Novel Mutants Define Genes Required for the Expression of Human Histocompatibility Leukocyte Antigen DM: Evidence for Loci on Human Chromosome 6p

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Summary

We and others have shown that the products of the HLA-DM locus are required for the intracellular assembly of major histocompatibility complex class II molecules with cognate peptides for antigen presentation. HLA-DM heterodimers mediate the dissociation of invariant chain (Ii)-derived class II–associated Ii peptides (CLIP) from class II molecules and facilitate the loading of class II molecules with antigenic peptides. Here we describe novel APC mutants with defects in the formation of class II–peptide complexes. These mutants express class II molecules which are conformationally altered, and an aberrantly high percentage of these class II molecules are associated with Ii-derived CLIP. This phenotype resembles that of DM null mutants. However, we show that the defects in two of these new mutants do not map to the DM locus. Nevertheless, our evidence suggests that the antigen processing defective phenotype in these mutants results from deficient DM expression. These mutants thus appear to define genes in which mutations have differential effects on the expression of conventional class II molecules and DM molecules. Our data are most consistent with these factors mapping to human chromosome 6p. Previous data have suggested that the expression of DM and class II genes are coordinately regulated. The results reported here suggest that DM and class II can also be differentially regulated, and that this differential regulation has significant effects on class II–restricted antigen processing.

Conventional class II molecules of the MHC are polymorphic cell surface glycoproteins which bind peptide Ags and display them on the surface of APC for recognition by $CD4^+$ T cells. Though MHC class II molecules are mainly associated with peptides derived from endogenous proteins (1–3), in terms of host defense, class II molecules function primarily to display peptides derived from exogenous Ags. The selectivity of class II molecules for exogenous Ags derives from a distinct intracellular trafficking pathway of class II molecules and distinct intracellular sites in APC for processing of exogenous Ags (4–7).

In the class II pathway, class II α and β chains assemble in the endoplasmic reticulum (ER)[1] with the invariant chain (Ii). A short region of Ii encoded by exon 3 prevents premature binding of peptides in the ER to class II α/β by sterically blocking the Ag binding groove (8, 9). This portion of Ii also appears to act as a surrogate peptide to promote egress of nonamer $(\alpha/\beta\text{–Ii})_3$ complexes out of the ER (10, 11). Ii targets α/β–Ii to the endosomal compartments where Ii is selectively removed, in part by acid proteolysis (12–16). The complete dissociation of Ii allows access of the α/β binding groove to exogenous peptides for functional α/β–peptide complex assembly (17). Specialized intracellular vesicles within APC, termed MIIC (5) or CIIV (18), have been implicated as putative sites for final Ii dissociation and peptide loading (6, 18–20). Until recently, details of the molecular nature of peptide loading within this compartment were unknown.

Using a somatic cell mutant approach, we and others have shown that the products of the HLA-DM locus have a critical function in the assembly of MHC class II molecules with cognate peptides (21–24). HLA-DMA and -DMB genes map to the class II region of the HLA complex (25) and encode subunits of an unconventional MHC class II heterodimer. HLA-DM mutants are defective in antigen processing; although these mutants express normal levels of class II molecules (22, 26–29), they fail to assemble normal α/β–peptide complexes (30–32). This defect in DM mutants is a direct consequence (33) of an aberrantly high percentage of class II molecules that remain complexed with

[1] *Abbreviations used in this paper*: B-LCL, B-lymphoblastoid cell lines; EMS, ethyl methane sulfonate; ER, endoplasmic reticulum; Ii, invariant chain; PI, propidium iodide.

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Ii-derived class II–associated Ii peptides (CLIP) (30–32). CLIP are a nested set of peptides derived from amino acids 81–104 of Ii. CLIP-class IIα/β complexes are normal intermediates in the MHC class II biosynthetic route (34). DM mutants have a block in a step required for removal of CLIP from class II molecules. Recently, the DM heterodimer has been shown in cell-free systems to catalyze the dissociation of CLIP from class II molecules, and to facilitate loading of cognate peptides (23, 35–37). Presumably this activity of DM is restricted to the peptide loading compartment(s) of APCs. Indeed, HLA-DM has been shown to localize intracellularly to MIIC (38, 39). How DM expression is regulated, how its activity is coordinated with the expression of conventional class II molecules, and what other factors might interact in the class II pathway remain important unresolved problems. However, recent available data suggest that the DM genes are regulated coordinately with class II genes under most conditions (52).

To further analyze the pathway of class II antigen processing, we have isolated additional mutant APCs defective in MHC class II–peptide complex assembly. Here we describe a set of mutants which display a novel class II–deficient phenotype resembling that of DM mutants. However, these mutants are complementary with T2, a cell that is homozygously deleted for HLA-DM and the entire class II region (34, 40, 41). Therefore, the lesions in these new mutants cannot be in the HLA-DM genes, and the affected genes map outside the HLA class II region. We provide evidence that these mutants fail to express sufficient levels of DM heterodimers to effect normal DM function. The results suggest that DM and class II can be differentially regulated, and that this differential regulation has significant effects on MHC class II–cognate peptide assembly.

Materials and Methods

Cell lines and Derivation of Mutants. Mutants 2.2.93 and 2.7.93, and mutants 3.6.95 and 3.4.95, were isolated from ethyl methane sulfonate (EMS) mutagenized 3.1.0/DR3 and 6.3.6/DR3, respectively, by immunoselection with anti-DR3 mAb 7.3.19.1 (42) and rabbit complement. To derive 3.1.0/DR3 and 6.3.6/DR3, a 1.2-kb DRB1.0301 cDNA was cloned from B-LCL 8.1.6 into pGEM-4 (Promega Corp., Madison, WI). This was then subcloned into the EcoRI site of pBluescript II KS +/- (Stratagene Inc., La Jolla, CA) to make pDR3B1.121. A HindIII/SmaI DRB1.0301 cDNA fragment from pDR3B1.121 was gel purified and subcloned into the HindIII/ HpaI site of pLNCx, a retroviral expression vector (43). This was used to obtain clones of the packaging line PA317 (43) to transduce B-LCL 3.1.0 and 6.3.6. DR3-expressing clones were obtained by 3-d coculture of B-LCL with the transfected PA317 packaging line in the presence of 4 μg/ml polybrene (Sigma Chem. Co., St. Louis, MO). After coculture, G418 (1 mg/ml) resistant cultures of transduced cells were cloned and screened by flow cytometry for stable DR3 expression to obtain 3.1.0/DR3 and 6.3.6/DR3. In addition, to derive 6.3.6/DR3, an EcoRI/BamHI DRB1.0301 cDNA fragment from pDR3B1.121 was gel purified and subcloned into the EcoRI/BamHI site of pLxSHD, a similar retroviral expression vector (44) containing *hisD*. This was used also to derive a clone of the packaging line PA317, which was used to super-infect 6.3.6 for high-level DR3 expression. Transduced 6.3.6 was doubled selected in 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD) and 4 mM l-histidinol (Sigma) to obtain a 6.3.6/DR3 clone. Both progenitors were mutagenized with EMS to induce point mutations before immunoselection (45).

Somatic cell hybrids were prepared by PEG fusion as described (26) and were selected using methotrexate (2×10^{-7} M) and G418 (1 mg/ml). All hybrids are uncloned, stable lines. For stable transfection of 2.7.93 with DMA, 2.7.93 was transduced with a DMA retroviral packaging line as described for 2.2.93 (22). 2.7.93/DMA and 2.2.93/DMA were obtained by transduction of 2.7.93 and 2.2.93, respectively, with a DMA retroviral packaging line as described for 2.2.93 (22). Stable clones were obtained by selection in 4 mM l-histidinol.

Flow Cytometric Analysis. For immunofluorescent flow cytometry, 0.5×10^6 cells were stained for 1 h at 4°C with saturating amounts of the indicated mAbs in 50 μl RPMI/5% FBS, washed and further stained 1 h at 4°C with 1:100 fluoresceinated goat $F(ab')_2$ anti–mouse IgG Fc (FITC-GAM) (Sigma). After staining, cells were washed, resuspended in 25 μg/ml propidium iodide (PI) for dye exclusion (viability) gating, and run through FACScan® (Becton-Dickinson) and analyzed with REPROMAN. Cells in each panel were analyzed in parallel. Histograms represent immunofluorescence on viable, triple-gated events (forward scatter FSC, side scatter SSC, and PI exclusion).

Antibodies. For indirect immunofluorescent flow cytometry, primary mAbs used were: mAb L-243 (anti DR, reference 1); mAb VI-15 (anti-DR, reference 46); mAb UK8.1 (anti-DR3, 5, 6, reference 47); mAb 62.74 (anti-DR1); mAb 16.23 (anti-DR3; reference 48); mAb 4AA7 (anti-DQ1, reference 49); mAb 13.3.B4 (anti-DP4.1, reference 50); mAb 1A3 (anti-DQ); mAb SPV-L3 (anti-DQ); mAb B7/21 (anti-DP); mAb W6/32 (anti–class I); mAb 4D12 (which recognizes a TAP-dependent determinant on HLA-B5 class I molecules); mAb CER.CLIP.1 which recognizes Ii-derived CLIP associated with HLA-DR molecules (51); and mAb I-5 which recognizes CLIP associated with HLA class II molecules (E. Mellins and Y. Paterson, personal communication). For immunoselections, mAb 7.3.19.1 was used. mAb 7.3.19.1 binds an epitope which blocks mAb 16.23 binding (S.P. Fling unpublished observations) and, like mAb 16.23, exhibits reduced binding on DM mutants as a result of peptide-induced serological changes in HLA-DR3 molecules.

To obtain DMα-specific anti-serum 8338H, a recombinant fusion protein was constructed by subcloning, from pDMA (22), a 570-bp cDNA fragment encoding the DMα1 and DMα2 in frame into pMal-C2 (N.E. Biolabs) for bacterial expression. Recombinant fusion protein was purified as described (NEB Instruction manual, Version 3.01. Protein fusion and expression system. 1993) and was used to immunize and boost NZW rabbits for production of anti-serum 8338H.

MHC Class II Dimer Stability Assays. Western immunoblots were stained with a combination of mAb DA6.147, which recognizes HLA-DRα monomers and HLA-DRα/β dimers (53), and mAb HB10.A, which recognizes HLA-DR3β monomers and HLA-DRα/β dimers (54). For dimer stability assays, nondenatured, NP-40 extracted whole cell lysates were prepared and 1×10^6 cell equivalents per sample were run in SDS-PAGE and transferred to PVDF. Membranes were stained with mAb followed by polyclonal rabbit anti–mouse IgG and ^{125}I protein-A.

Cell Sorting. To fractionate 2.7.93 into mAb 16.23^{low} and mAb 16.23^{null} subpopulations, 10^7 cells of a 2.7.93 clone were stained with saturating levels of mAb 16.23 in RPMI/5% FBS, washed and stained with FITC-GAM, and then resuspended in

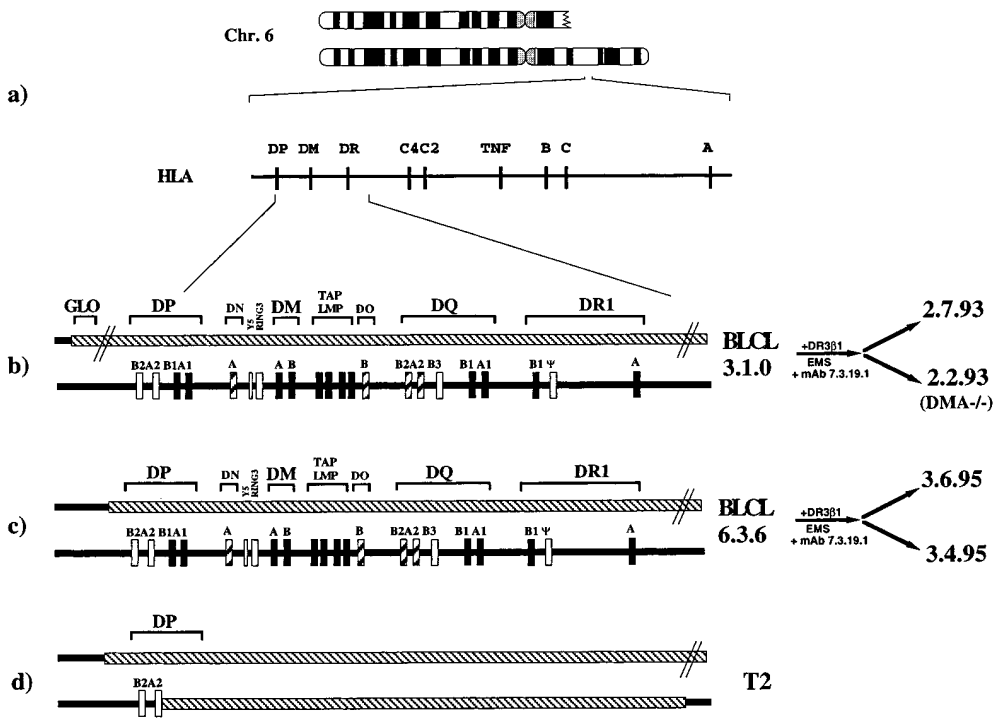


Figure 1. Derivation of mutant cell lines. (*a*) Regions of chromosome 6p hemizygously deleted in progenitors 3.1.0 (~40 Mb) and 6.3.6 (~30 Mb) and their positions relative to the HLA complex. (*b*) The HLA class II region haplotypes of B-LCL 3.1.0, the progenitor of mutant 2.7.93 and mutant 2.2.93 (DMA null) (22). (*c*) The HLA class II region haplotypes of B-LCL 6.3.6, the progenitor of mutants 3.6.95 and 3.4.95. Deletions are indicated by horizontal hatched bars on the deleted haplotype and have been previously described for 3.1.0 and 6.3.6 (71). Vertical bars indicate approximate locations of genetic loci and details of the HLA class II region (72). (*d*) The 1-Mb class II region HLA homozygous deletion in T2 (T × B hybrid line: 721.174 × CEM.T2) (40). The centromeres are to the left. Not to scale.

PI for viability gating. Cells were sorted by FACStar® (Becton-Dickinson) by setting collection gates to collect the 10% of the stained population expressing the lowest levels, or highest levels, of the 16.23 epitope to obtain mAb 16.23^{null} and mAb 16.23^{low} cells, respectively. Sorted cells were expanded for 2 wk in RPMI/10% BCS. Cell lysates from the indicated cell lines were then run in dimer stability assays and were stained with a combination of mAb DA6.147 and mAb HB10.A. Subclones of the mAb 16.23^{low} and mAb 16.23^{null} subpopulations were obtained by cloning in soft-agar and were analyzed for mAb 16.23 expression by indirect flow cytometry.

Western Immunoblots. Boiled, detergent (NP-40) extracted cell lysates from 10^6 cells/lane or 0.5×10^6 hybrids/lane were prepared and run in reducing SDS-PAGE and transferred as described (55). Membranes were stained with anti-DMα anti-serum 8338H followed by biotinylated goat anti–rabbit IgG (GIBCO BRL) and Streptavidin-HRP (GIBCO BRL) followed by Lumiglo substrate (Kirkegaard and Perry Labs. Gaithersburg, MD).

Transient DM Expression Assays. For transient expression experiments, cDNA encoding HLA-DMB and cDNA encoding HLA-DMA were cloned into the expression vector pcDNAI/AMP (Invitrogen Corp., Carlsbad, CA) to obtain plasmids pDMB and pDMA, respectively, as described (22). pcDNAI/AMP contains Polyoma and SV40 origins of replication, allowing for episomal replication. For transient transfections, 2.5×10^7 of the indicated cells were resuspended in 0.5 ml RPMI with 15–25-μg circular pDMB or pDMA or both and were electroporated using 2.2 kV. Samples were cultured for 72 h to regain high viability and then were stained and analyzed by flow cytometry as described in Fig. 2.

Northern Analysis. Total cellular RNA was isolated and northern blots were prepared by standard techniques (56, 57). Probes used for hybridization were labeled with [^{32}P]dCTP using random primers (Boehringer Mannheim Biochemicals, Indianapolis, IN). PCR was used to generate full-length probes from DMA and DMB cDNAs as described (22). The actin probe was a cDNA in a linearized plasmid (58).

Results

Derivation of Mutants 2.7.93, 3.6.95, and 3.4.95. EBV transformed human B-lymphoblastoid cell lines (B-LCL) with hemizygous MHC deletions have been useful as progenitors for immunoselecting recessive mutants which are defective in MHC class II/peptide assembly, including DM mutants previously isolated (21, 22, 26, 27). Using this approach, we derived mutant 2.7.93 from progenitor 3.1.0, which contains an ~40-Mb hemizygous deletion in chromosome 6p, removing one HLA haplotype (59) (Fig. 1). Mutants 3.6.95 and 3.4.95 were similarly derived from progenitor 6.3.6, which contains a smaller, ~30 Mb hemizygous deletion in chromosome 6p, which is overlapped by the 3.1.0 deletion (59) (Fig. 1). As a basis for immunoselection of these mutants, progenitors 3.1.0 (DR1+/DR3-) and 6.3.6 (DR1+/DR3-) were first transduced with DRB1.0301. HLA-DR3 molecules exhibit reduced binding of certain mAbs, e.g., mAb 16.23 and mAb 7.3.19.1, as a result of changes in occupancy of the peptide binding groove (22, 26). The 3.1.0/DR3 and 6.3.6/DR3 progenitors were mutagenized with EMS and were immunoselected with mAb 7.3.19.1. After immunoselection, surviving clones were screened for loss of mAb 16.23 binding and further analyzed for class II alterations suggestive of defects in MHC class II/peptide assembly. Mutants 2.7.93, 3.4.95, and 3.6.95 were isolated from mutagenized progenitors at frequencies of $\sim 10^{-4}$, consistent with observed frequencies for EMS-induced recessive mutations in other hemizygous loci using equivalent EMS concentrations and standard protocols (55, 60).

Mutants 2.7.93, 3.6.95, and 3.4.95 Express Conformationally Altered MHC Class II Molecules at the Cell Surface. The class II cell surface phenotypes of the new mutants were characterized by FACS® analysis using a panel of mAbs

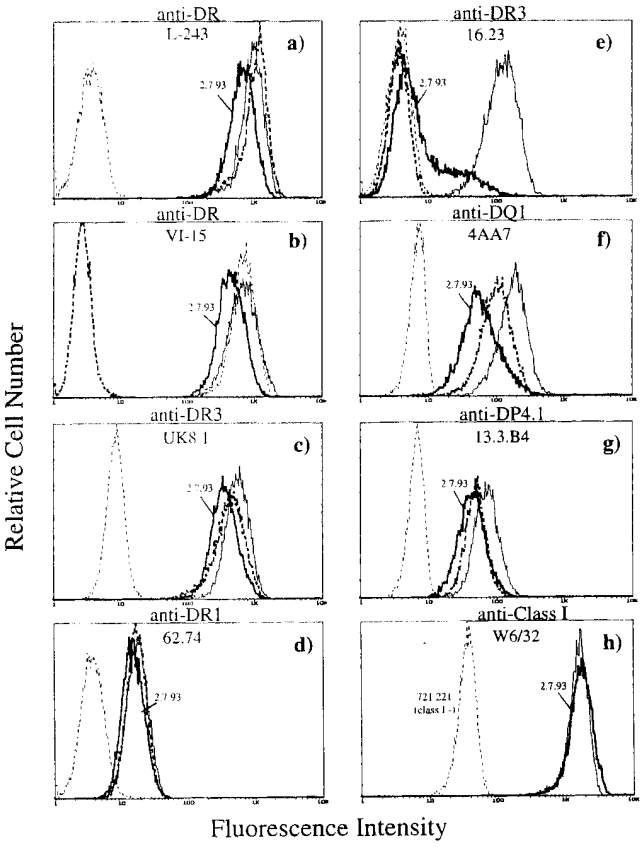


Figure 2. Mutant 2.7.93 expresses conformationally altered class II molecules. Cell surface staining of MHC molecules on mutant 2.7.93. Mutant 2.7.93 (*dark line*), its progenitor, 3.1.0/DR3 (*thin line*), control DMA null mutant, 2.2.93 (*heavy dashed*), and negative control, T2 (*thin dashed*), were stained in indirect immunofluorescent flow cytometry using class II conformation-independent mAbs: (*a*) L-243 (*anti-DR*); (*b*) VI-15 (*anti-DR*); (*c*) UK8.1 (*anti-DR3, 5, 7*); (*d*) 62.74 (*anti-DR1*); and class II conformation-sensitive mAbs: (*e*) 16.23 (*anti-DR3*); (*f*) 4AA7 (*anti-DQ1*); (*g*) 13.3.B4 (*anti-DP4.1*); and mAb to class I: (*h*) W6/32. Only mutant 2.7.93 is labeled to highlight its profile. In *b* only, T2 is heavy dashed and 2.2.93 is thin dashed.

to polymorphic and monomorphic determinants. As shown in Fig. 2, new mutant 2.7.93 has a pattern of diminished binding of antibodies to polymorphic class II determinants which resembles that of DM null mutants. Specificially, mutant 2.7.93 expresses levels of class II HLA-DR molecules similar to its progenitor 3.1.0/DR3, but exhibits a major reduction in the binding of DR3-specific mAb 16.23 (Fig. 2 *e*). Anti-DR3,5,6 mAb UK8.1 staining indicates that the reduction in expression of the mAb 16.23 determinant is not a result of loss of DR3B1 transgene expression (Fig. 2 *c*). Mutant 2.7.93 also exhibits reductions in the binding of DQ1-specific mAb 4AA7 and of DP4.1-specific mAb 13.3.B4 (Fig. 2). The pattern of expression of the determinants recognized by these mAbs in mutant 2.7.93 is similar to that seen in DMA null mutant 2.2.93 (Fig. 2). These determinants, like that recognized by mAb 16.23, have previously been shown to be DM dependent (22). Thus the lesion in mutant 2.7.93 extends to determinants on all class II isotypes. These changes in MHC expression are limited to class II, as the expression of MHC class I appears unaffected (Fig. 2 *h*).

Mutants 3.6.95 and 3.4.95 have similar phenotypes to mutant 2.7.93, although they were derived from a different progenitor, 6.3.6/DR3 (Fig. 1), and thus are independent of 2.7.93. Mutants 3.6.95 and 3.4.95, like 2.7.93, exhibit substantial loss of the mAb 16.23 epitope (Fig. 6 *a*), but they also retain levels of MHC HLA-DR and of the DR3 transgene comparable to those of progenitor 6.3.6/DR3 (Table 1).

Although these changes resemble those of DM null mutants, there are features of the mAb binding profile that distinguish the new mutants from DMA mutant 2.2.93 and all other DM mutants. In particular, whereas DM null mutants are essentially mAb 16.23^{null}, mutant 2.7.93 has the mAb 16.23^{null} peak but also a reproducible mAb 16.23^{low} shoulder (Figs. 2 *e* and 5). Mutants 3.4.95 and 3.6.95 also exhibit this unusual, bimodal distribution of mAb 16.23 staining (Fig. 6 *a*), although relative to 2.7.93, mutant 3.4.95 consistently displays a greater proportion of mAb 16.23^{null} cells and 3.6.95 consistently displays a greater proportion of mAb 16.23^{low} cells in their respective populations. Thus, these mutants have related, yet distinguishable phenotypes. The bimodal mAb 16.23 staining pattern in these mutants persists despite repeated subcloning (see below and Fig. 5).

A second significant difference between the new mutants and DM null mutants is evident from cell surface binding patterns with a broader panel of class II mAbs, as shown in Fig. 2 and summarized in Table 1. Whereas DM null mutant 2.2.93 is affected principally in the binding of antibodies recognizing class II polymorphic determinants (the DP monomorphic determinant recognized by mAb B7/21 being the only exception), the new mutants are affected not only for these class II polymorphic determinants but for class II monomorphic determinants as well. For example, HLA-DR levels measured by mAb VI.15 and L-243 staining are diminished ($\sim$75% of wild type) on mutant 2.7.93. This pattern differs from that of a DM null mutant 2.2.93 (also 3.1.0/DR3-derived), in which HLA-DR levels measured by mAb VI.15 and L-243 staining are virtually unchanged (reference 22 and Table 1). In addition, the expression on mutant 2.7.93 of DQ determinants recognized by mAbs SPVL3 and 1A3 is $\sim$25–50% of wild type, and is reduced to levels lower than in DMA null mutant 2.2.93 (Table 1).

In summary, both DM-dependent and DM-independent class II cell surface epitopes are affected in the new mutants. However, there is a greater reduction in the expression of DM-dependent as compared to DM-independent epitopes. These data suggest that the genetic lesions in these mutants have pleiotropic effects: a major effect on DM activity and a lesser effect in cell surface class II expression.

Mutants 2.7.93, 3.6.95, and 3.4.95 Express MHC Class II Molecules Which Are Unstable in SDS. To assess the extent of cognate peptide binding to class II molecules in these new mutants, we analyzed the stability of their class II dimers in SDS. Cognate peptide binding to class II molecules induces conformational changes in class II dimers which enhance their stability in SDS (61–63). Thus, impaired cognate

Table 1. *MHC Class II mAb Staining of Mutants 2.7.93, 3.4.95, 3.6.95*

Cells	mAbs (Antigenic Specificity)										
	L-243 (DR)	VI.15 (DR)	16.23 (DR3)	7.3.19.1 (DR3)	UK8.1 (DR3)	62.74 (DR1)	4AA7 (DQ1)	SPVL3 (DQ)	Ia3 (DQ)	13.3.B4 (DP4.1)	B7/21 (DP)
310/DR3	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
2.7.93	0.72 ± 0.03	0.74 ± 0.08	0.15 ± 0.04	0.46 ± 0.04	0.64 ± 0.09	0.93 ± 0.17	0.29 ± 0.03	0.26 ± 0.03	0.45 ± 0.05	0.42 ± 0.03	0.47 ± 0.07
2.2.93*	1.08 ± 0.04	1.08 ± 0.20	0.04 ± 0.01	0.57 ± 0.04	0.81 ± 0.08	1.53 ± 0.18	0.63 ± 0.14	0.63 ± 0.12	1.15 ± 0.16	0.73 ± 0.11	0.70 ± 0.03
636/DR3	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
3.4.95	0.77 ± 0.07	0.92 ± 0.15	0.15 ± 0.05	0.45 ± 0.20	0.77 ± 0.08	0.60 ± 0.03	0.53 ± 0.06	0.42 ± 0.12	0.52 ± 0.03	0.51 ± 0.25	0.49 ± 0.06
3.6.95	0.87 ± 0.14	0.88 ± 0.15	0.30 ± 0.04	0.66 ± 0.01	0.88 ± 0.02	0.66 ± 0.11	0.75 ± 0.19	0.67 ± 0.05	0.83 ± 0.10	0.66 ± 0.04	0.58 ± 0.08

Cell surface class II levels were determined by staining mutants and their progenitors in indirect immunofluorescent flow cytometry. Data are expressed as a proportional value, relative to wild type (1.00), ± SE of replicate experiments.
*2.2.93 is a DM null mutant (22) included for reference staining.

peptide binding in DM null mutants results in the expression of SDS unstable class II dimers. Class II dimers from 2.7.93, like class II DR dimers from DM mutants, dissociate into DRα and DRβ monomers in SDS-PAGE, whereas dimers extracted from progenitor 3.1.0/DR3 remain stable (Fig. 3). Similarly, dimers from mutants 3.6.95 and 3.4.95, but not progenitor 6.3.6/DR3, dissociate into DRα and DRβ monomers in SDS-PAGE (data not shown). Notably, these changes in dimer stability are less severe than in DM null mutants (see Fig. 5 *a*) suggesting that the factor(s) required for class II–peptide complex assembly are significantly reduced, but not entirely absent in these mutants.

MHC Class II Molecules from Mutants 2.7.93, 3.6.95, and 3.4.95 Are Associated with Increased Levels of CLIP Peptides. The altered conformation of class II dimers expressed by these new mutants is similar to that of DM null mutants, in which the alterations in class II conformation result from the presence of CLIP and the absence of cognate peptides in the binding groove. To determine if the new mutants express aberrantly high levels of CLIP–class II complexes, we measured cell surface levels of CLIP using mAbs CER.CLIP1 and mAb I-5, both of which recognize CLIP associated with MHC class II molecules. Mutants 2.7.93, 3.4.95, and 3.6.95, like DM null mutants, exhibit marked increases in cell surface levels of class II molecules associated with CLIP peptides (Figs. 4 *b* and 6 *b*). However, the levels of anti-CLIP staining on these mutants are not as high as those of DM null mutants 2.2.93 or 9.5.3 (Figs. 4 and 6).

Class II Dimer Stability Correlates with mAb 16.23 Staining in 2.7.93 Subpopulations. An interesting aspect of the 2.7.93-type mutants is the bimodal distribution of mAb 16.23 staining described above. To determine if this unusual distribution of mAb 16.23 staining correlates with different levels of class II stability in SDS, we sorted mutant 2.7.93 by FACS® into mAb 16.23^{null} and mAb 16.23^{low} subpopulations. Cell lysates from these two subpopulations were then run in dimer stability assays. As demonstrated in Fig. 5 *a*, mAb 16.23^{null} and mAb 16.23^{low} subpopulations of 2.7.93-type mutants express different levels of stable HLA-DR dimers. Whereas the majority of HLA-DR molecules in both subpopulations are in an SDS-unstable state, there is a significantly higher proportion of stable HLA-DRα/β dimer in the mAb 16.23^{low} than in the mAb 16.23^{null} subpopulation. Therefore, the level of expression of the mAb 16.23 epitope in these mutants correlates with the level of expression of stable dimers. Insofar as class II dimer stability in SDS is a measure of DM activity, the bimodal mAb 16.23 phenotype of these may result from variable levels of DM expression.

The Phenotype of 2.7.93-type Mutants Appears Genetically Stable. The bimodal phenotype of the new mutants appears genetically stable, in that mutants maintain their characteristic bimodal distribution in continuous culture (data not shown). Furthermore, the bimodal distribution of the mAb 16.23 epitope is regenerated in sub-clones of both mAb 16.23^{null} and mAb 16.23^{low} sorted subpopulations. This was demonstrated by isolating sub-clones of the mAb

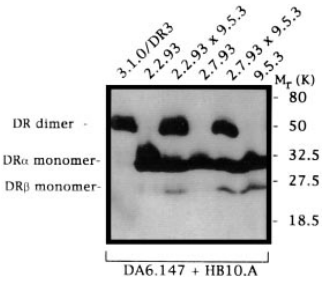


Figure 3. Mutant 2.7.93 expresses unstable class II dimers, and the defects in dimer stability in mutant 2.7.93 and DM mutant 9.5.3 are complementary. Cell lysates from mutant 2.7.93, and the indicated control cell lines, DMA mutant 2.2.93 and DMB mutant 9.5.3, and lysates from somatic cell hybrids were run in dimer stability assays essentially as described (55). Western blots were stained with a combination of mAb DA6.147, which recognizes DRα monomer and DRα/β dimers, and HB10.A, which reacts with DR3β1 and DR3β3 in monomeric form and in DRα/β dimers.

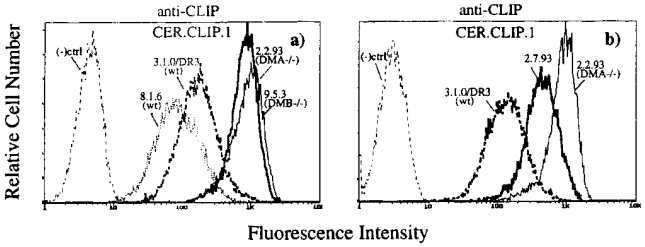
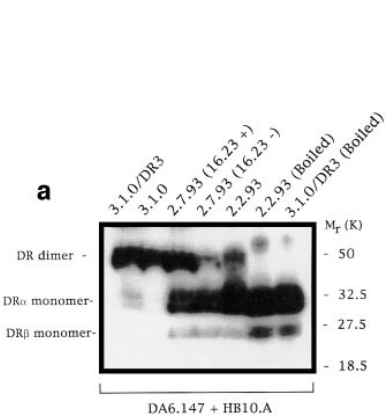


Figure 4. Mutant 2.7.93 expresses increased levels of class II which are associated with CLIP. (*a*) Control cells, DMA null mutant 2.2.93 and DMB null 9.5.3, their progenitors 3.1.0/DR3 and 8.1.6, and negative control T2, were stained in indirect immunofluorescent flow cytometry with mAb CER.CLIP.1, which recognizes CLIP associated with HLA-DR molecules. (*b*) Mutant 2.7.93, control cell DMA mutant 2.2.93, their progenitor, 3.1.0/DR3, and negative control T2, were stained in indirect immunofluoresence with mAb CER.CLIP.

16.23 sorted subpopulations and analyzing them for mAb 16.23 expression. All sub-clones from both populations reveal a variable, but bimodal distribution of mAb 16.23 staining (Fig. 5 *b*). Within sub-clones, changes in the percent distribution of cells into either population are slow, on the order of 1–5% per week of continuous culture (data not shown). While the proportion of cells in each subpopulation varies, the overall phenotype is stable. Finally, it should be noted that the mean mAb 16.23 staining of the mAb 16.23^{low} subpopulations in these mutants is consistently $<$50% of the mean of that of the progenitors (Figs. 2 *e* and 6 *a*).

The HLA Class II Homozygous Deletion Mutant T2 Complements the Defect in the New Mutants 2.7.93 and 3.6.95. 3.1.0 and 6.3.6, the progenitors of the new mutants, contain large hemizygous deletions of chromosome 6p which encompass the HLA-DMA and DMB loci (Fig. 1). Thus, the DM genes could be considered candidate site for the mutations because only a single mutational hit in a gene mapping to the hemizygous region would generate a recessive phenotype. However, as described above, these mutants manifest subtle characteristics distinct from those of DM null mutants. Preliminary observations also suggested that the lesions in these new mutants might not be in the HLA-DM genes. We observed that 2.7.93 and the DMB null mutant 9.5.3 are complementary in somatic cell fusions (2.7.93 × 9.5.3 lane in Fig. 3, and data not shown). In addition, stable transduction of 2.7.93 with DMA alone, using a retroviral vector containing a DMA cDNA, fails to complement the 2.7.93 defect (data not shown), though this same construct complements the DMA null mutant 2.2.93 (22). These data tended to exclude mutations in either DMA or DMB alone.

To determine if the mutations of the new mutants map to either the DMA or DMB locus, we made stable somatic cell hybrids between 2.7.93 and T2, and between 3.6.95 and T2. T2 is homozygously deleted for the entire class II region (41) (Fig. 1) and can contribute neither conventional class II molecules nor DM molecules to somatic cell hybrids. Control hybrids between a 3.1.0/DR3-derived DM null mutant 2.2.93 and T2 are noncomplementary (Fig. 6), as expected. However, both mutants 2.7.93 and 3.6.95 are complemented by T2 in somatic cell hybrids. This is evidenced by restoration to wild-type levels of mAb 16.23 binding (Fig. 6), by a dramatic reduction to wild-type levels of cell surface CLIP (Fig. 6), and by the appearance of SDS-stable class II dimers in cell lysates from these hybrids (data not shown). Therefore, the lesions in these new mutants are recessive, cannot be in the HLA-DM genes, and



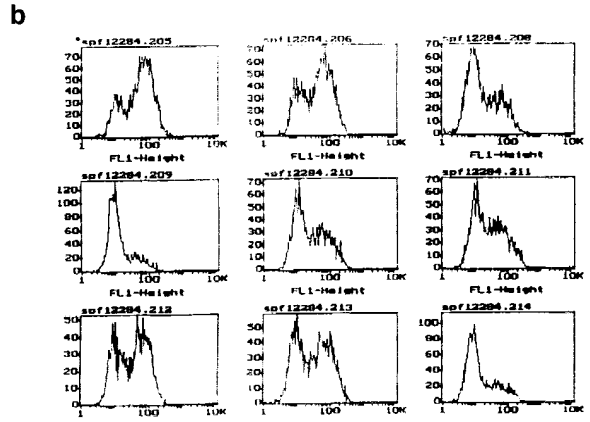


Figure 5. mAb 16.23^{null} and mAb 16.23^{low} sorted subpopulations of 2.7.93-type mutants express differential levels of stable HLA-DR dimers. (*a*) SDS stability of HLA-DR molecules from cell-sorted mAb16.23^{null} and mAb 16.23^{low} subpopulations of mutant 2.7.93. Western blots were stained with a combination of mAbs DA6.147 and HB10.A. (*b*) Bimodal reexpression of the mAb 16.23 epitope in subclones of the 2.7.93 mAb 16.23^{null} sorted subpopulations. 2.7.93 cells were stained in indirect immunofluorescence with mAb 16.23. Each panel represents mAb 16.23 staining of an individual subclone of 2.7.93 (mAb 16.23^{null}); subclones of the mAb 16.23^{low} subpopulation show similar bimodal reexpression of the mAb 16.23 epitope (data not shown).

map outside the 1 Mb HLA class II region. To verify that these somatic hybrids contain the MHC haplotypes of both input partners, we stained them and controls with the TAP-dependent, HLA-B5–specific antibody mAb 4D12. Stable hybrids 2.7.93 × T2 and 3.6.95 × T2 each express the epitope recognized by mAb 4D12 (data not shown). This reciprocal complementation of the TAP defect in T2 by the 2.7.93 and 3.6.93 partners can only be achieved in *trans* by contributions from both the T2 (TAP−; B5+) and 2.7.93 or 3.6.95 (TAP+; B5−) MHC haplotypes. Thus, these somatic hybrids contain the chromosome 6p haplotypes of both input partners. Stable hybrids between 3.4.95 and T2 have not yet been obtained.

Mutants 2.7.93, 3.6.95 and 3.4.95 Express Diminished Levels of DM Proteins. The T2 complementation results indicate that the lesion(s) in these new mutants map outside the HLA class II region and thus are neither in the HLA-DM structural genes nor in *cis*-acting DM regulatory elements. Because the phenotype of these mutants resembles that of DM null mutants, these mutants might have regulatory lesions affecting the expression of DM which could explain their unusual phenotype. Western immunoblots of lysates from the mutants and their progenitors were stained with rabbit polyclonal antisera against DMα (Fig. 7) and DMβ (data not shown) to assess relative levels of DM protein expression. Mutant 2.7.93 expresses no detectable DMα monomer; mutants 3.6.95 and 3.4.95 exhibit significantly reduced (1/5 to 1/10 of wild type) levels of DMα monomer (Fig. 7). Western blots with rabbit polyclonal antiserum raised against DMβ cytoplasmic tail similarly reveal that mutants 2.7.93, 3.6.95, and 3.4.95 have reduced DMβ monomer (data not shown). This suggested that the phenotype of these mutants could be explained by deficient DM expression.

DMα monomer is increased in western immunoblots in lysates from 2.7.93 × T2 and 6.95 × T2 hybrids (Fig. 7), consistent with the ability of T2 to complement the other phenotypic defects in these mutants, as shown above. Because T2 cannot contribute DM molecules in these somatic cell hybrids, the restoration of DM expression must result from provision of a transactive factor by T2.

Both HLA-DMA and -DMB Are Required to Complement the 2.7.93 Defect. As shown above, both DMA and DMB proteins are significantly reduced in 2.7.93, and increased levels of these proteins are observed in lysates made from hybrids of mutants × T2. Therefore, we asked if transfections of DMA and DMB driven by heterologous promotors could alter the 2.7.93-type mutant phenotype. In transient transfection assays, cell surface expression of the mAb 16.23 epitope on mutant 2.7.93 was significantly enhanced by transfection of both DMA and DMB in combination, but not

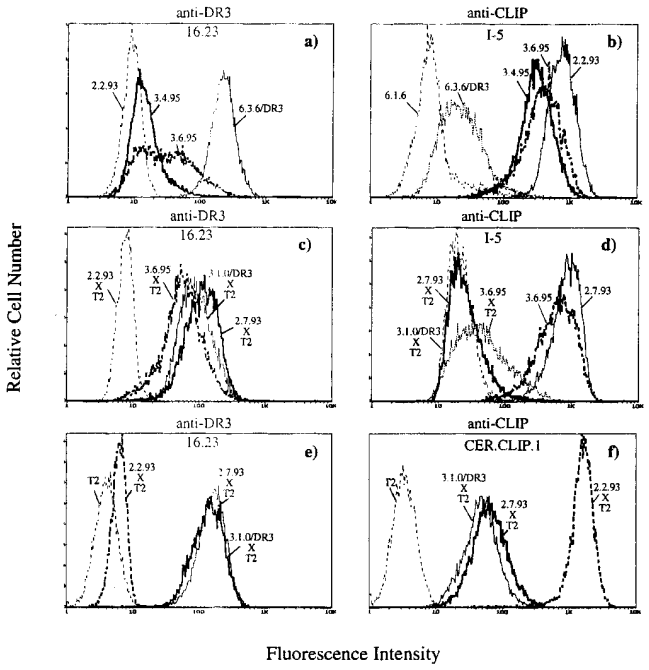


Figure 6. Cell surface staining of MHC class II and MHC class II/CLIP complexes on 3.4.95 and 3.6.95, and on (*2.7.93 × T2*) and (*3.6.95 × T2*). (*a* and *b*) Cell surface expression of the mAb 16.23 epitope and of MHC class II–CLIP complexes on 6.3.6/DR3-derived mutants 3.4.95 and 3.6.95. (*a*) Negative control DMA null mutant 2.2.93, mutants 3.6.95 and 3.4.95, and progenitor 6.3.6/DR3, were stained by indirect immunofluorescence with mAb 16.23. Subclones of 3.4.95 and 3.6.95 manifest consistent bimodal expression of the mAb 16.23 epitope as seen in mutant 2.7.93 (data not shown). (*b*) Negative control 6.1.6 (71), progenitor 6.3.6/DR3, positive control, DMA null mutant 2.2.93, and mutants 3.6.95 and 3.4.95, were stained by indirect immunofluorescence with anti-class II–CLIP mAb I-5, which with HLA class II molecules. (*c–f*) Cell surface expression of the mAb 16.23 epitope and of MHC class II–CLIP complexes on somatic cell hybrids. (*c*) Negative control hybrid (*2.2.93 × T2*), positive control hybrid (*3.1.0/DR3 × T2*), and hybrids (*2.7.93 × T2*) and (*3.6.95 × T2*); and (*d*) control hybrid (*3.1.0/DR3 × T2*); and hybrids (*2.7.93 × T2*) and (*3.6.95 × T2*) and mutants 2.7.93 and 3.6.95; and (*e* and *f*) negative control (*T2*), negative control somatic cell hybrids (*2.2.93 × T2*), positive control hybrids (*3.1.0/DR3 x T2*); and hybrids (*2.7.93 × T2*) were stained in indirect immunofluorescent flow cytometry using the indicated mAbs.

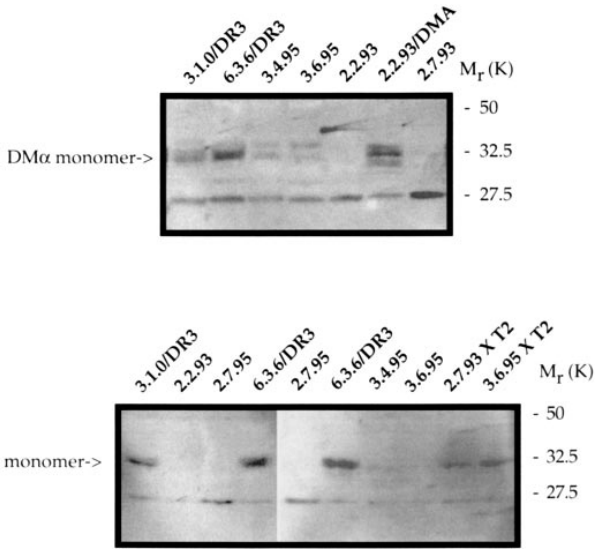


Figure 7. DM protein levels are reduced in 2.7.93-like mutants.Whole cell lysates from the indicated cells and somatic cell hybrids were run in denaturing SDS-PAGE and analyzed in Western immunoblots by staining with 8338H, a rabbit polyclonal anti-DMα antiserum which recognizes HLA-DMα monomer, as described in Materials and Methods.

Table 2. *Transient DM Expression Assay: Restoration of the mAb 16.23 Epitope*

Cells	Genotype	Transfected DNA (Transient)	No. trials	Δ %16.23$^+$ cells*
2.2.93‡	DMA−/−	pDMA	$n = 15$	4–19
2.2.93	DMA−/−	pDMB	$n = 15$	0
9.5.3‡	DMB−/−	pDMA	$n = 12$	0
9.5.3	DMB−/−	pDMB	$n = 12$	3–15
2.7.93	??	pDMA	$n = 3$	0
2.7.93	??	pDMB	$n = 2$	0
2.7.93	??	pDMA + pDMB	$n = 1$	11
2.7.93/DMA§	DMA wt	pDMB	$n = 2$	11–13

*Cells were stained and analyzed by indirect immunofluorescent flow cytometry: Δ%16.23$^+$ cells = % cells in R2 (mAb 16.23$^+$) of test population −% cells in R2 of negative control (untransfected) population.
‡DM null mutants 2.2.93 and 9.5.93 (22) included as controls.
§2.7.93 stably transduced with DMA.

by transfection of DMA or DMB individually, (Table 2). This is consistent with the finding that stable transfection of 2.7.93 with DMA cDNA alone failed to complement the 2.7.93 defect. Furthermore, this result is consistent with the complementation observed between 2.7.93 and the DMB mutant 9.5.3 (data not shown).

Mutants 2.7.93, 3.6.95, and 3.4.95 Express Diminished Levels of DM mRNA. The transfection results, described above, suggested that DM mRNA levels might be deficient in these mutants. Therefore, DM mRNA levels in the mutants were analyzed by Northern blotting (Fig. 8). These studies confirmed that DM levels were altered in the mutants, and also indicated a phenotypic difference between 2.7.93 on the one hand, and mutants 3.4.95 and 3.6.95 on the other. In 2.7.93, both DMA and DMB levels were markedly reduced, to 10–20% of the levels of progenitor 3.1.0/DR3 (Fig. 8, and data not shown). In mutants 3.4.95 and 3.6.95, DMB levels were likewise reduced to the ~10% level, but DMA levels were only modestly reduced. Thus, the primary defect in 2.7.93 appears to be a reduction in both DMA and DMB mRNAs whereas in 3.4.95 and 3.6.95 the principal defect appears to be a reduction in DMB mRNA. These reductions in DM mRNAs provide a reasonable basis for the reductions in DM protein and the secondary manifestations of DM deficiency in the mutants, described above. It is likely that the reduction in DMα protein in 3.4.95 and 3.6.95 results in part from accelerated turnover of DMα protein beacause of the relative deficiency of the DMβ subunit, as is common when one subunit of a dimeric protein is absent (74).

Discussion

The molecular requirements for the association of antigenic peptides with MHC class II molecules have been more clearly elucidated in the past few years. In particular, the accessory molecule HLA-DM has been shown to provide a critical role in the maturation of functional class II–peptide complexes (21, 22, 24, 54). However, the factors involved in regulating DM expression and its catalytic activity remain to be determined. The importance of DM in proper class II presentation, the presence of typical class II promotor elements in the DM regulatory regions, and the fact that γ-interferon induces DM expression, all imply coordinate expression of DM with other class II molecules. We describe here a set of novel APC mutants which exhibit discoordinate expression of conventional MHC class II molecules and the accessory molecule HLA-DM.

The mutants described in this paper have a phenotype that bears a marked resemblance to that of DM null mutants. In particular, their cell surface class II molecules are predominantly associated with CLIP. Our data also suggest that the phenotype of these mutants directly results from a deficiency in DM expression. However, the defects in two of the mutants tested so far do not map to the HLA-DM loci; in somatic cell hybrids, these mutants are complementary with T2, a fusion partner that contains a homozygous deletion encompassing HLA-DMA and -DMB and the entire HLA class II region. Thus the lesions for 2.7.93 and 3.6.95 cannot be in the HLA-DMA or -DMB structural genes nor in any genes in the class II region, including the conventional class II genes. Complementation of the mutant defect in *trans* with T2 also rules out mutations in DM *cis*-regulatory regions.

The locations of the genes affected in these mutants are not known with certainty. However, their observed frequency in EMS mutagenized progenitors 3.1.0 and 6.3.6 is $\sim 10^{-4}$; this is the expected frequency for monozygous loss, and is several orders of magnitude higher than expected for dizygous loss (60). Thus, the genetic lesions in these mutants most likely map to the regions of hemizygous deletions in 3.1.0 and 6.3.6. The hemizygous deletions in 3.1.0

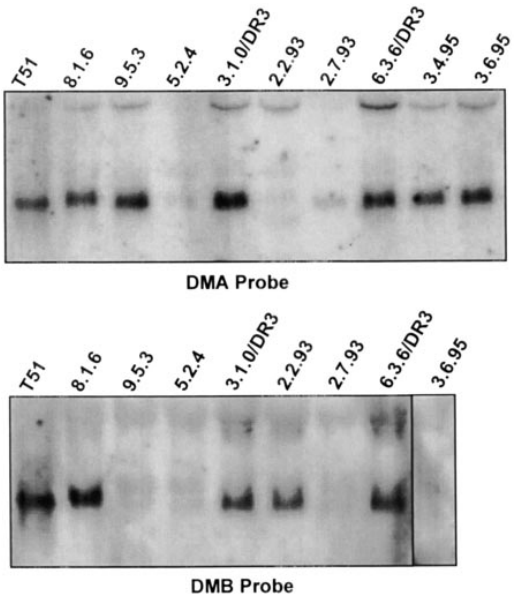


Figure 8. DMA and DMB mRNA levels are reduced in 2.7.93-like mutants. Total cellular RNA from the indicated cells were analyzed in Northern blots. Full-length probes from DMA and -B cDNAs were labeled with [^{32}P]dCTP and used for hybridization. Reprobing of Northerns with actin (not shown) indicated equal loading of RNA to all lanes.

and 6.3.6 encompass ~40 Mb and ~30 Mb, respectively, of chromosome 6p (Fig. 1); therefore the affected gene(s) likely map to these regions of chromosome 6p, but outside the class II region.

Although the defects in mutants 2.7.93 and 3.6.95 do not map to the DM loci, the dramatic reductions in DM proteins and mRNAs suggest that their phenotype results from deficient DM expression. This is evidenced by the fact that class II molecules are expressed but are unstable in SDS, that class II molecules remain complexed with CLIP peptide, and that there is a marked reduction of DM-dependent mAb epitopes on class II molecules which exceeds the reduction in class II levels. Thus, class IIα/β–Ii complexes are being expressed in the absence of adequate levels of DM for removal of CLIP. Consistent with this, transient transfections of 2.7.93 with DMA and DMB together, but not singly, lead to an increase in the level of mAb 16.23 binding (Table 2). Although all measures of DM activity indicate that each mutant has a major deficiency in DM expression, the lesions also modestly affect class II expression, as indicated by the decreased levels of cell surface class II molecules (Fig. 2 and Table 1) and class II mRNA's (Muczynski, K., and B. Arp, unpublished data). These reductions in class II expression are generally not seen in DM structural gene mutants, such as mutant 2.2.93 (Table 1). It appears from these results that the lesions in the new mutants differentially affect DM versus conventional class II expression.

The complementation data suggest that the disproportionate loss of DM expression relative to conventional class II expression in these mutants results from recessive mutations in a transactive factor(s). Previous reports have indicated that DM and class II genes are coordinately regulated. Both class II and HLA-DM transcription are induced by IFN-γ (25, 64, 65), and it has been proposed that the S-X-Y regulatory regions of DM genes function similarly to class II regulatory elements (52). Furthermore, HLA-DM and conventional class II transcription are coordinately regulated in all known cases of class II immunodeficiency (66), in in vitro class II transcription mutants (67, 68), and in mutants defective in IFN-γ–induced class II transcription (69). The mutants described here thus provide evidence suggesting that DM and class II expression can be differentially regulated by a *trans*-acting factor. The existence of an MHC class II isotype-specific *trans*-acting factor has also been recently described for HLA-DQ (73).

While mutants 2.7.93, 3.6.96, and 3.4.95 all express essentially undetectable levels of DMB mRNA, they express different levels of DMA mRNA, suggesting that different transactive factors may be affected in this set of mutants. Consistent with this, preliminary complementation studies suggest that at least two different factors are affected in the three mutants (Rak, J., unpublished data). How these factors differentially regulate DM and class II mRNA abundance is currently under investigation.

Perhaps the most surprising and distinguishing aspect of the mutants described here is the bimodal mAb 16.23 staining pattern observed in clonal populations. Our data show that expression of the mAb 16.23 epitope on DR3 molecules on these mutants is distributed into distinct 16.23^{null} and 16.23^{low} sub-populations. This bimodal mAb 16.23 staining profile is specific for the DR3 expressed by these mutants; it is not observed with other DM sensitive mAbs to DQ and DP, nor with mAbs recognizing class II-CLIP. Altered levels of expression of the mAb 16.23 determinant by sub-populations of these mutants do, however, correlate with differences in other measures related to DM activity, i.e., SDS-stability of both DR1 and DR3 dimers (Figs. 3, 5) and of DQ and DP dimers (data not shown). Thus, the bimodality observed for 16.23 expression by these mutants is most likely related to their reduced levels of DM expression. This is consistent with preliminary data which indicate that the bimodality for the 16.23 epitope in 2.7.93 is correlated with bimodality for DM expression (Anderson, S., S. Fling, and D. Pious, manuscript in preparation). The biphasic phenotype is suggestive of a threshold effect at the level of DM expression.

The bimodal expression of a DM-dependent, DR3 class II determinant is not unprecedented; it is similar to that described for T2/DR3 transfected with DM (51). Clonal populations of T2/DR3 transfected with DM also stain in a bimodal distribution with a DM-sensitive mAb, in this case mAb CER.CLIP.1 (51). This bimodal profile is specific for DR3 expressed in T2; it is not observed in T2/DR4 nor T2/DR11. As with the staining profile of the mutants described here, the bimodal staining profile of T2/DR3/DM with the DM-sensitive mAb CER.CLIP.1 cannot be removed by repeated subcloning (51). Anti-CLIP staining on the mutants described here is not distributed into clearly

distinct CLIP+/− subpopulations, unlike anti-CLIP staining on T2/DR3/DM. This difference most likely results from the ability of the anti-CLIP mAbs used in our study to recognize CLIP in association with each of the four class II alleles (DR1, DR3, DQ1, DP4.1) expressed by these mutants; in contrast, T2/DR3/DM expresses only DR3. The bimodal distribution of DM-dependent epitopes seen specifically on DR3 molecules in these mutants and in T2/DR3/DM could be related and may suggest that the requirements for DM may differ for DR3 and other class II molecules. Differences between DR3 and other class II molecules in their respective affinities for CLIP have been shown (70).

The mutants described in this report appear to define a gene or genes required to maintain coordinated expression of HLA-DM and conventional class II molecules in APC. For the majority of cells in a clonal population of these mutants, the expression of DM appears to be below a threshold required to remove CLIP from class II molecules, and to catalyze their loading with cognate peptides. Overall, our data show that even modest perturbations in the coordinated expression of DM and class II can result in profound differences in the level of functional class II–peptide complexes ultimately expressed by APC. The significant influence of DM on the spectrum of peptides associated with MHC class II suggests the interesting possibility that such dysregulation of DM expression may contribute to some level of immune dysfunction. Identification of the factors deficient in these mutants should help to identify components which regulate expression of DM. That the genes defective in these mutants have effects on both DM and class II expression suggests the defective genes may also regulate other components of the MHC class II antigen processing pathway.

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