Immunogenetics 34: 28–38, 1991

Immuno-
genetics

Developmental and tissue-specific expression of the $Q5^k$ gene

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Received November 12, 1990; revised version received January 24, 1991

Abstract. Expression of the $Q5^k$ gene was examined by northern blot analysis and polymerase chain reaction (PCR) in the AKR mouse and various cell lines, each of the $H\text{-}2^k$ haplotype. Our results show that $Q5^k$ mRNA is present during the whole postimplantational development of the AKR embryo/fetus (gestation day 6 to 15). In the juvenile mouse (week 2 to 4) transcription of the $Q5^k$ gene persisted in all organs examined. In contrast, in the adult animal expression of the $Q5^k$ gene was limited to the thymus and uterus of the pregnant mouse. Upon malignant transformation, the amount of $Q5^k$-specific mRNA increased dramatically in thymus and could also be observed in the spleen of thymoma bearing animals. Expression of the $Q5^k$ gene was also detectable in several transformed mouse cell lines. Mitogen stimulation or treatment with cytokines induced $Q5^k$ expression in primary spleen cell cultures. A possible explanation for the tissue-restricted expression in the adult AKR mouse is discussed.

Introduction

The majority of the mouse major histocompatibility complex (Mhc) class I genes are encoded by the *Q/TL* region, localized telomeric to the *H-2* complex. Both the *Q* and *TL* subregions vary significantly among different inbred mouse strains. The *Q* region contains between one and ten loci (Weiss 1987). Class I gene products of this region are similar to the H-2K antigen in respect of nucleotide homology, antigenic crossreactivity, association with β_2-microglobulin, and peptide structure (Michaelson et al. 1977; Stanton and Hood 1980; Soloski et al. 1981; Klein 1986). However, the *Q* genes differ from classical transplantation genes, which function as restriction elements for cytotoxic T lymphocytes, in several respects. Unlike the classical class I antigens all of which are transmembrane proteins, the *Q* region products are found in two different forms: as secreted molecules (Kress et al. 1983; Devlin et al. 1985a) and as cell surface molecules anchored in the membrane via phosphatidylinositol (PI) linkage (Stiernberg et al. 1987; Stroynowski et al. 1987; Waneck et al. 1988). In addition, their expression is limited to only a few tissues of hematopoietic origin. Compared with the highly polymorphic *H-2* alleles, the *Q* region genes display no polymorphism (Steinmetz et al. 1981; Mellor et al. 1984; Devlin et al. 1985b; Klein 1986; Weiss 1987; Flaherty et al. 1990; Tine et al. 1990).

The exact function of genes mapping to the *Q/TL* region, or that of the gene products is so far unknown, and there is little information available on their expression pattern in adult and embryonic tissues (Fahrner et al. 1987). It seems to be evident, however, that T-cell receptor (Tcr) $\gamma\delta$-positive T lymphocytes can recognize nonclassical class I antigens of the *TL* region (Haas et al. 1990; Ito et al. 1990), possibly induced by transformation and infection (Janeway et al. 1988).

The *Q* region of the AKR ($H\text{-}2^k$) mouse is comprised of six genes: $Q1^k$, $Q2^k$, $Q3^k$, $Q4^k$, $Q5^k$, and $Q10^k$ with the *Q5* gene being directly linked to the *Q10* gene (Weiss et al. 1989). The organization and structure of the AKR-*Q* family is therefore virtually identical to the *Q* genes of the C3H ($H\text{-}2^k$) mouse (Watts et al. 1989). The $Q5^k$ gene of the AKR mouse has some unique features: It codes for a classical transmembrane domain almost identical to the hydrophobic membrane anchor of the $H\text{-}2D^d$ gene, and thus, it should determine an integral cell surface glycoprotein. Since the unique restriction map of the AKR $Q5^k$

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gene points to the absence of a direct counterpart in the C57BL/10 haplotype, this gene might constitute the first polymorphic class I gene localized in the *Q* region.

To further elucidate the biological and functional role of the $Q5^k$ gene, it is essential to first investigate the expression of this gene in the AKR mouse. Therefore, we studied the expression of the $Q5^k$ gene in a wide spectrum of different juvenile and adult AKR tissues, primary tumor, tissue, and tumor, respectively tumor producing cell lines and one embryonic cell line. In addition, we focused on the developmentally regulated activation in postimplantation mouse embryos. A regulational mechanism possibly responsible for restricted $Q5^k$-expression in the adult animal was also studied.

Materials and methods

Cell culture. The high tumor producing (sarcoma) cell line NCTC 2472 (ATCC CCL 11, derived from connective tissue culture of a male C3H/HeN mouse) and low tumor producing cell line NCTC 2555 (ATCC CCL 12, derived from connective tissue of a male C3H/HeN mouse), the mouse lymphoma cell line RI.I (ATCC TIB 42, derived from a spontaneously arising thymoma of a C58/J mouse, $H\text{-}2^k$), the embryonic cell line C3H/10T1/2 (ATCC CCL 226, derived from a line of C3H mouse embryo cells), the AKR-derived spontaneous leukemia cell lines 110, 339, 344, 365, 369, 389, 422, 424, and 439 (AKR-cell lines, originally established by P. Kramer, Heidelberg, were obtained from W. Schmidt, Essen), and the human Jurkat T-cell line were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco-BRL, Eggenstein, FRG). Mouse spleen cell cultures were grown in RPMI 1640 supplemented with 10% FCS, 2 m*M* L-glutamine, and 5×10^{-5}M β-mercaptoethanol. For the stimulation of the mouse cell lines recombinant mouse IFN-γ (Genzyme, Boston, MA) was used at a final concentration of 100–1000 U/ml, and recombinant murine IFN-α/β (Genzyme) at a final concentration of 250 U/ml (for 6, 12, and 24 h). Phytohemagglutinin (PHA; Sigma, Deisenhofen, FRG) at a final concentration of 2 µg/ml, lipopolysaccharide (LPS; Sigma) at a final concentration of 1 µg/ml, and Concanavalin A (ConA; Sigma) at a final concentration of 2.5 µg/ml were used for the stimulation of mouse spleen cells.

The Jurkat cell line was stimulated with PHA at a final concentration of 10 µg/ml, and with PMA at a final concentration of 50 ng/ml.

Embryonic material. Spontaneously ovulating female Balb/c ($H\text{-}2^d$) mice were caged with AKR ($H\text{-}2^k$) males overnight and examined for the presence of a vaginal plug the following morning. The day of plug detection was taken as day 0 of embryo development and the approximate age of embryos calculated accordingly. Pregnant females (obtained from two animal facilities) were killed by cervical dislocation and several stages of embryonic development from postimplantation pregnancy were examined. Entire conceptuses were dissected from the decidual shells of the 6-, 8-, 9-, 10-, 14-, and 15-day pregnant mouse uterus and placed in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. Conceptuses from day 14 on were dissected into extra-embryonic tissues and embryo proper, 15-day embryos were dissected in embryonic liver, the main hematolymphopoietic organ, and body trunk. Immediately after dissection, embryos were frozen and kept at a temperature of −70 °C until RNA preparation was performed.

Northern blot analysis. Total RNA from embryonic tissues, adult tissues, and cell lines was prepared according to the guanidinium thiocyanate procedure as described by Chirgwin and co-workers (1979) and by sedimentation through a cesium chloride gradient. Alternatively, RNA from cell cultures was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). Poly $(A)^+$ RNA was purified by oligo (dT) cellulose chromatography. 10–20 µg total RNA or 1 µg poly $(A)^+$ RNA were separated on formaldehyde/1% agarose gels and then transferred to Hybond-N membranes (Amersham, Braunschweig, FRG).

Restriction fragments. The 3′ and 5′ *Bam* HI fragments of the D^b gene (Weiss et al. 1984); human 1.6 kilobase (kb) actin cDNA; murine β_2m cDNA (Morello et al. 1982); β_2m has been shown to be synthesized even in early cleavage stage embryos (Sawicki et al. 1981) were labeled according to Feinberg and Vogelstein (1984) and hybridization and washing of the filters were performed following standard protocols (Church and Gilbert 1984). Purification of the Q5*- (derived from exon 4 of the $Q5^k$ gene, Fig. 7B), the K^k- (TGGCGATCTGCGTGTTCC, derived from exon 2 of the K^k gene), and the D^k- (TTCAACTT-TAGATCTGGGGTGAT, derived from exon 4 of the D^k gene) oligonucleotides and hybridization at a temperature of 60 °C for the Q5*- and D^k-, respectively 55 °C for the K^k-oligonucleotide was done as described by Cate and co-workers (1986). The probes were labeled with terminal desoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, FRG) and α^{32}P-dATP (Amersham; Collins and Hunsaker 1985). Washing was done at hybridization temperature with 3 × standard sodium citrate (SSC), 10 m*M* NaH_2PO_4/Na_2HPO_4, 5% sodium dodecyl sulfate (SDS) for 2 × 20 min and with 1 × SSC, 1% SDS for 20 min. The K^k-oligonucleotide is specific for the K^k gene, since it does not hybridize to liver RNA isolated from a Balb/c ($H\text{-}2^d$) mouse and also gives no signal with cell line 422, shown not to transcribe the endogenous K^k gene (Fig. 6). The D^k-oligonucleotide was shown to be D^k-specific by hybridization to isolated AKR class I genes and to liver RNA of various inbred mouse strains (data not shown).

Reverse transcription (RT) and polymerase chain reaction (PCR). RNA was transcribed into cDNA by the following standard reaction. The listed reagents were dispensed 0 °C into a microcentrifuge tube: 10 µl of 5 × M-MLV reverse transcriptase (RT) buffer (250 m*M* Tris/HCl, pH 8.3, 375 m*M* KCl, 50 m*M* DDT, and 15 m*M* $MgCl_2$); 1 µl (5 µg/µl) of nuclease free borine serum albumin (BSA), 2.5 µl of nucleotide solution (10 m*M* each of dATP, dGTP, dCTP, dTTP in 10 m*M* Tris/HCl, pH 7.5), 5 µl RT-primer (100 pM), 1–2 µg of heat denatured RNA in sterile destilled H_2O, sterile destilled H_2O to 49 µl, and 1 µl of M-MLV RT (BRL: Darmstadt, FRG, 200 U/µl.) The reverse transcription assay was incubated at 37 °C for 1 h. Standard PCR was performed as described by Saiki and co-workers (1988). A portion of the RT-reaction (5 µl) was mixed with 1 unit of *Taq* polymerase (8 units/µl, US Biochemicals, OH), 5′ and 3′ sequence specific oligonucleotide primers (0.4 µ*M*), nucleotide solution (0.1 m*M* each of dATP, dGTP, dCTP, dTTP) in a buffer containing 10 m*M* Tris/HCl, pH 8.4, 1 m*M* $MgCl_2$, 50 m*M* KCl in a 50 µl volume. The mixture was overlaid with mineral oil to prevent evaporation, and after denaturation (95 °C, 5 min) 30 cycles of amplification were carried out on a programmable heating block (Biometra, Göttingen, FRG), followed by 10 min final extension at 72 °C (temperature profile for the $Q5^k$ amplification: 94 °C – 1 min denaturation, 62 °C – 1 min annealing, 72 °C – 1 min extension). Booster PCR was performed as described by Ruano and co-workers (1989). In this biphasic amplification strategy primers were diluted to low concentrations (pM) during the first amplification step, and then raised to 0.1 µ*M* at the beginning of the second round of amplification. A portion of the PCR mixture (10–15 µl) was added to a loading dye mix and electrophoresed in a 80 V constant field in 2% agarose gels. DNA separated on agarose gels was transferred to Hybond-N + membrane (Amersham). The filters were hybridized with the $Q5^k$-specific Q5*- oligonucleotide as described.

RT primer. CCACCTGTGTTTCTCCT, spanning exon 5 and 6 of the $Q5^k$ gene. $Q5^k$-amplification primer: 5′-primer (Q5 I): ATGTGTGAA-

GAGGCTCCTGGAT, derived from exon 3 of the $Q5^k$ gene, 3′-primer (Q5 II): GTGTTAGTCTTGGTGGATGAAG, derived from exon 5 of the $Q5^k$ gene (Fig. 7B).

Electrophoretic mobility shift assay (EMSA). Stimulated phytohemagglutinin (PHA 5 µg/ml, TPA 50 ng/ml, for 4 h) and nonstimulated Jurkat T-cells were harvested, washed in ice-cold phosphat-buffered solution (PBS), and then lysed in hypotonic buffer A (Dignam et al. 1983). Nuclear and cytosolic fractions were prepared as described (Baeuerle and Baltimore 1988). Annealed DNA-primers were labeled with α^{32}P-dATP (Amersham) by the Klenow enzyme (Boehringer Mannheim), and then electrophoretically purified on 3% low melting agarose gels, extracted with Tris (TE) EDTA saturated phenol, followed by chloroform/isoamylalcohol (24:1) extraction, precipitation, and finally dissolved in 10 m*M* Tris/HCl, pH 7.5, 0.5 m*M* ethylenediaminetetraacetate (EDTA). Conditions for electrophoretic mobility shift assays were essentially as described by Sen and Baltimore (1986). The purification of NF-kB from human placenta is described by Zabel and coworkers (1990), the isolation of p50 and p65 from affinity-purified NF-kB by SDS-gel electrophoresis and subsequent renaturation is described by Baeuerle and Baltimore (1989). The amounts of the two DNA-binding forms of NF-kB were calculated from the amount of protein-DNA complex, which was formed in EMSAs with a DNA probe of known specific activity under saturating concentrations of DNA. The sequences used for the investigations are: A (Fig. 8): $Q5^k$ motif (Q): 5′AGCTTCCCAGGGCTAGGGATTCCCCATCTCCCCAG3′, *HLA-A2* motif (A2): 5′AGCTTCCCAGCCTTGGGGATTCCCCAACTCCGCAG3′, kB motif (kb): 5′TCTGAAGCTTCAGAGGGACTTTCCGAGAGGTCGA3′ (Sen and Baltimore 1986); minimal binding site in the center. B (Fig. 9): $Q5^k$ motif: 5′TCGAGGGCTAGGGATTCCCCATCT3′, $H\text{-}2K^b$ motif: 5′TCGAGGGCTGGGGATTCCCCATCT3′.

Results

Expression of the $Q5^k$ gene in the juvenile and adult AKR mouse. Investigation of tissue expression of the $Q5^k$ gene was performed using the previously described $Q5^k$-specific Q5*-oligonucleotide (Weiss et al. 1989). This sequence is unique to the *Q5* gene encoded by $H\text{-}2^k$ haplotypes. Total RNA preparations of different organs from two, three, and four week old and adult (ten weeks or older) AKR mice were tested by northern blot analysis. Control hybridizations were carried out with a mouse class I probe [either the 3′ or 5′ *Bam* HI fragments of the D^b gene or with the K^k-specific oligonucleotide (K^k)]. Integrity and amount of mRNA applied was determined by hybridization with an actin or a β_2-microglobulin probe.

While constant and low levels of the $Q5^k$ message were observed in the heart, liver, and spleen of two, three, and four week old AKR mice (Fig. 1, lanes 1 to 8), in the thymus, $Q5^k$ mRNA level dramatically increased from week 2 to 3 (Fig. 1, lanes 9 to 11).

Adult AKR tissues could be divided into two groups: tissues without detectable $Q5^k$ expression (lung, liver, heart, kidney, uterus, and skeletal muscle) and tissues with $Q5^k$ expression (uterus of the pregnant animal, three animals of different pregnancy duration were tested; thymus; thymomas, isolated from two animals; spleen of the animals with thymomas; Fig. 2). Even after over-exposure no signal was detectable in negative tissues. Notably, the highest levels of $Q5^k$ message were detected in the thymomas and spleen cells of adult AKR mice with thymomas (Fig. 2, lanes 3, 4, and 12).

$Q5^k$ gene expression in mouse cell lines. The high level of $Q5^k$ mRNA in the primary AKR thymomas led us to investigate $Q5^k$ transcription in cell lines established from primary AKR leukemias (cell lines 110, 339, 344, 365, 369, 389, 422, 424, and 439; Fig. 3). The level of K^k mRNA correlated with the even lower amount of D^k

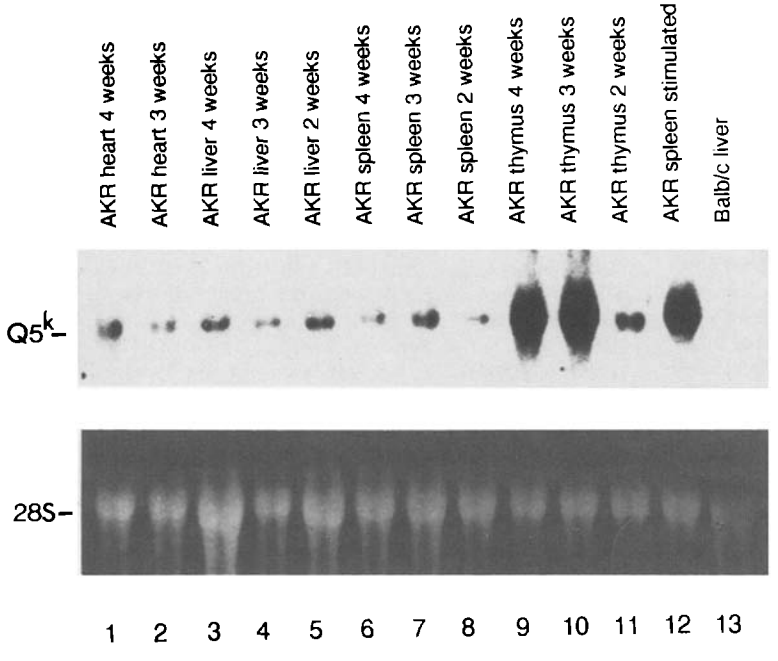


Fig. 1. Detection of $Q5^k$ mRNA in different organs (heart, liver, spleen, and thymus) of juvenile AKR mice (week 2 to 4). 8–12 µg of total RNA were probed with the Q5*-oligonucleotide (*upper panel*). Ethidium bromide staining of the 28S is shown in the *lower panel*. Liver RNA isolated from an adult BALB/c ($H\text{-}2^d$) mouse (lane 13) was applied as negative control. RNA isolated from adult AKR splenocytes (stimulated with PHA) was loaded as positive control (lane 12).

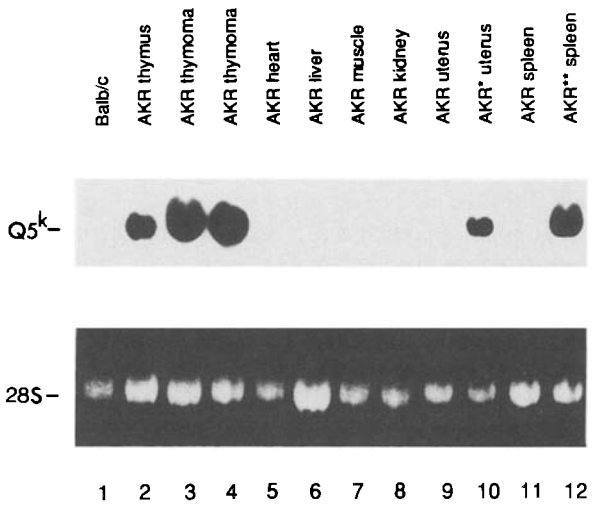


Fig. 2. Expression of the $Q5^k$ gene in tissues of adult AKR mice. 8–12 µg total RNA isolated of thymus, heart, liver, skeletal muscle, kidney, uterus, and spleen was probed with the Q5*-oligonucleotide (*upper panel*); in the *lower panel* ethidium bromide staining of the 28S is shown as control of the amount of RNA applied. In addition to normal AKR tissue, primary thymoma (isolated from two different animals; lane 3 and 4), spleen tissue removed from one of the animals with thymoma (**, lane 12), and uterus from one pregnant animal (*, lane 10) were also tested for $Q5^k$ expression.

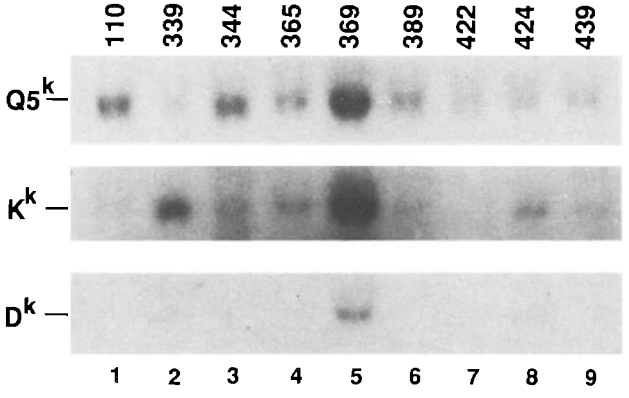


Fig. 3. Expression of the $Q5^k$, $H\text{-}2K^k$, and $H\text{-}2D^k$ genes in AKR leukemic cells. 10 µg total RNA per lane was tested by northern blot analysis with the K^k-oligonucleotide (*middle panel*). After removal of the probe the filter was rehybridized with the D^k-oligonucleotide (*lower panel*), and finally with the Q5*-oligonucleotide (*upper panel*).

transcripts (see Fig. 3, compare cell lines 339, 369, 424). This result is in agreement with the previously observed $H\text{-}2K^k$ and $H\text{-}2D^k$ cell surface expression (Schmidt et al. 1985), but no correlation was found with regard to the amount of $Q5^k$ mRNA detected in these AKR leukemias. Additional tumor, respectively tumorigenic cell lines, and one embryonic cell line derived from different mouse strains each of the $H\text{-}2^k$ haplotype were also tested (RI.I, NCTC 2472, NCTC 2555, and C3H; Fig. 4). Northern blot hybridizations with the Q5*-oligonucleotide revealed that the $Q5^k$ gene is transcriptionally active in all these cell lines, with the highest mRNA level detectable in the

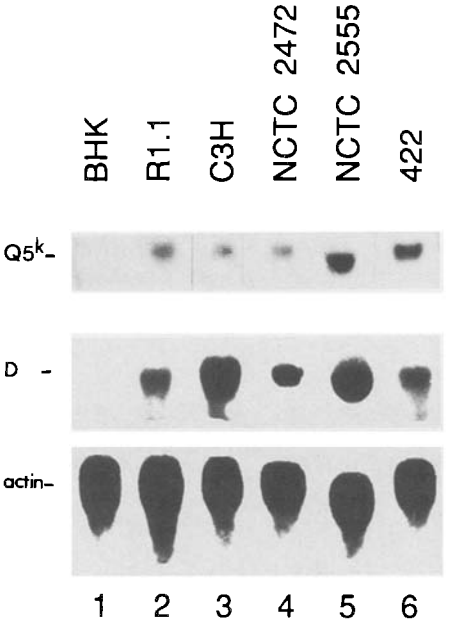


Fig. 4. Northern blot analysis of total RNA (8–12 µg per lane) from different mouse cell lines (lane 2 to 6). The filter was first hybridized with the Q5*-oligonucleotide (*upper panel*), then rehybridized with a class I probe (*middle panel*), and finally with an actin probe (*lower panel*). RNA of the baby hamster kidney cell line (BHK, lane 1) was used as negative control for the class I hybridization.

AKR leukemia cell lines, and the low tumor producing cell line NCTC 2555, followed by the lymphoma cell line RI.I, the high tumor producing cell line 2472, and the embryonic cell line C_3H. In addition, L929 cells were also shown to express the $Q5^k$ gene (data not shown).

To investigate whether transcription of the $Q5^k$ gene could be induced in spleen cells and augmented in the tested mouse cell lines, AKR splenocytes and the cell lines 422 and NCTC 2555 were stimulated with mitogens or cytokines. A uniformly weak induction of the $Q5^k$ gene expression was detected in primary spleen cell cultures treated with Con A or PHA for 48 h, or LPS for 24 h compared to the high increase of class I mRNA (Fig. 5). In the NCTC 2555 cell line recombinant mouse IFN-γ or -α/β increased $Q5^k$ message, with the highest increase observed after IFN-γ treatment (Fig. 5, lane 10, 12 h-stimulation).

Expression of the $Q5^k$ gene in postimplantation embryos. We also focused on the expression of the $Q5^k$ gene during embryonic development, whereby we concentrated our investigation on postimplantational development. Because contamination of the embryonic tissue with maternal material cannot be avoided in early postimplantation stages, we chose BALB/c ($H\text{-}2^d$ females) X AKR ($H\text{-}2^k$ males) F_1 embryos (generated by two separate breeding facilities) four our study. To determine the onset of $Q5^k$ gene transcription, embryos from day 6 (egg

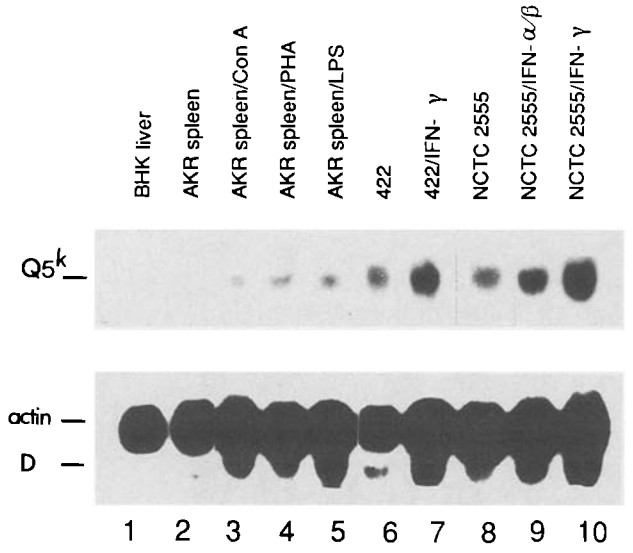


Fig. 5. Stimulation of $Q5^k$ expression in AKR spleen cell cultures and transformed mouse cell lines. For northern blot analysis 12–15 µg total RNA was tested with the Q5*-oligonuleotide (*upper panel*), and in a second step the same filter was rehybridized with both, an actin and a class I probe (marked in the *middle* and *lower* panel). Splenocytes of the AKR mouse were tested either unstimulated (lane 2) or stimulated with Con A, PHA, LPS (lane 3 to 5). Cell lines were stimulated with IFN-γ (422, lane 7; NCTC 2555, lane 10) or IFN-α/β (only shown for NCTC 2555, lane 9) for 12 h. Lane 1: total RNA of BHK.

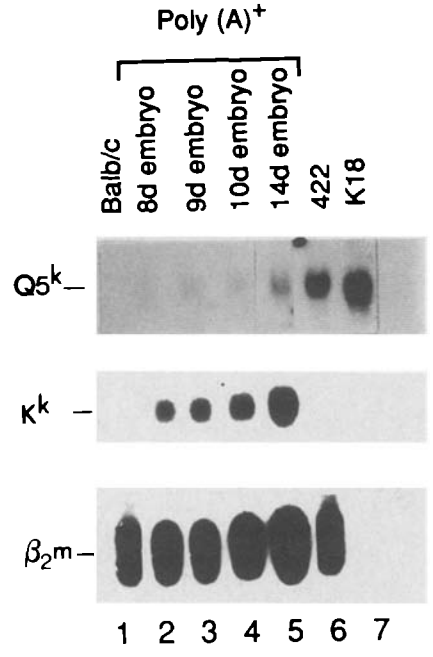


Fig. 6. $Q5^k$ expression in postimplantation embryos. 1 µg poly $(A)^+$ RNA was isolated from F1 embryos (BALB/c/AKR) of gestation day 8, 9, 10, and 14, and 15 µg total RNA from cell lines 422 and K18 ($Q5^k$-BHK-transfectant clone; Weiss et al. 1989) were first probed with the K^k specific oligonucleotide (*middle panel*), then rehybridized with the Q5*-oligonucleotide (*upper panel*), and finally hybridized with the β_2m probe (*lower panel*).

cylinder stage) to day 15 of gestation were examined. Preparations of both total (material from gestation day 6 to 15) and poly $(A)^+$ (material from gestation day 8, 9, 10, and 14) RNA were tested by northern blot analysis. A faint band at 1.6 kilobase (kb) was detectable only in poly $(A)^+$ RNA preparations from day 8 and older embryos (Fig. 6).

A second approach was used to study th onset of transcription of the $Q5^k$ gene. Total RNA from embryonic material of gestation day 6 on was used to prepare single stranded cDNA, which provided the substrate for specific amplification of part of the $Q5^k$ sequence by PCR. We employed the booster PCR procedure, since only a few template copies might be present in the early postimplantation tissue. Probing the reaction products with the Q5*-specific oligonucleotide revealed that all tested embryonic postimplantation stages and tissues expressed the expected 372 base pair (bp)-$Q5^k$ product (Fig. 7A). These results prove that $Q5^k$ message is present even in embryos from day 6, although it was not detectable by northern blot analysis of total cellular RNA.

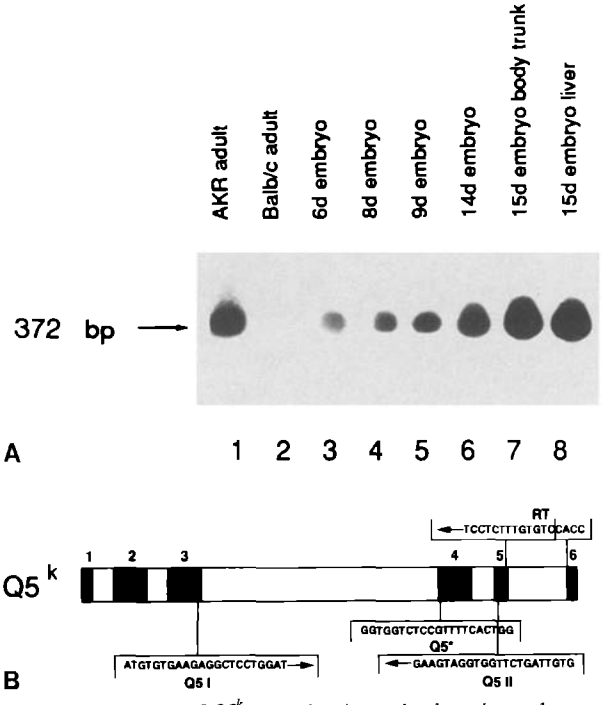


Fig. 7 A, B. Detection of $Q5^k$ transcripts in postimplantation embryos. **A** Amplification products were separated on 2% agarose gels, transferred to Hybond-N^+ membranes, and hybridized with the Q5*-oligonucleotide. RNA of 6, 8, 9, 14 day embryos was tested (lane 3 to 6) and 15 day embryos were dissected into body trunk and liver (lane 7 and 8). Splenocytes stimulated with phytohemagglutinin (PHA; Fig. 5) and Balb/c liver RNA were analyzed accordingly as positive and negative control (lane 1 and 2). In all lanes (except the negative control) the amplification product of the expected size of 372 bp hybridized with th Q5*-oligonucleotide. **B** Total RNA of embryonic material was reverse transcribed into cDNA priming with a class I specific RT-primer, followed by a $Q5^k$-specific booster PCR (Q5I, Q5II).

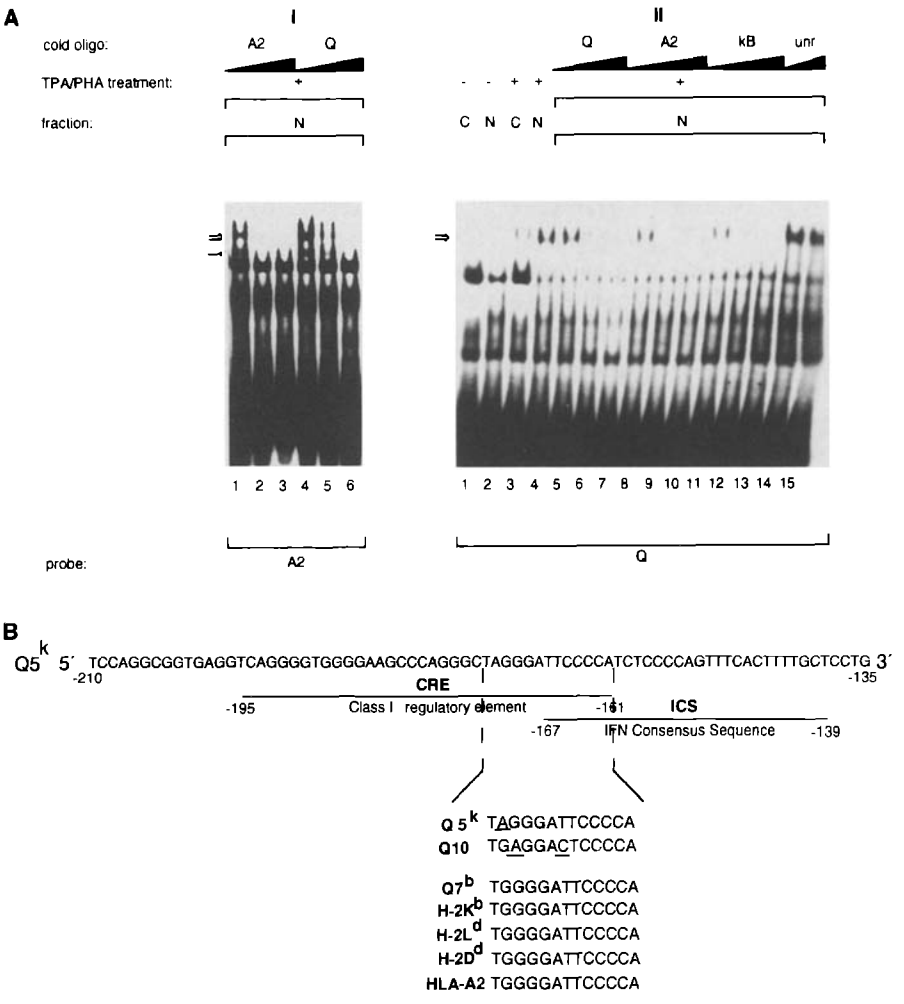


Fig. 8 A. Interaction of NF-kB and H2TF1/KBF1 with the $Q5^k$ regulatory sequence: Analysis of nuclear factor binding using an electrophoretic mobility shift assay. Radioactively labeled $Q5^k$-probe (Q), respectively *HLA-A2* (A2) probe, being sequence identical to H-2K^b for the binding region, were incubated with nuclear (N) or cytosolic (C) extracts of either stimulated (+) or unstimulated (−) Jurkat cells, as indicated. The binding reactions contained either no competitor DNA (lane 1–4, *right panel*) or increasing excess (2.5, 25, 250 molar) of the unlabeled Q5^k oligonucleotide (lane 4–6, *left panel*; lane 5–7, *right panel*), A2 oligonucleotide (lane 1–3, *left panel*; lane 8–10, *right panel*), kB oligonucleotide (lane 11–13, *right panel*), unrelated oligonucleotide (unr), derived from the plasmid pKK 223-3 (Pharmacia Freiburg, FRG), encompassing 56 bp upstream of the *Eco* RI site of the polylinker. The *thick arrow* indicates the position of the specific protein-DNA-complex formed with NF-kB. The *thin arrow* indicates the position of the specific protein-DNA-complex formed with KBF1/H2TF1. **B** Comparison of the CRE sequences of different *Mhc* class I genes revealed that the $Q5^k$ gene exhibits a nucleotide exchange localized in the palindrome sequence (G-A), a site which is normally conserved in the promoter of classical class I genes of mouse and human: *H-2K^b*, *H-2L^d*, *H-2D^d*, *Q7^b*, and *HLA-A2* (Kimura et al. 1986; Korber et al. 1987; Devlin et al. 1985; Zachow and Orr 1989).

Regulation of $Q5^k$ expression. Extensive studies on the regulation of class I expression have confirmed the important role of a 13 bp palindromic sequence localized within the class I regulatory element (CRE) which is conserved in mouse and human. This upstream promoter element is required for constitutive (KBF1/H2TF1; Baldwin and Sharp 1987; Israel et al. 1987; Yano et al. 1987) as well as inducible (NF-kB; Baldwin and Sharp 1988; Israel et al. 1989) class I expression.

Comparison of CRE sequences revealed that the $Q5^k$ gene has a mutation (a G to A exchange), localized in the core binding site (Fig. 8B), which might explain the lack of constitutive expression. Using electrophoretic mobility shift analysis, we therefore investigated whether this single bp exchange influences nuclear factor binding to the CRE of the $Q5^k$ gene. Nuclear extracts from stimulated Jurkat T cells were used, which as previously shown contain both regulatory proteins, NF-kB and KBF1/H2TF1 (Messer et al. 1990). One prominent complex was formed when the $Q5^k$ motif fragment was incubated with the extract of stimulated Jurkat cells, but no complexes were detected using extracts from unstimulated Jurkat cells (Fig. 8A). Formation of the labeled complex (thick arrow) was abolished by adding an excess of unlabeled Q5^k-oligonucleotide, indicating that this complex is due to the binding of a sequence-specific factor.

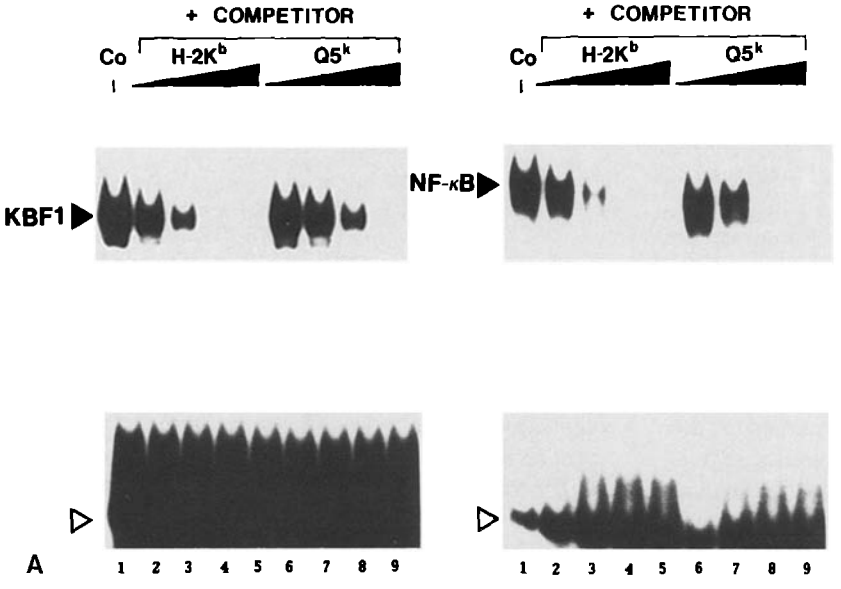
To test whether the interacting factor of the stimulated cells is NF-kB, competition analysis was performed with the k-immunoglobulin enhancer DNA NF-kB motif. Increasing amounts of this kB-probe, as well as the NF-kB binding sequence of the *H-2K^b/HLA-A2* (Messer et al. 1990) genes inhibited complex formation by the radioactively labeled $Q5^k$ probe. An unrelated DNA probe in 25 and 250 molar excess had no effect on the binding. The comigration of the $Q5^k$ specific complex with well characterized shifted bands obtained with NF-kB binding sequences (kB/A2), and the fact that this factor is only present in stimulated Jurkat cell might indicate that the $Q5^k$-motif specifically binds to the NF-kB transcription factor, though with a lower affinity than the kB/A2 motifs. No additional specific complex was observed with the $Q5^k$ motif using extracts of unstimulated or stimulated

Jurkat cells, which would indicate the interaction with KBF1/H2TF1. The *H-2K^b/HLA-A2* probe, however, presented two distinct DNA binding activities, NF-kB in addition to KBF1/H2TF1 (thin arrow), interacting with the conserved palindromic sequence.

In order to confirm the results obtained with cell extracts, demonstrating that the $Q5^k$ motif does not interact effectively with KBF1/H2TF1, but can still bind NF-kB, we used purified NF-kB (heterotetramer, $p50_2$ $p65_2$) and purified KBF1/H2TF1 ($p50_2$, the dimeric form of the NF-kB DNA binding subunit (Kieran et al. 1990)) for gel shift analysis (Fig. 9). Compared to a homologous competition with the unlabeled *H-2K^b/HLA-A2* motif, the $Q5^k$ motif had a much lower efficiency to inhibit binding of KBF1 than of NF-kB to the labeled *H-2K^b/HLA-A2* motif. These results show that the $Q5^k$ sequence has a significantly reduced affinity for the binding of $p50_2$/KBF1, whereas it still binds effectively to $p50_2$ $p65_2$/NF-kB. This might explain why only NF-kB was detectable with the $Q5^k$ probe in nuclear extracts from Jurkat cells.

Discussion

Transcription of H-2 class I genes is developmentally regulated. The status of expression of these genes on preimplantation mouse embryos remains controversial and it is not clear yet whether the failure to detect H-2 antigen reflects a true absence of these determinants or is caused by the limited sensitivity of the techniques employed (Searle et al. 1976; Webb et al. 1977; Sawicki et al. 1981). Some recent data point to a start of class I transcription in the preimplantation embryo (Morello et al. 1985). On postimplantation stages, however, H-2 class I gene expression is clearly detectable (Morello et al. 1985; Ozato et al. 1985; Fahrner et al. 1987).



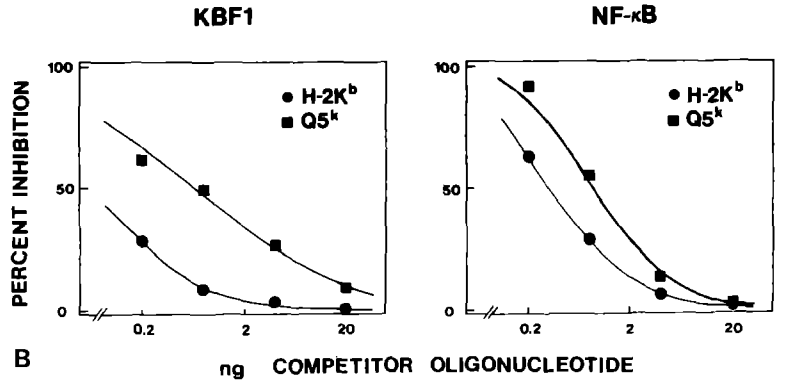


Fig. 9 A, B. Competition binding analysis. **A** Purified NF-kB and KBF1 were incubated with a ^{32}P-labeled DNA probe encompassing the 13 bp palindrome of the class I gene *H-2K^b*. Control: lane 1. Increasing amounts of oligonucleotides with the same motif (lane 2 to 5) or the $Q5^k$ motif (lane 6 to 9) were incubated with the labeled probe. *Filled arrowhead* indicates the position of the protein-DNA-complex formed with KBF1 (*left panel*) or NF-kB (*right panel*). The *open arrowhead* indicates the position of the labeled *H-2K^b* probe. **B** Quantitation of competition efficiencies. The amount of radioactive protein-DNA-complex obtained in the presence of unlabeled competitor oligonucleotides is plotted as percentage of the control.

In this study we were able to detect $Q5^k$ message in embryos of gestation day 6 and onward. The fact that we were studying the situation in embryos growing in vivo and the usage of the highly sensitive RT-PCR technique might explain the detection of $Q5^k$ transcripts in embryos of gestation day 6 and 8, while Ozato and co-workers (1985), showed detectable levels of class I mRNA only after midsomite stage on gestation day 9 and older embryos (C3H × Balb/c, F1). By hybridization with a H-2K^k specific oligonucleotide, K^k transcripts were reproducibly found in eight day embryos, a result which has also been described by Fahrner and co-workers (1987) for 8.5 day embryos using RNAse protection analysis. By S1 nuclease mapping (Morello et al. 1985) detected H-2D^d mRNA even in preimplantation embryos (blastocysts). Thus, it is likely that $Q5^k$ activation might also take place even before implantation of the embryo.

Only low levels of classical class I message and surface antigen are detectable in embryos, but their expression increases dramatically during the first weeks of postnatal development. In contrast, the $Q5^k$ gene, which is expressed at low but almost equal rates in all examined juvenile tissues (week 2 to 4), is inactivated in the adult AKR mouse with the exception of the thymus. Although not examined in detail, overall tissue expression of the $Q5^k$ gene in the juvenile AKR mouse is probably a continuation of embryonic and fetal $Q5^k$ gene expression. Thus, the $Q5^k$ gene might be transcribed in nearly every embryonic tissue and stage of development, like the *Q7* and *Q9* genes, but in contrast to *Q10* (Fahrner et al. 1987). This *Q* gene, encoding a class I protein secreted by the adult liver, apparently retains its tissue specificity throughout embryonic development.

In the adult AKR mouse $Q5^k$ gene expression is clearly tissue restricted. All tested organs are negative for $Q5^k$ message, except the thymus. Because of multiple retroviral insertions adult AKR mice often develop tumors, particularly thymomas. Tumor cells frequently show quantitative or qualitative alterations in their expression of class I molecules which is discussed to be a selective advantage for tumor progression (Jones and Bodmer 1980; Festenstein and Schmidt 1981; Schmidt and Festenstein 1982; Lampson et al. 1983; Doyle et al. 1985; Schmidt et al. 1986). In particular, AKR derived leukemic T-cell lines have been shown to express new H-2 class I specificities (Labeta et al. 1989). Notably, the highest levels of Q5^k mRNA are found in AKR thymomas and also in all established AKR leukemia cell lines studied. Therefore, while classical class I antigen expression is often reduced or even absent (K^k- and D^k- expression in some AKR leukemias (Fig. 3; Henseling et al. 1990), and in primary AKR thymomas (data not shown) $Q5^k$ expression seems to be augmented in the transformed tissue respectively cell lines. Thus, the Q5^k gene product might represent the novel H-2 class I "allo" specificity, characterized by Labeta and co-workers (1989) on $Q5^k$ expressing tumor cell lines. Furthermore, $Q5^k$ expression is not restricted to these AKR leukemia derived cell lines, $Q5^k$ message is also detectable in other transformed and tumor producing cell lines.

Since $Q5^k$ mRNA was not detectable in normal adult tissue we examined whether the treatment with cytokines and mitogens might induce $Q5^k$ transcription. All factors tested were able to induce $Q5^k$ gene expression in primary mouse spleen cell cultures and to increase the expression in established cell lines. Thus, $Q5^k$ message can be readily induced as a result of cell activation. Perhaps a direct or indirect hormonal influence is responsible for $Q5^k$ expression in the uterus of pregnant mice, whereas the $Q5^k$ expression in the spleen of thymoma bearing animals could either be due to the presence of a second tumor in the mouse (perhaps leukemic cells), or simply to the status disease.

Evidence is accumulating for a nonimmunological function of Mhc class I antigens, and it has been proposed that the nonpolymorphic class I molecules might play a role in morphogenesis and cell-to-cell interaction in embryonic development, based on the finding of Warner and co-workers (1987), that preimplantation cleavage rate is controlled by a gene of the *Qa-2* region. The recent study by Zijlstra and co-workers (1990), showing that mice unable to express the β_2m gene develop normally, argues strongly against an essential influence of class I molecules in embryonic development, and also against an important nonimmunological function of these antigens. Although evidence has been presented that β_2m is not required for cell surface expression of some class I antigens [shown for H-2D^b by Allen and co-workers (1986), and for an HLA class I-like antigen by Schnabl and co-workers (1990), no function could be assigned to these heavy chains. Moreover, the β_2m-less H-2D^b antigen was not recognized as alloantigen. Since the function of nonclassical H-2 class I molecules remains obscure, and does perhaps not depend on cell surface expression and on association with β_2m, it is still formally possible that a $Q5^k$ molecule could function in β_2m negative mice.

Several reports indicate that Mhc class I molecules interact in a possibly locus and allele-specific manner with a variety of cell surface proteins (Kittur et al. 1987; Sharon et al. 1988; Solano et al. 1988; Burlingham et al. 1989). Particularly, H-2 class I antigens were shown to control the expression and function of the insulin receptor (Hansen et al. 1989; Stagsted et al. 1990). Current evidence indicates that complex formation with β_2-microglobulin might also be necessary for these interactions. Recently a more general nonimmunological function of Mhc class I molecules has been suggested in the control of the regulation of hormone receptors upon activation by their corresponding ligands (Stagsted et al. 1990). Thus, quantitative or qualitative changes of class

I expression in tumor cells could contribute to the transformed phenotype via the regulation of receptor activity. Therefore, it is still conceivable that a $Q5^k$ gene product might influence both morphogenesis and cell-to-cell interaction during embryonic development, as well as cell growth and differentiation in the adult mouse.

Recent data showed that the DNA binding subunits of NF-kB (p50) and KBF1 are identical and display sequence homology with rel oncoproteins and the *Drosophila* dorsal gene (Ghosh et al. 1990; Kieran et al. 1990). The binding characteristics of p50 and KBF1 are indistinguishable, but differ from those of the NF-kB and v-rel contact points on the Mhc class I site (Kieran et al. 1990). NF-kB-like v-rel contacts the four G residues of the palindrome site, but the external G is less critical than the following three.

Gel shift analysis with the $Q5^k$ motif revealed, that in contrast to the results published for the conserved perfect palindrome of the H-2K^b/HLA-A2 promoter, the asymmetrical Q5^k site with one differing nucleotide (TAGGGATTCCCCA) seems to bind only NF-kB. These results were confirmed by competition analysis with purified KBF1 and NF-kB, indicating that the constitutive factor KBF1 binds with much lower affinity to the $Q5^k$ motif than to the classical sequence. Thus, for constitutive factor binding, all four G residues are critical, and this might explain that the nucleotide exchange in the external G position (G-A) of the Q5^k promoter prevents KBF1/H2TF1, but not NF-kB binding to this site. Although it has not been shown so far that this region is also important in the regulation of the $Q5^k$ gene, the restricted binding activity of the $Q5^k$ fragment might account for the expression data described above, where most adult tissues have no constitutive $Q5^k$ expression, and a significant increase can be observed after induction, possibly mediated, at least in part, by NF-kB. A similar situation has been described for the *Q10* gene (Handy et al. 1989), where a two nucleotide difference (TGAGGACTCCCCA) in this motif is responsible for the loss of constitutive expression.

However, the presented binding affinity studies of the $Q5^k$ motif cannot necessarily explain the activity of the $Q5^k$ gene in embryonic, juvenile, and transformed tissues. It would be highly interesting to investigate a possible influence by the oncogenic members of the rel related protein family on tumor associated $Q5^k$ induction. Additional regulatory sequences, which also might be of essential importance for $Q5^k$ regulation, also contribute to class I gene expression and therefore must be included in further studies focusing on the in vivo situation and on functional data. Comparison between protein-DNA interaction in gene expressing vs non-expressing tissues, embryonic vs adult tissue, methylation pattern analysis correlated with gene activation or inactivation, will be necessary to understand the complex regulatory system.

Acknowledgments. We wish to thank Georg Honold and Gerald Messer for their help in establishing PCR technique respectively gel shift analysis, and Dr. J. Johnson for critical review of the manuscript. Dr. K. Ozato, Laboratory of Developmental and Molecular Immunity, Bethesda, is acknowledged for useful discussion. We also thank Dr. M. Nüssel, GSF, München, for cooperation for the animal breeding and Dr. G. Arnold, Genzentrum, München, for providing the oligonucleotides. This work was supported by grants from the BMFT (Genzentrum, München) and by the Deutsche Forschungsgemeinschaft (postdoctoral fellowship).

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