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Immunology

Alternative protein products with different carboxyl termini from a single class I gene, *H-2K^b*

(pre-mRNA splicing/lariat branch point)

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ABSTRACT **In addition to mRNA encoding the canonical form of the murine class I antigen H-2K^b (348 amino acid residues), mRNA that would encode a shortened form of H-2K^b (missing 9 amino acids from the C-terminus) has been identified in C57BL/6 spleen cells by RNase-protection studies. The alternative transcripts of H-2K^b arise through the use of different AG acceptor splice sites for exon VIII. The existence of a shortened H-2K^b protein was demonstrated by sequential immunoprecipitation. Lysates of spleen cells that had been labeled with [^{35}S]methionine and [^{3}H]histidine were precleared with rabbit anti-peptide serum reactive with the C-terminus of the canonical H-2K^b. The shortened form of H-2K^b was immunoprecipitated from this lysate with H-2K^b alloantiserum. Both forms of H-2K^b were isolated by NaDodSO$_4$/PAGE. Tryptic peptide mapping confirmed that these molecules differed only at their C-terminus. The shortened form of H-2K^b is also found in a B-cell line (R8) but not in three cloned T-cell lines or in a T-cell lymphoma (EL4), suggesting that regulatory events are involved in producing the two forms of H-2K^b. Putative lariat branch points involved in these alternative splicing events for the 3′ coding region of H-2 class I pre-mRNAs are proposed.**

The classical class I molecules (transplantation antigens) encoded by the major histocompatibility complex possess a great degree of polymorphism. There are multiple loci that encode transplantation antigens (reviewed in ref. 1) and for each locus multiple alleles exist, possibly as many as 100 for *H-2K* in the mouse (2). In addition to this polymorphism, there is great diversity among both allelic and nonallelic classical class I genes; homologies range from 80% to 94% for the gene products.

While the function of class I molecules has not been precisely defined, it is likely that their extensive polymorphism and diversity are important for species survival. Foreign molecules, such as viral antigens that are expressed on the host cell surface, are recognized by cytotoxic T lymphocytes (CTLs) in the context of the host's own class I antigens, a phenomenon known as restricted recognition (3). This suggests a possible role for the extensive diversity and polymorphism of classical class I molecules, in that they may serve to expand the repertoire for restricted recognition; e.g., certain class I molecules might be more suitable to present a particular antigen than other products of that same or another locus (1).

Although the primary source of the diversity is the availability of multiple alleles and loci, recent studies have indicated that this may not be the only means of diversification in the class I antigen system. Sequence analyses of cDNA clones indicated that H-2 class I molecules vary in length, so that H-2K^b is 348 residues long, whereas H-2D^b is 338. Comparison of these sequences with those of genomic H-2 clones (4) indicated that these differences in length may arise from the utilization of alternative splicing sites for exon VIII and that most class I molecules have within their genomic sequence the information for each type of C-terminus (Fig. 1*A*). Although studies at the mRNA level have indicated that alternative splicing exists for individual class I genes (5–8), evidence for protein products translated from these alternatively spliced mRNAs has only been reported for *in vitro*-deleted class I genes (9).

The study reported here demonstrates by RNase protection analysis that C57BL/6 spleen cells express two forms of H-2K^b mRNA, one that encodes the canonical, 348-residue form of the H-2K^b molecule (H-2K$^b_{348}$) and another that encodes a shortened form of H-2K^b (H-2K$^b_{339}$). The corresponding protein encoded by each mRNA was demonstrated by sequential immunoprecipitation and tryptic peptide mapping. Examination of some *H-2^b* cell lines indicated that the relative amounts of the H-2K$^b_{348}$ and H-2K$^b_{339}$ proteins differ in different cell types. Moreover, scrutiny of the nucleotide sequences of H-2 class I genes allowed identification of sequences containing potential pre-mRNA lariat branch points that may be involved in these splicing events.

MATERIALS AND METHODS

RNase Protection. A cDNA clone of H-2K^b (pH202) was a gift from B. Wallace (Beckman Research Institute, Duarte, CA) (16). From this clone a 345-base-pair *Pst* I–*Pvu* II fragment, which spans from the 3′ third of exon IV to just beyond exon VIII, was directionally cloned into pGEM2 (Promega Biotec, Madison, WI). The vector was digested with *Sma* I and *Pst* I and dephosphorylated prior to ligation (17). The *Hin*dIII-linearized form of this clone was used as the substrate for the synthesis of an RNA probe uniformly labeled with uridine 5′-[α-[^{35}S]thio]triphosphate, using SP6 polymerase (18). This linearized clone is schematically represented in Fig. 2*A* (the *Pst* I site is regenerated such that 350 nucleotides are complementary to *H-2K^b*). As a control, an H-2D^d probe was generated using similar technology. RNA was extracted from the spleens of C57BL/6 and BALB/c mice by the guanidinium thiocyanate method (20) and RNase protection analysis was performed (18). In brief, spleen RNA and probe were coprecipitated, redissolved, denatured, and allowed to hybridize overnight at 45°C. Single-stranded RNA was digested by RNase A and RNase T1 for 1 hr at 30°C. The

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Abbreviations: CTL, cytotoxic T lymphocyte; αK-C, antiserum against a peptide corresponding to the canonical H-2K^b carboxyl-terminal residues 337–348; αK^b, alloantiserum against H-2K^b.

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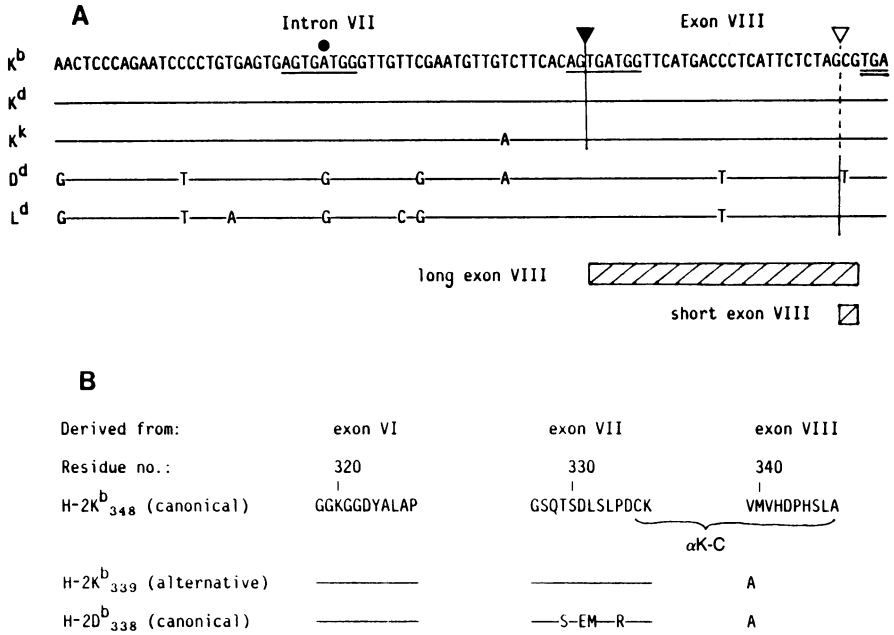

FIG. 1. (*A*) Sequence comparison of exon VIII and part of intron VII from H-2 class I genes. Five genomic sequences that have been published are shown (10–14). The canonical AG acceptor splice site is indicated by a vertical solid line, and the putative alternative acceptor splice site, by a broken line. Double underline indicates the termination codon TGA. The adenosine (filled circle) that is 28 nucleotides upstream from the canonical acceptor splice site of *H-2K^b* (filled triangle) is proposed in the *Discussion* as the lariat branch point for splicing; the repeated sequence AGTGATGG containing this putative lariat branch point is underlined. (*B*) C-terminal amino acid sequences for the known form of H-2K^b as determined by protein (15) and clone pH202 cDNA (16) sequence analyses, and the putative alternative form as predicted by alternative splicing. This alternative form has the same number of residues corresponding to exon VIII as H-2D^b (H-2D^b is one amino acid shorter than the alternative form of H-2K^b, due to a deletion in exon V). Also indicated is the portion of H-2K^b to which αK-C is directed. Standard one-letter amino acid abbreviations are used in *B*.

mixture was fractionated by electrophoresis in a 6% polyacrylamide denaturing gel. The size markers used were deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate-labeled fragments generated by dideoxy-sequencing reaction of genomic clone HLA-A3 (a gift from E. P. Cowan, National Institutes of Health, Bethesda, MD), starting at position 1772 as designated by Strachan *et al.* (21).

Synthesis of Peptide and Preparation of Antisera. A peptide corresponding to the sequence of canonical H-2K^b from position 337 to 348 (Fig. 1*B*) was synthesized by the solid-phase method (22), using a Beckman 990 peptide synthesizer. The peptide was coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (23) and this conjugate was injected into rabbits. The antiserum obtained was designated αK-C.

The alloantiserum against H-2K^b (αK^b) designated SN80 was generously provided by S. Nathenson (Albert Einstein College of Medicine, New York). The serum was prepared in (BALB/c × A/J)F$_1$ mice by multiple injections of HTI spleen and lymph node cells. The Y-3 monoclonal antibody (24), which is specific for the H-2K^b molecule, was produced in ascites fluid.

Metabolic Labeling. Spleen cells (10^8) from a C57BL/6 mouse were incubated at 37°C for 6 hr in 10 ml of RPMI 1640 medium (without methionine and histidine) containing 10% fetal bovine serum, [^{35}S]methionine (250 μCi/ml, 1000 Ci/mmol, Amersham; 1 Ci = 37 GBq), and, where indicated, [^{3}H]histidine (250 μCi/ml, 58 Ci/mmol, Amersham). Cells were lysed at 4°C for 1 hr with 1 ml of 0.5% Nonidet P-40 in 0.9% NaCl containing 10 mM Tris, 1.5 mM $MgCl_2$, 30 μg of aprotinin (Boehringer Mannheim) per ml, and 0.5 mM phenylmethylsulfonyl fluoride (Sigma) at pH 7.4. The lysate was centrifuged at 60,000 × *g* for 30 min.

Surface Labeling by Lactoperoxidase-Catalyzed Radioiodination. Washed cells (4×10^7) were resuspended in 50 μl of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS). Lactoperoxidase (30 μl of a 2-mg/ml solution in PBS; L8257, Sigma), Na^{125}I (1 mCi, Amersham), and 10 μl of 0.05% H_2O_2 in PBS were added. After 5 min, 15 μl of 0.05% H_2O_2 in PBS was added and incubated for 10 min. A further 20 μl of 0.05% H_2O_2 was added, followed by incubation for 5 min. Cells were washed three times with PBS and lysed in 0.5% Nonidet P-40 as described above.

Cell Lines. EL4 is a T-cell lymphoma derived from a C57BL/6 mouse. R8 and three cloned CTL lines were gifts of J. Bluestone (National Institutes of Health). R8 is an Abelson virus-transformed pre-B-cell line derived from a (C57BL/6 × BALB/c)F$_1$ mouse. Two T-cell lines were derived from C57BL/6 mice with the *bm10* mutation, and the third from C57BL/6 mice with the *bm11* mutation. These mutations are in exons III and II, respectively, and so are not expected to have any bearing on these studies.

Preclearance and Immunoprecipitation. For preclearance, the labeled lysate (400 μl) was incubated with 250 μl of the preclearing serum for 1 hr at room temperature. One-half milliliter of 50% (vol/vol) protein A-agarose (Bethesda Research Laboratories) in NETT buffer (0.15 M NaCl/5 mM EDTA/50 mM Tris/1% Triton X-100/0.02% NaN_3, pH 8.2) was added and incubation proceeded for 1 hr with mixing. After centrifugation, the supernatant was used for immunoprecipitation.

Immunoprecipitation was done in a similar manner with the exception that the volumes of antigen, serum, and protein A-agarose were generally 100, 10, and 20 μl, respectively. The agarose beads were washed five times in NETT buffer. The bound proteins were analyzed by NaDodSO$_4$/PAGE under reducing conditions, followed by fluorography or autoradiography.

Electroelution. Preparative NaDodSO$_4$/PAGE was performed by using the middle lanes of the slab gel for eventual electroelution and the flanking lanes for fluorography. The middle lanes were sliced into horizontal strips (0.4 cm wide) and stored frozen. The strips corresponding to the appropriate fluorographic band were electroeluted (25) using sample cups (ISCO) as the electroelution cells.

Tryptic Peptide Analysis. The electroeluted samples were digested with trypsin, and the resulting peptides were resolved by reversed-phase HPLC (26).

RESULTS

A Shortened mRNA of H-2K^b. To determine whether alternative splicing of H-2K^b pre-mRNA occurs in spleen cells, we performed RNase-protection studies. Total cellular RNA was hybridized with an excess of a 383-nucleotide [^{35}S]labeled antisense RNA probe derived from a canonical H-2K^b cDNA clone (Fig. 2*A*). Duplex hybrids of RNA molecules in which the canonical (long) exon VIII is present would be expected to yield a 350-nucleotide fragment after RNase digestion. Hybrids formed with transcripts with a

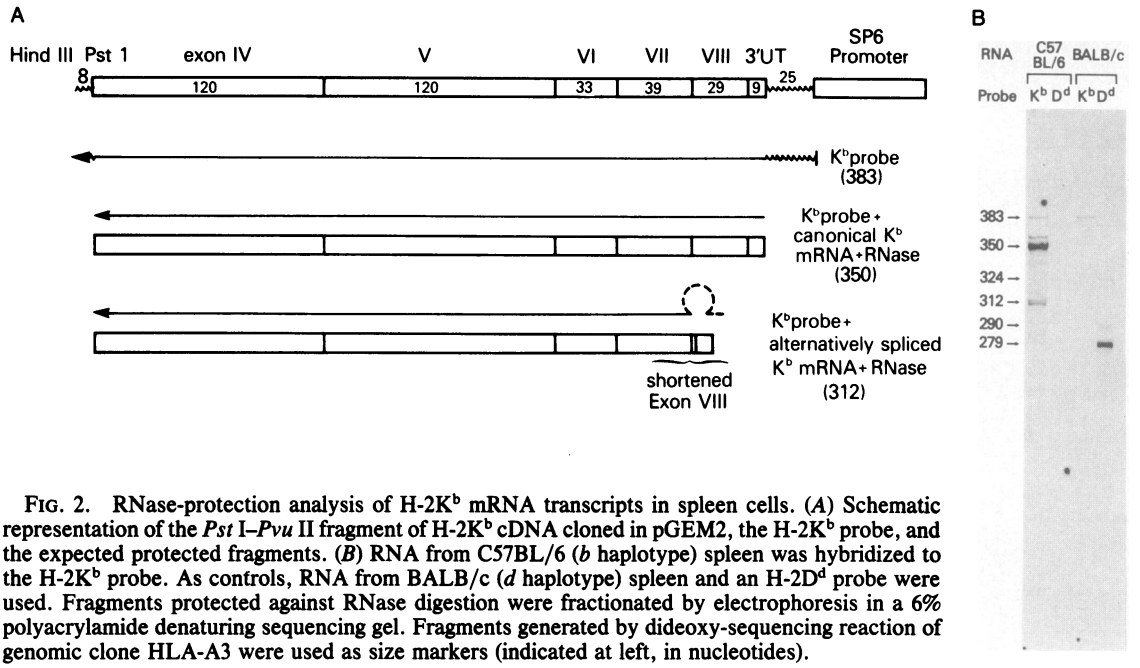

FIG. 2. RNase-protection analysis of H-2K^b mRNA transcripts in spleen cells. (*A*) Schematic representation of the *Pst* I–*Pvu* II fragment of H-2K^b cDNA cloned in pGEM2, the H-2K^b probe, and the expected protected fragments. (*B*) RNA from C57BL/6 (*b* haplotype) spleen was hybridized to the H-2K^b probe. As controls, RNA from BALB/c (*d* haplotype) spleen and an H-2D^d probe were used. Fragments protected against RNase digestion were fractionated by electrophoresis in a 6% polyacrylamide denaturing sequencing gel. Fragments generated by dideoxy-sequencing reaction of genomic clone HLA-A3 were used as size markers (indicated at left, in nucleotides).

shortened exon VIII would generate a 312-nucleotide RNase-resistant fragment and a 38-nucleotide fragment which is unlikely to be detected. As shown in Fig. 2*B*, the H-2K^b probe is protected by RNA from spleen of the C57BL/6 (b haplotype) mouse so that several fragments are produced. The size of these fragments was estimated by comparison with a DNA sequence ladder. Hence, the major band protected corresponds to the canonical H-2K^b mRNA (size, 350 nucleotides). A second band corresponds to the expected 312 nucleotides of a shortened H-2K^b mRNA. The ratio of canonical to shortened H-2K^b RNA is 10:1 by densitometry. The minor band at 383 corresponds to undigested H-2K^b probe. Another minor band, at 358, probably represents an incompletely digested probe fragment in which the *Hin*dIII–*Pst* I area has not been digested.

As controls, the H-2D^d probe does not hybridize to RNA from *b*-haplotype spleen, nor does the H-2K^b probe to RNA from *d*-haplotype spleen (Fig. 2*B*). In both cases, only the bands corresponding to undigested probe can be detected. The H-2D^d probe (324 nucleotides) hybridizes to RNA of *d*-haplotype spleen as expected and produces two major fragments, corresponding to a canonical H-2D^d transcript (279 nucleotides) and an alternatively spliced form in which exon VII is missing (290 nucleotides). It should be noted that the D^d probe was derived from a cDNA clone in which exon VII was missing (i.e., the alternatively spliced form), unlike the K^b probe, which was derived from canonical H-2K^b; thus, a longer fragment is protected by the alternatively spliced form than by the canonical form.

Detection of an H-2K^b Protein That Does Not Have the Canonical C-Terminus. Immunoprecipitation of [^{35}S]methionine-labeled-cell lysate with the alloantiserum against H-2K^b produces two bands of proteins with M_r of about 47,000 and 45,000 (Fig. 3, first lane). However, if the spleen cell lysate is first precleared with the anti-C-terminus serum (αK-C), the alloantiserum only detects the M_r 45,000 molecule (third lane). There is 7-fold more of the high M_r form than the low M_r form, as determined by densitometry of the fluorograph (taking into account that the low M_r form has only seven methionine residues, whereas the high M_r form has eight). This ratio is roughly consistent with the RNase protection studies. The band at M_r 29,000 probably corresponds to class II protein, as this particular alloantiserum has anti-I-A^b activity. Similar results were obtained when a monoclonal antibody against H-2K^b (Y-3) was used instead of the αK^b alloantiserum, except that there was no band at M_r 29,000 (data not shown).

Comparison of the High and Low M_r Forms of H-2K^b by Tryptic Peptide Mapping. In order to prove that the two forms of H-2K^b could be attributed to differences in their C-termini, tryptic peptide mapping was performed. Inspection of the complete amino acid sequence of the H-2K$^b_{348}$ molecule reveals that the C-terminal decapeptide resulting from trypsin cleavage at Lys-338 should be the only peptide in a tryptic digest containing both histidine and methionine and that this

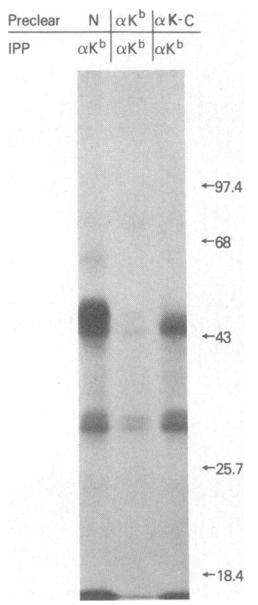

FIG. 3. Detection of a molecule that reacts with alloantiserum against H-2K^b but not with antiserum against a peptide corresponding to the canonical C-terminus of H-2K^b. A Nonidet P-40 lysate of [^{35}S]methionine-labeled spleen cells of a C57BL/6 mouse was subjected to sequential immunoprecipitation. Samples were precleared with various sera (N, normal rabbit serum; αK-C, antiserum against a peptide corresponding to the canonical C-terminus; αK^b, alloantiserum against H-2K^b), and the second, immunoprecipitating (IPP) antibody was αK^b. The resulting immune complexes were analyzed by NaDodSO$_4$/10% PAGE followed by fluorography. Markers at right indicate $M_r \times 10^{-3}$ of standard proteins run in parallel.

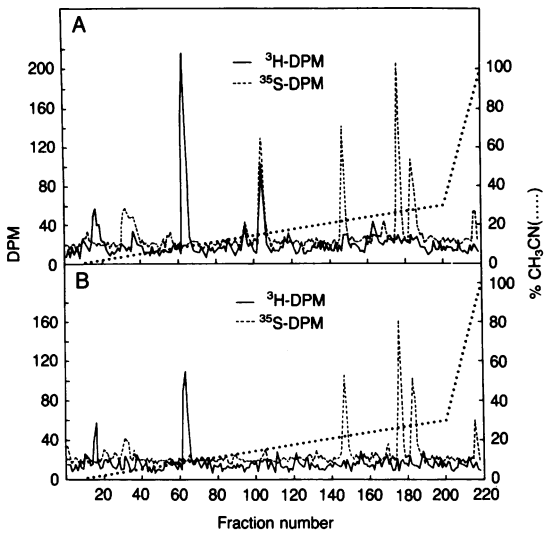

FIG. 4. Tryptic peptide map comparisons of the higher M_r (*A*) and lower M_r (*B*) forms of [^{3}H]histidine/[^{35}S]methionine-labeled H-2K^b. Both molecules were digested simultaneously and analyzed by reversed-phase HPLC.

peptide should be missing in the tryptic map of H-2K$^b_{339}$ (see Fig. 1*B*). Therefore, we isolated the two M_r forms of H-2K^b by preparative NaDodSO$_4$/PAGE after radiolabeling with [^{3}H]histidine and [^{35}S]methionine and analyzed their respective tryptic peptides by reversed-phase HPLC. Fig. 4*A* illustrates the tryptic peptide map of the higher M_r form of H-2K^b and Fig. 4*B* shows the peptide map of the lower M_r species. Comparison of these two peptide profiles reveals that the only peptide difference is the lack of a peptide at fraction 104 in the lower M_r form. Furthermore, this is the sole peptide containing both histidine and methionine. These results are therefore consistent with the hypothesis that the lower M_r form of H-2K^b does not possess the normal H-2K^b C-terminal amino acid sequence—i.e., it corresponds to the H-2K$^b_{339}$ molecule.

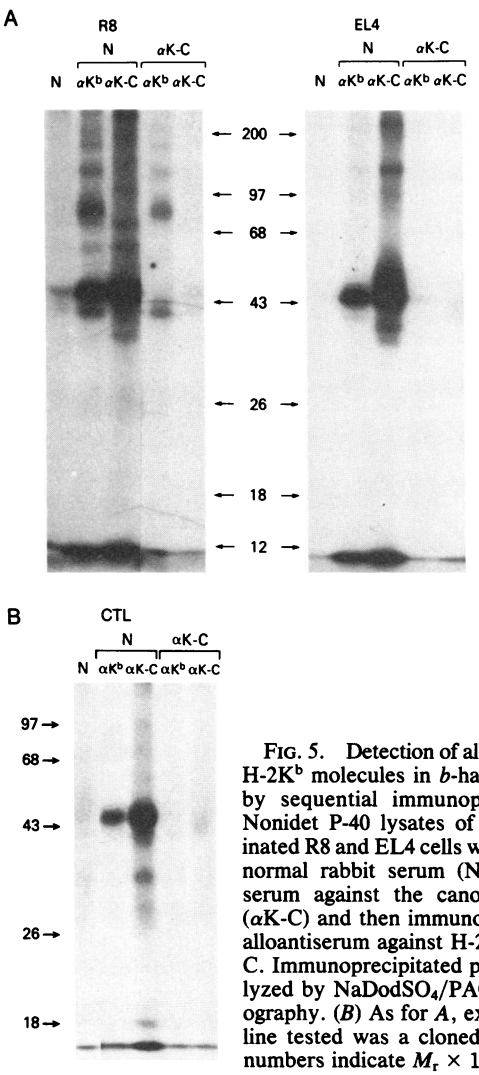

FIG. 5. Detection of alternatively spliced H-2K^b molecules in *b*-haplotype cell lines by sequential immunoprecipitation. (*A*) Nonidet P-40 lysates of surface-radioiodinated R8 and EL4 cells were precleared by normal rabbit serum (N) or anti-peptide serum against the canonical C-terminus (αK-C) and then immunoprecipitated with alloantiserum against H-2K^b (αK^b) or αK-C. Immunoprecipitated proteins were analyzed by NaDodSO$_4$/PAGE and autoradiography. (*B*) As for *A*, except that the cell line tested was a cloned CTL line. Scale numbers indicate $M_r \times 10^{-3}$.

Sequential Immunoprecipitation of Lysates of Cultured Cell Lines Expressing H-2K^b. In order to see whether protein products due to alternative splicing occur in tissue culture cell lines, R8 (a pre-B-cell line), EL4 (a T-cell lymphoma), and three cloned T-cell lines were surface-labeled with ^{125}I and studied by sequential immunoprecipitation as described above. For R8, after preclearance with the anti-peptide serum directed against the canonical C-terminus, the lower M_r form could be immunoprecipitated by the anti-H-2K^b alloantiserum (Fig. 5*A*). Therefore, R8 expresses both forms of H-2K^b. In contrast, despite several attempts, the lower M_r form (due to alternative splicing) could not be demonstrated in EL4 cells (Fig. 5*A*) or in the three cloned T-cell lines (the results for one of these cloned T-cell lines are shown in Fig. 5*B*).

DISCUSSION

Sequence analyses of H-2 class I cDNA clones indicated that different H-2 molecules were variable in length primarily because of differences at their C-termini (4), which could be explained by the utilization of two alternative splice sites during the generation of mRNA (see Fig. 1). In this study, we have shown that a given H-2 molecule, H-2K^b, can be expressed with either of these C-termini. RNase-protection studies showed that spleen cells express two forms of H-2K^b mRNA that differ by containing either a long or a short exon VIII. The presence of both forms of protein was demonstrated by showing that, after removal of the canonical H-2K$^b_{348}$ from spleen cell lysates by immunoprecipitation with antiserum specific for its C-terminus, a smaller H-2K^b molecule remained that could be precipitated with alloantisera (conventional or monoclonal) specific for H-2K^b. Tryptic peptide mapping indicated that this molecule lacked the C-terminal peptide encoded by the long exon VIII (see Fig. 1*A*) and indicated that the smaller H-2K^b molecule was a product of an mRNA containing the short exon VIII.

Available evidence based on cDNA sequences and our results above, suggests that *H-2D* subregion transcripts always utilize a short exon VIII, whereas *H-2K* subregion transcripts can utilize either a short or a long exon VIII. Hence, we considered mechanisms whereby splicing preferentially occurs at the upstream acceptor splice site for H-2K (to produce a long exon VIII) but at the downstream site for H-2D (to produce a short exon VIII) (Fig. 1*A*). Lariat formation during pre-mRNA splicing requires as its branch point an adenosine at about 18–37 nucleotides upstream from this acceptor splice site (27, 28). It can be seen from Fig. 1*A* that the only adenosine in this region that is found in H-2K but not H-2D or -L is the adenosine (indicated by a filled circle in Fig. 1*A*) 28 nucleotides 5′ from the upstream acceptor splice site (filled triangle) of H-2K. Furthermore, the sequence AGTGATGG (underlined) that includes this adenosine is repeated further downstream, 22–29 nucleotides 5′ from the downstream acceptor splice site (open triangle). Since this putative downstream lariat formation sequence is identical in H-2D and H-2L, as well as H-2K, this may explain why H-2D and H-2L genes appear to only translate a short exon VIII, whereas H-2K can translate both long and short forms of exon VIII. Furthermore, the fact that a set of 8

nucleotides is repeated suggests that it contains other signals for these splicing events besides the lariat branch-point adenosine. Site-directed mutagenesis would be an expeditious way of testing our hypothesis.

The existence of mRNAs encoding a given class I molecule with different C-termini was shown by Kress *et al.* (5). In a cDNA library made from liver of *H-2*^{q}-haplotype mice, they found four *H-2K*^{q} clones that encoded a predicted molecule of 347 residues, which was typical for *K*-region molecules, while a fifth *H-2K*^{q} clone encoded a molecule of 338 residues. An alternatively spliced form of H-2K^k mRNA, which would encode a shortened molecule of 339 residues, has also been described (29). Although these data suggest that given class I genes may utilize two forms of exon VIII, data by Brickell *et al.* (6, 7) suggest that other types of alternative splicing may occur. These investigators obtained four H-2D^d cDNA clones that represented identical mRNAs, with the exception that two of the clones did not contain sequence corresponding to exon VII but did retain the exon VIII sequence. Furthermore, other investigators (8) have shown that alternative splicing events can also occur at the 5′ end of class I genes.

Despite the considerable evidence in favor of alternative splicing events resulting in multiple mRNAs from a given class I gene, the expression of alternatively spliced mRNA at the protein level has not been easy to demonstrate. Previously, we were able to show that L cells transfected with a truncated *H-2D*^{d} gene expressed protein products derived from alternative splicing (9); more recently, we have found that this also occurs for the entire gene (unpublished results). In the present work, we found about 7 times more H-2K$^b_{348}$ than H-2K$^b_{339}$ in spleen cell lysates, which indicates that one reason for the difficulty in finding these proteins is that they may be much less abundant than the canonical proteins. In agreement with this, Transy *et al.* (8) found that the 5′-altered K^d message was one-tenth as abundant as the normal message, and Kress *et al.* (5) found four cDNA clones for H-2K^q that utilized a long exon VIII but only one that utilized short exon VIII. A further difficulty in detecting products of alternatively spliced class I messages may be that they occur predominantly in certain cell types and/or at specific times during the cell cycle. The fact that alternative splicing was found in R8 cells but not in EL4 cells or three cloned T-cell lines indicates that only certain cell types express the shortened form of H-2K^b. If this is representative of what may occur *in vivo*, it suggests that alternative splicing may be regulated among different tissues or cell types, as one might expect if the alternative protein structures were functionally important. R8 is of B-cell lineage while the other four cell lines are of T-cell lineage, but more cell lines need to be tested to see whether it generally applies that the shortened form is found in B cells but not T cells.

Attempts to discover the function of the C-terminal region by transfecting L cells with class I genes containing altered or deleted exons encoding the cytoplasmic domain have, in most cases, shown no effect on expression of the class I molecule nor any qualitative effect on its interaction with CTLs (30). However, Murre *et al.* (31) found that CTL lysis of vesicular stomatitis virus-infected cells was significantly reduced when the region encoding the cytoplasmic domain of H-2L^d was removed. This reduction was not observed for influenza-specific CTL restricted by the altered H-2L^d molecule. Like Zuniga *et al.* (30), Murre *et al.* (31) found that the altered H-2L^d molecule was expressed at the L-cell surface in similar amounts to the canonical H-2L^d molecule and, moreover, found that the capping properties of both molecules were similar.

Although the function of the cytoplasmic domain of class I molecules with regard to CTL lysis of virus-infected cells remains equivocal, class I molecules may have broader functions than those of immune recognition. For instance, there is some evidence that class I molecules act as receptors for certain hormones (32, 33). In this context, there is at least one example that shows that alteration of the cytoplasmic domain of a receptor leads to functional effects: the low density lipoprotein receptor with a mutant cytoplasmic domain does not cluster properly and so fails to transport the lipoprotein into cells (19). It is possible that the role of the cytoplasmic domain (and hence class I molecules altered in this region) will remain elusive until the functions of the class I molecules in the wider pathophysiological and developmental context have been elucidated.

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