DIFFERENTIAL EXPRESSION OF H-2D^d AND H-2L^d HISTOCOMPATABILITY ANTIGENS

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The murine major histocompatability (H-2) antigens are polymorphic cell surface glycoproteins that play a critical role in the interaction of many T cells with their target cells (1). The H-2 antigens are present on nearly all cell types of the mouse but are differentially expressed depending on the species of mouse studied and the tissue examined (2, 3). For example, mice of the H-2^b haplotype express more H-2D antigen on their cell surfaces than H-2K. Similarly, H-2^d haplotype mice express more H-2K on the surface of their cells than H-2D or H-2L, and more H-2D than H-2L. Understanding the mechanisms that regulate the amount of H-2 antigen on the cell surface may be important to our understanding of the control of the immune system, as well as the mechanisms controlling cell surface glycoprotein synthesis.

Each H-2 antigen gene consists of seven or eight exons (see Fig. 1) (4). We and others (5–7) have cloned the genes encoding the H-2L^d and H-2D^d antigens. The cloned genes contain all the recognition signals required for the expression of the H-2 antigens, since mouse L cells transfected with these genes express functional H-2D^d and H-2L^d antigens on their cell surface (5, 7–9). The ability to modify H-2 genes in vitro, reintroduce them into L cells, and study the expression of the H-2 antigens has proven useful for identifying the features of H-2 genes (10, 11).

We have used just such an assay to examine how the expression of H-2D^d and H-2L^d genes could be altered when the normal H-2 promoters were replaced with an exogenous promoter, the metallothionein gene promoter (12). L cells transfected with such hybrid genes express normal H-2D^d and H-2L^d antigens on their cell surface but do not express both antigens at the same level. That is, H-2D^d antigen expression is two to fourfold higher than H-2L^d antigen expression, analogous to the different amounts of the two antigens on the surface of H-2^d cells. Molecular analyses of L cells transfected with these hybrid genes indicated that the amount of exogenous H-2 antigen expressed on the cell surface is independent of the amount of H-2-specific mRNA and H-2-specific cytoplasmic protein in the cells (12). Thus, there must be a region of the H-2D^d protein that enhances its surface expression relative to H-2L^d antigen, presumably during the maturation of the protein after translation. To determine if such a region exists, we examined the expression levels of H-2 antigens in which the H-2L^d and H-

$2D^d$ domains were exchanged. We determined that the $H-2D^d$ N domain enhances expression of the $H-2D^d$ antigen.

Materials and Methods

Plasmid Constructions.   The metallothionein-promoted H-2 genes, DM and LM, are $H-2D^d$ and $H-2L^d$, respectively, in which the normal H-2 promoters have been replaced with the metallothionein gene promoter (12). Mouse L cells transfected with these hybrid genes express functional $H-2D^d$ and $H-2L^d$ antigens on the cell surface. In addition, these cells contain 20–60 times the normal amount of H-2 mRNA and the saturating amount of H-2 antigen that may be placed on the cell surface (12).

Hybrid genes formed by exchanging segments of the $H-2L^d$ and $H-2D^d$ genes have been described elsewhere (10). The plasmids pG24 and pG18 that encode these hybrid genes are diagrammed in Fig. 1, *top*. Additionally, the metallothionein gene–promoted hybrid gene, pDL, which contains all exons of the $H-2L^d$ gene except the N domain (see Fig. 2, *top*), has been previously described (11).

Hybrid genes between the DM and LM plasmids were made by digesting these plasmids to completion with BamH1, isolating the two DNA fragments generated by each plasmid, and ligating the larger fragment from one plasmid with the smaller from the other plasmid, and vice versa. The correct orientation of the inserts was determined by restriction mapping. Manipulation of DNA fragments is described in Maniatis et al. (13).

Cell Culture, Transfection, and Radioimmunoassay.   Mouse L cells (Tk⁻) were maintained in Dulbecco's minimum essential medium (DMEM)[1] supplemented with 10% fetal calf serum. After transfection, cells were selected for the presence of the exogenously added herpes simplex virus thymidine kinase gene (HSV TK) by selection in HAT (hypoxanthine, aminopterin, thymidine) media (14).

Cells were transfected as previously described (5, 12). Briefly, 5 μg of plasmid DNA was mixed with 500 ng HSV TK and 30 μg of C3H carrier DNA and precipitated on mouse L cells by calcium phosphate precipitation. After 48 h in nonselective media, cells were selected in HAT media. Colonies were picked 3 wk later and analyzed for expression of the transfected H-2 antigens by radioimmunoassay using monoclonal antibodies specific for the different H-2 antigens (15, 16).

The presence and amount of the transfected and endogenous H-2 antigens on the surface of transfected and normal L cells was determined by an indirect radioimmunoassay as previously described (12, 17). Specifically, the appropriate cell cultures were lightly trypsinized and washed, and suspended cells counted. Aliquots of the cells were added to 1 ml DMEM and centrifuged. The cell pellets were resuspended in 50 μl of a 1:100 dilution of the appropriate antibody (15, 16) and incubated at 4°C for 4 h. The cells were then washed twice and the pellets resuspended in 50 μl of ^{125}I–protein A (New England Nuclear, Boston, MA). After incubating 12 h at 4°C, the cells were washed three times and counted in a gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Each antibody was previously titrated against a constant cell number to insure saturation of the cells with antibody. Background binding of the ^{125}I–protein A was determined by incubating control L cells (untransfected) with the specific antibodies. These backgrounds, usually <500 cpm, were subtracted from the specific binding of the transfected L cells.

Northern Blot Analysis.   Total cellular RNA was isolated from the appropriate cell lines as described (18). Equal amounts of RNA were denatured, electrophoresed in a formaldehyde-agarose gel, and transferred to nitrocellulose as described (19). H-2-specific mRNA was detected by hybridization with a ^{32}P-labeled probe that cross-reacted with the $H-2D^d$, $H-2L^d$, $H-2K^k$, and $H-2D^k$ transcripts.

Results

Previously (12), we have demonstrated that, when the $H-2L^d$ and $H-2D^d$ antigen genes are placed under the control of mouse metallothionein gene

[1] *Abbreviations used in this paper:*   DMEM, Dulbecco's minimum essential medium; HAT, hypoxanthine, aminopterin, thymidine; HSV, herpes simplex virus; TK, thymidine kinase.

promoter, normal H-2 antigens are expressed on the surface of transfected cells. However, the levels of H-2 antigens expressed are not equal since the amount of H-2D^d antigen on the cell surface is two- to fourfold, more than that of H-2L^d antigen. Induction of the metallothionein gene promoter increases the level of H-2-specific mRNA (and H-2-specific protein) in the transfected cells several fold, but not alter the level of surface expression of H-2 antigens (12). This indicates that the amount of H-2 mRNA made from the metallothionein gene–promoted hybrid genes saturated the transfected cells for the expression of H-2 antigens. Thus, the amount of H-2D^d and H-2L^d on the surface of uninduced, transfected DM and LM cells, respectively, was the highest that could be achieved.

Both DM and LM cells were saturated for the expression of H-2 antigens, and this saturation was clearly posttranslational; yet the H-2D^d antigen was present on the cell surface in greater quantities than the H-2L^d antigen. We therefore expected that hybrid genes containing portions of both the H-2D^d and H-2L^d genes should determine which protein domains caused enhanced expression of H-2D^d over H-2L^d antigen. Initially, we examined cells transfected with H-2 genes in which the first three exons had been switched from H-2D^d to H-2L^d, and vice versa (Fig. 1, top) (10). The cell lines compared were those transfected with metallothionein-promoted H-2D^d (DM-1) (12), metallothionein-promoted H-2L^d (LM-1) (12), H-2-promoted H-2D/L gene (pG24:37.2.1) (10), and H-2-promoted H-2L/D gene (pG18:37.1.3) (10). Quantitative radioimmunoassay analysis of the amounts of the normal or hybrid H-2 antigens on the surface of transfected cells indicated that the first three exons of H-2D^d antigen gene cause the enhanced expression of H-2D^d antigen (Fig. 1, bottom). Monoclonal antibodies specific for the C2 domains of the H-2 antigens were used (10); however, other monoclonal antibodies specific for H-2D^d and H-2L^d antigens demonstrate the same level of expression (12 and data not shown). The 37.1.3 and 37.2.1 cells showed depressed levels of endogenous H-2 antigens (H-2K^k and H-2D^k) (indicative of high levels of expression of the transfected H-2 genes; 12), and the H-2-specific mRNA levels in the transfected cells were both elevated equally over the amount seen in the L cell controls (data not shown). However, because of the possibility of promoter differences, we repeated these experiments with new hybrid genes in which the normal H-2 promoter had been replaced with the metallothionein gene promoter.

To eliminate the possibility that the different amounts of hybrid H-2 antigens expressed on the surface of 37.2.1 and 37.1.3 cells was due to subtle differences in H-2D^d and H-2L^d gene promoters, we prepared H-2 hybrid molecules identical to those described above except that the H-2 promoter was replaced by the metallothionein gene promoter (Fig. 2, top). Transfection of these metallothionein-promoted hybrid H-2 genes into mouse L cells resulted in cell clones that stably expressed and maintained the MDL gene but not its reciprocal (MLD). The reciprocal MLD gene contains the metallothionein gene promoter followed by the L, N, and C1 domains of the H-2L^d gene and the C2, M, I1, I2, and I3 domains of the H-2D^d gene. Cell clones expressing the hybrid H-2 gene encoded by the MLD gene were originally isolated but were unstable in culture and quickly lost the ability to express the hybrid gene (data not shown). We also examined the cell surface expression levels of a previously described (11) metal-

L N C_1 C_2 M $I_1 I_2 I_3$

DM-I

37.2.I

37.I.3

LM-I

3

$CPM \times 10^{-4}$

2

1

37.2.I
DM-I

LM-I
37.I.3
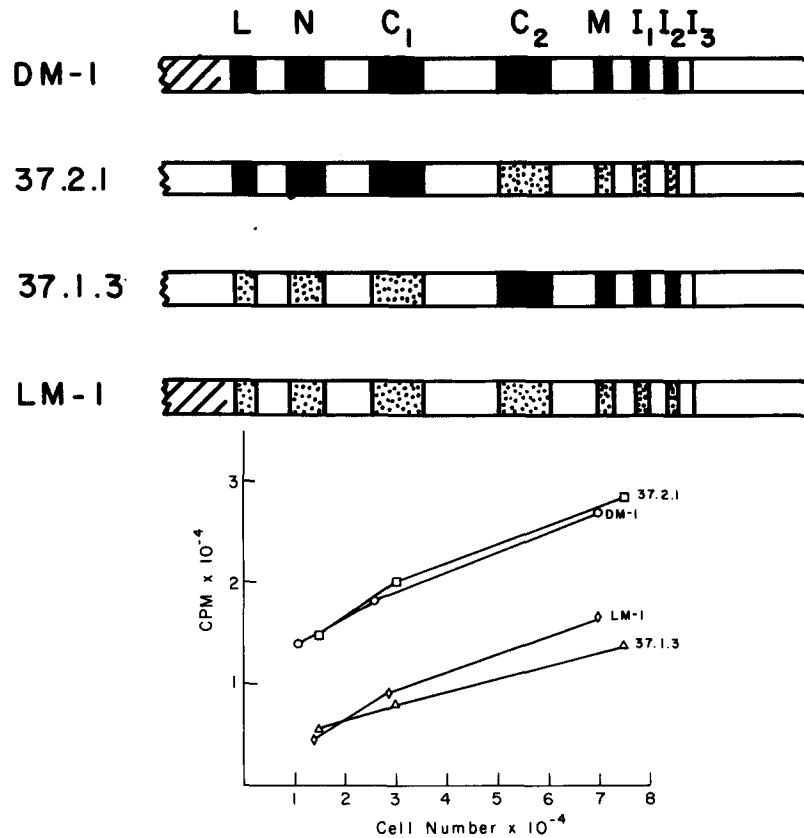
1 2 3 4 5 6 7 8
Cell Number x 10^{-4}

FIGURE 1. Radioimmunoassay of transfected mouse L cells expressing hybrid H-2 antigens. (Top) Diagram of the hybrid H-2 genes. The DM-1 and LM-1 genes are $H-2D^d$ and $H-2L^d$ genes, respectively, in which the normal H-2 promoters have been replaced with the metallothionein gene promoter (12). The 37.2.1 and 37.1.3 constructs represent previously described hybrid genes (10) in which the last five coding blocks of the $H-2D^d$ gene have been replaced with the analogous sequences from the $H-2L^d$ gene (37.2.1) and vice versa (37.1.3). The black areas represent $H-2D^d$ domains and the stippled areas represent $H-2L^d$ domains. Diagonal lines represent the metallothionein promoter in place of the H-2 promoter. (Bottom) Radioimmunoassay L cell clones transfected with genes described above. Monoclonal antibodies used in this assay were 34.2.12 (specific for the C2 domain of $H-2D^d$ molecule) (γ2A) for the DM-1 and 37.1.3 cell lines, and 28.14.8 (specific for the C2 domain of the $H-2L^d$ molecule) (γ2a) for the LM-1 and 37.2.1 cell lines (10).

lothionein gene–promoted hybrid H-2 gene (pDL), in which only the N domain of the $H-2L^d$ gene had been replaced by the N domain of the $H-2D^d$ molecule (Fig. 2, top).

To determine the levels of normal and hybrid H-2 antigens expressed on the surface of transfected L cells, a quantitative radioimmunoassy was performed using an $H-2D^d$ C2-specific monoclonal antibody, for the DM-1 cell, and an $H-2L^d$ C2-specific monoclonal antibody for the LM-1, MDL, and pDL transfected cells (Fig. 2, bottom). Again the expression of hybrid H-2 antigen containing the first three domains of the $H-2D^d$ molecule (MDL) was identical to that of the normal $H-2D^d$ antigen produced from the metallothionein-promoted $H-2D^d$ gene (DM-1). Also, the metallothionein gene–promoted hybrid H-2 antigen that
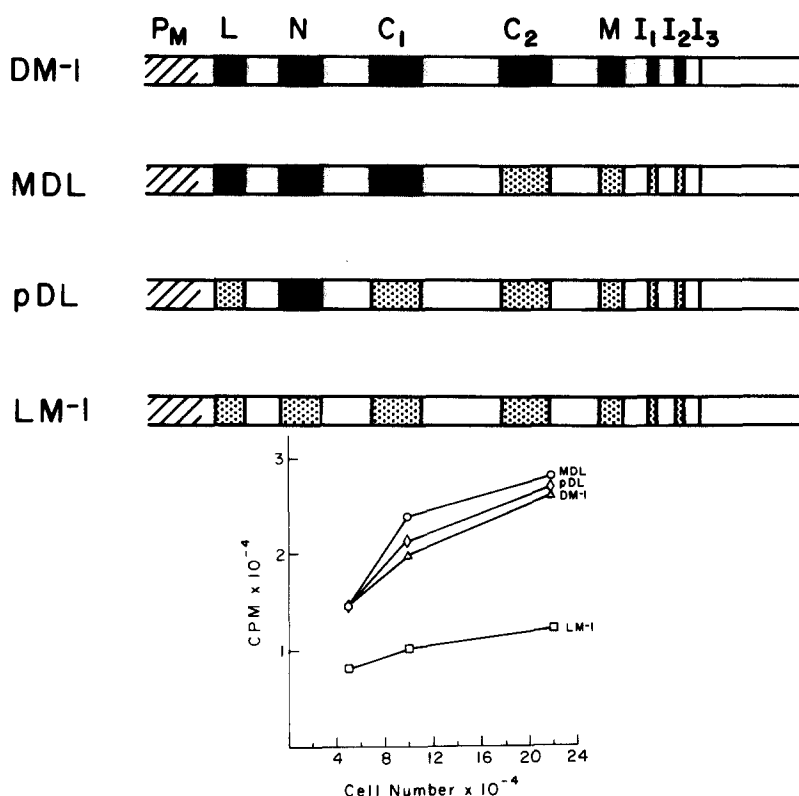
FIGURE 2. Construction and expression of metallothionein-promoted H-2 hybrid molecules. (*Top*) Diagram of hybrid molecules MDL and pDL constructed from the DM and LM metallothionein-promoted H-2 molecules. (*Bottom*) Radioimmunoassay of the DM-1-, LM-1-, pDL-, and MDL-transfected L cells. Expression of the foreign H-2 antigens was determined using H-2D^d- and H-2L^d-specific monoclonal antibodies (see Fig. 1).

contained only the N domain of the H-2D^d gene was expressed on the surface of the transfected L cells at a level identical to that seen for H-2D^d antigen expressed on the surface of DM-1.

Although all H-2 genes analyzed in Fig. 2 contained the metallothionein gene promoter and were therefore under the same transcriptional control, these cells do not contain the same number of transfected H-2 genes (data not shown). Thus, the level of H-2-specific mRNA in the recipient cells may vary with the copy number of the transfected gene. Previously (12), we have shown that the variability of copy number inherent in DNA transfections does not appear to influence the cell surface expression of H-2 antigens transcribed under the control of the metallothionein gene promoter, since the mouse L cells are easily saturated for cell surface expression of new H-2 antigens, presumably due to a limitation of β_2-microglobulin (12). However, to insure that the LM-1, DM-1, MDL, and pDL cells were making comparable levels of metallothionein-promoted, H-2-specific mRNA, total RNA was extracted from these cells and examined by Northern blot analysis using an H-2-specific probe (Fig. 3). Equal amounts (as determined by optical density and ethidium bromide staining) of
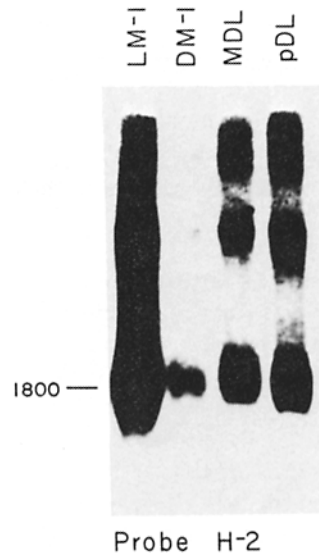
Probe H-2

FIGURE 3. Northern blot analysis of H-2-specific mRNA in cells transfected with the metal-lothionein-promoted H-2 hybrid genes. Total RNA was extracted from DM-1-, LM-1-, MDL-, and pDL-transfected cells and analyzed by Northern blot hybridization with an H-2-specific probe. Equal amounts (5 μg) of total RNA were loaded per lane.

total RNA were loaded into the gel. All four samples had the same amount of β_2-microglobulin mRNA, as determined by rehybridizing a β_2-microglobulin-specific probe to the same nitrocellulose filter. The LM-1 cell contained the greatest amount of H-2-specific mRNA and DM-1 the least. Both MDL and pDL cells contained H-2-specific mRNA levels below that of the LM-1 cell. Thus, mRNA levels alone do not account for the enhanced expression of the hybrid H-2 antigens (encoded by the MDL and pDL genes) over that of the H-2L^d antigen.

Discussion

The expression of the major histocompatability antigens (H-2 antigens) is constitutive on the surface of most cells of the mature mouse (3). Apparently, H-2 expression occurs soon after the first steps of cellular differentiation of the developing mouse embryo, since molecular analysis (20, 21) of murine embryonal carcinoma cells demonstrates the absence of β_2-microglobulin and H-2 mRNA in the undifferentiated cell, but the presence of class I antigen complexes on the surface of differentiated cells. Additional experiments show that the level of class I antigens expressed on the surface of mature cells may vary with the mouse strain or specific cell types examined, or with the effects of external stresses on the cells. For example, increased levels of class I antigens on the cell surface are apparently important in the resistance of certain strains of mice to plasmodium infections (22), while decreased levels of class I antigens on leukemic T cells may provide these cells with a mechanism to escape from the animal's normal immune surveillance network (23). In addition, the level of major histocompatability antigens may be altered by the expression of different class I antigens, such as

the TL antigens. Antigenic modulation experiments demonstrate that, when the TL antigen is removed from the cell surface, the levels of H-2D increase but the surface levels of H-2K remain the same (24, 25). More recently, it has been shown that adenovirus 12 infections apparently block the synthesis of class I antigens in infected cells (26) and that SV-40-transformed cells may express a new TL-Qa-like transcript (27). Thus, it is important and useful to define the regulation of class I antigen expression and what factors determine the relative amounts of various class I antigens.

In this paper, we demonstrate that the relative level of expression of two class I antigens, H-2D^d and H-2L^d, is not dependent on the level of mRNA transcription or protein translation, but on a particular domain of the molecule. By moving protein domains from the H-2L^d molecule to the H-2D^d chain, and vice versa, we demonstrated that the enhanced cell surface expression of the H-2D^d molecule over the H-2L^d antigen is due to the N domain of the H-2D^d antigen.

The mechanism the cell uses to discriminate between H-2L^d and H-2D^d N domains is not clear. A large degree of heterogeneity of H-2 antigens is found in the N domain. In the wild mouse population, this heterogeneity may provide an immune response against a variety of infections (28). Our data suggest this polymorphism may also control the level of different H-2 antigens (H-2K, H-2D, and H-2L) on the cell surface.

Although the level of expression of β_2-microglobulin can limit class I antigen expression on the cell surface (12, 29, 30), it is unlikely that differential competition for the available β_2-microglobulin allows H-2D^d molecule expression to be higher than H-2L^d. The N domain presumably does not bind the β_2-microglobulin chain (the C2 domain is associated with the β_2-microglobulin chain after tryptic fragmentation of the mature H-2 chain) (31). Also, both DM-1 and LM-1 cells show depressed levels of expression of endogenous H-2 antigens (12) (as do MDL, pDL, 37.2.1, and 37.1.3 cells) (data not shown), indicating that both H-2L^d and H-2D^d antigens compete well with endogenous H-2 antigens for the available β_2-microglobulin. Thus, the lower expression of H-2L^d antigen relative to H-2D^d antigen is probably not due to the inability of the H-2L^d antigen to successfully compete for β_2-microglobulin.

Instead, the enhanced expression of H-2D^d antigen may result from the H-2D^d N domain determining the number of sites available to that protein molecule in the cell membrane (32). As previously noted (12), the amount of endogenous H-2 antigens (H-2K^k and H-2D^k) is reduced >90% on the surface of mouse L cells into which DM and/or LM plasmids have been introduced (data not shown). The introduction of additional copies of the mouse β_2-microglobulin gene (33) into the DM cell line does not significantly alter the amount of H-2D^d on the cell surface. However, cell surface expression levels of H-2K^k and H-2D^k antigens return to those on the surface of control mouse L cells (Weis and Seidman, unpublished data). This suggests that H-2D^d antigen has already occupied all sites on the cell surface available to the protein, and cannot occupy more even in the presence of additional β_2-microglobulin molecules. Thus, the H-2K^k and H-2D^k antigens are able to fill the remaining sites, since β_2-microglobulin is no longer limiting. These data, together with the enhanced expression of H-2D^d over H-2L^d antigen, suggest the N domain may determine what sites in the cell

surface membrane are filled by a particular class I molecule, i.e., the N domain acts as a recognition site for a cell sorting mechanism. That such a sorting mechanism exists has been previously proposed by Guan and Rose (34), for the secretion of proteins through the cell surface, and by von Figura and Weber (35), for the localization of lysosomal hydrolases from the Golgi apparatus into primary lysosomes. To fully test whether such an H-2-sorting mechanism exists, further study is required, using additional hybrid molecules constructed of H-2 genes from several other H-2 haplotypes. Analysis of the expression of these hybrid genes in the mouse L cell should determine if the N domains of other class I antigens can control the level of antigen expression or if the control mechanisms in the expression of the H-2D^d and H-2L^d antigens are unique to those molecules.

Summary

To determine why the H-2D^d antigen is expressed on the surface of transfected cells at a rate several-fold higher than an analogously transfected H-2L^d molecule, we analyzed both previously described and new H-2 hybrid genes. These genes were constructed by exchanging domains between H-2 genes. Quantitative radioimmunoassay indicates that the region of the H-2D^d molecule responsible for its enhanced expression resides in the polymorphic N domain, the first domain of the mature class I molecule.

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