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Immunology

HMT, encoded by *H-2M3*, is a neoclassical major histocompatibility class I antigen

(gene expression/interferon γ/site-directed mutagenesis/peptide presentation/cytotoxic T lymphocytes)

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ABSTRACT ***H-2M3* encodes HMT, the major histocompatibility complex (MHC) class I heavy chain of the maternally transmitted antigen (Mta). Like classical MHC class I genes, the expression of *M3* can be stimulated by γ-interferon and its message can be detected from mid-gestational embryos (day 8) through adulthood. HMT^b, a nonimmunogenic allelic form of HMT, differs from the common HMT^a molecule by four amino acids, of which only two (residues 31 and 95) are located in the α1 and α2 domains that form the peptide-binding groove. Recognition of site-directed mutants by Mta-specific cytotoxic T lymphocytes was hardly affected by the substitution of Met for Val^{31} but was abolished by the substitution of Gln for Leu^{95}, which is located in the β-sheet floor of the peptide-binding groove. Thus a single amino acid difference is responsible for the immunological silence of HMT^b.**

Mta, the maternally transmitted antigen of mice, is an unusual histocompatibility antigen because it is maternally inherited and recognized by specific cytotoxic T lymphocytes (CTLs) that are not H-2-restricted (1). Mta is a complex of MTF, HMT, and β_2-microglobulin (2); MTF is a polymorphic, N-formylated, hydrophobic peptide derived from the amino terminus of the mitochondrially encoded ND1 protein (3), and HMT is a major histocompatibility complex (MHC) class I antigen encoded by *H-2M3* (formerly *Hmt*) (4), which is telomeric of the *H-2T* region (5). We use HMT to refer to the class I molecule and its phenotype as detected by CTLs, and we refer to the gene itself and its nucleic acid products (mRNA or cDNA) as *M3*.

A few alleles of *M3* (*Hmt*) have been distinguished by CTL reactivity. The *a* allele is associated with an Mta^+ phenotype and is present in most laboratory and wild mice. The *b* allele, which behaves as a null allele (Mta^-), is found in *Mus musculus castaneus*. Other alleles have been identified in *Mus spretus*, *Mus musculus bactrianus*, and *t*-haplotype mice (2, 6). The null phenotype does not result from a deletion of the *M3* gene or transcriptional failure but resides in the HMT^b molecule itself (4). The predicted protein sequence of HMT^b differs from HMT^a by four amino acids.

All vertebrates have one, two, or three classical, polymorphic class I antigens that are expressed at high levels and dominate antigen presentation to $CD8^+$ T cells. All other class I molecules have been considered nonclassical because of their low-level expression and polymorphism, their limited tissue distribution, and the lack of evidence that they present peptide antigens. Because HMT binds N-formylated mitochondrial and bacterial peptides (7, 8), it can play a role in host defense against intracellular prokaryotic pathogens. Thus, mice infected with *Listeria monocytogenes* generate $CD8^+$ CTLs that recognize a *Listeria* peptide presented by HMT (9, 10). It is therefore of interest to know whether expression of HMT and formation of Mta parallel those of classical class I antigens. Here we show that HMT should be considered a neoclassical class I antigen.

MATERIALS AND METHODS

***M3* DNA Clones.** Cosmid 14RB-A, isolated from a BALB/c (Hmt^a) cosmid library in the pWE15 vector, contains the complete $H\text{-}2M3^d$ class I gene (4). An *M3* cDNA clone was isolated from a thymus cDNA library from $M3^{b/k}$ mice, also Hmt^a, and extended to full length as described (4).

After the completion of these experiments, we have come to realize that this cDNA construct carries Cys rather than Trp at position 244 in the α3 domain. This mutation was already present in one of the two original cDNA clones used to make the construct, but it is not found in the $M3^{cas3}$ genomic sequence (4). Furthermore, position 82 in the α2 domain is Leu (codon CTC) and not His (CAC) in both $M3^{cas3}$ and $M3^{b/k}$; we regret this typographical error (4).

RNA Isolation and Northern Analysis. Total RNA from various tissues was prepared by the guanidinium/acid/phenol method described by Chomczynski and Sacchi (11). Formaldehyde/agarose gel electrophoresis and Northern transfer were performed according to Fourney *et al.* (12). The filter was blocked for 4 hr in 1 M NaCl/1% SDS/10% dextran sulfate with salmon sperm DNA (100 μg/ml) at 60°C and hybridized overnight in the same solution with a DNA probe labeled by random priming (13). After hybridization, the filter was washed for 5 min in 2× standard saline citrate (SSC) at room temperature and twice for 30 min in 2× SSC/0.1% SDS at 60°C and exposed for autoradiography.

Polymerase Chain Reaction (PCR) Amplification of *M3* mRNA. To synthesize cDNA, 2 μg of total RNA, 50 pmol of the M3-3′ primer (5′-GACTAGCAACGATGACCATGATGAC-3′, which anneals to the sense strand of *M3* exon 5), and 100 units of Moloney murine leukemia virus reverse transcriptase (BRL) in 20 μl were incubated for 45 min at 37°C. The reverse transcriptase was then heat-inactivated by a 5-min incubation at 95°C. The reaction volume was increased to 100 μl by addition of 75 pmol of the M3-5′ primer (5′-TGGAGCCCACTATCAGGCTGCGTAT-3′, which anneals to the antisense strand of *M3* exon 3), 25 pmol of M3-3′ primer, and reverse transcriptase buffer. PCR was performed with an initial denaturation at 95°C for 10 min, followed by 30 cycles of extension at 65°C for 3 min and denaturation at 94°C for 1 min.

γ-Interferon (IFN-γ) Treatment. Cells were grown in 75-cm^2 tissue culture flasks in RPMI 1640 medium with 10% fetal bovine serum. When the cells reached half confluency, they

Abbreviations: CTL, cytotoxic T lymphocyte; IFN-γ, γ-interferon; MHC, major histocompatibility complex; Mta, maternally transmitted antigen.

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received IFN-γ (Amgen Biologicals) to a final concentration of 500 units/ml and were incubated for 72 hr.

Site-Directed Mutagenesis. The *M3* (*Hmt*^{a}) cDNA fragment was subcloned into an M13 bacteriophage vector (4). Site-directed mutagenesis with synthetic oligonucleotides SM31, 5′-GTTGACGACATGCAGTTTCA-3′, and SM95, 5′-TCTCACATCCAGCAGTGGAT-3′ (mutated nucleotides are underlined), was carried out as described by Kunkel *et al.* (14), except that *Escherichia coli* DH5αF′ cells (BRL) were transformed. Positive plaques were identified by probing the filters with ^{32}P-labeled oligonucleotides (15). In all cases, the mutagenized region was checked by single-stranded DNA sequencing (16). The mutagenized fragments were subcloned behind the metallothionein promoter in the pMT-neo expression vector (provided by R. Taussig, Univ. of Texas Southwestern Medical Center at Dallas), which contains the neomycin-resistance gene.

Transfection. The mutated gene constructs were introduced by calcium phosphate (17) or cationic liposome-mediated (18) transfection into a B10.CAS2 fibroblast cell line as described previously (4). At least two transfected lines were assayed for each of the mutant constructs. Transfectants with wild-type cosmid or cDNA were cloned by limiting dilution, and the results were confirmed by using these clones as target cells.

Nomenclature and CTL Assay. Mta is determined jointly by *Mtf*, the mitochondrial gene which determines the sequence of the MTF peptide ligand (3), and by *H-2M3* (*Hmt*) (6). The Mta antigen of an *Mtf*$^\alpha$ *Hmt*^{a} cell is designated [α,a] for short. The recognition of target cells by CTLs specific for HMT, MTF$^\alpha$, or MTF$^\beta$ was determined in a standard ^{51}Cr-release assay (3).

Mice and Embryonic Tissue. Mice used in this study were bred and maintained in our colony and have been described (3, 5) or were purchased from The Jackson Laboratory. Two sexually mature female Swiss mice were placed with one male in each breeding box, and the appearance of a vaginal plug in the morning marked day 0 of pregnancy. Embryos were collected from days 8, 10, 12, 14, and 17 of gestation. A dissecting microscope and fine forceps were used to remove the embryonic sacs completely, and the embryos were rinsed in buffered saline to remove blood.

RESULTS

The *M3* Promoter Directs the Synthesis of HMT. We previously demonstrated that an *Hmt*^{b}, Mta$^-$ cell line can express an HMT^a molecule and present the MTF peptide to form Mta after transfection with constructs of *M3* cDNA from *Hmt*^{a} mice under control of the metallothionein or human β-actin promoters (4). If HMT functions as a classical class I antigen, the *M3* promoter itself must be able to direct the synthesis of HMT. Cosmid 14RB-A, containing the entire *M3* gene from BALB/c (*Hmt*^{a}) mice, was used to transfect B10.CAS2 [α, b] fibroblasts. The TC-2D transfectants became susceptible to lysis by CTLs raised in a strain combination that differs for the entire *Hmt* region ([α, b] anti-[α, a]) (Fig. 1*A*). This alloantigen is indeed Mta (see also Fig. 4*A*), rather than the product of an unidentified gene in the cosmid, which contains no other class I gene (9), because the transfectants were also lysed by CTLs raised in a combination that differs for *Mtf* only ([β, a] anti-[α, a]) (Fig. 1*B*). The transfectants were lysed by Mta-specific CTLs at levels equivalent to the control *Hmt*^{a} targets (e.g., L929); thus, *Hmt*^{b} cells are not defective in trans-acting factors required for M3 expression. This is consistent with our finding the *M3* mRNA in cells from *Hmt*^{b} mouse strains (4).

***M3* Expression Is Increased by IFN-γ.** Expression of all classical class I genes can be induced or increased by IFN-γ, but some nonclassical class I genes are refractory to IFN-γ

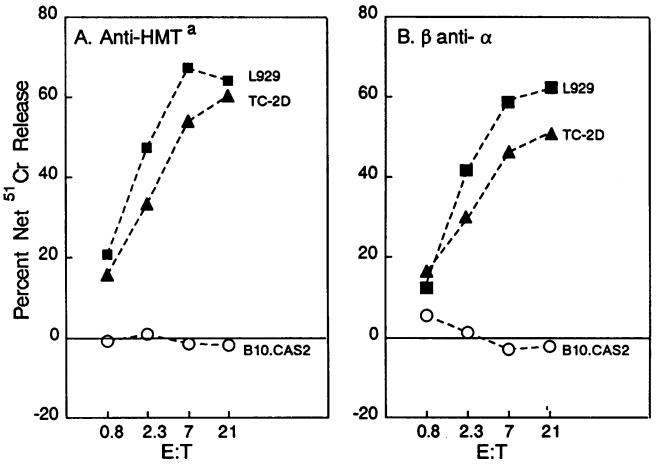

FIG. 1. CTL assay of genomic *M3*^{d} (*Hmt*^{a}) transfectants. TC-2D (▲) cells are B10.CAS2 [α, b] fibroblasts transfected with the 14RB-A cosmid from *Hmt*^{a} BALB/c mice. (*A*) Anti-HMT^a effectors from B6.CAS3(R4) mice (*Mtf*$^\alpha$ *Hmt*^{b} *H-2*^{k}, or [α, b; k]) were primed *in vivo* with C3H/HeJ [α, a; k] and restimulated *in vitro* with B10.BR [α, a; k] spleen cells. (*B*) Anti-MTF$^\alpha$ CTLs from (BOM × B10.BR)F$_1$ [β, a; q/k] mice were immunized and restimulated with B10.BR [α, a; k] spleen cells. Control target cells were B10.CAS2 [α, b; w17] (○) and L929 [α, a; k] (■) fibroblasts. E:T, effector/target cell ratio.

(19). Northern analysis showed that *M3* expression is up-regulated by IFN-γ both in L929 cells and in TR8.4 transfectants with the *M3* cosmid (Fig. 2*A*). As judged by densitometry, the level of *M3* mRNA in IFN-γ-treated cells was about twice that in untreated cells. The *M3* mRNA in B10.CAS2, a fibroblast cell line derived from the kidney of a newborn B10.CAS2 mouse, was detectable only by PCR and did not appear to respond to IFN-γ treatment. B10.CAS2 mice express the same level of *M3* mRNA in their liver (data not shown) as do *Hmt*^{a} mice (4). Hybridization with an actin probe confirmed that similar amounts of RNA were present in all lanes (Fig. 2*B*).

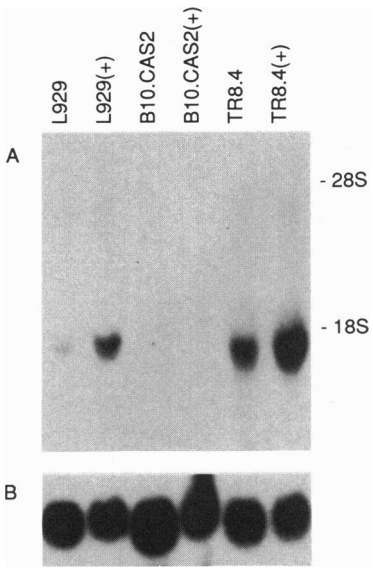

FIG. 2. Northern blot analysis of *M3* expression after IFN-γ treatment. RNAs from L929 or B10.CAS2 control cells or 14RB-A transfectants (TR8.4a) without or with (+) IFN-γ treatment were fractionated in a 1.5% agarose/formaldehyde gel, transferred to nylon membrane, and hybridized with the *M3*-specific probe A2-7 (4) (*A*) or an actin probe (*B*). Positions of 18S and 28S rRNA are indicated.

Expression of *M3* mRNA in Embryos. Classical class I antigens are expressed early in development. We monitored *M3* expression during embryonic development by PCR with primers from exons 3 and 5 to determine when *M3* is transcribed and whether the exons are properly spliced. The size of the product amplified from cDNA is predicted to be 530 bp, and the product amplified from genomic DNA or unspliced cDNA will be 1623 bp. *M3* transcripts could be detected as early as day 8 of gestation and were continuously expressed afterwards (Fig. 3*A*). Southern blot analysis with an *M3*-specific probe confirmed the identity of the amplified product (Fig. 3*B*). Of the tissues from newborn mice, thymus yielded the most intense band, consistent with the findings that, in the adult, the *M3* message is most abundant in the thymus (4) and that Mta is easily detected on thymocytes from newborn mice (K.F.L., unpublished work).

Effects of Amino Acid Substitutions on CTL Recognition. Many amino acids that were identified as important elements in the three-dimensional structure of the classical class I molecules HLA-A2, Aw68, and B27 are conserved in HMT (20–23), and it is therefore possible to build a model of HMT on the assumption that it has the same overall structure (4). If HMT is structurally and functionally a neoclassical class I antigen, then we can use this model to predict the interaction of HMT with its cognate peptides. The immunologically null form, HMT^b, differs from HMT^a by four amino acids: (*i*) an Arg → Cys (*a* → *b*) substitution in the leader sequence, (*ii*) a Val → Met substitution at position 31 in the α1 domain between two β strands in the loop pointing toward the α3 domain, (*iii*) a Leu → Gln substitution at position 95 in the α2 domain pointing up from the β-sheet floor into the peptide-binding groove, and (*iv*) a deletion of one Val at the end of the transmembrane domain.

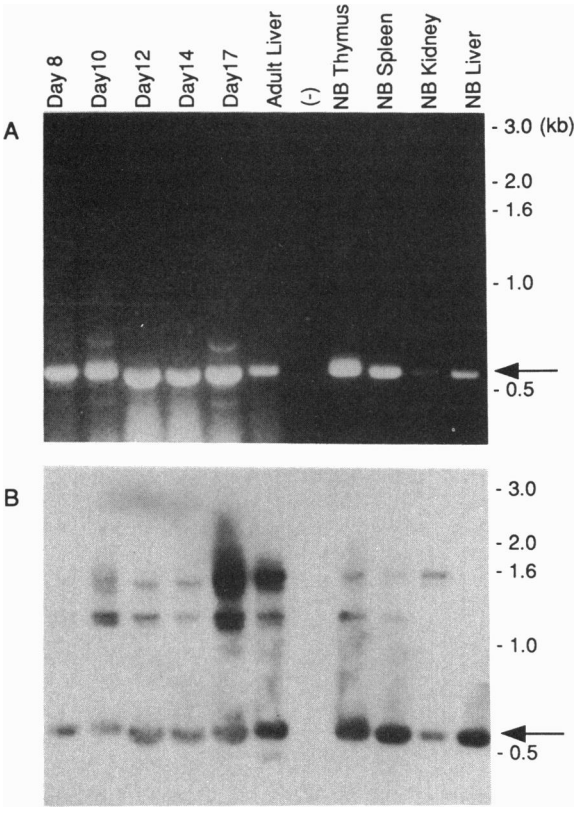

FIG. 3. PCR analysis of *M3* expression during embryonic development. RNA from day-8 to day-17 embryos and from tissues of newborn (NB, <1-day-old) Swiss mice was used for cDNA synthesis and PCR amplification. The PCR products were separated in a 1.5% agarose gel, transferred to a nylon membrane, and hybridized with probe A2.7. PCR mixtures without cDNA (−) served as the negative control. Size markers (1-kb ladder, GIBCO) are indicated at right. Ethidium bromide staining (*A*) and Southern blot analysis (*B*) are shown. Arrows point to a PCR band for fully spliced *M3* mRNA; two larger bands detected by hybridization may represent partially spliced mRNA (≈1.4 kb) and unspliced mRNA or genomic DNA (≈1.6 kb).

To determine which amino acid substitution in the HMT^b molecule affects the recognition by Mta-specific CTLs, we constructed three *M3* mutants: M31 (Val^{31} → Met in α1), Q95 (Leu^{95} → Gln in α2), and the double mutant M31Q95. Each was transfected into the B10.CAS2 (*Mtf*$^{\alpha}$, *Hmt*^{b}; Mta^-) cell line, and CTLs were used to test whether these transfectants expressed HMT^a determinants and could present an MTF peptide. In a typical assay, anti-HMT^a-specific CTLs reacted as well with mutant M31 as with wild-type (*Hmt*^{a}) transfectants, but they did not react with the Q95 or M31Q95 transfectants (Fig. 4*A*). The Q95 and M31Q95 mutants had also lost the ability to present an endogenous MTF^{α} peptide (Fig. 4*B*). By contrast, M31 transfectants remained sensitive to MTF^{α}-specific CTLs. Furthermore, the Q95 and M31Q95 mutants were also unable to present an exogenous synthetic peptide that mimics MTF^{β} (Fig. 4*C*), whereas M31 transfectants treated with β peptide were recognized almost as effectively as the wild-type (*Hmt*^{a}) transfectants given the β peptide.

DISCUSSION

The three main regulatory sequences of classical class I genes were previously identified in *M3* (4): two enhancer-like elements, homology A and homology B (24), and an interferon-responsive consensus sequence (ICS) (25). Two nucleotides are substituted and two nucleotides inserted in the ICS of *M3*^{d} (AGGTTTCACT/TC/TCT) relative to the *H-2K*^{b} ICS (CAGTTTCACT//TCT). Nevertheless, expression of *M3* was still stimulated 2-fold by IFN-γ, a degree of inducibility comparable to that of classical class I mRNA (26). The ability to respond to a lymphokine, such as IFN-γ, is consistent with the notion that *M3* and its product, HMT, may have a biological function in host defense (9, 10).

The class I genes from the *H-2K, D, Q*, and *T* regions are differentially expressed during embryonic development (27). Similar to the classical class I genes *H-2K* and *H-2D* (28), *M3* is transcribed as early as day 8 of gestation and continues to be transcribed thereafter. Some class I genes located telomeric to *H-2D* are expressed in a tissue-specific manner, but *M3* and Mta are widely expressed in a variety of adult tissues (4, 29, 30). The early expression of *M3* and the high level in the thymus suggest that HMT may be active in thymic selection of the T-cell receptor repertoire.

Of the four amino acid changes between HMT^a and HMT^b, one is a substitution in the leader peptide, which is cleaved from the mature molecule, and one alters the length of the transmembrane region by a single residue. Only two substitutions occur in the α1 and α2 domains, which contain almost all T-cell epitopes in classical class I molecules (31–34). The mutation at position 95 completely eliminated the T-cell epitopes of Mta, while mutation of residue 31 had no significant effect. This is concordant with the structure of HMT predicted from classical HLA molecules (20–23). Position 31 is in the loop between two β-strands, pointing away from the peptide-binding groove toward the α3 domain and, therefore, not likely to affect the binding of MTF or T-cell recognition. Position 95 is located on the floor of the peptide-binding groove, pointing into the binding site. Changing Leu to Gln, with a larger and polar side chain, may prevent the binding of MTF to HMT^b or alter its conformation into a nonimmunogenic form. These two possibilities should be discriminated by a peptide binding assay (35, 36). The importance of residue 95 in peptide binding and allorecognition has also been observed in HLA-A2 variants (37, 38).

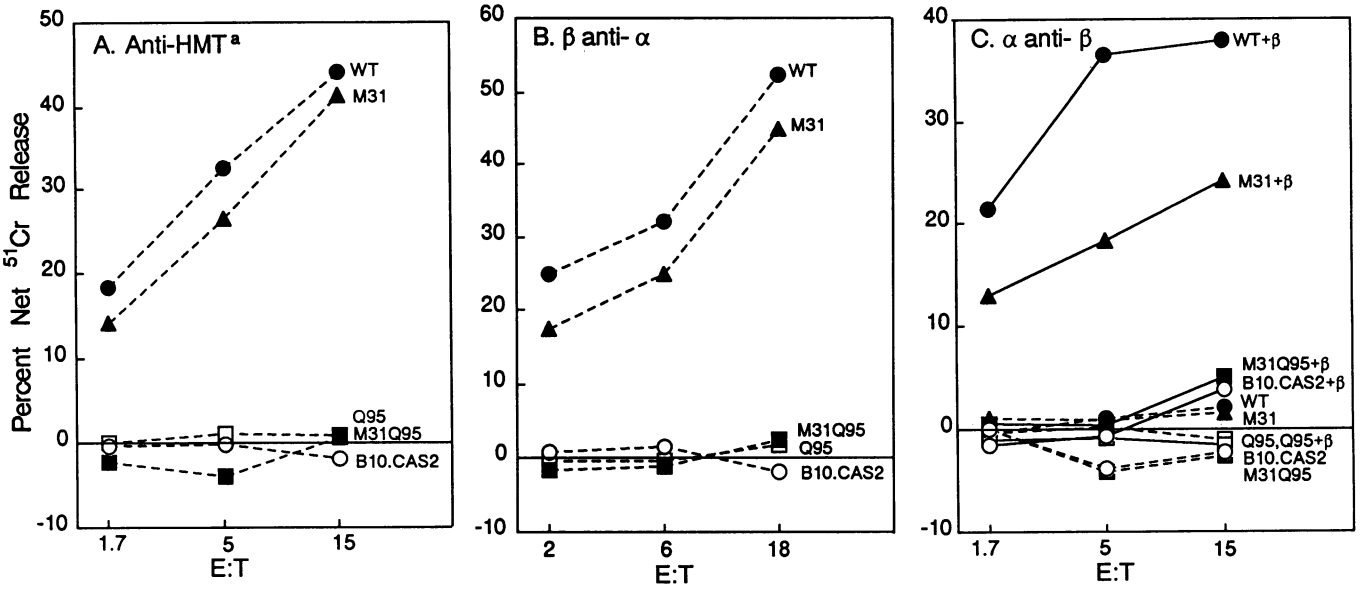

FIG. 4. CTL assays of M3 mutants. B10.CAS2 [α, b] fibroblasts were transfected with wild-type (WT, *Hmt^a*) cDNA or the M31, Q95, or M31Q95 mutant cDNA. Anti-HMT^a (*A*) and anti-MTF$^\alpha$ (*B*) CTLs were generated as described in Fig. 1. Anti-MTF$^\beta$ CTLs (*C*) were from (C57BL/10 × BOM)F$_1$ mice [α, a; b/q] primed and restimulated with BOM [β, a; q] cells. Some targets, marked "+β" in *C*, were incubated overnight before the assay with 1 μM of ND1β1-17 peptide (fMFFINΔLTLLVPILIAM; the Ile → Ala change from MTF$^\alpha$ to MTF$^\beta$ is underlined, and fM represents *N*-formylmethionine) (3). E:T, effector/target cell ratio.

The Q95 mRNA, like the other mRNAs, could be translated in an *in vitro* system to a product of the expected size (data not shown). A direct assessment of wild-type and mutant HMT molecules on the cell surface was not feasible, because our two antisera raised against HMT-specific peptides fail to stain mouse cells expressing *M3*, whether from tissues or cell lines (unpublished data). Although we doubt that Gln^{95} precludes surface expression of HMT, we are not aware of another MHC class I molecule with Gln in this position.

We conclude that HMT is a neoclassical class I antigen. It was omitted from the classical class I antigens, not because of any fundamental difference in structure, function, or expression, but because of three fortuitous circumstances: (*i*) *M3* is, in molecular terms, far removed from the classical *H-2* locus; (*ii*) HMT polymorphism is low, and most laboratory strains are HMT^a; and (*iii*) the formylated peptides bound by HMT are not generated by processing the viral or minor histocompatibility antigens classically used to study antigen presentation to CTLs.

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