The EMBO Journal vol.4 no.9 pp.2225-2230, 1985

Tissue-specific, inducible and functional expression of the $E_\alpha{}^d$ MHC class II gene in transgenic mice

C.A.Pinkert, G.Widera[1], C.Cowing[2], E.Heber-Katz[3], R.D.Palmiter[4], R.A.Flavell[1] and R.L.Brinster

Laboratory of Reproductive Physiology, School of Veterinary Medicine, [2]Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, and [3]The Wistar Institute, Philadelphia, PA 19104, [1]Biogen Research Corporation, 14 Cambridge Center, Cambridge, MA 02142, and [4]Howard Hughes Medical Institute, Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

Communicated by R.A.Flavell

We have introduced the class II $E_\alpha{}^d$ gene into (C57BL/6 × SJL) F2 mice which do not express their endogenous E_α gene. The mRNA expression of the $E_\alpha{}^d$ gene shows the same tissue distribution as the endogenous class II genes except in the case of one mouse, which carried 19 copies of the $E_\alpha{}^d$ gene. In this mouse expression of $E_\alpha{}^d$ mRNA was seen in all tissues tested. Expression of the transgene was induced by γ-interferon in isolated macrophages from the transgenic mice. In addition, fluorescence activated cell sorter (FACS) analysis, mixed lymphocyte response and antigen-presentation experiments showed that the product of the transferred gene is expressed on the cell surface and functions as a major histocompatibility complex restriction element. Transmission of the gene occurred only with female transgenic mice, all males were infertile or did not transmit the gene, suggesting an effect of the transferred DNA sequence on male reproductive function.

Key words: class II genes/I-$E_\alpha{}^d$ immune response-associated gene/MHC/transgenic mice

Introduction

The major histocompatibility complex (MHC) of the mouse is located on chromosome 17 and is comprised of a series of genes mapped to four regions: K, I, S and D. The I region codes for the MHC class II molecules, associated with the generation and regulation of immune responses. Genes of the A and E subregions encode cell surface glycoproteins [I-A and I-E immune response-associated (Ia) antigens] that are heterodimers composed of two polypeptide chains of 33-35 kd (α chain) and 27-29 kd (β chain). Each chain possesses two extracellular globular domains (a polymorphic α1 or β1 domain and a conserved immunoglobulin-like α2 or β2 domain), a transmembrane domain and a short carboxy-terminal cytoplasmic domain. The E_α gene is not polymorphic and is located in the E subregion, whereas the other three genes, A_β, A_α and E_β, are highly polymorphic and reside in the A subregion (see Klein *et al.*, 1981; Hood *et al.*, 1983; Steinmetz and Hood, 1983; Flavell *et al.*, 1985).

The expression of Ia antigens is normally restricted to a subset of cells of the immune system, in particular to B lymphocytes, to antigen-presenting cells such as macrophages, dendritic cells and Langerhans cells and to thymic epithelium (see Moller, 1976). In addition, Ia has been observed in several non-hematopoietic tissues: e.g., renal tubule cells (Hart and Fabre, 1981), astrocytes (Wong *et al.*, 1984), vascular endothelium (Pober *et al.*, 1983) and thyroid follicular cells (Hanafusa *et al.*, 1983).

Ia molecules located on the surface of antigen-presenting cells, in concert with other foreign antigens, activate helper T cells (T_H cells). This T_H cell activation is required for induction of most cell-mediated and B cell antibody responses (Nagy *et al.*, 1981; Corley *et al.*, 1985). In the thymus, class II molecules are expressed on epithelial cells, dendritic cells and macrophage-like cells and play an important role in the generation of class II MHC restriction and self-tolerance of T_H cell precursors (Kast *et al.*, 1984). Although constitutively expressed by these cells, as well as by mature B cells (Kearney *et al.*, 1977), Ia antigen expression is found in some macrophages and in non-hematopoietic cells only after induction by γ-IFN (see Unanue *et al.*, 1984).

Although the I region has been extensively characterized for several haplotypes at the molecular level (Steinmetz *et al.*, 1982; Steinmetz, 1984; Widera and Flavell, 1985), the molecular mechanisms governing the complex developmental and tissue-specific expression of class II genes are not yet understood. Transgenic mice, produced by microinjection of a foreign gene into fertilized mouse eggs and implantation of the microinjected embryos into foster mothers (Gordon *et al.*, 1980; Brinster *et al.*, 1981; E.Wagner *et al.*, 1981; T.Wagner *et al.*, 1981; Harbers *et al.*, 1981), have proven to be useful tools in the investigation of gene expression *in vivo*. Appropriate and tissue-specific expression of the microinjected DNA has now been achieved for several genes (Brinster *et al.*, 1983; Grosschedl *et al.*, 1984; Storb *et al.*, 1984; Swift *et al.*, 1984; Shani, 1985; Townes *et al.*, 1985). In search of a system which could be used to investigate the regulation and consequences of expression of genes which are normally tissue-restricted and inducible, we introduced the class II $E_\alpha{}^d$ gene into C57BL/6 × SJL (H-2^b haplotype × H-2^s haplotype; b × s) F_2 mice. These mice do not express the endogenous E_α gene due to a deletion in the promoter region of this gene in both parental haplotypes (Mathis *et al.*, 1983). Because of the failure to express an E gene product, the I-E heterodimer does not appear on the cell surface even though the E_β genes are expressed and proteins are present in the cytoplasm (Jones *et al.*, 1981).

Here we describe the analysis of transgenic (b × s) F_2 mice carrying a cloned E_α gene of the H-2^d allele. We have examined the transcription of the foreign E_α gene in various tissues of several of these mice and compared it with the transcriptional pattern of the endogenous class II A_β gene. In addition, we have examined the cell surface expression of the I-E heterodimer in the transgenic mice by investigating self-tolerance, allorecognition and antigen presentation by the expressed I-E molecule.

Results

One hundred and fifteen one-cell eggs microinjected with the cloned $E_\alpha{}^d$ gene were transferred into pseudopregnant foster females. The $E_\alpha{}^d$ gene used in this experiment is located on cosmid I^d-1 (Hyldig-Nielsen *et al.*, 1983; Figure 1). The cosmid

© IRL Press Limited, Oxford, England.

was linearized by *Cla*I digestion and ~46 copies of the cosmid were injected per fertilized mouse egg. Fifteen pups were weaned and four of these integrated the $E_\alpha{}^d$ DNA. The number of genes integrated into the cells of each mouse ranged from 2 to 19 copies per cell (Table I).

As a first step in the investigation of expression of the inserted gene, we analyzed spleen RNA for $E_\alpha{}^d$ specific mRNA sequences. All four mice carrying the $E_\alpha{}^d$ gene expressed the gene in this tissue, most likely within B cells. The size of the $E_\alpha{}^d$ mRNA was ~1300 nucleotides, the same as the E_α mRNA in BALB/c mice (Figure 2). In three of these four mice, the level of expression was comparable with the level of expression of the A_β gene, which we monitored in parallel as an endogenous control (Table I, Figure 2). Mouse 25-4, which contained 19 copies of the $E_\alpha{}^d$ gene, expressed the gene at a level of ~10-fold higher than the internal control.

Tissue-specific and inducible expression of the transgene.

Four of the transgenic mice carrying the $E_\alpha{}^d$ gene were tested for tissue-specific expression of the transferred gene. An equal amount of RNA isolated from different tissues was analyzed by Northern blotting for the presence of E_α mRNA and, as an internal control, for A_β mRNA. Table I and Figure 2 summarize the results obtained from these experiments. The ratio of E_α mRNA and endogenous A_β mRNA was constant in all tissues of mice 24-3, 27-2 and 27-3 (Table I), therefore the $E_\alpha{}^d$ gene appears to be expressed in a normal tissue-specific fashion. In contrast, mouse 25-4 had a level of E_α gene expression that was elevated in relation to endogenous A_β.

To determine whether the expression of the transgene in macrophages was induced by γ-IFN, we isolated peritoneal exudate cells (PECs) from three mice. Analysis of equal amounts of RNA from PECs with and without γ-IFN treatment showed a very low level of E_α-specific mRNA in PECs without γ-IFN treatment, but a large increase in the level of this mRNA from the γ-IFN treated cells (Table I, Figure 2b).

We tested tissue specificity and inducibility of the transgene in three offspring from mouse 27-2, all of which contained the same gene copy number as the mother. Tissue-specific and inducible expression of the E_α gene was observed in all three animals.

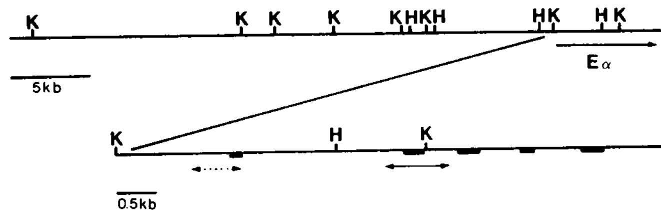

Fig. 1. Map of cosmid I^d-1 (Hyldig-Nielsen *et al.*, 1983) used for injections. The location and 5'-3' orientation of the E_α gene on this cosmid is indicated by the arrow-line, the exon-intron organization is shown in enlarged scale underneath the cosmid map. The hatched double-arrow indicates the ~600-bp deletion in this gene in $H\text{-}2^b$ and $H\text{-}2^s$; the probe used for detection of E_α mRNA (a 1-kb *Pst*I-*Bgl*II fragment) is shown by the solid double arrow. (H: *Hpa*I, K: *Kpn*I; the vector containing the unique *Cla*I site used for linearization is not shown).

Functional expression of the $E_\alpha{}^d$ protein

Membrane expression of the $E_\alpha{}^d$ transgenic protein was assessed by immunofluorescence. Spleen cells from the four transgenic mice and from a control (b × s) F_1 mouse were incubated with monoclonal antibodies (MAbs) specific for I-A^b, I-A^s, and a non-polymorphic determinant (Ia.7) on the E_α chain and subsequently analyzed by flow microfluorometry. The fluorescence profiles (Figure 3) indicated normal membrane expression of an I-E gene product ($E_\beta{}^bE_\alpha{}^d$ and/or $E_\beta{}^sE_\alpha{}^d$) in two of the four mice, 25-4 and 27-2. In these two animals, both the percent of spleen cells expressing I-E molecules (74.2% and 61.5%, respectively) and the approximate membrane density of I-E molecules were similar to those of I-A^b (74.5% and 72.5%, respectively) and I-A^s (75.9% and 72.7%, respectively) molecules. In one of the four mice, 24-3, the percent of cells expressing I-E (26.8%) was significantly lower than that for I-A^s (74.3%); however, this mouse also had a reduced cellular expression of I-A^b molecules compared with the (b × s) F_1 (72.8%). Mouse 27-3 also had an unusually low expression of the I-A^b allele (43.6%) compared with I-A^s (80.7%), but this animal may not have expressed any I-E gene product, because the percentage of cells stained with the anti-Ia.7 MAb (14.9%) was not different from the (b × s) F_1 control (11.3%).

In addition to membrane expression of the $E_\alpha{}^d$ transgene product, we wanted to know whether the product functioned normally as a self-tolerogen, an alloantigen and a restriction element for T_H cells. Spleen cells from two second-generation progeny of mouse 27-2 (27-2-4-1 and 27-2-4-6) were used as both responders and stimulators in a primary mixed leukocyte response (MLR) and as antigen-presenting cells (APC) for two T_H hybridomas specific for a herpes simplex virus glycopeptide in the context of I-E. The high level of [^{3}H]thymidine incorporation and the high stimulation index in the MLR indicate that both progeny express an alloantigen capable of stimulating a primary proliferative response in (b × s) F_1 mice (Table II, lines 1 and

Table I. Expression of H-2 $E_\alpha{}^d$ gene in transgenic mice

Mouse number[a]	Gene copies[b]	$E_\alpha{}^d/A_\beta$ mRNA level[c]								Macrophage induction	Transmission frequency[d]
		Spleen	Thymus	Heart	Kidney	Liver	Muscle	Brain	Bone marrow		
24-3♂	4	+/+	+/+	−/−	tr/tr	tr/tr	tr/tr	−/−	tr/tr	Yes	0/22
25-4♂	19	++/+	+/+	+/−	+/tr	+/tr	nd	nd	nd	nd	Infertile
27-2♀	2	+/+	+/+	−/−	tr/tr	tr/tr	tr/tr	−/−	tr/tr	Yes	6/11
27-3♂	2	+/+	+/+	−/−	tr/tr	tr/tr	tr/tr	−/−	tr/tr	Yes	0/17
Control	−	−/+	−/+	−/−	−/tr	−/tr	−/tr	−/−	−/tr	nd	

[a]These are the founder mice that integrated the $E_\alpha{}^d$ gene.
[b]Gene copies per cell as determined by dot hybridization.
[c]Levels of mRNA for the injected $E_\alpha{}^d$ gene are in the numerator and for the endogenous class II A_β gene in the denominator, as determined by hybridization intensity following Northern analyses. ++ indicates 10 times greater intensity than normal class II mRNA for spleen or thymus; + indicates intensity similar to normal class II mRNA for spleen or thymus; tr indicates barely detectable mRNA; nd, not determined.
[d]Fraction is the number of offspring with the $E_\alpha{}^d$ gene compared with the total number of offspring examined.

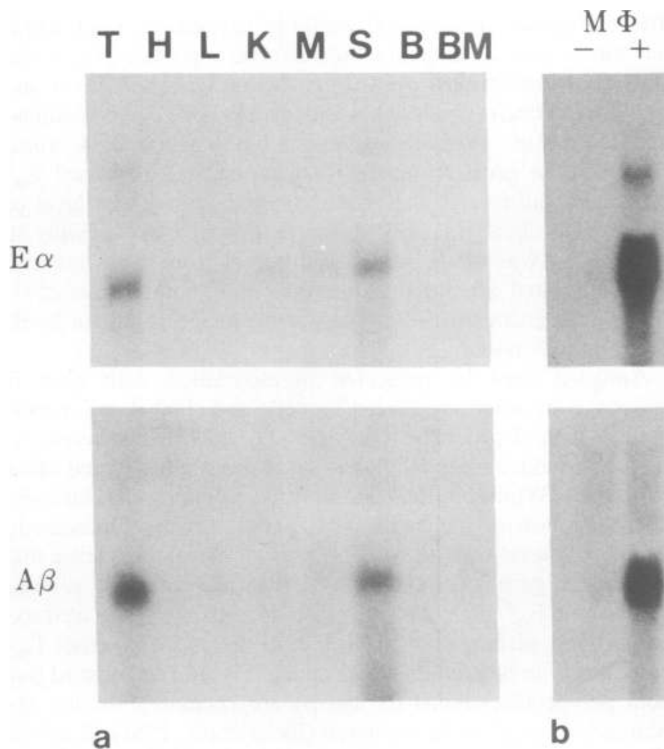

Fig. 2. Northern-blot analysis of total RNA from different tissues of mouse 27-2. 15 μg of total RNA were applied per lane and hybridized with E_α and A_β specific probes, respectively. (**a**) T: thymus; H: heart; L: liver; K: kidney; M: muscle; S: spleen; B: brain; BM: bone marrow. (**b**) MΦ: macrophages; −: incubation without γ-IFN; +: incubation with 100 U/ml of mouse γ-IFN in the medium.

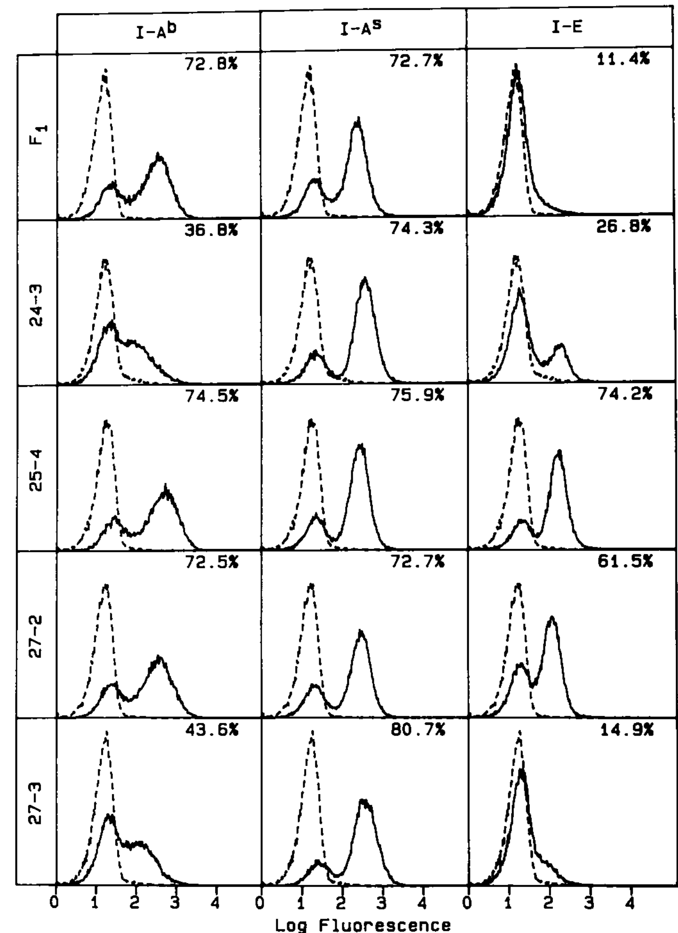

Fig. 3. Cell surface protein products. Spleen leukocytes from a C57BL/6 × SJL F_1 mouse (F_1) and from four individual transgenic animals were incubated with biotinylated MAbs BP107 (anti-I-A^b), 10.3.6.2 (anti-I-A^s) or 14.4.4 (anti-I-E_α) and fluorescein-avidin, then analyzed on a fluorescence-activated cell sorter (FACS-IV) equipped with a log-amplifier. Animal designations are shown on the left of each row, for identification see Table I. The surface antigen examined, by appropriate MAbs, is labelled at the top of each column. Within each diagram, the units on the abscissa represent the logarithm of fluorescence intensity, reflecting the number of molecules on the cell surface. The ordinate represents the relative number of cells. The hatched lines illustrate cells stained with fluorescein-avidin alone (— — —), while the solid line illustrates those cells stained with MAb, then fluorescein-avidin (——). The percent positive cells are given in the upper right-hand corner of each diagram and were defined as cells fluorescing >99% of the cells stained with fluorescein-avidin alone.

2). Both transgenic progeny reacted against the other (Table II, lines 4 and 6) indicating that the two were homozygous for the same haplotype. When these same mice were used as APC donors for the T_H hybridomas restricted to I-E, both transgenic progeny had functional APCs while the (b × s) F_1 did not (Table III). The T cell hybridomas, in the presence of antigen and spleen cells expressing the appropriate MHC restriction element, will produce IL-2. If the MHC restriction element or the antigen are absent no IL-2 will be produced. The supernatants following incubation were added to HT-2 cell cultures containing [^{3}H]TdR. In response to IL-2, the HT-2 cells proliferated and incorporated the [^{3}H]TdR. The stimulation indices show spleen cells from both transgenic mice presented antigen in the context of $E_\beta{}^bE_\alpha{}^k$ to both hybridomas (E_α is not polymorphic, therefore $E_\alpha{}^k$ and $E_\alpha{}^d$ are synonymous alleles). However, the control B.10S(9R), ($E_\beta{}^sE_\alpha{}^k$), did not present antigen to the 2.3.20.21 hybridoma but did present antigen to the second hybridoma (2.3.7.10). These data indicate that the two transgenic offspring analyzed were of the b haplotype and could present antigen to the T cell hybridomas by virtue of an expressed $E_\beta{}^bE_\alpha{}^d$ gene product.

Differential germ line penetrance and fertility

The founder female transgenic mouse was fertile and transmitted the $E_\alpha{}^d$ gene. In contrast, one of the males was infertile and two did not transmit the gene. A total of 39 progeny were produced by the two fertile males (two litters per male) and none of the progeny contained the $E_\alpha{}^d$ gene. These litters were of a size comparable with control matings in our colony. Although male 25-4 mated and vaginal plugs were formed, he was infertile. No sperm were evident in ejaculates and squash preparations of the testis and epididymis revealed no normal sperm and only occasional sperm heads devoid of midpiece and tail.

Six of 11 offspring of female 27-2 inherited the $E_\alpha{}^d$ gene, suggestive of normal Mendelian inheritance. Four first generation offspring were males and they have not fathered any offspring in spite of nine separate matings that included vaginal plug formation. Both female offspring have raised normal litters. Furthermore, second-generation males from these daughters have mated hybrid control females but again no pregnancies have occurred.

Discussion

Our findings demonstrate the functional and tissue-specific expression of a microinjected class II histocompatibility gene in mice that have a defective endogenous E_α gene. Tissue-specific expression of microinjected DNA has been demonstrated convincingly with other genes. Tissue specific expression of the injected

Table II. Mixed leukocyte response[a]

Responder	Stimulator	[^{3}H]TdR incorporation[b]	
		△ c.p.m.	S.I.
(b × s) F_1	27-2-4-1[c]	10400	7.7
(b × s) F_1	27-2-4-6[c]	5700	4.3
27-2-4-1	(b × s) F_1	10700	3.9
27-2-4-1	27-2-4-6	0	<1
27-2-4-6	(b × s) F_1	18200	15.7
27-2-4-6	27-2-4-1	300	1.2

[a]Lymph node cells (2.5 × 10^5/well) from individual responder mice were incubated with their own irradiated (1000 rad) spleen leukocytes (2.5 × 10^5/well) or with those of individual stimulator mice in 200 μl/well of a 96-well round-bottom microtiter plate, at 37°C for 5 days. [^{3}H]TdR (0.5 μCi/well) was added for the final 18 h. C57BL/6 × SJL hybrid F_1 mice were used as controls and are designated (b × s) F_1.
[b]Data are expressed as mean c.p.m. [^{3}H]TdR incorporated into DNA in triplicate wells. △ c.p.m. = (c.p.m. in cultures of responder + stimulator) − (c.p.m. in cultures of responder + responder). S.I., stimulation index = (c.p.m. in cultures of responder + stimulator) ÷ (c.p.m. in cultures of responder + responder).
[c]These mice are F_2 generation progeny of founder mouse 27-2 (see Table I).

Table III. I-E restricted antigen presentation[a]

T cell hybridoma	Spleen cells	[^{3}H]TdR incorporation[b]	
		△ c.p.m.	S.I.
2.3.20.21	27-2-4-1[c]	11900	9.5
2.3.20.21	27-2-4-6[c]	5400	4.9
2.3.20.21	B10.A	13600	4.1
2.3.20.21	B10.S(9R)	600	1.4
2.3.20.21	(b × s) F_1	700	1.6
2.3.7.10	27-2-4-1	25600	17.0
2.3.7.10	27-2-4-6	12900	5.0
2.3.7.10	B10.A	13600	3.8
2.3.7.10	B10.S(9R)	6300	7.3
2.3.7.10	(b × s) F_1	400	1.3

[a]The T cell hybridomas were incubated for 2 days with irradiated (1500 rad) spleen cells with and without antigen (75 μg/ml peptide 1 − 12(H) from herpes simplex virus). Supernatants from triplicate cultures were assayed for IL-2 activity by addition to cultures of HT-2 cells. C57BL/6 × SJL hybrid F_1 mice were used as controls and are designated (b × s) F_1. Alleles of MHC regions and subregions (K, I-A, I-E, I-C, S and D) for the strains used: C57BL/6, bbbbbb; SJL, ssssss; B10.A, kkdddd; B10.S(9R), sskddd.
[b]Data are expressed as mean c.p.m. [^{3}H]TdR incorporated into DNA of HT-2 cells. △ c.p.m. = (c.p.m. in cultures containing supernatants from hybridoma and spleen cells + antigen) − (c.p.m. in cultures containing supernatants from hybridoma + spleen cells). S.I., stimulation index = (c.p.m. in cultures containing supernatants from hybridoma + spleen cells + antigen) ÷ (c.p.m. in cultures containing supernatants from hybridoma + spleen cells).
[c]These mice are F_2 generation progeny of founder mouse 27-2 (see Table I).

$E_\alpha{}^d$ gene occurred in three mice (24-3, 27-2, 27-3) because the $E_\alpha{}^d$ mRNA levels corresponded to A_β mRNA endogenous control levels. This indicated that the transgene was transcribed and that the level of expression was similar to endogenous class II genes, despite evidence that integration of the microinjected gene is thought to be random. Mouse 25-4, with 19 copies of DNA per cell, had the highest level of expression; mice with low to moderate DNA integration had normal levels of mRNA. As anticipated, the highest mRNA expression was observed in primary and secondary lymphoid organs, the thymus and spleen. In the spleen, normally the greatest proportion of cells are B cells, and therefore, expression of E_α was correspondingly high. Likewise, thymic expression of class II would be expected in thymic epithelium as well as resident dendritic cells and macrophage-like cells. The low level of class II expression in kidney, liver and muscle may be the result of blood or lymph node cell contamination (Storb *et al.*, 1984; Townes *et al.*, 1985). Mouse 25-4, which contained the greatest number of gene copies, expressed $E_\alpha{}^d$ message in all tissues analyzed; however, the highest level of $E_\alpha{}^d$ mRNA level was still observed in spleen tissue. Only in mouse 25-4 was the E_α message different from the A_β levels. This suggested abnormal expression of $E_\alpha{}^d$ in mouse 25-4, because the endogenous A_β gene was expressed at normal levels in the tissues tested.

Antigens must be presented in association with class II molecules in order to activate T_H cells and elicit B cell activation. A natural product of activated T_H cells, γ-interferon (γ-IFN) can induce class II expression in macrophages and other cell types. While control macrophages contain very little E_α mRNA, following incubation with γ-IFN, a striking increase in the mRNA level was found. This provides strong evidence that the inserted gene is responding to appropriate biological stimuli.

Since the $E_\alpha{}^d$ gene was expressed appropriately, we next examined cell surface class II protein to determine whether $E_\alpha{}^d$ participated in heterodimer formation. It is well established that both polypeptide chains (α and β) are necessary for the appearance of a cell surface antigen (Jones *et al.*, 1981). Employing monoclonal antibodies we were able to detect the combination of $E_\alpha{}^d$ with the endogenous E_β and the presence of class II I-E molecules on the cell surface (Figure 3). Two of the mice, 25-4 and 27-2, had a normal level of $E_\alpha{}^d$ on the surface. Mouse 25-4 had 10 times the normal level of $E_\alpha{}^d$ mRNA but exhibited equal I-A and I-E cell-surface protein. It is possible that the excess $E_\alpha{}^d$ protein is degraded or sequestered in the cytoplasm and that E_β was the limiting factor in controlling membrane expression of the complete antigen. The results from mice 24-3 and 27-3 are different from 25-4 and 27-2. Mouse 24-3 had a normal density of molecules on the cell surface because the peak log fluorescence of I-E (~2.5) was similar to 25-4 and 27-2. However, the number of cells expressing I-E was lower and only 26.8% of cells in mouse 24-3 contained I-E antigen. These data suggest that animal 24-3 could have been mosaic for the $E_\alpha{}^d$ gene. About 10 − 15% of transgenic mice are mosaic (Palmiter *et al.*, 1984; Brinster *et al.*, 1985). The I-$E_\alpha{}^d$ and I-A_β mRNA levels, however, were similar in the total cell population. Consequently other factors related to translation or surface expression of the antigen may be involved. Mouse 27-3 displayed little or no I-E antigen on the cell membrane despite normal levels of mRNA in spleen cells. It is possible that an abnormal $E_\alpha{}^d$ protein was made. Occasionally animals containing growth hormone fusion genes produce specific mRNA and/or hormone that must be abnormal because there is no biological function (Palmiter *et al.*, 1983; Hammer *et al.*, 1984). Normal expression of MHC alleles is thought to be co-dominant, but both 24-3 and 27-3 had diminished cell surface expression of the I-A^b allele. In both animals, fewer spleen leukocyte expressed I-A^b than I-A^s. Furthermore, those that did express, had a lower density of surface antigen molecules. It is curious that these same mice were the ones that had low I-E expression from the injected gene. The number of animals involved is too low to determine whether the relationship is valid, but we are examining progeny from mouse 27-2 to determine if the pattern is repeated.

Since the $E_\alpha{}^d$ protein was present on the cell membrane, we next asked whether it was functional by measuring the mixed leukocyte response and the antigen-presenting ability of cells from

progeny of 27-2. The stimulation of a proliferative response in lymph node cells of (b × s) F_1 control mice by spleen leukocytes of the transgenic mice indicated that the injected gene was indeed functional. Genetically, the only basis for this stimulation would be a mutation in the MHC or, more likely, expression of an I-E gene product containing the E_α^d protein. However, since neither transgenic mouse reacted against the other and both gave a response against the (b × s) F_1 stimulator, it is less likely that the stimulation was the result of an MHC mutation. These data imply that both animals had the same MHC haplotype and the alloantigenic stimulus for the (b × s) F_1 was shared by both progeny. In addition, spleen cells from both of these mice were able to present antigen to I-E restricted T cell hybridomas. Thus, the progeny of mouse 27-2 not only expressed the gene but also formed a completely functional I-E molecule of the cell surface.

Lastly, a very interesting abnormality in reproductive function was observed in male mice. The males were either infertile or did not transmit the injected gene to progeny. It is improbable that the abnormality would be coincidental or a sex chromosome-linked phenomenon since integrations are believed to occur randomly following microinjection, and all of the integrations appeared to result in abnormal male reproductive function. Abnormal expression of the E_α^d gene on sperm cells may have resulted in destruction of sperm carrying the gene. This would prevent transmission of the gene to progeny of 24-3 and 27-3. Animal 24-3 contained a high gene copy number which may have reflected integration on more than one chromosome. Such double integrations do occur in 10 – 15% of transgenic mice (Palmiter *et al.*, 1984; Brinster *et al.*, 1985). If the deleterious effect of the injected DNA is in the haploid sperm cells and the gene segregates, because of multiple integrations, to most sperm cells, the animal may have too few viable sperm to be fertile. Alternatively, an involvement of the T locus may be related to the reproductive phenomenon observed (Sherman and Wudl, 1977; Figueroa *et al.*, 1985). Whether the T locus, in this study, is suppressing male fertility is conjectural, but the appearance of only a few abnormal sperm in the testis and epididymis is similar to findings in some T locus-related male infertility (see Sherman and Wudl, 1977). The DNA fragment that we injected, 43 kb long, may contain elements other than the E_α^d gene that result in infertility, possibly by interacting with genes in the H-2 or T locus. Perhaps it is relevant that three defective mechanisms have been reported to affect E_α expression in mice (Mathis *et al.*, 1983); however, in the analogous human HLA-DR gene no such defects exist. It could be that some I-E alleles expressed in conjunction with certain T locus alleles lead to male infertility and that mutational changes have evolved to offset this reproductive disturbance in mice.

Materials and methods

Embryo microinjection

The pronuclei of fertilized eggs derived from C57BL/6 × SJL (H-2^b × H-2^s; b × s) hybrid females mated to identical hybrid males were microinjected with ~46 copies of the E_α^d gene, by the method of Brinster *et al.* (1985). The E_α^d gene used in this experiment is located on cosmid I^d-1 and was described by Hyldig-Nielsen *et al.* (1983). The cosmid includes the structural gene with 35 kb of 5'-flanking DNA and ~0.5 kb of DNA, 3' of the polyadenylation signal of the gene (Figure 1). Eggs that survived microinjection were implanted into the oviducts of pseudopregnant Swiss foster females.

Analysis of DNA and mRNA

A small piece of tail from weaned mice was analyzed for the presence of the E_α^d DNA by dot hybridization using pBR as a probe, as previously described (Brinster *et al.*, 1985).

RNA from different tissues was isolated using the guanidinium-isothiocyanate method as described by Maniatis *et al.* (1982). Northern analysis of total RNAs was done following procedures described by Broome and Gilbert (1985) with the exception that 0.8% agarose was used in the gels. E_α-specific mRNA was detected by hybridization with a nick-translated probe containing the $\alpha 1$ domain coding exon 2 of the E_α^d gene; A_β-specific mRNAs were detected by hybridization with a probe coding exons 1 and 2 of the A_β^b gene (Larhammar *et al.*, 1983).

Macrophage induction

Mice were injected i.p. with 3.5 ml of 3% thioglycollate, and peritoneal exudate cells were collected 3.5 days after induction in Hank's balanced salt solution supplemented with 0.1% bovine serum albumin (BSA). Cells were washed and plated at 5 × 10^6 cells per 10 cm plate in RPMI-1640 supplemented to contain 10% fetal calf serum (FCS). Cells were allowed to adhere for 2 h, washed gently, twice, then fresh medium was added. γ-IFN-induced cultures received 100 U/ml of mouse γ-IFN (Genentech, Inc.). Cells were harvested for RNA analysis after 2 days in culture.

Fluorescence-activated cell sorter (FACS) analysis

Spleen fragments were removed from mice that had been previously hemisplenectomized. Cell suspensions were depleted of erythrocytes by treatment with Tris-ammonium chloride, and viable leukocytes were isolated by centrifugation on Ficoll-Hypaque. The latter procedure was necessitated by poor viability of the splenocytes and may have distorted the normal ratio of T and B cells. The cells were suspended in iced phosphate-buffered saline (pH 7.5) containing 2% w/v BSA and 0.02% sodium azide (PBA). Cells were incubated for 30 min at 0°C, at a density of 1 × 10^6/100 μl PBS containing 1 μg of biotinylated MAb. MAbs included: 10.3.6.2 (anti-I-$A^{k,f,r,s}$, Ia.7, Oi *et al.*, 1978). BP 107 (anti-I-$A^{b,d,p,q,u}$, Symington and Sprent, 1981), 14.4.4 (anti-I-$E^{k,d}$, Ozato *et al.*, 1980), and MK-D6 (anti-I-$A^{d,p,q}$, Kappler *et al.*, 1981). The cells were washed and re-incubated at 0°C in 100 μl PBA containing 1 μg fluorescein (FITC)-avidin. After 30 min, the cells were washed again, resuspended in PBA and analyzed on a FACS IV (Becton-Dickinson) equipped with a log amplifier. FITC was excited by an argon laser at 488 nm (200 mW) and fluorescence signals brighter than those seen on 99% of control cells (FITC-avidin alone) were considered positive. Data are expressed as relative cell number *versus* $\log_{10}$ fluorescence intensity.

Mixed leukocyte response

Lymph nodes and spleens were removed from individual mice and leukocyte suspensions were made in Hy medium (Kennett *et al.*, 1978) containing 10% FCS (Hyclone, defined), glutamine and antibiotics. Spleen cells were irradiated with 1000 rads., then 2.5 × 10^5 lymph node cells were mixed with 2.5 × 10^5 spleen cells in 200 μl of medium and plated in triplicate in 96-well round-bottom microtiter plates (Costar). After 4 days of incubation at 37°C in 5% CO_2 and air, 0.5 μCi [^{3}H]TdR was added to each well and the cultures reincubated for an additional 18 h. Plates were harvested and DNA synthesis was measured by liquid scintillation in a beta-counter. Data are expressed as mean c.p.m. [^{3}H]-TdR incorporated in triplicate wells. Δ c.p.m. designates c.p.m. in cultures containing lymph node and spleen cells from different mice minus c.p.m. in wells containing lymph node and spleen cells from the same mouse, while the stimulation index (S.I.) is a ratio of the two.

Antigen-presentation

T cell hybridomas were made by fusing B10.A T cells, specific for the herpes simplex virus gD peptide 1-23 (H), to thymoma BW 5147 (Heber-Katz *et al.*, 1985). Hybridoma, clone 2.3.20.2, was selected for use in asessing the functional expression of I-E, because it had previously been shown to be restricted to $E_\beta^k E_\alpha^k$ and $E_\beta^b E_\alpha^k$. Clone 2.3.7.10 was selected to assess $E_\beta^s E_\alpha^d$ function because it is restricted to $E_\beta^k E_\alpha^k$, $E_\beta^b E_\alpha^k$ and $E_\beta^s E_\alpha^k$. Activation of both clones are blocked by MAb 17.3.3 which is specific for a determinant composed of $E_\beta E_\alpha$ complexes (Ozato *et al.*, 1980). Irradiated (1500 rad) spleen cells from 27-2-4-1, 27-2-4-6, B10.A, B10.S(9R) and (b × s) mice were used as antigen-presenting cells in microtiter cultures containing 75 μg/ml of peptide 1-23 (H). After 2 days of culture, supernatants were removed and assayed for IL-2 content by incubation with the IL-2 dependent cell line, HT-2. Data were obtained as c.p.m. of [^{3}H]TdR incorporated into HT-2 DNA and are expressed in the same manner as the MLR rate.

Acknowledgements

We acknowledge G.Waneck for help with the macrophage induction experiments, M.Trumbauer for DNA microinjection, C.Pope for secretarial assitance and Genentech, Inc. for providing murine γ-interferon. This work was supported by grants from Biogen N.V., NIH (AI-16044) and ACS (IM-347) to C.C., NIH (HD-09172) to R.D.P. and NIH (HD-17321) to R.L.B. C.A.P. was supported by an NIH training grant (HD-07155) and G.W. was supported by a fellowship from the Deutsche Forschungsgemeinschaft.

References

Brinster,R.L., Chen,H.Y., Trumbauer,M., Senear,A.W., Warren,R. and Palmiter,R.D. (1981) *Cell*, **27**, 223-231.

Brinster,R.L., Ritchie,K.A., Hammer,R.E., O'Brien,R.L., Arp,B. and Storb,U. (1983) *Nature*, **306**, 332-336.

Brinster,R.L., Chen,H.Y., Trumbauer,M, Yagle,M.K. and Palmiter,R.D. (1985) *Proc. Natl. Acad. Sci. USA*, in press.

Broome,S. and Gilbert,W. (1985) *Cell*, **40**, 537-546.

Corley,R.B., LoCascio,J., Ovnic,M. and Haughton,G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 516-520.

Figueroa,F., Golubic,M., Nizetic,D. and Klein,J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2819-2823.

Flavell,R.A., Allen,H., Huber,B., Wake,C. and Widera,G. (1985) *Immunol. Rev.*, in press.

Gordon,J.W., Scangos,G.A., Plotkin,D.J., Barbosa,J.A. and Ruddle,F.H. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7380-7394.

Grosschedl,R., Weaver,D., Baltimore,D. and Costantini,F. (1984) *Cell*, **38**, 647-658.

Hammer,R.E., Palmiter,R.D. and Brinster,R.L. (1984) *Nature*, **311**, 65-67.

Hanafusa,T., Chiovato,L., Doniach,D., Pujol-Borrell,R., Russell,R.C.G. and Bottazzo,G.F. (1983) *Lancet*, **2**, 1111-1115.

Harbers,K., Jahner,D. and Jaenisch,R. (1981) *Nature*, **293**, 540-542.

Hart,D.N.J. and Fabre,J.W. (1981) *J. Immunol.*, **126**, 2109-2113.

Heber-Katz,E., Hollosi,M., Dierzschold,B., Hudecz,F. and Fasman,G.D. (1985) *J. Immunol.*, in press.

Hood,L., Steinmetz,M. and Malissen,B. (1983) *Annu. Rev. Immunol.*, **1**, 529-568.

Hyldig-Nielsen,J.J., Schenning,L., Hammerling,U., Widmark,E., Heldin,E., Lind,P., Servenius,B., Lund,T., Flavell,R., Lee,J.S., Trowsdale,J., Schreier,P.H., Zablitzky,F., Larhammar,D., Peterson,P.A. and Rask,L. (1983) *Nucleic Acids Res.*, **11**, 5055-5071.

Jones,P.P., Murphy,D.B. and McDevitt,H.O. (1981) *Immunogenetics*, **12**, 321-337.

Kappler,J.W., Skidmore,B., White,J. and Marrack,P. (1981) *J. Exp. Med.*, **153**, 1198-1214.

Kast,W.M., DeWaal,L.P. and Melief,C.J.M. (1984) *J. Exp. Med.*, **160**, 1752-1766.

Kearney,J.F., Cooper,M.D., Klein,J., Abney,E.P., Parkhouse,R.M.E. and Lawton,A.R. (1977) *J. Exp. Med.*, **146**, 297-301.

Kennett,R.H., Denis,K.A., Tung,A.S. and Klinman,N.R. (1978) *Curr. Top. Microbiol. Immunol.*, **81**, 77-91.

Klein,J., Juretic,A., Baxevanis,C.N. and Nagy,Z.A. (1981) *Nature*, **291**, 455-460.

Larhammar,D., Hammerling,U., Denaro,M., Lund,T., Flavell,R.A., Rask.L. and Peterson,P.A. (1983) *Cell*, **34**, 179-188.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.

Mathis,D.J., Benoist,C.O., Williams,V.E.,II, Kanter,M.R. and McDevitt,H.O. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 273-277.

Moller,G., ed. (1976) *Transplantation Reviews*, Vol. **30**, published by Munksguard, Copenhagen.

Nagy,Z.A., Baxevanis,C.N., Ishii,N. and Klein,J. (1981) *Immunol. Rev.*, **60**, 59-83.

Oi,V.T., Jones,P.P., Goding,J.W., Herzenberg,L.A. and Herzenberg,L.A. (1978) in Melchers,F., Potter,M. and Warner,N.L. (eds.), *Current Topics in Microbiology and Immunology*, Springer, NY, pp. 115-129.

Ozato,K., Mayer,N. and Sachs,D.H. (1980) *J. Immunol.*, **124**, 533-540.

Palmiter,R.D., Norstedt,G., Gelinas,R.E., Hammer,R.E. and Brinster,R.L. (1983) *Science (Wash.)*, **222**, 809-814.

Palmiter,R.D., Wilkie,T.M., Chen,H.Y. and Brinster,R.L. (1984) *Cell*, **36**, 869-877.

Pober,J.S., Collins,T., Grimbrone,M.A.,Jr., Cotran,R.S., Gitlin,J.D., Fiers,W., Clayberger,C., Krensky,A.M., Burakoff,S.J. and Reiss,C.S. (1983) *Nature*, **305**, 726-729.

Shani,M. (1985) *Nature*, **314**, 283-286.

Sherman,M.I. and Wudl,L.R. (1977) in Sherman,M.I. (ed.), *Concepts in Mammalian Embryogenesis*, MIT Press, Cambridge, MA, pp. 136-234.

Steinmetz,M. (1984) *Trends Biochem. Sci.*, **9**, 224-226.

Steinmetz,M. and Hood,L. (1983) *Science (Wash.)*, **222**, 727-733.

Steinmetz,M., Minard,K., Horvath,S., McNicholas,J., Srelinger,J., Wake,C., Long,E., Mach,B. and Hood,L. (1982) *Nature*, **300**, 35-42.

Storb,U., O'Brien,R.L., McMullen,M.D., Gollahon,K.A. and Brinster,R.L. (1984) *Nature*, **310**, 238-241.

Swift,G.H., Hammer,R.E., MacDonald,R.J. and Brinster,R.L. (1984) *Cell*, **38**, 639-646.

Symington,F.W. and Sprent,J. (1981) *Immunogenetics*, **14**, 53-61.

Townes,T.M., Lingrel,J.B., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1985) *EMBO J.*, **4**, 1715-1723.

Unanue,E.R., Beller,D.I., Lu,C.Y. and Allen,P.M. (1984) *J. Immunol.*, **132**, 1-5.

Wagner,E., Stewart,T. and Mintz,B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 5016-5020.

Wagner,T.E., Hoppe,P.C., Jollick,J.D., Scholl,D.R., Hodinka,R.L. and Gault,J.B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6376-6380.

Widera,G. and Flavell,R.A. (1985) *Proc. Natl. Acad. Sci. USA*, in press.

Wong,G.H.W., Bartlett,P.F., Clark-Lewis,I., Battye,F. and Schrader,J.W. (1984) *Nature*, **310**, 688-691.

Received on 18 June 1985