Comparison of the promoter regions of H-2K^b and H-2K^{bm1} class I MHC genes

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ABSTRACT

The 2.0 kb-long nucleotide sequences of the promoter regions of two closely related class I genes of the mouse major histocompatibility complex (H-2K^b and H-2K^{bm1}) have been determined and compared. The promoter sequence of the H-2K^{bm1} gene differs from that of the H-2K^b gene by a single deletion of a 'C' at position -456 in the upstream region of H-2K^{bm1} gene. The actual existence of this deletion of a single base in genomic DNA has been verified by genomic DNA hybridization, using oligonucleotide probes specific for H-2K^{bm1} or H-2K^b respectively. The effect on the enhancer activity of H-2K^{bm1} promoter region of the difference at position -456 has been analyzed by the chloramphenicol acetyltransferase (CAT) assay, using appropriate *DdeI* fragments (-533 to -408 for H-2K^{bm1}; -534 to -408 for H-2K^b) cloned downstream of pH-2(367)CAT gene construct. The CAT activity determined by the H-2K^{bm1} fragment was about 3-fold higher than that of H-2K^b, a result which probably accounts for the higher level of the H-2K^{bm1} transcript and antigen in lymph node cells.

INTRODUCTION

Spontaneous mutations affecting genes in the H-2 major histocompatibility complex (MHC) were discovered by Egorov, Bailey, Kohn and Melvold (1-4). Mice carrying such mutations display altered characteristics not only with respect to graft-rejection but also in cell-mediated lymphocytotoxicity, mixed lymphocyte reactivity, and H-2 associated recognition (5, 6). These mutations affect mainly the H-2K^b gene. The study of the K^{bm} series of mutants is relevant to attempt to define the origin of polymorphism in the MHC (1, 7, 8). For example, comparison of the structure of K^{bm} variant molecules with other class I sequences has suggested that the variants have been generated by block transfers of genetic information, possibly via gene conversion events (9). Weiss *et al.* (10) and Schulze *et al.* (11) have independently cloned and sequenced both the H-2K^b and the H-2K^{bm1} genes. Differences between coding sequences result

in three amino acid substitutions located on the edge of the peptide binding pocket (12). These substitutions account for the differential immunological properties of the H-2K^{bm1} antigen. However, differences are not restricted to the gene itself and its product, and can be found at the level of its expression. As shown in this paper, H-2K^{bm1} gene is 3 to 4 times more expressed than the original H-2K^b gene both at the mRNA and at the cell surface levels.

Recently, the promoter of mouse MHC H-2K^b class I gene was dissected in detail and various elements responsible for basal and inducible activities were characterized, together with the proteins that bind to these regions (13-18). Kimura *et al.* (13) have determined the nucleotide sequence of the H-2K^b gene up to 700 bp from the site of initiation of transcription. Elements controlling negatively MHC class I gene expression have been assigned to upstream regions (19, 20). In order to understand the origin of the enhanced expression of the H-2K^{bm1} gene, we have determined the nucleotide sequence of the promoter regions (about 2 kb) of the H-2K^b gene (clone C.1.4.1) from the mouse strain C57BL/6 and that of the mutant counterpart H-2K^{bm1} (clone E107.1.1). We have also compared the transcriptional activity of these promoter sequences.

MATERIALS AND METHODS

Cells and reagents — All restriction enzymes, T4 polymerase, T4 DNA ligase, Klenow large fragment (Pol IK) and T7 DNA polymerase, were obtained from Takara Shuzo Co., Kyoto, Japan. T4 polynucleotide kinase, dideoxy- and deoxy-nucleotides were obtained from Toyobo Co., Tokyo. Plasmid vector Bluescript SK II (-), exonuclease III and mung bean nuclease were from Stratagene, La Jolla, CA. Mouse 3T6 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco), L-glutamine (2 mM) and antibiotics.

H-2K^b and H-2K^{bm1} genomic clones (C1.4.1 and E107.1.1, respectively) have been kindly provided by Dr Nathenson and Dr Wallace (see ref. 12). *DdeI* fragments (-533 to -408 of the H-2K^{bm1} promoter region; -534 to -408 of the H-2K^b

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Figure 1. Compared cell surface expression of H-2K^b and H-2K^b^{m1} antigens. Panel A: MIH 030-210 anti-H-2K^d mAb (—); MIH 030-110 anti-H-2K^b mAb (---) on C57BL/6 K^b^{m1} lymph node cells. Panel B: MIH 030-110 anti-H-2K^b mAb assayed on C57BL/6 cells (—) and on C57BL/6 K^b^{m1} cells (---). Panel C: B8-24-3 anti-H-2K^b mAb assayed on C57BL/6 cells (—) and on C57BL/6 K^b^{m1} cells (---).

promoter region), which both contained the enhancer sequence III (13) were filled in with Pol IK and cloned in the *Bam*HI site of the plasmids pH-2K^b^{m1} (367)CAT, pH-2K^b (367)CAT (20) which contained both H-2 enhancer and promoter elements, and pTKCAT (ref. 14), to generate pH-2K^b^{m1} (367)CAT125, pH-2K^b (367)CAT126, pTKCAT126 and pTKCAT125, respectively.

Nucleotide sequencing, DNA and RNA analysis — C.14.1 and E107.1.1 DNAs were digested with *Hind*III and *Nru*I to permit isolation of the 2 kb promoter regions of each clone, and these were inserted into Bluescript SKII(–). Nucleotide sequences of the promoter regions of the H-2K^b and the H-2K^b^{m1} genes were determined by the exonuclease III deletion method (21) and dideoxynucleotide sequencing (22).

Oligonucleotide probes 20 mer specific for H-2K^b^{m1} (5'-GCT-TGCAGCCAGCGCCTTAA-3'), for H-2K^b, (5'-GCTTGCAG-CCCAGCGCCTTA-3') and common to all H-2K genes (5'-CGGTGGATGGAGCAGGAGG-3') (23) were synthesized on an automated solid-phase synthesizer (Applied Biosystem Inc.) by the standard phosphoramidite coupling method (24). Aliquots of 10 µg of genomic DNAs isolated from spleens C57BL/6 H-2K^b (4 weeks, males) and C57BL/6 H-2K^b^{m1} (4 weeks, males) mice were digested with *Eco*RI and transferred to a nitrocellulose membrane filter. The probe was radiolabeled with (γ-³²P)-ATP and T4 polynucleotide kinase to a specific activity

Figure 2. Comparative studies of the expression of H-2K^b genes. Northern blot analysis of total RNAs from C57BL/6.Kh (H-2^b) and C57BL/6.Kh (H-2K^b^{m1}) spleen cells probed with an H-2K specific probe. Lanes 1 and 3, 40 µg of total RNA from C57BL/6 (H-2K^b) mouse spleen cells; lanes 2 and 4, 40 µg of total RNA from C57BL/6 (H-2K^b^{m1}) mouse spleen cells. Left panel shows the class I RNA (1.6 kb) and right panel shows the β-actin RNA (1.9 kb).

Figure 3. Nucleotide sequence (*HindIII-NruI* fragment) of the promoter region of the H-2K^{bm1} gene (E107.1.1) from C57BL/6.Kh.H-2K^{bm1} mouse spleen cells. The difference is boxed. The cap site is +1 and sequences I to VIII are underlined, as are the TATA box and CAAT box (13). The actual sequence data are shown in panel B.

of $1-5 \times 10^8$ cpm/ μ g. Hybridization was performed for 20 hr at 59°C in $6 \times \text{SSC}$ (0.15 M NaCl, 0.01 M sodium citrate), 0.1% SDS, $6 \times \text{Denhardt's}$ buffer. The filter was washed three times for 30 min at 59°C in $6 \times \text{SSC}$ and 0.1% SDS. Total cytoplasmic RNA isolated by the guanidinium isothiocyanate method (25) was subjected to Northern blotting in the presence of 2.2 M formaldehyde (40 μ g of RNA per lane). The gel was blotted onto a nitrocellulose filter and probed with in H-2K cDNA specific probe (26) radiolabeled with (α - ^{32}P)-dCTP, using Amersham's random primer labeling kit (specific activity: 5×10^8 cpm/ μ g). Hybridization was performed in 50% (v/v) formamide at 42°C for 20 hr. The filter was washed three times at 65°C in the presence of $6 \times \text{SSC}$ and 0.1% SDS and autoradiographed on Fuji RX-film for 24 hr at -80°C .

Transfection and CAT assay — 3T6 cells were transfected with 18 μ g of pH-2K^b(367)CAT126 or pH-2K^{bm1}(367)CAT125 and 2 μ g of pCH110 (27) by the calcium precipitation method (28). Forty eight hours after transfection, cell extracts were assayed for both CAT activity and β -galactosidase activity as described elsewhere (29, 30). Activity was expressed as the percentage of chloramphenicol converted to the 3-acetyl form by 25 μ l of extract, in 1 hr at 37°C , and the ratio of CAT activity to β -galactosidase activity was used for normalization of the results.

Flow cytometry analysis — Single cell suspensions were prepared from mesenteric lymph nodes of C57BL/6.Kh H-2K^b mice (4 weeks, male) and from the mutant counterpart C57BL/6.Kh H-2K^{bm1} (4 weeks, males). The cells were washed twice in RPMI-1640 and once in PBS with 3% FCS. The following monoclonal antibodies were used for staining: FITC-conjugated anti-H-2K^d mAb (MIH 030-210, Meinyu, Inc., Tokyo), FITC-conjugated B8-24-3 anti-H-2K^b mAb (ATCC, Rockville, MA) and FITC-conjugated anti-H-2K^d mAb (MIH 030-110, Meinyu, Inc., Tokyo). Incubation and washings were carried out at 4°C . Cells (5×10^5) were incubated twice in PBS 3% FCS and analyzed for the expression of H-2K^b and H-2K^{bm1} antigens on a FACStar II flow cytometry (Becton Dickinson, Mountain View, CA) gated to exclude non viable cells.

RESULTS

Although C57BL/6.Kh (H-2K^b) and (H-2K^{bm1}) mice share the same genetic background, the level of H-2K^{bm1} class 1 antigen detectable at the surface of lymph node cells is 2–4 times higher than the level of H-2K^b antigen, as shown by the results of flow cytometry analysis on age and sex matched animals (Fig. 1). This FACS analysis was repeated three times, using anti-H-2K^d mAb as a control (panel A) and two different anti-H-2K^b mAbs both independent of the bm1 mutation, thus assuming to detect H-2K^b and H-2K^{bm1} antigens in the same manner. Moreover, the amount of the H-2K^b and H-2K^{bm1} mRNAs probed with an H-2K specific DNA probe, is constantly found higher in H-2K^{bm1} than in H-2K^b cells (Fig. 2).

Since H-2K^{bm1} gene differs from H-2K^b by several mutational events within the coding sequences (10, 11), the regulatory region of H-2K^{bm1} could also have been structurally altered. Thus, the DNA sequences of the promoter regions of both H-2K^b and H-2K^{bm1} genes were determined. The nucleotide sequence of the H-2K^{bm1} promoter is shown in Figure 3. It differs from that of the promoter of the H-2K^b gene by a single deletion of 'C' at position -465. The rest of the H-2K^{bm1} promoter is identical to that of H-2K^b. To confirm the presence of this single deletion in the H-2K^{bm1} promoter of the uncloned gene, several specific oligonucleotide 20-mers were synthesized as probe, and used in Southern blot hybridization of genomic DNAs. Figure 4 demonstrates that the *Eco*RI 10.5 kb restriction fragment generated from H-2K^{bm1} gene hybridized with the H-2K^{bm1} specific oligodeoxynucleotide probe (5'-GCTTGCAGCCAGCGCCTTAA-3'), whereas the *Eco*RI digested DNA from a H-2K^b mouse did not (Figure 4A). A Southern blot hybridization experiment using the H-2K^b specific oligodeoxynucleotide probe (5'-GCTTGCAGCCAGCGCCTTAA-3') revealed the same 10.5 kb restriction band in H-2K^b DNA and not in H-2K^{bm1} DNA (Figure 4B). The oligodeoxynucleotide probe (5'-CGGTGGATGGAGCAGGGAGGG-3') common to both H-2K^b and H-2K^{bm1} genes revealed an identical restriction fragment in both H-2K^b and H-2K^{bm1} DNAs (Figure 4C). These results indicate that the deletion of 'C' at position -456 of the H-2K^{bm1} promoter (or the insertion of a 'C' in the H-2K^b

Figure 4. Southern blot analysis of DNAs from C57BL/6.Kh.(H-2^b) and C57BL/6.Kh.(H-2^{bm1}) mouse spleen cells. DNA (20 μ g) was digested with *Eco*RI. Lanes 1 and 3, DNA from C57BL/6.H-2K spleen cells; lanes 2 and 4, DNA from C57BL/6.H-2K^{bm1} spleen cells. Panel A, the ethidium bromide staining of DNAs. The nitrocellulose filter was probed with a ³²P-labeled oligonucleotide 20-mer, specific for H-2K^{bm1} (panel B, lanes 1, 2), H-2K^b (panel B, lanes, 3, 4) and common H-2K (panel C) respectively.

promoter) is indeed present at the mouse genomic DNA level and thus is not the result of propagation of the cloned gene in bacteria.

In an attempt to correlate this deletion with the enhanced expression of the H-2K^{bm1} gene, we have constructed a set of fusions with the coding region of the chloramphenicol acetyltransferase (CAT) gene and tested these constructs in a short-term transfection assay (29). We isolated the respective *DdeI* fragments (−533 to −408 for the H-2K^{bm1} promoter, −534 to −408 for the H-2K^b promoter) which each contained the enhancer sequence III, without and with the 'C' of interest. Each fragment was inserted into the plasmid pH-2K^b(367)CAT (20) to generate the pH-2K^b(367)CAT126 and pH-2K^{bm1}(367)CAT125, respectively, and transfected into mouse 3T6 cells. As shown in Figure 5B, the cloned fragments further stimulate the basal level obtained with the pH-2K^b(367)CAT construct, and the stimulation caused by the H-2K^{bm1} fragment is about 3-fold higher than that generated by the H-2K^b fragment (Figure 5). This result indicates that the deletion of 'C' at position −456 is probably responsible for the higher promoter activity of the H-2K^{bm1} gene. The site of initiation of transcription of each construct was determined by the primer extension method and found to be identical to the previously determined one (13).

DISCUSSION

The bm1 mutant was the first affecting the MHC to be described. It was detected in a (C57BL/6 By X.BALB/.cBy) heterozygote (2). The mutation which alters the H-2K^b gene has been transferred onto the C57BL/6.Kh background and is by now usually maintained as C57BL/6.Kh H-2K^{bm1} strain, its 'normal' counterpart being therefore the C57BL/6.Kh H-2K^b mouse strain. The amino acid substitutions that distinguish the variant H-2K^{bm1} glycoprotein from the standard H-2K^b glycoprotein have been deduced from the nucleotide sequences of the genes.

A block of seven nucleotide differences were detected in H-2K^{bm1}, which accounts for three amino acid substitutions: glutamine to alanine at position 152; arginine to tyrosine at position 155; leucine to tyrosine at position 156. No other difference was found within the genes proper (10, 11). In a variety of immunological assays (such as cell mediated cytotoxicity, mixed-lymphocyte reactivity, and tissue grafting (31), changes in the ability to present antigen in viral (32), chemical (33) and minor histocompatibility antigen assays (34)), the bm1 variant exhibits reactivity that differs from that of the parent. These immunological differences are accounted for by the above mentioned differences in the coding sequence which alters locally the edges of the peptide binding pocket of the H-2K molecule (12). In addition, H-2K^{bm1} gene is about 3 times more actively transcribed than H-2K^b gene. We show in this paper that the deletion of a single 'C' at position −465 is the sole difference between the two promoter regions derived from the H-2K^{bm1} and H-2K^b genes present in C57BL/6.Kh H-2K^{bm1} and H-2K^b, respectively. Among the several possible reasons for this enhanced expression of H-2K^{bm1} gene, one most probably lies within the previously undetected enhancer-like region (−533 to −408) which is about 3 times more active when the C at −456 is deleted, like in the H-2K^{bm1} promoter which we report here. The H-2K^{bm1} antigen is also more expressed at the surface of lymph node cells. The enhanced expression can be directly caused by enhanced transcription of the gene. Differences in the structure of the protein might also lead to a differential expression of the genes production.

The origin of the single mutational event we have found in the 5' promoter region of the H-2K^{bm1} gene is unclear. The clustered changes within the coding sequence are believed to originate from Q10 gene (10). However, no sequence homologous to the nucleotide sequence surrounding the deleted 'C' can be found in 2.0 kb of the Q10 promoter region (David-Watine, unpublished). Thus, this mutational event is unlikely to have been caused by the same genetic event which created the

Figure 5. CAT activity detected after transfection of the constructs into mouse fibroblast 3T6. *DdeI* fragments were isolated from either H-2K^b (−534 to −408) or H-2K^{bm1} (−533 to −408) genes and ligated with either pH-2K^b(367)CAT or pTKCAT (ref. 13) by addition of *Bam*HI linkers. The CAT activity was determined as described elsewhere (29). Lane 1, pH-2K^b(367)CAT126; lane 2, pH-2K^{bm1}(367)CAT125; lane 3, pTKCAT126; lane 4, pTKCAT125; lane 5, pH-2K^b(367)CAT; lane 6, pH-2K^{bm1}(367)CAT; lane 7, pSVOCAT; lanes 8 and 9, pActinCAT. The relative activities are indicated in the lower part of the figure.

bm1 mutations. It might well have preexisted to the bm1 mutation in the C57BL/6. By animals used and, therefore, be independent of the bm1 mutation itself. Anyhow, further investigations are necessary to characterize the molecular and cellular effects of this deletion: is it related to the binding (or the absence of binding) of a specific transacting factor? Does this region interact specifically with the proximal H-2K^b promoter region (0 to -367) or with any other promoter? Whatever the answers are, these observations can clearly account for the higher transcriptional activity of H-2K^{bm1} gene.

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