KBF1 (p50 NF-κB Homodimer) Acts as a Repressor of H-2K^b Gene Expression in Metastatic Tumor Cells

By Daniel Plaksin,* Patrick A. Baeuerle,‡ and Lea Eisenbach*

*From the *Department of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel; and the ‡Laboratorium für Molekulare Biologie der Ludwig-Maximilians-Universität München, D-8033 Martinsried, Germany*

Summary

Downregulation of major histocompatibility complex class I expression is causally related to high malignancy and low immunogenicity of certain murine tumors. In this study, we have analyzed the roles of the nuclear factors KBF1/p50 and p65 in regulation of class I expression in high and low metastatic tumor cells. Low class I–expressing cells show at higher levels of KBF1/p50 and NF-κB (p50/p65) binding activity than high class I–expressing cells. However, an excess of KBF1 over NF-κB is observed in low expressing cells, while an excess of NF-κB over KBF1 is observed in high expressing cells. Stable transfection of a p65 expression vector into low class I–expressing cells activated H-2 transcription and cell surface expression, while stable transfection of p50 expression vector into high expressing cells suppressed H-2K^b transcription and cell surface expression. Our studies suggest that KBF1 has the potential of downregulating class I gene expression, whereas dimers containing the p65 subunit are activators of class I gene expression.

Class I molecules of the MHC present peptide fragments from intracellularly processed antigens to CTL (1, 2). Reduced levels of expression of H-2 class I proteins were observed in spontaneous, chemically induced, and virally transformed murine tumors (3–8), human small cell carcinomas (9), neuroblastomas (10), eccrine protocarcinomas (11), cervical carcinomas (12), and Burkitt's lymphoma lines (13). Moreover, examination of 44 primary tumors and autologous metastases showed significant downregulation of class I in metastases (14). In many of the experimental tumor systems, the lack of H-2 expression determined reduced immunogenicity and high tumorigenicity.

The Lewis lung carcinoma (3LL) tumor that originated spontaneously in a C57Bl, H-2^b mouse almost lacks cell surface expression of H-2K^b antigen (3). Screening a large number of newly generated single cell clones of 3LL showed that the higher the metastatic phenotype of a clone, the lower the expression of H-2K^b glycoprotein; H-2D^b, on the other hand, is expressed to some extent on most clones (15). The low metastatic H-2K^b-positive clones were shown to be significantly more immunogenic both in syngeneic and in allogeneic hosts, compared with high metastatic H-2K-negative cells (15). It therefore appeared that putative peptides derived from tumor antigens could be recognized by CTL only in association with H-2K^b gene products, while H-2D^b molecules do not serve as a restriction element for antitumor CTL (16). In vitro treatment by IFN-γ transiently activated the expression of H-2K^b gene in the metastatic clones (17). When cells treated for 5 d with IFN-γ were injected intravenously into mice, a significant decrease in their metastatic competence was observed (17–19). Since IFN-γ is known to affect many different functions of the cells, we tested whether the reduction of metastatic competence by IFN-γ can be attributed directly to the induction of H-2K^b expression, by introducing H-2K^b gene into cells of a highly metastatic, K^b-negative clone, called D122. We demonstrated that cells of a highly metastatic clone, transfected with H-2K^b gene, reverted to low or nonmetastatic phenotypes. This was correlated with the acquisition of H-2K-restricted immunogenic properties (20, 21). Low levels of class I cell surface expression in most tested tumors, of human or murine origin, are the result of low mRNA levels, and only rarely are defects in protein transport, peptide processing, or MHC-peptide assembly described (22, 23). The unique model of the 3LL carcinoma enables a direct analysis of the transcriptional mechanism responsible for the differential class I expression in high and low expressing clones.

Analysis of the mouse MHC class I promoter has revealed several regions important for the expression and regulation of these genes through specific binding of proteins (24–34). The promoter-enhancer region of class I genes includes two main enhancer sequences, A and B. Enhancer A overlaps with the IFN-responsive sequence (IRS) (−163, −137). Both enhancers are conserved within the promoter of several genes coding for classical transplantation antigens. In most cells or tissues of human, rat, or mouse origin, a nuclear factor called

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KBF1, interacts specifically with two *cis*-acting GC-rich palindromes, within the A enhancer (25, 35, 36). This constitutive nuclear factor also binds to a similar sequence, within the promoter of the mouse β_2-m gene (35). KBF1 was thus expected to play a role in the coordinate up regulation of class I and β_2-m gene expression (35).

Another factor binding to the palindrome sites in class I gene enhancers and the β_2-m promoter is NF-κB (28, 37). This inducible factor is thought to displace the constitutive KBF1 upon treatment of cells with TNF and phorbol ester, thereby replacing a weakly *trans*-activating factor by a strong activator (36). NF-κB is composed of two DNA binding subunits called p50 and p65 (38–40). A third subunit, called IκB, serves to inhibit the DNA binding and nuclear translocation of the p50-p65 heterodimer (41). The release of IκB in response to a multitude of external stimuli provides the molecular basis for the inducible activation of NF-κB (reviewed in reference 42). p50 and p65 share sequence homology and are members of a novel class of transcription factors also including the Rel protooncogene (for review, see reference 43). KBF1 is, by all criteria tested, identical to a homodimer of the p50 subunit of NF-κB (44–46), p50 homodimers escape inducible control because they cannot detectably interact with purified IκB proteins, unlike dimers containing p65. A unique feature of p50/KBF1 is its synthesis of a large non-DNA binding precursor molecule, p105, with endogenous IκB activity in its COOH-terminal portion (47–49).

KBF1 (p50 NF-κB homodimer) is present in most differentiated cells where MHC class I antigens are expressed. However, its binding activity is absent in undifferentiated embryonal carcinoma (EC) cells, where class I and β_2-m genes are silent. It is induced when the cells are triggered to differentiate (28), and the appearance of KBF1 binding activity in EC cells parallels the onset of H-2 class I and β_2-m gene expression (mRNA and proteins). KBF1-like binding activity is undetectable in brain nuclear extracts, which correlates with the lack of H-2 class I antigens in this organ (50). Its absence from fetal mouse liver nuclear extracts is however in contrast to class I expression from midsomite stage of gestation onwards (50). In stage-specific T cell lines, the level of class I transcription has been shown to correlate with the amount of KBF1-like binding activity (51). Another factor, H-2 transcription factor (H2TF1), binds also to enhancer A at exactly the same nucleotide residues as KBF1 (52), but its relationship to KBF1 is unclear at present. Recent reports show that homodimers of p50 (KBF1) lack significant *trans*-activating potential in transient transfection assays but rather have the potential of downregulating κB-specific gene expression (53–55). In contrast, in vitro transcription studies showed that p50 dimers act as activators of gene transcription (56, 57).

These conflicting observations prompted us to test whether stable expression of KBF1 in 3LL carcinoma cell lines positively or negatively influences class I gene expression. Our data suggest that KBF1 is a negative regulator of class I expression, whereas the p65 subunit of NF-κB is a positive regulator. These results suggest that the balance between the two NF-κB subunits is crucial for the level of class I expression.

Materials and Methods

Tumor Cells. Tumor cells were maintained in DME, 10% FCS, and supplements described elsewhere (4). The Lewis lung carcinomas (3LL) is a malignant tumor that originated spontaneously in C57BL/6 (H-2^b) mice (4). A9 and D122 are low and high metastatic clones, respectively, cloned from the 3LL carcinoma cells by limiting dilution (4).

Gene Expression. RNAs were prepared from 1–3 × 10^8 cells, propagated in tissue culture, by the method of Chirgwin et al. (58). Northern blots were prepared from formaldehyde-containing agarose gels loaded with 40 μg total RNA per lane as described (21) and assayed by hybridization to ^{32}P-labeled probes. The following probes were used: for the H-2K^b probe, the H-2K^b-specific 30-mer oligonucleotide, which codes for amino acids 290–299 of the H-2K^b protein; for the H-2D^b probe, the H-2D^b-specific 30-mer oligonucleotide, which codes for amino acids 293–302 of the H-2D^b protein; for the p50 probe, a 1.5-kbp insert of pBL50 (a gift of S. Ruben, Roche Institute, NJ) (59); for the p65 probe, a 2.0-kbp insert of pBL65 (a gift of S. Ruben) (59); for the β-actin probe, a 4.3-kbp insert of pAC18.1 (a gift of U. Nudel; Weizmann Institute, Rehovot, Israel) (60). Hybridizations were performed in 50% formamide at 42°C. Blots were washed in 0.1× SSC, 0.1% SDS at 60°C. Blots hybridized to oligonucleotide probes were washed in 0.5× SSC, 0.1% SDS at 50°C.

Plasmids, Constructs, Antibodies, and Proteins. Plasmids RcCMV-p50$^{1-502}$ and RcCMV-p65$^{1-550}$ were described previously (53). PUC-365-CAT was prepared by cloning of a BamHI-XbaI restriction fragment from pH-2-CAT (a gift of A. Israel, Pasteur Institute, Paris) (61) into a Puc19 vector. PUC-142-CAT and PUC-190-CAT were subcloned from p138H-2K CAT and p190H-2K CAT (a gift from A. Baldwin, Massachusetts Institute of Technology, Cambridge, MA) (52), respectively, into a Puc19 vector (52). p38-H-2K-CAT (a gift from A. Baldwin) and the β galactosidase–containing expression vector PCH110 (Pharmacia Fine Chemicals, Piscataway, NJ). Recombinant p50 and p65 were prepared by the MAXIBAC baculovirus expression system (a gift of D. Baltimore, The Rockefeller University, New York) (56), and antibodies to KBF1 α2 and α3 were a gift of A. Israel (44). IκB recombinant MAD-3 was expressed in bacteria and purified as described (61a). Antibodies to c-*rel* (a gift of I. Verma, The Salk Institute, San Diego, CA), and antibodies to v-*rel* (a gift of M. Hannink, University of Missouri, Columbia, MO) were used.

Preparation of Stable Transfectants. Transfections of H-2K^b, 2.9-K^b, 4.1-K^b, and RcCMV-p65 expression vectors into the high metastatic clone D122 (3LL) and transfection of RcCMV-p50 into the low metastatic clone A9 (3LL) were performed by the calcium phosphate technique, in cotransfection with a 1:9 ratio of PSV$_2$-neomycine resistance gene (PSV$_2$neomycin) (21). Control transfection was done with PSV$_2$neo alone. To increase the efficiency of transfection, a 3-min treatment with DME, 15% glycerol was preformed after incubation with DNA precipitates. Colonies growing in 400 μg/ml (D122 cells) and 800 μg/ml (A9 cells) G418 (GIBCO BRL, Gaithersburg, MD) 4 wk after transfection were expanded and analyzed for expression of the inserted genes. Control transfectants carrying pSV$_2$neo show hybridization patterns, and MHC class I expression equal to parental cells. Positive clones were checked for plasmid DNA integration by Southern blots.

Cell Surface and Cellular H-2 Proteins. To determine cell surface H-2 expression, protein A–purified mAbs 20–8–4 (αK^b) and 28–14–8 (αD^b) (62) were iodinated by chloramine T. 500 ng of labeled antibody was mixed with 5 × 10^5 freshly trypsinized cells in 0.1 ml PBS in BSA-coated tubes. Triplicate samples were incubated

at 0°C for 90 min. After four washings in PBS, 0.05% BSA, 0.02% sodium azide, samples were monitored in a gamma scintillation counter. Immunoprecipitation of class I proteins from [^{35}S]methionine-labeled cells were described previously (4).

Transient Expression Assays. For transient assays, 10^6 cells were plated in 10-cm dishes, 24 h before DNA transfection. Cells were incubated for 12 h with calcium phosphate–precipitated plasmid DNAs (20 μg of the CAT derivative plus 4 μg of pCH110). Then, after a 3-min glycerol (15%, 37°C) shock, the cells were rinsed once, reefed with fresh medium, and 24–48 h later the cells were processed for enzymatic assays. CAT activity, using 30 μg of total cell extract protein, was determined as described by Schüle et al. (63) and β-galactosidase activity was determined as described by Nielson et al. (64). Experiments were repeated at least three times. Activity of CAT was normalized to activity of β-galactosidase to correct for difference in transfection efficiency.

Gel Retardation Assay. Nuclear extracts were prepared from 10^7 cells and used in gel retardation assays as described by Schreiber et al. (65). Protein concentration was determined using the Bradford method and ranged from 4 to 7 mg/ml. A synthetic double-stranded 52-bp oligodeoxynucleotide containing the entire enhancer A (−205 to −154) of the H-2K^b promoter, a 66-bp oligonucleotide containing the entire enhancer B (−123 to −58) of the H-2K^b promoter, a 13-bp oligonucleotide containing the NF-κB/KBF1 binding domain, and a 375-bp fragment containing the entire enhancer A and B (XbaI-HindIII-restricted fragment from pH-2-CAT) were used as probes. The nonlabeled competitor oligonucleotides used in the binding assay were: AP1 (−203 to −190), AP1AX (−205 to −184), AP2 (−184 to −172), AP2X (−188 to −169), class I regulatory element (CRE) (−172 to −160), CREX (−175 to −154), and AP1B (−106 to −92).

DNA–protein binding was conducted in 20-μl volumes. Nuclear extracts (3–5 μg) were incubated with 3 μg of poly(dI:dC) (Pharmacia Fine Chemicals) for 15 min at room temperature. Approximately 0.1 pmol of ^{32}P-labeled DNA (~10,000 cpm) was added to the preincubated nuclear extracts. Unlabeled competitor DNAs were added to the binding reactions 2 min before the labeled oligomer. Rabbit anti-p50 antiserum was added to the nuclear extracts for 15 min at 4°C, before ^{32}P-labeled DNA addition. No disruption of the nucleoprotein complex binding was observed when a control antisera was added to the extract. DNA–protein complexes were resolved on 4% polyacrylamide gel (acrylamide/bisacrylamide, 39:1) in 0.4× TBE (1× TBE is 50 mM Tris-borate [pH 8] 1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −70°C.

Construction of Genomic D122 DNA Library. 10–20-kbp fragments of D122 genomic DNA were ligated to predigested λ dash/EcoRI arms (Stratagene, La Jolla, CA). The library was plated out at a density of 20,000 plaques per plate (20 plates). Replica filters were prepared from each plate. After denaturation and neutralization, the filters were hybridized with an H-2K^b oligomer probe. Two specific clones were isolated. EcoRI-digested fragments from the two clones, containing the whole H-2K^b gene, were subcloned into Bluescript plasmid for further analysis.

Results

Differential Expression of H-2K^b Molecules in High and Low Metastatic 3LL Clones. K^b cell surface expression was shown to be much higher on low metastatic clones than in high metastatic clones of 3LL (15). Low metastatic A9 and high metastatic D122 cells were compared for cell surface, cellular protein, and mRNA levels of the K^b gene. Fig. 1 shows high levels of transcript, total cellular heavy chain, and cell surface antigen on A9 as compared with D122. H-2D^b cell surface expression is readily detectable on D122 cells and it is approximately threefold lower on D122 cells as compared with A9 cells (see Figs. 6 *D* and 7 *C*) (21). We have shown before that transfection of D122 cells with a plasmid containing the K^b gene cloned from a normal genomic library and driven by an autologous promoter yielded K^b-positive clones, provided that a high copy number of the gene was inserted (21). To test whether the D122 endogenous K^b gene can be expressed under similar conditions, we cloned the gene from a D122 genomic library (λdash). Restriction analysis with a variety of restriction enzymes indicated that no gross mutations, deletions, or rearrangements occurred in the K^b genomic clones. Two genomic clones, 2.9-K^b and 4.1-K^b, 10.5 kbp in length, which include the entire K^b promoter, were subcloned into Bluescript and were used for transfection into D122 cells. The H-2K^b clone derived from a normal library was transfected in parallel. Fig. 2 shows that either of the two D122-derived plasmids or the normal K^b were all expressed in D122 cells at similar levels. No significant changes in D^b expression were observed as compared to parental cells. DNA analysis indicated that multiple copies of the plasmid were inserted in the different clones (not shown). Thus, the endogenous genes seem to be intact in structure

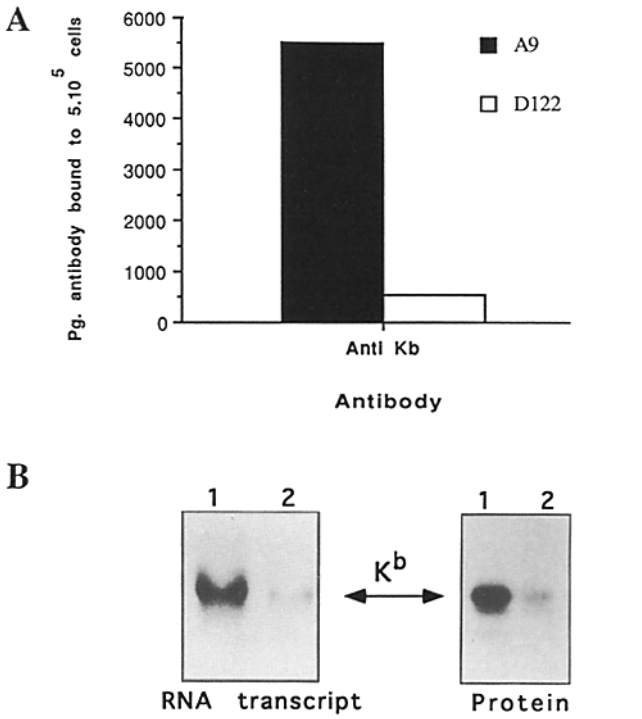


Figure 1. H-2K^b expression on A9 and D122 cells. (*A*) Cell surface expression was determined by RIA. (*B*) RNA and cytoplasmic K^b heavy chain in A9 (lanes *1*) and D122 (lanes *2*) cells. Northern blot and hybridization were performed as described in Materials and Methods. Immunoprecipitation was performed with antibody 20–8–4 as described (4).

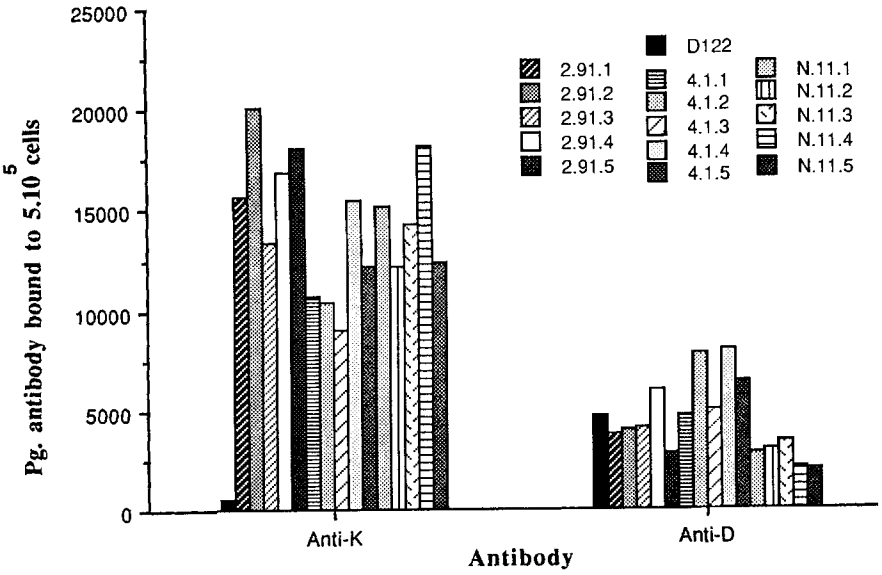


Figure 2. H-2 cell surface expression of D122 clones transfected by K^b genes. D122-derived genomic K^b clones 2.91 and 4.1 and genomic normal K^b clone N.11 were transfected with PSV_2neo as described in Materials and Methods. Isolated single cell clones were monitored for K^b and D^b expression by RIA.

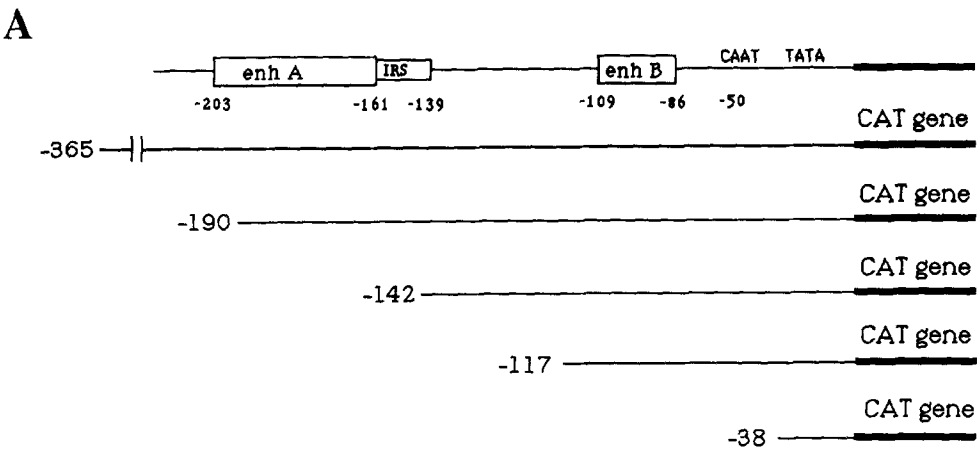
and able to be functionally expressed, even in the original D122 cells.

Differential Activity of Enhancer A in A9 and D122 Cells. Since the endogenous K^b promoter can be activated in D122 cells upon transfection of multiple copies of the gene, it seems reasonable to assume that *trans*-acting silencer factors exist in D122 cells and these are diluted by excess promoter sequences. To test the activity of enhancer elements of the K^b gene in A9 and D122 cells, deletions of the K^b promoter were fused to the bacterial CAT gene (Fig. 3). These recombinants were transiently cotransfected into A9 and D122 cells with pCH110, which express the bacterial β-galactosidase under the regulation of the SV40 promoter, and served as an internal control. CAT activity was normalized to β-galactosidase activity for each transfection. Plasmids containing enhancer B, with endpoints at −117 and −142, showed low and similar activities upon transfection into the two cell types (Fig. 3 *B*). In contrast, the plasmid containing the endpoint at −190 was at least twofold more active in A9 than in D122 cells, and the plasmid containing the endpoint at −365 produced a threefold increase in activity between the two cell types (Fig. 3 *B*). These data suggest the participation of at least two enhancer elements in the differential regulation of the K^b gene in D122 cells, one between −142 and −190 and the other between −190 and −365.

K^b Promoter DNA Binding Proteins in Nuclear Extracts of A9 and D122 Cells. To examine the nuclear factors binding to the K^b promoter, nuclear proteins were prepared from A9 and D122 cells, and examined by electrophoresis mobility shift assay (EMSA),[1] using as a probe the domain −365 to +10. Three main complexes were detected (Fig. 4, *B* and *C*). The fastest migrating complex binds to enhancer A only, and is competed efficiently with excess enhancer A (−205 to −154), AP2X (−188 to −169) CREX (class I regulatory element) (−175 to −154), and CRE (−172 to −160), (Fig. 4, *B* and *C*, lanes *1*, *8*, *4*, and *3*, respectively), indicating that this complex binds to the NF-κB domain, CRE. This complex exists at higher levels in nuclear extracts from D122 cells (Fig. 4 *C*) than extracts from A9 cells (Fig. 4 *B*). The intermediate band represents proteins binding to enhancer B only, because this protein–DNA complex is competed with an oligomer of enhancer B (−123 to −58) (Fig. 4, *B* and *C*, lane *2*) and is similarly expressed in both cell lines. The slowest migrating complex seems to be able to bind both enhancers as it is competed with oligomers comprising either enhancer A or B (Fig. 4, *B* and *C*, lanes *1* and *2*). This complex is also slightly more abundant in D122 extracts.

To further analyze protein binding to subdomains of the promoter, analysis was repeated with enhancer A as a probe. Six complexes were identified by EMSA (Fig. 4, *E* and *F*). Complexes A1 to A5 but not A6 were more abundant in D122 extracts (compare Fig. 4 *E*, lane *1*, to *F*, lane *8*). Competition analysis indicated that A3 and A4 bind to the NF-κB binding domain and are competed by the minimal 13-bp CRE (Fig. 4 *E*, lane *5*, and *F*, lane *4*); moreover, when CRE is used as a probe, it binds only complexes A3 and A4 (Fig. 4 *F*, lane *8*, and *F*, lane *1*). Complexes A1, A2, and A6 are bound at the domain partially overlapping CRE and AP2, since they are competed by CREX (Fig. 4 *E*, lane *2*, and *F*, lane 7), by AP2X (Fig. 4 *E*, lane *4*, and *F*, lane *5*), and to some extent with CRE (Fig. 4 *E*, lane *5*, and *F*, lane 4). A5 binds at the AP1A domain and is competed mainly by AP1AX (Fig. 4 *E*, lane *3*, and *F*, lane *6*) and AP1A (Fig. 4 *E*, lane *6*, and *F*, lane *3*). Analysis of protein binding to enhancer B (−123 to −58) reveals similar binding proteins in extracts of both clones (Fig. 4 *D*, lanes *2* and *3*). The slowest migrating proteins are competed by the AP1B domain (Fig. 4 *D*, lanes *5* and *6*). Nuclear extracts from HeLa cells were used as controls (Fig. 4 *D*, lanes *1* and *4*). Since the same

[1] *Abbreviation used in this paper:* EMSA, electrophoresis mobility shift assay.



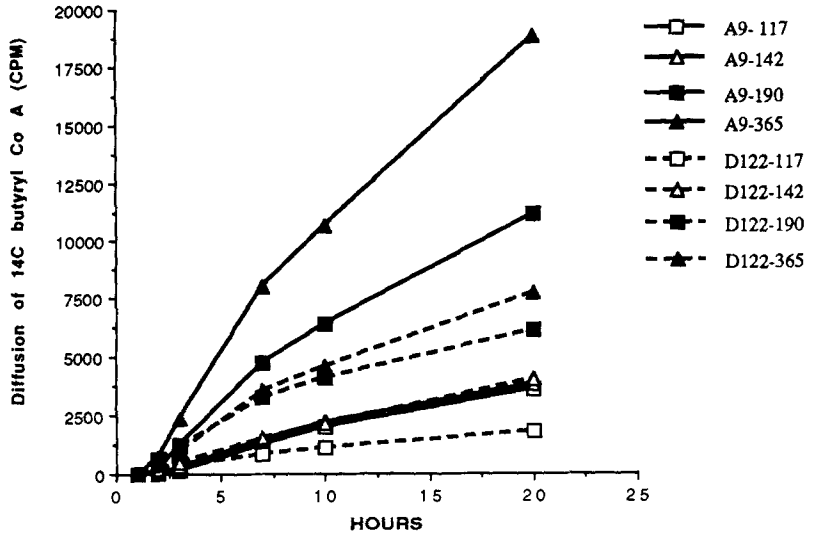


Figure 3. Activity of K^b promoter-CAT constructs in A9 and D122 cells. (*A*) Schematic representation of the K^b promoter and the various deletion constructs used in the CAT assay. (*B*) A9 and D122 cells were transiently transfected by the various CAT constructs, and pCH110 was cotransfected as a control gene. 40 h later, extracts were prepared and CAT and β-galactosidase activity were monitored as described. A 20-h kinetics was performed from all samples. p38-CAT construct gene gave no CAT activity in the two cell lines (not shown). This experiment is one of six experiments that gave equal data.

quantities and batches of nuclear extracts were used for binding of the different probes, these data indicate that a number of enhancer A binding proteins are more abundant in D122 cells than in A9 cells. Analysis of nuclear extracts with CRE as a probe showed that levels of complex A3 and A4 (NF-κB and KBF1) are similar in A9 cells while D122 cells higher levels of A4 than A3 are detected (Fig. 4 *G*).

Characterization of Proteins Bound to κB Motif in the H-2K^b Promoter. The NF-κB motif in the H-2K^b promoter was shown to bind NF-κB (p50-p65 heterodimer), KBF1 (p50-p50 homodimer), and other related and nonrelated factors. To test whether complexes A3 and A4 comprise one of the known factors, we used anti-KBF1-specific antibodies nos. 2 and 3, which are specific for the p50 homodimer (44). Fig. 5 *A* shows that incubation of nuclear extracts with antibody no. 3 and to a lesser extent no. 2 shifted the p50 dimer–DNA complex to a slower mobility antibody–KBF1–DNA complex, and eliminated band A4 in EMSA (Fig. 5 *A*, lanes *3*, *4*, 7, and *8*). An unrelated antibody did not change the mobility of the p50 homodimers (Fig. 5 *A*, lanes *2* and *6*). Again the differential quantitative levels of KBF–DNA binding activity are obvious between the two cell types. Further support for the A4 complex containing a p50 homodimer is shown in Fig. 5 *B*. Recombinant shortened p50, which migrates slightly faster than p50 from D122 cells, reacted similarly with the no. 3 antibody to the cellular p50 homodimer (Fig. 5 *B*, compare lanes *5* and *4* to *2* and *1*). Complex A4 was unaffected by addition of human recombinant MAD-3 (Fig. 5 *B*, lane *6*), an IκB protein cloned from human macrophages (66). This indicates the absence of p65 or c-Rel from the A4 complex.

Recombinant p65 (Fig. 5 *B*, lane *3*) could only weakly bind to the class I κB motif. Incubation of D122 nuclear extracts with recombinant MAD-3 eliminated complex A3 (Fig.

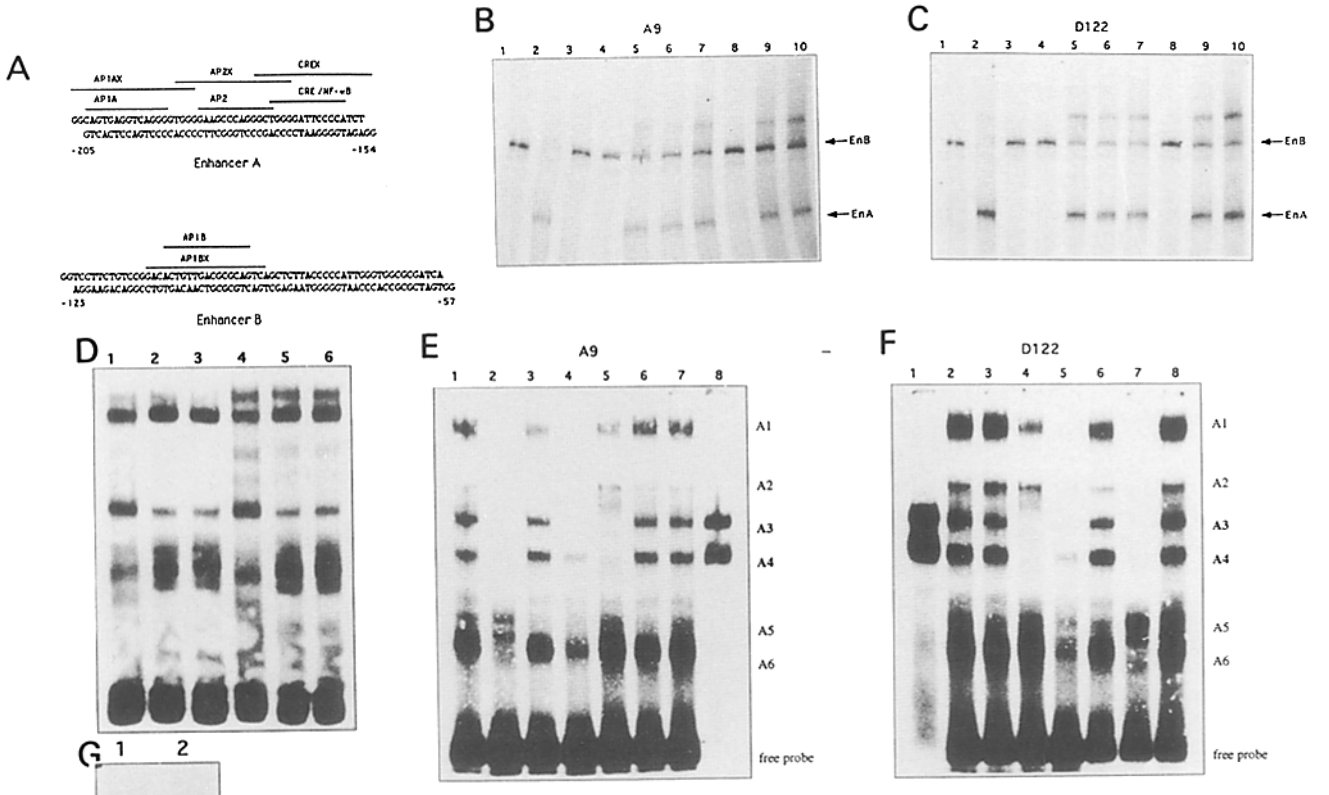


Figure 4. K^b promoter activity in nuclear extracts of A9 and D122. (*A*) Schematic representation of enhancer A, enhancer B, and various fragments used as probes or as competitors. (*B* and *C*) A ^{32}P-labeled HindIII-Xba fragment of puc-365-CAT, encompassing the region from −365 to +10, was used as a probe. Nuclear extracts from A9 cells (*B*) or D122 cells (*C*) were incubated in presence or absence of competitors: no competitor (lanes *10*); enhancer A (lanes *1*); enhancer B (lanes *2*); CRE (lanes *3*); CREX (lanes *4*); AP1A (lanes *5*); AP1AX (lanes *6*); AP2 (lanes *7*); AP2X (lanes *8*); AP1BX (lanes *9*). (*D*) A ^{32}P-labeled enhancer B was incubated with nuclear extracts of Hela cells (lanes *1* and *4*); A9 (lanes *2* and *5*); D122 (lanes *3* and *6*) in presence (lanes *1–3*) or absence (lanes *4–6*) of AP1B competitors. (*E* and *F*). A ^{32}P-labeled enhancer A probe was incubated with extracts of A9 cells (*E*) and D122 cells (*F*). Control (*E*, lane *1*; *F*, lane *8*); CREX (*E*, lane *2*; *F*, lane *7*); AP1AX (*E*, lane *3*; *F*, lane *6*); AP2X (*E*, lane *4*; *F*, lane *5*); CRE (*E*, lane *5*; *F*, lane *4*); AP1A (*E*, lane *6*; *F*, lane *3*); AP2 (*E*, lane *7*; *F*, lane *2*); ^{32}P-CRE was used as a probe (*E*, lane *8*; *F*, lane *1*). (*G*) A ^{32}P-labeled CRE (KBF1/NF-κB binding site) probe was incubated with extracts of A9 cells (lane *1*) and D122 cells (lane *2*). The mixtures were analyzed by EMSA.

5 *B*, lanes *5* and *6*). Although MAD-3 was shown to bind both p65 and c-*rel*, complex A3 was not shifted or eliminated by specific c-*rel* or v-*rel* antibodies (not shown), indicating that this complex represents the NF-κB p50-p65 heterodimer. In conclusion, higher levels of KBF1 are found in nuclear extracts of D122 cells as compared with A9 cells. In D122 nuclear extracts, KBF1 seems to be in excess over NF-κB while in A9 extracts NF-κB is in some excess over KBF1.

Stable Expression of p50 Suppresses Class I Expression while Stable Expression of p65 Increases Class I Expression. The data, so far, suggested that the ratio and relative amounts of KBF1 and NF-κB may regulate the expression of H-2K^b in the two cell lines. Northern blot analysis showed that p105 mRNA levels are lower in A9 than in D122 cells (Fig. 6 *A*). To test whether increased KBF1 levels affect K^b transcription, we have stably transfected A9 cells with CMV-p50$^{1-502}$ (53). By only expressing the NH_2-terminal 502 amino acids of the KBF1 precursor p105, we could circumvent the negative control imposed by the IκB-γ contained in the COOH-terminal portion of p105. Clones expressing the truncated p105 mRNA showed, in addition to a novel shorter mRNA, elevated levels of the p105 mRNA (Fig. 6 *B*). This is in accordance with the observation that p50 can upregulate its own transcription (67). Thus, p50 may act as an inducer of transcription in variant κB sequences as those in the promoter of p105. The same clones, however, show the same p65 mRNA levels as parental A9 cells (Fig. 6 *B*), indicating that the balance between p50 and p65 subunits of NF-κB, rather than absolute amounts, controls class I expression. Three clones overexpressing p50 mRNA showed a threefold decrease in K^b-specific mRNA levels (Fig. 6 *B*) and revealed strongly increased KBF1 DNA binding activity in EMSA (Fig. 6 *C*). These cells also showed a two- to threefold decreased cell surface expression of the H-2K^b antigen (Fig. 6 *D*, *left*). Since the H-2D^b promoter also contains an NF-κB site, we tested whether A9-p50 transfectants are downregulated in H-2D^b expression; a 20–40% decrease was observed for the three (Fig. 6 *D*, *right*). To test whether overexpression of p65 might

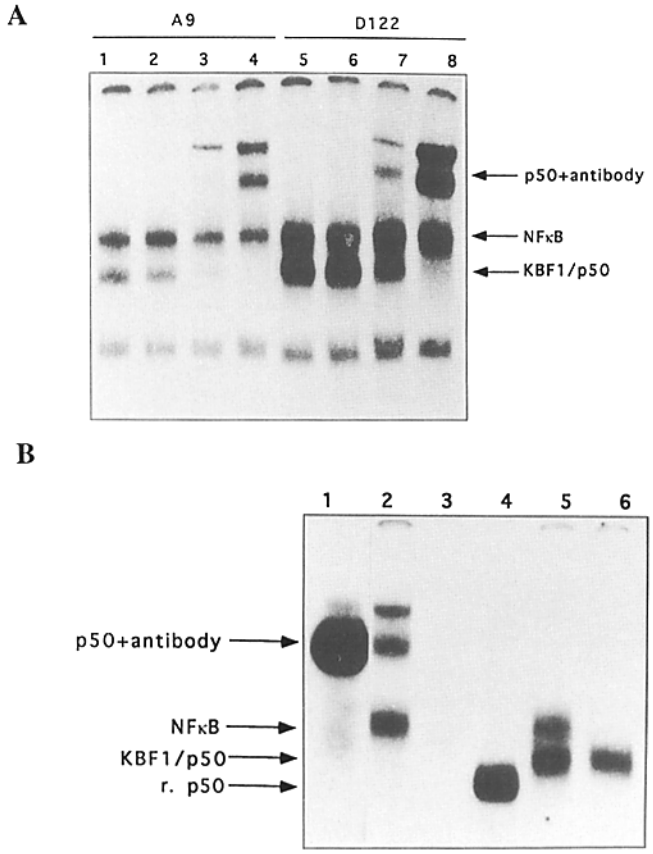


Figure 5. Characterization of κB domain binding proteins. (*A*) A modified CRE probe (−172 to −148) was incubated with nuclear extracts of A9 (lanes *1–4*) and D122 (lanes *5–8*) in the presence of: no antibody (lanes *1* and *5*); anti-H-2D^b (lanes *2* and *6*); anti-p50 (α2) (lanes *3* and *7*); anti-p50 (α3) (lanes *4* and *8*). (*B*) CRE probe was incubated with nuclear extracts from D122 cells (lanes *2*, *5*, and *6*) with recombinant p65 (lane *3*); recombinant p50 (lanes *4*) recombinant p50 and α3 antibody (lane *1*); nuclear extract and α3 antibody (lane *2*); and nuclear extract and recombinant IκB (MAD-3) (lane *6*). The mixtures were analyzed by EMSA.

upregulate class I expression of D122 cells, cells were stably transfected by CMV-p65. Fig. 7 *A* shows that elevation of p65 mRNA levels resulted in increased NF-κB binding activity in EMSA, and in almost complete disappearance of KBF1 from nuclear extracts of the transfectants (Fig. 7 *B*). The transfectants showed upregulation of K^b mRNA (Fig. 7 *A*) and, in accordance, a 2.5–4-fold elevation in cell surface K^b expression (Fig. 7 *C*, *left*). D^b cell surface expression was elevated approximately twofold (Fig. 7 *C*, *right*). Transfection with control plasmids of both A9 and D122 did not change K^b or D^b cell surface expression (not shown). Thus, changing the balance between NF-κB and KBF1 modulates transcriptional activity of the K^b promoter. Elevated NF-κB complex is correlated with activation of the genes while elevated KBF1 is correlated with suppression of transcription.

Discussion

The downregulation of MHC class I gene expression in malignant cells has been shown to be an important mechanism for tumor escape from immune surveillance. In this study the molecular basis underlying class I deficiency was investigated by comparing a high K^b expresser clone to a low expresser K^b clone. We have first demonstrated that the endogenous H-2K^b gene is structurally intact and can be expressed in the parental D122 cells similarly to a normal genomic clone, provided multiple copies of the gene are inserted (Fig. 2). A similar conclusion was reached in some other tumor systems. For example, the IC9 clone of the T10 sarcoma (H-2^b × H-2^k) does not express K^b, K^k, and D^k antigens, however, the cloned K^b gene from these cells could be expressed in Ltk$^-$ cells and also in the original IC9 cell line from which it was derived (68). Similarly, the K^b gene isolated from nonexpresser cells, embryonal carcinoma PCC4-aza-RI, was functionally expressed when transfected into differentiated cells (69).

We have further shown that suppression of the K^b gene is at the transcriptional level; low RNA levels correlated with the low amount of H-2K^b protein in the cytosol and on the cell surface of nonexpressor cells (Fig. 1). CAT assays, using various deletions mutants of the K^b promoter region, indicated that the domain downstream to nucleotides −142, which encompasses enhancer B (containing TATA and CAAT boxes), is similarly activated in expresser and in nonexpresser cells (Fig. 3). Moreover, nuclear extracts of the two cell lines revealed similar factors binding to enhancer B at similar concentrations (Fig. 4, *B–D*). This region, however, shows very low activity in A9 and D122 cells. Similar results were obtained with constructs containing enhancer B alone in BALB/c 3T3 cells and in Ltk$^-$ cells (25, 52). CAT constructs containing enhancers A and B show differential activities in A9 and D122 cells; a twofold activation is observed in the p190 CAT that contains the NF-κB binding motif, and a threefold activation is observed in the activity of the p365 CAT construct between the two clones (Fig. 3). The active element upstream of the NF-κB is probably a CREB/ATF/AP1-like binding motif between −210 and −188. Purified AP1, cyclic adenosine monophosphate–inducible transcription factors, as well as a member of the nuclear hormone receptor superfamily, H-2RIIBP, were shown to bind this motif (24, 36, 70). We have shown before that A9 cells expressed higher levels of c-*fos* and c-*jun* than D122 cells and that stable transfectants of either c-*fos* alone or c-*fos* + c-*jun* expression vectors increased H-2 expression (71, 72). Transient transfections of CAT constructs into c-*fos* + c-*jun* D122 over expressers showed high activation of the p365 and low activation of the p190 constructs, indicating also participation of an API complex in upregulation of class I in these cells (Yamit-Hezi, A., D. Plaksin, and L. Eisenbach, manuscript submitted for publication).

Gel shift analysis indicated that some enhancer A binding proteins were more abundant in nuclear extracts from D122 as compared with nuclear extracts from A9 cells (Fig. 4, *E* and *F*). Most prominent were complexes A3 and A4, which bind specifically to minimal NF-κB domain −172 to −160 (Fig. 4 *E*, lane *8*, and *F*, lane *1*). By the use of specific anti-p50 antibodies, which react only with the p50 homodimer KBF1, and recombinant IκB (MAD-3), which binds to the

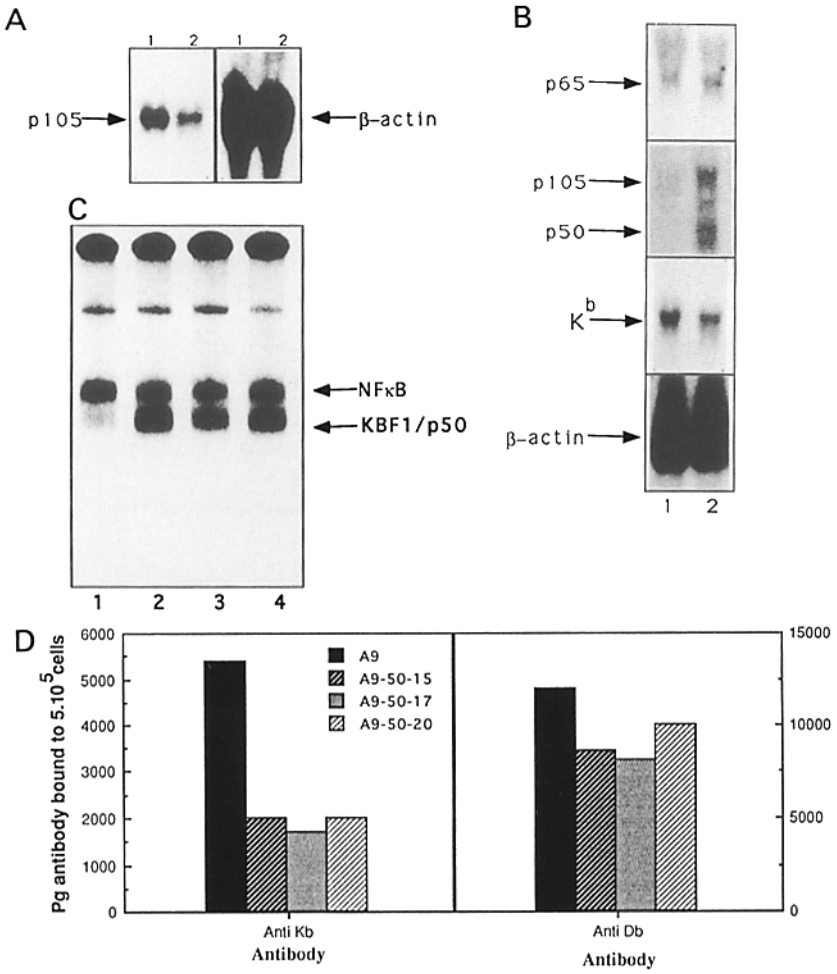


Figure 6. Effect of p50 transfection on K^b expression in A9 cells. (*A*) Endogenous mRNA levels of p50 precursor (p105), in D122 (lane *1*) and A9 (lane *2*) cells. β-actin was used as control for equal amounts of RNA. (*B*) mRNA levels of p65, p50, p105, K^b, and β-actin of A9 (lane *1*) and A9-p50 transfectant. (lane *2*) (*C*) A ^{32}P CRE probe was incubated with nuclear extracts of A9 (lane *1*) and A9-p50 transfectants (lanes *2–4*). The mixtures were analyzed by EMSA. (*D*) H-2K^b and H-2D^b cell surface expression of A9 and A9-p50 transfectants: H-2K^b (*left*) and H-2D^b (*right*). Cell surfaces were analyzed by RIA as described in Materials and Methods.

p65 subunit and inhibits binding of NF-κB complex to the DNA, we found that complex A3 is NF-κB and complex A4 represents KBF1 (Fig. 5). *c-rel* and v-*rel* antibodies did not react with any of the complexes, indicating that p65 and p50 are probably the only subunits in A3 and A4 complexes. The fact that both NF-κB and KBF1 activities are increased in D122 cells is in apparent discrepancy with a number of studies that showed a correlation between class I expression and activity of both KBF1 and NF-κB (73, 74). However, recently it was demonstrated that the presence of NF-κB but not KBF1 seems necessary for class I expression in a variety of human tumor cell lines (75), suggesting that it is the balance between NF-κB and KBF1 binding that might be the determinant for expression of K^b in 3LL clones. In this study, we therefore transfected K^b expresser A9 cells with a p50 expression vector, and low expresser (D122) cells with a p65 expression vector. Elevated K^b mRNA and cell surface protein in D122-p65 transfectants correlated exclusively with binding of NF-κB to the A enhancer motif (Fig. 7). On the other hand, increased KBF1 binding in A9-p50 transfectants correlated with decreased K^b mRNA and cell surface expression (Fig. 6).

Controversial results were obtained in various studies as to the role of KBF1 in activation of κB-controlled promoters. KBF1 was implicated in the constitutive basal expression of the MHC class I (36) and TNF-α gene in murine macrophages (76). Recombinant p50 and p65 subunits of NF-κB were shown to independently activate transcription in vitro from various κB motifs (56, 57). In contrast, transient coexpression of p50 with κB-dependent reporter constructs in transient CAT assays indicated that $(p50)_2$ suppresses the *trans*-activation by NF-κB in monkey COS and mouse L cells (53). Similarly, transient cotransfection of excess p50 with low amounts of p65 expression vectors decreased expression of HIV-κB CAT reporter (77). In nontransformed $CD4^+$ T lymphocytes, antigenic stimulation, which upregulates IL-2 gene expression, decreased the levels of KBF1 and increased the levels of NF-κB, indicating a physiological function for $(p50)_2$ homodimers in suppression of IL-2 expression (55). Another study using cell hybrids containing only KBF1 but not active NF-κB did not activate the IL-2 receptor κB enhancer (78). Similarly, coexpression of $(p50)_2$ with a reporter plasmid containing HIV-1 κB motif caused no activation of the reporter gene (54). Unlike KBF1, homodimers of p65 clearly have *trans*-activating function although it is not clear whether p65 homodimers actually exist in intact cells (53). Moreover, $(p65)_2$ homodimers have a reduced affinity for all κB motifs tested so far (56). Also, a heterodimer of p50 and

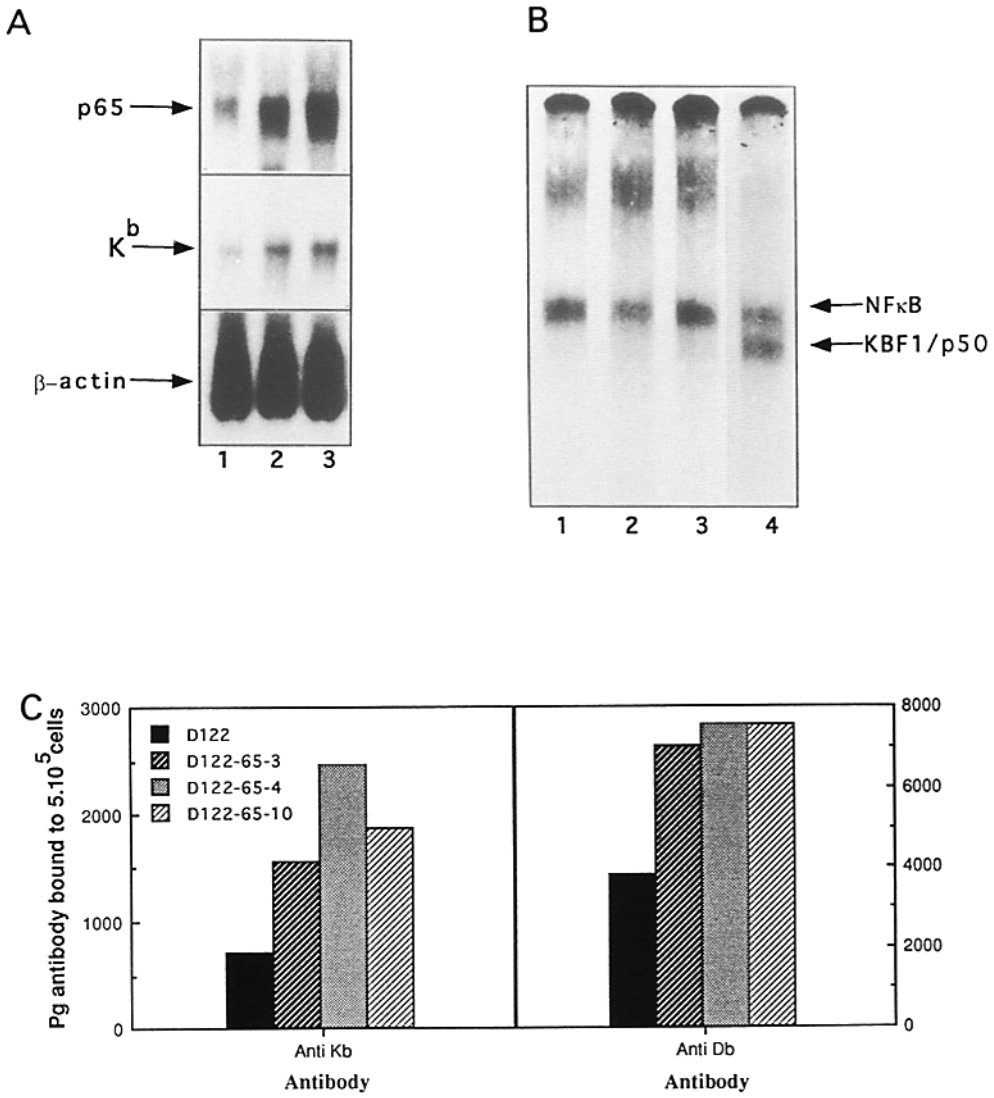


Figure 7. Effect of p65 transfection on K^b expression in D122 cells. (*A*) mRNA levels of p65 (*top*); K^b (*middle*); and β-actin (*bottom*) of D122 (lane *1*) and D122-p65 transfectants (lanes *2* and *3*). (*B*) A ^{32}P CRE probe was incubated with nuclear extracts of D122 (lane *4*) and D122-p65 transfectants (lanes *1–3*). The mixtures were analyzed by EMSA. (*C*) H-2K^b and H-2D^b cell surface expression of D122 and D122-p65 transfectants: H-2K^b (*left*) and H-2D^b (*right*). Cell surfaces were analyzed by RIA as described in Materials and Methods.

p65 (NF-κB) showed activating properties both in vitro and in vivo (53, 55–57). It is surprising however that the low expresser clone D122 shows higher levels of NF-κB than the high expresser clone A9. A possible explanation may reside in the relative affinities of NF-κB and KBF1 in the H-2 κB motif. Recently it was shown that KBF1 has a higher affinity to the H-2-κB motif than NF-κB, with K_d values of 6.2 and 10.9 pM, respectively (56). KBF1, which is highly expressed in D122 nuclear extracts, might therefore prevent binding of *trans*-activating NF-κB by occupying κB binding sites. Transfection of multiple copies of the K^b promoter may dilute the excess of KBF1 and enable activation of K^b expression. Elevating the concentration of NF-κB by overexpressing p65 has a similar effect on activation of K^b expression.

In conclusion, this study demonstrates that KBF1 might have an inhibitory function on class I promoters containing κB motif in vivo, and that the balance between the p50 and p65 subunits of NF-κB is crucial for class I expression.

Although the K^b gene is barely expressed in D122 cells, the H-2D^b gene is expressed at detectable levels. In A9 cells the D^b gene is expressed at approximately threefold the density than it is on D122 cells. How can these allele-specific differences between K^b and D^b expression in D122 cells be explained? Comparison of promoter sequences in the two alleles showed that enhancer A is highly homologous between the two promoters. Indeed, transcription by p65 elevated also D^b expression and transfection by p50 suppressed partially D^b expression. Enhancer B on the other hand shows 14/66 differences in nucleotide sequences (25, 79). It is conceivable that different factors may bind to enhancer B and affect differentially basal level expression of class I alleles. Such differences were observed in EMSA using enhancer B sequences of K^b and D^b and nuclear extracts from A9 and D122 cells (R. Goodenow, personal communication). Allele-specific down-regulation of class I alleles was reported in several human tumors (80–84). It will be of interest to further investigate mechanisms involved in allele-specific regulation of class I genes.

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Address correspondence to Lea Eisenbach, Department of Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel.

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