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Immunology

Expression in L cells of transfected class I genes from the mouse major histocompatibility complex

(genomic clones/*H-2* region/restriction fragment length polymorphism)

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ABSTRACT **One of the major surprises of the molecular analysis of major histocompatibility complex (MHC) genes is the large number of class I (*K/D*)-related sequences in the genome. Both restriction fragment length polymorphisms and cosmid cloning experiments showed them all to be closely linked to the MHC. Until now little information was available concerning either their expression or recognition by the immune system. Here we report that these non-*K/D* genes can provoke antibody responses and be recognized by cytolytic T cells. Immunization of C3H mice with L cells transfected with class I genomic clones resulted in antisera that reacted preferentially with cells from strain B10.P (the gene donor). Thus, these genes can be expressed by L cells. These products were recognized by cytolytic T cells produced by mixed lymphocyte culture with B10.P stimulators. One gene, represented in clone λ3a, was chosen for further analysis. A restriction fragment length polymorphism, detected between B10.P (K^pD^p) and B10.F(14R) (K^bD^p) and between B10 (K^bD^b) and B10.F(13R) (K^pD^b), has enabled us to map the λ3a sequence to the *D* or *Tla* region. Restriction endonuclease mapping of the λ3a clone shows that the gene is intact and that, although many restriction sites are conserved, the gene in λ3a differs from other class I genes. When the λ3a clone was transfected into mouse L cells, a new product was expressed. Cells expressing this product (designated L3a cells) were killed by primary *D*-end-reactive, allospecific cytolytic T lymphocytes. The L3a cells were unreactive with monoclonal antibodies specific for the K^p,D^p,Qa-2, Tla.3, and Tla.5 molecules.**

The classical class I histocompatibility molecules of the mouse, H-2K, H-2D, and H-2L, are 45-kDa membrane-bound glycoproteins. These molecules are found on the cell membrane in association with β_2-microglobulin. The K, D, and L class I molecules function in the immune recognition of viral cell-surface antigens as well as allogeneic cells by cytolytic T lymphocytes (CTLs) (1, 2). This obligatory self-recognition is termed H-2 restriction. Localized telomeric to the *D* gene are the *Tla* and *Qa* regions, which contain other members of the class I gene family. These genes code for the Tla, Qa-1, and Qa-2 antigens which are expressed primarily on hematopoietic cells (3). Qa antigens are found on thymocytes, peripheral T and B cells, and lymphoblasts, whereas Tla antigens are found on intrathymic T cells and some T-cell leukemias. Unlike H-2 K, D, and L antigens, Qa and Tla do not elicit a primary mixed-lymphocyte response (MLR) or function as target cells of primary CTLs (4, 5). Qa and Tla antigens can, however, function as unrestricted CTL targets and can be detected by *in vivo* "priming" followed by *in vitro* "boosting" (6). By virtue of their limited expression compared to K or D, Qa and Tla antigens are thus tissue-specific class I antigens (3).

Molecular analysis has shown that *K, D, L,* and *Qa/Tla* represent only a fraction of the class I gene family. Southern blot analysis and cosmid cloning have shown that the mouse class I gene family has 25–35 members, all encoded on chromosome 17 (7). It is not known which, if any, of the non-*K/D/L/Qa/Tla* class I sequences are transcribed or have any biological function.

The possibility that the *Tla* and *Qa* genes are also expressed in nonhematopoietic tissues has been suggested (8–10). The DNA sequence of the cDNA clone pH16 suggests an unusual peptide, which lacks a transmembrane region and would probably be a secreted product. Mellor *et al.* (11) determined the nucleotide sequence of a class I genomic clone (Q10), located in the *Qa-2,3* region, to be identical to the pH16 cDNA sequence.

The isolation of genomic DNA clones encoding class I molecules and their subsequent transfer and functional expression in mouse L cells has allowed us and others to characterize cell surface major histocompatibility complex (MHC) molecules and to perform immunologic experiments on the role of class I gene products (12–19). We have begun to use DNA-mediated gene transfer to characterize a series of genomic clones isolated from a mouse strain B10.P sperm library. Here we describe evidence for the expression of several of these genes in L cells, and a more detailed analysis of one of the clones (λ3a). Immunization of C3H/HeJ ($H\text{-}2^k$) mice with transfected L cells resulted in the production of antibodies specific for B10.P ($H\text{-}2^p$) spleen cells. Similarly B10 anti-B10.P killer cells recognize L cells transfected with B10.P class I genes but not L cells transfected with the herpesvirus thymidine kinase (TK) gene. The λ3a product therefore functions, as do the K and D class I molecules, as a target for primary allospecific CTLs. The functional significance of the λ3a gene and related class I genes is discussed.

MATERIALS AND METHODS

Mice. All mice (except C3H/HeJ) were bred and housed in our animal colony at the University of North Carolina. The C3H/Fre subline was descended from breeders provided by F. Bang (Johns Hopkins, Baltimore, MD). C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

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Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; RFLP, restriction fragment length polymorphism; bp, base pair(s); kb, kilobase(s).

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Cell Lines. The C3H (*H-2*^{k}) mouse fibroblast cell line LTK$^-$ was derived from a thymidine kinase-deficient mutant of L_{929} cells. The Rat-2 cell line is a TK$^-$ fibroblast line derived from Fischer rats. L3a, L5b, L12a, L15a, and L17a are cloned cell lines derived by the cotransfection of LTK$^-$ cells with the herpes TK gene and class I genomic clone λ3a, λ12a, λ15a, or λ17a. The genomic DNA clones were selected from a B10.P sperm DNA library as described (12). Transfection of LTK$^-$ cells was as described (12). Cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum, 10 mM Hepes, penicillin and streptomycin, and hypoxanthine/aminopterin/thymidine (20).

Monoclonal Antibodies and Alloantisera. All H-2^p-specific monoclonal antibodies used have been described (21). All monoclonal antibodies were elicited by intraperitoneal injection of lymphoid cells. Alloantiserum (C3H/HeJ anti-L-cell antibodies) was produced by multiple intraperitoneal immunizations with 5×10^7 irradiated (3000 rads) transfected L cells. Fischer rats were immunized by intraperitoneal injection of 2.5×10^7 transfected R3a.6 cells. Qa-specific alloantisera were kindly provided by L. Flaherty (New York State Department of Health, Albany), and Tla-specific monoclonal antibodies by F. W. Shen (22, 23).

Restriction Enzyme Mapping of the λ3a Gene. The cloned λ3a DNA was characterized by digestion with a variety of restriction endonucleases [New England Biolabs or International Biotechnologies (New Haven, CT)]. λ3a DNA was digested with appropriate enzymes for 3 hr at 37°C and then electrophoresed for 500 volt·hr in 0.8% agarose gel and blotted to nitrocellulose according to the method of Southern (24). Blots were hybridized with the class I cDNA probes pH-2III (5′) and pH-2IIa (3′), as described (12, 25).

Mapping of Restriction Fragment Length Polymorphisms. RFLPs were studied by mapping genomic DNAs prepared from livers of B10.P (K^pD^p), B10.F(13R) (K^pD^b), B10.F(14R) (K^bD^p), and B10 (K^bD^b) mouse strains. The genomic DNAs along with λ3a DNA were digested with *Sac* I for 3 hr at 37°C and then electrophoresed in agarose gels. Gels were blotted and the blots were hybridized with the nick-translated cDNA clone pH-2IIa.

Analysis of CTL Target Function. Alloreactive CTLs were produced according to published procedures (26, 27). Erythrocyte-depleted spleen cells were mixed at equal concentrations (2.5×10^7 each) of responders and irradiated (2000 rad) stimulators, and cultured for 5 days at 37°C in a humidified 7.5% CO_2 atmosphere in 20 ml of Click's medium with 5% fetal bovine serum. CTL activity was measured in a standard 4-hr ^{51}Cr-release assay (27). Percent specific lysis was calculated as [(cpm experimental − cpm spontaneous)/(cpm maximal − cpm spontaneous)] × 100.

Complement-Mediated Microcytotoxicity Analysis. The reactivity of alloantiserum from C3H/HeJ anti-L3a immune mice was studied with a panel of inbred and *H-2* recombinant inbred mouse strains in a two-step complement-dependent microcytotoxicity assay (28). Selected rabbit serum was used as a source of complement. Splenocytes were isolated after sedimentation with Lympholyte M (Cedarlane Laboratories, Hornby, ON) and the test was performed in duplicate in Terasaki plates. Complement-only (control) cytotoxicity was always <10%.

RESULTS

Transfected Cells Induce H-2^p Reactive Antibodies. Several class I genomic clones have been isolated from a B10.P library that were found not to encode classical K or D antigens. To determine whether these cloned class I genes can be expressed, we used L cells transfected with class I genes to provoke an antibody response in C3H mice. It has been shown that such an approach is feasible and that immunization of C3H mice with transfected L cells evokes an antibody response against the transfected-gene product (29). When C3H mice, which are syngeneic with the original L_{929} cell line, were immunized with *H-2*^{p}-transfected L cells, antibodies with B10.P derived cells were evoked. Fig. 1 shows the results of a cytotoxicity test with C3H anti-L3a, C3H anti-L5b, C3H anti-L12a, C3H anti-L15a, and C3H

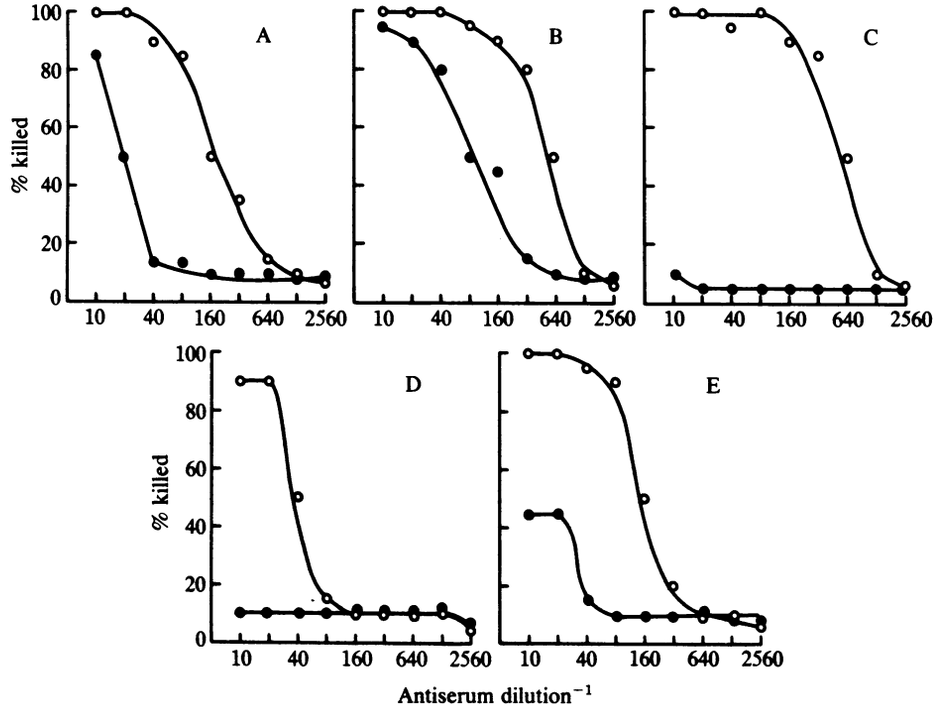

FIG. 1. Direct cytotoxicity test of antisera raised against L-cell transformants. Target cells were from B10.P (○) or C3H (●) mice. (*A*) C3H anti-L3a. (*B*) C3H anti-L5b. (*C*) C3H anti-L12a. (*D*) C3H anti-L15a. (*E*) C3H anti-L17a. Complement-alone controls all gave ≤10% killing.

anti-L17a antisera and mesenteric lymph-node cell targets. All sera showed preferential reactivity with B10.P cells over C3H cells. C3H anti-L12a was included as a positive control, since λ12a has been shown to carry the D^p gene (12). This serum shows specific reactivity for B10.P cells. Three of the remaining four sera showed variable but clear-cut, specific reactivity. The fourth, C3H anti-L5b showed only slight specific reactivity with B10.P cells. These results show that the transfected cells express a product that can provoke antibodies reactive with cells from the gene donor. Further analysis of these sera revealed that they are complex and contain antibodies broadly crossreactive among the antigens encoded by the *H-2* class I gene family (unpublished data). For example, the B10 anti-L3a serum reacts with both B10 and B10.P lymph node cells. Surprisingly this serum failed to react with B10.F(14R) (K^bD^p) cells but did react with B10.F(13R) (K^pD^b) cells. This pattern suggests crossreactivity of the λ3a product with K^p and/or $D^b/Tla^b/Qa^b$ but not the Qa^p products. Adsorption shows that B10.P cells completely adsorb the reactivity for B10 cells. Either the only antibody present is the λ3a product K^p-crossreactive antibody or the λ3a product cannot be detected by direct test but the H-2^b-reactive antigen can.

The high and variable activity against C3H targets by the antisera may be due to variable crossreactivity of the antibodies with the transfected product and K^k/D^k expressed on the C3H cells. Alternatively, an autoantibody might be induced by immunization with antigen similar to a self-antigen. This is likely since the *TL/Qa*-region-encoded class I molecules are much less polymorphic than K or D.

A similar antiserum was prepared by immunizing Fischer rats with Rat-2 cells transfected with the λ3a gene. This serum showed reactivity with L3a cells and Rat-2 cells transfected with the λ3a gene but not with untransfected LTK^- or Rat-2 cells (data not shown).

As has been seen often, immunization with tumor cells results in the production of high titers of antibodies to the 70-kDa glycoprotein gp70 and other associated products (e.g., anti-fetal calf serum). Because of the high concentrations of these antibodies, attempts to produce gene-specific antibodies by adsorption using transfected L cells were unsuccessful.

To eliminate the possibility that we had cloned *Qa-2* or *Tla* genes from $H\text{-}2^p$ mice, we tested the transfected lines with anti-Tla and anti-Qa-2 antibodies. Qa-1 is not expressed in $H\text{-}2^p$ strains. In direct binding tests using transfected L-cell targets, no reactivity was seen with anti-Tla.3 or -Tla.5 or anti-Qa-2 antibodies (data not shown).

Transfected Cells are Recognized by Allospecific CTLs. We wished to determine whether the products of the class I genes transfected into L cells could be recognized by allospecific CTLs. We prepared B10 anti-B10.P; B10 anti-B10.K; and B10.K anti-B10.P effector cells. These cells were tested on uncloned L3a, L12a, and L15a and on LTK^+ cells. As seen in Table 1, L12a (L cells transfected with the D^p gene) were efficiently killed by B10 anti-B10.P effectors. L3a and L15a were also killed, but LTK^+ cells were not. All cells tested were killed by B10 anti-B10.K killer cells, indicating that the cells expressed endogenous H-2^k antigen and could be lysed by CTLs. Third-party specific killers (B10.K anti-B10) were ineffective. Thus both L3a and L15a could be recognized by B10.P-specific killer cells, demonstrating that the product was recognized by T cells and that the product was expressed on the cell surface.

Table 1. Killing of transfected cells by allospecific CTLs

Effector cells	% specific ^{51}Cr release of targets			
	L3a	L12a	L15a	LTK^+
B10 anti-B10.P	35	28	19	9
B10 anti-B10.K	42	47	46	50
B10.K anti-B10	8	9	5	10

All effectors were tested at 50:1 effector/target ratio.

We chose to focus on the λ3a gene because (*i*) cells transfected with the λ3a gene provoked an antibody response, (*ii*) L cells transfected with the gene were recognized by CTLs, and (*iii*) a convenient RLFP was available for mapping (see below).

L3a Cells Act as CTL Targets for *D*-End-Specific Killer Cells. We wished to know whether the λ3a product expressed in L3a cells could be recognized by subregion-allospecific CTLs. CTLs were produced *in vitro* by use of B10 responders and B10.F(14R) stimulators. These effectors are *D*-end-specific because both B10.F(14R) and B10 mice *K* and *I* regions are K^bI^b. These effectors then were assayed for cytolytic activity in a standard 4-hr chromium-release assay using ^{51}Cr-labeled Con A-stimulated lymphoblasts, LTK^+ cells, L12a cells (L cells transfected with the D^p gene), or the L3a cells as target cells (Fig. 2). B10.F(14R) lymphoblasts, L12a cells, and L3a cells all were efficiently killed by the B10 effector cells. B10, B10.F(13R) (K^pD^b), and LTK^+ cells were not recognized. This result is consistent with mapping of the specificity to the *D* end of $H\text{-}2^p$. Similar results were obtained with B10.F(13R) anti-B10.F(14R) effector cells (data not shown).

Thus, L3a cells express an $H\text{-}2^p$-derived *D*-end gene product that can be recognized by CTLs. Since B10 ($H\text{-}2^b$) and L3a cells ($H\text{-}2^k$ + λ3a) share no MHC-derived components, the recognition of the L3a cells must not be *H-2*-restricted. Thus, the λ3a product shares the property of other class I genes as an unrestricted CTL target. It cannot, however, be assumed that the stimulating antigen on B10.P and B10.F(14R) spleen cells is the same molecule as that expressed on L3a cells. Nevertheless, our results show that the λ3a gene product is expressed on the cell surface and can be recognized by B10.P- and B10.F(14R)-specific CTL.

Restriction Map of λ3a. The restriction endonuclease mapping of genomic DNA clones containing class I genes isolated from a B10.P sperm DNA library has been described for the $H\text{-}2D^p$ gene (12). A restriction map of the cloned insert in λ3a (Fig. 3) shows a general homology with the restriction maps of other class I genes (7, 11, 17, 19, 30). Although there is a conservation of restriction sites at the 3′ end of the molecule (*Bam*HI, *Bgl* II), there are considerable differences in the 5′ end. There are no *Eco*RI, *Hin*dIII, or *Kpn* I sites within the entire insert, nor are there any 5′-end *Bam*HI sites. Weiss *et al.* (30) have described 26 class I genes in the B10

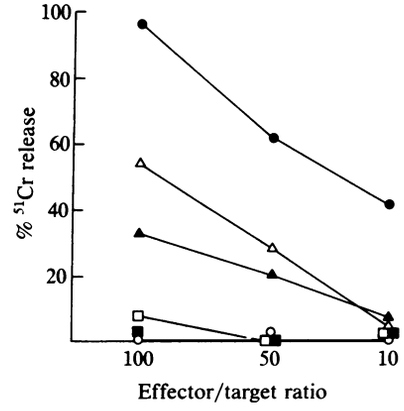

FIG. 2. B10 anti-B10.F(14R), *D*-end-specific CTLs recognize L3a cells. Target cells were from mouse strain B10.F(14R) (●), B10 (■), or B10.F(13R) (K^pD^b) (□) or cell line L3a (▲), L12a (△), or LTK^+ (○).

FIG. 3. Restriction map of clone λ3a. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; P, *Pst* I; S, *Sac* I. There are no *Eco*RI, *Kpn* I, *Xba* I, or *Hin*dIII sites within the 3a insert. The coding and intervening sequences were determined by hybridization with cDNAs as well as homology with mapped and sequenced class I genes.

genome. The restriction endonuclease analyses of K^b, D^b, *Q1–Q10*, *T1–T5*, *T10*, and *T13* reveal that each has at least one *Kpn* I site. In addition, the *T6*, *T7*, and *T9* genes have *Hin*dIII sites. These restriction sites are also found among the BALB/c ($H\text{-}2^d$) genes (7, 30).

Another interesting feature of the λ3a clone is an apparent 700-base-pair (bp) interruption in the 3′ end of the coding sequence. Comparison of the λ3a restriction map with that of Q10 shows conservation of *Pst* I sites through the exons encoding the cytoplasmic regions of the polypeptide. The 700-bp *Pst* I fragment immediately 3′ to these sequences does not hybridize to either of the class I-specific cDNA probes pH-2IIa and pH-2III. A 2.1-kilobase (kb) *Pst* I fragment 3′ to this fragment hybridizes to pH-202 (a full-length cDNA) but not pH-2IIa. Together, these data suggest interruption of the 3′ untranslated region with a 700-bp sequence. We do not know whether the region is transcribed, but preliminary experiments using this fragment as a probe suggest this fragment contains a highly repeated sequence (unpublished data). It is too long to be the single B2 repeat insertion found in only *D*-region class I genes (33). The B2 sequence is found in the D^p gene (unpublished data). We are currently sequencing this region of λ3a and thus far have not found any homology to B2.

Mapping 3a Sequences by RFLPs. We located the map position of the λ3a gene by use of a RFLP. We can identify, on a genomic Southern blot of B10.P DNA, the band that corresponds to the λ3a gene. This is accomplished by digesting both B10.P genomic DNA and λ3a DNA with *Sac* I, which cuts twice within the mouse DNA insert. As in Fig. 4, a 3.0-kb band is seen in the λ3a and B10.P lanes. When DNA from B10 ($H\text{-}2^b$) is treated identically, although many bands are present, the 3.0-kb band is absent. A slightly faster band of much lower intensity can sometimes be seen in blots of DNA from B10 and B10.F(13R). The absence of the 3-kb band signals the change of a *Sac* I site in or near the B10 equivalent of λ3a. Thus, the presence of the 3-kb band can be used to signal the presence of the λ3a *p* allele. When DNA from the reciprocal recombinant strains B10.F(13R) (K^pD^b) and B10.F(14R) (K^bD^p) is treated similarly, only B10.F(14R) DNA shows the 3-kb band. Therefore the λ3a gene maps telomeric to the recombination in B10.F(14R) in the *D* or *Tla/Qa* regions.

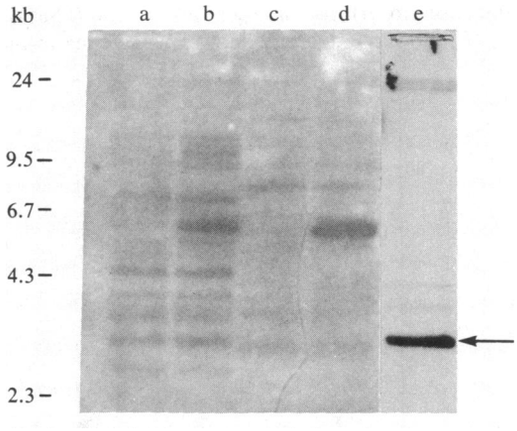

FIG. 4. RFLPs map the λ3a clone to the *D* end of $H\text{-}2^p$. *Sac* I-digested genomic DNA samples from congeneic and recombinant mouse strains were analyzed by Southern blot hybridization using pH-2IIa as probe. *Sac* I-digested λ3a DNA electrophoresed coincidently with *Sac* I-digested genomic DNAs shows a band at 3.0-kb (arrow). Lanes: a, B10.P (K^bD^b); b, B10.F(14R) (K^bD^p); c, B10.F (13R) (K^pD^b); d, B10 (K^bD^b); e, λ3a.

DISCUSSION

We have shown that several class I genes can be expressed in transfected cells, provoke antibodies reactive with MHC molecules, and be recognized by allospecific CTLs. We have not determined the detailed specificity of the antibodies, but crossreactivity among class I molecules is well documented, both between K and D, and between *Qa-2/Tla*-region-encoded antigens and K. This is expected since the class I gene family is highly homologous, and many polymorphic features should be conserved among members of the family. This is especially likely to be true if gene conversion events move homologous sequences among the members of the class I family. This type of mechanism is supported by the finding that the putative donor gene sequence of the K^{bm1} mutation can be found in a *Q*-region gene (31, 32).

We have demonstrated that the λ3a genomic DNA clone, which was isolated from an $H\text{-}2^p$ genomic DNA library, encodes a novel class I MHC gene. RFLP as well as CTL analysis indicates that λ3a maps in the *D–Tla* interval. L cells expressing the λ3a gene can both provoke antibody formation and be recognized by CTLs.

These experiments were directed at uncovering the role of the previously unsuspected class I sequences identified by molecular genetic analysis. The structure of the λ3a gene is similar to other previously known class I genes in terms of its general organization of introns and exons. The restriction map of λ3a differs from other, previously described class I genes in several ways. It lacks both internal *Kpn* I and *Eco*RI sites. Perhaps more interesting is that the λ3a clone has an apparent insertion of the coding sequence near the 3′ end. A 700-bp insertion in the 3′ untranslated region was found. The λ3a gene shares many features with other class I genes, including a *Bam*HI site in intron 3, but shows multiple differences with all the previously described class I genes. *D*-region class I genes share a common insertion of an ≈180-bp B2 repetitive element (33). This insert contains both functional splice acceptor sites and a polyadenylylation signal. It has been suggested that these may be important in the independent regulation of *D*-end genes. The insertion in λ3a is too large to be a single B2 repeat but might also function in the regulation of λ3a.

One explanation for our results would be that the $H\text{-}2^p$ haplotype expresses a multiple *D*-region product (L^p, R^p, etc.). Several lines of evidence argue against this. D^p, like other *D*-region molecules, has a B2 repeat. This repeat is not present in λ3a. Further, no evidence of multiple D^p-region products is suggested by serology. None of the 11 D^p-specific monoclonal antibodies reacts with the 3λa-transfected cells

(data not shown). In contrast, many monoclonal antibodies crossreact with L^d and D^d. Further, the sequence of D^p is more closely related to L^d and D^b than to D^d (unpublished observation), thus suggesting only a single D molecule in *H-2*^{p} as in *H-2*^{b}.

The crucial question in understanding class I sequences is their function. Two major functions have been suggested for class I determinants. First, it has been shown that they can function as the targets for *H-2*-unrestricted cytolytic lymphocytes. This means that they are able to be killed by specific effector cells regardless of shared target/effector cell expression of K and D antigens. This seems to be a universal property of class I gene products and is true for the product expressed by line L3a. Our results, which show that B10 anti-B10.P cells are able to kill the *H-2*^{k} L cells transfected with the λ3a gene but not untransfected LTK^- cells, demonstrate unrestricted killing. The crucial question remains whether this is a fortuitous event caused by a similarity in structure to classical antigens. There may be CTL crossreactivity among the λ3a product, K^p, D^p, and other class I products. It would be interesting to raise CTL specific for the λ3a product by stimulating CTL cells *in vitro*. However, we have been unsuccessful in eliciting responses to L^d, D^p, or even the endogenous L-cell $H\text{-}2^k$ molecules by use of L cells as stimulators.

One intriguing possible explanation for the function of the class I genes such as that in λ3a is that they represent tissue-specific class I genes. Schultz *et al.* (34) have found some evidence for tissue-specific expression of class I genes. They used grafts of liver to kidneys and showed that the hierarchy of graft rejection in these liver grafts was different than for the classically defined skin grafts (34). Thus strain combinations that showed rapid skin rejection sometimes showed slow rejection of liver, and vice versa. One explanation for this difference is a differential expression of different class I genes in skin versus liver. Since these different class I genes would have different sequences and hence be recognized as different antigens, it is conceivable that different rejection rates would be possible.

Many other questions still remain regarding these other class I sequences. Does the sequence homology correlate with function? Is there regulation of expression during development? Are these molecules involved in self-recognition and tolerance? If these genes have no correlation between structure and function, why are the gene sequences conserved? Is this conservation selected? Alternatively, is the class I sequence conservation simply related to the clustering of class I genes on chromosome 17 and the influence of the *t* locus where stability by recombination suppression is forced? The analysis of the class I gene in L3a indicates that some of these genes encode functional proteins that may play important roles in the immune response.

Note Added in Proof. We have found, by hybridization of synthetic oligonucleotide corresponding to a λ3a sequence, that λ3a corresponds to either the *Q3* or the *Q5* gene of BALB/c.

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