DIFFERENT STRUCTURAL CONSTRAINTS FOR RECOGNITION OF MOUSE H-2K^d AND -K^k ANTIGENS BY ALLOIMMUNE CYTOLYTIC T LYMPHOCYTES

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Class I genes of the mouse MHC encode cell surface glycoproteins that are crucial for the cellular immune response. These antigens, called H-2K, -D, and -L, consist of an H chain (mol wt 45,000) noncovalently associated with β_2-microglobulin (mol wt 12,000; reviewed in reference 1). The H chain is integrated in the plasma membrane, with the largest part exposed on the cell surface and ~30–40 amino acids protruding on the cytoplasmic side. The extracellular part can be divided into three domains of similar size (α1, α2, and α3) each comprising ~90 amino acids (2–4).

Several H-2 class I genes have been cloned and characterized: the L^d gene (5, 6), the K^d gene (7), the K^b gene (8), the K^k gene (9), and the D^d gene (10). These genes all have eight exons, which correlate with the domains of the corresponding protein. The first exon encodes the signal sequence (11); exons 2, 3, and 4 correspond to the three outer domains α1, α2, and α3; the fifth exon encodes the transmembrane domain; and the remaining exons (6, 7, and 8) encode the cytoplasmic domain and the 3′ noncoding region (reviewed in reference 12).

It has been shown (13) that during viral infection, H-2 antigens play an important role in the T lymphocyte response. CTL have the ability to recognize and selectively kill the infected cells. The specificity of the CTL is not directed against the viral antigen per se, but rather requires in addition self-determinants present on H-2 class I antigens. This phenomenon is known as H-2 restriction (13).

Several of the cloned H-2 class I genes have been reintroduced into cells by transfection. These genes are transcribed and translated, and their protein products can function as restricting elements in CTL assays (14–16). By using genetically altered H-2 genes we and others (17–21) have identified the regions

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of the H-2 antigens recognized by CTL, both during virus infection and in an allogeneic response.

Recently, we have analyzed (19) a large number of individual T cell clones directed against either the H-2K^d or -K^k. That study confirmed that very few CTL recognize individual domains on the H-2 molecule (19). In the present report we describe a new series of H-2 hybrid antigens that have the COOH-terminal half of the α2 domain (α2B) exchanged between H-2K^d, -K^k, and -K^b. Our results show that the α1 domain and the NH_2-terminal half of the α2 domain (α2A) are required for recognition by CTL directed against the H-2K^k antigen. In contrast, for the H-2K^d antigen, the α2B domain plays a crucial role in T cell recognition.

Materials and Methods

Cell Culture and DNA Transfection of Cells. The recipient cell line used in this study was 1T 22-6 (H-2^q). Cells were grown in DMEM containing 10% FCS serum. Cells were transfected by a modification of the method of Wigler et al. (22), which has been described earlier (9). The neophosphotransferase gene (23) was used as the selectable marker, together with the antibiotic G-418 (Gibco Laboratories, Grand Island, NY).

FACS Analysis and Immunoprecipitation. For these techniques we have used the mAb H100-27.55 (24) to detect the H-2K^k antigen, as well as the products of the hybrid genes pJ2 and pJ5. For the analyses of the H-2K^d antigen and the hybrid gene products pJ1, pJ3, and pJ4, the mAbs K9-18 and 20-8-4S (Hämmerling, G., unpublished results and reference 25) were used. The method for labeling cells with these antibodies has been outlined before (26), and the cells ($1-2 \times 10^4$) were analyzed on a FACS II flow cytometer gated to exclude nonviable cells (27).

Construction of Hybrid Genes. The hybrid genes pJ1–pJ5 were constructed as described in the text and shown in the legend to Fig. 1. The gene pJ5 was constructed by using an endogenous Nco I site in the intron separating exons 3 and 4. The generation of the different subclones was done according to Frischauf et al. (28), and has been described in detail (9).

Generation and Assay of CTL. CTL from normal and alloimmune mice were generated as described in detail previously (29). Briefly, 25×10^6 spleen cells from normal mice (C3H or BALB/c) or alloimmune mice (A/J or BALB/c immunized intraperitoneally 3–4 wk previously with 10^8 viable BALB/c or A/J spleen cells, respectively) were cultured for 5 d with an equal number of irradiated (2,000 rad) stimulator spleen cells in 20 ml of DME containing additional amino acids, 5% FCS, and 5×10^{-5} M 2-ME. Recovered viable cells were assayed for cytotoxicity at various E/T cell ratios in a 3-h assay using ^{51}Cr-labeled transfected fibroblasts as targets. Percent specific ^{51}Cr release was calculated as described (29). Spontaneous ^{51}Cr release (in the absence of effector cells) was 10–20% of total incorporated radioactivity.

Other Materials. Restriction enzymes and other DNA-modifying enzymes were from Boehringer Mannheim, Schwalbach, Federal Republic of Germany; New England Biolabs, Beverly, MA; or Bethesda Research Laboratories, Bethesda, MD. Geneticin (G-418) was obtained from Gibco Laboratories. Radioactive isotopes were from Amersham Corp., Amersham, United Kingdom.

Results

Construction and Expression of H-2K Hybrid Genes. We have used the same method as described previously (28) to construct five new hybrid H-2K genes. To construct the hybrid gene pJ1, we used a subclone of a deletion in the 5′ to 3′ direction of the *H-2K*^{d} gene (9). This subclone, pK^d-6b was digested with the restriction enzymes Eco RI and Cla I (Fig. 1). This generates a DNA fragment

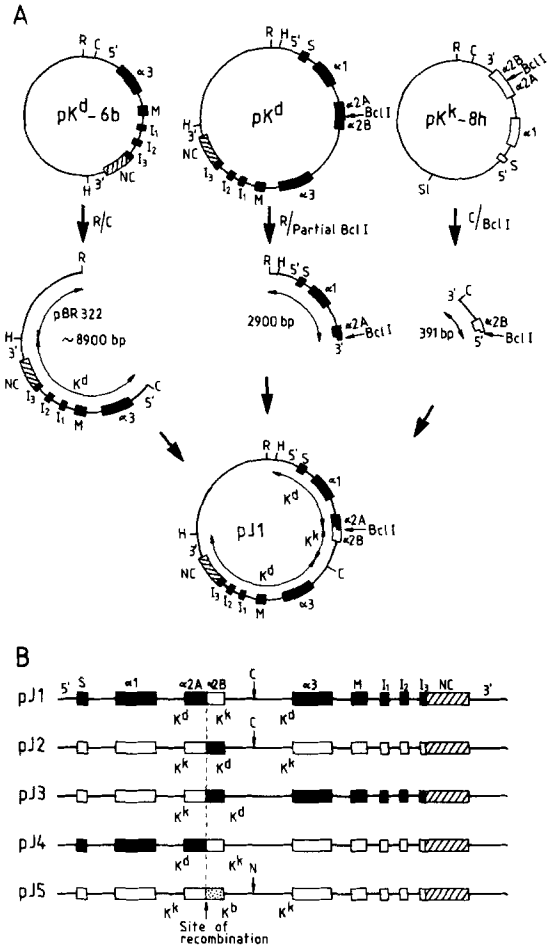

FIGURE 1. Construction of the H-2K hybrid genes pJ1-pJ5. (*A*) A subclone of the pK^d plasmid, pK^d-6b, was digested with Eco RI and Cla I restriction enzymes to generate a 8,900-bp fragment. This fragment contains exons 4–8 of the *H-2K*^{d} gene, as well as the entire pBR322 plasmid except for 23 bp between the Eco RI and Cla I sites. The pK^d plasmid was partially digested with Bcl I, followed by complete cut with Eco RI. This generates a 2,900-bp fragment containing the entire two first exons and part of the third of the K^d gene (α2A region). The 391-bp fragment containing the α2B region of H-2K^k origin was generated by digestion of subclone pK^k-8h (9) with Cla I and Bcl I. The three fragments were ligated together to yield pJ1. (*B*) We constructed plasmids pJ2, pJ3, pJ4, and pJ5 in much the same way as in *A*. (*A* and *B*) *Filled boxes* denote sequences (exons) of H-2K^d origin, whereas *open boxes* denote H-2K^k exons. The *hatched box* is the 3′ noncoding region. The plasmid pJ5 has its α2B region of H-2K^b origin (indicated by *dots*). The exon encoding the signal sequence is indicated by an *S*; exons 2, 3, and 4 are called α1, α2, and α3; the membrane spanning sequence is denoted by an *M* and the three cytoplasmic exons are called I_1, I_2, and I_3. The 3′ noncoding region is denoted *NC*. Restriction enzymes were: *R*, Eco RI; *C*, Cla I; *Sl*, Sal I; *H*, Hind III; and *N*, NcoI. Allele-specific sequences are indicated. See text for further details.

with the length of ~8,900 bp, and it contains exons 4–8 of the *H-2K*^{d} gene. In addition, it has almost the entire pBR322 plasmid sequences retained between the Hind III and Eco RI sites, and can therefore be used as a vector fragment for replication in *Escherichia coli*. The second fragment was generated from a

complete Eco RI cut of the pK^d plasmid, which had previously undergone partial digestion with the Bcl I enzyme. This 2,900 bp fragment contains the complete exons 1 and 2 and part of the third exon (the $\alpha 2$ domain). The break point in the third exon is at the Bcl I site at codon 142 of the mature H-$2K^d$ antigen. The second half of the $\alpha 2$ domain (COOH-terminal end) was isolated from a subclone of the *H-2K*^{k} gene, pK^k-8h, and is a Bcl I/Cla I fragment with a length of 391 bp. The three fragments were ligated together and we isolated recombinant plasmids. We determined the DNA sequences of the recombination sites and surrounding sequences. No unexpected rearrangements were found. The recombinant plasmid pJ1 carries H-$2K^d$ sequences, except for the COOH-terminal half of the $\alpha 2$ domain (codons 142–182), which is of H-$2K^k$ origin. Fig. 1 *A* shows a schematic outline of the procedure.

In a similar way we have constructed the plasmids pJ2, pJ3, pJ4, and pJ5 (Fig. 1 *B* and Materials and Methods). The hybrid genes were introduced into 1T 22-6 cells (H-2^q), together with the neophosphotransferase gene as a selectable marker. Cell clones resistant to the antibiotic G-418 were further examined for their expression of hybrid H-2K antigens. This was done by using two different techniques. First, the cells were analyzed in a FACS and, secondly, the molecular weights of the hybrid antigens were determined by SDS-PAGE. For both methods, we used mAbs against either H-$2K^d$ or -K^k. The first approach verified cell surface expression of H-2 hybrid gene products for all five gene constructs (Fig. 2). The second method revealed that the hybrid antigens had a correct molecular weight (data not shown). Thus, no major rearrangements had occurred in those regions of the gene that were not sequenced after the construction had been finished. From these experiments we conclude that H-2K hybrid molecules are expressed on the surface of the recipient cell line 1T 22-6 in amounts similar to the parental H-$2K^k$ and -K^d antigens (Fig. 2, compare *B* with *C* and *D*; compare *F* with *G*, *H*, and *J*).

Recognition of the Hybrid H-2K Antigens by CTL. When the entire $\alpha 2$ domain of either H-$2K^k$ or -K^d antigens is nonsyngeneic with the $\alpha 1$ domain, the cytolytic response is almost totally abolished for both influenza A–specific and allogeneic T cells (9, 19). To more precisely localize the region(s) that is crucial for CTL recognition, we have analyzed alloreactive T cells for their ability to lyse transfected cells expressing the new hybrid antigens.

Mice of the C3H strain (H-2^k) were immunized in vitro with BALB/c (H-2^d) splenocytes to generate CTL against the H-$2K^d$, -D^d, and -L^d antigens. Similarly, BALB/c mice were immunized with C3H cells to generate CTL against H-$2K^k$ and D^k. These two sets of primary CTL were analyzed for their ability to lyse ^{51}Cr-labeled target cells expressing the hybrid antigens.

Target cells expressing the pJ2 gene (J2-21 cells) were as well recognized by CTL directed against H-2^d as were those expressing the parental K^d antigen (Fig. 3 *A*). Control 1T 22-6 cells and cells expressing the pJ1 gene (J1-13 cells) (see Fig. 1 *B*) were not recognized by these CTL, whereas cells expressing the H-$2K^d$ antigen were lysed slightly more than background. The CTL directed against H-2^k specifically lysed K^k-expressing target cells and J2-21 cells (Fig. 3 *B*). Thus, the J2-21 cells were lysed by T cells directed against both H-$2K^d$ and K^k, despite the fact that only the COOH-terminal half of the $\alpha 2$ domain ($\alpha 2$B) was of K^d

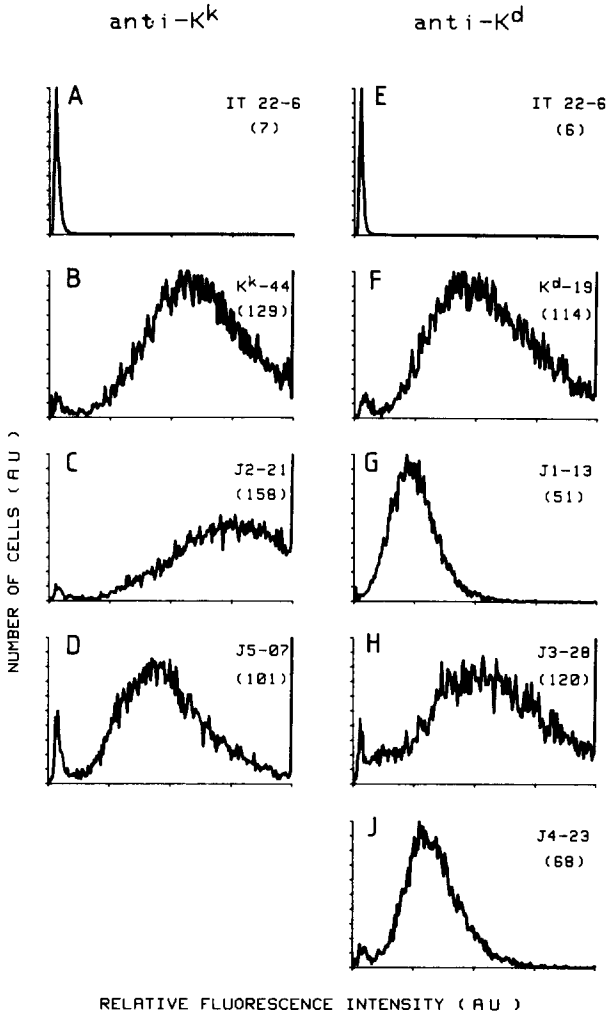

FIGURE 2. Cell surface expression of hybrid genes. Transfected 1T 22-6 cells were stained by indirect immunofluorescence using mAbs H100-27.55 (*A–D*) or 20-8-4S and K9-18 (*E–J*), and were analyzed by flow microfluorometry. Cell clones examined are indicated. The values given in brackets correspond to the mean fluorescence intensity.

origin. J1-13 cells, IT 22-6 cells, and cells expressing the H-2K^d antigen were not lysed by the anti–H-2K^k CTL.

As these CTL were raised in mouse strain combinations that differed also in the H-2D gene product, we could not exclude the possibility that anti–H-2D CTL crossreacted with the hybrid antigens. To rule out this possibility we raised additional CTL specific only for H-2K antigens (i. e., A/J anti-BALB/c [H-2K^k, D^d anti-H-2K^d/D^d] and BALB/c anti-A/J). In this experiment we included the J3-28 and J4-23 cells expressing the pJ3 and pJ4 genes, respectively, as additional controls. Also, to increase assay sensitivity, the T cells were primed in vivo before being restimulated in vitro.

The results clearly showed that J2-21 cells were lysed by anti-H-2K^d CTL

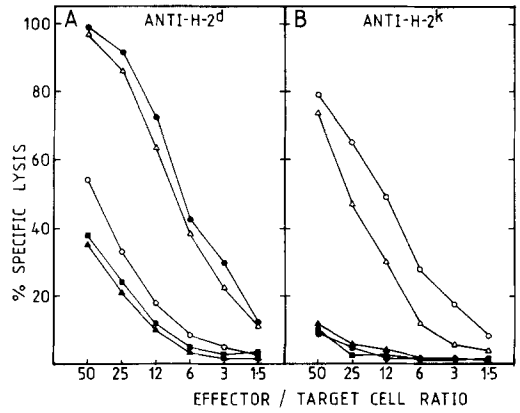

FIGURE 3. CTL recognition of cells expressing hybrid antigens. (*A*) Splenocytes from C3H mice immunized in vitro with irradiated BALB/c stimulator cells were assayed for cytotoxicity against the following targets cells: K^d-19 (*filled circle*); K^k-44 (*open circle*); J1–13 (*filled triangle*); J2-21 (*open triangle*); 1T-22-6 (*filled square*). (*B*) Splenocytes from BALB/c mice immunized as above with C3H cells, were assayed as in *A*. Symbols are the same as in *A*.

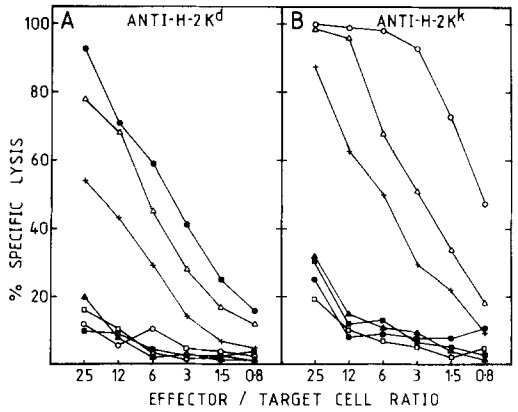

FIGURE 4. Analysis of cell lines expressing H-2K hybrid antigens with H-2K^d- and -K^k-specific CTL. (*A*) Splenocytes from A/J alloimmune mice were restimulated in vitro with BALB/c cells and were analyzed for their ability to lyse target cells expressing hybrid H-2K antigens. Target cells were: K^d-19 (*filled circle*); K^k-44 (*open circle*); J1-13 (*filled triangle*); J2-21 (*open triangle*); J4-23 (*open square*); J3-28 (*X*); 1T22-6 (*filled square*). (*B*) Spleen cells from BALB/c alloimmune mice were restimulated with A/J cells and analyzed as above. Symbols are the same as in *A*. See text for further details.

almost as efficiently as K^d-expressing cells (Fig. 4*A*). Furthermore, the J3-28 cells were lysed significantly over background. These cells express the pJ3 gene that contains the same α2B domain as the pJ2 gene (see Fig. 1*B*). The control 1T 22-6 cells, as well as J4-23 cells and K^k-expressing cells, were not lysed (Fig. 4*A*). Thus, this experiment excludes the participation of immunization to H-2D^d antigens as an explanation for the lysis of J2-21 cells by anti-H-2^d CTL. In a reciprocal experiment (Fig. 4*B*), CTL raised against H-2K^k lysed both J2-21 and J3-28 cells (although to a lesser extent than K^k-expressing cells), while control IT 22-6 cells and K^d-expressing cells were not lysed.

These results indicate two important structural differences between the K^d

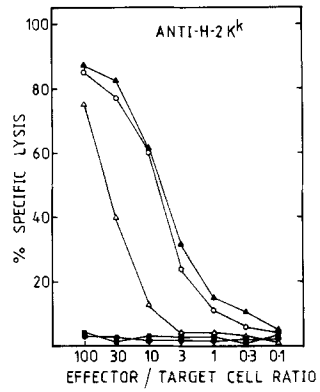

FIGURE 5. Influence of the α2B domain in recognition by anti-H-2K^k. Cytotoxic T cells were raised against the H-2K^k antigen by immunizing BALB/c mice with splenocytes from A/J mice. Target cells were: K^d-19 (*filled circle*); K^k-44 (*open circle*); J2-21 (*open triangle*); J5-07 (*filled triangle*); 1T22-6 (*filled square*). See text for further details.

and K^k antigens: (*a*) the α1 and α2A domains of the H-2K^k antigen can constitute a target molecule for anti-K^k CTL; and (*b*) the α2B domain of the H-2K^d antigen seems to play an essential role for recognition by anti-H-2K^d CTL.

Role of the α2B Domain for Anti-H-2K^k CTL. The results described above suggest that the α2B region of the second domain of the H-2K^k antigen is of little importance for recognition by anti-H-2K^k CTL. We decided to examine this further by analyzing J5-07 target cells expressing the pJ5 gene (Fig. 1*B*) where this region of the *K^k* gene has been replaced by the homologous region from the *H-2K^b* gene.

Interestingly, these target cells were lysed as efficiently as cells expressing the parental *H-2K^k* gene by CTL directed against K^k (Fig. 5). J2-21 cells were also lysed but to a lesser extent. 1T 22-6 cells and K^d-expressing cells were not lysed. This result indicates that the α2B region of the α2 domain is not of crucial importance for recognition by K^k-specific CTL or that the H-2K^k and -K^b antigens share considerable homology in this region. This will be discussed further below.

Discussion

In this paper we describe the construction of a novel series of hybrid genes involving H-2K^d and -K^k. The site of recombination is situated in the middle of the third exon encoding the α2 domain (Fig. 1, *A* and *B*). We have arbitrarily called the NH_2-terminal half of the α2 domain, α2A, whereas the COOH-terminal half is called α2B. We have introduced these new genes into the genome of 1T 22-6 cells (H-2^q) and established cell clones that stably express the corresponding H-2K hybrid antigens on their surface (Fig. 2).

Our aim in constructing the hybrid genes was to be able to analyze in greater detail the structural requirements for H-2K antigens to constitute target molecules for CTL. Therefore we examined cells expressing the hybrid antigens for their susceptibility to lysis by both anti-H-2K^d and anti-H-2K^k CTL. Surprisingly, J2-21 cells, which express the hybrid gene pJ2 and have H-2K^d sequences only in the α2B region of the α2 domain, were lysed by both types of CTL (Figs. 3 and 4).

We draw the following conclusions from these results: (*a*) the α1 and α2A domains of the H-2K^k molecule are important for recognition by alloreactive T cells, whereas the α2B domain seems of less importance; (*b*) the same domains (α1 and α2A) of the H-2K^d molecule do not seem crucial for recognition by anti-K^{d}T cells. This does not mean that these regions can be deleted or are not participating in structural domains recognized, but rather reflects a flexibility of the α2B region of the K^d antigen to accept a high degree of variability in the α1 and α2A domains; and (*c*) the α2B domain of the H-2K^d antigen is of crucial importance for alloreactive CTL directed against the K^d antigen. Cells expressing the pJ1 gene (Figs. 1*B*, 3*A*, and 4*A*) are not recognized by anti-K^d CTL, thus confirming the importance of the α2B domain. Similarly, J4-23 cells, which express the pJ4 gene, are not recognized by either set of T cells.

It is surprising that the product of the pJ2 gene is so readily recognized by anti-K^d CTL, as our previously described gene, PC35 (9, 19), which is of H-2K^d origin in the entire α2 domain, is not. This probably indicates that the pJ2 gene product has undergone a conformational change due to the α2A region being of H-2K^k origin. Such a change presumably does not alter the overall three-dimensional structure of the molecule, as evidenced by the fact that it is still recognized by T cells, but rather may expose structures of crucial importance for T cell recognition. We believe that further analyses of the product of the pJ2 gene might eventually lead to a better understanding of what T cells do recognize.

The α2B region of H-2K^k influences the recognition by alloreactive CTL only to a limited degree (Figs. 3*B* and 4*B*). Furthermore, by substituting the homologous segment of the *H-2K^b* gene for this region in J5-07 cells, lysis by anti-H-2K^k CTL was not affected (Fig. 5). In contrast, J2-21 cells are lysed (three to four times) less efficiently than J5-07 cells by anti-K^k CTL (Fig. 5). A careful analysis of the amino acid sequence for the three H-2K alleles in the α2B region reveals that H-2K^b and -K^k share several residues that are different from the H-2K^d antigen (Fig. 6; residues 144, 145, 155, 163, and 177). Three of these residues (positions 155, 163, and 177) represent nonconservative shifts and include charged amino acids. We consider it likely that one or several of these amino acids are involved either directly or indirectly (via a conformational change) in the different recognition pattern seen for the anti-K^k CTL in Fig. 5 (c.f., J2-21 cells and J5-07 cells). Interestingly, one of these residues (position 155) has been shown to be responsible for T cells being able to distinguish between H-2K^b and its mutant K^{bm1} (30–32). The total number of amino acid differences between K^b and K^k in the α2B region is five, of which at least four are nonconservative changes (Fig. 6; positions 152, 156, 173, and 174). The corresponding number for an H-2K^d/-K^k comparison is eight, with at least four involving charged amino acids (Fig. 6; positions 155, 156, 163, and 177). Given this limited number of possibilities, we hope that site-directed mutagenesis of some of these amino acids will allow us to identify the residue(s) responsible for allospecific T cell recognition in this system.

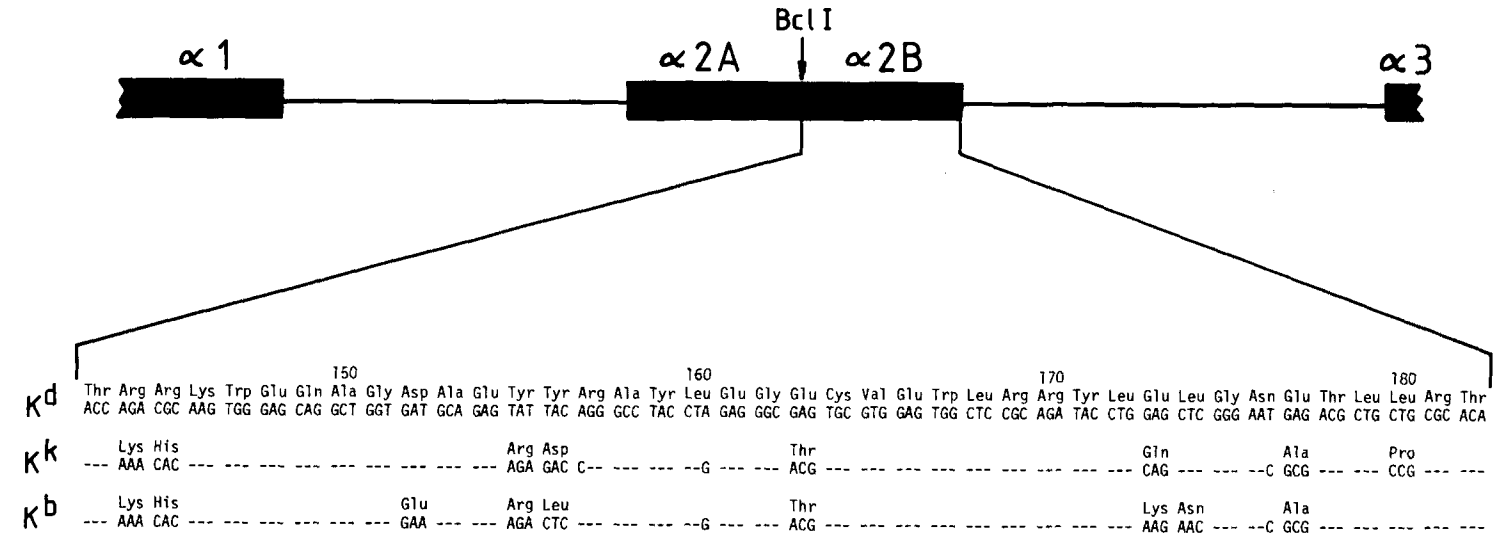

FIGURE 6. Amino acid comparison of the α2B domain of three H-2K alleles. Nucleotide and deduced amino acid sequences of the H-2K^d, -K^k, and -K^b antigens were compared for residues 142–182. Amino acid substitutions are indicated above the nucleotide sequences, whereas *dashes* denote identity with the H-2K^d sequence. Single nucleotide changes are also indicated.

Summary

We have constructed a new series of hybrid genes among the H-2K^d, -K^k, and -K^b. The site of recombination occurs in the third exon, encoding the α2 domain, and divides this domain into two parts, α2A and α2B. The novel genes differ only in the COOH-terminal half of the α2 domain, i. e., the α2B region. This region, comprising residues 142–182, contains a limited number of amino acid differences between the three alleles. The hybrid genes have been introduced into 1T 22-6 cells (H-2^q), and cell surface expression of hybrid antigens was verified. Cells expressing different types of hybrid antigens have been examined for their susceptibility to lysis by cytotoxic T lymphocytes directed either against the H-2K^d antigen or the H-2K^k antigen. Our results show that the α1 and α2A domains of the H-2K^k antigen can constitute target molecules for alloimmune anti–K^k T cells, whereas the α2B region, when exchanged for K^d or K^b sequences, plays only a limited role. In contrast, the α1 and α2A domains of K^d are not sufficient to be recognized by alloimmune anti–K^d T cells. In this instance, the α2B domain seems to play an essential role. This region has undergone several amino acid substitutions involving charged residues.

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