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Immunology

Primary structural studies of an H-2L molecule confirm that it is a unique gene product with homology to H-2K and H-2D antigens

(histocompatibility antigens/radiolabeling/immunoprecipitation/amino acid sequence)

JOHN E. COLIGAN*, THOMAS J. KINDT*, RODERICK NAIRN†, STANLEY G. NATHENSON†, DAVID H. SACHS‡, AND TED H. HANSEN‡§

*Laboratory of Immunogenetics, Building 8, Room 100, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205; †Departments of Microbiology and Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461; and ‡Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Insitutes of Health, Bethesda, Maryland 20205

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ABSTRACT **Radiochemical methodology has been used in the isolation and preliminary biochemical characterization of the murine *H-2L*^{d} major histocompatibility complex gene product. The radiolabeled molecule was isolated by immunoprecipitation from the glycoprotein fraction of detergent-solubilized *H-2*^{d} tumor cells. Six major CNBr fragments were isolated from a papain fragment of this molecule; three of the fragments are connected by disulfide bonds. Due to the high degree of homology between major transplantation antigens, it was possible to align the fragments by comparison of their amino acid sequences to that of the *H-2K*^{b} gene product. Of the positions available for comparison between *H-2L*^{d} and *H-2K*^{b}, *H-2D*^{d}, and *H-2K*^{d} gene products, 61 out of 80 (78%), 45 out of 55 (82%), and 12 out of 15 (80%), respectively, are identical. Differences between the L^d and K^b and D^d molecules are distributed throughout the amino acid sequence. These data indicate that the *H-2L*^{d} gene product is a molecular species distinct from, but homologous to, the *H-2K* and *H-2D* gene products.**

The major histocompatibility complex contains genes encoding products involved in a diversity of immunologic phenomona, including allograft rejections, humoral immune responses, and complement activity (1, 2). Homologous gene complexes have been described in several species; the most thoroughly characterized are the *H-2* gene complex of mice and the *HLA* gene complex of humans. The serologically detected products that originally defined the major histocompatibility complex are the classical transplantation antigens, cell surface glycoproteins that determine the fate of allogeneic tumor or tissue transplants (1, 2). Classical transplantation antigens might also have an important role in host immune surveillance, as suggested by cellular cytotoxicity studies with chemically modified (3) and virus-infected (4) syngeneic cells.

The *K* and *D* loci encoding the classical transplantation antigens have been genetically mapped to the major histocompatibility region of the murine 17th chromosome and the *HLA-A, B,* and *C* loci have been mapped to the human 6th chromosome. A striking feature of the *H-2K/D* and *HLA-A/B/C* loci is their extremely high level of genetic polymorphism, as demonstrated by serological and functional assays and by chemical characterizations (1, 2). Furthermore, these studies indicate that despite their polymorphic nature, the *H-2K* and *H-2D* genes. as well as the *HLA-A, B,* and *C* genes, encode structurally homologous molecules. These observations suggest that these major histocompatibility loci arose from a common primordial gene by gene duplication (5, 6).

Recent cocapping (7) and immunochemical (8, 9) studies have shown that at least certain *H-2* haplotypes possess a third locus coding for classical transplantation antigens in addition to *H-2K* and *H-2D*, and this locus has been termed *H-2L*. Because the *H-2D* and *H-2L* loci have not been separated by an observed recombination, they are probably very tightly linked. The L molecule has been shown to be similar to the K and D molecules by serologic testing (10), polyacrylamide gel electrophoresis of immunoprecipitates (8, 9), and analysis of cytotoxic T-cell responses (11–14). However, serologic (15) and T-cell functional differences (11, 13) have also been reported for the L molecule in comparison to K and D molecules.

The structural studies reported here were undertaken to determine whether the L molecule is the product of a separate locus (loci) and, if so, whether this molecule has a primary structure similar to the previously characterized K and D molecules. Furthermore, it was hoped to gain insights into possible evolutionary relationships between the *H-2L*, *H-2K*, and *H-2D* genes.

MATERIALS AND METHODS

Antiserum. The alloantiserum used to isolate the H-2L^d glycoprotein was prepared by immunizing mice of the mutant strain BALB/c-*H-2*^{dm2} (16) with tissues from BALB/c mice, the wild-type strain. A detailed description of this procedure and the characterization of this anti-L^d serum has been published elsewhere (10).

Preparation of Radiolabeled H-2 Antigens. Radiolabeled amino acids were incorporated by a minor modification (R. Nairn and S. G. Nathenson, unpublished data) of previously published techniques (17). In essence, *H-2*^{d} tumor cells (C14 line, obtained from Sloan-Kettering Institute, New York, NY) in rapid growth phase were harvested by low-speed centrifugation at room temperature and resuspended in labeling medium consisting of a group of radiolabeled amino acids (in this case, either Phe, Ile, Val, Arg, and Tyr or His, Ile, Thr, Val, Trp, and Phe) in Dulbecco's modified Eagle's medium lacking these amino acids but containing 10% fetal calf serum, 1% glutamine, and antibiotics. Cells were cultured for 8 hr at 37°C in a humidified atmosphere of 5% CO_2/95% air, harvested by low-speed centrifugation at room temperature, washed, and then extracted with 0.5% Nonidet P-40 (Shell Chemical). Nuclei and other cell debris were removed by centrifugation ($10^5 \times g$, 90 min) and the supernatant was retained for isolation of the H-2L antigen.

Isolation of H-2L^d Glycoprotein. The H-2L^d glycoprotein was isolated by indirect immune precipitation of lentil-lectin-purified Nonidet P-40 extracts of cells, followed by papain digestion and subsequent Sephadex G-75 column chromatography as described (18). For the glycoprotein extract labeled with tritiated His, Ile, Thr, Val, Trp, and Phe, the H-2D^d alloantigen had been removed for other studies by indirect im-

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Abbreviation: Gdn·HCl, guanidine hydrochloride.

§ Present address: Immunology Department, Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, NJ 07065.

mune preciptation prior to the precipitation of the H-2L^d molecule.

Cleavage with CNBr. The lyophilized, radiolabeled H-2L^d glycoprotein and carrier protein (mouse and goat immunoglobulins remaining from immunoprecipitation) were dissolved in 70% HCOOH at 10 mg/ml and treated with a 25-fold excess (wt/wt) of CNBr (Eastman Kodak, Rochester, NY) in the dark for 1 hr at room temperature and for 23 hr at 4°C. The reaction mixture was diluted with a 10-fold (vol/vol) excess of distilled H_2O and freeze-dried.

Reduction and Alkylation. Disulfide bonds were reduced by dissolving CNBr fragments containing 2 mg of horse cytochrome *c* as a carrier protein in 3 ml of 6 M guanidine hydrochloride (Gdn·HCl)/0.3 M Tris·HCl/1 mM EDTA/0.1 M dithiothreitol under N_2 for 3 hr at 37°C. The Cys residues were alkylated in the dark at 20°C by adding solid iodoacetamide to a final concentration of 0.25 M and adjusting the pH to 8.5. After 20 min, the reaction was halted by adding 2-mercaptoethanol to a final concentration of 0.75 M.

Desalting of Peptides. All material pooled from columns equilibrated in 6 M Gdn·HCl were desalted on a Sephadex G-15 column (40 × 2 cm) in 2 M HCOOH and were subsequently lyophilized. Recovery of peptides ranged from 80 to 100%.

Automated Amino Acid Sequence Analysis. Details of the radiochemical amino acid sequencing methodology have been published (19, 20). Radioactive phenylthiohydantoin-amino acids were identified by cochromatography with unlabeled standard phenylthiohydantoin amino acid derivatives on a high-pressure liquid chromatograph, with the isocratic elution conditions of Gates *et al.* (21). The peaks were collected and the radiolabeled phenylthiohydantoin amino acid residue was identified by liquid scintillation counting of the freeze-dried samples in 5.0 ml of Biofluor (New England Nuclear).

RESULTS

Isolation of Papain Fragments. Fig. 1 shows the Sephadex G-75 column profile of the H-2L^d molecule after treatment with papain. Earlier studies (22) on the release of H-2 molecules from intact cells revealed that the H-2 antigenic activity resided in two large fragments having M_r similar to those estimated for Pap. I ($M_r \approx 37{,}000$) and Pap. II ($M_r \approx 28{,}000$) in Fig. 1. Papain digestion of immunoprecipitates of radiolabeled H-2K^b molecules generates two similar fragments that differ from intact

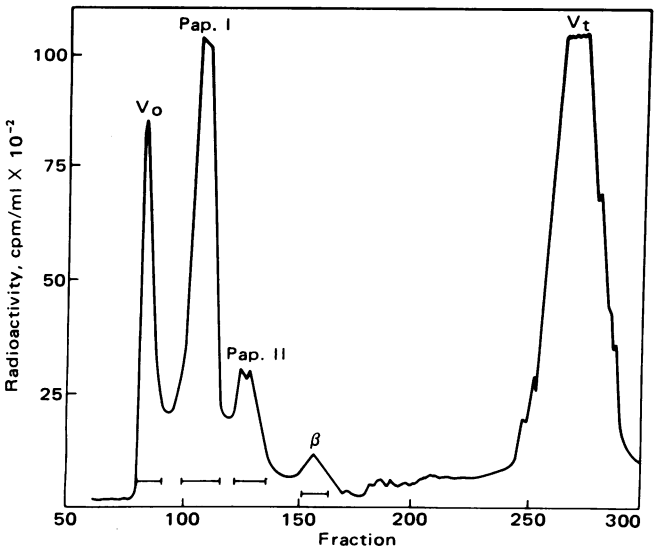

FIG. 1. Chromatography of papain-digested H-2L^d labeled with tritiated Phe, Ile, Val, Arg, and Tyr on Sephadex G-75 (190 × 2 cm) in 1.0 M HCOOH. Fraction size was 2.4 ml; flow rate was 15 ml/hr.

H-2K^b molecules by missing portions of the COOH terminus of the molecule (18, 23). The papain fragments elute from the column well separated from both the undigested material near the excluded volume (V_0) and from that in pool β. This latter material elutes in a position identical to that of β_2-microglobulin, which has been isolated on the same column after papain treatment of radiolabeled H-2D^d. However, the yield of pool β relative to the yield of the papain fragment is only approximately 15% of the yield of the β_2-microglobulin isolated from the H-2D^d immunoprecipitate. The large peak of radioactivity eluting at the column volume (V_t) is due to the presence of small peptides created by the digestion.

Isolation of CNBr Fragments. After CNBr digestion, the Pap. I material was chromatographed on Sephacryl S-200 in 6 M Gdn·HCl, and fractions were pooled as indicated (Fig. 2A). From previous experience with other H-2 molecules—namely, H-2K^b (24), H-2D^d (R. Nairn, S. G. Nathenson, and J. E. Coligan, unpublished data), and H-2D^b (W. L. Maloy, J. M. Martinko, and J. E. Coligan, unpublished data)—pool L1 was thought to consist of several peptides linked by disulfide bonds. Pools L3, L4, and L5 appeared to be pure peptides when examined by automated amino acid sequence analysis and are discussed further in subsequent sections. Essentially identical chromatography patterns were seen whether the H-2L^d mol-

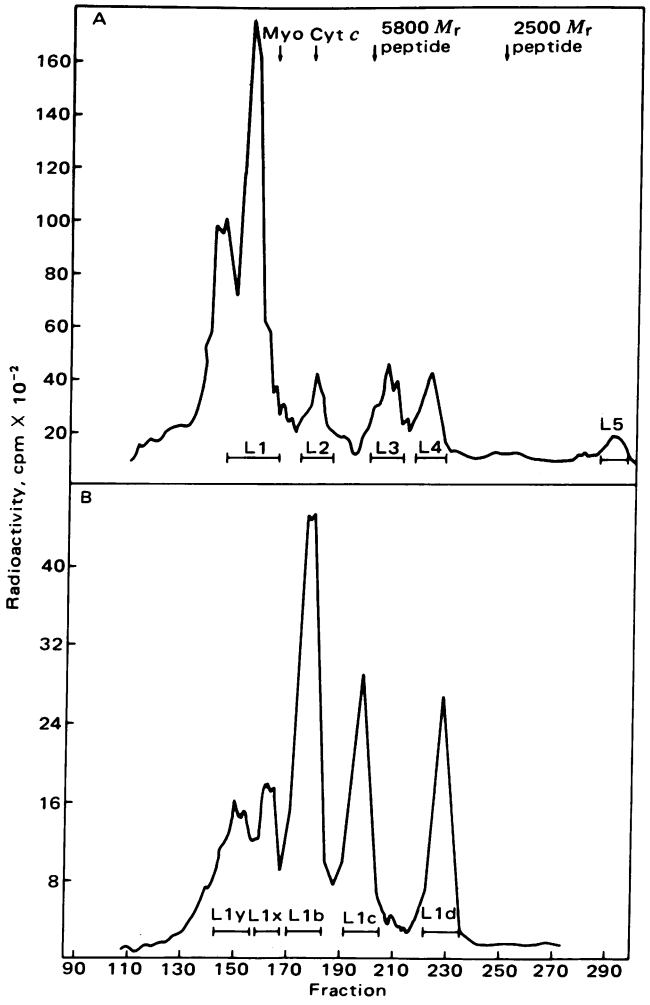

FIG. 2. (*A*) Chromatography of CNBr fragments from H-2L^d Pap. I labeled with tritiated His, Ile, Thr, Val, Trp, and Phe on a Sephacryl S-200 column (193 × 2 cm) in 6 M Gdn·HCl. The fraction size was 2.0 ml and the flow rate was 18 ml/hr. (B) Rechromatography of pool L1 after reduction and alkylation. M_r markers are sperm whale myoglobin (Myo), horse cytochrome *c* (Cyt *c*), and peptides of known M_r from H-2K^b (24).

Table 1. NH_2-Terminal amino acid sequences of radiolabeled peptides from H-2L^d

Peptide	Sequencer step	Amino acid	Peptide	Sequencer step	Amino acid
Pap. II	3	His	L5	3	His
	6	Arg	L1b	4	Ile
	7	Tyr		5	Thr
	8	Phe		6	Arg
	10	Thr		7	Arg
	12	Val		9	Trp
	14	Arg		17	Tyr
	21	Arg		18	Tyr
	22	Tyr		19	Arg
	23	Ile		21	Tyr
	25	Val		27	Val
	27	Tyr		29	Trp
	28	Val		31	His
	33	Phe		32	(Arg)
	34	Val		33	(Tyr)
	35	Arg	L1c	3	Val
	36	Phe		5	Thr
	44	Arg		6	Arg
	45	Tyr		12	Thr
L2	1	Arg		13	Phe
	2	Tyr		16	Trp
	3	Phe		19	Val
	7	Val		20	Val
	9	(Arg)		21	Val
	16	(Arg)		29	Tyr
	17	Tyr		30	Thr
L3	7	Tyr		33	Val
	8	Trp		34	Tyr
	10	Arg		35	His
	11	Ile	L1d	1	Tyr
	12	Thr		5	Val
	14	Ile		10	Arg
	21	Trp		13	Arg
	22	Phe		15	Tyr
	23	(Arg)		18	Phe
	24	Val		20	Tyr
	27	(Arg)		25	Tyr
	28	Thr		26	Ile
	41	His		34	(Thr)
L4	1	Arg		35	Trp
	2	Tyr			
	3	Phe			
	5	Thr			
	7	Val			
	9	Arg			
	16	Arg			
	17	Tyr			
	18	Ile			
	20	Val			
	22	Tyr			
	23	Val			
	28	Phe			
	29	Val			
	30	Arg			
	31	Phe			

Parentheses indicate that the radioactivity was less than 10 cpm above background for this residue when measured after fractionation by high-pressure liquid chromatography.

ecule was labeled with tritiated Phe, Ile, Val, Arg, and Tyr or with His, Ile, Thr, Val, Trp, and Phe. This was also true for chromatographic patterns of L1 after reduction and alkylation (see below).

Gel Filtration of Reduced and Carboxamidomethylated CNBr Fragments of Pool L1. The reduced and carboxamidomethylated components of pool L1 were separated by rechromatography on Sephacryl S-200 in 6 M Gdn·HCl. (Fig. 2*B*). Pools L1x and L1y consisted of mixtures of peptides of M_r greater than 17,000, as indicated by their elution volumes. Examination of similar high M_r peaks obtained from cleavage of other H-2 molecules has shown them to consist mainly of CNBr partial cleavage products (ref. 24; J. E. Coligan and R. Nairn, unpublished data). Automated amino acid sequence analysis of pools L1b, L1c, and L1d indicated that each contained a single peptide (Table 1).

NH_2-Terminal Amino Acid Sequence and Alignment of Peptides. Table 1 lists the amino acid residues determined for the amino terminus of H-2L^d (Pap. II fragment) and for each of the CNBr fragments. All radiolabeled phenylthiohydantoin-amino acids were identified by high-pressure liquid chromatography. After correction for the relative specific activities of the radiolabeled amino acids, calculation of repetitive yields for the automated amino acid sequence analysis yielded values of 90–93%. Fig. 3 depicts the postulated alignment of the CNBr fragments based on homology to H-2K^b as shown in Fig. 4. The locations of the Met residues as predicted by CNBr cleavage points in H-2L^d differ from H-2K^b in their presence at positions 5 and 98 and in the absence of a Met residue at position 23. However, the Met residue at position 5 is present in both HLA-A2 and B7 (27), and the one at position 98 has been observed in both HLA antigens and all other major histocompatibility complex antigens of the mouse *b* and *d* haplotypes (ref. 24; J. E. Coligan, W. L. Maloy, R. Nairn, and E. S. Kimball, unpublished data).

The M_r, NH_2-terminal sequence, and low yield relative to the smaller fragments (Fig. 2*A*) support the conclusion that fragment L2 is derived from failure to obtain complete cleavage of the Met at position 52 during CNBr treatment. Although they are approximately the same length, peptides L3 and L4 are resolved on Sephacryl S-200 because peptide L3 probably possesses a carbohydrate moiety as predicted by analogy to H-2K^b (19) and HLA-A2 and B7 (27).

NH_2-Terminal amino acid sequence analysis (12 degradation cycles) of pool β indicated that it contained several peptides, one of which appeared to have an amino-terminal sequence like that reported for murine β_2-microglobulin (28). The low yield of this pool prevented any significant further investigation.

Homology to Other H-2 Alloantigens. Of the positions available for comparison between H-2K^b and H-2L^d, which include positions where Met is predicted in H-2L^d, 62 out of 80 (78%) are identical. Positions that possess different residues are indicated by italics in H-2K^b in Fig. 4. This degree of homology is similar to that observed in comparisons of NH_2-terminal sequences of H-2 molecules (Fig. 5).

Recent sequence information obtained for the H-2D^d molecule (R. Nairn, S. G. Nathenson, and J. E. Coligan, unpublished data) allows comparison to the H-2L^d molecule at 55 positions. Of those positions, 10 (18%) are different between the molecules (the positions of difference are indicated by the alternative H-2D^d residues in Fig. 4). In many cases where differences in amino acid sequence occur between the H-2L^d and H-2D^d molecules, the alternative residue is not known because the appropriate radiolabeled amino acids have not been incorporated into each of the molecules. The magnitude of the difference, as well as the distribution of these amino acid differences, observed thus far between H-2L^d and H-2D^d is similar to that between H-2L^d and H-2K^b.

NH_2-Terminal amino acid sequence data for H-2K^d allow comparison to the H-2L^d at 15 positions. Of these positions, 3 (20%) are different, as shown in Fig. 5.

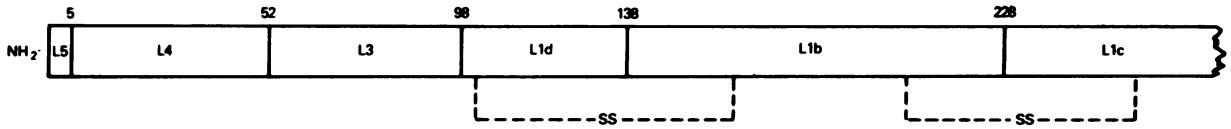

FIG. 3. Proposed alignment of the major CNBr fragments from H-2L^d based on homology to H-2K^b (see Fig. 4). The numbers indicate the proposed locations of Met residues. L1b, L1c, and L1d are connected by disulfide bridges (SS), and the proposed location of these bridges is also based on homology to H-2K^b (J. M. Martinko and S. G. Nathenson, unpublished data).

DISCUSSION

The partial amino acid sequence of the L^d molecule shows striking homology to other murine transplantation antigens (Figs. 4 and 5). For example, the L^d molecule has the identical residue at 78%, 82%, and 80% of the analyzed positions when compared with K^b, D^d, and K^d molecules, respectively. Therefore, these comparisons of primary structure substantiate the classification of L^d as an H-2 molecule in the same category as the K and D molecules, the classical transplantation antigens of mice.

In spite of similarity in the structures of their products, the loci coding for the murine major transplantation antigens exhibit extensive polymorphism. Serologic studies of H-2 antigens have shown that each K or D molecule of every haplotype of independent origin determines a unique combination of alloantigenic specificities (1). Structural studies, including both amino acid sequence data (summarized in Figs. 4 and 5) and peptide mapping comparisons (17, 35), have confirmed this high degree of polymorphism by demonstrating that each H-2K and H-2D allelic product has a characteristic primary structure. For the limited amino acid sequence data thus far available, the number of sequence differences between different K alleles is similar to the number of differences between K alleles and D alleles (i.e., there is no evidence for "K-ness" or "D-ness").

The studies reported here indicate that the L^d molecule is likewise distinct, showing sequence differences from both K^d and D^d. Ten of 55 positions where the amino acid has been identified distinguish L^d from D^d molecules and 3 out of 15 distinguish L^d from K^d molecules. Therefore each of the three *H-2* gene products, (K^d, D^d, and L^d), all synthesized by the same cell, has a unique primary structure and each is thus undoubtedly the product of a separate locus. Furthermore, comparison of the NH$_2$-terminal sequence of H-2L^d to limited NH$_2$-terminal data reported for other H-2 molecules indicates that the H-2L^d amino acid sequence differs from all others except that of H-2D^q and H-2D^b (Fig. 5). Although the available data permit direct comparison at only 15 positions, the similarity between the L^d and D^q molecules supports previous serological (36) and peptide map comparsions (S. Rose, T. H. Hansen, and S. E. Cullen, unpublished data) which also found L^d and D^q molecules similar to one another and quite different from D^d molecules. Sears and Polizzi (37) have also shown peptide map differences between the L^d and D^d molecules.

Because the amino acid sequence of L^d is no more like that of D^d than it is like any other previously described H-2 molecule, the *H-2L* gene was probably not the result of a recent duplication of the *H-2D^d* gene. On the other hand, the *H-2L^d* gene could have resulted from a recent duplication in another haplotype and *H-2^d* could have acquired *H-2L* from this other haplotype by recombination. For example, such a recent du-

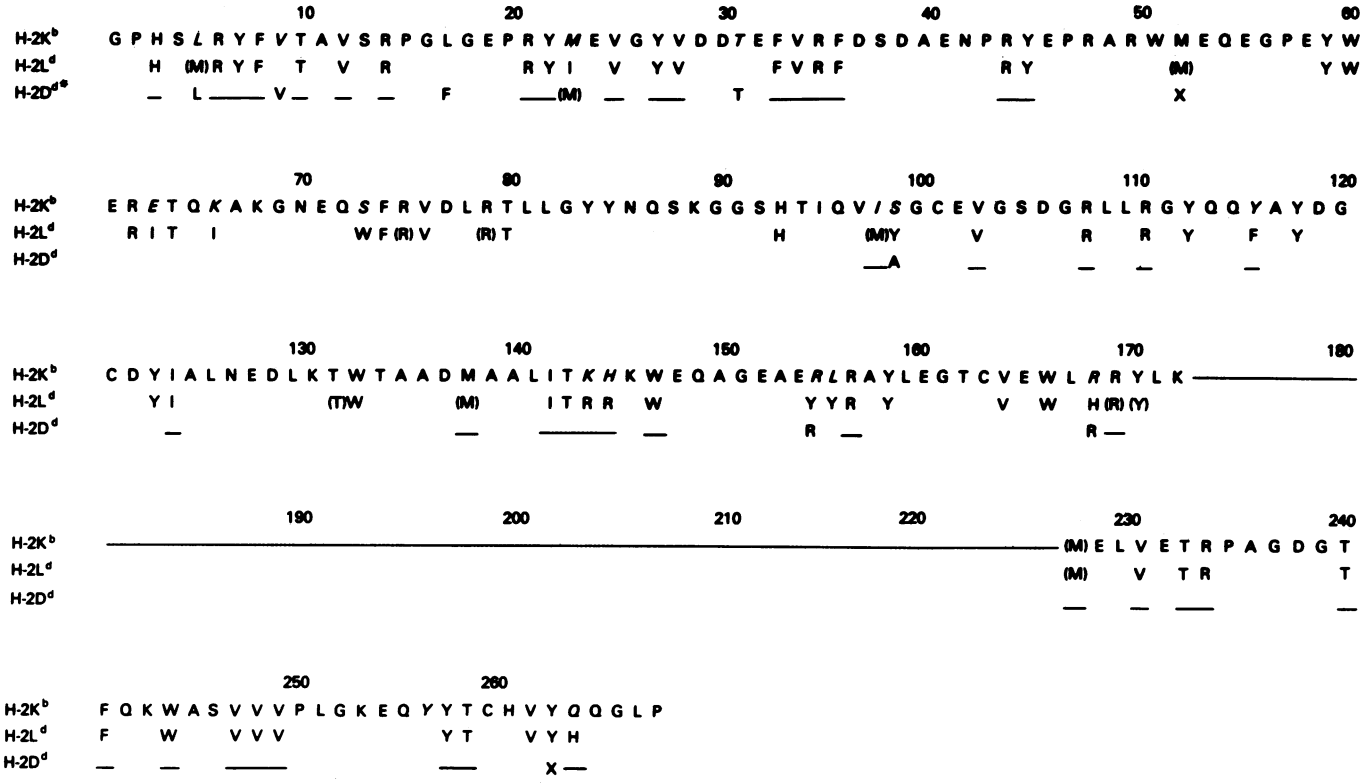

FIG. 4. Alignment of H-2L^d CNBr peptides by homology to H-2K^b and comparison of the H-2L^d sequence with those of H-2K^b and H-2D^d. The H-2K^b sequence is from refs. 20 and 25; the H-2D^d sequence is from R. Nairn, S. G. Nathenson, and J. E. Coligan (unpublished data). The NH$_2$-terminal amino acid sequence was determined by amino acid sequence analysis of Pap. II. The standard one-letter symbols (26) for amino acids are used. Asterisk, the H-2D^d sequence is compared to H-2L^d with dashes indicating homology and letters differences.

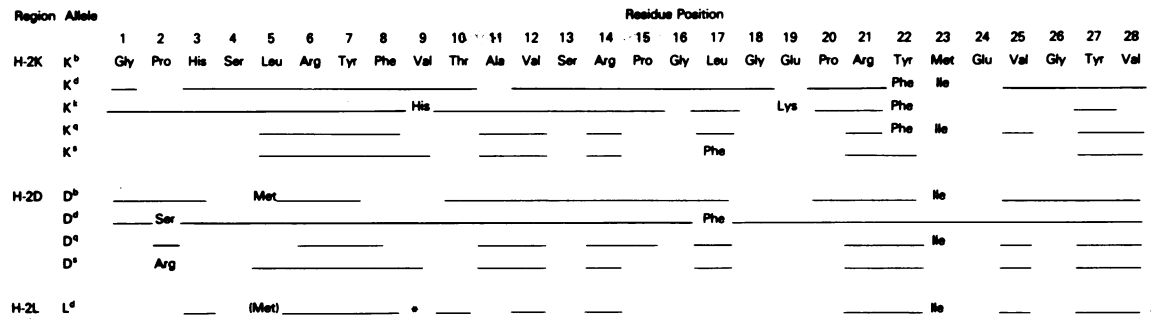

FIG. 5. Partial amino acid sequences of murine transplantation antigens. Data included are from ref. 19 for K^b; ref. 29 for K^d, K^k, and D^b; ref. 30 for K^k and D^b; ref. 18 for D^b; ref. 31 for K^d; ref. 32 for K^k; R. Nairn, S. G. Nathenson, and J. E. Coligan (unpublished data) for D^d; E. S. Kimball, J. E. Coligan, and S. G. Nathenson (unpublished data) for K^d; and W. L. Maloy, S. G. Nathenson, and J. E. Coligan (unpublished data) for D^b; ref. 33 for K^q; and ref. 34 for K^q, K^s, D^q, and D^s. Asterisk, residue is not a Val or His.

plication might have occurred in the *H-2*^{q} haplotype, which would explain the similarities found between the L^d and D^q molecules (see above).

Another possible model to explain the existence of *H-2L* is that the required duplications occurred before speciation, and that all species have three major histocompatibility loci. By this model, *H-2L* might be considered the homologue of *HLA-C*. On the other hand, the finding that K, D, and L molecules are more similar to each other in primary structure and antigenic specificity (38, 39) than they are to the HLA A or B antigens (see ref. 27 for amino acid sequences of HLA-A2 and HLA-B7) would suggest that this duplication either occurred independently in these two species or that there has been parallel (i.e., nonrandom) evolution of subloci in each species. More primary structural data and further information concerning the selective forces influencing evolution of histocompatibility antigens will be needed in order to distinguish between these models. Whichever model proves correct, the amino acid sequence differences demonstrated among H-2L^d, H-2K^d, and H-2D^d verify that the H-2L^d molecule is the product of a third murine locus encoding major transplantation antigens.

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