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IDENTIFICATION OF MURINE H-2D^b HISTOCOMPATIBILITY ANTIGENS IN CELLS TRANSFECTED WITH CLONED H-2 GENES

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Abstract—Clones of mouse L-cells transformed with 21 cosmids containing 15 major histocompatibility complex class I genes of C57BL10 (H-2^b) sperm cell DNA were analyzed for the expression of their transfected H-2 and Qa/Tla genes. Three cosmids contained a single gene, mapping to the H-2D region. This gene encodes the H-2D^b alloantigen: mouse L-cells transfected with cosmids containing this gene reacted with monoclonal antibodies and alloantisera specific for the H-2D^b antigen and expressed a 46-kd H-2 heavy chain associated with β_2-microglobulin in their cell membranes. Furthermore, these transfected cells were stimulators of, and targets for, anti-H-2D^b cytotoxic T-lymphocytes. Eighteen cosmids contained 14 different genes mapping to the Qa and Tla regions. L-cells transfected with these genes did not express class I genes reacting with alloantisera or monoclonal antibodies against Qa2, Qa4 or TL differentiation antigens. In particular, the Qa2,3 gene of C57BL10 was not identified.

INTRODUCTION

The MHC§ plays a central role in the regulation of immune responses in mammals. The class I antigens, the polymorphic transplantation antigens initially defined by allograft rejection, and expressed on most somatic cells, are involved in the T-cell recognition of cells altered by viral infection or neoplastic transformation (Zinkernagel and Doherty, 1980).

Both the H-2 class I antigens and the related Qa- and TL-antigens are integral membrane glycoproteins of 39,000–45,000 mol. wt and non-covalently associated with β_2m (Nathenson *et al.*, 1981; Vitetta *et al.*, 1975; Michaelson *et al.*, 1981).

Genomic DNA clones from BALB/c and C57BL10 DNA libraries containing MHC class I genes were isolated by recombinant DNA technology (Steinmetz *et al.*, 1982; Mellor *et al.*, 1982). Transformed cells with cloned H-2 genes which express alloantigenic H-2 molecules on their surface have been used for further immunogenetic analysis of the role of MHC antigens in the immune response (Goodenow *et al.*, 1982; Mellor *et al.*, 1982; Margulies *et al.*, 1983).

In this paper we describe the expression and functional properties of a class I gene from the H-2D region of C57BL10 mice transfected into L-cells. We show that this gene encodes the H-2D^b transplantation antigen.

§MHC, major histocompatibility complex; β_2m, β_2-microglobulin; CTL, cytotoxic T-lymphocyte(s); SDS, sodium dodecylsulfate.

MATERIALS AND METHODS

Cloning of H-2 genes and establishment of L-cell transformants

The isolation and characterization of genomic clones containing H-2 genes used in this study have been described in detail elsewhere (Weiss *et al.*, 1984). The establishment of stable L-cell transformants was performed as described previously (Mellor *et al.*, 1982). Ltk$^-$ and transformed L-cells were grown in RPMI medium supplemented with antibiotics and 10% fetal calf serum.

Mice

C3H, B10, B10.BR and B10.A(4R) mice as well as F_1 hybrids were bred in our animal colonies.

Serology

Complement-dependent cytotoxic tests using monoclonal antibodies and alloantisera were performed as previously described (Schmidt *et al.*, 1981).

Radiobinding studies were done in 96-well microtiter plates. 10^5 cells were incubated with antibody in 50 μl of RPMI medium plus 10% foetal calf serum for 1 hr at 4°C on a shaker, washed with MEM and incubated for 30 min with ^{125}I-labelled protein A (100 μCi/μg). Cells were washed 3 times and protein A binding measured in a gamma counter. Alloantisera D2 {[B10.A(5R) × LP.RIII]F_1 anti-B10}, D33 [(B10.D2 × A)F_1 anti-B10.A(5R)], and D28.b [(B10.BR × LP.RIII) anti-B10.A(2R)] were obtained from the NIH, Bethesda, MD. Monoclonal antibodies 100-5, 141-30, 166-32, 172-93 and B22-249

were obtained from G. Hämmerling, Heidelberg, F.R.G.; monoclonal antibodies 15-5-5, 20-8-6, 28-8-4 and 28-14-8 were from D. Sachs, Bethesda, MD; D3-262 ascites (anti-Qa2) was from L. Flaherty, New York; anti-TLm4 was from E. A. Boyse, New York; and B16-146 ascites (anti-Qa4) was obtained from Camon Ltd, Wiesbaden, F.R.G. The specificities of these reagents have been described previously (Lemke *et al.*, 1979; Lynes *et al.*, 1982; Ozato *et al.*, 1982; Hämmerling, unpublished; Shen *et al.*, 1982).

Immunoprecipitation and SDS gel electrophoresis

Cell surface proteins were radiolabelled with ^{125}I by the glucose oxidase–lactoperoxidase technique (Hubbard and Colin, 1972) and lysed with 0.5% NP40 in Tris–HCl buffer, pH 7.4, containing 0.15 *M* NaCl, 1 m*M* $MgCl_2$ and 1000 KIE/ml of soy bean trypsin inhibitor. Aliquots of the cell lysates were immunoprecipitated with monoclonal antibodies and alloantisera using *Staphylococcus aureus* and separated on 12.5% SDS–polyacrylamide gels as described previously (Schmidt *et al.*, 1981).

Cell-mediated cytotoxicity

Responder mice were injected once or repeatedly at 14-day intervals with 10^8 B10.A(4R) lymphocytes or 3×10^7 LB1.1 cells. Fourteen days after the last immunisation the spleen cells (2×10^7) were restimulated *in vitro* with 10^7 B10.A(4R) lymphocytes irradiated with (2000 rad) or 2×10^6 LB1.1 cells (4000 rad) for 5 days. Cytotoxic activity was assayed in a 4-hr ^{51}Cr-release assay using L-cell transformants and PHA-stimulated lymphocytes as targets.

RESULTS

Cell surface expression of transfected H-2D^b gene

A cluster of cosmids containing a single H-2 gene was isolated from a DNA library from C57BL10 mouse spleen cells and mapped into the H-2D region of the MHC (Fig. 1) (Flavell *et al.*, 1983).

Mouse L-cells were transformed with cosmids B1.1, B1.1.1, B1.28 and B3.2G by calcium phosphate mediated DNA transfer and selected for stable transformants in HAT medium or in the presence of the G418 antibiotic (Mellor *et al.*, 1982). Transformants LB1.1, LB1.1.1, LB1.28 and LB3.2G obtained with these cosmids respectively reacted in a radiobinding assay with monoclonal antibody against H-2D^b antigens. None of the transformants reacted with monoclonal antibody against H-2K^b antigens (not shown).

The transformants were further analysed in a radiobinding assay using a panel of monoclonal antibodies, reacting with different epitopes of the H-2D^b antigens and with alloantisera directed against private and public H-2^b specificities. Titrations with monoclonal antibodies directed against different sites of the H-2D^b molecule were performed and established that all epitopes present in H-2D^b alloantigens of B10 lymphocytes (Hämmerling, unpublished results) are present in transformants LB1.1 and LB3.2G. Radiobinding data with a panel of different anti-H-2^b monoclonal antibodies are summarized in Table 1. All antibodies were used at saturating amounts. Ltk$^-$ cells, LH8 cells transfected with the H-2K^b gene (not shown) and Lb1.1.1 cells transfected with a Qa-region gene were negative with the anti-H-2D^b reagents.

In addition, the transformants reacted with alloantisera D2 (anti-H-2D^b private specificity) and D28 (anti-H-2D^b public specificity) in radiobinding (Table 1) and complement-dependent cytotoxic assay (Table 2). Reactions of the antisera were specific, because pre-absorption with spleen cells from B10 (H-2^b) mice blocked binding and cytotoxicity whereas absorption with B10.A (H-2^k, Qa2,3-positive) did not. There was no reaction of LB1.1 cells with monoclonal antibodies specific for Qa2 and Qa4 (Table 2).

All transformants continued to express H-2K^k and H-2D^k antigens.

Expression of class I genes from the Qa/Tla region

Molecular heterogeneity of the H-2D region products has been reported by Demant *et al.* (1981). Since the H-2D-region cosmids from C57BL10 DNA only contained a single gene, we investigated L-cells transfected with cosmids carrying genes from the Qa and Tla regions. We attempted to identify the Qa2,3 from C57BL10 (Qa2,3^a, Tla^b) and to find additional genes reacting with antisera and monoclonal antibodies directed against the H-2D^b antigen.

Table 2 shows the reactivity of LBl.11 cells carrying the Q8 gene from the Qa region. The reaction of this cell line is representative of the other transformants from this cluster. The Qa-specific monoclonal antibodies D3-262 (Qa2) and B16-146 (Qa4) did not react with the transformants.

Table 3 summarizes the reactivity of L-cells transfected with 18 cosmids carrying 14 different class I genes from the Qa and Tla regions (Weiss *et al.*, 1984). None of the cell lines reacted with monoclonal anti-Qa2 and anti-Qa4 antibodies, which were lytic

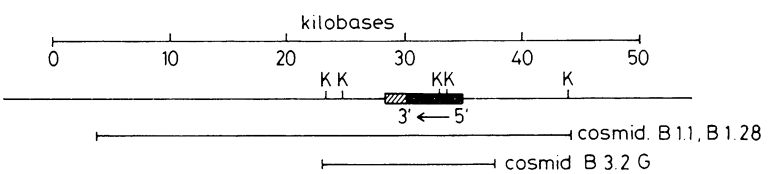

Fig. 1. Physical DNA map of H-2D^b gene deduced from restriction enzyme analysis of cosmids containing the gene. K = restriction site for KpnI. Orientation of the gene (5′ to 3′) is indicated by the arrow.

Table 1. Cell surface expression of H-2D^b antigenic sites on L-cells (H-2^k) transfected with H-2D^b genes

		^{125}I-protein A binding (Δ cpm/10^5 cells)[c]				
		Ltk$^-$	LB1.1	LB3.2G	LB1.1.1	B10[d]
			Transfected H-2 gene			
Antibody[a]	H-2 specificity[b]		D^b	D^b	Qa	—
Monoclonal antibody						
100/5	K^k	143,689 ± 5724	113,779 ± 2723	153,023 ± 891	103,661 ± 2466	1303 ± 784
15-5-5	D^k	45,712 ± 220	46,718 ± 482	51,454 ± 610	45,661 ± 2243	ND
20-8-4	K^b	1185 ± 156	1002 ± 549	1277 ± 111	80 ± 23	81,830 ± 4025
28-8-6	K^b, D^b	237 ± 498	27,281 ± 1898	12,399 ± 1552	94 ± 6	19,400 ± 71
28-14-8	D^b	591 ± 185	105,133 ± 2126	48,577 ± 877	36 ± 151	47,954 ± 4933
141-30	D^b site I	1502 ± 405	117,080 ± 606	74,171 ± 1911	ND	43,308 ± 1127
166-32	D^b site I	2128 ± 781	103,418 ± 3858	49,189 ± 2392	ND	44,752 ± 3472
172-93	D^b site II	1716 ± 328	66,661 ± 4778	33,281 ± 2518	ND	17,816 ± 816
B22-249	D^b site II	1146 ± 113	101,355 ± 4123	44,119 ± 1965	ND	37,944 ± 442
Antisera						
D33	K^b	1584 ± 61	1214 ± 347	ND	2733 ± 498	64,385 ± 2960
D2	D^b	1323 ± 441	45,138 ± 3753	ND	9229 ± 1544	36,020 ± 2205
D2 B10.A absorbed		ND	37,865 ± 3425	ND	ND	38,767 ± 2828
D2 B10 absorbed		ND	3480 ± 711	ND	ND	3158 ± 2257
D28.b	Public	430 ± 143	37,196 ± 3818	ND	1945 ± 322	32,269 ± 2734

[a]Monoclonal antibodies from either hybridoma culture supernatants or ascites were used at saturating concns; for derivation of reagents see Materials and Methods.
[b]See Lemke *et al.* (1979), Ozato and Sachs (1981), and Hämmerling (unpublished results).
[c]Normal mouse serum controls (medium: 1008, range: 995–1248) have been subtracted.
[d]Δ cpm/10^6 cells is given for B10 binding.

Table 2. Antibody-dependent cytotoxicity assay of transfected cells

Antibody[a]	H-2 specificity	% specific ^{51}Cr release[b] Ltk⁻	LB1.1 Transfected H-2 gene D^b	LB3.2G D^b	LB1.1.1 Qa	B10 —
Monoclonal antibody						
100-5	K^k	39.9	38.0	38.2	34.0	0.3
20-8-4s	K^b	3.4	2.3	ND	ND	50.7
28-14-8s	D^b	0.9	31.4	51.0	3.6	50.8
D3-262	Qa2	0.4	0.8	2.6	2.1	22.7
B16-146	Qa4	0.3	0.3	ND	0.9	39.1
Antisera						
D2	D^b	4.5	34.9	41.9	10.6	41.0
D2 B10.A absorbed		ND	34.0	ND	ND	37.6
D2 B10 absorbed		ND	4.6	ND	ND	3.4
D28.b	H-2.28 (public)	9.1	33.5	47.7	1.3	43.1

[a]Monoclonal antibodies were used at a final concn of 1/500 (antisera 1/50).
[b]% specific ^{51}Cr release was measured as [(test cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100. The SEM values were less than 5%.

for B10 lymphocytes, as expected, or with anti-TL monoclonal antibody, which was negative for B10 (Tla^b) but positive for B10.A (Tla^a) thymocytes (not shown). Antiserum D28.b and another anti-H-2.28 serum (ASA30), both of which contained additional anti-Qa2,3 antibodies (Flaherty *et al.*, 1978; Demant and Roos, 1982), also failed to react with L-cells transfected with class I genes from the Qa region. However, all L-cells transfected with Qa-region genes showed a weak reaction with D2 antiserum, which, due to the strain combination used, should not contain anti-Qa2 antibody. This could indicate either a cross-reactivity of the anti-D^b serum with unidentified class I genes from the Qa region or the presence of additional Qa-reactive antibodies in the serum.

Demonstration of H-2D^b molecules on the cell surface

L-cells transformed with cosmids B1.1 and B3.2G were cell surface labelled with ^{125}I, lysed with NP40 and the cell extracts immunoprecipitated with either alloantisera or monoclonal antibodies directed against the H-2D^b antigen. Immunoprecipitates were analysed by SDS–polyacrylamide gel electrophoresis and the results with transformant LB1.1 are shown in Fig. 2 (identical results were obtained with B3.2G-transformed L-cells).

The H-2D^b-specific alloantisera and the monoclonal antibodies precipitated cell surface polypeptides of mol. wts 11,000 and 46,000, β_2m and H-2 heavy chain respectively. The latter comigrated with the H-2D^b heavy chain precipitated from EL4 lysates ($H\text{-}2^b$) which were run in parallel on the same gel (not shown). Preabsorption of the anti-H-2D^b alloantisera with B10 prevented precipitation of the H-2D^b heavy chain and the β_2m molecule from LB1.1. Absorption of the anti-H-2D^b sera with AKR lymphocytes, to remove antiviral antibodies, or with B10.A lymphocytes, to remove Qa2,3-reactive antibodies, did not inhibit the precipitation (not shown). No polypeptides were precipitated from LB1.1 cells by anti-H-2K^b-specific monoclonal antibodies or antisera. Anti-H-2D^b antibodies and antisera failed to precipitate any polypeptides from untransformed Ltk⁻ cells or from L-cells transformed with cosmids containing H-2K^b (LH8) or Qa genes (LB1.11) (not shown).

Cytotoxic T-cell killing

To test whether the product of the transfected H-2D^b gene in LB1.1 cells is recognized as a target

Table 3. Reactivity[a] of L-cells transfected with cosmids containing Qa/Tla genes

Antibody	Specificity	Location: Class I gene Cosmid	Qa Q2,3 H26	Qa Q6,7 H16	Qa Q8,9 B4.8 H19	Tla T1,2 H4, H13, H14	Tla T3,4,5 H9, H18	Tla T11,12,13 B2.8 H6, H43
100-5	K^k			+ +			+ +	
20-8-4s	K^b			−			−	
28-14-8s	D^b			−			−	
D2 serum	D^b			+			ND	
D28.b serum	H-2.28			−			ND	
D3-262	Qa2			−			−	
B16-146	Qa4			−			−	
TLm4	$TL^{a,c,d,f}$			−			−	

[a]Reactivity in antibody-dependent cytotoxicity assay (% specific ^{51}Cr release). −, 5%; +, 5–15%; + +, 15–50%, ND, not done.

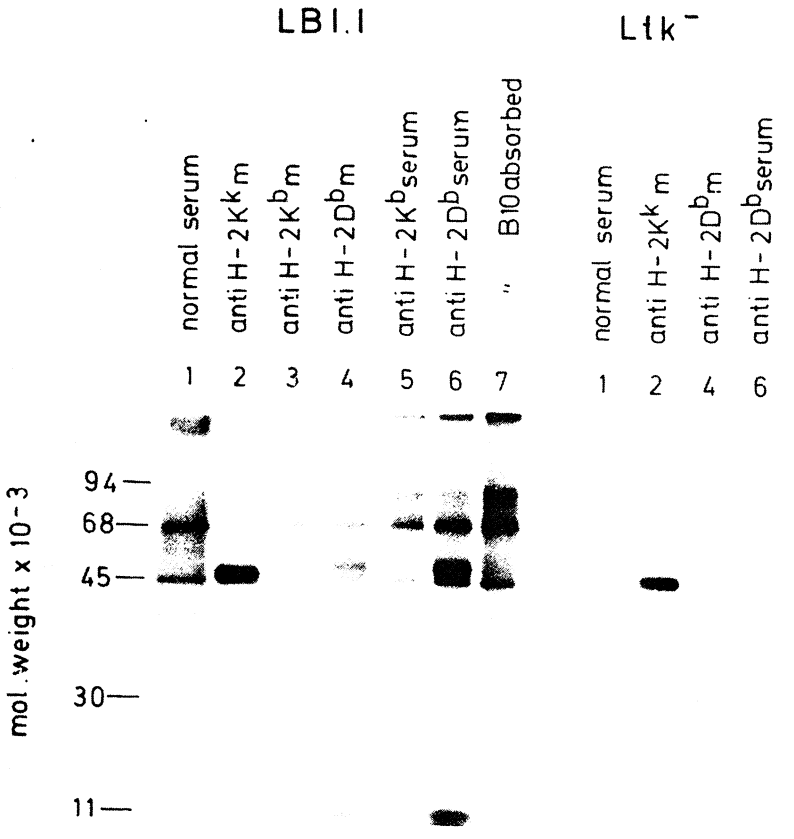

Fig. 2. Immunoprecipitation of radiolabelled cell surface antigens. NP40 extracts of cell surface iodinated LB1.1 and control Ltk$^-$ cells were immunoprecipitated with normal mouse serum (1), anti-H-2K^k monoclonal antibodies (m) (100-5) (2), anti-H-2K^b m (20-8-4) (3), anti-H-2D^b m (28-14-8) (4), anti-H-2K^b serum (5), anti-H-2D^b serum, either unabsorbed (6) or absorbed with B10 lymphocytes (7). Both anti-H-2D^b monoclonal antibodies and antiserum precipitated a 46,000 mol. wt heavy chain and the 11,000 mol. wt β_2m from LB1.1 cells.

structure by cytotoxic T-cells, we generated CTL directed against H-2K^b and H-2D^b antigens and tested their ability to kill various target cells (Table 4). Whereas the anti-H-2K^b CTL did not lyse LB1.1 cells, but lysed LH8 cells (containing the H-2K^b gene) (not shown), the anti-H-2D^b CTL specifically lysed LB1.1 cells, but not LH8, LB3.5 (tranformed by a Qa gene) or untransformed L-cells. All L-cell lines were lysed by anti-H-2^k CTL (not shown). These data suggest that the H-2D-region gene transfected into

Table 4. Cytotoxic effectors specific for H-2D^b antigens lyse L-cells (H-2^k) expressing H-2D^b antigens after transfection with H-2D^b genes

			% specific ^{51}Cr release[c] (effector:target cell ratio)			
Responder[a]	Stimulator	Target[b]	30:1	10:1	3:1	1:1
B10.BR	B10.A(4R)	B10 (D^b)	40.1	26.6	8.6	4.4
K^k D^k	K^k D^b	B10.A(4R) (D^b)	43.4	25.7	12.0	5.0
		LH8 (K^b)	4.8	1.7	−1.1	−1.6
		LB1.1 (D^b)	36.5	20.1	9.4	4.3
(B10.BR × C3H)F$_1$	B10.A(4R)	LB1.1.1 (D^b)	81.1	76.0	64.8	37.3
K^k D^k	K^k D^b	LH8 (K^b)	1.5	2.3	2.2	0.6
		LB3.5 (Qa)	6.1	0.8	2.6	0.7
		Ltk$^+$	5.0	2.6	2.9	2.0
		B10.A(4R) (D^b)	72.7	68.2	53.8	30.1

[a]B10.BR mice injected intraperitoneally once, and (B10.BR × C3H)F$_1$ mice, injected twice with 10^8 spleen cells from B10.A(4R) mice 14 days apart. After a further 14 days, their spleen cells (2×10^7) were cultured for 5 days with 10^7 B10.A(4R) lymphocytes irradiated with 2000 R.

[b]Target cells from the indicated mouse strains were transformed with PHA for 3 days. LB1.1 and LB1.1.1 cells transfected with D^b, LH8 cells with K^b, and LB3.5 with Qa2, 3 gene, and Ltk$^+$ control fibroblasts with thymidine-kinase gene only.

[c]Spontaneous ^{51}Cr release in the 4-hr ^{51}Cr-release assays ranged from 13 to 30%.

Table 5. Stimulation of anti-H-2D^b cytotoxic lymphocytes by LB1.1 cells[a]

Target cells	H-2 specificity	% ^{51}Cr release (effector:target cell ratio) 50:1	16:1	5:1
C3H	K^k D^k	13 ± 1	7 ± 2	1 ± 1
B10	K^b D^b	50 ± 2	43 ± 4	28 ± 3
B10.A(5R)	K^b D^d	21 ± 1	29 ± 11	6 ± 5
B10.A(4R)	K^k D^b	62 ± 5	50 ± 6	34 ± 6

[a]C3H mice were immunized with 3×10^6 LB1.1 cells 4 times. Spleen cells were restimulated in *in vitro* cultures with 4000-R irradiated LB1.1 cells at a ratio of 10:1. Four-hour ^{51}Cr-release assay. Spontaneous release: 14–33%.

and expressed by the LB1.1 cells is recognized as target antigen by H.2D^b-specific CTL.

We then tested whether the H-2D^b gene product on LB1.1 cells could stimulate anti-H-2D^b CTL precursors. We immunized female C3H mice 4 times by i.p. injection of LB1.1 cells and spleen cells were restimulated *in vitro* with irradiated LB1.1 cells.

The resultant effector cells lysed B10. and B10.A(4R) target cells (both H-2D^b), but not B10.A(5R) targets (H-2K^b) (Table 5). These data show that the H-2D^b gene transfected into LB1.1 cells induced H-2D^b-specific CTL.

DISCUSSION

A series of cosmids containing 15 MHC class I genes mapping to the D-, Qa and Tla regions of the H-2^b genotype have been isolated and transfected into L-cells (Weiss *et al.*, 1984). Only a single gene mapped to the H-2D region. The identity of this gene product with the H-2D^b gene product on B10 cells was shown by several criteria. (1) L-cells transformed with cosmids containing the gene express a cell surface antigen which carries all antigenic determinants characteristic of the H-2D^b molecule. All epitopes defined by a set of monoclonal antibodies and alloantisera are encoded by the cloned H-2D^b gene. (2) The antigenic determinants are represented on a glycopeptide of 46,000 mol. wt, which can be precipitated by monoclonal antibodies in association with β_2m, the invariant polypeptide of all known MHC class I antigens. (3) The H-2D^b molecules expressed in transformed L-cells can serve as targets for complement-dependent lysis by alloantisera and for allogeneic H-2D^b-specific CTL. (4) Transformed L-cells can specifically induce proliferation of H-2D^b-specific CTL.

Finally, Townsend *et al.* (1983) have shown that the gene products of one of the H-2D^b transfectants (LB1.1) acted efficiently as a restriction element for cloned influenza virus specific H-2D^b-restricted CTL lines after influenza virus infection of the transformed cells. We have analyzed 14 class I H-2^b genes which mapped to the Qa or the Tla region (Weiss *et al.*, 1984). Although B10 mice are Qa2,3-positive, we failed to identify the gene(s) coding for the Qa2,3 molecules. These molecules have a limited tissue distribution, and although fibroblasts do not normally express them, the Qa2,3-gene product from BALB/c mice was identified after transfer of this gene into L-cells (Goodenow *et al.*, 1982). We were nevertheless able to identify a functional gene in the Qa2,3 region. L-cells transfected with this gene did not react with anti-Qa2 or anti-Qa4 monoclonal antibodies, but could be used to raise antisera which specifically reacted with the transfected cells and with lymphocytes from certain B10 congenic mice (Alonzo *et al.*, in preparation).

Restriction mapping of the D-end cosmids failed to reveal an H-2L gene (Weiss *et al.*, 1984), nor could we find an L-molecule on L-cells transfected with D-, Qa or Tla cosmids. This is in agreement with immunogenetic studies (Demant *et al.*, 1981) and in contrast to the findings in BALB/c mice, in which 2 additional MHC class I genes have been identified in the H-2D region, 1 of which is the H-2L^d gene (Goodenow *et al.*, 1982; Winoto *et al.*, 1983). In addition, the genetic basis for M and R molecules has not yet been established. Although positive reactions of H-2D^b transfectants were obtained with anti-M and anti-R antibody (Demant, personal communication), it is not known whether the reactions are directed against the H-2D^k or the H-2D^b molecule. In addition, unexpected reactivities with Qa-region genes were obtained with anti-H-2D^b antisera (Tables 2 and 3).

Furthermore, it is not known whether, due to alternative splicing of the 5′-moiety of certain H-2D transcripts (Transy *et al.*, 1984), there are different-size mRNA transcribed which could generate distinct but antigenically related polypeptides. Further experiments to clarify this point are in progress. In contrast to the D-region, the genetic organisation of the H-2K region of C57BL10 and BALB/c as well as AKR/J mice is similar (Mellor *et al.*, 1982; Goodenow *et al.*, 1982; Arnold *et al.*, 1984a).

The transfected H-2D^b gene is biologically active and may thus be used to further elucidate the biological role and the structure–function relationship of this molecule (Zinkernagel *et al.*, 1983). Already several cloned MHC class I genes have been shown to act as restriction elements for specific CTL after transfection into appropriate recipient cells (Örn *et al.*, 1982; Mellor *et al.*, 1982; Levy *et al.*, 1983; Margulies *et al.*, 1983; Townsend *et al.*, 1983).

Furthermore, the construction of hybrid H-2 genes should help not only to map serological determinants (Evans *et al.*, 1982) but also to define functional domains of the H-2 molecule, such as those involved in interaction with foreign antigens or CTL recognition (Arnold *et al.*, 1984*b*; Ozato *et al.*, 1983).

The transfer of cloned H-2 genes into tumour cells should help to understand the role of these molecules in tumour growth and metastasis (Hui *et al.*, 1984).

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