

## cDNA clone coding for part of a mouse H-2<sup>d</sup> major histocompatibility antigen

(hybrid selection/membrane protein)

S. KVIST\*, F. BREGEGERE†, L. RASK†‡, B. CAMI†, H. GAROFF\*, F. DANIEL†, K. WIMAN§, D. LARHAMMAR§, J. P. ABASTADO†, G. GACHELIN†, P. A. PETERSON§, B. DOBBERSTEIN\*, AND P. KOURILSKY†

\*European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, 6900 Heidelberg, Federal Republic of Germany; †Unite de Biologie Moleculaire du Gene, Equipe de Recherche, Centre National de la Recherche Scientifique 201 and Sous-Contrat National Institut National de la Santé et de la Recherche Médicale 20, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris, France; and §Department of Cell Research, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

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**ABSTRACT** mRNA coding for mouse major transplantation antigens of the *d* haplotype was partially purified, copied into double-stranded cDNA, and cloned in *Escherichia coli*. Clones were selected by their ability to hybridize specifically with mRNA coding for H-2K, D, or L antigens. One of these clones, pH-2<sup>d</sup>-1, carries a 1200-base-pair insert, comprising the noncoding region, including poly(A) at the 3' end and part of the coding region. A partial sequence of the latter region showed extensive homology with the known amino acid sequences of H-2K<sup>b</sup>, K<sup>k</sup>, and HLA-B7 antigens. From this comparison, it appears that the coding region extends from amino acid 133 in the second domain, through the third domain, to the cytoplasmic COOH-terminal region. A stretch of 24 hydrophobic or uncharged residues, located 31 amino acids from the COOH-terminal end, could represent the segment that spans the membrane. This is followed on the cytoplasmic side of the membrane by a cluster of basic amino acids and a possible phosphorylation site on a threonine residue.

The major histocompatibility complex of the mouse, termed H-2, is located on chromosome 17 (for review, see ref. 1). This complex governs the expression of several structurally distinct classes of molecules, including cell surface proteins and secretory proteins (2). A variety of immune phenomena, such as graft rejection, antibody production, and interactions among different subpopulations of lymphocytes, are associated with these antigens. The classical transplantation antigens (H-2K, D, and L) are cell surface glycoproteins that span the membrane. They comprise a heavy chain ( $M_r$  44,000) noncovalently linked to  $\beta_2$ -microglobulin ( $M_r$  12,000) (3, 4). The latter is not coded by the major histocompatibility complex (4). On a genetic level, the H-2K, D, and L antigens are represented by distinct loci separated by unknown lengths of DNA containing the loci for at least two other classes of antigens.

One of the most remarkable properties of the H-2 loci is their extensive polymorphism. Based on serological differences among the antigens, it has been possible to define  $\approx 50$  different alleles at each of the K and D loci (2). Limited amino acid sequences of antigens from different loci and alleles suggest that the polymorphism, at least in part, can be explained by differences in the primary structure (5-9). Extensive protein sequence determination of products from several loci and alleles would most likely show where in the molecules the differences are located. This, in turn, could give clues to the functional implication of the polymorphism. However, extensive protein sequence determination has been hampered by the scarcity of the antigens.

This problem can be overcome by cloning DNA sequences coding for H-2 antigens. A similar approach has already been used for HLA antigens (10), the human counterpart to H-2.

We report here the isolation and characterization of a recombinant plasmid carrying a DNA sequence complementary to a mRNA coding for an H-2<sup>d</sup> histocompatibility antigen. The identification of this clone is based on extensive homology with known amino acid sequence data of mouse and human histocompatibility antigens.

### MATERIALS AND METHODS

**Isolation of mRNA.** SL2 lymphoma cells were grown in DBA/2 mice (Bomholtgaard, Denmark) and harvested as described (11). The cells were washed in ice-cold 50 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM MgCl<sub>2</sub> containing cycloheximide at 100  $\mu$ g/ml and broken by using a Dounce homogenizer in 20 mM Tris·HCl, pH 7.5/10 mM KCl/5 mM MgCl<sub>2</sub> containing cycloheximide at 100  $\mu$ g/ml. An equal volume of 20 mM Tris·HCl, pH 7.5/300 mM KCl/5 mM MgCl<sub>2</sub> was then added. Cell debris and nuclei were removed by centrifugation at 5000  $\times g$  for 10 min. From the resulting supernatant, crude rough microsomes were pelleted by centrifugation for 1 hr at 30,000  $\times g$  in a Sorvall SS34 rotor. The pellet was suspended in 50 mM Tris·HCl, pH 7.5/120 mM NaCl/5 mM EDTA/2% NaDodSO<sub>4</sub> (wt/vol). RNA was extracted, and polyadenylated RNA was purified over oligo(dT)-cellulose as described (11, 12). mRNA coding for H-2 antigens was further enriched by centrifugation in an 8-20% aqueous sucrose gradient (11) for 12 hr at 40,000 rpm in a Beckman SW 41 rotor at 20°C. Fractions containing mRNA coding for H-2 antigens were identified by cell-free translation and immunoprecipitation as described (11). These enriched fractions were pooled and used for all subsequent experiments.

**Construction of cDNA Clones.** The enriched mRNA fraction was transcribed into cDNA with avian myeloblastosis virus reverse transcriptase (a kind gift of W. Beard, National Institutes of Health) using oligo(dT) as primer. Conditions for first-strand synthesis were as described by Rougeon and Mach (13), except for the addition of 4 mM sodium pyrophosphate (14). The second strand was synthesized with avian myeloblastosis virus reverse transcriptase (15), and the resulting molecules were treated with nuclease S1 (a gift of F. Rougeon) (13). After fractionation in a 5-20% sucrose gradient (in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/1 M NaCl) for 5 hr at 50,000 rpm in a Beckman SW 50.1 rotor at 15°C, the longest molecules [ $>500$  base

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Abbreviation: bp, base pair(s).

‡ Present address: The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden.

pairs (bp)] were used for tailing with deoxycytidine by terminal deoxynucleotidyl transferase in the presence of  $\text{Co}^{2+}$  (13). The pBR322 vector (16) was digested by *Pst* I (17) and tailed with deoxyguanosine in the presence of  $\text{Mg}^{2+}$  (13, 18). The tailed molecules were hybridized and used to transform *Escherichia coli* 803  $r^-_k m^-_k$  (17, 19).

**Purification of Plasmid DNA.** After growth in L broth and chloramphenicol amplification (200  $\mu\text{g}/\text{ml}$ ), cleared lysates were prepared (20). Plasmids were purified by centrifugation in a CsCl/ethidium bromide density gradient. For restriction mapping and sequence determination, plasmid DNA was further fractionated in a 5–40% sucrose gradient (21).

**mRNA Selection by Hybridization.** Plasmid DNA was broken by sonication into pieces of 0.5–1 kilobase. Alkali-denatured DNA (10  $\mu\text{g}$ ) was immobilized onto 25-mm-diameter nitrocellulose filters (Schleicher & Schull BA85). Hybridization and elution of mRNA (1–2  $\mu\text{g}$  per half filter) were performed as described by Ricciardi *et al.* (22).

**Cell-Free Synthesis.** The eluted mRNA was concentrated (22) and translated in a rabbit reticulocyte lysate in the presence of dog pancreas microsomes (11). Microsomes were prepared and freed from ribosomes (23). After translation, membranes were pelleted by centrifugation at  $12,000 \times g$  for 30 min in an Eppendorf centrifuge and solubilized in 10 mM Tris-HCl, pH 7.5/150 mM NaCl/2 mM EDTA/1% Nonidet P-40. H-2 antigens were precipitated with a rabbit anti-H-2 antiserum (24) as described (11). Precipitated proteins were characterized by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (25) and fluorography using EN<sup>3</sup>HANCE (New England Nuclear).

**Construction of Restriction Map and Nucleotide Sequence Determination.** An endonuclease restriction map was constructed as described (26, 27). Isolation of DNA fragments for sequence determination was based on the restriction map. Briefly, DNA was cleaved by using a suitable restriction endonuclease (see Fig. 2). After labeling both 3' and 5' ends or only 5' ends, the DNA fragments were cleaved a second time to produce fragments with only one labeled end (28). All DNA fragments were isolated by electrophoresis in agarose gels and subsequent electroelution into hydroxyapatite (29). DNA sequence determination was done as described by Maxam and Gilbert (28). We used five reactions: guanosine, guanosine plus adenosine, cytidine plus thymidine, cytidine, and cytidine plus adenosine.

**Materials.** Terminal deoxynucleotidyl transferase from calf thymus was purchased from Bethesda Research Laboratories (Rockville, MD); DNA polymerase I was from Boehringer Mannheim. Restriction enzymes were from New England BioLabs, Bethesda Research Laboratories, and Boehringer Mannheim. Polynucleotide kinase was a gift from H. Lehrach and Anna-Maria Frischauf. Oligo(dT)-cellulose (T2 and T3) was from Collaborative Research (Waltham, MA). Rabbit reticulocyte lysate and [<sup>35</sup>S]methionine were from New England Nuclear. Low-temperature gelling agarose (type VII) was from Sigma. Nitrocellulose filters (BA85) were purchased from Schleicher & Schuell. [ $\alpha$ -<sup>32</sup>P]Deoxynucleotides (>400 Ci/mmol; 1 Ci =  $3.7 \times 10^{10}$  becquerels) and [ $\gamma$ -<sup>32</sup>P]deoxyadenosine (>2000 Ci/mmol) were from Radiochemical Centre (Amersham, England).

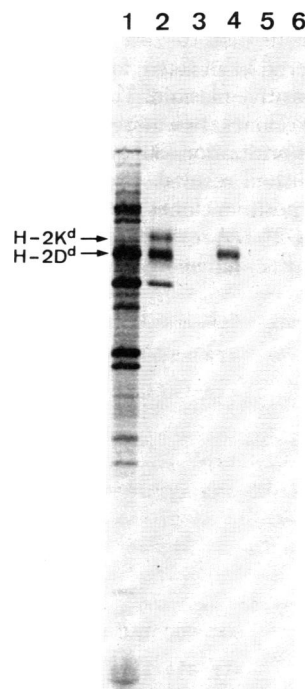
**Biosafety.** The work described here was done in accordance with the French and German guidelines for recombinant DNA research.

## RESULTS

**Construction of Clones and Isolation of pH-2<sup>d</sup>-1.** mRNA coding for H-2 antigen heavy chains was enriched in two steps. First, H-2 antigens are known to be assembled on membrane-

bound ribosomes (11) and so crude rough microsomes from SL2 cells were used as the source of mRNA. Second, this mRNA was size fractionated on a sucrose gradient. mRNA in the 17S region contained the appropriate mRNA as determined by cell-free translation and immunoprecipitation with a rabbit H-2 antigen antiserum (24). About 0.5–1% of the [<sup>35</sup>S]methionine incorporated into the proteins in the cell-free system was specifically precipitated by this antiserum, rather than the  $\approx 0.01$ –0.02% when total mRNA was used as a template (not shown). This indicated an  $\approx 50$ -fold enrichment and suggested that H-2 antigen-specific mRNA was 0.5–1% of that fraction.

Enriched mRNA (6  $\mu\text{g}$ ) was copied into double-stranded cDNA and cloned in *Escherichia coli* as described in *Materials and Methods*. Double-stranded cDNA molecules (75 ng) were recombined at the *Pst* I site of pBR322 and, after transformation, this yielded 400 tetracycline-resistant, ampicillin-sensitive colonies (17). Plasmid DNA, purified from individual colonies and immobilized onto nitrocellulose filters, was used to select mRNA by hybridization. The bound mRNA was eluted and then translated in a cell-free system. As the heavy chains of H-2 antigens are membrane proteins, translation was performed in the presence of membranes that have been shown to incorporate and glycosylate the antigens and cleave their signal sequences (11). After translation, the membranes were isolated and analyzed for newly synthesized H-2 antigens by immunoprecipitation and subsequent gel electrophoresis. Out of 80 clones



**Fig. 1.** Identification of pH-2<sup>d</sup>-1 by hybridization selection and cell-free translation. Plasmid DNAs were immobilized on nitrocellulose filters and hybridized with SL2 mRNA (16–20S). After elution, mRNA was translated in a cell-free system in the presence of dog pancreas microsomes, and antigens were characterized by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis and autoradiography. Lane 1, translation products of the mRNA (200 ng) used for hybridization. Lane 2, antigens synthesized from the same amount of mRNA precipitated from membranes by a rabbit anti-H-2 antigen antiserum. The lower band represents a contaminant that often coprecipitates. Other lanes, translation products of 1  $\mu\text{g}$  mRNA hybridized with pBR322 DNA (lane 3), pH-2<sup>d</sup>-1 DNA (lane 4), and two other recombinant plasmids having unidentified inserts (lanes 5 and 6). Arrows indicate the positions of authentic H-2D<sup>d</sup> and H-2K<sup>d</sup> molecules as found in rough endoplasmic reticulum in SL2 cells.

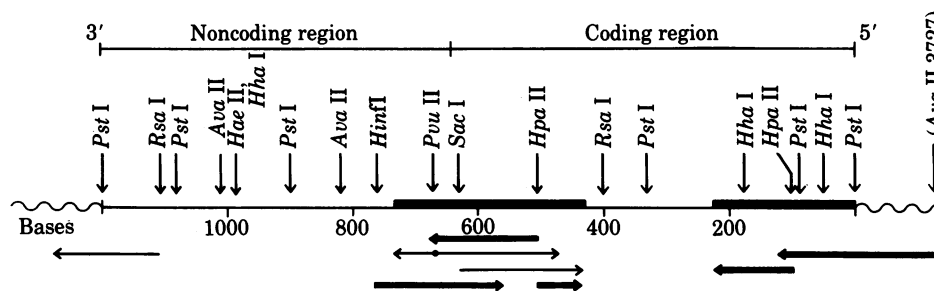


FIG. 2. Restriction map of the pH-2<sup>d</sup>-1 cDNA insert. The 1200-bp insert is drawn as a straight line, flanked by pBR322 sequences as it is oriented in the plasmid (with the *EcoRI* site close to the right-hand side of the insert). The restriction map was constructed as described (26, 27). Coding and noncoding regions are indicated and oriented as 5'→3'. The parts whose sequences were determined are shown as thick lines and the strategy is indicated by arrows. Thick arrows indicate fragments of DNA where both strands have been sequenced. Thin arrows indicate 5' end sequences. Fragments were isolated from the pH-2<sup>d</sup>-1 as follows: (i) Cut with *Ava* II, label, recut with *Hinf*I, isolate 870-bp fragment; (ii) cut with *Hinf*I, isolate 2136-bp fragment, cut with *Hpa* II, label, recut with *Hha* I or *Rsa* I, and isolate 355-bp or 310-bp fragments, respectively; (iii) cut with *Hpa* II, label, recut with *Hha* I, and isolate 490-bp fragment; (iv) cut with *Sac* I, label, recut with *Eco*RI, and isolate 1359-bp fragment; (v) cut with *Pvu* II, label, recut with *Taq* I and isolate 1071-bp and 1564-bp fragments; (vi) cut with *Hinf*I, label, recut with *Eco*RI, and isolate 1505-bp fragment; (vii) cut with *Rsa* I, label, recut with *Hpa* II, and isolate 940-bp fragment.

tested, one was found to be positive. The eluted mRNA directed the synthesis of a membrane-associated polypeptide of ≈43,000 daltons that comigrated with authentic core-glycosylated H-2D<sup>d</sup> antigen (11) (Fig. 1, lane 4). The other plasmids and the vector (lanes 3, 5, and 6) did not yield any immunoprecipitable proteins. Nonhybridized mRNA from the enriched fraction (lane 1) showed, after immunoprecipitation (lane 2), protein bands in the region of H-2D<sup>d</sup> and H-2K<sup>d</sup> antigens (11). By comparison, we estimate that 10–20% of the input H-2 antigen mRNA was recovered in an active form after hybridization and elution with the positive plasmid. This plasmid was named pH-2<sup>d</sup>-1. From the 400 clones, two more positive clones were identified by colony hybridization (30) and mRNA hybrid selection (as above; unpublished results). From these data, the overall frequency of H-2 positive clones is ≈1%.

**Structure of pH-2<sup>d</sup>-1.** A restriction map of the pH-2<sup>d</sup>-1 cDNA insert is shown in Fig. 2. The insert is 1200 bp long. *Pst* I sites

were reconstituted at both ends, and four *Pst* I sites are found within the insert. Single *Hinf*I, *Pvu* II, and *Sac* I sites were useful for DNA sequence determination as were other sites indicated in Fig. 2. There are no *Eco*RI, *Bam*HI, *Bgl* II, or *Hind*III sites in the insert.

Preliminary sequence determinations were carried out from both extremities of the insert. They revealed a stretch of poly(A) residues close to the left *Pst* I site on the map. This indicated that the 3' noncoding region lies to the left in the insert and the coding region lies to the right.

A sequence of 220 nucleotides at the right-hand side of the insert is shown in Fig. 3. The only open reading frame encodes a sequence of amino acids that shows extensive homology with the published sequences of H-2K<sup>b</sup>, D<sup>d</sup>, and L<sup>d</sup> molecules, in the region of amino acids 133–173 (7).

Unglycosylated, processed H-2D<sup>d</sup> antigen has an apparent size on NaDodSO<sub>4</sub>/acrylamide gels of ≈38,000 daltons (11).

pH-2 <sup>d</sup> -1	5'	G <sub>28</sub>	TGG ACG GCG GCG GAC ATG GCG GCG CAG ATC ACC CGA CGC AAG TGG GAG CAG GCT GGT GCT GCA GAG AGA GAC	
			trp-thr-ala-ala-asp-met-ala-ala-gln-ile-thr-arg-arg-lys-trp-glu-gln-ala-gly-ala-ala-glu-arg-asp-	
H-2K <sup>b</sup>			trp-thr-ala-ala-asp-met-ala-ala-leu-ile-thr-lys-his-lys-trp-glu-gln-ala-gly-glu-ala-glu-arg-leu-	
H-2D <sup>d</sup>			met	ile thr arg arg trp
H-2L <sup>d</sup>			trp (met)	ile thr arg arg trp
			133	140 150
pH-2 <sup>d</sup> -1	5'		CGG GCC TAC CTA GAG GGC GAG TGC GTG GAG TGG CTC CGC AGA TAC CTG AAG AAC GGG AAT GCT ACG CTG CTG	
			arg-ala-tyr-leu-glu-gly-glu-cys-val-glu-trp-leu-arg-arg-tyr-leu-lys-asn-gly-asn-ala-thr-leu-leu	
H-2K <sup>b</sup>			arg-ala-tyr-leu-glu-gly-thr-cys-val-glu-trp-leu-arg-arg-tyr-leu-lys	
H-2D <sup>d</sup>			arg	arg arg
H-2L <sup>d</sup>			arg tyr	val trp his (arg) (tyr)
			160	170 180
pH-2 <sup>d</sup> -1	5'		CGC ACA GAT CCC CCA AAG GCC CAT GTG ACC CAT CAC CGC AGA CCT GAA	
			arg-thr-asp-pro-pro-lys-ala-his-val-thr-his-his-arg-arg-pro-glu	
				190 196

FIG. 3. Nucleotide sequence of the 5' end of the insert in pH-2<sup>d</sup>-1 and predicted amino acid sequence. The sequence is compared with the available amino acid sequences of H-2K<sup>b</sup>, D<sup>d</sup>, and L<sup>d</sup> antigens (7). Differences between the amino acid sequence deduced from pH-2<sup>d</sup>-1 and the protein sequences of H-2K<sup>b</sup>, D<sup>d</sup>, and L<sup>d</sup> are indicated by the boxed residues. A possible glycosylation site is indicated by an asterisk. Stop codons are underlined. Numbers refer to amino acid positions in the H-2K<sup>b</sup> molecule (7).

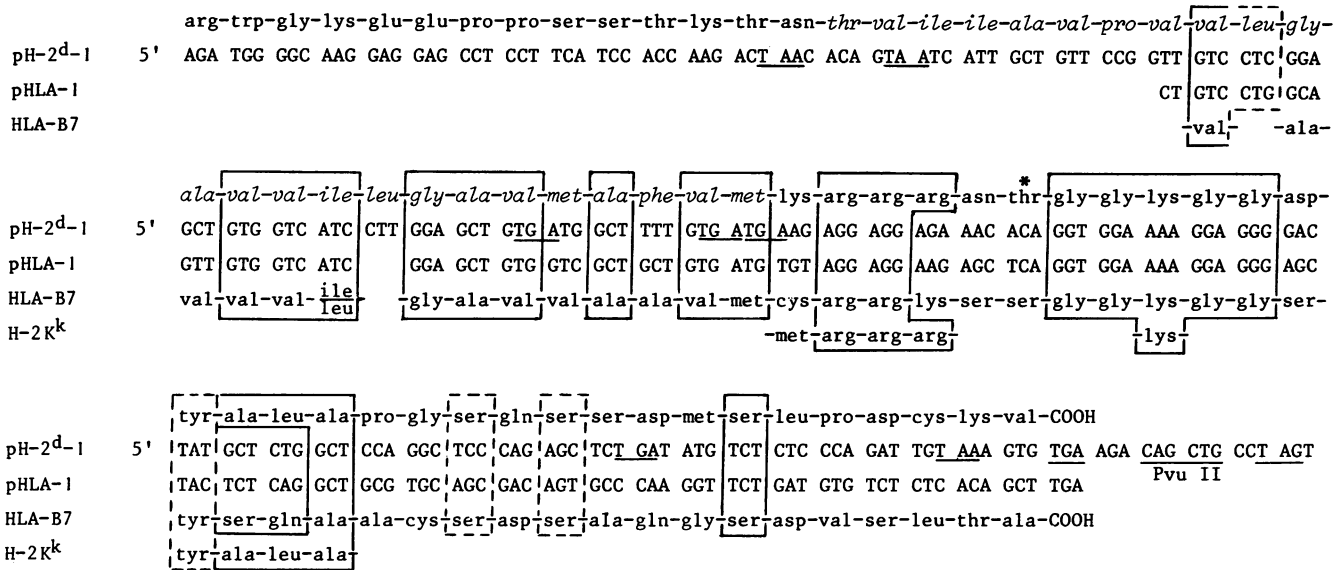


FIG. 4. Nucleotide sequence of the middle portion of the insert and predicted amino acid sequence of the COOH-terminal end of the coded molecule. The sequence is compared with the amino acid sequence of H-2K<sup>k</sup> and HLA-B7 antigens (8, 31) as well as with the nucleotide sequences that code for part of the HLA-A, B, or C antigens (10). Regions identical in both nucleotide and amino acid sequences are shown in closed boxes. Positions with identical amino acids but different codons are shown in boxes with broken lines. The stretch of 24 hydrophobic or uncharged amino acids possibly spanning the membrane is written in italics. The possible phosphorylation site is indicated by an asterisk. Stop codons are underlined.

Therefore, the processed heavy chain is expected to be ≈350 amino acids long (3). Based on this estimate and on the alignment with the amino acid sequences of H-2 and HLA antigens, we predicted that the COOH terminus would be coded by a region near the *Pvu* II site on the restriction map. The sequence of this region is shown in Fig. 4. The open reading frame yields an amino acid sequence that shows a high degree of homology with the partial amino acid sequence known from the COOH terminus of an H-2K<sup>k</sup> antigen (8). In addition, it resembles the COOH terminus of HLA-B7 at 22 positions out of 46 (31). A stop codon at the COOH terminus occurs close to the *Pvu* II site, at the same position as was found in pHLA-1 (10). Furthermore, a stretch of 24 hydrophobic or uncharged amino acids is found 31 residues from the COOH terminus. This was expected because the heavy chains of H-2 antigens span the membrane close to their COOH termini (11, 32).

DISCUSSION

H-2 antigens are membrane proteins that are synthesized on membrane-bound ribosomes. We took advantage of this property to select a cDNA clone specific for H-2 antigens. mRNA coding for H-2 antigens was enriched by isolating crude rough microsomes, and microsomes from dog pancreas were used to characterize hybrid-selected mRNA in the cell-free system. Both of these procedures should be applicable to other membrane proteins synthesized on membrane-bound ribosomes.

The cDNA clone, pH-2<sup>d</sup>-1, has the following properties. (i) It hybridizes with the mRNA that encodes a membrane glycoprotein of 43,000 daltons specifically precipitated by a rabbit H-2 antigen antiserum. (ii) Partial DNA sequence of the coding region in the cDNA shows extensive homology with the known amino acid sequences of several H-2 antigens and HLA-B7 antigen. We thus conclude that pH-2<sup>d</sup>-1 represents a DNA corresponding to one of the H-2<sup>d</sup> transplantation antigens. Fig. 5 shows a schematic outline of an H-2 antigen. The regions from which the amino acid sequences were deduced are indicated.

There are only limited amino acid sequence data available on H-2<sup>d</sup> antigens. In two positions, the pH-2<sup>d</sup>-1 sequence is inconsistent with the available H-2L<sup>d</sup> sequence (7). In contrast,

it fits the known information on H-2D<sup>d</sup> antigen (7). Nevertheless, this does not allow a distinction from an H-2K<sup>d</sup> molecule nor from H-2 antigen-like molecules derived from loci that are as yet unknown (2).

The homology with H-2K<sup>b</sup> antigens in the region of amino acids 133-173 is 85%. The differences are not unexpected for H-2 antigens from different haplotypes and have already been noted in the NH<sub>2</sub>-terminal region of several H-2 antigens (5-9). Uehara *et al.* have suggested that amino acid residue 176 in the H-2K<sup>b</sup> antigen is a potential glycosylation site (6). We find an asparagine residue at amino acid number 176, followed by alanine and threonine. This triplet is known to be a recognition sequence for glycosylation (33).

The available COOH-terminal sequences of the H-2K<sup>k</sup> antigen and HLA-B7 antigen were matched with the postulated corresponding one for pH-2<sup>d</sup>-1. Of the nine positions known for the H-2K<sup>k</sup> antigen, eight are identical to those predicted from the pH-2<sup>d</sup>-1. In the case of HLA-B7 antigen (31), perfect homology was found at 22 residues out of 46 (with one possible amino acid deletion in HLA-B7) and 8 additional residues could be interchanged by single base substitutions. Less homology was found with HLA-A2 antigen (31). Matching the partial nucleotide sequence of pHLA-1 (10) with pH-2<sup>d</sup>-1, 64% homology was found. In addition, the stop codon in pH-2<sup>d</sup>-1 was found at exactly the same position as for pHLA-1, corresponding to the COOH terminus of HLA-B7. This indicates that the high degree of homology between H-2 antigens and HLA-A, -B, and -C antigens (6, 34) extends through the COOH-terminal region at the amino acid, as well as at the nucleotide level.

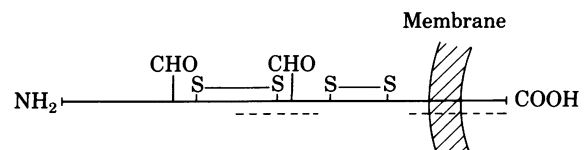


FIG. 5. Schematic outline of H-2 antigens as deduced from published data (5-9) and those presented here. Broken lines indicate portions for which the amino acid sequence was derived by determining part of the nucleotide sequence contained in pH-2<sup>d</sup>-1.

The uninterrupted stretch of 24 hydrophobic or uncharged amino acids found 31 amino acids before the COOH terminus in pH-2<sup>d</sup>-1 has the expected characteristics for a segment that spans the membrane: its length is similar to that found in other membrane proteins (20–30 amino acids) (35) and it is terminated on the cytoplasmic side by a cluster of four basic amino acids (Lys-Arg-Arg-Arg). These might function in anchoring the protein in the membrane. Spanning segments are thought to be  $\alpha$ -helical and it is interesting that a proline residue is found within this region, 17 amino acids from the four basic residues. As proline is known to disrupt the  $\alpha$ -helix (36), the presence of this proline may change the secondary structure of the spanning segment. Of the 24 hydrophobic or uncharged amino acids, 8 are valine. Such a predominance of valine residues is also found for HLA antigens (10).

H-2 antigens as well as HLA antigens are phosphorylated in their cytoplasmic parts (8, 37). Phosphorylation is known to occur at serine or threonine residues (38–40). In the case of phosphoserine, one or two basic amino acids are found close to the NH<sub>2</sub>-terminal side of this residue (38, 39). The sequence Arg-Arg-Asn-Thr is found three amino acids from the hydrophobic stretch and might constitute a phosphorylation site.

The availability of the pH-2<sup>d</sup>-1 probe described here will facilitate obtaining complete amino acid sequences of H-2 antigens from different alleles, as well as from different loci. This will permit us to map the polymorphism at the molecular level. Furthermore, it will be possible to analyze the genes for histocompatibility antigens in mouse and possibly in other organisms such as man on both a structural and a functional level.

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