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Immunology

T-cell receptor variable region gene usage in T-cell populations

(major histocompatibility complex restriction/ribonuclease protection assay)

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ABSTRACT **We have examined T-cell receptor α- and β-chain variable (V) region gene usage in T-cell populations predicted to have different major histocompatibility complex-restriction specificities. Using a sensitive ribonuclease protection assay to measure T-cell receptor mRNA levels, we found no striking differences in the usage of three V_α genes and three V_β genes in T-cell populations from three congeneic *H-2*-disparate strains of mice and between the mutually exclusive Ly2$^+$ L3T4$^-$ and Ly2$^-$ L3T4$^+$ T-cell subpopulations. These results suggest that major histocompatibility complex restriction cannot be explained by the differential usage of nonoverlapping V_α or V_β gene pools. In contrast, striking but unpredictable differences were seen in *V* gene usage in populations of T cells selected by activation with particular alloantigens.**

The recognition of foreign antigens by T lymphocytes occurs only when the antigen is presented by cells that express appropriate major histocompatibility complex gene products (MHC restriction; refs. 1–4). Most helper and cytotoxic T lymphocytes (CTL) recognize antigens in the "context" of class II or class I MHC determinants, respectively, and appear to be developmentally influenced toward recognition of antigens in the context of the products of the particular MHC alleles encountered in the thymus during T-cell differentiation ("thymus education"; refs. 5–7). The α and β chains of the antigen receptor on T cells have recently been isolated and characterized (8–10). cDNA clones encoding the α (11, 12) and the β (13–15) chains have been isolated, and the genomic organization of these genes has been determined (16–18). As in the heavy and light chain immunoglobulin genes (19), the genes encoding the α and β chains of the T-cell receptor are composed of discrete variable (*V*), joining (*J*), and constant (*C*) region germ line segments that rearrange during development to form a functional transcription unit.

In the present study we asked whether known differences in the MHC specificity of various T-cell populations could be correlated with differential usage of several *V* region genes. If a particular germ line V_α or V_β gene segment alone is responsible for recognizing MHC determinants, one might expect that certain of these segments would be preferentially used in certain T-cell populations. Using a sensitive ribonuclease protection assay (20, 21), we found no major differences in the levels of mRNAs corresponding to three different V_α and three different V_β genes in total RNA from the Ly2$^+$ L3T4$^-$ (largely class I restricted) and the Ly2$^-$ L3T4$^+$ (largely class II restricted) T-cell subpopulations, suggesting that MHC restriction cannot be explained by selective usage of nonoverlapping subsets of either V_α or V_β gene segments. We reached a similar conclusion concerning the usage of these *V* genes by T cells from three *H-2*-disparate congeneic mouse strains, which are expected to differ in MHC specificity due to thymus education. In contrast, large differences in *V* gene usage were seen in populations of T cells selected by stimulation with particular MHC alloantigens. However, no simple correlation between the *V* gene used and the stimulatory alloantigen could be made. Taken together, the data suggest that neither the germ line V_α segment nor the germ line V_β segment alone determines the restriction specificity of a particular T-cell receptor.

MATERIALS AND METHODS

Animals, Cell Lines, and Culture Medium. All mice used were bred and maintained in the Center for Cancer Research Animal Facility, M.I.T. Cells were cultured *in vitro* in RPMI 1640 medium containing either 5% or 10% fetal calf serum. The alloreactive CTL clone 2c (BALB.B anti-L^d) was kindly provided by D. Kranz and H. Eisen, M.I.T.

Preparation of T-Cell Populations. T cells were purified by treatment of lymph node cells with anti-Ia (BP107 and/or 14.4.4) monoclonal antibodies plus complement (22, 23). In some cases, these cells were cultured for 6 days in the presence of concanavalin A (Con A) at 2 μg/ml and 50% mouse Con A supernatant before purification (see figure legends). Some of these purified T cells (>90% pure) were further treated with anti-L3T4 (GK1.5) or anti-Ly2 ([AD4(15) plus 3.168.8] monoclonal antibodies plus complement (24, 25) to prepare Ly2$^+$ and L3T4$^+$ subpopulations (>95% pure), respectively. Some of the Ly2$^+$ T cells were cultured for 7 days with recombinant interleukin 2 at 10 units/ml (Amgen Biologicals, Thousand Oaks, CA) in the presence of 1% (vol/vol) Sepharose bead-bound anti-T-cell receptor antibody (F23.1) coupled at 2 mg of protein per ml of bead suspension (26). F23.1 ascites fluid was kindly provided by M. Bevan and U. Staerz, Scripps Clinic and Research Foundation, La Jolla, CA. Ly2$^+$ T cells from one-way mixed lymphocyte cultures (MLC) were prepared by using spleen cells from BALB.B (*H-2^b*), BALB/c AnN (*H-2^d*), and BALB.K (*H-2^k*) mice. Responder spleen cells were cultured in all six one-way combinations with the appropriate γ-irradiated (3000 rads; 1 rad = 0.01 Gy) stimulator spleen cells (1:1). The cells were restimulated on day 10 (responder/stimulator = 1:5). On day 14, Ly2$^+$ cells were prepared as described above; these cell preparations were 80–90% Ly2$^+$. After all antibody plus complement treatments, viable cells were isolated using Ficoll/Isopaque. In all cases cell populations were prepared from cells pooled from the spleens or lymph nodes from 10–50 mice to control for variation among mice.

Molecular Cloning and DNA Sequence Analysis. cDNA was prepared from poly(A)$^+$ RNA (27, 28) obtained from BALB/c AnN spleen cells cultured for 24 hr in the presence of Con A at 2 μg/ml, and a cDNA library was constructed in the λgt11 vector by using standard methods (29). The library was screened with ^{32}P-labeled, nick-translated C_α or C_β

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Abbreviations: MHC, major histocompatibility complex; V, J, C, variable, joining, constant regions of T-cell receptor genes; MLC, mixed lymphocyte culture; CTL, cytotoxic T lymphocytes.

(pHDS58 or pHDS11; refs. 12 and 15) cDNA as described (30). Clones positive in two further screenings were considered positive for α- or β-chain cDNA. DNA sequences were determined from overlapping restriction fragments of several of these clones by using the chemical cleavage method of Maxam and Gilbert (31) or by subcloning restriction fragments into Gemini plasmids and sequencing both strands using SP6 or T7 promoter primers in dideoxy chain-termination analysis as described by Sanger *et al.* (32) according to the manufacturer's protocol (Promega Biotec, Madison, WI).

RNA Preparation and Ribonuclease Protection Analysis. Total cellular RNA was isolated from cell populations using the guanidinium/cesium chloride method (33). RNase protection analysis was performed as described (21) with minor modifications. Restriction fragments containing T cell receptor V, J, and/or C region cDNA sequences were subcloned in SP6 or Gemini plasmids (Promega Biotec) and ^{32}P-labeled RNA probes were prepared by transcription of linearized plasmids. Full-length probes were eluted from 6% polyacrylamide/7 M urea sequencing gels by incubating gel slices at 37°C for 4 hr in 0.5 M ammonium acetate/1% NaDodSO$_4$/3 mM EDTA containing 20 μg of yeast soluble RNA per ml as carrier. Hybridization was carried out at 35°C for 12 hr and followed by treatment with RNase at 14°C for 30 min.

RESULTS

V_α and V_β cDNA Clones. For these analyses, three V_α and three V_β region genes were used; two have been described previously (12, 15) and four additional *V*-region genes (11α, 41α, 3β, 12β) were cloned in this laboratory. The V_α11 gene is a member of the V_α1 subfamily described by Arden *et al.* (34) and is 98% homologous at the nucleotide level with their V_α1.2 sequence. The V_α41 gene is identical over the region of the published V_α4.2 sequence (34). The cDNA clone from which the V_α41 gene was obtained does not contain a J segment but is in a productive translational reading frame with the downstream C region sequences. A splicing event deleting the J segment could have occurred if a 5′ splice site occurred near the *V–J* junction of the particular rearranged gene from which the mRNA was transcribed. Ribonuclease analysis of RNA from T-cell populations with a V_α41-C probe (Fig. 1*c*) revealed the presence of a protected fragment corresponding to a V_α41-C$_\alpha$ transcript. Since it is possible that the protected V–C fragment results from looping out of the J segment from mRNA molecules containing a J segment, the relative abundance of V_α41-C$_\alpha$ transcripts lacking a J segment cannot be determined from the present data. The V_β3 gene is identical with the V_β1 sequence reported by Behlke *et al.* (36) and with the V_β6 sequence published by Barth *et al.* (37). The V_β12 cDNA clone did not extend to the 5′ end but was identical with the 3′ half of the V_β8.2 gene of Barth *et al.* (37) and the V_β4 sequence of Behlke *et al.* (36).

Measurement of mRNA Levels Using RNA Probes Containing V, J, and C Sequences. We measured α- and β-chain mRNA levels in total RNA using a sensitive ribonuclease protection assay (20, 21). In some experiments separate V

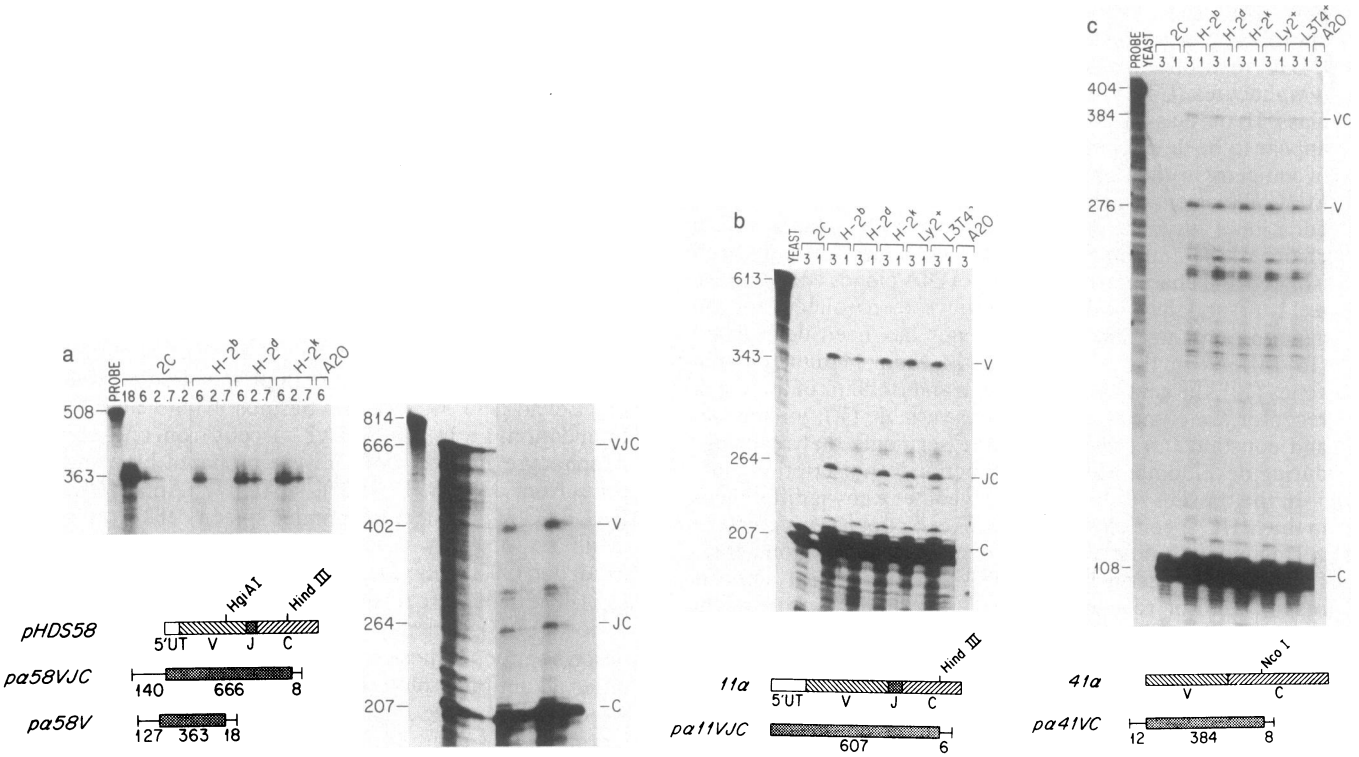

FIG. 1. Ribonuclease protection analysis of α-chain mRNA levels in T cells from *H-2*-disparate congeneic mice and in T-cell subpopulations. Four probes were used: pα58V and pα58VJC (*a*), pα11VJC (*b*), and pα41VC (*c*). The indicated amounts (0.2–18 μg) of total cellular RNA were analyzed; samples containing less RNA than the highest amount indicated for each experiment were supplemented to that level with yeast tRNA. 2C, RNA from the CTL clone 2C; H-2^b, H-2^d, and H-2^k, RNA from purified lymph node T cells from BALB.B, BALB/c AnN, and BALB.K mice, respectively; Ly2$^+$, RNA from BALB/c AnN Ly2$^+$ lymph node T cells; L3T4$^+$, RNA from BALB/c AnN L3T4$^+$ lymph node T cells; A20, RNA from the A20 B-lymphoma cell line of BALB/c origin. A20 and yeast-soluble RNA served as negative controls. The lengths of the probes derived from the cDNAs are indicated in the diagrams (blacked in) as well as the length of plasmid sequences included in the transcripts (flanking lines). Regions of the probes corresponding to variable (V), joining (J), and constant (C) sequences are indicated. Undigested probe and ^{32}P-labeled markers were run on each gel; digestion of the plasmid-derived sequences confirms the specificity of the assay. In the case of the V_α58 data, an α-constant region probe (α58C, ref. 35) was used as a control for the level of α mRNA in the test samples in a parallel experiment (data not shown). For the V_α58 and V_α41 data, purified T cells were cultured for 6 days in the presence of Con A at 2 μg/ml and 50% mouse Con A supernatant before purification. Numbers on the left of the gels and below the probe schematics represent lengths of RNA fragments (no. of nucleotides).

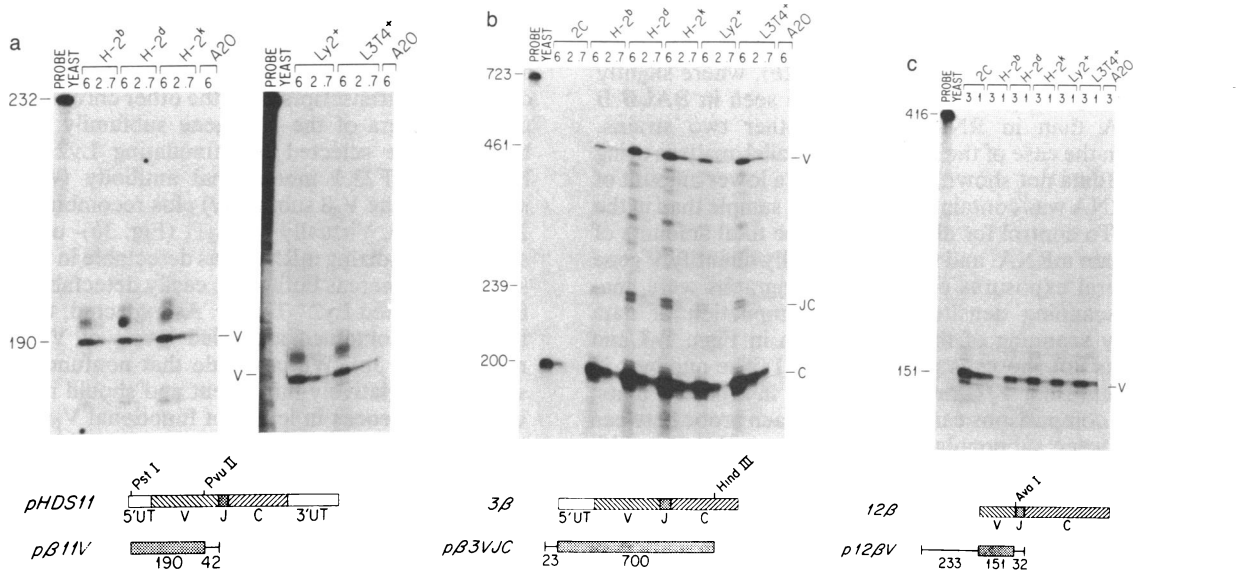

FIG. 2. Ribonuclease protection analysis of β-chain mRNA levels in T cells from *H-2*-disparate congeneic mice and in T-cell subpopulations. Three probes were used: pβ11V (*a*), pβ3VJC (*b*), and pβ12V (*c*). The same RNA samples as in Fig. 1 were analyzed. In the case of the V_β11 data, a C_β11 probe was used as a control for the level of β-chain mRNA in the test samples (data not shown). As shown, RNA from the 2C cell line we used does not hybridize with the V_β11 probe but does hybridize with the V_β12 probe [containing the 3'-most half of a V_β gene in the V_β8 (38) subfamily], suggesting that this cell line uses a V_β gene in the V_β8 subfamily. For the 3β and 12β data, purified T cells were cultured for 6 days in the presence of Con A at 2 μg/ml and 50% mouse Con A supernatant before purification. Numbers on the left of the gels and below the probes represent lengths of RNA fragments (no. of nucleotides).

and C probes were used to determine the level of α- or β-mRNA in the test sample. In most experiments we used probes containing contiguous V–J–C sequences for the analyses. Ribonuclease protection analysis with V–J–C probes allows determination of the ratio of transcripts containing a particular V region segment to transcripts containing C region segments in the same lane of a gel in an internally controlled fashion. For example, as shown in Fig. 1*a Right*, ribonuclease protection analysis using the V–J–C probe constructed from cDNA clone pHDS58 resulted in at least four bands in the lanes corresponding to RNA from T-cell populations. The band labeled "C" corresponds to a fragment of the probe protected from ribonuclease digestion by hybridization with transcripts containing the C_α sequence but not the J_α58 sequence. The band labeled "JC" corresponds to C_α-containing transcripts that also contain the J_α58 sequence, and the band labeled "V" corresponds to V_α58-containing transcripts. Presumably because of the extensive diversity of the nucleotide sequences at V–J junctions, a full-length V–J–C fragment is not detectably protected by RNA from heterogeneous populations of T cells, but it is an abundant protected fragment when RNA from the 2C cell line is similarly analyzed; the cDNA clone pHDS58 was isolated from the 2C cell line. The strong band(s) seen between the V and JC bands with this and some other probes presumably corresponds to transcripts containing *V* sequences related to but distinct from the *V* segment of the probe—i.e., a *V* gene of the same subfamily. Some of the minor bands in this region of the gels are due to radiolysis of the protected *V* fragment. Note that the proportion of full-length C-containing or JC-containing transcripts cannot be ascertained by ribonuclease protection analysis. However, RNA blot analysis using a C_β probe demonstrated that the ratio of V-region containing transcripts (1.3 kilobases) to transcripts without V regions (1.0 kilobases) was approximately the same in all of our RNA preparations (data not shown).

Table 1. Relative levels of V_α and V_β transcripts in various T-cell total RNA preparations as measured by the ribonuclease protection assay

	V_α			V_β		
	11	41	58	3	11	12
Strain						
H-2^b	8.14	2.97	9.10	2.45	10.36	3.69
H-2^d	5.93	3.37	6.08	3.15	6.23	3.46
H-2^k	6.13	4.59	9.05	5.52	5.32	6.18
Subpopulation						
Ly2$^+$/L3T4$^-$	7.39	4.48	7.50	6.54	2.83	7.61
Ly2$^-$/L3T4$^+$	4.89	4.65	8.70	4.56	1.98	3.40
MLC Ly2$^+$						
b anti-d	11.58	3.83	4.22	6.82	10.33	3.46
b anti-k	3.39	2.66	3.91	3.32	3.93	12.30
d anti-b	7.12	3.12	6.02	9.44	14.53	5.91
d anti-k	32.26	7.70	5.03	12.43	15.76	5.27
k anti-b	8.10	3.95	6.96	11.47	12.43	3.77
k anti-d	17.83	5.37	10.48	18.02	11.85	5.10

Several exposures of autoradiographs from ribonuclease protection assays were analyzed by scanning densitometry. Relative levels of V transcripts (in arbitrary units) were obtained by normalizing each test preparation for C_α- or C_β-hybridizing mRNA.

V_α and V_β Gene Usage in Congeneic *H-2*-Disparate Mice and in T-Cell Subpopulations. In our experiments, we studied the usage of V_α and V_β gene segments in peripheral T cells from three *H-2*-disparate congeneic strains of mice that have the same *V*-region gene pools from which to draw. Since the MHC region among these animals differs, the T cells in each strain learn to recognize antigen in the context of different MHC products. We also chose to study *V* gene usage in two mutually exclusive peripheral T-cell subpopulations (Ly2$^+$ L3T4$^-$ and Ly2$^-$ L3T4$^+$) that in general have specificity for class I or class II MHC molecules, respectively (24). If a particular V_α or V_β gene segment alone is responsible for recognizing MHC determinants, one might expect that certain of these segments would be preferentially used in certain T-cell populations. Figs. 1 and 2 show results of ribonuclease protection analysis of total RNA from various T-cell populations using V_α or V_β hybridization probes; visual inspection of the autoradiographs suggests that there are no striking

differences in the usage of any of the V_α or V_β gene segments. The largest apparent differences are in the usage of the $V_\alpha 58$ gene (Fig. 1*a*) and of the $V_\beta 3$ gene (Fig. 2*b*), where slightly lower amounts of hybridizing mRNA are seen in BALB.B T-cell RNA than in RNA from the other two strains. However, in the case of the $V_\alpha 58$ data, parallel analysis using a C_α probe (data not shown) revealed that a lower amount of α-chain mRNA was contained in this RNA sample than in the other two. To control for differences in the total amounts of α- and β-chain mRNA, and to more carefully quantify *V* gene usage, several exposures of the autoradiographs were analyzed by scanning densitometry. A compilation of data obtained by scanning of the films shown in Figs. 1–3 and several films not shown is given in Table 1. The numbers in this table represent *V/C* ratios expressed in arbitrary units; meaningful comparisons can be made for each probe between strains, between subpopulations, and between MLC combinations. The levels of usage of particular *V* gene segments are quite similar between strains and between subpopulations; the largest differences detected were slightly more than 2-fold. For example, the $V_\beta 12$ gene is used slightly more than twice as frequently in $Ly2^+$ $L3T4^-$ T cells than in $Ly2^-$ $L3T4^+$ T cells, a result in agreement with data previously published by Roehm *et al.* (38). Those authors showed that the monoclonal antibody KJ16-133, which reacts with an epitope determined by a small subfamily of V_β genes (39), of which $V_\beta 12$ is a member, binds to nearly twice as many $Ly2^+$ T cells as $L3T4^+$ T cells. This result provides independent confirmation of the modest difference in $V_\beta 12$ expression in these two populations of T cells and suggests that quantitation of V-region mRNA in cell populations can accurately predict the proportion of cells that functionally express this *V*-region gene segment. Thus, the differences in *V* gene usage on the whole are small when comparing cell populations predicted to have different MHC-restriction specificities but random antigen specificities.

V_α and V_β Gene Usage in Activated T-Cell Populations. One potential problem with the interpretation of the results in Figs. 1 and 2 is the possibility of the existence of nonfunctional, full-length transcripts containing V, J, and C sequences. cDNAs with out-of-frame joining of the V and J segments have been described (34), although the majority of reported sequences are in frame. If the percentage of these nonfunctional transcripts in T-cell populations were high enough, the detection of different levels of functional transcripts would be masked. To address this issue, we attempted to find situations in which striking differences in *V* gene usage could be demonstrated in populations of T cells. In one approach, we asked whether T cells that functionally express a particular V_β gene also transcribe other V_β genes (e.g., out-of-frame transcripts from the other chromosome). T cells using members of the $V_\beta 8$ gene subfamily to which $V_\beta 12$ belongs were selected by stimulating $Ly2^+$ T cells with bead-bound F23.1 monoclonal antibody (which binds to members of the $V_\beta 8$ subfamily) plus recombinant interleukin 2 (26, 39, 40). Virtually no $V_\beta 11$ (Fig. 3*b*)- or $V_\beta 3$ (data not shown)-hybridizing mRNA was detectable in total RNA from such cells, whereas both were easily detectable in total RNA from unselected $Ly2^+$ T cells. As expected, total RNA from these cells contained amplified levels of $V_\beta 12$-hybridizing mRNA (Fig. 3*c*). We conclude that nonfunctional V_β transcripts are relatively infrequent and should not mask detection of differences in levels of functional V_β mRNAs.

Marked differences in the expression of V_α and V_β genes can also be detected in populations of T cells selected by stimulation in culture with particular alloantigens. Two representative experiments are shown in Fig. 3; data from experiments with all six probes are given in Table 1. The results show that up to 10-fold differences can be detected in the usage of particular *V*-region genes in T cells selected for reactivity with a particular alloantigen. For example, $V_\alpha 11$ is used nearly 10 times more frequently by $Ly2^+$ T cells from an H-2^d anti-H-2^k MLC than from an H-2^b anti-H-2^k MLC. In addition, certain MLC combinations (for example, H-2^d anti-H-2^k) appear to use a V_α gene segment related to, but different from, $V_\alpha 58$ (i.e., probably a member of the same V_α subfamily) at a high frequency (Fig. 3*a*), suggested by the presence of a strong band slightly above the JC band. Therefore, striking differences in V_α as well as V_β gene usage can be detected in populations of T cells, strengthening the data presented in Figs. 1 and 2. Interestingly, however, there is no obvious correlation between *V* gene usage and alloantigen specificity (Table 1).

DISCUSSION

Recently, it has been shown that two helper T-cell clones with different antigen and class II restriction specificities use the same V_β gene segment (41). In another study, it was shown that the same V_β gene is used in a class I-restricted CTL clone and in a class II-restricted helper T-cell clone (42). In addition, use of monoclonal antibodies directed against V_β gene products has shown in both the human (43) and the mouse (38) that V_β gene usage cannot be correlated with

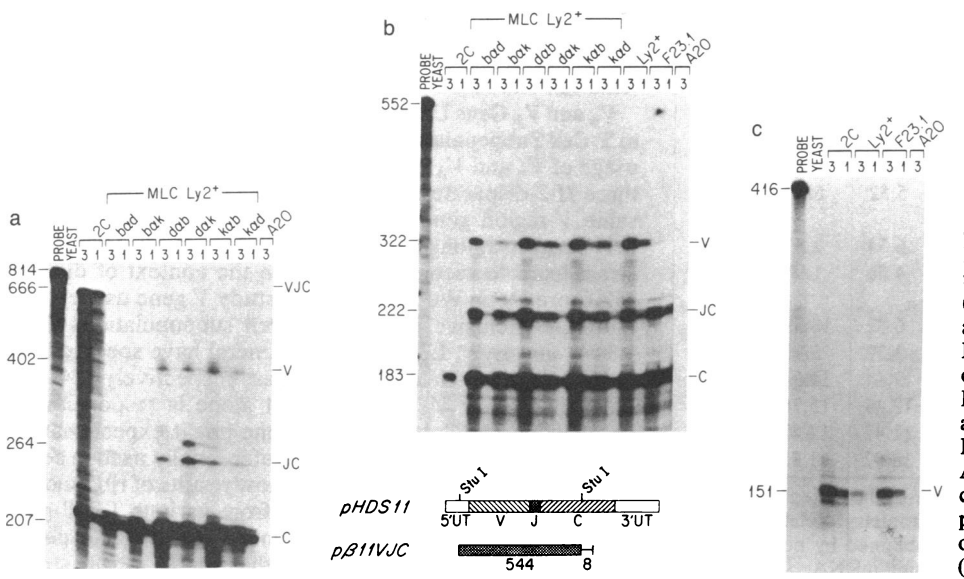

FIG. 3. Ribonuclease protection analysis of α- and β-chain mRNA levels in $Ly2^+$ T cells derived from allogeneic MLC or stimulated with anti-T-cell receptor antibody. Three probes were used: pα58VJC (*a*), pβ11VJC (*b*), and pβ12V (*c*). Total cellular RNA was analyzed as in Fig. 1. 2C, RNA from the CTL clone 2C; MLC $Ly2^+$ cells, RNA from $Ly2^+$ cells derived from one-way MLC using BALB.B (*H-2^b*), BALB/c AnN (*H-2^d*), and BALB.K (*H-2^k*) spleen cells as responders and stimulators; $Ly2^+$, RNA from BALB/c AnN $Ly2^+$ splenic T cells; F23.1, RNA from BALB/c AnN $Ly2^+$ T cells activated with bead-bound anti-T-cell receptor monoclonal antibody F23.1 and recombinant interleukin 2; A20, RNA from the A20 B-lymphoma cell line. In the case of the $V_\beta 12$ data, a C_β probe was used as a control for the level of β-chain mRNA in the test samples (data not shown).

phenotype or function, although approximately twice as many $Ly2^+$ T cells appeared to react with an antibody directed against gene products of a particular V_β gene subfamily than did $L3T4^+$ T cells. These experiments left open the possibility that V_α could be responsible for MHC restriction specificity. However, our data using three different V_α and three different V_β probes show that no striking differences can be seen in either V_α or V_β gene usage in situations where different MHC specificities are predicted. In addition, our data extend these observations to populations of T cells rather than to a few individual T-cell clones. These results argue that MHC restriction cannot be explained by the differential usage of nonoverlapping *V* gene pools of either the α-chain or the β-chain locus.

If MHC recognition is determined by the product of the α-chain or the β-chain gene individually, combinatorial diversity provided by other regions of the α or the β chain, such as the D, J, or N regions and possible junctional diversity may be critical in forming the MHC-binding site. We acknowledge the possibility that study of the usage of additional *V* genes may reveal instances of highly biased *V* gene expression in MHC-restricted populations with random antigen specificities. However, our results argue that this would not be a general phenomenon. Alternatively, the combining site(s) for both MHC products and antigen may be created by the complex interaction of complete α- and β-chain variable region protein sequences. The possible role in MHC restriction of a third T-cell receptor-like gene called γ remains to be determined (15, 35).

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