ORGANIZATION AND EVOLUTION OF D REGION CLASS I GENES IN THE MOUSE MAJOR HISTOCOMPATIBILITY COMPLEX

By DANIELLE STEPHAN,* HENRY SUN,‡ KIRSTEN FISCHER LINDAHL,* EVI MEYER,§ GÜNTER HÄMMERLING,§ LEROY HOOD,‡ AND MICHAEL STEINMETZ*

*From the *Basel Institute for Immunology, CH-4005 Basel, Switzerland; the ‡Division of Biology, California Institute of Technology, Pasadena, California 91125; and the §Institute for Immunology and Genetics, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany*

The MHC encodes highly polymorphic cell surface glycoproteins which are recognized by T cells alone or in association with foreign antigens on the surface of target cells (for reviews see 1–3). Most cytotoxic T cells recognize antigen in the context of MHC class I molecules, while most helper T cells do so in the context of MHC class II molecules. In the BALB/c mouse, three restriction elements of class I, termed K, D, and L, and two of class II, called A and E, have been identified. In addition to the K, D, and L restriction elements, which are present on virtually all somatic cells, a growing number of tissue-specific MHC class I molecules (Qa-1, Qa-2, TL) have been characterized, the function of which is unknown.

Mouse strains can be distinguished from one another by their particular sets of MHC alleles or haplotypes (4). Haplotypes are indicated by lower case letters, e.g., the BALB/c, C57BL/10, and AKR strains are representatives of the *d*, *b*, and *k* haplotypes. The MHC of the BALB/c mouse contains at least 33 class I genes, 2 in the *K* region, 13 in the D and Qa regions, and 18 in the Tla region (5–7) (Fig. 1). Among these the K^d, D^d, and L^d class I genes of BALB/c have been identified (15, 16). Two tissue-specific class I genes have been extensively characterized, namely the *Q10* gene (17–21), coding for a liver-specific secreted class I molecule, and the *Tla-1,2* gene (7, 12–14), coding for a T cell–specific molecule.

Serological and molecular genetic techniques have yielded conflicting results regarding the number of class I genes in the D region of the MHC. For instance, five D^d region class I molecules, D^d, L^d, R^d, M^d, and $L2^d$, have been identified by immunochemical methods (22–24), while only three class I genes (D^d, L^d, and one additional class I gene) were cloned from the D region of the BALB/c mouse (5, 6, 16). It is not clear at present whether the R^d, M^d, and $L2^d$ class I molecules are encoded by separate D^d region genes, some of which might not have been cloned so far, or are alternative splicing products or posttranslationally modified products of the known D region class I genes.

Serological and molecular evidence exists (22–30) for different numbers of

J. Exp. Med. © The Rockefeller University Press · 0022-1007/86/05/1227/18 $1.00 1227
Volume 163 May 1986 1227–1244

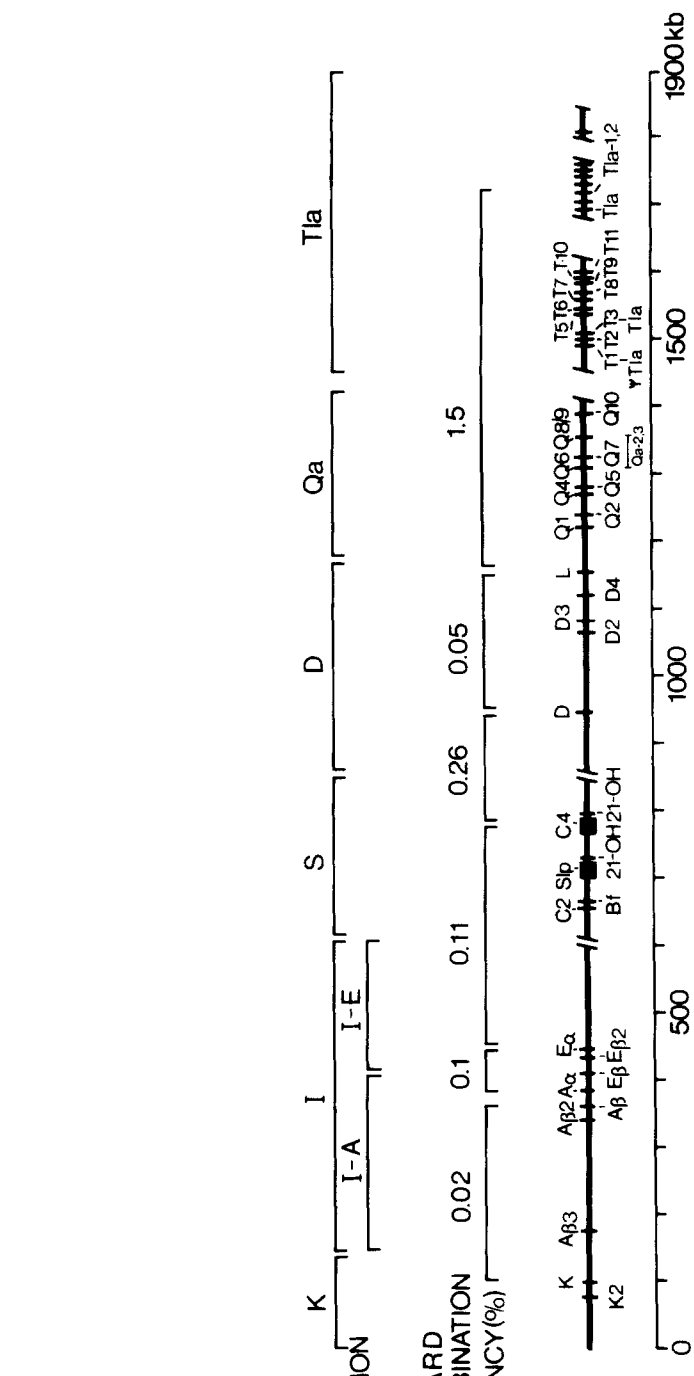


FIGURE 1. Molecular map of the MHC of the BALB/*c* mouse. The six gene clusters, containing a total of 46 genes and encompassing ~1,600 kb of DNA, are correlated with the genetic regions and subregions of the MHC defined by recombination events. A 600 kb gene cluster spans the K and the I region and contains two class I (*K2, K*) and seven class II ($A_{\beta 3}$, $A_{\beta 2}$, A_β, A_α, E_β, $E_{\beta 2}$, E_α) genes (8). Following a gap of unknown size, a 250 kb cluster contains four complement-related (*C2, Bf, Slp, C4*) and two 21-hydroxylase (*21-OH*) genes (9–11 and M. Steinmetz, unpublished results). This is followed at an unknown distance by the 500 kb class I gene cluster described in this paper. Finally, three gene clusters with 18 class I genes have been cloned from the Tla region (5–7) and 4 *Tla* genes; including the *Tla*-1,2 gene encoding the TL $1^+ 2^+ 3^-$ 4^- molecule, have been identified (7, 12–14).

class I genes and gene products in the D and Qa regions of different mouse strains. First, by immunoprecipitation, peptide map analysis, and cocapping techniques, three to five class I molecules have been shown to be encoded in the D^d and D^q, two in the D^{w16}, and one each in the D^b, D^{dx}, D^k, and D^p regions (22–27). Second, while three class I genes have been cloned from the D^d, only one has been isolated from the D^b region (28). Third, different mouse strains either do or do not encode a Qa-2 class I molecule in the Qa region (29). Fourth, over a stretch of 250 kb cloned from the Qa region of the C57BL/10 mouse, containing 10 class I genes, 2 genes have apparently been lost in BALB/c (Qa-2^+) (28), and presumably a third one has been lost in BALB/cBy (Qa-2^-) mice (30).

To address the question of how many class I genes occur in the D^d region of BALB/c, we have studied the molecular organization by chromosome walking. Contrary to previous findings (5, 6), we show that at least five class I genes are located in the D^d region. Comparison of the molecular map of the D^d region to similar maps of the D^b and D^k regions allows us to draw interesting conclusions about evolutionary mechanisms underlying the dynamic variation of the numbers of D and Qa region class I genes in different mouse strains.

Materials and Methods

Chromosome Walking. A BALB/c cosmid library, constructed using the vector pNNL (31) as previously described (32), was screened successively (see Fig. 2) with probes 13 (yielding clones II5.16 and II7.13 due to crosshybridization), 5 (yielding clone II2.20), 7 (yielding clone II6.18), 14 (yielding clone II1.10 and II4.19), 16 (yielding clones II6.17 and II3.20), and 1 and 3 together (yielding clones II3.5 and II4.12). The overlap between clone II3.20 and the previously isolated one, 2.1 (5), was confirmed by hybridization with probe 17 (see Fig. 2). A BALB/c phage λ library, constructed using the vector EMBL3 (33), was screened with probes 9 and 11, yielding clones λ1.2 and λ5.4 (see Fig. 2).

An AKR cosmid library (32) was screened successively with probes 13 (yielding clones k1.3, k2.4, k12.1, k14.2, k21.4, k13.2), 3 (yielding clone k9.4), and with probe D (a K^k region probe [32]), yielding clones k1.4, k6.2, and k11.1 due to crosshybridization (see Fig. 3).

Hybridizations were done as described (34), except that restriction fragments used as probes were isolated and labeled according to Feinberg and Vogelstein (35).

Hybridization Probes. Hybridization probes shown in Figs. 2 and 3 were derived from the following cosmid clones: 1, 2.5 kb Sac II-Kpn I fragment from k9.4; 2, 0.5 kb Sau 3A fragment from the 4.5 kb Bam HI fragment of clone dm1-38.1, described in Sun et al. (36); 3, 0.8 kb Hpa I fragment from k1.3; 4, probe 18.1B, described in Sun et al. (36); 5, 1.4 kb Sma I fragment from II7.13; 6, 0.5 kb Alu I fragment from the 3.5 kb Hpa I fragment of II2.20; 7, 6.2 kb Hpa I fragment from II2.20; 8, 0.2–0.5 kb Hpa I-Hind III fragment from the 1 kb Sma I-Sal I fragment of II6.18 (the Sal I site is located in the cosmid vector); 9, 1.8 kb Sma I fragment from 50.2; 10, 0.9 kb Sau 3A fragment from the 9 kb Xho I-Nru I fragment of BALB/c cosmid clone JS4.4 (H. Sun, unpublished data) (the Nru I site is located in the cosmid vector) extending from map position 185 to 226 kb (see Fig. 2); 11, Dpn I fragment from the 5.2 kb Nru I-Sma I fragment of 16.1 (the Nru I site is located in the cosmid vector); 12, probe 59.2B, described in Sun et al. (36); 13, probe 27.51, described in (5); 14, 1.2 kb Sma I fragment from 59.2; 15, 1 kb Kpn I-Sal I fragment from II1.10 (the Sal I site is located in the cosmid vector); 16, 3.2 kb Sma I-Sal I fragment from II4.19 (the Sal I site is located in the cosmid vector); 17, 1.1 kb Bam HI fragment from 2.1; 18, Dpn I fragment from the right end of the k11.1 (see Fig. 3).

D^k Gene Transfer and Expression. For some transfections, the 11 kb Eco RI fragment

of cosmid k12.1 was subcloned into pBR322, yielding plasmid clone pDK17. Transfections were performed as described previously (37). For transfection, the (C3H × C57BL/6)F_1 mouse fibrosarcoma cell line IC9 that expresses only the D^b molecule (37), or a B10.CAS2 mouse fibroblast cell line was used. Briefly, for the IC9 cell line class I DNA (10–20 μg) and pAG60 (1–4 μg) (38) and for the B10.CAS2 cell line class I DNA and pSV2neo (39) was coprecipitated with calcium phosphate, and added to ~10^6 adherent cells. After 5 h at 37°C the medium was replaced by 5 ml of 15% glycerol for 2 min to facilitate uptake of DNA by the cells. After overnight incubation with medium, the transfected IC9 cells were collected with EDTA buffer and distributed into two Costar 3524 multiwell plates in selection medium containing 700 μg/ml G418 (Gibco, Grand Island, NY). B10.CAS2 transfectants were selected with 1 mg/ml G418 and tested uncloned. IC9 transfectants were expanded, tested for expression of the D^k molecule, and then subcloned. H-2 expression on IC9 transfectants was determined in a cellular RIA using ^{125}I-labeled protein A, as described previously (40). Briefly, 10^6 cells were incubated in microtiter plates with specific anti-H-2 mAb. After washing, ^{125}I-labeled protein A was added and the amount of radioactivity determined. Values represent mean cpm of triplicate wells. The SD range is 5–10% (not shown). The following anti-H-2 mAb were used: H142-23, H100-27, H100-30, B22-249, H116-22, 18/20 (40); K7-309 (41); R1-21.2 (42); 15-5-5S, 16-1-2 (43). H11-3 was a generous gift of Dr. Svendsen, (Statens Seruminstitut, Copenhagen).

Results

500 kb of Cloned BALB/c DNA Link D and Qa Regions at the Molecular Level and Contain Five D and Eight Qa Region Class I Genes. We previously (5, 6) described five BALB/c class I gene clusters, termed clusters 1, 2, 6, 9, and 13, that were mapped into the D and Qa regions of the MHC, and which contained 13 class I genes. The single class I gene on cluster 13 and one of the two class I genes on cluster 2 encode the D^d and L^d molecules, respectively (16). Further characterization showed that clusters 1 and 9, both mapping into the Qa region, contained overlapping DNA sequences (44). To link the D and Qa regions, gene clusters 2, 6, 1 and 9, and 13 at the molecular level, we have now isolated a series of overlapping phage λ and cosmid clones from BALB/c liver DNA libraries by using hybridization probes derived from previously isolated BALB/c clones and from the 5′ flanking sequence of the D^k gene (see Materials and Methods).

The previously described clones together with the newly isolated ones allow us to define a stretch of 500 kb of DNA that spans the D and Qa regions of the MHC of the BALB/c mouse (Fig. 2). This stretch contains 13 class I genes, identified with the highly crossreactive class I cDNA clone pH-2IIa (5), namely five class I genes in the D region and eight class I genes in the Qa region. The three class I genes located between D^d and L^d are provisionally called *D2*^{d}, *D3*^{d}, and *D4*^{d}. The eight class I genes in the Qa region are denoted *Q1*, *Q2*, *Q4*, *Q5*, *Q6*, *Q7*, *Q8/9*, and *Q10*, in agreement with the nomenclature established by Weiss et al. (28) for the C57BL/10 Qa region class I genes. It appears that the *Q3* gene has been lost from the BALB/c mouse and that the *Q8* and *Q9* genes have been fused into a *Q8/9* hybrid gene (28). Gene *Q7* corresponds to the previously sequenced gene 27.1 (5, 45).

The stretch of 500 kb of DNA is defined by a total of 84 overlapping cosmid clones, 28 of which are shown in Fig. 2, and six phage λ clones, two of which are shown. Individual cosmid clones overlap by >10 kb, except for a gap between cosmid clones 50.2 and II4.8, which is bridged by the λ clones λ1.2 and λ5.4.

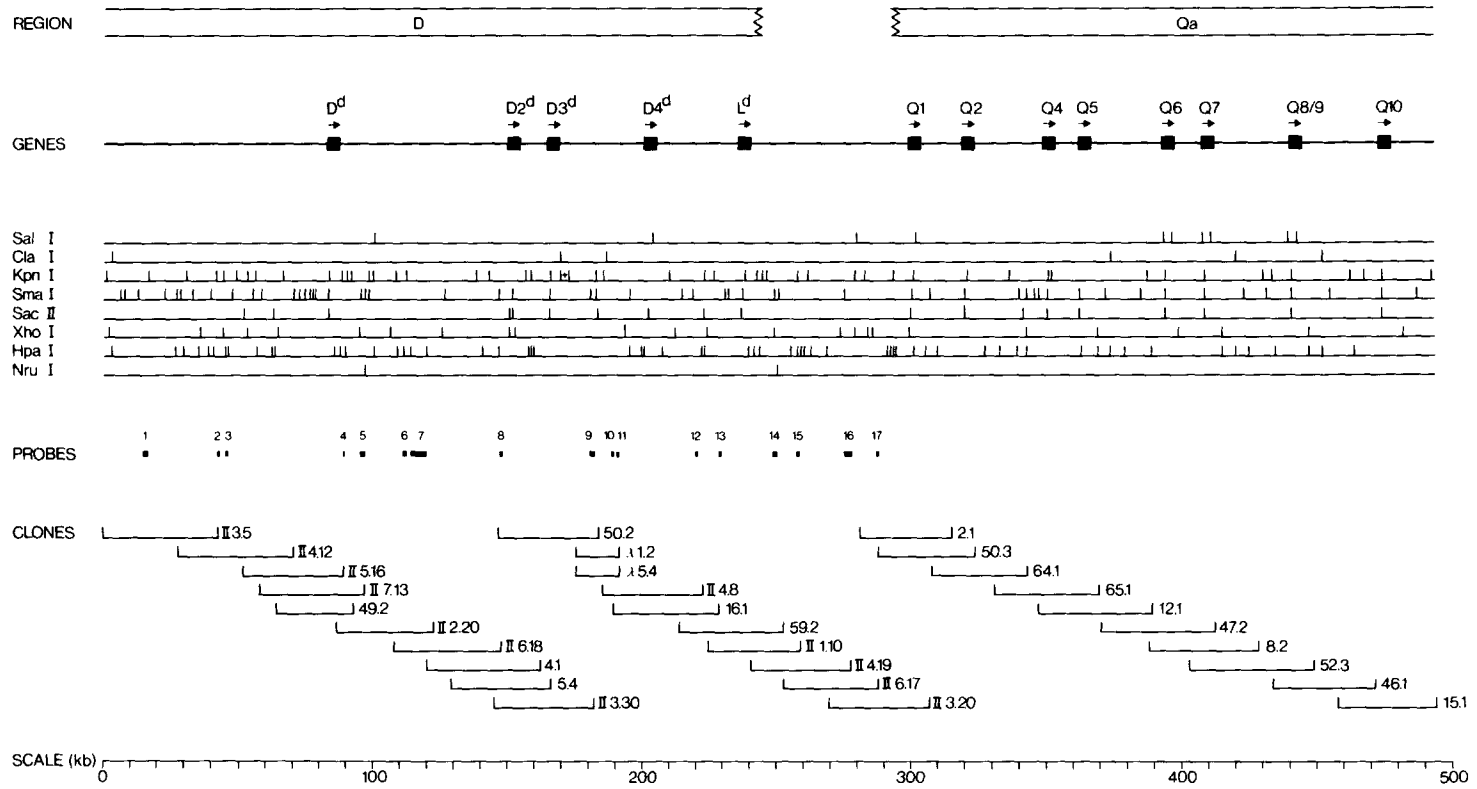


FIGURE 2. A gene cluster of 500 kb with 13 class I genes links the D^d and Qa regions of the BALB/c mouse. Genes are shown as boxes, but are not drawn to scale. The D^d, L^d, *Q7*, and *Q10* genes have been sequenced (19, 45–48). Restriction maps were determined by single and double (49), and by partial digestions (36). A + between two sites indicates the presence of more cleavage sites for the same enzyme. The hybridization probes were isolated as described in Materials and Methods. Cosmid and phage clones II3.35 to II7.13 (from left to right), II2.20, II6.18, II3.30, λ1.2, λ5.4, II4.8, and II1.10 to II3.20 were isolated in the course of this work, while cosmid clones 49.2 containing the D^d gene (36, 46), cosmid clones 4.1, 5.4, 50.2 constituting cluster 6 (5), cosmid clones 16.1, 59.2 constituting cluster 2 (5), cosmid clones 2.1 to 46.1 constituting cluster 1 (5), and cosmid clone 15.1 constituting cluster 9 (5) have been described previously. The linkage between clusters 1 and 9 was described previously (44).

These clones were isolated with probes 9 and 11 and share restriction maps with the cosmid clones as shown. The linkage was further confirmed by mapping Bam HI, Xba I, Sst I, and Hind III sites in the two λ clones and the overlapping segments of the cosmid clones (not shown).

130 kb of Cloned DNA Containing Two Class I Genes Link the D and Qa regions of the AKR Mouse at the Molecular Level. To compare the D region gene organization in BALB/c and AKR mouse strains, we screened an AKR cosmid library with probes from the 5′ flanking regions of the L^d and D^k genes and from the 3′ flanking sequence of the K^k gene (see Materials and Methods). Ten overlapping cosmid clones were obtained, which define a stretch of 130 kb of DNA with two class I genes identified by hybridization with the cDNA clones pH-2III and pH-2IIa specific for the 5′ and 3′ regions of class I genes, respectively (5) (Fig. 3). Comparison of the restriction maps of the two AKR class I genes with those of the BALB/c D and Qa region class I genes showed that the upstream AKR gene is homologous to D^d and L^d, while the downstream AKR gene is the counterpart to the *Q1* gene of BALB/c (compare Figs. 2 and 3). This result suggested that the upstream AKR class I gene is D^k and the downstream one is *Q1*. To confirm that the cloned region does indeed link the AKR *D* and *Qa* regions at the molecular level, we mapped the two single-copy probes 1 and 18 with respect to their location on the genetic map of the MHC. As shown in Fig. 4, the restriction fragment length polymorphism identified with probe 1 maps to the D region, while the polymorphic restriction site identified with probe 18 maps to the Qa region. Thus we conclude that the cloned stretch of 130 kb of DNA covers part of the D and Qa regions of the AKR mouse.

Identification of the D^k Gene by Gene Transfer and Expression. To confirm that the proximal class I gene in the 130 kb gene cluster corresponds to the D^k gene, we cotransfected the neomycin resistance gene together with each of the cosmid clones k1.3 and k12.1 into a B10.CAS2 mouse fibroblast cell line, and the same resistance gene together with cosmid clone k13.2 as well as a plasmid subclone (pDk17) of k12.1 into the mouse fibrosarcoma cell line IC9, which expresses only the D^b molecule (see Materials and Methods). Transfectants were selected in the presence of G418 and tested, either uncloned or cloned, by RIA with a panel of mAb and with D^k-alloreactive cytotoxic T cells (Tables I and II). Clearly three of the cosmid clones, when introduced into the cell lines, led to the appearance of D^k serological determinants on the surface of the transfected cells. Transfected IC9 cells were positive for all mAb, with reactivity against the D^k molecule. Furthermore, B10.CAS2 fibroblasts, transfected with cosmid clones k1.3 or k12.1 were killed by the D^k-specific cytotoxic T cells. Together with the genetic and physical mapping data we can therefore be sure that we have correctly identified the D^k gene.

Four of the Five D Region Genes of BALB/c Mice Appear to Be Missing in Some Strains and Can Be Deleted. A comparison of the D region gene organization in the three mouse strains that have been characterized by molecular cloning, BALB/c, AKR, and C57BL/10, is shown in Fig. 5. While five class I genes have been identified in the D region of BALB/c, only one has been found in AKR and C57BL/10. The D^d, D^k, D^b, and to a lesser extent L^d genes have very similar restriction maps for their 5′ flanking sequences. Also the 3′ flanking sequences

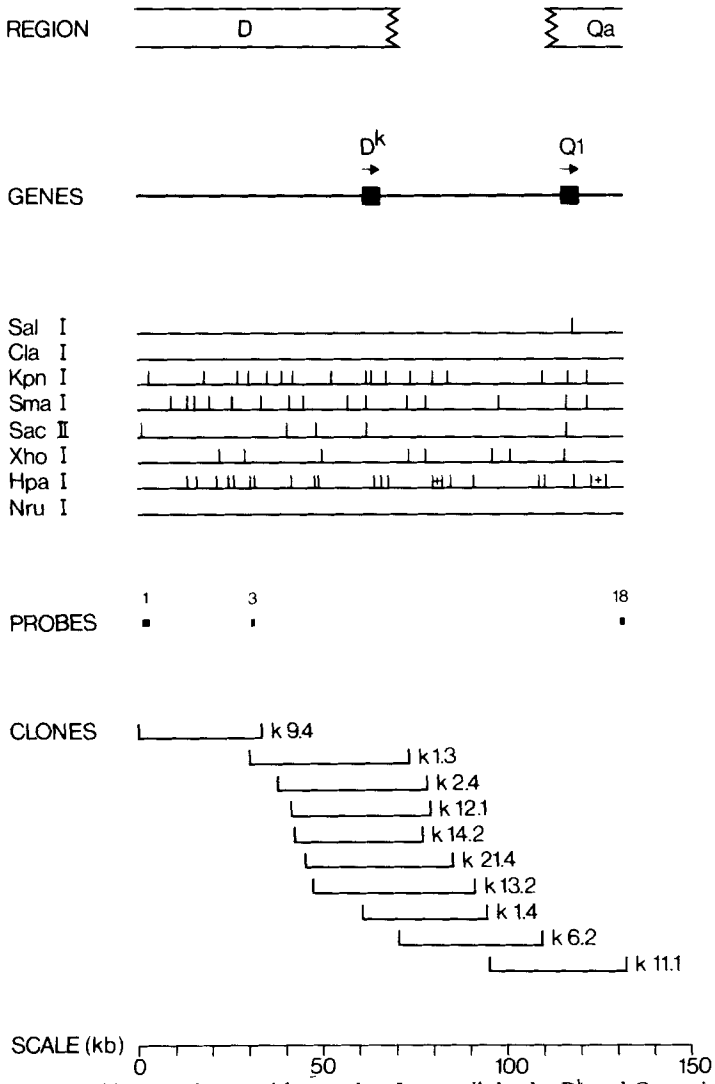
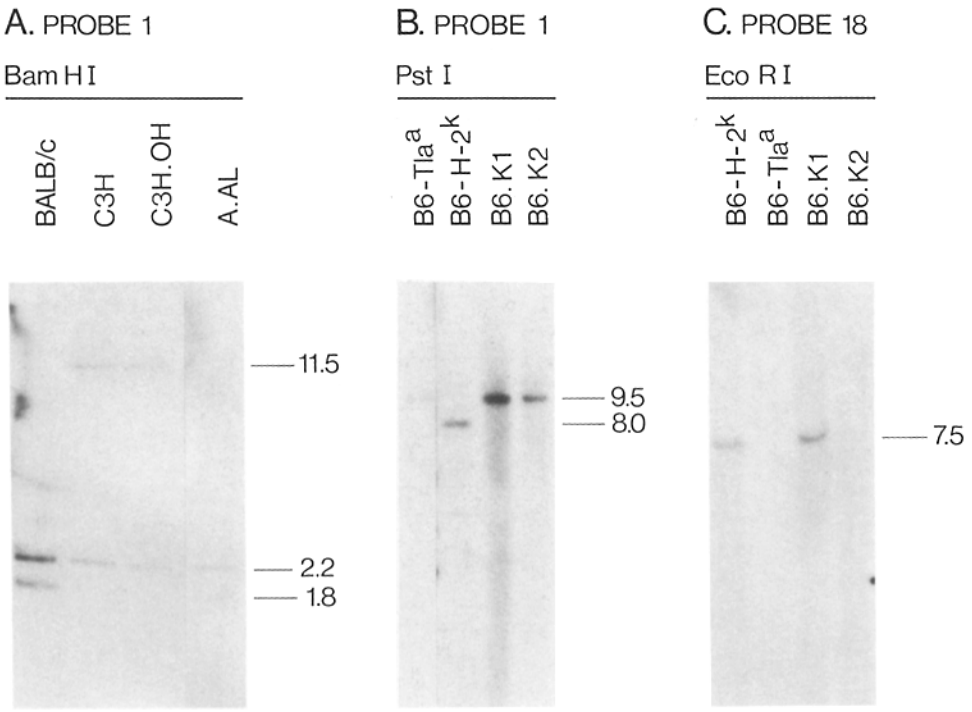


FIGURE 3. A 130 kb gene cluster with two class I genes links the D^k and Qa regions of the AKR mouse. Genes, restriction maps and hybridization probes are shown as in Fig. 2. Cosmid clones k9.4 to k11.1 (from left to right) were isolated as described in Materials and Methods.

of the D^d, D^k, D^b, and L^d genes are closely related (Fig. 5). These sequence homologies will be further discussed below.

The cloning studies indicated that four of the five BALB/c D region class I genes are missing in AKR and C57BL/6, and Southern blot hybridization confirmed that sequences located between the D^d and L^d genes are absent from AKR and C57BL/6 DNA. As shown in Fig. 6, hybridization probes isolated from the region between the L^d and D^d genes (probes 7 and 11) do not pick up complementary sequences in AKR and C57BL/10 DNA. On the other hand, probes 3 and 15, isolated from the 5′ and 3′ flanking sequences of the D^d and L^d genes, respectively, hybridize to the same restriction fragments in AKR and



D.

	S		D	Qa
BALB/c	d		d	a
C3H	k		k	b
C3H.OH	d	I	k	b
A.AL	k	I	d	a

E.

	D		Qa		Tla
B6-Tla^a	b		a^{AorB6}		a^{AorB6}
B6-H-2^k	k		b^{AKR}		b^{AKR}
B6.K1	b	I	b^{AKR}		b^{AKR}
B6.K2	b		a^{AorB6}	I	b^{AKR}

FIGURE 4. Typing of D- and Qa region–specific sequences from AKR by Southern blot analysis of polymorphic restriction sites. 10 μg of DNA per lane was cut with the indicated restriction enzymes, run on a 0.6% agarose gel and blotted to nitrocellulose filters. Hybridization with the probes indicated was done as described (8). *A*: probe 1, isolated from cosmid clone k9.4 (compare Fig. 3 and Materials and Methods) identifies a polymorphic Bam HI site in BALB/c and C3H mice. Analysis of the recombinant strains C3H.OH and A.AL maps the polymorphic site distal to the S region (compare with *D*). *B*: probe 1 also identifies a polymorphic Pst I restriction fragment in B6-*H-2*^{k} and B6-*Tla*^{a} mice. Analysis of the recombinants B6.K1 and B6.K2 maps the polymorphism proximal to the Qa region marker locus (compare with *E*). Together with the mapping result from Fig. 4A, this maps probe 1 to the D region. *C*: probe 18 (compare Fig. 3 and Materials and Methods) identifies a 7.5 kb Eco RI fragment in B6-*H-2*^{k} that is missing B6-*Tla*^{a}. Analysis of the recombinants B6.K1 and B6.K2 maps the polymorphism and therefore probe 18 to the Qa region (compare with *E*). *D* and *E*: H-2 alleles in inbred mouse strains used (4). Vertical bars identify recombination events.

TABLE I

AKR Cosmids k12.1 and k13.2 Encode the D^k Class I Molecule

Antibody	Specificity	Cells tested (cpm bound)			
		IC9‡	87-8-7.3§	87-1-6.3§	76D2.6‖
15-5-5 S	D^k, K^d	556	7,130	11,528	8,514
H11-3	D^k	986	7,742	13,958	9,484
H142-23	D^k, K^k, K^b, K^s	498	5,450	12,264	8,010
H100-27	D^k, K^k, r	908	3,956	6,172	4,494
H100-30	D^k, K^k, s, b, r, q	942	4,420	6,648	4,992
B22-249	D^b	6,538	5,348	6,854	5,048
K7-309	K^b	842	828	1,220	1,340
H116-22	K^k, t, q, s, r	1,264	1,470	1,536	1,228
R1-21.2*	D^k, K^k, K^b, K^s, K^f, K^q	1,072	7,912	17,108	13,878
R28-27*	I_i	1,272	1,308	1,460	1,980

Data are from cellular RIA. For mouse mAb, ^{125}I–protein A was used; for rat mAb, first anti–rat κ chain and then ^{125}I–protein A was used. Significant binding is underscored.

* R1-21.2 is a rat anti–mouse H-2 antibody and R28-27 is a rat anti–mouse invariant chain antibody.

‡ IC9 is a (C57BL/6 × C3H)F_1 fibrosarcoma line expressing only D^b.

§ 87-8-7.3 and 87-1-6.3 are independent clones isolated from transfection of IC9 cells with plasmid pDk17 derived from cosmid k12.1.

‖ 76D2 is a clone selected from IC9 cells transfected with cosmid k13.2.

TABLE II

AKR Cosmids k1.3 and k12.1 Encode the D^k Target Antigen

B10.CAS2 fibroblasts transfected with cosmid	Exp.	Lysis by CTL*		Binding of mAb‡		
		Anti-D^k	Anti-B10.CAS2	15-5-5 (anti-D^k)	16-1-2 (anti-D^k, D^{cas2})	18/20 (anti-TL)
k1.3	1	4.3	11.3	275	1,739	40
	2	16.6	10.7	1,016	2,155	ND
	3	ND	ND	263	2,107	25
k12.1	1	20.7	23.5	144	765	43
	2	28.8	22.1	438	436	ND
	3	ND	ND	160	2,105	44
Controls§	1	−6.8–−1.3	14.6–31.8	84–128	114–344	50–88
	2	−7.9–1.2	11.7–39.6	197–387	94	ND
	3	ND	ND	56–131	245–743	24–68

* Expressed as percent of total ^{51}Cr released above control at E/T = 100:1. Anti-D^k: (B10 × B10.CAS2)F_1 anti-B6.AK1; anti-B10.CAS2: C57BL/6 anti-B10.CAS2. Significant lysis underscored.

‡ [^{3}H]Leucine-labeled antibodies bound (cpm per 1–2 × 10^6 fibroblasts). Significant binding underscored.

§ Transfected with L^d, D^d or Tla region cosmids; 1–12 cosmids per test.

C57BL/10 as in BALB/c DNA, in agreement with the restriction map homologies described above. The 4.8 kb Bam HI fragment picked up by probe 7 in BALB/c, AKR, and C57BL/10 DNA is due to a crosshybridization of this probe to a sequence, labelled "7 cross", located in the 3′ flanking sequence of the L^d gene (Fig. 6 and see below).

Southern blot analysis of the D^p and D^q regions in mouse strains B10.F(14R) and B10.AKM, respectively, with the four D region hybridization probes shows that the D^q region is indistinguishable from BALB/c, while in the D^p region,

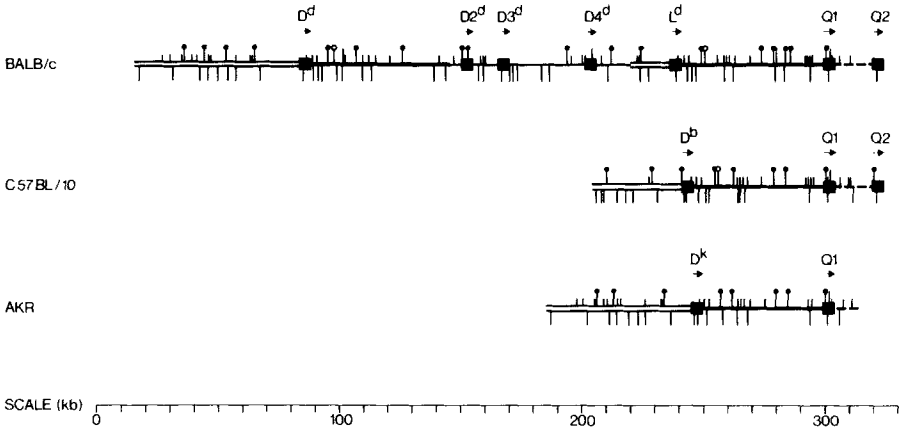


FIGURE 5. Comparison of restriction maps for D and Qa region sequences of BALB/c, C57BL/10 and AKR mouse strains. Restriction sites indicated (Sal I: ⊥, Kpn I: ⊤, Xho I: ♀, Hpa I: ⊥, Nru I: ♀) were taken from Fig. 2 (BALB/c), Fig. 3 (AKR), and Weiss et al. (28) (C57BL/10). Homologous segments based on restriction map similarity are shown by different symbols: 5′ flanking sequences of D^d, L^d, D^b, D^k: open boxed line; 3′ flanking sequences of D^d, L^d, D^b, D^k: thick line; 3′ flanking sequences of *Q1*: dotted line.

sequences 11 and 7 cross are apparently deleted (Fig. 6). Again, different strains of mice appear to have different sequence organizations in the D region of the MHC. With the D region probes, we have also analyzed three *d* haplotype mutant mouse strains, one with a mutation in the K locus (C.B6-*H-2*^{dm5}) (50), and two with mutations in the D region. One of the D region mutants has recently been shown (36, 51) to contain an H-2D/L hybrid gene (B10.D2-*H-2*^{dm1}), while the other one has lost the H-2L gene product (BALB/c-*H-2*^{dm2}) (52). As shown in Fig. 6, the K region mutant *dm5* is indistinguishable from BALB/c with the four D region probes, but the two D region mutants show deletions of probes 7 and 11. This is exactly the result expected for the *dm1* mutant if the mutation was caused by unequal crossing-over deleting the DNA between L^d and D^d (see 36). As with the *dm1* mutation, and in agreement with the hybridization data, the mutation in the *dm2* mutant might also be due to unequal crossing over, in this case deleting the *D2*^{d}, *D3*^{d}, *D4*^{d}, and L^d genes, leaving the D^d gene behind.

DNA Sequences from D^d Region Crosshybridize Most Strongly to Other D^d and to Qa Region Sequences. We used 11 restriction fragments isolated from the D region as hybridization probes to analyze the relationship of the D^d region DNA sequences to those of the other class I regions of the MHC of the BALB/c mouse. The 11 restriction fragments were labeled (35) and then hybridized to dot blots containing 33 cosmid clones, which covered a total of 1,000 kb and represented all cloned class I genes (not shown). Cosmid clones giving a positive signal were then analyzed by Southern blotting with the appropriate probes. Although the results obtained were complex, certain recurrent patterns can be seen (Fig. 7). First, the four probes, 4, 6, 7, and 8, isolated from the segment between genes D^d and *D2*^{d} all crosshybridize to sequences located between genes L^d and *Q1*, and the two probes, 14 and 15, isolated from the region between the L^d and *Q1* genes crosshybridize to the D^d-*D2*^{d} segment. The D^d-*D2*^{d}/L^d-*Q1* homologies, as seen by crosshybridization, are in perfect agreement with the restriction map

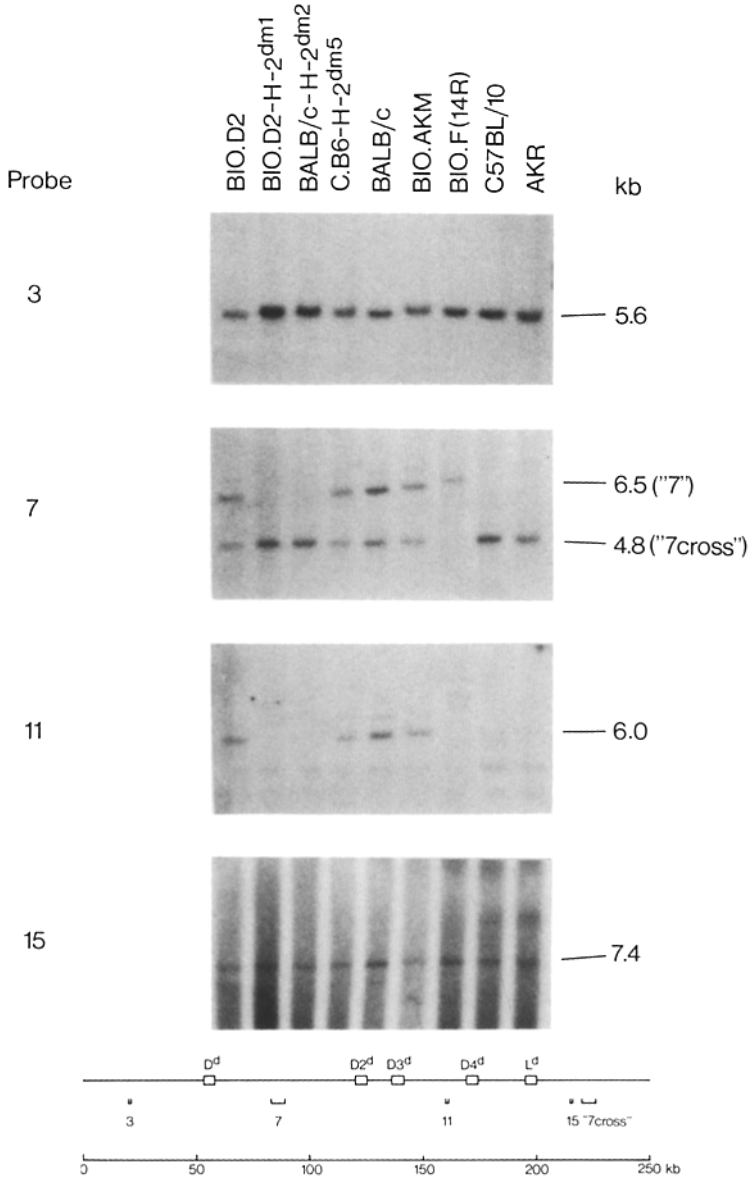


FIGURE 6. Southern blot analysis of different inbred strains of mice and three *d* haplotype mutant strains with D region probes. Location of the probes is shown at the bottom (compare Fig. 2). The "7 cross" indicates a sequence in the 3′ flank of L^d that crosshybridizes strongly to probe 7 (see also Fig. 7). 10 μg of DNA per lane was cut with Bam HI and analyzed as described in Fig. 4. After hybridization, filters were washed twice for 20 min each at 68°C in 0.1× SSC, 0.1% SDS.

homologies mentioned above. Second, the two probes, 9 and 10, isolated from the $D3^d$-$D4^d$ segment crosshybridize to the Qa region segment *Q1-Q4*, a relationship that was not seen by restriction map comparison. Third, the D^d-$D2^d$ segment probe, 8, also shows strong hybridization to the *Q5* to *Q10* genes as well as to

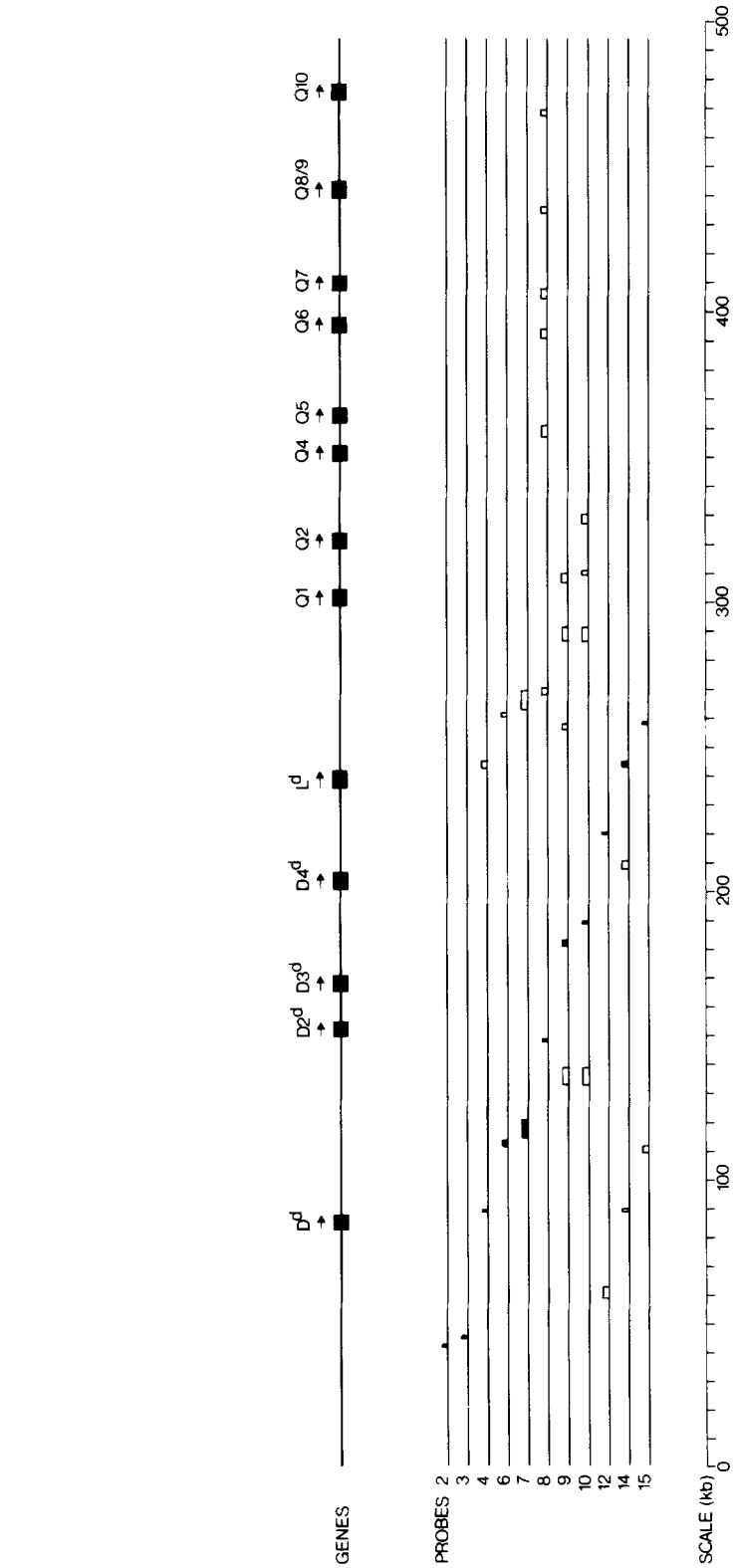


FIGURE 7. Crosshybridization patterns in the BALB/c D and Qa regions. Cross-hybridiation was checked on K, D, Qa, and Tla region cosmids as described in the text. Hybridization probes indicated by filled boxes are the same as in Fig. 2. Crosshybridizing sequences are indicated by open boxes on the same line. Probes 1 and 3 showed no crosshybridization to any of the K, D, Qa, and Tla region cosmids tested under the stringency conditions used (washes at 0.5× SSPE [45], 68°C, 1 h). In addition to the sequences shown, probes 8 and 14 also gave strong crosshybridization to K region sequences, and the following probes gave weak crosshybridization to: probe 4: 3′ of K^d, 3′ of *Q1*; probe 7: segments between *Q4-Q5*, *T3-T5*, *T6-T7*; probe 10: segments between *T3-T5*, *T6-T7*; probe 14: 3′ of *Q1*, 3′ of *Q2*.

the K^d and $K2^d$ genes. Fourth, the $D4^d$-L^d segment probe, 12, cross hybridizes ~21 kb upstream of the D^d gene, while the two probes, 2 and 3, located 32–40 kb upstream of the D^d gene show no crosshybridization to the 5′ flanking sequence of the L^d gene, and in fact, are single-copy probes. These sequence homologies have important evolutionary implications, as will be discussed below.

Discussion

Organization and Expression of Class I Genes in the D and Qa Regions of the BALB/c Mouse. 13 class I genes have been linked into a cluster of 500 kb of DNA extending from the D to the Qa region of the MHC of the BALB/c mouse. We have previously shown (6) that low-copy probes, isolated from the 5′ flanking sequence of the D^d and from the 3′ flanking sequence of the L^d gene, map into the D region, while probes isolated from the 3′ flanking sequence of genes *D2* and *D3*, respectively, map into the *Qa* region. These results are in agreement with the molecular linkage map except for genes $D2^d$ and $D3^d$, which constituted cluster 6, previously localized (6) to the Qa region. We now know that the $D3^d$ gene probe 9 used by Winoto et al. (6), crosshybridizes strongly to the 3′ flanking sequence of gene *Q1* (Fig. 7), and therefore maps to the Qa region in the recombinant B6.K1 which, since it contains the D^b region (4), does not possess genes *D2*, *D3*, and *D4*. The localization of genes $D2^d$ and $D3^d$ between genes D^d and L^d also explains their deletion in the *dm1* and *dm2* mutant strains, presumably through unequal crossing-over between the D^d and L^d genes. Weiss et al. (28) have mapped the recombination event in B6.K1 that separates the D from the Qa region to a 40 kb segment between D^b and *Q1*. Thus, there are at least five class I genes in the D region of BALB/c and eight in the Qa region. The localization of the D^d gene proximal to L^d by cloning is in agreement with the gene order recently determined (53) by genetic recombination.

Two of the five D^d region class I genes code for the D^d and L^d molecules (46–48). Whether the $D2^d$, $D3^d$, and $D4^d$ class I genes are functional genes remains to be determined. The identification of three D^d region class I genes in addition to the D^d and L^d genes should readily permit their characterization to determine whether they encode any of the additional D^d region class I molecules, termed M^d, R^d, and $L2^d$ (22–24). Five D region class I molecules have also been identified (25) in mouse strains carrying the D^q region which, according to Southern blot analysis (Fig. 6), appears to have a similar gene organization as the D^d region.

Of the eight class I genes located in the Qa region of the BALB/c mouse, four, *Q6*, *Q7*, *Q8/9*, and *Q10*, have been carefully analyzed (54, 55) for their expression in L cells after gene transfer. None of them could be shown to encode a cell surface class I molecule (54, 55), in contrast to a previous study (16). The lack of cell surface expression of these four genes appears to be due to structural defects or regulator sequences in their 3′ regions (54, 55). Only the *Q8/9* gene, however, appears to be a truly nonfunctional gene, while the *Q6*, *Q7*, and *Q10* genes might encode secreted class I molecules or contain tissue-specific control elements preventing their stable cell surface expression in L cells (54, 55). Indeed, the *Q10* gene of C57BL/10 mice has been shown (17–19) to encode a liver-specific, secreted class I molecule. Moreover, all of the *Q6*, *Q7*, *Q8*, and *Q9* genes of the C57BL/10 mouse appear to encode Qa-2 serological determinants (30).

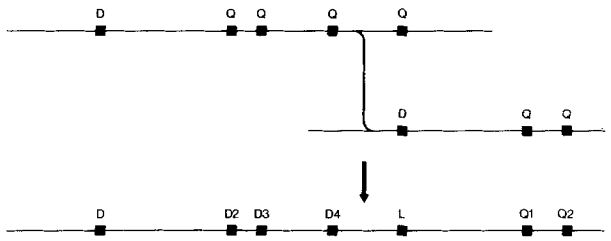


FIGURE 8. Proposed evolutionary scheme for the generation of the five D region class I genes of the BALB/c mouse by unequal crossing over. The placement of the recombination breakpoint is based on the sequence homologies between the 5′ flank of the D^d gene and the intergenic segment from genes $D4^d$ to L^d (compare with Figs. 5 and 7).

Evolution of D Region Class I Genes Occurs by Gene Expansion and Contraction. In addition to the accumulation of point mutations, at least three recombinational mechanisms contribute to the generation and maintenance of polymorphic DNA sequences in class I genes during evolution. First, gene conversion appears to be the primary genetic mechanism generating variability in the K^b gene and, presumably, other class I genes as well (56–59). Second, homologous and equal crossing-over events, occurring with a high frequency at certain sites termed recombinatorial hot spots, lead to relatively rapid shuffling of chromosomal segments of the MHC (8, 60). Recombination at hot spots therefore contributes to the generation of new MHC haplotypes and might be important for the maintenance of useful MHC alleles during evolution. Third, homologous and unequal crossover events apparently generate the class I gene duplications and deletions as seen in the Qa region (28, 30).

The homologies detected by crosshybridization of probes between the flanking sequences of genes $D2^d$, $D3^d$, $D4^d$ and the flanking sequences of the Qa region genes also suggest strongly that the organization of the D region class I genes in the BALB/c mouse is the result of unequal crossing-over. The unequal recombination event presumably occurred between two different unknown haplotypes in the 5′ flanking sequences of a D and a Qa region class I gene (perhaps *Q4*), generating an expanded chromosome with five class I genes in the D region, three of which were derived from the Qa region (Fig. 8). Clearly the restriction map and sequence homologies, as detected by crosshybridization between the flanking sequences of the D^d and L^d genes, are in agreement with this evolutionary pathway.

If the proposed evolutionary origin of the BALB/c D region genes is correct, then the presence of a single class I gene in mouse strains AKR and C57BL/10 reflects the older D region gene organization. However, it is of course also conceivable that some of the present-day strains, containing a single D region class I gene, originated from strains with multiple D region class I genes, through gene contraction. Apparently the *dm1* and *dm2* mutant strains were generated this way. The close homology that has been noted (61) between D^b and L^d class I genes might thus be due either to gene expansion, involving an $H\text{-}2^b$-like haplotype in the generation of the BALB/c D region (Fig. 8), or to gene contraction, in which case the D^b region would have originated from the D^d or a D^d-like region. It will be interesting to determine the sequence relationships

between the D^k and the D^d, L^d, and D^b genes (46–48, 61) to determine how the D^k gene might fit into the proposed evolutionary schemes.

It is reasonable to assume that the many examples of unequal crossing-over in the D and Qa regions arise from the close spacing, sequence homologies, and identical 5′ to 3′ orientation of the multiple class I genes in this part of the murine MHC.

Summary

Chromosome walking has been used to study the organization of the class I genes in the D and Qa regions of the MHC of the BALB/c mouse and in the D region of the AKR mouse. Five and eight class I genes are found in the D and Qa regions of the BALB/c mouse, respectively, while the AKR mouse contains only a single class I D region gene that has been identified by transfection as the D^k gene. Restriction map homologies and crosshybridization experiments suggest that the multiple class I genes in the D region of the BALB/c mouse have been generated by unequal crossing-over involving class I genes from the Qa region. The expanded D region of BALB/c and other *H-2*^{d} haplotype mouse strains appears to be metastable, since evidence for gene contraction in the D^d region has been found in two mutant strains. Thus the D region and also the Qa region class I genes are in a dynamic state, evolving by gene expansion and contraction.

We thank Drs. S. Carson and J. Kaufman for helpful comments, Ms. N. Bulbuc for technical assistance, and Ms. C. Plattner for preparation of the manuscript. L. Hood is supported by a grant from the National Institutes of Health, Bethesda, MD. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche, Basel, Switzerland.

Received for publication 18 December 1985.

References

1. Klein, J., F. Figueroa, and Z. A. Nagy. 1983. Genetics of the major histocompatibility complex: The final act. *Ann. Rev. Immunol.* 1:119.
2. Schwartz, R. H. 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann. Rev. Immunol.* 3:237.
3. Steinmetz, M., and L. Hood. 1983. Genes of the major histocompatibility complex in the mouse and man. *Science (Wash. DC).* 222:727.
4. Klein, J., F. Figueroa, and C. S. David. 1983. H-2 haplotypes, genes and antigens: Second listing. II. The H-2 complex. *Immunogenetics.* 17:553.
5. Steinmetz, M., A. Winoto, K. Minard, and L. Hood. 1982. Clusters of genes encoding mouse transplantation antigens. *Cell.* 28:489.
6. Winoto, A., M. Steinmetz, and L. Hood. 1983. Genetic mapping in the major histocompatibility complex by restricting enzyme site polymorphisms: Most mouse class I genes map to the Tla complex. *Proc. Natl. Acad. Sci. USA.* 80:3425.
7. Fisher, D. A., S. W. Hunt, III, and L. E. Hood. 1985. Structure of a gene encoding a murine thymus leukemia antigen and organization of Tla genes in the BALB/c mouse. *J. Exp. Med.* 162:528.
8. Steinmetz, M., D. Stephan, and K. Fischer Lindahl. 1986. Gene organizaton and recombinational hot spots in the murine major histocompatibility complex. *Cell.* In press.

9. Chaplin, D. D., D. E. Woods, A. S. Whitehead, G. Goldberger, H. R. Colten, and J. G. Seidman. 1983. Molecular map of the murine S region. *Proc. Natl. Acad. Sci USA.* 80:6947.
10. Amor, M., M. Tosi, C. Duponchel, M. Steinmetz, and T. Meo. 1985. Liver mRNA probes disclose two cytochrome P-450 genes duplicated in tandem with the complement C4 loci of the mouse H-2 S region. *Proc. Natl. Acad. Sci. USA.* 82:4453.
11. White, P. C., D. D. Chaplin, J. H. Weis, B. Dupont, M. I. New, and J. G. Seidman. 1984. Two steroid 21-hydroxylase genes are located in the murine S region. *Nature (Lond.).* 312:465.
12. Obata, Y., Y.-T. Chen, E. Stockert, and L. J. Old. 1985. Structural analysis of TL genes of the mouse. *Proc. Natl. Acad. Sci. USA.* 82:5475.
13. Chorney, M. J., J.-S. Tung, Y. Bushkin, and F.-W. Shen. 1985. Structural characteristics of Tla products. *J. Exp. Med.* 162:781.
14. Chen, Y.-T., Y. Obata, E. Stockert, and L. J. Old. 1985. Thymus-leukemia (TL) antigens of the mouse. Analysis of TL mRNA and TL cDNA from TL^+ and TL^- strains. *J. Exp. Med.* 162:1134.
15. Goodenow, R. S., M. McMillan, A. Örn, M. Nicolson, N. Davidson, J. A. Frelinger, and L. Hood. 1982. Identification of a BALB/c H-$2L^d$ gene by DNA-mediated gene transfer. *Science* (Wash. DC). 215:677.
16. Goodenow, R. S., M. McMillan, M. Nicolson, B. T. Sher, K. Eakle, N. Davidson, and L. Hood. 1982. Identification of the class I genes of the mouse major histocompatibility complex by DNA-mediated gene transfer. *Nature (Lond.).* 300:231.
17. Cosman, D., M. Kress, G. Khoury, and G. Jay. 1982. Tissue-specific expression of an unusual H-2 (class I)-related gene. *Proc. Natl. Acad. Sci. USA.* 79:4947.
18. Maloy, W. L., J. E. Coligan, Y. Barra, and G. Jay. 1984. Detection of a secreted form of the murine H-2 class I antigen with an antibody against its predicted carboxyl terminus. *Proc. Natl. Acad. Sci. USA.* 81:1216.
19. Mellor, A. L., E. H. Weiss, M. Kress, G. Jay, and R. A. Flavell. 1984. A nonpolymorphic class I gene in the murine major histocompatibility complex. *Cell.* 36:139.
20. Devlin, J. J., A. M. Lew, R. A. Flavell, and J. E. Coligan. 1985. Secretion of a soluble class I molecule encoded by the Q10 gene of the C57BL/10 mouse. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:369.
21. Barra, Y., K. Tanaka, K. J. Isselbacher, G. Khoury, and G. Jay. 1985. Stable transfer and restricted expression of a cloned class I gene encoding a secreted transplantation-like antigen. *Mol. Cell Biol.* 5:1295.
22. Hansen, T. H., K. Ozato, M. R. Melino, J. E. Coligan, T. J. Kindt, J. Jandinski, and D. H. Sachs. 1981. Immunochemical evidence in two haplotypes for at least three D region-encoded molecules, D, L, and R. *J. Immunol.* 126:1713.
23. Iványi, D., and P. Démant. 1979. Complex genetic effect of B10.D2(M504)(H-2^{dm1}) mutation. *Immunogenetics.* 8:539.
24. Iványi, D., and P. Démant. 1982. Molecular heterogeneity of D-end products detected by anti-H2.28 sera. II. B10.D2 (M504)(H-2^{dm1}) mutant fails to express one of the two H-2.4^-, H-2.28^+ D^d region molecules. *Immunogenetics.* 15:467.
25. Iványi, D., and P. Démant. 1984. Five serologically distinguishable D^q region molecules. *Immunogenetics.* 20:211.
26. Chatterjee-Das, S., S. K. Berg, and D. H. Sachs. 1985. Molecular and serologic analysis of products of the D region of H-2^q. *Transplant. Proc.* 17:722.
27. Lillehoj, E. P., N. B. Myers, D. R. Lee, T. H. Hansen, and J. E. Coligan. 1985. Structural definition of a family of L^d-like molecules distributed among four of seven haplotypes compared. *J. Immunol.* 135:1271.
28. Weiss, E. H., L. Golden, K. Fahrner, A. L. Mellor, J. J. Devlin, H. Bullman, H.

Tiddens, H. Bud, and R. A. Flavell. 1984. Organization of the class I gene family in the major histocompatibility complex of the C57BL/10 mouse. *Nature (Lond.)* 310:650.

29. Flaherty, L. 1981. IN: Role of the Major Histocompatibility Complex in Immunology. M. Dorf, editor. Garland Publishing Co., New York. 35–57.
30. Mellor, A. L., D. Antoniou, and P. J. Robinson. 1985. Structure and expression of genes encoding murine Qa-2 class I antigens. *Proc. Natl. Acad. Sci. USA.* 82:5920.
31. Grosveld, F. G., T. Lund, E. J. Murray, A. L. Mellor, H. H. M. Dahl, and R. A. Flavell. 1982. The construction of cosmid libraries which can be used to transform eukaryotic cells. *Nucleic Acids Res.* 10:6715.
32. Steinmetz, M., M. Malissen, L. Hood, A. Örn, R. A. Maki, G. R. Dastoornikoo, D. Stephan, E. Gibb, and R. Romaniuk. 1984. Tracts of high or low sequence divergence in the mouse major histocompatibility complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2995.
33. Frischauf A.-M., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. *J. Mol. Biol.* 170:827.
34. Steinmetz, M., D. Stephen, G. R. Dastoornikoo, E. Gibb, and R. Romaniuk. 1985. Methods in molecular immunology: Chromosomal walking in the major histocompatibility complex. *Immunol. Methods.* 3:1.
35. Feinberg, A. P., and B. Vogelstein. 1984. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266.
36. Sun, H., R. S. Goodenow, and L. Hood. 1985. Molecular basis of the dm1 mutation in the major histocompatibility complex of the mouse: A D/L hybrid gene. *J. Exp. Med.* 162:1588.
37. Wallich, R., N. Bulbuc, G. J. Hämmerling, S. Katzav, S. Segal, and M. Feldman. 1985. Abrogation of metastatic properties of tumour cells by *de novo* expression of H-2K antigens following H-2 gene transfection. *Nature (Lond.).* 315:301.
38. Colbère-Garapin, F., F. Horodniceanu, P. Kourilsky, and A.-C. Garapin. 1980. A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* 150:1.
39. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance and with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327.
40. Lemke, H., G. J. Hämmerling, and U. Hämmerling. 1979. Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TL region in mice. *Immunol. Rev.* 47:175.
41. Hämmerling, G. J., E. Rüsch, N. Tada, S. Kimura, and U. Hämmerling. 1982. Localization of allodeterminants on H-2K^b antigens determined with monoclonal antibodies and H-2 mutant mice. *Proc. Natl. Acad. Sci. USA.* 79:4737.
42. Koch, S., N. Koch, P. Robinson, and G. J. Hämmerling. 1983. Comparison of allo- and xenogeneic determinants on the H-2K^k molecule. *Transplantation (Baltimore).* 36:177.
43. Ozato, K., N. Mayer, and D. Sachs. 1980. Hybridoma cell line secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
44. Rogers, J. H. 1985. Family organization of mouse H-2 class I genes. *Immunogenetics.* 21:343.
45. Steinmetz, M., K. W. Moore, J. G. Frelinger, B. T. Sher, F.-W. Shen, E. A. Boyse, and L. Hood. 1981. A pseudogene homologous to mouse transplantation antigens: transplantation antigens are encoded by eight exons that correlate with protein domains. *Cell.* 25:683.

46. Sher, B. T., R. Nairn, J. E. Coligan, and L. Hood. 1984. DNA sequence of the mouse H-2D^d transplantation gene. *Proc. Natl. Acad. Sci. USA.* 82:1175.
47. Moore, K. W., B. T. Sher, Y. H. Sun, K. A. Eakle, and L. Hood. 1982. DNA sequence of a gene encoding a BALB/c mouse L^d transplantation antigen. *Science (Wash. DC).* 215:679.
48. Evans, G. A., D. H. Margulies, R. D. Camerini-Otero, K. Ozato, and J. G. Seidman. 1982. Structure and expression of a mouse major histocompatibility antigen gene, H-2L^d. *Proc. Natl. Acad. Sci. USA.* 79:1994.
49. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
50. Melvold, R. W., M. S. Sandrin, and I. F. C. McKenzie. 1982. The C.B6-H-2^{dm5} mutant. *Immunogenetics.* 15:331.
51. Burnside, S. S., P. Hunt, K. Ozato, and D. W. Sears. 1984. A molecular hybrid of the H-2D^d and H-2L^d genes expressed in the dm1 mutant. *Proc. Natl. Acad. Sci. USA.* 81:5204.
52. Hansen, T. H., S. E. Cullen, R. Melvold, H. I. Kohn, L. Flaherty, and D. H. Sachs. 1977. Mutation in a new H-2 associated histocompatibility gene closely linked to H-2D. *J. Exp. Med.* 145:1550.
53. Savarirayan, S., W. P. Lafuse, and C. S. David. 1985. Recombination between H-2D and H-2L genes: Identification, characterization, and gene order. *Transplant. Proc.* 17:702.
54. Stroynowski, I., J. Forman, R. S. Goodenow, S. G. Schiffer, M. McMillan, S. O. Sharrow, D. H. Sachs, and L. Hood. 1985. Expression and T cell recognition of hybrid antigens with amino-terminal domains encoded by Qa-2 region of major histocompatibility complex and carboxyl termini of transplantation antigens. *J. Exp. Med.* 161:935.
55. Straus, D. S., I. Stroynowski, S. G. Schiffer, and L. Hood. 1985. Expression of hybrid class I genes of the major histocompatibility complex in mouse L cells. *Proc. Natl. Acad. Sci. USA.* 82:6245.
56. Weiss, E. H., A. Mellor, L. Golden, K. Fahrner, E. Simpson, J. Hurst, and R. A. Flavell. 1983. The structure of a mutant H-2 gene suggests that the generation of polymorphism in H-2 genes may occur by gene conversion-like events. *Nature (Lond.).* 301:671.
57. Mellor, A. L., E. H. Weiss, K. Ramachandran, and R. A. Flavell. 1983. A potential donor gene for the bm1 gene conversion event in the C57BL mouse. *Nature (Lond.).* 306:782.
58. Pease, L. R., D. H. Schulze, G. M. Pfaffenbach, and S. G. Nathenson. 1983. Spontaneous H-2 mutants provide evidence that a copy mechanism analogous to gene conversion generates polymorphism in the major histocompatibility complex. *Proc. Natl. Acad. Sci. USA.* 80:242.
59. Schulze, D. H., L. R. Pease, S. S. Geier, A. A. Reyes, L. A. Sarmiento, R. B. Wallace, and S. G. Nathenson. 1983. Comparison of the cloned H-2K^{bm1} variant gene with the H-2K^b gene shows a cluster of seven nucleotide differences. *Proc. Natl. Acad. Sci. USA.* 80:2007.
60. Steinmetz, M., K. Minard, S. Horvath, J. McNicholas, J. Frelinger, C. Wake, E. Long, B. Mach, and L. Hood. 1982. A molecular map of the immune response region from the major histocompatibility complex of the mouse. *Nature (Lond.).* 300:35.
61. Reyes, A. A., M. Schöld, and R. B. Wallace. 1982. The complete amino acid sequence of the murine transplantation antigen H-2D^b as deduced by molecular cloning. *Immunogenetics.* 16:1.