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Inactivation of E_α and E_β expression in inbred and wild mice by multiple distinct mutations, some of which predate speciation within *Mus* species

Fabienne Tacchini-Cottier[1,2], Werner E Mayer[1,3], Ann B. Begovich[4] and Patricia P. Jones[1]

[1]Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA
[4]Department of Human Genetics, Roche Molecular Systems, Alameda, CA 94501, USA
[2]Present address: Department of Pathology, CMU, University of Geneva, 1211 Geneva 4, Switzerland
[3]Present address: Max-Planck-Institut fur Biologie, Abteilung Immungenetik, D-72076, Tübingen, Germany

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Abstract

The *H-2* MHC of mice encodes two functional class II heterodimeric proteins: $A_\alpha A_\beta$ (A) and $E_\alpha E_\beta$ (E). While failure to express the A protein has not been reported, a significant proportion of of *H-2* haplotypes in both inbred and wild mice do not express E proteins. We and others have previously characterized the molecular basis for defective E expression in haplotypes from *Mus domesticus* (*b, f, q, s*, from inbred strains) and *M. castaneus* (*w17*, wild-derived) species, identifying six distinct defects in the genes for E_α or E_β. In this report we have extended these studies to other E^- haplotypes, including several from *t*-haplotype-bearing *M. domesticus* mice (*w29, w57, w302*) and one derived from the Asian species *M. bactrianus* (*w301*). Analyses at the protein, RNA and DNA levels were employed to identify the defects in the genes for *Ea* and *Eb*. At least one new defect was identified that prevents E_β expression in a *t*-associated *H-2* haplotypes (*w57*), bringing the number of distinct mutations causing the E^- phenotype to seven. Another *t*-associated haplotype, *w302*, was found to share the same E_β defect with mice of the inbred *q* haplotype and of the *w17* haplotype from *M. castaneus*, while its *Ea* gene contains the deletion carried also by the inbred *b* and *s* haplotypes and by a number of wild haplotypes. The mutations in the *Ea* and *Eb* genes of the *w301* haplotype from *M. bactrianus* were found to be identical to those of the inbred *f* haplotype. This indicates that the origin of the mutations in the *Eb* genes of the *q*, *w17* and *w302* haplotypes and in the *Ea* and *Eb* genes of the *f* and *w301* haplotypes, predated speciation within *Mus*, thought to have occurred ~0.35–1 million years ago. Their maintenance in mouse populations suggests that in certain conditions the failure to express $E_\alpha E_\beta$ proteins may be advantageous and selected for.

Introduction

Class II MHC proteins are polymorphic membrane glycoproteins essential for presenting peptides generated by degradation of endocytosed foreign antigens to $CD4^+$ T cells (T_h). In the mouse there are two isotypic class II heterodimeric proteins, $A_\alpha A_\beta$ and $E_\alpha E_\beta$, and both can present processed peptides to T cells. However, mice carrying four of the 11 classical inbred *H-2* haplotypes (1), largely derived from *Mus domesticus*, and up to 50% of the haplotypes in various wild mouse populations (2) do not express $E_\alpha E_\beta$ proteins. We and others (3–8) have previously characterized the defects in the *Ea* and *Eb* genes of classical inbred haplotypes and found that six distinct mutations prevent the expression of the $E_\alpha E_\beta$ dimer, three in *Ea* and three in *Eb*. The *Ea* genes of the *b* and *s* haplotypes have the identical 627 nucleotide deletion encompassing the promotor region and the first exon (3). This mutation is also found in wild *M. domesticus* (9) and in the

Correspondence to: F. Tacchini-Cottier
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w17 haplotype derived from another species, *M. castaneus* (5,6,10). The Ea^f gene has a single base substitution that creates a stop codon at amino acid –2 in the leader sequence (8), while the Ea^q gene has a single nucleotide insertion at codon 64 which results in a frame shift generating a premature stop codon at residue 69 of the mature protein (8). The Eb^q and Eb^f genes have two distinct mutations in the RNA donor splice site of their first intron. The Eb^f gene has a single base substitution at position 5 while the Eb^q gene has a single base insertion at position 2 or 3 (6,7) The latter mutation is also found in the *M. castaneus*-derived *w17* haplotype (5).The $Eb^{31.1}$ allele of the $H\text{-}2^{w37}$ haplotype has a point mutation creating a stop codon in exon 2 (4).

We have now extended these studies to additional mutant *Ea* and *Eb* genes in *Mus* mice to explore the origins and functional significance of E⁻ phenotypes. We have characterized the molecular basis for the absence of $E_\alpha E_\beta$ expression in the *w301* haplotype derived from *M. bactrianus*, the house mouse of India and Pakistan. This haplotype, obtainable as the C57BL/10 congenic strain B10.BAC1, has null alleles for both *Ea* and *Eb*. We also analyzed the defects in a number of E⁻ haplotypes of *M. domesticus* mice carrying mutations in *t* haplotypes; these are a group of loci in the vicinity of the *H-2* complex on chromosome 17 that affect developmental and genetic processes, causing recombination suppression, segregation distortion, abnormal tail formation and arrested embryonic development in some *t/t* homozygotes (11). Recombination suppression and segregation distortion have been proposed to be responsible for the widespread distribution of *t* haplotypes in *M. musculus* and *M. domesticus* species (12). Klein and collaborators have made the striking finding that more than half of *t*-associated *H-2* haplotypes do not encode expressed E proteins; while most of these have the *Ea* 5′ deletion shared by the inbred *b* and *s* haplotypes, several do not (9). We have characterized the molecular defects in the *Ea* and *Eb* genes in *H-2* haplotypes associated with the E⁻ t^0, t^{w2}, t^{w8} and t^{Tuw8} haplotypes from *M. domesticus* and the *w301* haplotype from *M. bactrianus*.

Methods

Mice

C57BL/10 and BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and subsequently bred in our facilities at Stanford. B10.RFB2 and B10.LG mice were obtained from Dr C. David (Mayo Clinic, Rochester, MN), DBA/1 mice from Dr C. G. Fathman (Stanford), NOD mice from Dr H. McDevitt (Stanford) and B10.BAC1 mice as well as SSH2 were received from Dr Kirsten Fischer-Lindahl (Howard Hughes Institute, Dallas, TX). B10.CAS2, CRO437 (t^{Tuw8}), t^0 and t^{w2} mice were originally obtained from Dr J. Klein (Max Planck Institute for Biology, Tubingen, Germany) and then bred in our facilities. Mice carrying the t^{w5} and t^{w8} haplotypes were obtained from Dr C. Day (Rutgers University, Newark, NJ). Five wild-derived E⁻ haplotypes from mice trapped near Stanford (WM1, WM2, WM3 and WL4, WL6, WL7) were also characterized.

Genetic crosses of t mice

The *H-2* haplotypes associated with semi-lethal *t* haplotypes [t^{Tuw8} ($H\text{-}2^{w57}$); t^{w2} ($H\text{-}2^{w29}$) or the lethal haplotype t^o ($H\text{-}2^{w29\,1}$)] were maintained as *T/t* balanced lethal heterozygotes generated by crosses with *T* strains of varying *H-2* haplotypes. (t^{Tuw8} x $T(H\text{-}2^d)$)F_1, (t^{w2} × $T(H\text{-}2^d)$)F_1 and (t^o x $T(H\text{-}2^q)$)F_1. *T* mutations (Brachyury) cause reduction in the posterior tail vertebra. In general, *T/+* heterozygotes have a short tail, *t/+* heterozygotes have a normal tail and *T/t* heterozygotes are tailless. To be able to test the *H-2* haplotypes associated with *t* mutations for functional E_α and E_β products, *T/t* heterozygotes were crossed with +/+ (non-*t*) B10 ($H\text{-}2^b$ haplotype: E_α^-,E_β^b) and B10.RFB2 ($H\text{-}2^{fb2}$ haplotype: E_α^k,E_β^-) mice. In the progeny, normal tail mice would be $t/H\text{-}2^b$ or $t/H\text{-}2^{fb2}$, whereas mice of shorter tail would be $T/H\text{-}2^b$ or $T/H\text{-}2^{fb2}$. Both *T/+* and *t/+* progeny of at least three independent crosses with B10 or B10.RFB2 were analyzed. *T/t* mice carrying semi-lethal *t* haplotypes (t^{w2}, t^{w8} and t^{Tuw8}) were bred to obtain homozygotes; in the progeny, mice with normal tails correspond to homozygous *t/t* mice, whereas tailless mice correspond to *T/t* heterozygotes. Each mouse was further tested with fluorescent antibodies to confirm its *H-2* genotype.

Northern blot hybridization analysis

Splenic RNA was isolated by the guanidinium isothiocyanate procedure (13). Between 5 and 20 μg of total cellular RNA was electrophoresed on a 1% agarose gel and transferred to nylon membrane (Nytran; Schleicher & Schuell, NH). The E_β probe was a 321 bp *Hin*dIII–*Bgl*I genomic DNA fragment including exon 1. The E_α probe was a 3.4 kb *Sal*I genomic fragment containing exons 2, 3 and 4 (14). The A_β^k probe was a cDNA clone obtained from Dr H. McDevitt (Stanford University) (15). The hybridization conditions were. 42°C, 40% formamide, 4×SSC, 20 mM Tris (pH 7.5), 1×Denhardt solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA and 10% dextran sulfate. The probes were labeled using random hexamer primers and Klenow fragment DNA polymerase (17). Filters were washed at 25°C in 2×SSC, 0.1% SDS (twice for 15 min) and then at 55°C in 0.1×SSC (twice for 2 min). In order to normalize the amounts of RNA analyzed, filters were scanned with a LKB Ultrascan laser densitometer and vallues were adjusted to the amount of *Ab* RNA.

Southern blot hybridization analysis

DNA was extracted from mouse liver or tail (16) and digested with *Eco*RI and *Kpn*I restriction enzymes. The digested DNA was then electrophoresed on a 0.8% agarose gel and vacuum-blotted (Vacublot; America Bionetics, CA) to a nylon membrane (Nytran). The *Ea*-specific probe (see above) was labeled using random hexamer primers and Klenow fragment DNA polymerase (17).

PCR amplification and sequencing of Ea^{w301} and Ea^{w29} cDNA

Ea cDNA was synthesized from 10–30 μg of total splenic RNA, using MuLV reverse transcriptase (BRL). cDNA synthesis and amplification reactions were carried out as previously described (8) The 5′ and 3′ primers used were: AGTCTGCGAAAGCTTCTGAACCCACCA (corresponding to

the 5′ of exon 1) and TAATGCTGGAAACTGCAAGATGAGGTC (corresponding to the 3′ untranslated region). The amplified single-stranded products were cloned into the phages M13 mp18 and mp19. Three to four independent clones were sequenced using Sanger's chain termination reaction in the presence of [^{35}S]dATP (18). Accession numbers for these sequences in GenBank are U13648 for Ea^{w29} and U13649 for Ea^{w301}.

PCR amplification and sequencing of Eb exon1, including its flanking sequences, and Eb exon 2

Genomic DNA (1 μg) from different mouse strains was amplified in a 100 μl reaction, which included PCR buffer (50 mM KCl, 10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin), 250 μM of each deoxynucleoside triphosphate, 50 pM of each PCR primer: (exon 1, GTGTCTCCTCTCCTGCAGCATG and AGCACAAACATCCAGGCTTCTG; exon 2, CGGGCATCTTGTCGGCAGAGAAG and CCTCACCGTGGTTCCGCCCC) and 2 units of thermostable DNA polymerase from *Thermus aquaticus* (Taq; Perkin Elmer, Norwalk, CT) (19). Amplification was carried out for 30 cycles (denaturation, 94°C for 30 s; annealing, 54°C for 30 s; extension, 72°C for 2 min). The amplified products were cloned into M13 EcoK (20) and three or four independent clones were sequenced. GenBank accession numbers are for exon1: U13650 for *Eb*^{ar1}, U1365 for *Eb*^{w29}, U13652 for *Eb*^{w301}, U13653 for *Eb*^{w302}; and for exon 2: U1654 for *Eb*^{w302}, U13655 for *Eb*^{w301}, U13656 for *Eb*^{w29}.

Immunofluorescence analysis

After lysis of red blood cells, spleen cell suspensions were plated at a density of 10^5 cells/ml in 24-well plates (Costar, location, MA) and incubated for 24 h in the presence or absence of 15 units/ml recombinant IL-4 (generously provided by DNAX Research Institute, Palo Alto, CA). Cells were then stained with FITC-conjugated mAb 14-4-4 (anti-E_α) (21), Y17 (anti-$E_\alpha E_\beta^{b,k,s}$) (22), MK-D6 (anti-A^d) (23) and 10-3.6 (anti-$A^{k,f,r,s,u}$) (24) for 25 min on ice in RPMI 1640 medium containing 5% heat-inactivated newborn calf serum and 0.01% sodium azide. After 25 min, 2 μg/ml propidium iodide was added. The cells were then washed and resuspended in medium. The fluorescence of live cells was determined on a Coulter EPICS 753 flow cytometer (Hialeah, FL).

Radiolabeling, immunoprecipitation and two-dimensional (2D) PAGE analysis

Splenic lymphocytes were labeled with [^{35}S]methionine and immunoprecipitation was carried out as described previously (25) using 14-4-4 anti-E_α and a negative control (anti-dextran) antibody. Immunoprecipitated proteins were then separated by 2D PAGE, using non-equilibrium pH gradient electrophoresis (NEPHGE) for the charge separation and 10% polyacrylamide SDS slab gels for the size separation (25).

Results

Analysis of RNA from E⁻ mice

RNA from E⁻ mice was analyzed quantitatively and qualitatively by Northern blots, using probes for both *Ea* and *Eb*. Mice previously-identified as carrying E⁻ haplotypes were separated into two groups. The first group included two B10 congenic strains, B10.LG, which carries the *H-2*^{ar1} haplotype from inbred strain LG, and B10.BAC1, which carries an *H-2* haplotype derived from *M. m. bactrianus* (which we have designated *w301*). Also included in this group were F_1 progeny from crosses between the $E_\alpha^-E_\beta^-$ inbred *q* haplotype strain B10.G and five E⁻ wild mice trapped near Stanford, of which the *w305* haplotype is representative. Immunofluorescence analyses using anti-E antibodies had shown that about one-third of these wild-derived haplotypes (tested in the appropriate F_1 heterozygotes) failed to encode expressed E proteins (our unpublished results).

Splenic RNA from these E⁻ mice was electrophoresed on agarose gels, blotted onto a nylon membrane and hybridized with probes for *Ea* and *Eb* (representative data are shown in Fig. 1). Control RNA used were from E⁺ strain *d* haplotype from BALB/c mice and from the E⁻ strains of the *i4*, *w17*, *f* and *q* haplotypes, since the E⁻ mice carrying these haplotypes express no or aberrant *Ea* and *Eb* RNAs (6). None of the

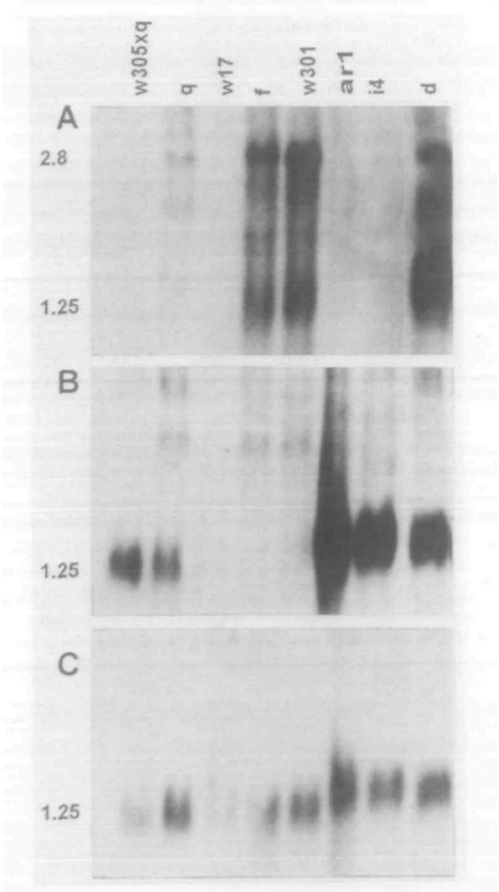

Fig. 1. Northern blot hybridization analysis of class II mRNA from *w301*, (*w305* × *q*)F_1 and *ar1* haplotypes. As controls, RNA was also included for *q*, ($E_\alpha^-E_\beta^-$), *w17*, ($E_\alpha^-E_\beta^-$), *f*, ($E_\alpha^-E_\beta^-$) *i4*, ($E_\alpha^-E_\beta^+$) and *d*, ($E_\alpha^+E_\beta^+$) haplotypes. The same blot was hybridized with probes for *Ea* (A), *Eb* (B) and *Ab* (C).

experimental mice analyzed expressed detectable levels of *Ea* RNA, with the exception of mice of the *w301* haplotype. The RNA pattern from this strain appeared quantitatively and qualitatively similar to the pattern seen with *Ea* RNA from *f* haplotype strains which is not translated due to a translation termination mutation (8) (Fig. 1A). Hybridization with the *Eb* probe revealed normal levels of expression in all the experimental RNAs analyzed, except for the *w301* haplotype (Fig. 1B). Only very low levels of Eb^{w301} RNA were detectable, as is also true for the *f* haplotype with this amount of total RNA. The Eb^{f} RNA is aberrantly processed due to a splice site mutation and is not translated. Identical results to those shown with the $(w305 \times q)F_1$ haplotype were obtained with all five locally-trapped E⁻ wild-derived haplotypes. They express *Eb* but not *Ea* mRNA. The same blot was stripped and reprobed with an *Ab* probe as a positive control for the amount of RNA (Fig. 1C).

Mice of the second group are all *M. domesticus* carrying *t* haplotypes. They include a mouse trapped near Stanford, WL4 of the *w308 H-2* haplotype, made heterozygous with the inbred *q* haplotype by crossing it with B10.G; homozygous *w57* haplotype (carrying t^{Tuw8}); w302 (carrying t^{w8}) and w29 (carrying t^{w2}) haplotype that carry semi-lethal *t* haplotypes; and the homozygous lethal t^{o} haplotype. F_1 heterozygotes of the t^{o} mice with inbred B10 ($E_\alpha^- E_\beta^b$) and B10.G (*H-2*^{q}, $E_\alpha^- E_\beta^-$) mice were used in these analyses. Figure 2(A) shows hybridization of RNA from mice of this group with the *Ea* probe. No *Ea* RNA was detected in the t^{w8} or $(w308 \times q)F_1$ haplotype (the low level of *Ea* RNA found in the F_1 is the low level of aberrant RNA contributed by the *q* haplotype). After normalization with the *Ab* hybridization signals, all other haplotypes appeared to express normal levels of *Ea* RNA, comparable with those of the expressed *d* haplotype. Hybridization using *Ea* probes detects two different RNA species, 1.25 and 2.8 kb in size. The 2.8 kb species is thought to result from the failure to splice out the second intron (3) and is also found in some of the E_α^+ haplotypes, such as *d* and in $E\alpha^-$ *f* haplotype (Fig. 2A).

Figure 2(B) shows the hybridization of this same blot with the *Eb* probe. Hybridization of RNA from *w29* and $(q \times w308)F_1$ haplotypes with the *Eb* probe reveals normal levels of a normal-sized 1.25 kb RNA species. It is difficult to evaluate the RNA from the $(q \times 29.1)F_1$ heterozygote, as a low level of *Eb* RNA (~5% of normal) would be contributed by the *q*

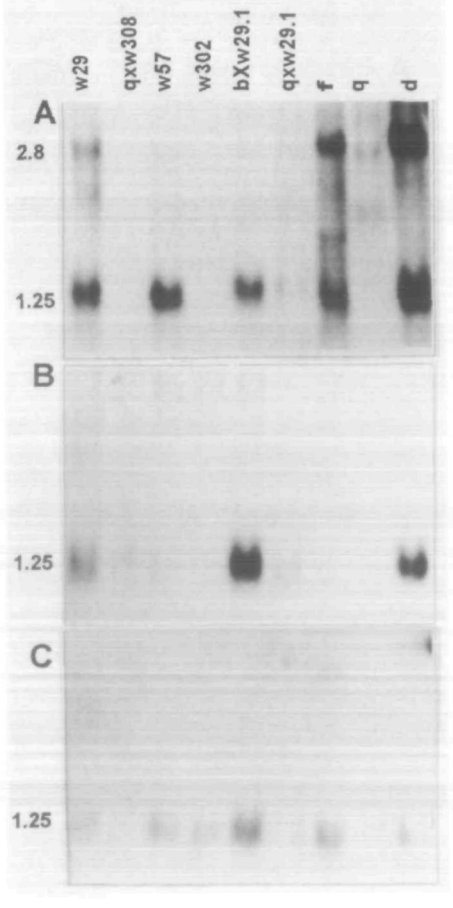

Fig. 2. Northern blot analysis of class II mRNA from mice carrying *t* haplotypes (and their corresponding *Ea*, *Eb* haplotypes *w29*, *w308/q*, *w57*, *w302*, *b/w29.1*, *q/29.2*) and representative control mice *f*, *q* and *d*. The same blot was hybridized with probes for E_α (A), E_β (B) and A_β (C).

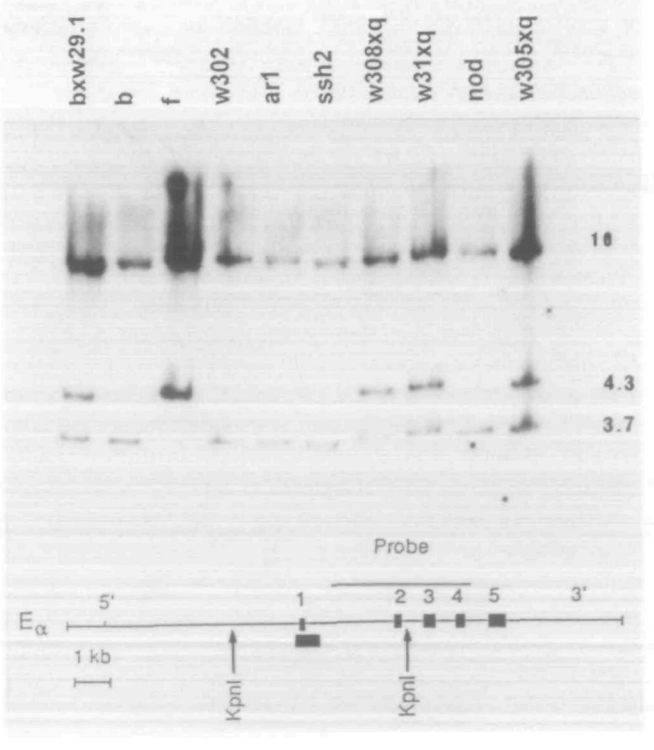

Fig. 3. Southern blot analysis of the *Ea* genes from selected E⁻ strains. Liver or tail DNA was analyzed after digestion with *Kpn*I restriction enzyme. The restriction sites for *Kpn*I are shown by arrows; the black box below the gene represents the site of the E_α^{01} deletion. The area covered by the *Ea* probe used is shown by a line above the gene. *Kpn*I digested DNA generates two fragments hybridizing with this probe, a 4.3 kb band (or 3.7 kb in DNA with the deletion) from the 5′ end of the gene and a 10 kb band from the 3′ end of the gene. The *Ea* gene is represented on the bottom of the figure. *Ea* exons are numbered 1–5.

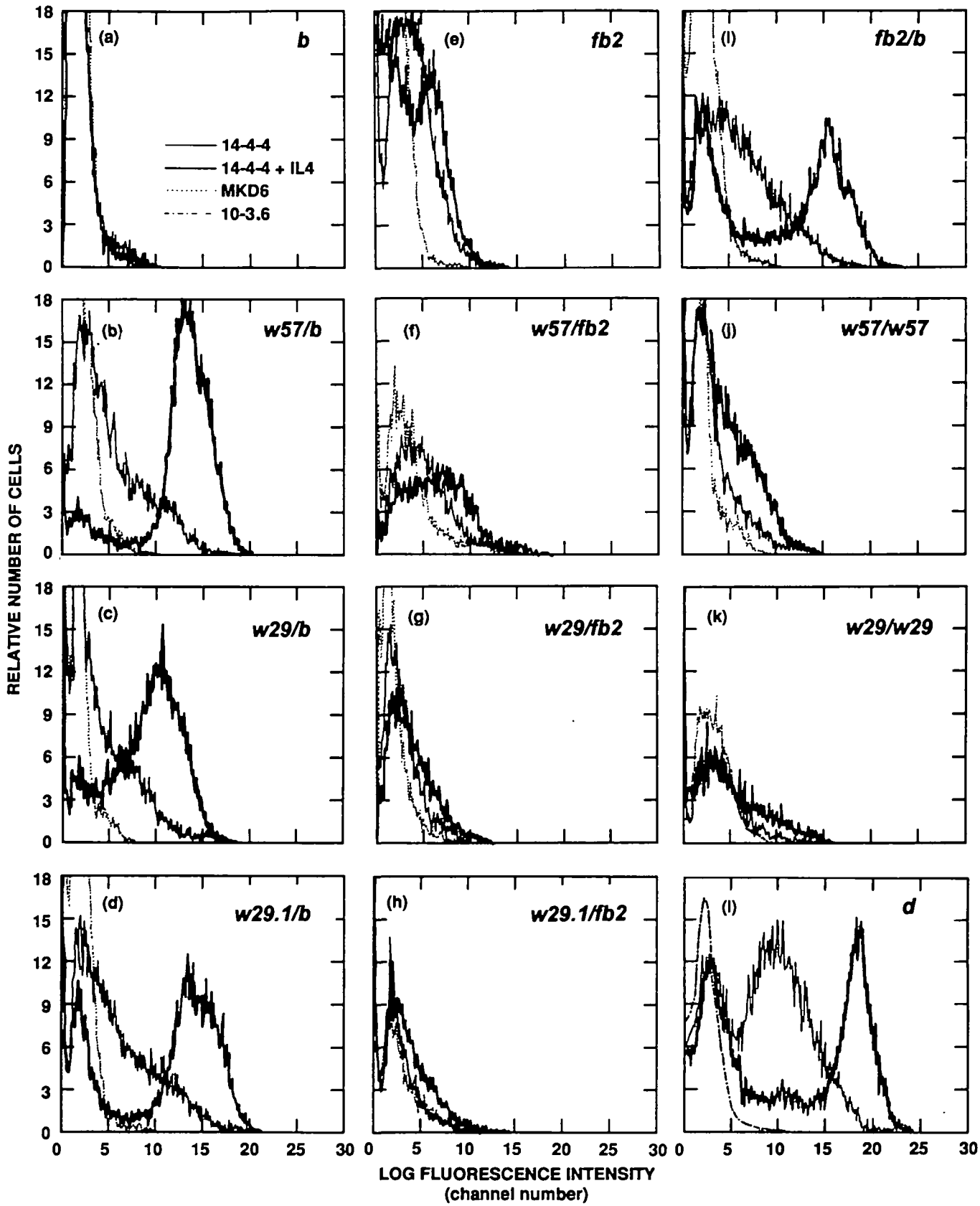

Fig. 4. Immunofluorescence analysis of cell surface class II proteins from (B10×*f*)F_1 and (B10.RFB2×*f*)F_1 mice. Splenic cells were stained with 14-4-4 (anti-*Ea*), MKD6 (anti-A^d) and 10-3.6 (anti-*A* of *k,r,f,s,u*) antibodies. 14-4-4 staining of cells cultured with IL-4 is represented by a bold line. (B10 x *f*) F1 data are shown in panels (b–d), whereas (B10.RFB2×*f*)F_1 data are shown in panels (f–h).

haplotype (6). Nevertheless, the intensity of the *Eb* hybridization appears higher than that of RNA from homozygous *q* mice (DBA/1), considering that similar amounts of RNA were loaded (Fig. 2C). Reduced levels of *Eb* hybridization were detected with *w302* and *w57* RNA.

These Northern blot hybridization analyses thus showed that all experimental mice in the first group (except those of the *w301* haplotype) lack *Ea* RNA but apparently have normal levels of *Eb* RNA. Ea^{w301} and Eb^{w301} RNA give patterns resembling those of *f* haplotype (in both cases the RNAs are defective and are not translated). Mice of the second group, carrying *t* haplotypes, show several patterns. Some haplotypes show normal levels of *Ea* RNA (*w57,w29.1,w29*), while others have none or very low levels of detectable *Ea* RNA (*w308* and *w302*). *Eb* levels appear normal in *w29.1, w29* and *w308*, but appear abnormally low in *w57* and *w302* haplotypes.

Screening for the Ea^0 deletion by Southern blot analysis

The E^- haplotypes that did not show detectable *Ea* RNA by Northern analysis (Figs 1A and 2A) were tested for the presence of the Ea^0 deletion. This 627 bp deletion encompasses the promoter and first exon in the *Ea* genes of the inbred *b* and *s* haplotypes (3) and is also present in about half of the *t*-bearing mice of the *M. domesticus* and *M. musculus* species (9). Genomic DNA was digested with *Kpn*I and subjected to Southern blot analysis using an *Ea* probe that identifies the presence of the deletion (Fig. 3). DNA from *b* haplotype was used as a positive control for the deletion; Ea^b DNA generates a 10 kb *Ea* fragment and a 3.7 kb fragment which spans the region of the deletion. DNA from Ea^f and others lacking the deletion generates the 10 kb fragment and a 4.3 kb fragment containing the region missing in *Ea* genes carrying the deletion. Representative data are shown in Fig. 3.

DNA from mice homozygous for *w302* haplotype clearly has the deletion, as does *the w31* haplotype [which has both the 3.7 and 4.3 kb band from DBA/1(*q*)]. As expected from the Northern analysis, the *w29.1*-derived DNA lacks the deletion; it has both the normal 4.3 kb band and the 3.7 kb band from B10. Homozygous *w29* and *w57* DNA also contained the 4.3 kb band (not shown) and therefore lack the Ea^0 deletion. Heterozygous (*w305*×*q*)F1 and (*w308*×*q*)F_1 DNA showed both a normal 4.3 kb fragment derived from the *q* DNA and a 3.7 kb fragment corresponding to apparent Ea^0 deletions in the haplotypes derived from the *w305* and *w308* wild mouse parents. DNA from *ar1, nod* and *ssh2* mice also produced a 3.7 kb fragment, indicating the presence of the Ea^0 deletion. Therefore, all haplotypes that did not show *Ea* RNA in the Northern blots apparently have the Ea^0 deletion. This was confirmed by Southern blots with another diagnostic restriction enzyme, EcoRI, (not shown). The *w301, w57, w29* and *w29.1* haplotypes were therefore the only E_α^- haplotypes analyzed that have defects other than the Ea^0 deletion.

Analysis of E_α and E_β polypeptide chain expression in $EaEb^{w57}$, $EaEb^{w29}$ and $EaEb^{w29.1}$ mice

Mice carrying these *t* haplotypes express normal levels of *Ea* RNA and both *w29* and *w29.1* haplotypes seem to express normal levels of *Eb* RNA. As no E proteins are detectable on the cell surface by immunofluorescence, E_α and/or E_β protein synthesis must be defective. We took advantage of genetic complementation for $E_\alpha E_\beta$ heterodimer expression in F_1 heterozygotes to determine whether mice carrying the *w57, w29* and *w29.1 H-2* haplotypes synthesize either E_α or E_β chains. Mice carrying these *t* haplotypes were crossed with B10 mice ($E_\alpha^- E_\beta^b$) to assess their ability to synthesize E_α chains and they were crossed with B10.RFB2 ($E_\alpha^k E_\beta^-$) to assess their ability to synthesize E_β chains (additional details about the crosses are provided in Methods). F_1 mice chosen for analysis were those carrying the *t* haplotype and its linked *H-2* complex carrying the defective *Ea* or *Eb* gene. The non-*t* haplotype-carrying F_1 littermates were also analyzed to confirm the representation of both *t* and non-*t* haplotypes in the F_1 progeny (data not shown).

Lymphocytes obtained from either spleen or peripheral blood of the *t*-carrying F1 heterozygotes were analyzed by flow cytometry for expression of $E_\alpha E_\beta$ proteins using FITC-conjugated 14-4-4 mAb. To increase levels of expression of class II [low in some heterozygotes due to gene dosage effects (26)] the cells were cultured with IL-4. Results of these experiments are shown in Fig. 4. Combination of all three *t*-associated *H-2* haplotypes with the *b* haplotype allowed cell surface expression of $E_\alpha E_\beta$ heterodimers, indicating that all

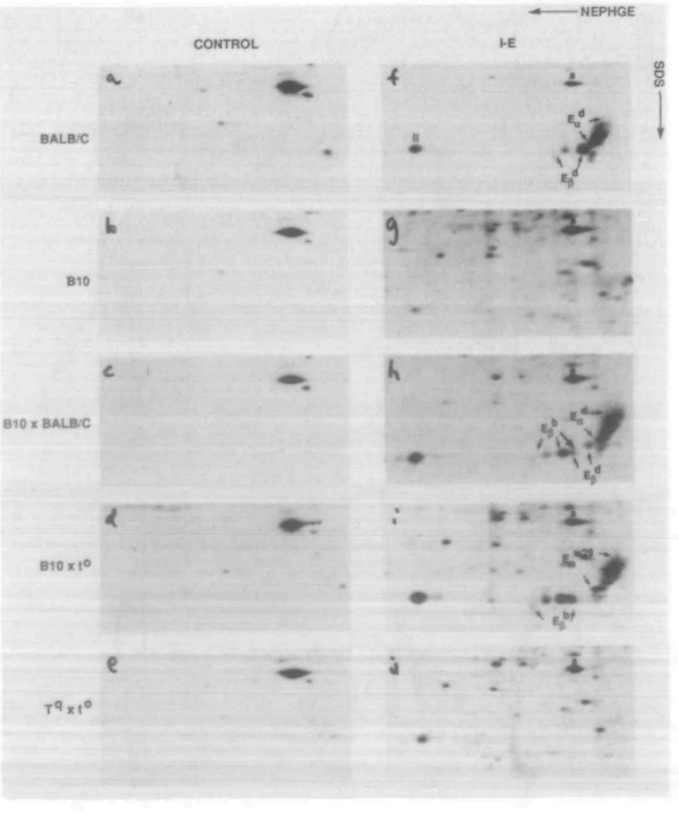

Fig. 5. 2D PAGE analysis of $E_\alpha E_\beta$ immunoprecipitates from t^0 mice carrying the *w29.1* haplotype. [^{35}S]Methionine-labeled E antigens were immunoprecipitated with a control antibody, anti-dextran (panels a–e) and with the 14-4-4 anti E_α mAb (panels f–j). The position of the invariant chain is indicated by Ii, the position of actin (43,000 mol. wt) is represented by the letter a. The spots corresponding to E_α and E_β are shown by the arrows. The panels show the portions of the fluorograms of 2D gels containing the immunoprecipitated proteins.

three haplotypes encode functional E_α polypeptide chains (Fig. 4b–d). The reduced levels of expression in these heterozygotes compared to homozygous $E_\alpha^d E_\beta^d$-expressing cells reflects gene dosage effects (only single copies of functional alleles for E_α and E_β are present in these F_1 mice).

In contrast, crosses between the three *t*-associated *H-2* haplotypes and the recombinant strain B10.RFB2 ($Ea^k Eb^{fb2}$) yielded F_1 mice whose cell surface of the $E_\alpha E_\beta$ heterodimer was equivalent to or less than the parental *fb2* haplotype, which expresses E_α chains but not E_β chains [which is attributed to some expression of free E_α chains (27)] (Fig. 4f–h) This suggests either that the synthesis of the E_β polypeptideis is defective in these haplotypes or that some structural feature prevents these E_β chains from associating with E_α^k chains (not known for any other expressed allelic form of E_β). Homozygous *w57* and *w29* haplotypes also express low but detectable levels of E_α, again probably in the absence of E_β chains. The control cross between strains B10 and B10.RFB2 did result in good levels of E expression, reflecting the association of E_α^k with E_β^b.

To confirm the presence of an expressable *Ea* gene and the absence of an expressable *Eb* gene in one of these E^- *t*-associated haplotypes, $E_\alpha E_\beta$ proteins were immunoprecipitated from [^{35}S]methionine-labeled (b×*w29.1*)F_1 cells and analyzed by 2D PAGE. The t^o haplotype ($H\text{-}2^{w29\ 1}$) is homozygous lethal, so this haplotype was maintained in crosses with B10 (*b*) or B10.G (*q*). In the (b×*w29.1*)F_1 immunoprecipitate (Fig. 5i), the E_β^b chain derived from the B10 parent is coprecipitated with an E_α chain that must be derived from the t^o parent ($E_\alpha^{w29.1}$) allele The E_β^b pattern is identical to that seen in the (*b* x *d*)F1 immunoprecipitate (Fig. 5h) However, unlike this immunoprecipitate, in which E_β^d spots from the BALB/c parent are also evident, only the E_β^b polypeptide is detectable in the (b×*29.1*) F_1 immunoprecipitate. This data provides additional evidence that the $Eb^{w29\ 1}$ gene associated with t^o has a mutation that prevents translation of the *Eb* message into a stable polypeptide chain.

Characterization of defective E_α and E_β expression in the w301 haplotype

Single point mutations in the *Ea* and *Eb* genes in the *f* haplotype result in a stop codon in exon 1 of *Ea* and an altered RNA splice site in *Eb* that produces defective RNA processing (6–8). Because the *w301* RNA patterns obtained by the Northern analyses described above appeared very similar to those of *f* RNA, we examined the nucleotide sequences of the *Ea* and *Eb* genes of the $H\text{-}2^{w301}$ haplotype. *w301* RNA was reverse transcribed and *Ea* cDNA was amplified by PCR using specific *Ea* oligonucleotide primers. The Ea^{w301} sequence was obtained and revealed a nucleotide substitution in codon –2, creating a translation stop codon (TGA) in the coding sequence for the leader peptide that would cause premature termination of translation. We showed previously (8) that an identical substitution was responsible for the lack of expression of E_α^f. The remainder of the *Ea* sequence is homologous to other *Ea* sequences and has no other defects that might affect expression. The putative amino acid sequence that would be encoded in the absence of the stop mutation is shown in Fig. 6, in comparison to other available *Ea* sequences. The only two other nucleotide substitutions in Ea^{w301} that distinguish it from Ea^f would result in changes in amino acid residues 86 and 208. As is apparent from Fig. 6, *Ea* coding sequences are extremely conserved among both expressed alleles [*k* (28), *d* (29), *u* (30), *w29* (see below)] and non-expressed alleles [*f*, *q* (*8*), *w301*, *w28* (31)].

The *Eb* defects reported so far are mutations in RNA splice sites in the first introns of the *f* and the *q* and *w17* (identical mutation) alleles that cause defective RNA splicing (5–7). The similar *Eb* RNA pattern for *w301* and *f* suggested that the defects might be related. This was examined by genomic sequencing. A DNA fragment containing exon 1 and its flanking regions was amplified from *w301* genomic DNA using PCR. The nucleotide sequence of the region including the *Eb* first exon is shown in Fig. 7. The sequence is identical to that of the Eb^f gene and has the same nucleotide substitution (G to A) at position 5 of the RNA donor splice site that is thought to cause defective RNA splicing. Therefore, the *Eb* genes of the *f* haplotype, derived from *M. domesticus* and the *w301* haplotype, derived from *M. bactrianus*, have the identical

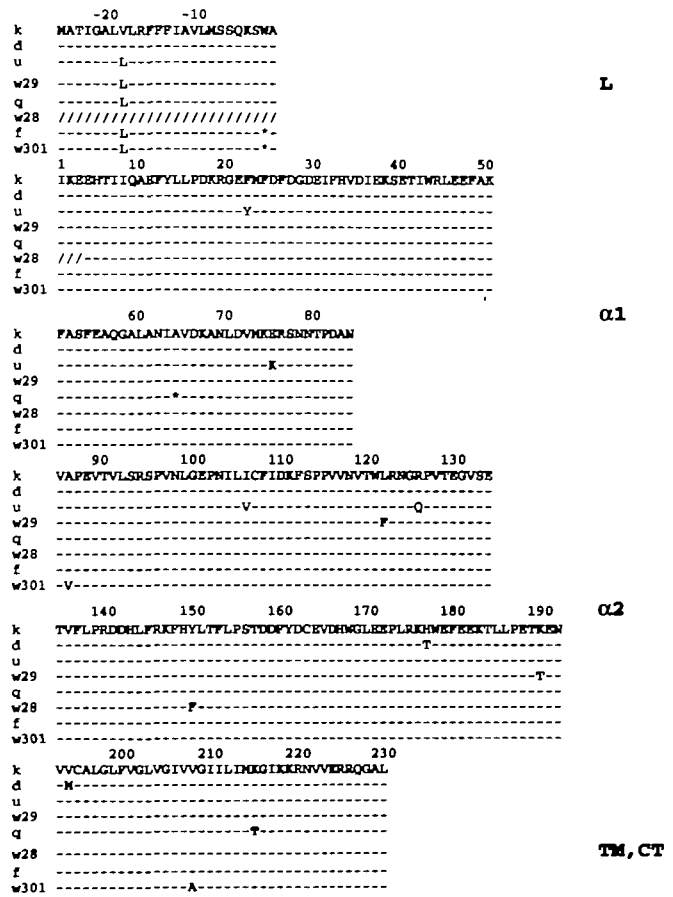

Fig. 6. Predicted amino acid sequences of E_α chains from B10.BAC1 (*w301*) and t^{w2} (*w29*). They are compared to the expressed E_α haplotypes, *k* (38), *d* (50) and *u* (5). The putative sequence of E_α^- haplotypes *q*, *f* (51) and *w28* (6) are derived from cDNA nucleotide sequences, disregarding the nucleotide mutations (*) in the *f*, *q* and *w301* haplotypes and the E_α^{o1} deletion (//////) in the *w28* haplotype.

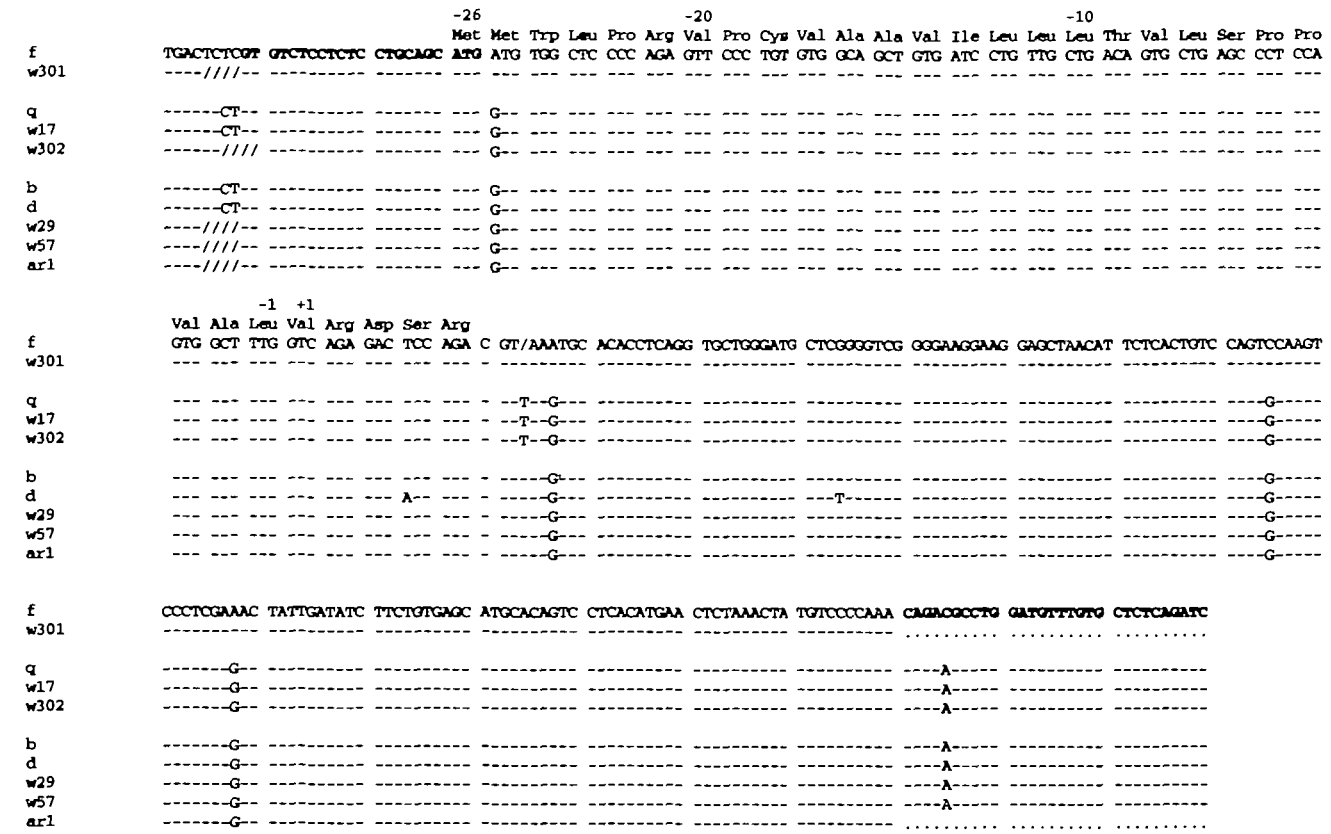

Fig. 7. Nucleotide sequences of *Eb* exon 1 and flanking regions in E_β^+ and E_β^- haplotypes. The PCR primers are depicted in bold characters Numbers above the sequence correspond to amino acid positions.

mutations in their *Eb* genes. These two allelic sequences are in general closely related, differing from the other alleles by three nucleotides in the first intron and one nucleotide at –75 in the 5′ untranslated region. The sequences of the other alleles shown in Fig. 7 will be discussed later.

The relatedness of the *Eb* genes of the *f* and *w301* haplotypes was also examined by obtaining the sequence of PCR-amplified *w301* exon 2 (encoding the polymorphic β1 domain) and its flanking intronic regions. The nucleotide sequence was determined and compared with other expressed (*b, d, k*) and non-expressed (*f, q, w17, w29, w31.1*) alleles in this region (Fig. 8). The sequences show considerable allelic differences among the expressed and non-expressed alleles. The *w301* sequence from B10.BAC1 is most similar to the *f* sequence in this region. However, they differ at two nucleotides; the substitution in codon 93 would be productive, while that in codon 95 would be non-productive. The differences between the *w301* and *f* and other alleles are tabulated in Table 1. The *w301* sequence is closely related to *f*, somewhat related to *q* and *w17* [which are non-expressed and closely related to each other (7)] and least related to the expressed *b* (32), *k* (33) and *d* (34) alleles.

Nucleotide sequences of Eb exon 1 and flanking DNA define three groups of E⁻ mutations

The *f, q, w17* (6) and *w301 Eb* genes all have mutations in the RNA donor splice site at the first exon–intron border. A genomic fragment containing the first *Eb* exon and its flanking sequences has been amplified by PCR from the E⁻ haplotypes of *w301* (described above), *w302, w29* and *w57* haplotypes. The nucleotide sequences obtained were compared with sequences of the expressed Eb^b and Eb^d genes and to those of the non-expressed Eb^{w17}, Eb^q and Eb^f genes (5,7) They are shown in Fig. 7 arranged into three groups by similarities. As mentioned above, the first group consists of the *f* and *w301* genes; their sequences are identical in the region analyzed, including the same mutation in the first RNA donor splice site. The second group contains the previously-analyzed *q* and *w17* genes, in which a thymidine insertion at position 2 or 3 in the first intron has been shown to cause aberrant RNA splicing and to prevent expression (5,7). Also belonging to this group is the Eb^{w302} gene from t^{w8} mice, which shares the same single base insertion. The remaining *Eb* sequences show normal RNA splice sites and are identical to the expressed Eb^b allele in this region. This establishes

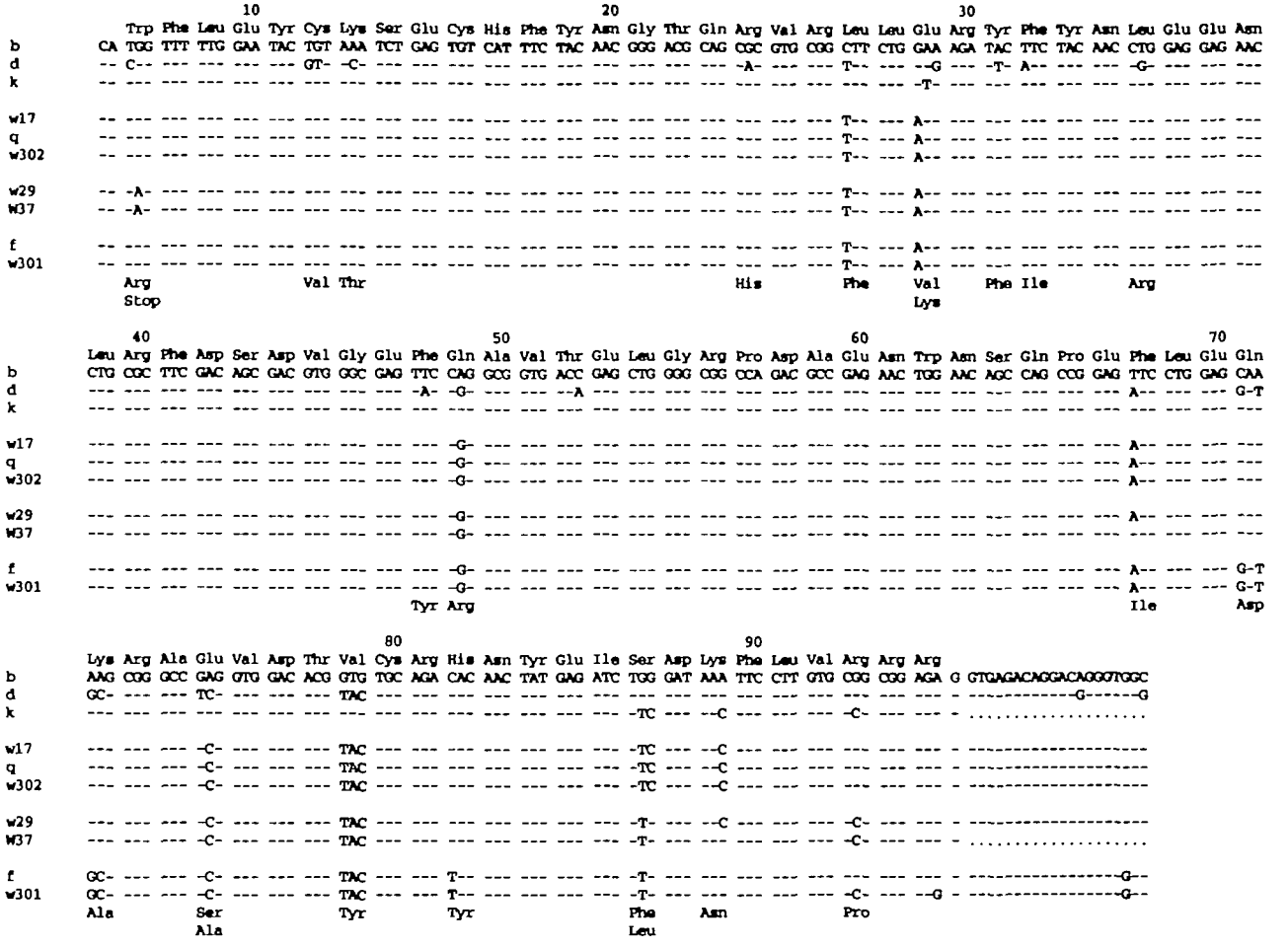

Fig. 8. Comparison of nucleotide sequences of *Eb* exon2 in *w302*, *w301* and *w29* haplotypes Their sequence was compared with the expressed *k* (33), *b* (32) and *d* (34), and non-expressed *w17* (5), *q* (7) and *w37* (4) haplotypes Only differences in nucleotides are noted. The amino acid sequence is that predicted from the *b* haplotype DNA sequence. Numbers above the line refer to amino acid positions, changes resulting in amino acid changes are noted below the sequences. The regions not available for comparison are depicted by (- - - -)

that the mutation(s) responsible for the defective expression of Eb^{w57}, $Ebw^{29.1}$ and Eb^{w29} haplotypes differ(s) from the two previously described *Eb* mutations.

A single nucleotide substitution creates a stop codon in the Eb^{w29} gene

The defect in E expression in the *w29* haplotype of t^{w2} mice apparently is in the expression of the E_β chain, since the E_α chain of this haplotype apparently can pair with $E_\beta{}^b$ in F_1 cells and be expressed (Fig. 4c). In addition, the Ea^{w29} cDNA was sequenced and shown to have no abnormalities (Fig. 6). The presence of essentially normal levels of Eb^{w29} RNA in t^{w2} cells (Fig. 2b) but no E_β protein (Fig. 4) suggests that the block in expression may be at the level of translation. Exon 1 and its flanking regions appear normal (Fig. 7). A fragment containing exon 2 and its flanking regions was amplified by PCR and sequenced as shown in Fig. 8. The sequence of exon 2 of the Eb^{w29} gene reveals a nucleotide substitution at

Table 1. Nucleotide differences between Eb^{w301}, EB^f and other alleles in the polymorphic *Eb* 1 exon

Alleles compared	Divergence (%)	
	Nucleotides (no. of differences)	Amino acids (no. of differences)
w301: b	5.9 (16)	11.5 (10)
w301: d	6 3 (17)	14.3 (13)
w301: k	6.7 (18)	12.2 (11)
w301: w17: and *q*	3.7 (10)	7.7 (7)
w301: f	0.7 (2)	1.1 (1)
f: b	4 8 (13)	10.0 (9)
f: w17	2.6 (7)	5.5 (5)
d: b	8.1 (22)	15.5 (14)

Eb^{w301} is compared with normal *Eb* (*b*, *d* and *k*) and non-expressed *Eb* genes (*w17*, *q* and *f*).

The number of nucleotides or corresponding amino acid differences between the amino acid compared is given in parenthesis.

nucleotide 3 of exon 2, creating a TAG stop codon in residue 7 which would cause translation termination.

This mutation had previously been identified in the *Eb* gene of the *H-2*^{w37} haplotype carried by *t*^{Tuw7} mice (this *Eb* gene is called the *w31.1* allele) (4). Presumably there is no expression of the $E_\beta^{w31.1}$ protein, although no data are available at this point. The *w29* and *w31.1 Eb* alleles differ by only two nucleotides, in codons 68 and 89, which are polymorphic positions among *Eb* alleles.

Eb defects in the w57 and w29.1 haplotypes

The complementation analyses carried out in F1 crosses with B10 and B10.RFB2 mice shown in Fig. 4 also indicate that the *w57* and *w29.1* haplotypes encode functional E_α chains but fail to express E_β chains. The *w57* haplotype found in *t*^{Tuw8} CRO437 mice expresses very low levels of *Eb* RNA (Fig. 2B). However, the sequence of PCR-amplified fragments containing exons 1 and 2 and their flanking regions failed to reveal any mutations that could contribute to its lack of expression. Thus it must be a different mutation from those described above for the *q*, *w17*, *w302*, *f*, *w301*, *w31.1* and *w29* alleles and would constitute the fourth independent *Eb* mutation.

In the *w29.1* haplotype of *t*^{0} mice *Eb* RNA is present [as indicated in Fig. 2B for the (*q*×*w29.1*)F_1 sample]. Given gene dosage effects, the level of *Eb* RNA expressed by *w29.1* may be comparable to that of the *w29* haplotype. The nature of the mutation has yet to be determined.

Discussion

The studies reported here were initiated to understand the origin and significance of null (non-expressed) alleles encoding E_α and E_β chains in mice. A summary of current knowledge of the molecular basis for defective E expression is presented in Table 2. As most of the *Ea* and *Eb* mutations are found in several alleles, each has been given a separate name (*Ea*$^{01-03}$, *Eb*$^{01-04}$).

Evidence now exists for seven distinct mutations, including at least one novel mutation described in this paper, *Eb*^{04}.

Table 2. Summary of current knowledge in $E_\alpha E_\beta$ defects

H-2 haplotype	Mouse name	*Mus* species	E_α	E_β	References
b, s, i4	B10, SJL, B10A(4R)	*domesticus*	E_α^{01}·627 bp deletion (promoter region, RNA init site, exon 1)	normal	(3)
nod	NOD[a]	*domesticus*	E_α^{01}	normal	Fig. 3
ar1	B10.LG[b]	*domesticus*	E_α^{01}	normal	(9), Fig. 3; 1
w303	WM1[c]	*domesticus*	E_α^{01}	normal	Fig. 3
w304	WM2[c]	*domesticus*	E_α^{01}	normal	Fig 3
w305	WM3[c]	*domesticus*	E_α^{01}	normal	Fig. 3
w306	WL6[d]	*domesticus*	E_α^{01}	normal	Fig. 3
w307	WL7[d]	*domesticus*	E_α^{01}	normal	Fig. 3
w308	WL4 (*t*?)[d]	*domesticus*	E_α^{01}	normal	Fig. 3
ssh2	SSH2[e]	*domesticus* or *castaneus*	E_α^{01}	normal	Fig. 3
q	B10.G	*domesticus*	E_α^{02}: nucleotide insertion in exon 2, frameshift, stop codon at codon 69	E_β^{02}: nucleotide insertion in intron 1 at position +2, or +3: defective RNA splicing	(6, 7, 8)
w17	B10.CAS2	*castaneus*	E_α^{01}	E_β^{02}	(9; 5)
w302	*t*^{w8}[g]	*domesticus*	E_α^{01}	E_β^{02}	Figs 3,6; 7,8
f	B10.M	*domesticus*	E_α^{03}· Nucleotide substitution in exon 1: stop codon at codon –2	E_β^{03}: Nucleotide substitution in intron 1 at position +5: defective RNA splicing	(8; 6,7)
fb2	B10.RFB[b]	*domesticus*	normal (k)	E_β^{03} (from f)	
w301	B10 BAC1[e]	*bactrianus*	E_α^{03}	E_β^{03}	Figs. 6; 7,8
w37(*Eb31.1*)	CRO435 (*t*^{Tuw7})	*domesticus*	normal	E_β^{01}: nucleotide substitution in exon 2 generates stop codon at codon +7	(4)
w29	*t*^{w2}[f]	*domesticus*	normal	E_β^{01}	Figs 7, 8
w29.1	*t*^{0}[f]	*domesticus*	normal	normal RNA, no protein (E_β^{01}?)	Figs 3, 5
w57(*Eb31*)	CRO437 (*t*^{Tuw8})[f]	*domesticus*	normal	E_β^{04}: low RNA level, no protein, nucleotides of exons 1 and 2 borders are normal	Figs 2, 7, 8

The haplotypes are grouped according to the nature of their defects.

Source of the mice: [a]Dr H. McDevitt (Stanford University, CA), [b]Dr C. David (Mayo Clinic, MN), [c]trapped in Palo Alto, CA, [d]trapped in Ladera, CA, [e]Dr K. Fischer-Lindahl (Howard Hughes Institute, Dallas, TX), [f]Dr J. Klein (Tübingen, Germany), [g]Dr C. Day (Rutgers University, NJ).

These mutations have been found in the four *Mus* species that have been evolving separately for 0.35×10^6 years (35) and that now occupy distinct geographical ranges. Four distinct mutations (E_α^{01}, E_α^{03}, E_β^{02} and E_β^{03}) have been found in both the Western European species of the highly commensal house mice, *M. domesticus*, and an Asian species, either *M. castaneus* or *M. bactrianus*. The most frequent defect preventing E expression in the mice of the *M. domesticus* species analyzed is the E_α^{01} deletion, a deletion described previously in the inbred *b* and *s* haplotype (3), at high frequencies in wild mice of this species (e.g. in 6/33 haplotypes from wild mice that we trapped) and in nearly half of the known *t*-bearing strains (9), including the *t*-associated *w302* haplotype as shown in this study. The Ea^{01} deletion was also previously reported in the *w17* haplotype of the Asian *M. castaneus* species (3). The Ea^{02} mutation, an insertion that causes a frameshift in exon 2, has been uniquely found in the inbred *q* haplotype (7). The Ea^{03} mutation, a substitution creating a stop codon in exon 1, has been detected in both the inbred *f* haplotype (7) and in the *w301* haplotype derived from *M. bactrianus* (this study). The first *Eb* mutation identified, Eb^{01}, is a nucleotide substitution generating a stop codon in exon 2, initially identified in the *w37* haplotype associated with t^{Tuw7} (4) In this study we have found the same mutation in the *w29* haplotype associated with t^{w2} and possibly also in the *w29.1* haplotype associated with t^0, as these haplotypes fail to encode expressed *Eb* chains and generate identical RNA patterns. Furthermore, the *w29* and *29.1 H-2* haplotypes are related and their associated *t* haplotypes belong to the same complementation groups (2,3). The Eb^{02} mutation has been found in three haplotypes, the inbred *q* haplotype, the *w17* haplotype from *M. castaneus* (6,7) and the *w302 t*-bearing haplotype, t^{w8}, described herein. The Eb^{03} mutation, initially described in the inbred *f* haplotype (6,7), has now been shown to be present in the *M bactrianus*-derived *w301* haplotype.

The sharing of identical mutations inactivating expression of E_α or E_β chains in more than one *Mus* species suggests that in each case the mutation may have occurred prior to speciation and may now be found in closely-related alleles derived from a common ancestor in which the mutation occurred. This hypothesis is difficult to test for *Ea* genes, which show minimal sequence polymorphism (reviewed in 36). However, *Eb* genes do show the extensive allelic polymorphism typical of most MHC genes, with most of the variability located in the second exon which encodes the Eβ1 domain that forms half of the peptide-binding groove. We therefore compared nucleotide sequences of exons 2 from alleles carrying the Eb^{01-03} mutations and from normal, expressed alleles. The alleles sharing identical defects are remarkably similar. This is particularly apparent for the Eb^{02} mutation, where the three alleles showing this defect (*q, w17, w302*) are completely identical through this region. For the *q* and *w17* alleles all exons and flanking intronic regions have been sequenced and differ by only 7/3108 nucleotides (0.23%) (7). The differences are all located in introns, mostly in the recombination hot spot in the second intron (7). The similarity of the *Eb* exon 2 sequences between the two *M. domesticus*-derived alleles, *q* and *w302* and the *M. castaneus*-derived *w17* allele, including their sharing the same Eb^{02} mutation, agrees with a divergence time of 0.35–1 million years, which is the time these two species diverged about (35). This suggests that the mutation occurred in a common ancestral *Eb* gene prior to the divergence of the *M. domesticus* and *castaneus* species. We have shown previously that these three haplotypes also include related alleles for the closely-linked *Aa* class II gene (37).

The *Eb* genes of the *f* and *w301* haplotypes, derived from *M. domesticus* and *M-bactrianus* respectively, also share the same defect, the Eb^{03} mutation and also have very similar nucleotide sequences in the polymorphic second exon, differing by only two nucleotides. In contrast, Eb^{w301} differs from other alleles by up to 18 nucleotides (Table 2) These results suggest that Eb^f and Eb^{w301} alleles could also have been derived from a common ancestral gene that diverged from other *Eb* alleles before the divergence of *M. domesticus* and *M. bactrianus*. Similarly, the Ea^{03} mutation common to these two haplotypes must also have predated subspeciation.

Given that these *Ea* and *Eb* mutations inactivate expression of one of the two isotypic forms of class II proteins in mice, the persistence of certain mutations through speciation within *Mus* and the high frequencies of E^0 haplotypes in current mouse population are a challenge to understand. One explanation proposed for the high occurrence of E^0 haplotypes is that they could have been maintained in mouse populations because of their linkage to the *t* haplotypes that are transmitted at high frequency due to segregation distortion (9). According to this model the *t* haplotypes serve as a reservoir for E^0 mutations, recombining at a low rate onto non-*t* chromosomes. However, recent views on the origins of *t* haplotypes suggest that this model probably does not explain the presence of individual Ea^0 or Eb^0 mutations in multiple *Mus* species. According to a recent review by Silver (12), the primordial *t* haplotype generated by the accumulation of inversions in the segment of chromosome 17 containing *H-2* predated speciation within *Mus* but has been maintained only in *M. domesticus* of the current species. (The presence of *t* haplotype in *M musculus* is thought to be due to introgression from *M. domesticus*.) Examination of the sequence divergence of these genes included in *t* haplotypes suggests that all current *t* haplotypes descended from a common ancestor in *M. domesticus* as recently as 10,000–100,000 years ago (12). Thus it is unlikely that the maintenance of several of the Ea^0 and Eb^0 mutations in multiple *Mus* species is due to their association with *t* haplotypes.

Instead, it appears that the origin of these E^0 mutations pre-dated speciation, providing additional examples of the trans-species origin of MHC polymorphism (38,39). However, understanding the selective pressures maintaining null E alleles through speciation is more difficult than understanding why multiple alleles of functional MHC proteins are maintained. The absence of E expression potentially would be disadvantageous, as some peptide antigens are recognized in the context of this class II protein. Expression of the E class II protein also influences the TCR repertoire through the positive and negative selection in the thymus of T cells with specific V_β segments (7,40,41). Clonal deletions in E-expressing mice appear to be caused by the preferrential binding and presentation by E proteins of endogenous mouse mammary tumor virus (MMTV)-encoded superantigens (42). Such deletional

effects have been shown to protect the host against infections by exogenous MMTVs (43,44) and possibly also from diseases caused by bacteria whose superantigens contribute to infectivity or pathology and have the same V_β specificity (45). Therefore it is puzzling to find so many distinct mutations preventing E expression, some of which have been maintained in mouse populations since before speciation and/or may be present at high frequencies.

These observations suggest that in certain situations expression of E proteins may be disadvantageous and therefore selected against. For example, E-expressing mouse strains may be more susceptible to infection by parasitic nematodes than some E^- strains (46). Perhaps more significantly, the binding of superantigens, in general more common in E than A class II proteins (42), itself may be harmful. The strong polyclonal *in vivo* T cell responses induced by superantigens can be extremely destructive, leading under some conditions to shock and death. In addition, recent studies in several laboratories have indicated that the degree of host susceptibility to MMTV infection *in vivo* is dependent on expression of E molecules, presumably reflecting superantigen-induced T cell activation which enhances infection of the T cells by the virus (47,48). Absence of E expression decreases infectivity by the virus and therefore is beneficial to the host. Interestingly, endogenous superantigens have been reported only in mice and endogenous MMTV proviral genomes have been integrated in the mouse genome only for an estimated 1–5 million years (49). Mice thus far are the only species described as having significant frequencies of null alleles that inactivate expression of a class II isotype. This correlation between the occurrence of endogenous superantigens presented actively by E proteins and the establishment of null E alleles suggests that these two phenomena may be related. The presence of endogenous MMTV proviruses and perhaps other superantigens as well, may have created the selective pressures responsible for the maintenence of E^0 mutations during speciations in *Mus* and at high frequencies in current mouse populations.

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Abbreviation

MMTV mouse mammary tumor virus

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