three of these members are closely related, while the remaining two members are less homologous. In the DNA from the fusion partner BW5147 and its parental AKR mouse liver DNA, only a single strongly cross-hybridizing member existed, along with two weakly cross-hybridizing species (Fig. 2A, lanes 1 and 2). Unique rearrangements were seen in both 42H11 and 5.3.18, suggesting that different V_α or J_α segments or both are used to construct the functional α-chain gene.

Isolation and sequencing of full-length cDNA clones from the 5.3.18 THy reveal that this V_α differs by only 4 bp from 42H11 V_α, which results in two amino acid substitutions. The sequence comparison shows a double-nucleotide change at bp 336 to 337 (Fig. 3), resulting in a Val-to-Glu difference between 42H11 and 5.3.18 at position 60. This difference is particularly significant because it lies in one of the classically defined hypervariable regions, previously identified for V_H (5), V_β (49), and V_α (4, 25). An additional Val-to-Met change between 42H11 and 5.3.18 at amino acid position 13 in the 5′ end of the V_α region does not appear to fall into a previously defined hypervariable region. The fourth base pair change is at position 394 and is silent.

The 42H11 J_α segment is virtually identical to the previously described TA37 J_α gene (4). However, there is an additional base pair (G, bp 445) in the 42H11 $V_\alpha J_\alpha$ junction which could be derived from the 42H11 V_α segment or from the insertion of an N-region nucleotide during the combinatorial process. The 5.3.18 J_α segment has also been previously described (TA31 J_α [4]), but it differs in length from that gene by 13 bp at the 5′ end. There are three fewer base pairs (one codon) in the 5.3.18 $V_\alpha J_\alpha$ junction when aligned with the 42H11 $V_\alpha J_\alpha$ junction (Fig. 3). To determine the origin of the heterogeneity between the related J_α sequences it will be necessary to compare them with the germ line sequence. This will also be necessary for the heretofore undescribed V_α 42H11 and V_α 5.3.18 gene segments to determine whether or not these germ line segments have the same length.

The high degree of homology between the 42H11 and 5.3.18 α-chain leader and 5′ untranslated region gene segments is noteworthy. Both leader sequences are identical at

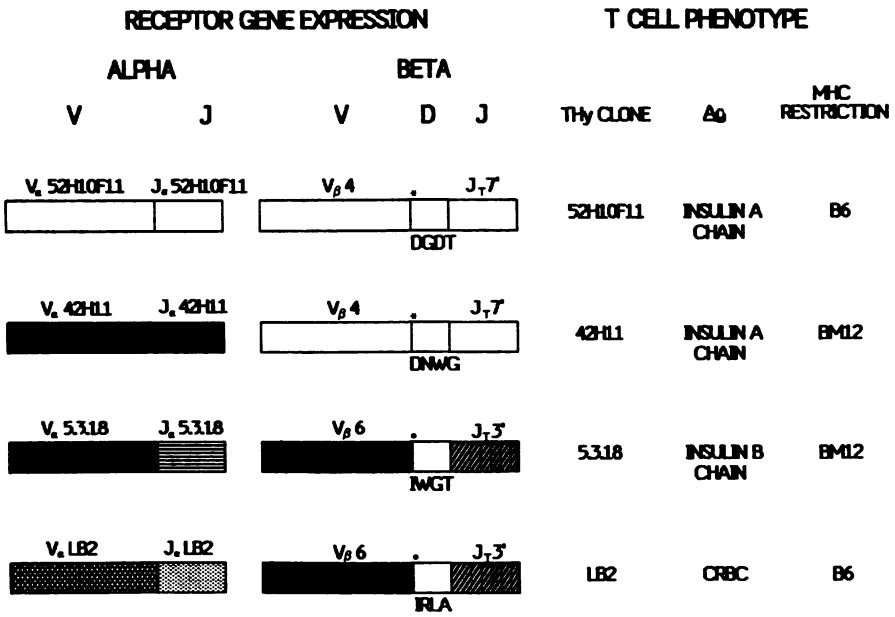

FIG. 1. Correlation of Tcr gene expression and THy phenotype. (Left panel) Summary of receptor genes expressed by insulin-reactive T-cell clones 52H10F11, 42H11, and 5.3.18 and the chicken erythrocyte-reactive cell line LB2 (49). Tcr α-gene segments are named according to the THy of their origin and are distinguished by their patterns. The 42H11 V_α differs from the 5.3.18 V_α by two amino acids (Fig. 3). Tcr V_β gene segments are named according to Barth et al. (5), and J_β segments are named according to Gascoigne et al. (19), and they are distinguished by their patterns. An asterisk denotes the identical amino acid at the VND junction in 52H10F11 and 42H11, and the four amino acids making up each of these regions are shown. A closed circle denotes the identical amino acid at the VND junction in 5.3.18 and LB2. (Right panel) Summary of functional reactivity for the insulin-reactive THys profiled in Table 2 and the chicken erythrocyte-reactive clone LB2 (49). Ag refers to the antigen recognized.

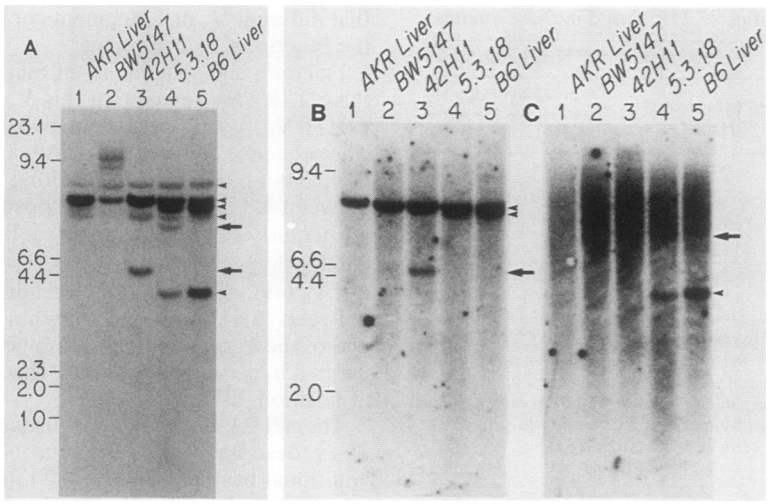

FIG. 2. Southern blot analysis of genomic DNA from 42H11 and 5.3.18 THys. DNA from the 42H11 and 5.3.18 THys and control DNA from AKR liver, B6 liver, and the tumor cell parent of the hybridomas, BW5147, was digested with *Eco*RI, electrophoresed, and blotted onto a nylon filter. (A) The blot was probed with the entire nick-translated 42H11 V_α gene segment. Arrows denote rearranged gene fragments in 42H11 and 5.3.18; arrowheads denote B6 liver V_α germ line genes. Note that the ~4.0-kilobase B6 liver V_α gene fragment is absent in 42H11 and presumably has been deleted during somatic rearrangement. Size standards are given in kilobases at the left side of the blot. (B and C) DNA from the sources listed from panel A was digested with *Eco*RI, electrophoresed, and blotted onto a nylon filter. Blot 1B was probed with the 42H11 V_α oligonucleotide probe that spans bp 330 to 350 in the 42H11 gene. Blot 1C was probed with the 5.3.18 V_α oligonucleotide probe that spans bp 330 to 350 in the 5.3.18 gene. Arrows denote the rearranged gene fragment in 42H11 and 5.3.18; arrowheads denote B6 liver germ line genes. Size standards are given in kilobases.

the amino acid level except for an additional Ala residue in the 42H11 leader sequence. These expressed V_α genes might, therefore, have been derived from two closely related germ line V_α gene segments or a single V_α subjected to somatic mutation to give rise to both of the V_α region genes. To resolve this issue we synthesized oligonucleotides spanning the double-nucleotide substitution between bp 330 and 350 in 42H11 and 5.3.18 and used these to probe Southern blots of genomic DNA. The results showed that an oligonucleotide probe to 42H11 V_α hybridizes to two of the three strongly cross-hybridizing bands originally seen with the 42H11 V_α-region probe in B6 liver (compare Fig. 2A, lane 5, and B, lane 5). This probe hybridized to a rearranged fragment found only in DNA from THy 42H11 (Fig. 2B, lane 3). Similarly, in the blot with the 5.3.18 V_α-specific oligonucleotide probe (Fig. 2C, lane 5), a single band was observed in the B6 liver lane, corresponding to the third strongly cross-hybridizing band seen in Fig. 2A, lane 5. Again, a single rearranged band was observed in the 5.3.18 DNA with the 5.3.18-specific oligonucleotide probe (Fig. 2C, lane 4). These results confirm that both V_α 42H11 and V_α 5.3.18 are distinct members of this B6 germ line V_α gene family.

Comparison of Tcr β-chain genes from functionally related THys. Our Tcr β-chain analysis began with the cloning and sequencing of a complete 42H11 β-chain cDNA. Northern blot analysis of a group of insulin-reactive B6- and bm12-restricted and autoreactive THys with a 429-bp *Xho*II-*Xho*II fragment derived from the 42H11 V_β region as a probe revealed a homologous Tcr mRNA from the 52H10F11 THy (data not shown). Both the 42H11 and 52H10F11 THys reacted specifically with the A chain of sheep and beef insulin. However, 42H11 is bm12 restricted, whereas the 52H10F11 clone is B6 restricted. Sequence analysis of full-length β-chain cDNA clones from 52H10F11 showed that they are derived from the same germ line V_β, D_β, and J_β gene segments as the 42H11 β-chain clone. Comparison of the nucleotide sequence and predicted amino acid sequence at the $V_\beta D_\beta J_\beta$ junction revealed a number of interesting features of the combinatorial process between these two clones. This comparison (Fig. 4A) also showed an unprecedented mechanism for generating diversity in Tcr β-chain genes; namely, a deletion of almost half of the 5′ end of the $J_\beta 7'$ (19) gene segment in the 52H10F11 Tcr β-chain gene.

A particularly striking feature of the 42H11 and 52H10F11 β-chain clones is the conserved use of N-region nucleotides at the $V_\beta J_\beta$ junction (Fig. 5A). The 42H11 gene contains four N-region nucleotides (GATA), whereas the 52H10F11 gene contans two (GA). This results in the introduction of an Asp at the VND junction of both β-chain genes. The D_β segments in both 42H11 and 52H10F11 (D_βN) introduce three contiguous amino acids that differ significantly in both clones. The Asn-to-Gly and the Trp-to-Asp substitutions involve changes in hydrophobicity and charge. The third substitution involves a Gly-to-Thr change which could also contribute to conformational changes in the formation of a Tcr-binding site. Of interest is the deletion of seven codons (21 bp; Fig. 4A) at the 5′ end of the J_β segment in the 52H10F11 gene. This deletion was observed in four independently isolated β-chain cDNA clones from 52H10F11, and an open reading frame was maintained despite this unusual event.

Comparison of β-chain genes from bm12- and B6-restricted THys with unrelated antigen specificities. We studied another pair of THys that utilize the identical V_β, D_β, and J_β germ line segments to construct a functional Tcr β-chain polypeptide. The β-chain cDNA from 5.3.18 was cloned and sequenced. Comparison with previously published Tcr β-chain sequences revealed an extremely close homology to the β chain of the B6-restricted, chicken erythrocyte-reactive cell line, LB2 (49). As observed in the previously discussed homologous β-chain pair (see above), we again see an example of the combinatorial process giving rise to an identical amino acid by insertion of N-region nucleotides. These nucleotide insertions result in an Ile codon at the VND junction in both 5.3.18 and LB2 (Fig. 4B). Though the

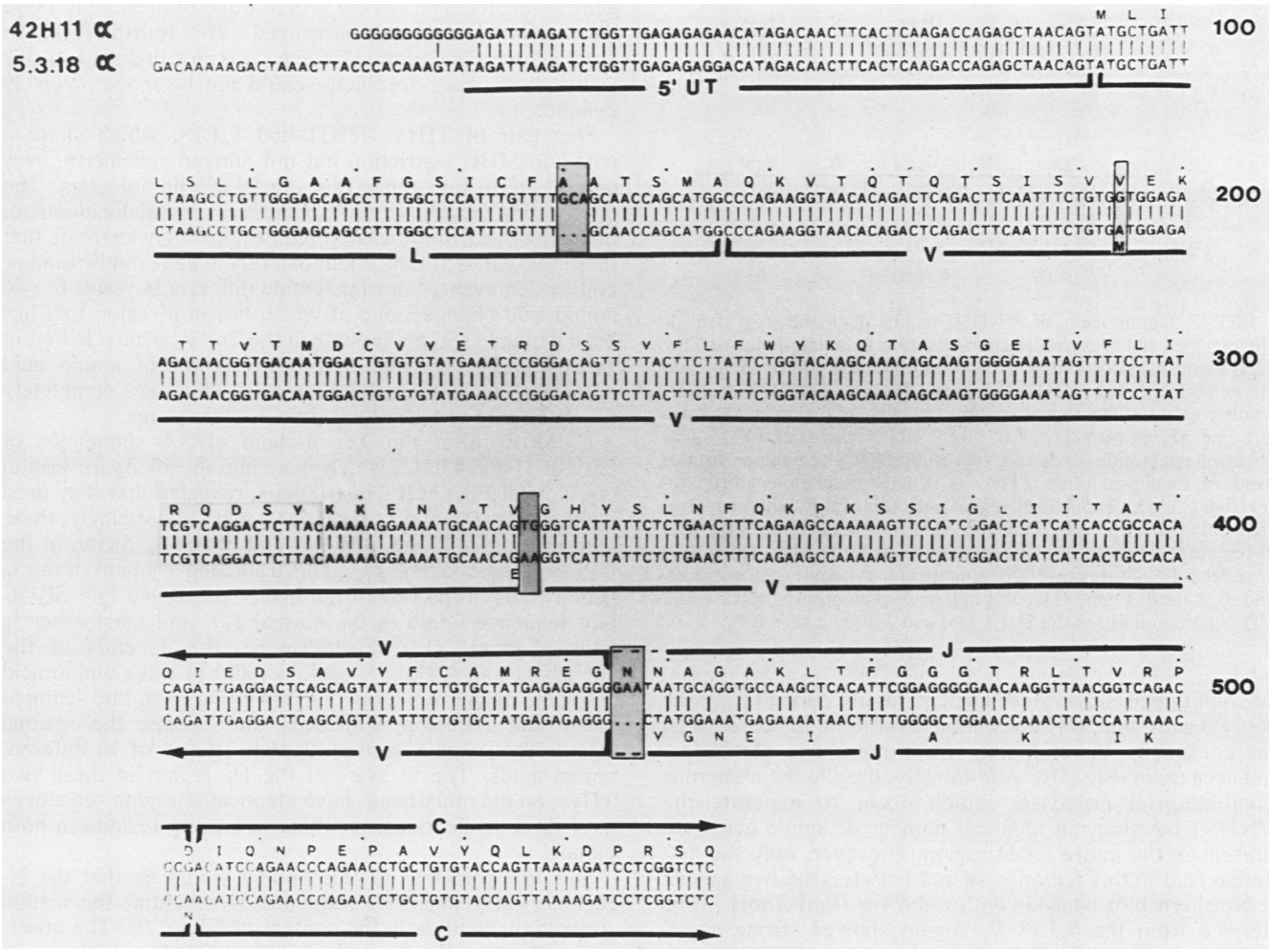

FIG. 3. Sequence comparison of the Tcr α-chain from insulin-reactive, bm12-restricted THy lines 42H11 and 5.3.18. Nucleotide sequence of 42H11 (top line) and 5.3.18 (bottom line). Shaded areas represent regions of diversity between the two clones. The leader peptide and V, J, and C regions are marked with arrowed lines. Amino acid position 1 in the V_α region has been assigned to the Gln (Q) residue at the leader-V_α junction. A potential site for N-linked glycosylation in both sequences is at amino acid positions 57 to 59 (see bp 326 to 334).

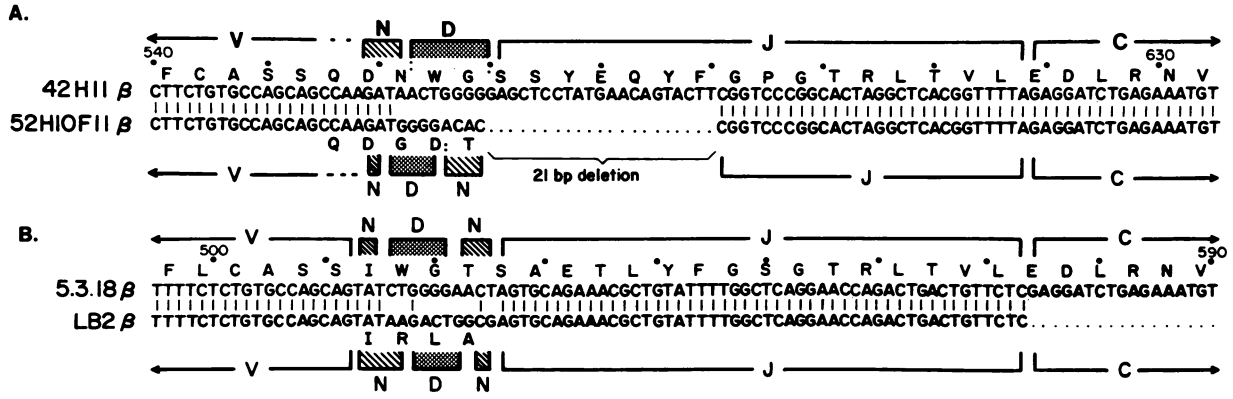

FIG. 4. (A) Sequence comparison of the Tcr β chain from insulin-reactive THy lines 42H11 and 52H10F11. Nucleotide sequence of 42H11 (top line) and 52H10F11 (bottom line). The V, J, and C regions are marked with arrowed lines. N-region nucleotides and the D segment in each gene are shown in brackets. The 21-bp deletion is noted in the 52H10F11 J_β segment. (B) Sequence comparison of the Tcr β chain from insulin-reactive THy 5.3.18 and chicken erythrocyte-reactive clone LB2. Nucleotide sequence of 5.3.18 (top line) and LB2 (bottom line). The V, J, and C regions are marked with arrowed lines. N-region nucleotides and the D-region gene segment are shown in brackets. The four amino acids making up the NDN region are shown in both genes.

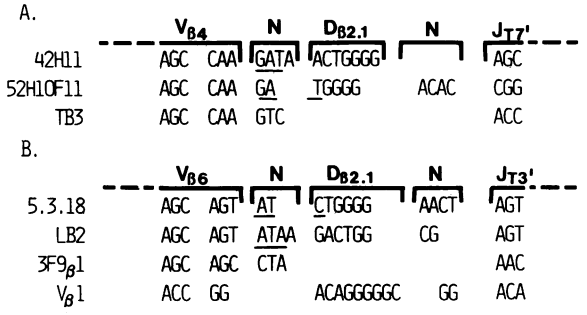

FIG. 5. Comparison of VNDNJ-region nucleotides of Tcr β-chain genes. (A) N-region nucleotides used in insulin-reactive THy 42H11 and 52H10F11 and from a B6 thymocyte-derived β-chain gene, TB3 (5). The 42H11 N-region GATA encodes an Asp (D) residue as does the recombination between N-region nucleotides GA and D segment TGGGG (GAT GGGG) in 52H10F11. TB3 N-region nucleotides encode a Val residue. (B) N-region nucleotides used in insulin-reactive THy 5.3.18 and chicken erythrocyte-reactive clone LB2. 5.3.18 N-region nucleotides AT combine with D segment CTGGGG (ATC TGGGG) to encode an Ile (I) residue. LB2 N-region nucleotides ATAA also encode an Ile residue. 3F9β1 (17) encodes a Leu at this position, contains no D-region gene segment, and uses the J_T1 gene (11). $V_\beta1$ has no N-region nucleotides at the VD junction and uses the $D_\beta1.1$ (55) and J_T1 gene segments.

possibility exists that these nucleotides are derived from the $V_\beta6$ (5) germ line, this does not appear to be the case based on consensus V_β endpoints from numerous cDNA sequences (5, 6) (Fig. 5B). Additionally, despite the numerous combinatorial processes which occur to generate the VNDNJ junction, an identical number of amino acids are present in the entire NDN region. However, only the first amino acid in this region is shared between the two genes.

Northern blot analysis with a 467-bp *Hha*I-*Xho*II probe derived from the 5.3.18 V_β region showed strong cross-reactivity with the bm12-autoreactive clone, 42F7 (data not shown). This result revealed that the Tcr β chains from these two THys are derived from the same V_β germ line gene. Since the specificity of the 42F7 autoreactive clone could not be precisely defined, however, no further analysis was performed.

DISCUSSION

We compared the Tcr structure of insulin-reactive THys that have clearly defined MHC restrictions. Special consideration was taken to analyze only THys that were derived from individual mice and were generated in separate fusions to avoid studying sublines of a given original T-helper line. In total, we analyzed 12 B6-derived THys (2 insulin A-chain specific, 9 insulin B-chain specific, and 1 autoreactive) and 9 bm12-derived THys (2 insulin A-chain specific, 5 insulin B-chain specific, 1 autoreactive, and 1 B6 and bm12 reactive).

Our analysis showed that insulin-specific T cells in B6 and bm12 mice use a diverse V gene repertoire. Although the Tcr gene pool is identical in B6 and bm12 mice and the Ia^b and Ia^{bm12} molecules are highly homologous, our structural findings complement functional studies (31, 36) to suggest that the unique Ia^{bm12} sequences are critical for the selection of the available self-reactive and antigen-specific T-cell repertoire, thus contributing to the genetic diversity observed. Despite the diverse V gene usage noted, we identified four insulin- and autoreactive THys that shared either V_α or V_β gene segments. The Tcr cDNAs of three of these four THys were then cloned and sequenced. The fourth THy, an autoreactive bm12-derived clone, was not included in this analysis, since its specificity could not be more precisely defined.

One pair of THys, 42H11 and 5.3.18, which share a common MHC restriction but not antigen specificity, uses two V_α germ line genes that are 98.6% homologous. The observation that these two V_α genes are essentially identical, including the 5′ untranslated regions, strongly suggests that these two genes are the result of a recent gene duplication or conversion event. Four nucleotide differences result in two amino acid changes, one of which lies in a region that has been defined as hypervariable in the Tcr V_α genes. Different germ line J_α segments that have significant amino acid homology, except for the 5′ regions which are completely divergent, are used by these two α-chain genes.

Comparison of the Tcr β-chain cDNA sequences of 52H10F11 and 42H11, which share fine specificity for insulin but have distinct MHC restrictions, revealed that they used identical V_β, D_β, and J_β germ line genes. Interestingly, there is a deletion of 21 bp at the 5′ end of the J_β region in the β-chain gene of 52H10F11. This truncated segment of the J_β gene results in the loss of the highly conserved Phe-Gly-X-Gly sequence found in the normal J_T7' and most other J_β gene elements (19). Furthermore, the 3′ ends of the 52H10F11 and 42H11 D_β regions differ at three amino acid residues, owing to various lengths of D_β germ line segment usage and N-region sequences that change the reading frame. This results in a contiguous stretch of 10 different amino acids. The 5′ sides of the D_β region in these two THys, on the other hand, have identical N-region sequences over three nucleotides, resulting in an Asp residue in both clones.

One interpretation of this finding would be that the N-region ($V_\beta D_\beta$ junction) sequence is essential for insulin A-chain recognition in the context of Ia^b-Ia^{bm12}. The observation that another B6-derived THy, TB3 (5), which has an unrelated antigen specificity but identical V_β gene segment, uses different N-region nucleotides is consistent with this idea (Fig. 5A). Precedence for this hypothesis comes from the findings in different immunoglobulin idiotype systems in which position 99 of the H chain (VD junction) is constant and specific for the antigen binding of the immunoglobulin molecule (8–10, 14, 20, 22, 35, 52, 57). A variant amino acid at that position has been shown, in two cases, to affect antigen binding, but not L-chain association or idiotype expression (54; T. Imanishi-Kari, unpublished observations). Our results must be interpreted with caution, however, as a second pair of clones, 5.3.18 and LB2, which use identical $V_\beta D_\beta J_\beta$ germ line genes, also has an identical amino acid encoded by N-region nucleotides at the $V_\beta D_\beta$ junction, although the rest of the D_β gene segment is in a different reading frame and encodes different amino acids. The 5.3.18 THy is specific for insulin B chain in the context of Ia^{bm12}, while LB2 is B6 derived and specific for chicken erythrocytes. Unfortunately, it is not possible to determine the exact antigen epitope in the multideterminant chicken erythrocyte antigen. If the N-region sequence is critical for antigen binding, the implication would be that the antigen epitope recognized by these two cell lines is similar. Finally, comparison of the N-region sequences of 5.3.18 and two other functionally unrelated BALB/c-derived T-cell clones that use the same V_β gene segment (Fig. 5B) showed usage of different N-region sequences. These clones show a polymorphism in the 3′ end of the V_β germ line sequences,

suggesting that the apparent use of conserved N-region nucleotides in 5.3.18 and LB2 stems from as yet unidentified germ line V_β or D_β gene elements.

Extensive diversity has not been noted in recent studies of Tcr V gene usage in hapten-specific cytotoxic T lymphocytes (29) and cytochrome *c*-reactive T-cell clones (18). In the former, approximately half of the *H-2K*^{b}-restricted hapten-specific cytotoxic T-cell clones studied used the the same V_β gene segment rearranged in each case to the same $D_\beta J_\beta C_\beta$ gene segment. In the latter study, all 16 B10.A-derived cytochrome *c*-specific T-cell clones expressed Tcr α chains encoded by the same V_α gene family and in association with β chains encoded by one of two different V_β genes. Furthermore, these V_α and V_β genes had rearranged to one or at most two different J regions. As these two studies (18, 29), in addition to our own, all report different degrees of common (or diverse) Tcr V gene usage, more extensive analysis must be undertaken in additional antigen-specific systems to determine the restriction (or lack thereof) placed on V gene usage for MHC-restricted T-cell recognition. However, an important point of agreement in the systems reported to date is that, as yet, a firm correlation cannot be made between V_α or V_β gene segments and recognition of a particular antigen or MHC molecule (21). This would imply that α and β chains do not independently form MHC or antigen-binding sites. Further analysis in the insulin system will be required to determine that within the diverse V gene usage, a particular V_α and V_β may correlate with MHC and antigen specificity, respectively.

Analysis of our cDNA clones does not provide evidence for somatic hypermutations in Tcr structural diversification. This mechanism, which is so effective in the generation of diversity and affinity maturation of immunoglobulin, therefore does not seem to play an analogous role in the generation of the diverse antigen-specific class II-restricted T-cell repertoire.

ACKNOWLEDGMENTS

We thank Susan Pagan and Dian L. Olson for expert technical assistance and Cindy Welch for careful preparation of this manuscript. We are grateful to S. Hedrick for providing us with the V_β BW5147 probe.

This work was supported by Public Health Service grants AI14910 and AI23031 from the National Institutes of Health, by a grant from the Cancer Research Institute (New York), and by Biogen N.V. D.H.S. is the recipient of a postdoctoral research fellowship from the Myron A. Bantrell Foundation, P.S.H. is the recipient of an Arthritis Investigator award and Public Health Service grant CA41539 from the National Institutes of Health and B.T.H. is the recipient of Research Career award AI00527 from the National Institutes of Health.

LITERATURE CITED

1. **Acuto, O., M. Fabbi, J. Smart, C. B. Poole, J. Protentis, H. D. Rogers, S. F. Schlossman, and E. L. Reinherz.** 1984. Purification and NH_2-terminal amino acid sequencing of the β subunit of the human T-cell antigen receptor. Proc. Natl. Acad. Sci. USA **81**:3851–3855.
2. **Acuto, O., S. C. Meuer, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz.** 1983. Peptide variability exists within α and β subunits of the T cell receptor for antigen. J. Exp. Med. **158**:1368–1373.
3. **Allison, J. P., B. W. McIntyre, and D. Bloch.** 1982. Tumor-specific antigen of murine T-lymphoma defined with a monoclonal antibody. J. Immunol. **129**:2293–2300.
4. **Arden, B., J. L. Klotz, G. Siu, and L. E. Hood.** 1985. Diversity and structure of genes of the α family of the mouse T-cell antigen receptor. Nature (London) **316**:783–787.
5. **Barth, R. K., B. S. Kim, N. C. Lan, T. Hunkapillar, N. Sobieck, A. Winoto, H. Gershenfeld, C. Okada, D. Hansburg, I. L. Weissman, and L. E. Hood.** 1985. The murine T-cell receptor uses a limited repertoire of expressed V_β gene segments. Nature (London) **316**:517–523.
6. **Behlke, M. A., D. G. Spinella, H. S. Chou, W. Sha, D. L. Hartl, and D. Y. Loh.** 1985. T-cell receptor β-chain expression: dependence on relatively few variable region genes. Science **229**:566–570.
7. **Ben-Nun, A., E. Choi, K. McIntyre, S. A. Leeman, D. J. McKean, J. G. Seidman, and L. H. Glimcher.** 1985. DNA-mediated transfer of major histocompatibility class II I-A^b and I-A^{bm12} genes into B lymphoma cells: molecular and functional analysis of introduced antigen. J. Immunol. **135**:1456–1464.
8. **Boersch-Supan, M. E., S. Agarwal, M. E. White-Scharf, and T. Imanishi-Kari.** 1985. Heavy chain variable region. Multiple gene segments encode anti-4-(hydroxy-3-nitrophenyl)acetyl idiotype antibodies. J. Exp. Med. **161**:1272–1292.
9. **Bothwell, A. L. M.** 1984. The genes encoding anti-NP antibodies in inbred strains of mice, p. 19–34. *In* M. I. Green and A. Nisonoff (ed.), The biology of idiotypes. Plenum Publishing Corp., New York.
10. **Bothwell, A. L. M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore.** 1981. Heavy chain variable region contribution to the NP^b family of antibodies; somatic mutation evident in a γ2a variable region. Cell **24**:625–637.
11. **Chien, Y., D. M. Becker, T. Lindsten, M. Okamura, D. I. Cohen, and M. M. Davis.** 1984. A third type of murine T-cell receptor gene. Nature (London) **312**:31–35.
12. **Chien, Y., N. R. J. Gascoigne, J. Kavaler, N. E. Lee, and M. M. Davis.** 1984. Somatic recombination in a murine T-cell receptor gene. Nature (London) **309**:322–326.
13. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
14. **Crews, S., J. Griffin, H. Huang, K. Calame, and L. Hood.** 1981. A single V_H gene segment encodes the immune response to phosphorylcholine: somatic mutation is correlated with the class of antibody. Cell **25**:59–66.
15. **Dembic, Z., W. Haas, S. Weiss, J. McCubrey, H. Kiefer, H. Von Boehmer, and M. Steinmetz.** 1986. Transfer of specificity by murine α and β T cell receptor genes. Nature (London) **320**: 232–238.
16. **Fabbi, M., O. Acuto, J. E. Smart, and E. L. Reinherz.** 1984. Homology of Ti subunit of a T-cell antigen-MHC receptor with immunoglobulin. Nature (London) **312**:269–271.
17. **Fabio, R., H. Acha-Orbea, H. Hengartner, R. Zinkernagel, and R. Joho.** 1985. Identical V_β T-cell receptor genes used in alloreactive cytotoxic and antigen plus I-A specific helper T cells. Nature (London) **315**:425–427.
18. **Fink, P. J., L. A. Matis, D. L. McElligott, M. Bookman, and S. M. Hedrick.** 1986. Correlations between T cell specificity and the structure of the antigen receptor. Nature (London) **321**:219–226.
19. **Gascoigne, N. R. J., Y. Chien, D. M. Becker, J. Kavaler, and M. M. Davis.** 1984. Genomic organization and sequence of T-cell β chain constant and joining region genes. Nature (London) **310**:387–391.
20. **Gearhart, P. J., N. D. Johnson, R. Douglas, and L. Hood.** 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. Nature (London) **291**:29–34.
21. **Goverman, J., K. Minard, N. Shastri, T. Hunkapillar, D. Hansburg, E. Sercarz, and L. Hood.** 1985. Rearranged β T cell receptor genes in a helper T cell clone specific for lysozyme: no correlation between Vβ and MHC restriction. Cell **40**:859–867.
22. **Gridley, T., M. N. Margolies, and M. L. Gefter.** 1985. The association of various D elements with a single immunoglobulin V_H gene segment: influence on the expression of a major cross-reactive idiotype. J. Immunol. **134**:1236–1244.
23. **Hannum, C. H., J. W. Kappler, I. S. Trowbridge, P. Marrack, and J. H. Freed.** 1984. Immunoglobulin-like nature of the α chain of a human T cell antigen/MHC receptor. Nature (London) **312**:65–67.
24. **Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P.**

Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. J. Exp. Med. **157**:1149–1169.

25. **Hayday, A. C., D. J. Diamond, G. Tanigawa, J. S. Heilig, V. Folsom, H. Saito, and S. Tonegawa.** 1985. Unusual organization and diversity of T-cell receptor α-chain genes. Nature (London) **316**:828–832.
26. **Hedrick, S. M., D. I. Cohen, E. A. Nielson, and M. M. Davis.** 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature (London) **308**:149–153.
27. **Hedrick, S. M., R. N. Germain, M. J. Bevan, M. Dorf, I. Engel, P. Fink, N. Gascoigne, E. Heber-Katz, J. Kapp, Y. Kaufmann, J. Kaye, F. Melchers, C. Pierce, R. Schwartz, C. Sorensen, M. Taniguchi, and M. M. Davis.** 1985. Rearrangement and transcription of a T-cell receptor β chain gene in different T-cell subsets. Proc. Natl. Acad. Sci. USA **82**:531–535.
28. **Hedrick, S. M., E. A. Nielson, J. Kavaler, D. I. Cohen, and M. M. Davis.** 1984. Sequence relationships bewteen putative T-cell receptor polypeptides and immunoglobulins. Nature (London) **308**:153–158.
29. **Hochgeschwender, U., H. U. Weltzien, K. Eichmann, R. B. Wallace, and J. T. Epplen.** 1986. Preferential expression of a defined T-cell receptor β-chain gene in hapten-specific cytotoxic T-cell clones. Nature (London) **322**:376–378.
30. **Hochman, P. S., and B. T. Huber.** 1984. A class II gene conversion event defines an antigen specific Ir gene epitope. J. Exp. Med. **160**:1925–1930.
31. **Hochman, P. S., and B. T. Huber.** 1984. Immune recognition of insulin by H-2^b mice: the mutation in the I-A^b_β gene of the B6.C-*H*-2^{bm12} mouse alters the self-I-A restricted T cell repertoire. Eur. J. Immunol. **14**:610–615.
32. **Hochman, P. S., G. Widera, R. A. Flavell, and B. T. Huber.** 1985. A gene conversion event in the I-A^b_β gene alters the self I-A restricted T cell repertoire of B6.C-*H*-2^{bm12} mice, p. 81–95. *In* H. Vogel and B. Pernis (ed.), The cell biology of the MHC. Academic Press, Inc., New York.
33. **Hood, L., M. Kronenberg, and T. Hunkapillar.** 1985. T cell antigen receptors and the immunoglobulin supergene family. Cell **40**:225–229.
34. **Huber, B. T., and P. S. Hochman.** 1984. B cell activation potential of insulin-reactive T cells in H-2^b mice. Eur. J. Immunol. **14**:1106–1110.
35. **Kaartinen, M., G. M. Griffiths, A. F. Markham, and C. Milstein.** 1983. mRNA sequences define an unusually restricted IgG response to 2-phenyloxazolone and its early diversification. Nature (London) **304**:320–324.
36. **Kanamori, S., W. D. Walsh, T. H. Hansen, and H. Y. Tse.** 1984. Assessment of antigen-specific restriction sites of Ia molecules as defined by the bm12 mutation. J. Immunol. **133**:2811–2814.
37. **Kappler, J., R. Kubo, K. Haskins, C. Hannum, P. Marrack, M. Pigeon, B. McIntyre, J. Allison, and I. Trowbridge.** 1983. The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. Cell **35**:295–302.
38. **Kappler, J., R. Kubo, K. Haskins, J. White, and P. Marrack.** 1983. The mouse T cell receptor: comparison of MHC receptors on two T cell hybridomas. Cell **34**:727–737.
39. **Kavaler, J., M. M. Davis, and Y. Chien.** 1984. Localization of a T-cell receptor diversity-region element. Nature (London) **310**: 421–423.
40. **Malissen, M., K. Minard, S. Mjolsness, M. Kronenberg, J. Goverman, T. Hunkapiller, M. B. Prystowsky, Y. Yoshikai, F. Fitch, T. W. Mak, and L. Hood.** 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the β polypeptide. Cell **37**:1101–1110.
41. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **McIntyre, B. W., and J. P. Allison.** 1983. The mouse T cell receptor: structure heterogeneity of molecules of normal T cells defined by xenoantiserum. Cell **34**:739–746.
43. **McIntyre, K. R., and J. G. Seidman.** 1984. Nucleotide sequence of the mutant I-A^{bm12}_β gene provides evidence for genetic exchange between mouse immune response genes. Nature (London) **308**:551–553.
44. **Mengle-Gaw, L., S. Connor, H. O. McDevitt, and C. G. Fathman.** 1984. Gene conversion between class II major histocompatibility complex loci. Functional and molecular evidence for the bm12 mutant. J. Exp. Med. **160**:1184–1194.
45. **Mengle-Gaw, L., and H. O. McDevitt.** 1983. Isolation and characterization of a cDNA clone for the murine I-E_β polypeptide chain. Proc. Natl. Acad. Sci. USA **80**:7621–7625.
46. **Meuer, S. C., O. Acuto, R. E. Hussey, J. C. Hodgdon, K. A. Fitzgerald, S. F. Schlossman, and E. L. Reinherz.** 1983. Evidence for the T_3-associated 90KD heterodimer as the T-cell antigen receptor. Nature (London) **303**:808–810.
47. **Meuer, S. C., K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz.** 1983. Clonotypic structures involved in antigen specific human T cell function. Relationship to the T_3 molecular complex. J. Exp. Med. **157**:705–719.
48. **Okayama, H., and P. Berg.** 1982. High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. **2**:161–170.
49. **Patten, P., T. Yokota, J. Rothbard, Y. Chien, K. Arai, and M. M. Davis.** 1984. Structure, expression and divergence of T-cell receptor β-chain variable regions. Nature (London) **312**:40–46.
50. **Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa.** 1984. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. Nature (London) **312**: 36–40.
51. **Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa.** 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequence. Nature (London) **309**:757–762.
52. **Schilling, J., B. Clevinger, J. M. Davie, and L. Hood.** 1980. Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangements in heavy chain V-region gene segments. Nature (London) **283**:35–40.
53. **Schwartz, R. H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3**:237–261.
54. **Sharon, J., M. Gefter, T. Manser, and M. Ptashne.** 1986. Site-directed mutagenesis of an invariant amino acid residue at the variable-diversity segments junction of an antibody. Proc. Natl. Acad. Sci. USA **83**:2628–2631.
55. **Siu, G., S. P. Clark, Y. Yoshikai, M. Malissen, Y. Yanagi, E. Strauss, T. W. Mak, and L. Hood.** 1984. The human T cell antigen receptor is encoded by variable, diversity, and joining gene segments that rearrange to generate a complete V gene. Cell **37**:393–401.
56. **Siu, G., M. Kronenberg, E. Strauss, R. Haars, T. W. Mak, and L. E. Hood.** 1984. The structure, rearrangement and expression of D_β gene segments of the murine T-cell antigen receptor. Nature (London) **311**:344–350.
57. **Slaughter, C. A., and J. D. Capra.** 1983. Amino acid sequence diversity within the family of antibodies bearing the major anti-arsonate cross-reactive idiotype of the A strain mouse. J. Exp. Med. **158**:1615–1634.
58. **Widera, G., and R. A. Flavell.** 1984. The nucleotide sequence of the murine I-E^b_β immune response gene: evidence for gene conversion events in class II genes of the major histocompatibility complex. EMBO J. **3**:1221–1225.
59. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82**:1585–1588.
60. **Yanagi, Y., Y. Yoshikai, K. Leggett, S. P. Clark, I. Alexander, and T. W. Mak.** 1984. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature (London) **308**:145–152.