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Functional and inducible expression of a transfected murine class II major histocompatibility complex gene
(antigen presentation/cosmid clones/γ-interferon/protoplast fusion/T-helper hybridoma)

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ABSTRACT Using the spheroplast fusion technique, we have introduced the cloned Eβ gene into two d haplotype cell lines, the B lymphoma line A20-ZJ and the macrophage tumor line P388D1. Analysis with a monoclonal antibody indicates that the product of the transfected Eβ gene associates with the endogenous Eα chain to form an Eα-Eβ complex. While expression of EαEβ is constitutive in A20-ZJ cells transfected with the Eα gene, surface expression of EαEβ is detected in transfected macrophage cells only after treatment of cells with culture supernatants from concanavalin A (Con A)-stimulated T cells. Transfected B lymphoma cells and transfected Con A supernatant-treated macrophage cells have acquired the ability to present antigen to Eα-Eβ-restricted T-cell hybridomas. The observed inducible expression of the transfected gene in the macrophage host indicates that sequences responsible for regulated expression of the Eβ gene may be associated with the transfected gene. In combination with directed mutagenesis, the system described here provides a means to study (i) Eβ sequences that are important in determining the restriction specificity of the E molecule and (ii) sequences associated with the Eβ gene that may be important in the regulation of Eβ chain expression.

The class II major histocompatibility antigens have been implicated in cell-cell interactions of the immune system (for review, see refs. 1 and 2). These polymorphic cell-surface glycoproteins control the level of immune response to certain antigens, probably through their role during presentation of antigen to class II-restricted antigen-specific T cells: a class II-restricted T cell recognizes specific antigen only when it is presented on the surface of a cell that expresses the appropriate allelic form of a class II molecule. Although the mechanism of restricted antigen presentation is not understood at a molecular level, it is clear that models for T-cell recognition of antigen should incorporate both a T-cell receptor (or receptors) and antigen in association with class II molecules on the surface of the presenting cell.

Expression of class II antigens is limited to certain cells of the immune system, including B cells and macrophages (3-5). A relatively high level of constitutive expression of class II antigens is observed in B cells. This can be increased by certain treatments but not by treatment with γ-interferon (unpublished observation). In macrophages, expression of class II antigens is modulated. Resting macrophages express low levels of surface class II antigen, and expression of these molecules can be induced with γ-interferon or with supernatants from Con A-stimulated T cells containing γ-interferon (5-7). Although induction by γ-interferon appears to operate at the level of transcriptional control (8, 9), virtually nothing is known about the mechanisms that regulate expression of class II molecules.

In mouse, two class II molecules, each consisting of an α and a β protein chain, have been defined (2, 10). The A molecule is designated as Aβ and the E molecule as EαEβ. Structural analysis of these molecules has been limited by a lack of protein sequence data. Recently, however, efforts from several laboratories have led to the isolation and characterization of cDNA and genomic clones for the Aβ, Aα, Eα, and Eβ chains (11-16). Protein sequences have been deduced and exon/intron organizations defined. Although sequence analysis provides some understanding of the organization of class II molecules and the genes encoding them, identification of functionally important regions remains tentative.

We have chosen to use a DNA transfection system, in combination with directed mutagenesis, to study the structure-function relationship of class II molecules and the regulation of their expression. Our initial goal has been to introduce a cloned class II gene into a host cell that has been shown (i) to express class II molecules and (ii) to be capable of presenting antigen to specific T-cell hybridomas in culture. We reasoned that a cell line that expresses endogenous class II genes would also express a transfected gene, provided appropriate DNA control elements were included in association with the transfecting gene. Once expression of a transfected gene has been established, we can begin to modify the gene prior to transfection and to determine the effect of defined modification on the expression and function of the class II molecule.

In this paper we report structural and functional evidence for the expression of the EαEβ gene after introduction into a B-cell lymphoma or a macrophage tumor. We also show that surface expression of the transfected EαEβ gene is inducible by Con A-activated T-cell supernatants after introduction into macrophage tumor cells.

MATERIALS AND METHODS
All cells were cultured in medium containing 10% fetal calf serum and 50 μM 2-mercaptoethanol. The BALB/c B lymphoma line A20-ZJ was previously designated L10.A2J but has been recently reidentified (and renamed) as a derivative of the A20 tumor (17). The P388D1 macrophage tumor line (18) was obtained from the American Type Culture Collection. The helper T-cell hybridomas BDK-38.2, BDK-44.5, and DO-11.10 have been described (19, 20). Two additional T-cell hybridomas, BDO-23.2 and BDO-34.1, were derived by established methods (21) and represent fusion products of normal T cells from ovalbumin-immunized (C57BL/6 × DBA/2) F1 mice and the AKR thymoma BW5147. Hybrids were screened for their ability to recognize ovalbumin presented by the A20-EαEβ-1 transfected cell line (see Results).

The Y-17 monoclonal antibody (22) that recognizes a variety of E molecules, including EαEβ but not EαEβ, was received from Donal Murphy (Yale University, New Haven, Conn.).

Abbreviations: IL-2, interleukin 2; kb, kilobase(s).
The anti-I-A\textsuperscript{d} specific MKD6 antibody is described by Kappler et al. (21). Hybriompas culture supernatants were used for immunofluorescence and immunoprecipitation. For immunofluorescence, the detection antibody was a fluoresceinated goat anti-mouse Fab antiserum (a gift from Henry Wortis, Tufts University Medical School, Boston, MA). Fluorescence was measured on an Ortho fluorescence-activated cell sorter, and immunoprecipitation of [\textsuperscript{35}S]methionine-labeled cell extracts was done as described by P. Jones (23).

Parental and transfected cell lines were tested for their ability to present antigen as described elsewhere (24). Briefly, \(1 \times 10^5\) presenting cells were cultured with \(1 \times 10^5\) responder T cells in the presence of 200 \(\mu\)g of antigen for 24 hr in 200 \(\mu\)l of culture medium. Interleukin 2 (IL-2) production by the T-cell hybridoma was assayed by the ability of the culture supernatant to support the growth of an IL-2-dependent T-cell line, HT-2 (25). Units of IL-2 were determined as described (24). For induction of P388D\(_1\) and derived lines, cells were incubated for 48 hr with culture supernatants from the T-cell hybridoma F57-20 at 1:8 dilution (26).

The C57BL/6 cosmId library was constructed and screened by established methods (27). To construct the pSV2-E\(_\beta\) plasmid, the pSV2gpt vector (28) was linearized with BamHI, treated with the Klenow fragment of Escherichia coli DNA polymerase I to create flush ends, and digested with calf alkaline phosphatase. A 22-kilobase (kb) Hpa I fragment isolated from cosmId 7-01-E\(_\beta\) was fused by blunt-end ligation to the pSV2gpt vector. Preparation of high molecular weight DNA was by standard methods (27). Southern blotting and hybridization were as described (29).

For transfection, bacteria containing pSV2-E\(_\beta\) or pSV2gpt plasmids were converted to protoplasts as described by Sandri-Goldin et al. (30). Protoplasts were fused to A20-21 or P388D\(_1\) cells with 50% polyethylene glycol (British Drug House) as described by Gillies et al. (31). After fusion, cells were plated and allowed to recover in normal growth medium for 48-72 hr, at which time an equal volume of selective medium was added. Selective medium contained xanthine at 250 \(\mu\)g/ml, hypoxanthine at 15 \(\mu\)g/ml, and mycophenolic acid at 2 \(\mu\)g/ml (for P388D\(_1\)) or 6 \(\mu\)g/ml (for A20-21). Xanthine and hypoxanthine were from Sigma. Mycophenolic acid was a gift from Eli Lilly. Plates were refed every 2-3 days with fresh selective medium. Colonies were picked for expansion after 2-3 wk.

RESULTS

As transfection hosts we have chosen the BALB/c B lymphoma cell line A20-21 and the DBA/2 macrophage tumor line P388D\(_1\). The A20-21 line constitutively expresses both A and E molecules of the d haplotype and presents antigen in a restricted fashion to T-cell hybridomas in culture (17). In normal growth medium, P388D\(_1\) expresses d haplotype class II molecules at low levels and presents antigen only poorly. However, when P388D\(_1\) is cultured with supernatant from Con A-stimulated T-cells, class II antigens are expressed at high levels and the induced culture presents antigen efficiently to restricted T-cell hybridomas (32). The E\(_\beta\) gene of d haplotype (E\(_\beta\)) was chosen as a transfecing gene, as specific reagents were available to assay expression and function of E\(_\beta\) and E\(_\beta\) hybrid molecules.

Isolation of the E\(_\beta\) Gene. The C57BL/6 cosmId library was screened with a probe derived from the first intron of the E\(_\beta\) gene (see Fig. 1A and ref. 16). Three overlapping clones were isolated, and cosmId clone 7-01-E\(_\beta\) was selected for restriction enzyme analysis (Fig. 1A).

Transfection of the E\(_\beta\) Gene into BALB/c Hosts. The pSV2-E\(_\beta\) plasmid was introduced into A20-21 or P388D\(_1\) cells by spheroplast fusion. For A20-21 cells, the frequency of stable transformation was \(5 \times 10^{-4}\) with pSV2-E\(_\beta\) and \(1 \times 10^{-4}\) with pSV2gpt. After transfection, A20-21 cells were

![Fig. 1. (A) Restriction enzyme map of the cosmId 7-01-E\(_\beta\). Only the structure of the cosmId insert is presented. Six exons, which are indicated by dark boxes, have been placed relative to conserved restriction enzyme sites by analogy to the E\(_\beta\) gene (16). The striped box indicates the a haplotype EcoR I-Pvu II fragment from phase lambda 1i.1. (16), which was used as a probe for the isolation of cosmId 7-01-E\(_\beta\). (B) Map of the recombinant plasmid pSV2-E\(_\beta\). The 22-kb Hpa I fragment from cosmId 7-01-E\(_\beta\) is indicated as a thin line with dark boxes representing exons of the E\(_\beta\) gene. Exons are indicated as follows: L, leader; \(\beta_1\), first external domain; \(\beta_2\), second external domain; TM, transmembrane; CP, cytoplasmic; 3'UT, 3' untranslated (see ref. 16). The Eco gpt gene (striped region), Simian virus 40 DNA sequences (dotted region), and ampicillin gene (shaded area) of the pSV2gpt vector are as indicated (see ref. 27). Modified BamHI sites are labeled as (Bam). Transcriptional orientations of E\(_\beta\) and Eco gpt genes are indicated by arrows.](image-url)

![Fig. 2. Southern blot analysis of E\(_\beta\)-transfected cell lines. High molecular weight DNA was digested with BamHI, separated by electrophoresis on 0.8% agarose gels, and blotted to nitrocellulose. Blots were probed with a nick-translated 1.2-kb EcoR I-Pvu II fragment isolated from a recombinant phage containing part of the E\(_\beta\) gene (see legend to Fig. 1A). The sizes of DNA bands are indicated in kilobases. (A) Data for A20-21 and A20-E\(_\beta\)-transfected lines: lane 1, A20-21; lane 2, A20-E\(_\beta\)-1; lane 3, A20-E\(_\beta\)-2; lane 4, A20-E\(_\beta\)-5. (B) Results for P388D\(_1\), lane 1) and the P388D\(_1\)-E\(_\beta\)-transfected population (lane 2).](image-url)
plated in 96-well culture plates, such that colonies were obtained in 1 of 10 wells. Colonies picked for analysis were therefore assumed to represent individual transfection events.

P388D1 transfecteds were selected on 60-nm tissue culture plates. Numerous colonies were visible after transfection with pSV2-Eβ or pSV2gpt. Plates were grown to confluence so that our transfected P388D1 lines represent populations of many individual transfecteds. Frequencies of transfection, subsequently determined for a subline of P388D1, were $3 \times 10^{-5}$ and $1.5 \times 10^{-4}$ with pSV2-Eβ and pSV2gpt, respectively.

Eβ DNA sequences are present in several A20-Eβ transfecteds and in the P388D1-Eβ population, as determined by Southern blot analysis (Fig. 2). Digestion is with BamHI endonuclease. The endogenous Eβ gene is seen as a 4.8-kb band, and the exogenous Eβ gene is represented by an 8.7-kb band. Clearly, the Eβ band is seen in all A20-Eβ transfected (Fig. 2A) and in the P388D1-Eβ population (Fig. 2B), whereas it is absent in DNA from the parental cell lines. Each of the A20-Eβ transfecteds contains an estimated 50–100 copies of the Eβ gene per haploid genome, and the P388D1-Eβ population contains an average of 3–4 copies per haploid genome. A third restriction enzyme fragment is seen at 6.5 kb in A20-2J and in A20-Eβ transfecteds. The origin of this fragment is unclear, as a fragment of this size is not expected from the restriction map of the Eβ gene (16).

Expression of Eβ in A20-2J Transfectants. Four A20-Eβ transfecteds and two A20-pSV2gpt transfecteds were examined for reactivity to the Y-17 monoclonal antibody by radioimmunoassay (data not shown). Surface expression of

![Fig. 3. Surface expression of Eβ, Eβ, in an Eβ, transfected A20 clone. A20-2J or A20-Eβ-5 cells were incubated with Y-17 culture supernatant, followed by incubation with fluoresceinated goat anti-mouse Fab' antisera. Fluorescence was measured on an Ortho fluorescence-activated cell sorter; 30,000 cells were analyzed per sample. The vertical axis indicates cell number and the horizontal axis indicates fluorescence intensity.](image)

Eα+Eβ was indicated for all A20-Eβ transfecteds tested, whereas the A20-2J parent and pSV2gpt transfecteds failed to react with Y-17. Total surface expression of E molecules was increased in Eβ-transfected lines, as measured with the monoclonal antibody 14-4-4S, which recognizes E molecules of all haplotypes (33). Reaction with the anti-I-Aα monoclonal antibody MKD6 indicated that surface expression of the A molecule was not altered by transfection.

The A20-Eβ-5 transfected cell line was further analyzed by fluorescence-activated cell sorting after reaction with Y-17. The results, as shown in Fig. 3, indicate that the vast majority of cells in the A20-Eβ-5 population are expressing surface Eα+Eβ. The expression of surface Eα+Eβ is similar to that of AαAβ, as seen by analysis with the MKD6 antibody (not shown).

To further characterize the product of the transfected Eβ gene, Nonidet P-40 extracts of [35S]methionine-labeled A20-2J or A20-Eβ-5 cells were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis after immunoprecipitation with the Y-17 antibody (Fig. 4). Labeled extracts of spleen cells from BALB/c or (BALB/c × C57BL/6) F1 mice expressing Eα+Eβ were used as controls. Specific products of the expected sizes are seen in the A20-Eβ-5 and F1 spleen samples. Eα and Eβ chains exist in several glycosylated

![Fig. 4. Immunoprecipitation of Eα,Eβ from A20-Eβ-5 cells. Nonidet P-40 extracts of [35S]methionine-labeled cells were immunoprecipitated with Y-17 antibody and inactivated Staphylococcus aureus bacteria. Extracts from 1 × 10⁶ cells were used for the A20-2J (lane 1) and A20-Eβ-5 (lane 2) samples. Extracts from 2.5 × 10⁶ spleen cells were analyzed for the BALB/c (lane 3) and (BALB/c × C57BL/6) F1 (lane 4) samples. Immunoprecipitates were analyzed by electrophoresis on a 12% polyacrylamide gel in NaDodSO4 buffer. The gel was fluorographed, dried, and exposed to Kodak XAR-5 film with an intensifying screen for 24 hr (lanes 1 and 2) or 2 wk (lanes 3 and 4). Molecular weight markers (×10⁻⁵) are indicated (lane M).

![Fig. 5. Surface expression of Eα,Eβ is induced in the P388D1-Eβ population by treatment with T-cell supernatants. P388D1 or P388D1-Eβ cells were incubated with the Y-17 or MKD6 (anti-I-Aα) antibody followed by incubation with fluoresceinated goat anti-mouse Fab' antisera. Fluorescence was measured on an Ortho fluorescence-activated cell sorter; 15,000 cells were analyzed per sample. Profiles are shown for uninduced (+IFN) and induced (+IFN) cultures as indicated (IFN, interferon). Cell number is indicated on the vertical axis and fluorescence intensity is along the horizontal axis. (A) P388D1 × anti-I-Aα; (B) P388D1 × Y-17; (C) P388D1 × anti-I-Aα; and (D) P388D1 × Y-17.

![Image](image)
Table 1. Antigen presentation by A20-2J and transfected A20-2J lines

<table>
<thead>
<tr>
<th>T-cell hybridoma</th>
<th>Ag/H-2 specificity</th>
<th>Ag in culture</th>
<th>Units of IL-2 per ml produced in response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO-11.10</td>
<td>cOVA/I-A(^d)</td>
<td>cOVA</td>
<td>ND</td>
</tr>
<tr>
<td>BDK-38.2</td>
<td>KLH/I-E(^{6b})</td>
<td>cOVA</td>
<td>&lt;10</td>
</tr>
<tr>
<td>BDK-38.2</td>
<td>KLH/I-E(^{6b})</td>
<td>KLH</td>
<td>640</td>
</tr>
<tr>
<td>BDK-44.5</td>
<td>KLH/I-A(^{6b})</td>
<td>KLH</td>
<td>320</td>
</tr>
<tr>
<td>BDO-23.2</td>
<td>cOVA/I-E(^{6b})</td>
<td>cOVA</td>
<td>320</td>
</tr>
<tr>
<td>BDO-34.1</td>
<td>cOVA/I-E(^{6b})</td>
<td>cOVA</td>
<td>80</td>
</tr>
</tbody>
</table>

Ag, antigen; ND, not done; cOVA, chicken ovalbumin; KLH, keyhole limpet hemocyanin. Ten units of IL-2 per ml is the minimum detectable by our assay.

*LB-15.13 is the F\(^+\)-I-A\(^b\)-bearing fusion product of A20-2J to C57BL/10 B cells (17).

Numbers in parentheses indicate units of IL-2 per ml produced when competing Y-17 antibody was added to the presentation assay.

F\(^+\)-I-A\(^6b\) refers to the hybrid molecule E\(_6\)bE\(_6\)b. I-A\(^6b\) refers to A\(_6\)bA\(_6\)b.

DISCUSSION

The class II antigens of the major histocompatibility complex affect the immune responsiveness to many antigens. To understand this phenomenon, it is important to determine at the molecular level (i) how class II molecules function in the presentation of antigen to restricted T cells and (ii) how the expression of class II molecules is regulated in antigen-presenting cells. These questions may be addressed through the use of directed mutagenesis and a defined DNA transfection system. To this end, we have introduced the cloned mouse E\(_6\)b gene into two d haplotype host cell lines—the B lymphoma line A20-2J and the macrophage tumor line P388D1. Using a monoclonal antibody that recognizes the hybrid E\(_6\)bE\(_6\)b molecule, we have shown the transfected gene to be constitutively expressed in the B lymphoma host. In contrast, surface expression of E\(_6\)bE\(_6\)b could be detected in the transfected P388D1-E6b macrophage line after, but not before, incubation of cells with supernatants from Con A-stimulated T cells. In addition, we have shown that transfected B lymphoma cells or transfected, induced macrophage cells can functionally present antigens to appropriate T-helper cell hybridomas. The ability of a transfected line to present antigen was consistent with the pattern of cell-surface expression of E molecules measured by antibody: A20-2J cells transfected with the E\(_6\)b gene effectively presented antigen to E\(_6\)bE\(_6\)b-restricted T-cell hybridomas, whereas the P388D1 cells transfected with the E\(_6\)b gene presented antigen optimally only after being cultured in T-cell-derived conditioned medium (with known inducing activity).

Functional expression of a transfected A\(_6\)b gene has recently been reported (35). However, it is not known whether A and E molecules play identical roles in the generation of immune response to antigens, and it will be important to understand how both A and E molecules function in cell-cell interactions of the immune system. The transfection system described here has the advantage that function may be assayed after introduction of a single class II gene into a recipient cell. In our system, transfected cell lines, which express E\(_6\)bE\(_6\)b (in addition to endogenous E\(_6\)bE\(_6\)b), are assayed against a panel of E\(_6\)bE\(_6\)b-restricted responder T-cell hybridomas. Because the polymorphism observed in E molecules is contributed principally by the E\(_6\) chain (E\(_6\) is the most highly conserved of the four class II polypeptides, whereas E\(_6\) is

Table 2. Inducible antigen presentation by P388D1 and transfected P388D1 lines

<table>
<thead>
<tr>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>cOVA/I-E(^{6b})</td>
<td>320</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

See legend to Table 1 for abbreviations.
highly polymorphic; see ref. 36), it is expected that analysis of E restricting elements can be carried out by modifying (and transferring) the Eβ gene alone. By contrast, both the α and β chains of the A molecule are polymorphic and both chains contribute to the determination of restriction specificity. Analysis of I-A-restricted cell-cell interactions may therefore be more complex. The restricting function of hybrid Eβ chains can be initially investigated by shuffling exons between Eβ' and Eβ' genes. The hybrid gene approach has been successfully applied to the study of class I major histocompatibility complex molecules (37, 38) and should allow localization of those regions of the Eα chain that are important in determining the restriction specificity of the E molecule.

Expression of class II major histocompatibility complex molecules is both positively and negatively regulated in macrophages. Positive regulation has been demonstrated in response to soluble products of stimulated T cells (6, 7), whereas negative regulation has been observed in response to prostaglandins (5). Recent data indicate that the T-cell factor responsible for induction is γ-interferon (7, 39). Results from human (8) and murine (9) systems suggest that regulation by γ-interferon may operate at the level of transcriptional control, in that increased levels of mRNA are observed after γ-interferon induction. In fact, HLA-DRα and HLA-DRβ (the human equivalents of Eα and Eβ, respectively) mRNA levels are simultaneously increased in response to γ-interferon treatment, indicating that transcriptional regulation by γ-interferon may operate on both α and β chain genes (8).

The data presented here indicate that the 22-kb DNA fragment containing the Eβ gene and its flanking regions may contain all of the sequence information required to make this gene susceptible to regulation by γ-interferon in macrophage cells. Premanipulation of this DNA fragment followed by transfection of P388D1 cells may lead to localization of one or more specific sequence element(s) required for the induction. In this context, it is interesting that an apparently tissue-specific transcriptional enhancer element is associated with the Eβ gene (unpublished observations). It is possible that the enhancer element itself is an indirect target for the inducing action of γ-interferon. A somewhat analogous involvement of an enhancer element in the modulation of gene expression by an externally provided agent has been seen in the glucocorticoid hormone-mediated induction of mouse mammary tumor virus genes (40).

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