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The β_1 domain of the mouse E_β chain is important for restricted antigen presentation to helper T-cell hybridomas

(class II major histocompatibility antigens/hybrid gene/exon shuffling/protoplast fusion)

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ABSTRACT **We have constructed a hybrid E_β gene by replacing the second exon of the E^d_β gene (which encodes the majority of the β_1 protein domain) with the corresponding exon from the E^b_β gene. The hybrid gene has been introduced into a *d* haplotype host, the lymphoma β-cell line A20-2J, and an $E_\alpha E_\beta$ dimer composed of the endogenous E^d_α chain and the product of the hybrid E^d_β/E^b_β gene was immunoprecipitated from extracts of transfected cells with an $E^d_\alpha E^b_\beta$-specific monoclonal antibody. Transfected cells have acquired the ability to present antigen to $E^d_\alpha E^b_\beta$-restricted helper T-cell hybridomas, indicating that the second exon of the gene for the E_β chain encodes sequences required for the restricted recognition of the antigen-presenting cell by the class II-restricted responder T-cell.**

The class II major histocompatibility antigens are polymorphic glycoproteins expressed on the surface of antigen-presenting B lymphocytes and macrophages (1, 2). The function of class II molecules is to direct the interaction of an antigen-presenting cell with a class II-restricted effector T cell. Thus, a class II-restricted T cell recognizes antigen only when it is presented on the surface of a cell that expresses the appropriate allelic form of a class II molecule.

In the mouse, two class II molecules, $A_\alpha A_\beta$ and $E_\alpha E_\beta$, have been defined. The genes encoding the α and β chains of these molecules have been cloned, and the E_α, E_β, and A_β genes have been well characterized (3–6). Although the gene for the E_α chain differs from the genes for the β chains in organizational detail, each gene is divided into exons that essentially correspond to the putative structural domains of the encoded protein chain. In the E_β gene, the first exon encodes the polypeptide leader sequence and the first five amino acids of the most external (β_1) protein domain. The second exon encodes the 90 amino acids that form the remainder of the β_1 domain, and the third exon encodes the 95 amino acids of the β_2 domain. The transmembrane and cytoplasmic peptides and the 3′ untranslated region are encoded by the fourth, fifth, and sixth exons (6).

Although the available sequence information allows general predictions about the structure–function relationship of class II molecules, the precise identification of functionally important regions requires more direct analysis. Such analyses may be achieved best through the use of defined DNA transfection systems and *in vitro* antigen presentation systems. We have recently reported the functional and inducible expression of a cloned E_β gene after DNA-mediated gene transfer (7), and several other groups have reported the functional expression of transfected A_β or A_α and A_β genes (8–10). We found that the BALB/c lymphoma B-cell line A20-2J acquired the ability to present antigen to $E^d_\alpha E^b_\beta$-restricted T-cell hybridomas following transfection with the cloned E^b_β gene. We have utilized the "exon shuffling" approach to identify a region of the E^b_β gene that is functionally important in the restriction of antigen presentation. Results presented here indicate that the second exon of the E^b_β gene encodes all of the determinants that are required for the class II-restricted presentation of antigen (by the *d* haplotype host) to three $E^d_\alpha E^b_\beta$-restricted T-cell hybridomas.

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MATERIALS AND METHODS

The BALB/c lymphoma B-cell line A20-2J was previously designated L10A.2J but has been reidentified as a derivative of the A20 tumor (11). The A20-2J line presents various antigens (in culture) to antigen-specific $E^d_\alpha E^d_\beta$- and $A^d_\alpha A^d_\beta$-restricted helper T cells and helper T-cell hybridomas. The helper T-cell hybridomas used in this study recognize antigen in the context of $E^d_\alpha E^b_\beta$ as described (7, 12). BDK-38.2 is specific for keyhole limpet hemocyanin, whereas BDO-23.2 and BDO-34.1 are specific for chicken ovalbumin. The A20-E^b_β-1 and A20-E^b_β-5 cell lines were derived from A20-2J after transfection with a plasmid containing a full-length copy of the E^b_β gene (7). Both cell lines contain a high copy number of the transfected E^b_β gene, and both lines present antigen to $E^d_\alpha E^b_\beta$-restricted T cell hybridomas.

The pSV2gpt plasmid vector contains the bacterial *Ecogpt* gene, which can be used as a dominant selectable marker in eukaryotic cells (13). This vector was used in construction of a hybrid E_β gene as described in the legend to Fig. 1. The hybrid gene construct was then introduced into recipient A20-2J cells by spheroplast fusion as described (14). After fusion, cells were plated in 96-well plates in normal growth medium (2×10^3 cells per well) and allowed to recover for 72 hr. Selective medium containing xanthine at 250 μg/ml (Sigma), hypoxanthine at 15 μg/ml (Sigma), and mycophenolic acid at 0.5 μg/ml (Eli Lilly) was then added. Plates were refed with selective medium every 3 days, and colonies were picked for expansion after 2 weeks. Preparation of high molecular weight DNA (from A20-2J and transfected cell lines) was by standard methods (15), and agarose gel blotting and hybridization was as described by Southern (16).

The Y-17 monoclonal antibody used in this study recognizes a variety of E molecules including $E^d_\alpha E^b_\beta$ but not $E^d_\alpha E^d_\beta$ (17). Y-17 culture supernatant was used for immunoprecipitation of [^{35}S]methionine-labeled cell extracts as described by Jones (18), and immunoprecipitates were analyzed by two-dimensional protein gel electrophoresis as described by O'Farrell *et al.* (19).

A20-2J and transfected derivative cell lines were tested for their ability to present antigen as described (20). Briefly, 1×10^5 presenting cells were cultured with 1×10^5 hybridoma T cells (± 1 mg of antigen per ml) for 24 hr in 250 μl of culture medium. Interleukin 2 production by the T-cell hybridoma was assayed by the ability of the culture supernatant to support growth of the interleukin 2-dependent cell line HT-2. Units of interleukin 2 are as defined (20).

RESULTS

A hybrid E_β gene was constructed in the pSV2gpt plasmid from cloned E^d_β and E^b_β genes. The resulting hybrid gene is essentially a *d* haplotype gene in which a 0.7-kb *Eco*RI fragment has been deleted from the first intron, and a 1.9-kb *Eco*RI fragment containing the second exon has been replaced by a corresponding 2.1-kb *Eco*RI from the E^b_β gene (Fig. 1). Thus, the pSV2-E^d_β/E^b_β plasmid contains an E_β gene that is predicted to encode an E_β molecule where the majority of the β_1 domain is *b* haplotype and the remainder of the molecule is *d* haplotype. The pSV2-E^d_β/E^b_β plasmid transformed A20-2J cells to mycophenolic acid resistance at a frequency of about 1×10^{-4}, and several individual transfected colonies were selected and expanded for analysis.

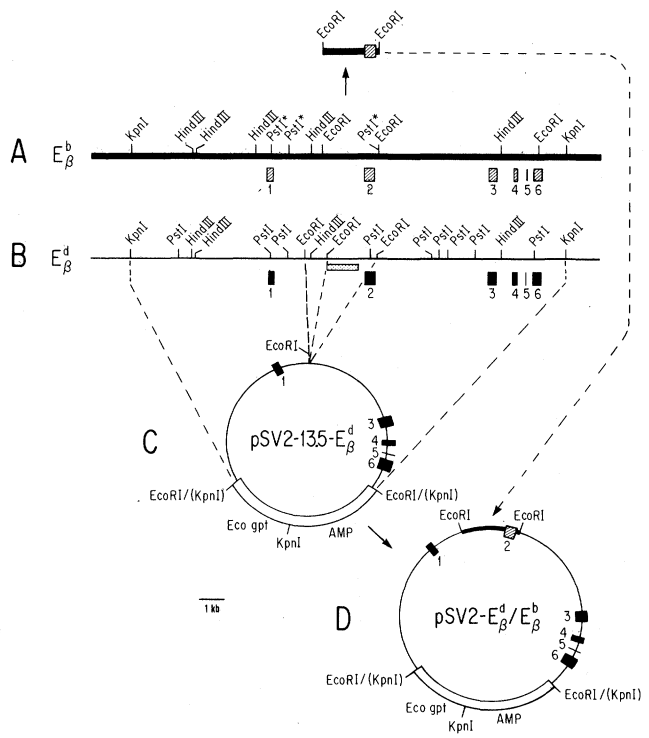

FIG. 1. (*A*) Restriction enzyme map of the E^b_β gene as determined from a cosmid clone containing the entire E^b_β gene (7). Exons are indicated by hatched boxes. *Pst* I sites are marked with asterisks to indicate that the *Pst* I map is incomplete. (*B*) Restriction enzyme map of the E^d_β gene that was deduced from four overlapping recombinant λ phage clones (6). Exons are indicated by black boxes. The probe used for Southern hybridization (Fig. 2) is indicated as a dotted box. (*C*) Construction of the pSV2-13.5-E^d_β plasmid. pSV2gpt vector DNA was prepared for construction by cleavage with *Eco*RI, reaction with the Klenow fragment of *E. coli* DNA polymerase to create flush ends, and treatment with calf intestinal alkaline phosphatase. A 6.5-kb *Kpn* I–*Eco*RI DNA fragment (from recombinant phage λHS1; ref. 6) was ligated *in vitro* to a 7.0-kb *Eco*RI–*Kpn* I DNA fragment (from recombinant phage λ 11.1; ref. 6). The ligated product was digested with *Kpn* I, and a 13- to 14-kb band was isolated from an agarose gel after electrophoresis. The 13- to 14-kb DNA band was assumed to contain all pairwise combinations of the 6.5-kb and 7.0-kb fragments. This DNA fraction was digested with T4 DNA polymerase to generate flush ends and was ligated to the pSV2gpt vector. The ligated product was used to transform competent *E. coli* cells, and bacterial colonies containing the pSV2-13.5-E^d_β plasmid were identified by restriction enzyme digestion of DNA minipreparations. (*D*) Construction of plasmid pSV2-E^d_β/E^b_β. Plasmid pSV2-13.5-E^d_β DNA was partially digested with *Eco*RI and was dephosphorylated with calf intestinal alkaline phosphatase. DNA was then digested to completion with *Kpn* I. Fragments of 10.0 kb and 8.8 kb were isolated by agarose gel electrophoresis and ligated to the 2.1-kb *Eco*RI fragment from E^b_β.

Southern blot analysis was used to distinguish the transfected E^d_β/E^b_β gene from the endogenous E^d_β gene and from a transfected E^b_β gene. The results (Fig. 2) indicate that two out of four E^d_β/E^b_β-transfected cell lines contain an apparently full-length copy (or copies) of the transfected hybrid gene. The endogenous E^d_β gene (A20-2J) is characterized by a 1.9-kb *Eco*RI fragment (Fig. 2*A*, lane 2), a 3.0-kb *Pst* I fragment (Fig. 2*B*, lane 2) and a 7.2-kb *Hin*dIII fragment (Fig. 2*C*, lane 2). Unidentified cross-hybridizing species were also detected with the probe, including the second band of the 1.9-kb *Eco*RI doublet, smaller *Pst* I fragments, and a *Hin*dIII band at 6.5 kb. The transfected E^b_β gene (A20-E^b_β-5) gives rise to a 2.1-kb *Eco*RI fragment, a 3.3-kb *Pst* I fragment, and a 7.2-kb *Hin*dIII fragment (lanes labeled 1). Two E^d_β/E^b_β-transfected cell lines (A20-E^d_β/E^b_β-3 and A20-E^d_β/E^b_β-5, lanes 4 and 6, respectively) contain the 2.1-kb *Eco*RI fragment characteristic of E^b_β, a smaller *Pst* I fragment (2.6 kb) due to deletion of the small *Eco*RI fragment from the intron during construction of the hybrid gene, and an 11-kb *Hin*dIII fragment (due to deletion of the *Hin*dIII site within the small *Eco*RI fragment). Although the integrated hybrid gene is not interrupted across the region assayed in A20-E^d_β/E^b_β-3 and A20-E^d_β/E^b_β-5, we have not assayed a 1.5-kb region at the 3′ end of the gene, and rearrangement within this region remains a possibility. We estimate that A20-E^d_β/E^b_β-3 contains one or two copies of the hybrid gene per cell, whereas A20-E^d_β/E^b_β-5 contains 5–10 copies of the hybrid gene per cell. The clone A20-E^d_β/E^b_β-4 appears to be rearranged within the coding region of the transfected gene, and clone A20-E^d_β/E^b_β-1 has deleted the majority of the hybrid gene sequences that are homologous to the DNA probe. The difference in the number of integrated gene copies in A20-E^b_β-5 as compared to the E^d_β/E^b_β-transfected cell lines is probably a result of a difference in the selective media used after transfection. A20-E^b_β-5 was selected with mycophenolic acid at 6 μg/ml (requiring more integrated copies of the transfecting plasmid for adequate Ecogpt production), whereas A20-E^d_β/E^b_β cell lines were selected with mycophenolic acid at 0.5 μg/ml.

The Y-17 monoclonal antibody was shown to recognize $E^d_\alpha E^b_\beta$ in A20-2J cells that had been transfected with the E^b_β gene (7). If the determinant recognized by Y-17 were encoded within the second exon of the E^b_β gene, then one would expect that an $E_\alpha E_\beta$ dimer composed of an E^d_α chain and the hybrid E^d_β/E^b_β chain should react with the Y-17 antibody. Hybrid gene-transfected cell lines that apparently contained a full-length copy (or copies) of the transfected gene were labeled with [^{35}S]methionine. Nonidet P-40 extracts of labeled cells were immunoprecipitated with Y-17 and analyzed by two-dimensional protein gel electrophoresis. Results were compared to those for the A20-2J parent and the A20-E^b_β-5 cell line (Fig. 3). As expected, no E_α or E_β chains were precipitated from A20-2J extracts with Y-17 (Fig. 3*A*), whereas E^d_α and E^b_β chains were precipitated from A20-E^b_β-5 (Fig. 3*B*). Although no E products were precipitated from A20-E^d_β/E^b_β-3 extract (Fig. 3*C*), species corresponding to the transfected hybrid E_β gene product and to the E^d_α gene product were clearly precipitated from A20-E^d_β/E^b_β-5 (Fig. 3*D*). With a few differences, the E^b_β species precipitated from A20-E^b_β-5 were superimposable with the hybrid E_β species precipitated from A20-E^d_β/E^b_β-5. A single spot seen in the A20-E^b_β-5 immunoprecipitate (Fig. 3*B*, arrow a) was absent from the A20-E^d_β/E^b_β-5 immunoprecipitate, and one or two minor spots (Fig. 3*D*, arrows b) were shifted in A20-E^d_β/E^b_β-5 relative to their respective positions in A20-E^b_β-5.

The lack of Y-17 precipitable product in A20-E^d_β/E^b_β-3 may indicate the absence of expression of a functional hybrid gene product in these cells. Functional expression of the transfected gene could be blocked by rearrangement within the coding region or by rearrangement within regulatory elements (21) associated with the transfected E_β gene. Immuno-

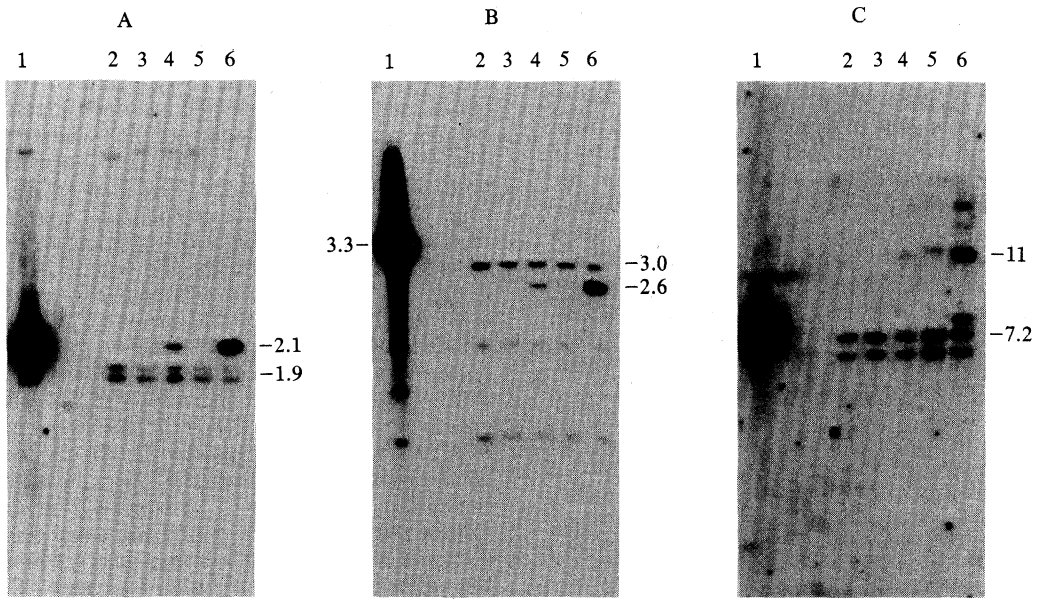

FIG. 2. Southern blot analysis of transfected cell lines. Restriction enzyme digestion was with *Eco*RI (*A*), *Pst* I (*B*), or *Hin*dIII (*C*). Digested DNA samples (10 μg per well) were run on 1.8% agarose (*A* and *B*) or 0.8% agarose (*C*) gels. Each blot was probed with a 1.2-kb probe derived from the first intron of the E^d_β gene, as indicated in Fig. 1*B*. Lanes: 1, A20-E^b_β-5 DNA; 2, A20-2J; 3, A20-E^d_β/E^b_β-1; 4, A20-E^d_β/E^b_β-3; 5, A20-E^d_β/E^b_β-4; 6, A20-E^d_β/E^b_β-5.

precipitation of labeled extracts with the 14-4-4S monoclonal antibody (which recognizes all E molecules regardless of haplotype; see ref. 22) indicated that A20-E^d_β/E^b_β-3 contains an apparently aberrant form of the E_β chain (data not shown). Therefore, we believe that the hybrid gene is interrupted near the 3′ end of the coding region as discussed above.

The hybrid gene-transfected cell lines were tested for their ability to present antigen to $E^d_\alpha E^b_\beta$-restricted T-cell hybrid-

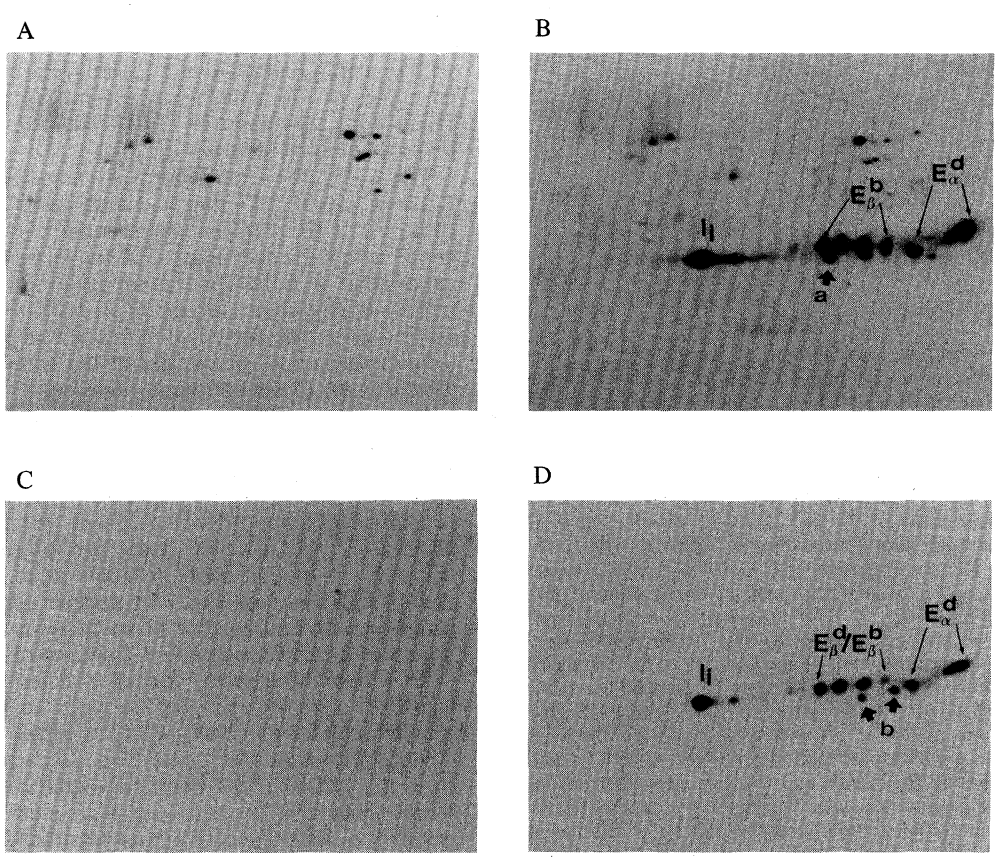

FIG. 3. Two-dimensional gel electrophoresis of Y-17 immunoprecipitates from [^{35}S]methionine-labeled cell extracts. The first dimension was nonequilibrium pH gradient electrophoresis, and the second dimension was sodium dodecyl sulfate/polyacrylamide gel electrophoresis (10% polyacrylamide). (A) A20-2J, (B) A20-E^b_β-5, (C) A20-E^d_β/E^b_β-3, (D) A20-E^d_β/E^b_β-5. The invariant chain is indicated I_i, and spots corresponding to various modified forms of E_α or E_β are bracketed by thin arrows. The short, thick arrow "a" in *B* indicates a species that is present in the A20-E^b_β-5 immunoprecipitate but absent in the A20-E^d_β/E^b_β-5 immunoprecipitate. The short, thick arrows labeled "b" in *D* indicate spots that appear shifted in A20-E^d_β/E^b_β-5 relative to A20-E^b_β-5.

Table 1. Antigen presentation by A20-E^d_β/E^b_β cell lines

T-cell hybridoma	Ag/H-2 specificity*	Ag in culture	Production of interleukin 2 in response to cell lines, units/ml				
			LB-15.13†	A20-2J	A20-E^b_β-1	A20-E^d_β/E^b_β-3	A20-E^d_β/E^b_β-5
BDO-23.2	cOVA/I-$E^{d/b}$	cOVA	320	<10	320	<10	160
BDO-23.2	cOVA/I-$E^{d/b}$	None	<10	<10	<10	<10	<10
BDO-34.1	cOVA/I-$E^{d/b}$	cOVA	1280	<10	1280	<10	1280
BDK-38.2	KLH/I-$E^{d/b}$	KLH	2480	<10	1280	<10	640

Ag, antigen; cOVA, chicken ovalbumin; KLH, keyhole limpet hemocyanin; IL-2, interleukin 2. Ten units of IL-2 per ml is the minimum detectable by our assay. Results for A20-E^d_β/E^b_β-1 and A20-E^d_β/E^b_β-4 are identical to those presented for A20-E^d_β/E^b_β-3. Assays were repeated at least three times. Although the magnitude of the measured response varied from experiment to experiment, the relative response levels are fairly consistent for the various presenting cell lines (against a given T-cell hybridoma line). The data presented are from a single experiment.
*I-$E^{d/b}$ refers to the hybrid molecule $E^d_\alpha E^b_\beta$.
†LB-15.13 is the I^b/I^d-bearing fusion product of A20-2J to C57BL/10 B cells (11).

omas. The results (Table 1) show that A20-E^d_β/E^b_β-5 (which expresses the hybrid gene product on the basis of reaction with the Y-17 antibody) has acquired the ability to present antigen to both chicken ovalbumin- and keyhole limpet hemocyanin-specific T-cell hybridomas that are restricted to $E^d_\alpha E^b_\beta$. In fact, the A20-E^d_β/E^b_β-5 line presents antigen to these T-cell hybridomas at least as efficiently as does the E^b_β gene-transfected cell line A20-E^b_β-1.

DISCUSSION

The general three-dimensional structure of a class II major histocompatibility complex molecule can be deduced from available DNA sequence information. Thus, each α or β chain is predicted to contain two external globular domains (α_1 and α_2 or β_1 and β_2), a transmembrane peptide, and a cytoplasmic peptide. Although the relationship of the predicted structural domains to the various functions of the class II molecule (including restricted T-cell recognition and interaction with antigen) is not known, one can predict that the amino-terminal (and most exposed) α_1 and β_1 domains may be functionally important in the recognition of the antigen-presenting cell by the effector T cell. To test this hypothesis, we have constructed a hybrid E^d_β/E^b_β gene from cloned E^d_β and E^b_β genes. The hybrid gene contains exon 2 (corresponding to the majority of the β_1 domain) from the *b* haplotype gene and exons 1, 3, 4, 5, and 6 from the *d* haplotype gene. After transfection with the hybrid E^d_β/E^b_β gene, BALB/c-derived A20-2J cells gain the ability to present antigen to three $E^d_\alpha E^b_\beta$-restricted helper T-cell hybridomas. The acquired presentation capability is not antigen specific in that A20-E^d_β/E^b_β transfected cells can present antigen to two chicken ovalbumin-specific hybridomas and one keyhole limpit hemocyanin-specific hybridoma. Thus we conclude that the β_1 domain of the E^b_β molecule determines recognition of the host cell by $E^d_\alpha E^b_\beta$-restricted helper T cells. This conclusion is supported by the recently reported comparison of E^d_β and E^b_β genomic DNA sequences, which indicates a total of 16 predicted amino acid differences between the two chains (23). Interestingly, 14 of the 16 predicted differences are encoded by the second exon, and several of these differences represent nonconservative changes. A single conservative change (serine compared to threonine) is seen in the first exon at amino acid position 4, and a more drastic difference is seen at position 140 (within the third exon, β_2 domain) where a lysine (E^b_β) is replaced by a glutamic acid (E^d_β). The product of the hybrid E^d_β/E^b_β gene is thus expected to differ from the E^b_β chain at only two positions. It will be interesting to see whether a T-cell hybridoma can be isolated that will distinguish the hybrid E^d_β/E^b_β chain from the E^b_β chain. Further investigation of E_β chain function in the restriction of cell–cell interaction will require either construction of hybrid β_1 domains or site-directed mutagenesis within the β_1 domain and possibly at amino acid 140.

Hybrid gene experiments from other laboratories indicate that the restricted recognition of class I major histocompatibility complex molecules operates principally through the first and second external domains of the mature protein (24, 25). These domains are structurally analogous to the α_1 and β_1 domains of a class II major histocompatibility complex molecule. The data presented here indicate that the β_1 domain of the E_β molecule is important in class II-restricted cell–cell recognition, suggesting a functional similarity between class I and class II molecules. However, further analysis is required to determine whether this functional analogy extends to the α_1 domain of the more highly conserved E_α chain.

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