EXPRESSION OF STRUCTURALLY DIVERSE Qa-2-ENCODED MOLECULES ON THE SURFACE OF CLONED CYTOTOXIC T LYMPHOCYTES

BY DAVID H. SHERMAN, DAVID M. KRANZ, AND HERMAN N. EISEN

From the Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Cell surface glycoprotein heterodimers in which the heavy chain (~40–45 kD) is encoded in the major histocompatibility complex (MHC)[1] and the light chain (13 kD) is beta-2-microglobulin (β-2M) are known as class I molecules and the genes that encode the heavy chains are known as class I genes (1, 2). Recent studies have revealed a surprisingly large number of class I genes in inbred mice (approximately 35 in the BALB/c strain and 26 in C57BL/10) (references 3, 4, and personal communication, Richard A. Flavell), all of them on chromosome 17 in three contiguous loci: H-2, Qa-2, and Tla. There appear to be 3–5 of these genes in H-2, 10 in Qa-2, and the rest in Tla. Class I genes of the H-2 locus are highly polymorphic and are expressed on virtually all nucleated cells, whereas the non-H-2 class I genes have only limited polymorphism and are expressed only on certain cell types of hematopoietic origin (5). Though the H-2- and non–H-2-encoded molecules bear regions of homologous sequences, suggesting a common ancestral origin (5–8), their products are functionally dissimilar. The H-2 class I molecules restrict recognition of antigens by cytotoxic T lymphocytes (CTL) (9), but neither this nor any other function has yet been identified for the Qa-2/Tla glycoproteins. In a recent study of immunoprecipitated cell surface molecules from CTL, we observed prominent class I molecules that were unusual in having relatively low molecular weight (10) and different apparent molecular weights in different clones. Because clonal variation of class I molecules within a given inbred mouse strain is unexpected, we have analyzed some biochemical properties of these molecules. Our results show that these unusual class I molecules are encoded in the Qa-2 locus, that they vary structurally in cloned CTL lines in respect to apparent molecular weight (M_r) and isoelectric focusing (IEF) pattern and that while some clones have one, others express two, distinct Qa-2-encoded molecules.

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[1] *Abbreviations used in this paper:* β-2M, beta-2-microglobulin; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; IEF, isoelectric focusing; mAb, monoclonal antibody; MHC, major histocompatibility complex; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Materials and Methods

Animals. BALB/cAnN (H-2^d), BALB.K (H-2^k), and BALB.B (H-2^b) mice were bred at the Massachusetts Institute of Technology Center for Cancer Research. Joan Press (Brandeis University) kindly provided B10.BR (H-2^k), C57BL/10 (H-2^b), B10.D2 (H-2^d), B10.S (H-2^s), B10.G (H-2^q), B10.A(5R) (K^bD^d) mice and Martin Dorf (Harvard Medical School) generously donated C3H.OL (K^dD^k) mice. BALB/cJ (H-2^d) and BALB/cByJ (H-2^d) mice were obtained from The Jackson Laboratory, Bar Harbor, ME.

Cloned CTL. To obtain cloned CTL, secondary alloreactive CTL were produced as previously described (10). Two of the cloned lines used here (G4 and 2C) were described previously (10, 11). 14 additional CTL clones were produced in essentially the same way. 10 were generated by inoculating BALB.B mice intraperitoneally with 2×10^7 P815 (H-2^d) cells, harvesting spleen cells from these mice 1 mo later, and stimulating them in culture with irradiated (2,000 rad) BALB/cAnN spleen cells. By limiting dilution, 10 clones were obtained and maintained by weekly stimulation with irradiated BALB/c spleen cells. To obtain the other four clones, RDM-4 (H-2^k) cells were used to elicit alloreactive CTL in vivo; the irradiated spleen cells used as stimulators to maintain these clones in culture were from BALB.K (H-2^k) mice. Thus, all 16 clones were of BALB.B (H-2^b) origin, 12 were specific for H-2^d cells and 4 for H-2^k cells.

Antisera and Monoclonal Antibodies. BALB.K antisera to CTL clone 2C were previously described (10). A rabbit antiserum to human β-2M was kindly provided by Howard Grey (National Jewish Hospital, Denver, CO). A mouse IgM monoclonal antibody (mAb) (D3.262) to the Qa-2 antigen was generously provided in ascites fluid by Lorraine Flaherty (Division of Laboratories and Research, New York State Department of Health, Albany, NY).

Radioiodination of Cells. Cell surface proteins were iodinated with ^{125}I, using lactoperoxidase as described (10).

Immunoprecipitations and SDS-PAGE. Nonidet P-40 (NP-40) extracts of the ^{125}I-labeled cells were precleared by incubating them three times successively with normal rabbit serum (40 μl per 10^7 cells) at 4°C for 30 min, then adding protein A–bearing Cowan I strain of *Staphylococcus aureus* and incubating the mixture at 4°C for 1 h with shaking. The precleared lysates were reacted with (*a*) BALB.K antisera to CTL clone 2C and (*b*) mAb to Qa-2. To the samples with mAb to Qa-2, rabbit anti-mouse IgM antiserum was added to permit binding of the immune complexes to protein A. After incubating the samples with antibodies for 30 min at 4°C, 0.1 ml of 20% *S. aureus* in phosphate-buffered saline (PBS) was added and incubation was continued with shaking for 1 h at 4°C. The bacteria were then pelleted by centrifugation, washed three times with PBS containing 0.5% NP-40, 2 mM methionine, 5 mM potassium iodide, and 0.02% sodium azide, and the absorbed immunoprecipitates eluted by heating the samples in a boiling water bath for 3 min in sodium dodecyl sulfate (SDS)-sample buffer (12). The samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) gels according to Laemmli (12). Gels were exposed to Kodak XAR-5 x-ray film using a Dupont Cronex Lightning-Plus intensifying screen. Exposure of film was for 3–4 d at −70°C. Analysis by two-dimensional gel electrophoresis was performed as described by O'Farrell (13) and modified by Jones (14); exposure of film was for 1–2 wk at −70°C.

Endoglycosidase F (Endo F) Digestion. Extracts of ^{125}I-labeled cells were prepared and immunoprecipitated with mAb to Qa-2 as described above. Washed *S. aureus* pellets containing bound Qa-2 immune complexes were incubated with 10 mU of Endo F (15) for 12–16 h at 37°C. Analysis by SDS-PAGE was then performed as described.

Enriched B and T Cell Populations. Subpopulations of BALB.B splenic lymphocytes were prepared as follows: (*a*) Enriched B cell populations were obtained by incubating 1×10^8 spleen cells with anti-Thy-1 mAbs 13–14 (16) and T-24 (17) for 10 min at 4°C and then with rabbit complement for 1 h at 37°C, followed by Ficoll-Paque purification to remove debris from lysed cells. (*b*) Enriched T cell populations were obtained by removing surface Ig^+ cells by "panning" (18, 19) (1×10^8 spleen cells were added to plates precoated with rabbit anti-mouse Ig and incubated for 90 min at 4°C. Nonadherent cells were collected and the process was repeated). (*c*) T cells were separated into Lyt-2^+ and Lyt-2^-

populations by panning on plates containing anti-Lyt-2 mAb: 2×10^8 surface Ig^- spleen cells were incubated with a mixture of four rat anti-Lyt-2 mAb (2.43.5, 3.155.2, 3.239.2, and 3.168.8, all generously provided by Frank Fitch and Marian Sarmiento, University of Chicago). After 30 min the cells were washed three times, added to two plates precoated with rabbit anti-rat Ig, and incubated for 90 min at 4°C. Nonadherent cells ($Lyt\text{-}2^-$) were collected, the plate rinsed, and adherent cells ($Lyt\text{-}2^+$) removed by gentle scraping. The enriched cell populations were ^{125}I-labeled and then extracted and subjected to immunoprecipitation and SDS-PAGE as described above.

Results

Previously, we described some cell surface molecules that were immunoprecipitated from three ^{125}I-labeled cloned CTL lines (10). The CTL were of BALB.B ($H\text{-}2^b$) (20) origin and were specific for target cells of the $H\text{-}2^d$ haplotype. Immunoprecipitations were carried out with alloantisera that had been raised by immunizing BALB/cAnN and BALB.K mice with some of the clones. Three of the four principle components precipitated were readily identified: (*a*) the class I heavy chains (~45 kD) encoded by the $H\text{-}2^b$ locus; (*b*) β-2M (~13 kD); and (*c*) concanavalin A (Con A) (25 kD) (21), a persistent contaminant resulting from the use of supernatants of Con A–stimulated spleen cells as a source of interleukin 2 (22). The fourth immunoprecipitated component is the subject of this study. It had an M_r of 37,000 in one clone (2C) and 38,000 in another clone (G4) (10). Because the 37–38,000 M_r components were also immunoprecipitated by rabbit antiserum to β-2M, they were evidently class I molecules. To determine whether these class I molecules of unusually low molecular weight are encoded in the Qa-2/Tla locus, we began the present study by looking for them in spleen cells of 12 recombinant and congenic strains of mice that differ in H-2, Qa-2, and Tla loci (23). As shown by the representative autoradiographs of Fig. 1 (lanes *1–4*) and the summary in Table I, a 37,000 M_r component was present in all $Qa\text{-}2^a$ strains and absent in all $Qa\text{-}2^b$ strains. Its presence or absence did not correlate with allelic variation at the other loci. Further evidence that this component is a class I heavy chain encoded at the Qa-2 locus was provided by the finding that it was immunoprecipitated from splenocytes of $Qa\text{-}2^a$ mice not only by antisera to clone 2C, but also by antisera to β-2M and by an mAb to the Qa-2 antigen. It is also notable that expression of the 37,000 M_r component in spleen cells of various BALB/c sublines correlated with their Qa-2 genotype: this component was apparent in the BALB/cJ subline ($Qa\text{-}2^a$), but was not apparent in Andervont-derived BALB/c sublines (i.e., BALB/cBy and a BALB/cAnN colony maintained for nine years at MIT). As expected, it was also present in the congenic BALB.B strain, which derives its H-2 locus from the C57BL/10 strain (Fig. 1, lanes *1–4*).

One of the striking differences between H-2 and non-H-2 class I molecules lies in their tissue distribution (5). To determine whether the expression of the Qa-2-encoded molecules precipitated by our antiserum to clone 2C were also limited to certain cells, spleen cells were subjected to immunoprecipitation with both BALB.K anti-clone 2C and with rabbit antiserum to β-2M. The results showed (Fig. 2) that Qa-2 was present in spleen cells that were enriched for T cells (both $Lyt\text{-}2^+$ and $Lyt\text{-}2^-$), but was virtually lacking in spleen cells that were enriched for B cells (Fig. 2, lanes *3* and *9*). Either B cells have only trace amounts of Qa-2-encoded molecules, or these cells lack them entirely and the trace amount

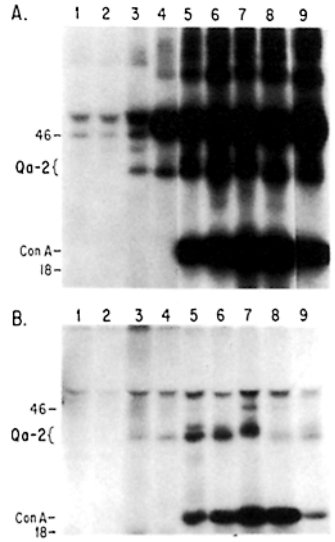

FIGURE 1. SDS-PAGE of Qa-2-encoded molecules from spleen cells of four inbred mouse strains and BALB.B-derived CTL. (*A*) Immunoprecipitation with BALB.K antisera to CTL clone 2C. (*B*) Immunoprecipitation with Lorraine Flaherty's mAb (D3.262) to Qa-2. Lane *1*, BALB/cByJ; lane *2*, BALB/cAnN; lane *3*, BALB/cJ; lane *4*, BALB.B; lane *5*; CTL clone 10B; lane *6*, clone 2C; lane *7*, clone G4; lane *8*, clone 2.1.1; lane *9*, clone 1.4.2. Qa-2-encoded heavy subunits appear in the bracketed region. H-2^b alloantigens appear at 46,000 M_r in panel *A*. To avoid confusion with B10 mice, CTL clone B10 (reference 31) is here designated clone 10B.

immunoprecipitated from them was due to contaminating T cells. We also did not detect Qa-2-encoded molecules in a B cell tumor of BALB/cJ origin (BCL1) (24) or in a hybridoma made from spleen B lymphocytes of the H-2^b haplotype (C57BL/6). Others (5, 25), however, using cytotoxicity assays, have reported that a Qa-2-encoded component is present on B cells (as well as on T cells). The discrepancy with B cells could mean that the cytotoxic assay is much more sensitive than the immunoprecipitation procedure used here, or that the cytotoxic assay more easily detects cross-reactive molecules.

We previously observed a difference of ~1,000 in apparent molecular weight between the Qa-2 components immunoprecipitated from CTL clones 2C and G4. To see if there are other clonal differences, we generated and examined 14 additional cloned CTL lines (10 were anti-H2^d and 4 were anti-H-2^k; see Materials and Methods). Clear variability in the cell surface Qa-2-encoded molecules was evident with five of these clones (30%), clones 10B, 2C, G4, 2.1.1 (H-2^b, anti-H-2^d), and 1.4.2 (H-2^d, anti-H-2^k). Extracts from each of these ^{125}I-labeled clones were immunoprecipitated with BALB.K antiserum to clone 2C, rabbit antiserum to β-2M, and anti-Qa-2 mAb. Qa-2 differences among the clones are illustrated in Fig. 1, lanes *5–9*. For instance, the apparent molecular weight was 38,500 for clone G4 and 36,500 for clone 2.1.1. Clones 10B and 1.4.2 each had two distinct Qa-2 components, whereas the other clones had only one component. In clone 2C it is not clear whether there are one or two components.

In two-dimensional gels, Qa-2-encoded molecules from various BALB.B CTL

TABLE I

Strain Distributions Suggest That the 37 M_r Band is Encoded in the Qa-2 Locus

Mouse strain	K	D	Qa-2‡	Qa-1‡	Tla‡	37 M_r band*
BALB/cJ	d	d	a	b	c	+
BALB/cByJ	d	d	b	b	c	−
BALB/cAnN (MIT)	d	d	b	b	c	−
BALB.B	b	b	a	b	b	+
BALB.K	k	k	b	b	b	−
B10.BR	k	k	b	a	a	−
B10	b	b	a	b	b	+
B10.D2	d	d	a	b	c	+
B10.S	s	s	a	b	b	+
B10.G	q	q	a	a	a	+
B10.A(5R)	b	d	a	a	a	+
C3H.OL	d	k	b	b	b	−

* Extracts of ^{125}I-labeled spleen cells were immunoprecipitated with normal mouse serum (control), or BALB.K anti-CTL clone 2C or rabbit anti-mouse β-2M.

‡ For a general summary of the distinctions among the allelic variants at each of these loci, see reference 16. The Qa-2 antigen is detectable in mice with the Qa-2^a locus but has not been detected in those with the Qa-2^b locus (6). Breeders for the BALB/cAnN(MIT) subline were originally obtained (in 1973) from Dr. Ralph Graff of Washington University School of Medicine, St. Louis, MO.

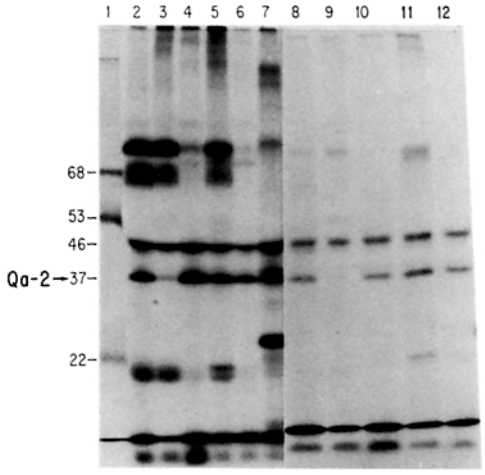

FIGURE 2. Tissue distribution of Qa-2 antigens in the BALB.B mouse. Lane *1*, molecular weight markers; lanes *2* and *8*, unfractionated spleen cells; lanes *3* and *9*, B cell–enriched spleen cells; lanes *4* and *10*, T cell–enriched spleen cells; lanes *5* and *11*, Lyt-2$^+$-enriched spleen cells; lanes *6* and *12*, Lyt-2$^-$-enriched spleen cells; lane *7*, CTL clone 2C. Lanes *2–7* immunoprecipitations were performed using BALB.K antisera to CTL clone 2C. Lanes *8–12* immunoprecipitations were performed using rabbit antisera to mouse β-2M (Miles Laboratories and courtesy of Howard M. Grey).

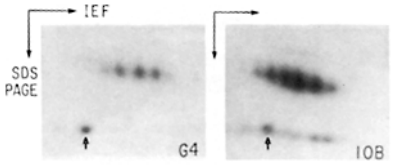

FIGURE 3. Two-dimensional analysis of the Qa-2-encoded components expressed on two cloned CTL lines. CTL were radiolabeled and immunoprecipitated with mAb to Qa-2 as described in Materials and Methods. The Con A standard is the spot (arrow) at lower left in each panel. Clone G4 (*left* panel) exhibits a single series of five spots all anodic to Con A. Clone 10B (*right* panel) shows two series of spots. The lower series of four spots are all anodic relative to Con A in contrast to the upper series of five spots.

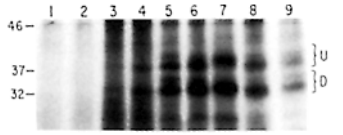

FIGURE 4. Endo F digestion of Qa-2-encoded alloantigens from spleen cells of inbred mice and cloned CTL lines. *U* in brackets shows region of undigested Qa-2-encoded components. *D* in brackets shows digested Qa-2 components with loss of 5,000–6,000 M_r. Complete digestion with total loss of the glycoslyated (heavier) species resulted only in components that appeared in the lower set of brackets. Material at still lower M_r was not observed. Lane *1*, BALB/cByJ; lane *2*, BALB/cAnN; lane *3*, BALB/cJ; lane *4*, BALB.B; lane *5*, CTL clone 10B; lane *6*, clone 2C; lane *7*, clone G4; lane *8*, clone 2.1.1; lane *9*, clone 1.4.2.

clones were seen to vary in the IEF, as well as the SDS-PAGE dimensions (Fig. 3). For instance, in clone G4 the single Qa-2 band in SDS-PAGE yielded a series of discrete spots in the IEF dimension that were all basic relative to the Con A internal standard, whereas in clone 10B there were two Qa-2-encoded components (in SDS-PAGE), each yielding a series of spots, with the lower series migrating more anodically relative to Con A than the upper (higher molecular weight) series. In contrast to this variability among CTL clones, Michaelson et al. (26, 27) found remarkable IEF uniformity in Qa-2-encoded molecules from normal spleen cells of 39 different Qa-2^a strains of mice (including wild mice). It also appears to us that the Qa-2-encoded molecules immunoprecipitated from normal spleen cells from diverse mouse strains (Qa-2^a) have a uniform M_r and migrate in SDS-PAGE as a more compact band than those of the CTL clones (Fig. 1, lanes *3–4* vs. *5–9*).

Since Qa-2 molecules on the CTL surface are expected to be glycoproteins, it seemed possible that their clonal diversity reflected oligosaccharide differences. To test this possibility, the immunoprecipitated Qa-2-encoded components from CTL clones 10B, 2C, G4, 2.1.1, and 1.4.2 were treated with the Endo F (15). As shown in Fig. 4, the apparent molecular weight of each Qa-2 component decreased by ~5,000–6,000. It is striking, however, that the molecular weight differences among the various Qa-2 components were retained, including the differences between the two distinct components in clone 10B and in clone 1.4.2. Hence, N-linked complex and high mannose glycans seem not to account for the structural differences between the Qa-2-encoded molecules in various CTL clones.

Discussion

What is the basis for the clonal variation of the Qa-2-encoded molecules? There appear to be 10 class I genes in the Qa-2 locus of the H-2^b and H-2^d haplotypes and it is possible that not all CTL clones express the same gene or genes. To answer this question we are currently preparing cDNA libraries from some of the CTL clones studied here in order to determine which non-H-2 class I genes are transcribed and ultimately expressed on the cell surface (work in progress in collaboration with R. A. Flavell and co-workers).

It is also possible that clonal variation of Qa-2 gene products arises from differential splicing of RNA transcripts in different CTL clones. This possibility must be considered because it has been recently reported that one of three exons that encode the cytoplasmic domain of both H-2 and non-H-2 class I genes is partially or totally missing from some transcripts (28–30). The splicing out of one of the cytoplasmic exons could account for the molecular weight difference (~1,500) between the two Qa-2 components of clone 10B and of clone 1.4.2 (Fig. 1, lanes *6* and *9*). Though our observations virtually rule out N-linked oligosaccharide diversity, it is possible that other sources of oligosaccharide diversity might account for the clonal differences in Qa-2, e.g., Endo F–resistant or O-linked sugars. Such differences, if they exist, could arise from variability in the amino acid sequence of Qa-2-encoded molecules.

The function of non-H-2 class I molecules encoded in the Qa/Tla locus remains an enigma. The apparent failure of Qa-2 genes to be expressed in Qa-2^b strains of mice (such as BALB/cAnN) is particularly puzzling. Are Qa-2 molecules truly absent, or are they present at low but functionally significant levels, perhaps in a serologically different form or different molecular weight than the Qa-2 molecules of Qa-2^a mice? In a preliminary experiment we observed that incubation of BALB/cAnN spleen cells in Con A supernatants (containing interleukin 2 and other growth factors) has led to the appearance on these cells of surface glycoproteins that are immunoprecipitable by an mAb to Qa-2. To evaluate these issues we are currently generating cloned CTL lines from BALB/cAnN and other, ostensibly Qa-2-negative (Qa-2^b) strains of mice.

Products of H-2 class I genes appear to be invariant "self-markers" in all cells of any particular mouse or inbred mouse strain. As shown here, however, the Qa-2 gene products differ among CTL clones from the same inbred mouse strain. Hence, the present findings add another difference to those thast are known to exist between products of H-2 and non-H-2 class I genes (5). Besides immunoprecipitating class I molecules encoded by the H-2 and Qa-2 loci, anti-β-2M antiserum immunoprecipitated from all of our cloned CTL lines a component of 55,000 M_r (data not shown). This component is probably a Tla region–encoded antigen (31), but positive identification awaits immunoprecipitation with the appropriate mAb. The structural variability of Qa-2-encoded molecules described here and the restricted tissue distribution of both Qa-2 and TL antigens (preferential expression on T cells) suggests that all of these non-H-2 class I molecules might have a special function for T cells.

Summary

Extracts of ^{125}I-labeled cloned murine cytotoxic T lymphocytes (CTL) were immunoprecipitated with alloantisera to the cloned CTL and rabbit antisera to

β-2 microglobulin. Polyacrylamide gel electrophoresis (PAGE) of the specific precipitates revealed, as expected, ^{125}I-labeled components that corresponded to products of class I genes of the major histocompatibility complex (MHC). However, additional class I gene products of relatively low apparent molecular weight (M_r) were also observed. Similar analyses of spleen cells from a variety of MHC-congenic mouse strains suggested that the class I molecules of relatively low M_r are encoded in the Qa-2 region of the MHC, and this was confirmed by immunoprecipitation with a monoclonal antibody to Qa-2. Surprisingly, however, the cell surface Qa-2 molecules of different CTL clones differed in M_r, in isoelectric focusing (IEF) pattern, and in the number of distinguishable molecules expressed per clone: some clones seemed to express only a single Qa-2-encoded molecule while others expressed two distinct ones. Treatment of the immunoprecipitated Qa-2 with endoglycosidase F (Endo F) resulted in a decrease in M_r of ~5,000–6,000, corresponding to the expected loss of N-linked oligosaccharides, but the decrease did not eliminate structural variability among the clones. Structural diversity of the Qa-2-encoded molecules expressed on CTL could arise because CTL clones differ (*a*) in the particular Qa-2 genes they express, (*b*) in the way they splice Qa-2 gene transcripts or, perhaps, (*c*) in Endo F–resistant oligosaccharides on their Qa-2 molecules.

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Note added in proof: The number of MHC class I genes in C57BL/10 mice, cited in the introduction to a personal communication from Richard A. Flavell, has been described in a recent publication (Weiss, E. H., L. Golden, K. Fahrner, A. L. Mellor, J. J. Devlin, H. Bullman, H. Tiddens, H. Bud, and R. A. Flavell. 1984. Organization and evolution of the class I gene family in the major histocompatibility complex of the C57BL/10 mouse. *Nature (Lond.).* 310:650).

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