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Immunology

Isolation of cDNA clones encoding a T-cell receptor β-chain from a beef insulin-specific hybridoma

(gene rearrangement/antigen recognition/amino acid sequence)

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ABSTRACT **cDNA clones coding for a T-cell receptor β-chain were isolated from a beef insulin/IA^d-reactive T-cell hybridoma, A20.2.15, and its complete amino acid sequence was deduced. This β-chain gene utilizes the same V_β segment as a thymocyte β-chain gene (86T1) and rearranges to the 5′ proximal *J–C* locus ($J_{\beta1}$–$C_{\beta1}$), thus providing definitive evidence of a β-chain gene from a functional hybridoma that utilizes $C_{\beta1}$. The amino acid sequence of the V_β gene in A20.2.15 is identical to 86T1, thus suggesting the absence of somatic mutation in the β-chain of A20.2.15. Southern blot analysis revealed a somatic DNA rearrangement unique to the A20.2.15 hybridoma. The expression of this gene in the hybridoma was confirmed by RNA dot hybridization. All 24 β-chain clones so far isolated from the A20.2.15 hybridoma contained $C_{\beta1}$, suggesting that the β-chain gene of the fusion partner BW5147 is not expressed in the hybridoma.**

The discovery of major histocompatibility complex (MHC) restriction led to the concept that foreign antigens must be seen by the T-cell receptor in the context of the Ir gene product. Helper T cells use Ia antigens (class II molecules of MHC) as restriction elements, whereas cytotoxic T lymphocytes are restricted to H-2K and D antigens (class I molecules of MHC). Recent success in generating anti-T-cell receptor antibodies has led to the identification of the T-cell receptor. These clonotypic antibodies were found to immunoprecipitate similar disulfide-bonded heterodimers with an intact molecular mass of ≈85 kDa and subunit molecular masses of 40–50 kDa (1, 2).

Hedrick *et al.* (3), using B-cell-subtracted cDNA libraries from T-cell hybridomas, were able to identify a gene that rearranged in T cells, was expressed only in T cells, and had variable (*V*) and constant (*C*) regions. At the same time a gene from a human T-cell line was identified that was expressed in T cells but not in B cells (4). Sequence analysis of these genes demonstrated that the murine and human analogues of the same T-cell receptor gene had been identified. Acuto *et al.* (5) obtained the NH_2-terminal sequence of the β-chain of the T-cell receptor and it corresponded exactly to the sequence reported by Yanagi *et al.* (4), thus strongly suggesting that the gene encoding the β-chain of the T-cell receptor had been identified. Analysis of the sequence components of the rearranged murine T-cell receptor β-chain by Chien *et al.* (6) indicated that the germ-line V_β, D_β, and J_β gene segments rearrange in T lymphocytes to form the complete V_β genes. These rearrangements are apparently mediated by recognition signals similar to those of immunoglobulin gene segments and in the β-chain gene are located 3′ of V_β gene segments, 5′ of J_β gene segments, and on both sides of the D_β gene segments. In this communication we report the sequence of the β-chain of the T-cell receptor obtained from a beef insulin-specific IA^d-restricted T-cell hybridoma, A20.2.15. The A20.2.15 β-chain cDNA sequence is encoded by 86T1 V_β, $J_{\beta1.1}$, and $C_{\beta1}$ elements. There is no evidence for somatic mutation in the 86T1 V_β used by A20.2.15.

MATERIALS AND METHODS

T-Cell Hybridomas. T-cell hybridomas were prepared as described (7). Briefly (BALB–B6)F_1 T-cell blasts specific for beef insulin were fused with BW5147, and hybridomas were screened by the antigen/IA-dependent interleukin 2 (IL-2) assay (8). Hybridomas of interest were subcloned at least twice by limiting dilution. One of these T hybridomas, A20.2.15, shown to be specific for beef insulin and restricted to IA^d (unpublished data), was used in the present experiments.

Preparation of cDNA Library from A20.2.15. Total RNA was prepared from A20.2.15 cells by the guanidinium, isothiocyanate procedure (9) and poly(A)$^+$ RNA was separated by oligo(dT)-cellulose chromatography (10). The first strand of cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase (11) and was converted to the double-stranded form by using the large fragment of *Escherichia coli* DNA polymerase I (12) or by incubating successively with reverse transcriptase (2 hr) and the large fragment of *E. coli* DNA polymerase I (3 hr) (13). The double-stranded cDNA was treated with S1 nuclease to remove the hairpin structure and oligo(dC) tracts were added to the 3′ ends by using calf thymus terminal deoxynucleotide transferase (14). The oligo(dC)-tailed DNA was hybridized to *Pst* I-digested, oligo(dG)-tailed plasmid pBR322, and the resultant recombinant plasmids were used to transform *E. coli* strain DH-1 (15). Transformed *E. coli* cells were screened by *in situ* colony hybridization (16) by using a ^{32}P-labeled mouse T-cell receptor β-chain cDNA clone, RBL5-17 (17), kindly provided by T. Mak.

DNA Sequence Analysis. Nucleotide sequences were analyzed by the Maxam–Gilbert procedure (18) and by the dideoxy chain-termination procedure (19) using M13 mp8 and mp9 phage vectors (20).

Southern Blot and RNA Blot Analysis. Southern blot hybridization and RNA blot hybridization were performed according to Southern (21) and Thomas (22), respectively, with some modifications. The hybridization mixture contained 50 mM Tris·HCl (pH 8.0), 1 M NaCl, 10 mM EDTA, 0.1% $NaDodSO_4$, 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 100 μg of salmon sperm DNA per ml, 50 μg of yeast tRNA per ml, and 0.5–5 × 10^7 cpm of

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Abbreviations: MHC, major histocompatibility complex; IL-2, interleukin 2; V, variable; D, diversity; J, joining; C, constant; bp, base pairs; kb, kilobase(s).

^{32}P-labeled probe per ml. After the hybridization at 65°C for 15–20 hr, the filters were washed with 15 mM NaCl/1.5 mM sodium citrate and 0.1% $NaDodSO_4$ for 90 min at 65°C. RNA dot hybridization was carried out using total RNA denatured with formaldehyde, according to White and Bancroft (23), in the presence of 125 μg of polyadenylic acid per ml in the hybridization mixture described above.

RESULTS

Nucleotide Sequence of A20.2.15 β1 and A20.2.15 β2. The screening of 110,000 independent transformants by *in situ* colony hybridization provided 24 clones that hybridized to the *Pst* I insert of RBL5-17, a mouse T-cell receptor β-chain cDNA (17). Two clones, A20.2.15 β1 and A20.2.15 β2, contained cDNA inserts larger than 1000 base pairs (bp). Analysis of these clones with restriction endonucleases showed that they were very similar except that digestion with *Pst* I released one fragment from A20.2.15 β1, but released two from A20.2.15 β2 (Fig. 1). A20.2.15 β2 contained an insert that was longer than that of A20.2.15 β1 by 74 nucleotides. Its nucleotide sequence was similar to that of A20.2.15 β1 except for the 5′-most 265 nucleotides. On reversing this portion of the DNA, A20.2.15 β2 became identical with A20.2.15 β1 in the nucleotide sequence except at position 280. Base pairing between 5′ AGACGG 3′, at position 254–259, and 5′ CCGTCT 3′, at position 6–11, could form a hairpin loop. Subsequent nicking would lead to the sequence inversion seen in A20.2.15 β2. The occurrence of nucleotide sequence rearrangements in double-stranded cDNA synthesis has been reported (27–30). In addition, A20.2.15 β2 contained a base substitution 15 nucleotides downstream of the rearrangement point, probably caused by a mismatch error that has been reported to occur in the reaction catalyzed by avian myeloblastosis virus reverse transcriptase (31) and *E. coli* DNA polymerase I (32). We conclude that A20.2.15 β1 and A20.2.15 β2 represent the same mRNA sequence.

Organization of Functional Segments. Fig. 2 shows the nucleotide sequence of A20.2.15 β1 with the extra 74 nucleotides of A20.2.15 β2 added to the 5′ end. The amino acid sequence deduced from the nucleotide sequence shows that the first 114 amino acid residues are identical to those of 86T1, a T-cell receptor β-chain cDNA isolated from a mouse thymocyte cDNA library (3). This segment is believed to represent the leader sequence and the *V* region of the β-chain (V_β). Two silent base substitutions were detected at nucleotides 48 and 121, which may reflect the different strains of mice used [BALB/c for 86T1, $(BALB/c–B6)F_1$ for this experiment]. The precise assignment of the *V–D* join is not possible because the germ-line sequence for the 86T1 V_β gene is not available. Since $D_{\beta1.1}$ (33) has been identified as the *D* region used in 86T1 (34), it was possible to identify seven nucleotides, TGGGGAG, in the A20.2.15 cDNA sequence that could be encoded by a germ-line *D* region. This is consistent with the high guanosine content reported for the two *D* regions identified (33, 34). The A20.2.15 *D* gene either could be an unidentified *D* gene present 5′ of $D_{\beta1.1}$ or could be encoded by $D_{\beta1.1}$, with one 5′ nucleotide and two 3′ nucleotides inserted by an N-region insertion mechanism as part of the joining process (35).

The J_β coding region for A20.2.15 mRNA was assigned to nucleotides 373–418 and was identical with $J_{\beta1.1}$, the 5′-most *J* segment situated upstream of the $C_{\beta1}$ gene (24). The nucleotide sequence from position 419 to 936 was identical with that of the germ-line $C_{\beta1}$ coding segment (24). Nucleotide 625 in A20.2.15 differs from that in 86T1, resulting in a difference in the amino acid residue at 200—tyrosine in A20.2.15 and histidine in 86T1. The $C_{\beta1}$ sequence of A20.2.15 is followed by a termination codon, TGA, and a 219-bp 3′ noncoding sequence. Two nucleotides at positions 942 and 1103 in the 3′ noncoding sequence were found to be different from those in the germ-line sequence (24). The AATAAA hexanucleotide starts at nucleotide 1131. Even though this is the only typical poly(A)-addition signal in this region, polyadenylylation of mRNA apparently can occur at two different sites. As shown in Fig. 2, A20.2.15 β2 has a poly(A) tail attached at the cytidine residue at position 1149, whereas A20.2.15 β1 has a poly(A) tail attached at the guanine residue 6 nucleotides further downstream. This may be due to the low

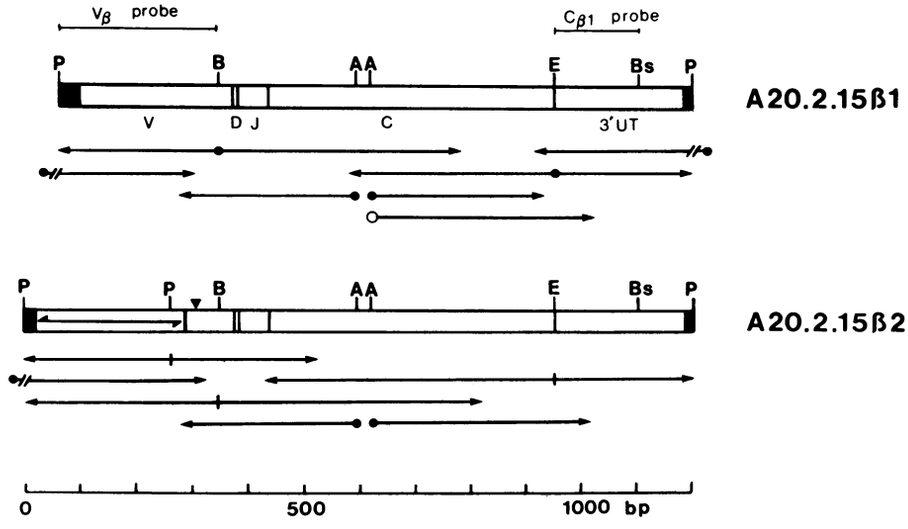

FIG. 1. Restriction endonuclease maps of A20.2.15 T-cell receptor β-chain cDNA clones and the sequencing strategies. Open bars and closed bars represent the β-chain cDNA inserts and poly(G)·poly(C) tracts, respectively. The arrow with an open circle and arrows with closed circles indicate the nucleotides determined by the Maxam–Gilbert procedures (24) by labeling the 3′ end and the 5′ end of the fragment, respectively. Arrows with vertical bars represent the nucleotides determined by the dideoxy chain-termination procedure (25) using M13 phage vectors (26). The parts of the mRNA that code for the *V*, diversity (*D*), joining (*J*), and *C* regions and 3′ untranslated region (3′ UT) are indicated below the map of A20.2.15 β1. The regions from where V_β probe (*Pst* I/*Bam*HI fragment) and $C_{\beta1}$ probe (*Eco*RI/*Bst*NI fragment) were derived are shown above the map of A20.2.15 β1. A closed triangle (▼) indicates the position of base substitution in A20.2.15 β2. Only those restriction endonuclease sites used in the experiments are presented. A, *Ava* II; B, *Bam*HI; Bs, *Bst*NI; E, *Eco*RI; P, *Pst* I; ⇀↽, DNA inversion.

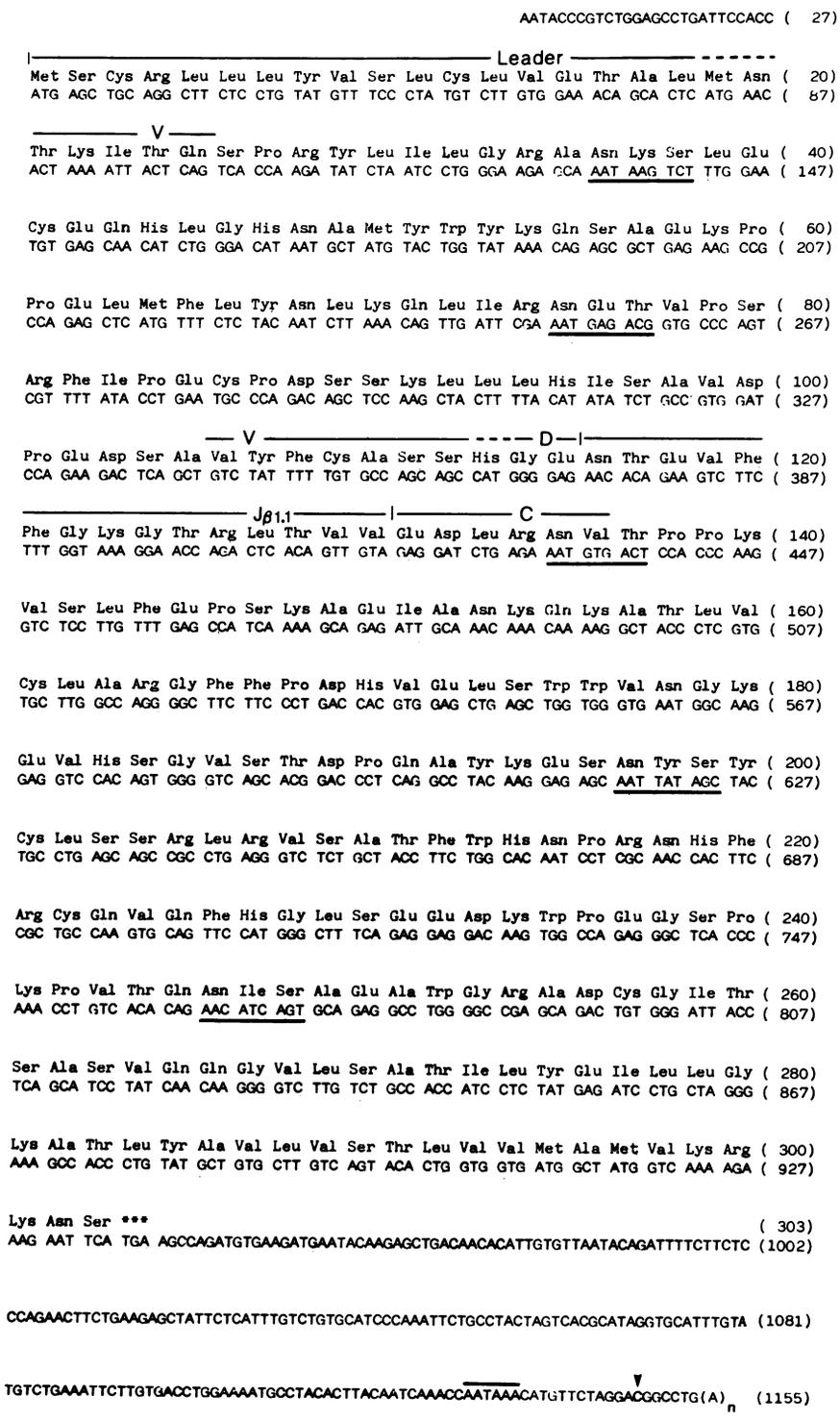

FIG. 2. The nucleotide sequence of T-cell receptor β-chain mRNA from A20.2.15 hybridoma and the amino acid sequence deduced from the nucleotide sequence. The poly(A) signal, AATAAA, is overlined. The arrowhead (▼) indicates the cytosine residue to which a poly(A) tract is attached in A20.2.15 β2. Five potential N-glycosylation sites in the polypeptide chain are underlined.

specificity of the enzymes involved in the polyadenylylation reaction. For example, a sequence similar to the authentic poly(A) signal, such as AATCAAA situated 9 nucleotides upstream of AATAAA, may be recognized. Alternatively, the secondary structure surrounding the poly(A) site may affect the selection of the poly(A) site. The functional significance of the two poly(A) sites in this β-chain mRNA is not apparent.

Expression of the A20.2.15 T-Cell Receptor β-Chain Gene. To confirm that the isolated T-cell receptor β-chain gene is expressed in A20.2.15 hybridoma, we performed blot hybridization analysis of RNA with the total insert of A20.2.15 β1 as a probe; a 1.3-kilobase (kb) band was detected in RNA preparations from both A20.2.15 and BW5147 (Fig. 3). Dot hybridization analysis using the V_β segment as a probe (see Fig. 1) also showed positive hybridization with both A20.2.15 and BW5147 RNAs (Fig. 4*A*). On the other hand, when the 3′ noncoding sequence of the A20.2.15 β1 was used as a probe, only A20.2.15 RNA showed hybridization (Fig. 4*B*). This result is consistent with the observation that the *V*

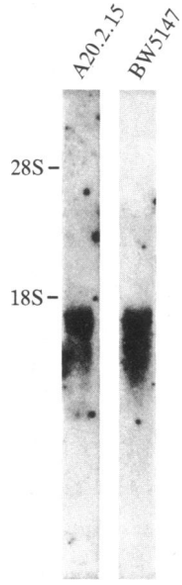

FIG. 3. RNA blot analysis of RNA from A20.2.15 hybridoma and BW5147. Total RNA (20 μg) was electrophoresed on a 1.4% agarose gel, transferred to a nitrocellulose filter, and hybridized with the ^{32}P-labeled *Pst* I insert from A20.2.15 β1. The positions of 18S and 28S rRNAs run in the neighboring slot as size markers are indicated.

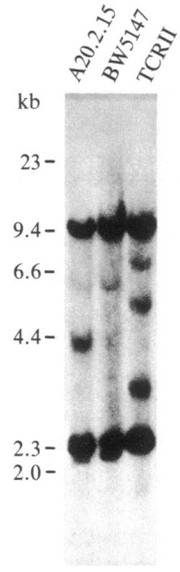

FIG. 5. Southern blot analysis of genomic DNA homologous to the A20.2.15 β1 insert. Total genomic DNA (20 μg) was digested with *Eco*RI endonuclease, separated on a 1% agarose gel, and subjected to Southern blot hybridization using the ^{32}P-labeled *Pst* I insert from A20.2.15 β1 as a probe. The positions of *Hin*dIII-digested phage λ DNA used as size markers are indicated on the left.

segment of the β-chain gene of the T-cell receptor of BW5147 is the same as that of 86T1, but the C_β segment is different (24). The 3′ noncoding sequence of $C_{\beta 2}$ is sufficiently different from that of $C_{\beta 1}$, so that the $C_{\beta 1}$ 3′ noncoding sequence probe hybridizes only to $C_{\beta 1}$. These results demonstrate that the cloned β-chain sequence is expressed in A20.2.15 but not in the parental cell line BW5147. DNAs of all 24 β-chain clones isolated from the hybridomas based on the positive hybridization with the total insert of RBL5-17 were analyzed for their ability to hybridize with the $C_{\beta 1}$-specific (3′ noncoding) probe. The β-chain gene of BW5147 containing $C_{\beta 2}$ should not hybridize with this probe. It was found that all 24 DNA preparations hybridized to the $C_{\beta 1}$ probe (data not shown). It is, therefore, unlikely that the β-chain gene of BW5147 is expressed in the hybridoma.

DNA Rearrangement in the β-Chain Gene. Fig. 5 shows the result of Southern blot analysis of DNAs from A20.2.15, BW5147, and TCRII (a B-cell hybridoma) that were digested with *Eco*RI and hybridized with the total insert of A20.2.15 β1. DNA from both A20.2.15 and BW5147 showed hybridization patterns different from that of TCRII, suggesting DNA rearrangements of β-chain genes in these cell lines. The 2.3-kb and 3.3-kb fragments in the *Eco*RI digest of TCRII have been seen in the *Eco*RI digests of liver DNA from BALB.B, BALB/c, B10A, AKR, and C57BL/6 mice (24–26, 33). The lack of the 3.3-kb band in the DNA from A20.2.15 and BW5147 suggests DNA rearrangements involving the region between $C_{\beta 1}$ and $J_{\beta 2}$, probably a partial $D_{\beta 2}$ to $J_{\beta 2}$ rearrangement. The β-chain gene in BW5147 has been reported to be rearranged at the $J_{\beta 2}$ region in both alleles (36). The A20.2.15 DNA showed a 4.2-kb hybridizable band, which is absent in BW5147, indicating the occurrence of a DNA rearrangement unique to the donor T cell.

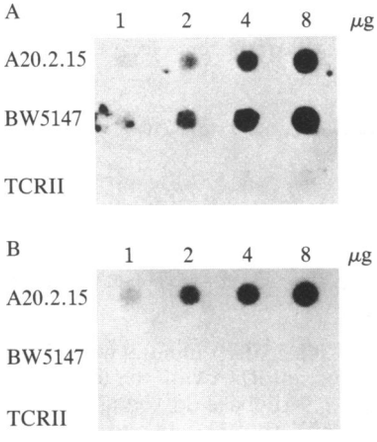

FIG. 4. Dot hybridization analysis of expression of the V_β and C_β regions in A20.2.15 hybridoma and BW5147. The indicated amount of total RNA was applied at each spot on nitrocellulose filters and hybridized with a ^{32}P-labeled V_β probe (*A*) or $C_{\beta 1}$ probe (*B*) prepared from A20.2.15 β1 (see Fig. 1). Total RNA from TCRII (a B-cell hybridoma) was included as a control.

DISCUSSION

The germ-line configuration for the β-chain of the T-cell receptor consists of separate *V*, *D*, *J*, and *C* region segments (6). The two *C* genes in the β-chain cluster differ by only four amino acids, all in the carboxyl-terminal region (24). The gene segments are arranged in a tandem sequence of *J–C/J–C* within a 15-kb region (24). The two clusters of *J* regions each contain seven distinct elements (6, 24).

The A20.2.15 β transcript is derived from the 5′-most locus ($J_{\beta 1}$–$C_{\beta 1}$). Although cDNA clones in the spleen and thymus appear to rearrange equally well to either $C_{\beta 1}$ (5′ locus) or $C_{\beta 2}$ (3′ locus) (3, 37), all of the published cDNA sequences derived from functional T-cell lines and hybridomas are derived from the $C_{\beta 2}$ locus (6, 26, 37). Expression of $C_{\beta 1}$ in the A20.2.15 cDNA sequence suggests that some mature antigen-reactive T cells do use $C_{\beta 1}$, and the $C_{\beta 2}$ preference seen earlier may have been due to the sample size and/or the antigen specificity of the T cells screened (6, 26, 37). For instance, the antigen specificity of the T cell could secondarily create a $C_{\beta 2}$ bias by selecting for a particular *J* segment associated with $C_{\beta 2}$.

Our demonstration of $C_{\beta 1}$ expression in A20.2.15 in contrast to the use of $C_{\beta 2}$ in 2B4, both being Ia-restricted IL-2-secreting hybridomas, suggested that the two C_β genes do not show T-cell subset-specific expression. This is in agreement with earlier results (37, 38).

The V_β gene in the A20.2.15 transcript is the 86T1 V_β gene earlier identified in a thymocyte library. The A20.2.15 V_β amino acid sequence is identical to the published sequence of the 86T1 V_β gene (3), thus suggesting the absence of somatic mutation in A20.2.15 at the V_β locus. This conclusion is in agreement with the earlier results on the 2B4 hybridoma (6).

The mRNAs expressed in both the fusion partner BW5147 and A20.2.15 are 1.3 kb in length, which corresponds to the products of *V*, *D*, and *J* rearrangements (36, 38). We have also shown that both mRNAs were transcribed from the same 86T1 V_β gene (3), but the C_β segment is different. The β-chain gene of the parental cell line (BW5147) was not expressed in this hybridoma. These observations are at variance with those of Chien *et al.* (34), who reported the isolation of two types of β-chain cDNA clones from the 2B4 hybridoma: one specific to the 2B4 hybridoma and the other derived from the fusion partner BW5147. A possible explanation could be the loss of the BW5147 β-gene chromosome in A20.2.15. This is consistent with the observed frequent loss of chromosomes in T-cell hybridomas.

Patten *et al.* (37) investigated the relative contributions of five *V* regions to the T-cell receptor repertoire by screening a BALB/c thymocyte library. Two kinds of frequencies were obtained. Four *V* regions—86T1, C5 (anti-dinitrophenylated/ovalbumin/IA^b), EI (anti-trinitrophenyl/IA^d), and LB2 (anti-chicken erythrocyte/H-2^b)—were expressed at a high frequency. The 2B4 *V* region (anti-pigeon cytochrome *c*/IE^k) was not detectably expressed in the thymocyte library. Since cytochrome *c* is a weak antigen, whereas the other three antigens (trinitrophenyl, chicken erythrocyte, and dinitrophenyl) are antigens with high-background reactivity, it is possible that the high frequency of expression could be a reflection of evolutionary selection for high expression in the thymus. Our demonstration of the 86T1 V_β gene in A20.2.15, a beef insulin/IA^d-reactive hybridoma, would seem to exclude such a straightforward explanation, since beef insulin is a weak antigen and this *V* region would not be selected in evolution for reactivity with beef insulin. An alternative explanation could implicate D_β/J_β and/or the α-chain with antigen recognition. The latter explanation is consistent with the observation that the same V_β gene is expressed by T cells with completely different antigen specificities (37, 39).

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