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Immunology

Structural studies on protein products of murine chromosome 17: Partial amino acid sequence of an H-2K^b molecule*

(histocompatibility antigens/radiolabeling/immunoprecipitation)

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ABSTRACT **By use of single internal radiolabels, 17 of the NH_2-terminal 27 amino acids of the murine H-2K^b molecule have been assigned. When the amino acid sequence is compared to that of the murine H-2K^k molecule, there is a minimum of six amino acid differences in 19 positions. This high degree of structural diversity confirms, at the level of amino acid sequence, the known polymorphism of the murine *H-2* complex. Significant primary structural homology is evident when the murine H-2K^k and H-2K^b sequences are compared to the recently reported partial amino acid sequences of human transplantation antigens. There is modest homology with β_2-microglobulin and immunoglobulins, but the available sequence information is insufficient for a satisfactory evaluation of its significance.**

The most thoroughly studied major histocompatibility complex is the *histocompatibility-2* (*H-2*) complex of the house mouse, *Mus musculus*. The loci of the *H-2* complex can be divided into five regions, *K*, *I*, *S*, *G*, and *D*, separable by genetic recombination (1–3). The regions appear to be functionally differentiated in the following way. The *K* and *D* regions seem to play a major role in destruction of virally or otherwise modified cells (4); the *I* region is primarily concerned with regulation of the immune response (5); and the *S* region is mainly involved in complement activation (6–8); (almost nothing is known about the function and properties of the *G* region; see refs. 9 and 10).

The molecules coded for by the *K* and the *D* regions are hydrophobic glycoproteins with a molecular weight of about 44,000 (11, 21). They are integral constituents of the plasma membrane of practically all somatic cells, although their concentration in different tissues varies, the highest being in cells of the lymphocytic series. In the membrane, the molecules are noncovalently associated with β_2-microglobulin chains (12–16).

The genes coding for the K and D molecules are extremely polymorphic (17): by typing the available inbred strains, about a dozen alleles have been identified at the *H-2K* and *H-2D* loci; the presence of many more alleles at the two loci is suggested by the typings of wild mice. The extreme genetic variability of the H-2K and H-2D molecules raises some intriguing questions. Are these molecules products of true alleles or are the *K* and *D* regions pseudoallelic clusters? Is the variability restricted to a single region of the H-2K or H-2D molecule or is it characteristic of the whole molecule? What is the relationship between the serological variability and the primary structure of the molecule? What is the genetic mechanism responsible for the generation and maintenance of the H-2K and H-2D polymorphism? In an attempt to answer at least some of these questions we have undertaken a comparison of the primary structure of two molecules encoded by alleles at the *H-2K* locus. In a previous communication (18) we reported the partial amino acid sequence of the NH_2-terminal end of the H-2K^k molecule. In this report we describe the partial primary structure of the NH_2-terminal end of the H-2K^b molecule, as well as make an additional assignment in the H-2K^k molecule.

Abbreviation: *H-2*, histocompatibility-2.

* This is paper no. II in a series. Paper no. I is ref. 18.

MATERIALS AND METHODS

Labeling of Murine Splenocytes. ^{3}H- and ^{35}S-Labeled Amino Acids. Each cell culture, consisting of 3 to 7 × 10^8 cells, was prepared from spleens of three to six adult C57BL/6J (*H-2^b*) mice (The Jackson Laboratory, Bar Harbor, Me.) (18, 19). To prepare each medium for labeling with a single essential radioactive amino acid, we deleted the one amino acid from the medium. When a nonessential amino acid was to be used for labeling, all the nonessential amino acids were deleted from the medium. All media were sterilized by filtration immediately before use and were supplemented with 100–200 μCi/ml of the particular isotope used for labeling. Cells were cultured for 4–5 hr.

Radioiodination. Cells (5 to 10 × 10^7) from C57BL/6J (*H-2^b*) mice or A/J (*H-2^a*) mice were radioiodinated (20) with 1–2 mCi of carrier-free, thiosulfate-free Na^{125}I (Amersham-Searle). Cells labeled with either isotopic amino acids or ^{125}I were washed, lysed in 0.5% Nonidet P-40 (Shell), and centrifuged to remove the nuclei.

Antisera. Rabbit antibody against mouse Ig, goat antibody against rabbit Ig, and goat antibody against mouse Ig were as described previously (18). Antiserum against H-2.33 was produced by hyperimmunization of (D2.GD × B10.D2) F_1 hybrids with cells from the thymus, spleen, and lymph nodes of C57BL/6J mice. Serological analysis of the antiserum (Table 1), however, revealed that it was monospecific, directed against H-2.33, the private antigen of *H-2K^b*.

Cytotoxic Tests. The microcytotoxic test was performed as described elsewhere (9).

Immunoprecipitation of Cell Lysates. After dialysis, lysates were centrifuged, the total radioactive protein was assayed (20), and the labeled Ig was removed by immunoprecipitation with rabbit anti-mouse Ig and goat anti-rabbit Ig (19). The supernatant of the immunoprecipitate was treated with a saturating amount of antibody against H-2.33 (100 μl/10^8 cell equivalents) and the complexes were precipitated with a slight excess of goat anti-mouse Ig. Precipitates were washed, dissolved in 1% sodium dodecyl sulfate containing 8 M urea, pH 8.4 (15, 17), and reduced with 0.1 M 2-mercaptoethanol. Multiple aliquots were electrophoresed for 16 hr on 12.5-cm 7.5% sodium dodecyl sulfate-polyacrylamide gels at 4 mA per gel. Gels were fractionated, the H-2 peaks were located and pooled, and radioactivity was determined as described (18).

Amino Acid Analysis. To assure that extensive interconversion of a single isotopic amino acid had not occurred during the period of cell culture, we combined a portion of each immunoprecipitate with a standard mixture of amino acids and

Table 1. Reactivity in the cytotoxic test of antiserum (D2.GD x B10.D2)F_1 against C57BL/6

Strain	*H-2* haplotype	Reciprocal of
C57BL/10Sn	*b*	10,240
B10.D2	*d*	0
B10.M	*f*	0
D2.GD	*g2*	0
B10.A(2R)	*h2*	0
B10.A(3R)	*i3*	10,240
B10.BR	*k*	0
B10.AKM	*m*	0
C3H.AH	*o2*	0
B10.P	*p*	0
B10.Q	*q*	0
B10.RIII(71NS)	*r*	0
B10.S	*s*	0

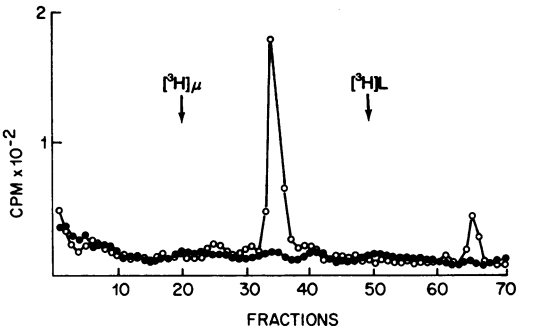

FIG. 1. Acrylamide gel electrophoresis of H-2K^b antigens precipitated from lysates of radioiodinated splenocytes. Precipitates were dissolved, reduced, mixed with [^{3}H]μ and [^{3}H]L chain markers, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In plotting the two different gel patterns, the marker proteins were aligned. ○, C57BL/6; ●, A/J.

hydrolyzed at 110° in 6 M HCl under reduced pressure for 18 hr (18). The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer; each amino acid was collected in a scintillation vial. The results of these analyses are indicated in column 7 of Table 2. Some [^{3}H]amino acids "exchange" the ^{3}H with the HCl such that some amino acids were not recovered in the analysis (tyrosine). In others, as much as 20% of the radioactivity was not detected in the amino acid peak. However, the remaining radioactivity was either present in the fallthrough (first wash) of the column or was present at buffer change positions in the analysis. In only one instance (alanine) was any single isotopically labeled amino acid interconverted to another definable amino acid in greater than 10% yield.

Determination of Sequence. A Beckman 890C sequencer was used. During this investigation significant improvements in the procedure described previously (18) were instituted such that not all samples were studied in an identical fashion. The sequencer was programmed with a DMAA program. Two-second delays have been instituted before each high vacuum step and before S3 delivery. In addition, the low vacuum step before S4 delivery has been allocated 100 sec. Since histidine was detected at position 3 but at no other position in the H-2K^b molecule, Quadrol buffers were not used for these analyses but rather the modified DMAA program. As shown in the upper left panel of Fig. 2, the histidine reported in position 3 shows virtually no "preview" such as that evident in the lower left portion of Fig. 2, which represents the result obtained with the H-2K^k molecule. For the range of experiments reported herein, the repetitive yield in the sequencer, as calculated with a myoglobin standard, ranged between 94.8 and 97.1%.

Step 1 was analyzed without phenylisothiocyanate (PITC) to remove any nonspecific radioactivity remaining in the sample. These washing procedures were necessary to assure a low background at the early stages of sequence analysis and gain reproducibility at the NH_2-terminus.

With the study of histidine, tyrosine, phenylalanine, and methionine, at least one radioactive sample was further analyzed on a Waters high pressure liquid chromatograph to confirm the presence of the particular amino acid in the positions indicated.

RESULTS

Analysis of Molecules Reacting with Antiserum against H-2K^b. Analysis of anti-H-2K^b precipitates prepared from lysates of C57BL/6 splenocytes revealed two peaks after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1).

Table 2. Isotopic labeling and extraction of H-2K*

Isotope	(1) No. cells labeled x 10^{-8}	Radioactivity in: (2) Protein cpm x 10^{-6}	(3) H-2K^b, cpm	(4) H-2 peak recovered from gel, cpm	% of total protein-associated radioactivity in: (5) Immunoprecipitate	(6) Gel extract	(7) % of radioactivity recovered from amino acid analyzer present in amino acid used for labeling†
L-[3-^{3}H]Alanine	3.0	18.3	315,000	23,875	1.7	0.13	50
L-[3-^{3}H(N)]Arginine	7.0	111.7	610,800	86,720	0.54	0.07	97
L-[2,5-^{3}H]Histidine	6.0	86.1	656,600	100,000	0.76	0.11	100
L-[4,5-^{3}H(N)]Isoleucine	5.0	74.0	574,600	114,000	0.76	0.15	Not done
L-[4,5-^{3}H]Leucine	3.9	172.7	991,400	193,600	0.5	0.08	87
L-[4,5-^{3}H(N)]Lysine	5.0	116.8	818,000	88,600	0.70	0.07	Not done
L-[^{35}S]Methionine	5.0	496.5	19,040,200	1,053,300	3.8	0.21	100
L-[4-^{3}H]Phenylalanine	3.9	142.1	1,680,800	259,425	1.2	0.18	100
L-[3,4-^{3}H(N)]Proline	5.0	14.5	142,900	18,983	0.98	0.13	100
L-[3,5-^{3}H]Tyrosine	5.0	195.0	9,206,400	856,620	4.7	0.44	0
L-[2,3-^{3}H]Valine	6.7	25.9	1,124,000	26,840	4.3	0.10	93
Average (11 amino acids)	5.0	132.1	3,196,427	256,542	1.81	0.15	91

* A representative experiment of 2–5 that were done for each isotope.
† Based on an hydrolysis and amino acid analysis of the precipitate.

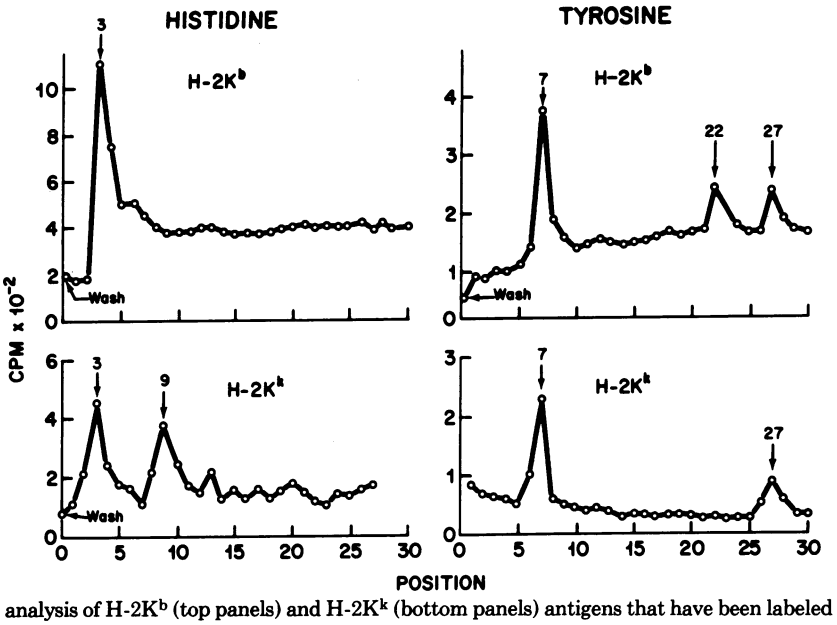

FIG. 2. Sample sequence analysis of H-2K^b (top panels) and H-2K^k (bottom panels) antigens that have been labeled with histidine (left panels) and tyrosine (right panels).

These peaks corresponded to molecules of 44,000 and 12,000. Occasionally, molecules of 22,000 were observed (not shown in the figure). None of these peaks was observed when equal aliquots of A/J lysates from cells labeled to the same specific activity (cpm/10^7 cells) were used. The 12,000 peak represents β_2-microglobulin (12–16).

Cell Labeling and Recovery of H-2K. When different amino acids were used for labeling, an average of 1.3×10^8 cpm were obtained for 5.1×10^8 cells (Table 2, columns 1 and 2). Of this radioactivity, 1.82% could be precipitated with antiserum against H-2K^b (column 5), consistent with our previous report on *H-2K^k* (18). An average of 8% of this radioactivity could be recovered from the extracted and desalted gel peak (column 6/column 5). This represented 0.15% of the initial acid-precipitated radioactivity (column 6) obtained from the cells.

Amino Acid Analyses. When a culture period of 4–5 hr was used, virtually all of the radioactivity was recovered in the particular precursor amino acid used in those tested (Table 2, column 7). Except for L-alanine, these results are analogous with our previous report (18) on H-2K^k and indicate that, in general, sufficient radioactivity can be obtained during short-term culture without any substantial interconversion.

Sequence Analysis. Fig. 2 (top left) shows the results of the [^{3}H]histidine analysis of H-2K^b. Very clearly, a histidyl residue is present in position 3. This is in contrast to the H-2K^k molecule (18), in which histidine was found in both positions 3 and 9 (Fig. 2, bottom left). In the present study valine was found in position 9 in the H-2K^b molecule. Similarly, tyrosine was assigned to positions 7, 22, and 27 in the H-2K^b molecule (Fig. 2, top right), but only to positions 7 and 27 in H-2K^k (Fig. 2, bottom right).

Fig. 3 shows the amino acids assigned in the H-2K^b molecule in this study compared to the sequence of the H-2K^k molecule previously determined in this laboratory (18). The blank spaces indicate either amino acids not tested or those tested and not found. Thus, the positions indicated by blanks (4, 10, 13, 16, 18, 19, 20, 24, 25, and 26) are presumably occupied by the amino acids, serine, threonine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, cysteine, or tryptophan. Carbohydrate, of course, may be present and obscure analyses of certain of these positions.

In virtually all of the positions only a single amino acid was detected; however, certain discrepancies were noted, especially with the assignment of methionine. In both the H-2K^k and H-2K^b molecules, methionine was detected in position 1 in every experiment. However, in 4 of 7 experiments it was detected in position 4, in 2 of 7 experiments in position 5, and in 6 of 7 experiments in position 23. Since leucine has been assigned to position 5, this represents the first time we have noted two different amino acids at a single position. These seven different sequencer runs with [^{35}S]methionine were separate cell culture and labeling experiments.

DISCUSSION

Comparison between Two H-2K Molecules. Thirty-six amino acid residues have been assigned to the NH_2-terminus of two murine H-2K molecules, allowing a comparison between the products of two presumably allelic genes in the murine histocompatibility complex. Certain of the residues reported in this publication have been reported by other workers independently (refs. 22–24; see legend to Fig. 3).

Fig. 3 compares the H-2K^b and H-2K^k sequences; several striking features are apparent. Thirteen of the NH_2-terminal 27 positions are identical and establish significant amino acid homology between these two H-2K gene products. In eight positions (4, 10, 13, 16, 18, 24, 25, and 26) no assignment was made in either molecule. Many of these assignments are likely to be identical because the number of amino acids remaining

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                    5                   10                  15
K^k  Met-Pro-His-——-Leu-Arg-Tyr-Phe-His——-Ala-Val——- Ile-Pro-

K^b  Met-Pro-His-——-Leu-Arg-Tyr-Phe-Val——-Ala-Val——-Arg-Pro-

                   20                  25
K^k  ——-Leu——-Lys-Pro-Phe-Ala-Met-————-——-Tyr

K^b  ——-Leu——-·——·——-Arg-Tyr-Met-————-——-Tyr
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FIG. 3. A comparison of the partial amino acid sequences of the H-2K^k and H-2K^b molecules. Our data are consistent with assignments made at positions 2, 3, and 5–8, for K^b and 3, 6, and 7 in K^k by Henning *et al.* (22); at positions 2, 4, 7, 9, 11, 12, 15, 17, and 22 for K^b and 2, 5, 7, 11, 12, 15, 17, and 19 for K^k by Silver and Hood (23); and 5, 6, 14, 17, and 21 for K^b by Ewenstein *et al.* (24).

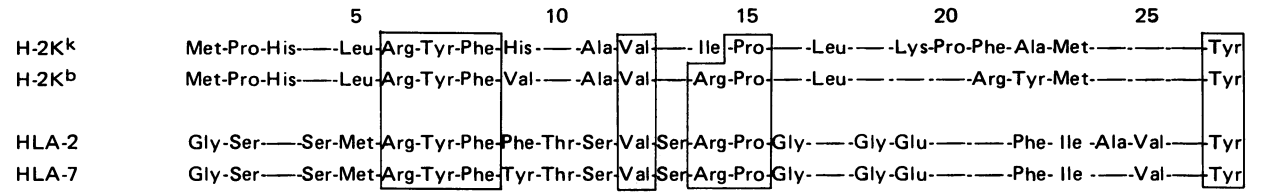

FIG. 4. A comparison of the partial amino acid sequence of two protein products of the *K* region and two human HLA antigens (25).

(see above) is limited.

There are four positions (9, 14, 21, and 22) in which alternative amino acids at each position have been assigned. The striking feature of these substitutions is that two of the four require three base changes in the genetic code (histidine-valine, position 9; phenylalanine-arginine, position 21). One requires a two-base change (alanine-tyrosine, position 22), whereas only one (isoleucine-arginine, position 14) requires a single-base change in the genetic code.

Thus, *in toto*, there are at least six positions in the NH_2-terminal 27 residues in which the products of these two presumably allelic genes differ in their amino acid sequence, whereas at least 13 positions are identical.

All *H-2K* alleles may not differ by as many amino acids as *H-2^k* and *H-2^b*. The *H-2^b* haplotype is only distantly related to other *H-2* haplotypes present in inbred strains (1), including *H-2^k* and *H-2^d*. Although its origin cannot be reconstructed with as much detail as one would wish, it is known that the C57-family of strains carrying the *H-2^b* haplotype was derived from asiatic mice, most probably from a subspecies of *Mus musculus*, distinct from the one that gave rise to most other inbred strains. Furthermore, the house mouse is an extremely variable species and, therefore, some of its subspecies differences might be actually the beginning of a new set of species characteristics. The major histocompatibility complex might be one of such characteristics. The comparison of *H-2^b* with other *H-2* haplotypes, therefore, probably maximizes the allelic variability when different *H-2K* gene products are compared.

Comparison between H-2 and HLA. Fig. 4 compares the sequence of the two murine histocompatibility antigens with the reported NH_2-terminal sequence of two human transplantation antigens recently described (25). There is no obvious homology in the first five positions. Starting with position 6, the next three amino acids are common to both the human and mouse transplantation antigens. A striking feature of the amino acid sequences shown in Fig. 4 is that in virtually every position in which assignments have *not* been made in the murine H-2K^k and/or H-2K^b molecules in our laboratory, the human transplantation antigens contain an amino acid that has not been tested in our system (for example, serine in positions 4 and 13, threonine in position 10, glycine in positions 16 and 18). Thus, the degree of homology between the human and murine transplantation antigens may be even higher than is evident from the presently available data.

Polymorphism of the H-2 System. A comparison of the structure of the two allelic products of the murine *K* region reveals an unusual distribution of the amino acid differences. Thus, commencing at position 19, the next four amino acids are different between the two *H-2K* gene products. It is noteworthy that in this portion of the molecule (positions 19–22) there is no obvious homology between the murine and human transplantation antigens. This clustering of structural differences between these transplantation antigens is reminiscent of the clustered differences between different immunoglobulin variable regions (26).

This unprecedented degree of variation between the products of presumably allelic genes is at one level surprising, but may indeed be reflective of the extraordinary polymorphisms that have been noted in the histocompatibility system. Recent results suggest that certain aspects of the antibody-forming system share some of these features. The extraordinary number of differences in amino acid sequence between molecules bearing the various group a allotypic markers in the rabbit is well established. In addition, extensive sequence differences exist between b4, b6, and b9 constant regions of rabbit light chains (27–31). Recently, Gutman *et al.* (32) have demonstrated that the kappa allotypes in the rat have multiple amino acid differences in the constant region. Recent observations by Strosberg *et al.* (33) indicate that certain rabbits may express three group a and three group b "alleles" after hyperimmunization. These findings in the rabbit have suggested to some that what were formerly thought to be alleles really represent pseudoallelic clusters. It is possible, as Bodmer originally suggested (34), that the H-2 haplotypes correspond to products of different, closely linked genes present in every animal and that a genetic polymorphism resides in the control of the expressed gene.

Comparison between H-2 and Ig Domains. Fig. 5 displays the sequences of the H-2K^k and H-2K^b molecules derived in this laboratory, of the human HLA transplantation antigens (25), and of the NH_2-terminal 27 residues of rat β_2-microglobulin (M. D. Poulik, C. J. Shinnick, and O. Smithies, manuscript in preparation). β_2-Microglobulins have extraordinarily conserved sequences, with the exception of the region around the NH_2-terminus. The rat sequence is displayed because the sequence of the same portion of the molecule has been determined in the mouse (23), and it is typical of β_2-microglobulin

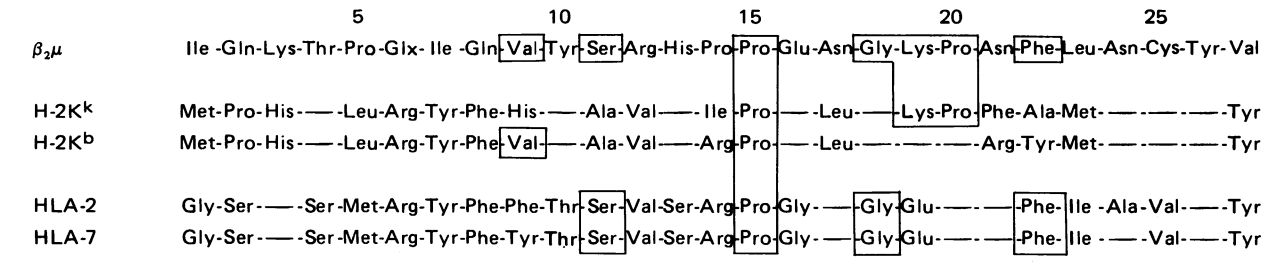

FIG. 5. Amino acid sequence comparison of rat β_2-microglobulin ($\beta_2\mu$) (M. D. Poulik, C. J. Shinnick, and O. Smithies, submitted for publication) and murine and human transplantation antigens. Homologous positions have been outlined.

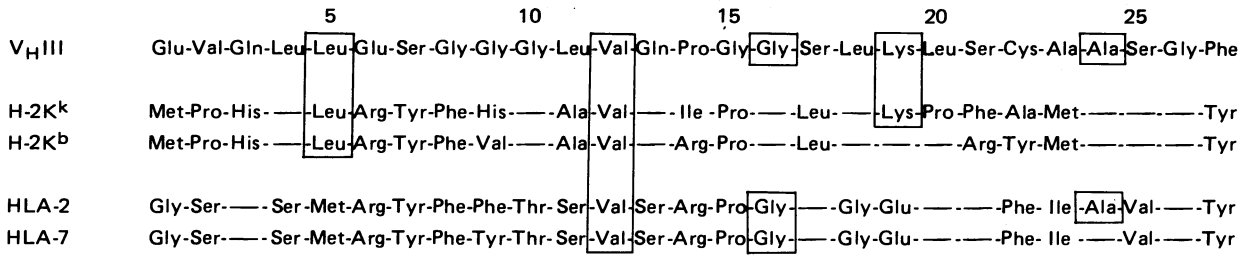

FIG. 6. Amino acid sequence comparison of a prototypic murine V_HIII heavy chain and murine and human transplantation antigens. Homologous positions have been outlined.

sequences. There are seven positions in β_2-microglobulin in which either the mouse or human transplantation antigens have an identical residue. The fact that β_2-microglobulin is selectively found in physical association with both human and mouse transplantation antigens might suggest that their structures would display some rudimentary structural similarities based on the necessity for their association. On the other hand, if additional homologies are noted between the transplantation antigens and β_2-microglobulin, the possibility of their having evolved from a common primordial gene must be seriously entertained.

Fig. 6 compares the amino acid sequence of a prototypic murine immunoglobulin V_HIII heavy chain (26, 35) with the human and murine transplantation antigens. Without introducing insertions or deletions in any of the chains, there is one position (position 5) in which the murine V_HIII prototype is identical to both murine H-2K gene products, and one position (position 21) in which the murine V_HIII prototype has the same residue as one of the *H-2K* alleles. In two additional positions [16 and 24] the murine V_HIII prototype has the same amino acid as both human transplantation antigens. In position 12 *all* murine V_HIII proteins, *both* murine transplantation antigens, and *both* human transplantation antigens contain valine. In a computer search (National Biomedical Research Foundation) a human V_HIII heavy chain emerged as the best "fit" of over 800 known sequences in the computer bank. This modest degree of amino acid sequence homology between immunoglobulin V regions and murine and human transplantation antigens can only be definitively established by additional amino acid sequence data on the gene products of the major histocompatibility complexes of both species.

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