The Transcription Factor NF-κB/p50 Interacts with the blk Gene during B Cell Activation*

(Received for publication, March 27, 1998, and in revised form, May 11, 1998)

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The B cell-specific transcription factor Pax-5 has been shown previously to interact with the promoter of the blk gene in vitro. blk encodes a tyrosine kinase associated with the B cell receptor, which is expressed during the early but not the final stages of B cell development. To investigate whether Pax-5 regulates expression of the blk gene in vivo during B cell development and/or activation, Pax-5a was overexpressed in B cell lines. Increases in blk promoter activity using a chloramphenicol acetyltransferase reporter gene system suggested a role for Pax-5a as a transcriptional activator. Subsequent site-specific mutagenesis studies showed that mutations of the Pax-5 binding site on blk significantly alter promoter activity, although results suggested that other factors could bind to this region as well. Using mobility shift assays, we detected an inducible transcription factor that interacts strongly with a sequence overlapping the Pax-5 site on the blk promoter and identified this as a homodimer of NF-κB/p50, a member of the NF-κB/Rel family of transcription factors. This factor was present at high levels in lipopolysaccharide-activated normal B cells and in plasma cell lines but either at low levels or undetectable levels in resting normal B cells or pre-B or mature B cell lines. In contrast, lipopolysaccharide induction of a pre-B cell line (703/Z) induced a complex that contained both NF-κB/p50 and p65. These studies suggest that different NF-κB complexes are able to interact with a sequence overlapping the Pax-5 site on the blk promoter and that the relative levels of “bound” factor influence levels of blk expression. Since p50 homodimers and p50/p65 heterodimers of the NF-κB complex should have opposing effects on blk transcription, this could provide a mechanism to differentially regulate blk expression during B cell development and activation.

The activation and subsequent proliferation and differentiation of mature B cells into plasma cells is perhaps the most critical event within the B cell differentiation pathway, because it leads to the production of antibodies necessary for the elimination of foreign antigens. B cell activation is typically initiated by cross-linking of the B cell antigen receptor complex, but activation signals can also be generated through binding of certain cytokines or the B cell mitogen lipopolysaccharide (LPS). Activation involves a cascade of events associated with proliferation and differentiation, eventually resulting in the presence of plasma cells, which secrete large amounts of antibodies and memory B cells. In order to elucidate the molecular pathways involved in B cell development and activation, we have taken the approach of investigating specific transcription factors and the roles they play in the modulation of gene activation and/or repression during these processes.

A number of Src family kinases have been reported to be associated with the B cell receptor complex, including the B cell-specific Blk kinase, encoded by the blk gene (1). In this study, we use blk as a B cell-specific target gene whose expression is regulated, at least in part, by transcription initiation (1). Blk transcripts are present in all pro-B, pre-B, and mature B cell line studied but are undetectable at the plasma cell stage (1). As has been observed in many developmentally regulated genes, the 5′-flank of the blk gene contains at least four transcription start sites but no functional TATA box (1). Earlier work indicated that a 320-nucleotide (nt) promoter region of blk can promote transcription of a heterologous gene after transcription into B cell lines (2), although its activity is low. At least one B cell-specific transcription factor, the B cell-specific activator protein (BSAP) has been shown to interact specifically with a 5′ blk sequence in vitro (2, 3).

BSAP is encoded by the Pax-5 gene and is a member of the paired domain family of transcription factors. BSAP (also named Pax-5) protein has been detected in B cells, adult testis, and the developing midbrain (3–5). Within the B cell lineage, Pax-5 transcripts and its encoded products have been detected in pro-B, pre-B, and mature B cell lines, whereas either very low or undetectable levels were found in the various plasma cell lines studied (3–5). Pax-5 is alternatively spliced, and at least four isoforms have been detected in nuclear extracts of B cell lines, although the expression levels of the full-length form, Pax-5a, are higher than any of the alternatively spliced forms Pax-5b, -5d, or -5e (3). The amino acid sequence of isoform Pax-5a is identical to that of BSAP (3).

The in vivo function of Pax-5 has been investigated through targeted gene disruption in mice (6). Homozygous mutant mice display a complete developmental block at the late pro-B cell stage, suggesting essential regulatory functions in early B cell development. Many putative target sequences for Pax-5 have been reported, including the promoters of CD19, CD20, μ, Vpre-B, J-chain, blk, and mb-1 (2, 4, 7–11) as well as the λ5′-α enhancers and switch regions of immunoglobulin (Ig) heavy chain.

**The abbreviations used are:** LPS, lipopolysaccharide; nt, nucleotides; BSAP, B cell-specific activator protein; WT, wild type; CAT, chloramphenicol acetyltransferase; BSA, bovine serum albumin.

* The work was supported by National Science Foundation Research in Undergraduate Institutions Grant MCB-9419067. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ”advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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chain genes (12, 13). Based on the expression pattern of Pax-5 target genes, it is likely that Pax-5 is required during later B cell stages as well. Some evidence to support this comes from a recent report showing that overexpression of isoform Pax-5a in plasma cell lines can repress expression of two of its target genes, the J-chain and the Ig heavy chain gene (10, 14, 15).

Recent studies suggest that Pax-5a can act as an activator, a docking protein, or a repressor, depending on the target gene and possibly the developmental state of the B cell. For example, the expression patterns of the CD19 and Pax-5 genes in B cells are identical, which suggests that Pax-5 is a positive regulator of the CD19 gene (4). This possibility is further supported by the observation that CD19 expression has ceased completely in homozygous Pax-5 mutant mice (6). In the regulation of the B cell-specific mb-1 gene, Pax-5a functions as a docking protein to recruit a group of more widely expressed transcriptional activators, the Ets proteins (16). In contrast, a suppressor function has been suggested by studies of both J-chain and Ig heavy chain genes, although the exact mechanisms by which this is accomplished by Pax-5a are not well understood (10, 12, 14, 17, 18). In regard to the regulation of blk gene expression, it has not yet been established what in vivo role, if any, Pax-5a plays. Notably, the levels of blk RNA in the pro-B cells from homozygous Pax-5 knockout mice were reportedly identical to those of their wild type (WT) littermates, which either implies that Pax-5a does not regulate blk levels during the earliest stages of B cell development or that the absence of Pax-5a in knockout mice leads to an artificial situation of redundancy in which other factors can take over Pax-5a functions.

In this study, we wished to investigate whether Pax-5a influences blk expression during B cell development and/or activation. We show here that overexpression of Pax-5a in B cell lines leads to an increase of blk promoter activity as measured using a reporter gene system, which suggests that it acts as a transcriptional activator. Subsequent site-specific mutagenesis studies of the blk promoter showed that mutations of the Pax-5 binding site can significantly alter blk promoter activity in vivo, although the results suggest that other factors with binding sites close to or even overlapping with the Pax-5 site may influence blk transcription as well. Using normal B cells activated with LPS, we subsequently identified an inducible transcription factor that binds strongly to a sequence overlapping the Pax-5 site in activated B cells.

This inducible factor was identified as a homodimer of NF-κB/p50, a member of the NF-κB/Rel family of transcription factors that in mammals contains the protein members p50, p65, p52, c-Rel, and RelB; these subunits can form either homodimers or heterodimers (19–22). NF-κB is present in the cytoplasm during periods when it is not needed, forming an inactive ternary complex with the so-called inhibitory (IκB) factor, which prevents NF-κB from moving into the nucleus. The NF-κB pathway can be activated in B cells by IgM cross-linking or by the addition of LPS (19–22). In such situations, the IκB protein is degraded, and the remaining (active) NF-κB dimer is translocated to the nucleus, providing a system that is able to respond very rapidly to cell activation events. Interestingly, p50 homodimers lack a transactivating domain, and it has been implied that such dimers are involved in repression of transcription initiation (22–25). In contrast, p50 heterodimers are thought to be involved in transcriptional activation (22–25).

Unexpectedly, LPS induction of a pre-B cell line 703/Z did not induce formation of the p50 homodimer complex but instead induced a complex containing both p50 and p65. Further analysis in B cell lines representing different stages of B cell differentiation indicated that the relative levels of p50 homodimers, p50 heterodimers, and Pax-5a bound to the blk promoter strongly influence the levels of blk transcripts in these lines.

These studies suggest that different NF-κB complexes are able to interact with the blk promoter and that these complexes compete for binding with Pax-5a. Because NF-κB/p50 homo- dimers and heterodimers have opposite effects on transcription initiation, this may provide a mechanism to differentially regulate blk expression after cell activation, depending on the developmental state of the B cell.

MATERIALS AND METHODS

Cell Lines—All cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum (Bio whitelistaker, Inc.), 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol.

DNA Constructs—The construction of the p-191blkB/CAT2 μE reporter construct has been described elsewhere (2). A promoterless chloramphenicol acetyltransferase (CAT) construct, ΔCAT2 μE, was derived from p-191blkB/CAT2 μE by restriction digestion with the endonucleases SstI and SalI, resulting in the specific deletion of blk sequence only. The effector construct pcDNA3.Pax-5a was made by digesting both the expression vector pcDNA3 (Invitrogen) and the pBS-1.2 plas- mid (2), containing the full-length cDNA sequence of Pax-5a, with the restriction endonuclease NotI, followed by gel purification of the 1.2-kb NotI insert. The Pax-5a insert was then ligated directly into calf intestine phosphatase-treated, NotI-digested pcDNA3, and the orientations of the resulting clones were determined using dideoxy sequencing. This resulted in clone pcDNA3.Pax-5a, which expressed Pax-5a protein as determined by in vitro transcription and translation in the presence of [35S]methionine followed by SDS-polyacrylamide gel electrophoresis and radiography. The pcDNA3 construct was used as a negative control effector construct, and the HBIICAT construct (2) was used as a control for transfection efficiency.

Transfections and CAT Assays—The B cell lines A20/2J, M12, and S194 were transiently transfected using DEAE-dextran, and chloramphenicol acetyl transferase was assayed as described elsewhere (2). The generation of clones S194-T1 and S194-T4, which are stable transfectants containing the full-length isoform Pax-5a in S194 (clone T1 expresses low levels of Pax-5a; clone T4 expresses high levels) has been described elsewhere (10, 14). The relative CAT conversion was determined by calculating the ratio of the acetylated to the acetylated plus unacetylated counts per minute, subtracting the activity of the mock transfection (transfection without DNA), followed by subtraction of the promoterless construct (ΔCAT2 μE) and normalization for protein content as determined by the Bradford assay. To enable comparison of different cell lines, values were normalized for transfection efficiency by each as determined by the relative CAT conversion of the HBIICAT control construct (see above). Relative CAT activity values for each transfection were calculated as -fold change compared with the activity of the wild type (−191blkB/CAT2 μE construct).

In Vitro Transcription and Translation of Pax-5a—The plasmid (pBluescript) pBS-1.2 (containing full-length Pax-5a cDNA; see Ref. 2) was transcribed in the sense direction with T3 RNA polymerase. Translation was carried out using a rabbit reticulocyte lysate (Promega) according to the supplier’s directions. Between 1 and 2 μl of extract was used for mobility shift assays.

Antibodies—To generate an antibody against Pax-5a isoforms, the paired domain sequence of the Pax-5 gene was cloned downstream from the glutathione S-transferase sequence in the vector pGEX2T (Amer sham Pharmacia Biotech). The fusion protein containing 148 amino acids of the paired domain sequence of Pax-5 was purified using glutathione-agarose beads. New Zealand White rabbits were immunized intradermally with 500 μg of purified protein, followed by five booster immunizations of 100 μg each that were administered every other week beginning 21 days after the initial immunization. Antiserum, named Ed-1, was obtained at 12 weeks, analyzed by enzyme-linked immuno- nusorbert assay, and used at a 1:2000 dilution in Western blot analysis or 1:5 or 1:10 dilution for the mobility shift assays. Antibodies to p65, p50, and Sp1 were purchased from Santa Cruz Biotechnology, Inc., and used at a 1:5000 dilution in Western blot analysis or 1:5 in mobility shift assays. The anti-p65, anti-Pax5, and anti-Sp1 antibodies were detected using a horseradish peroxidase-conjugated donkey-anti-rabbit secondary antibody (Amersham Pharmacia Biotech). The p50 antiserum was directed against the nuclear localization domain of p50, and a rabbit anti-goat, horseradish peroxidase-conjugated antibody was used as its
RESULTS

The murine blk promoter contains a Pax-5 binding site in the region covering nt -68 to -37, as determined previously in vitro. We wished to determine whether this sequence represents a functional site on the blk promoter. Initially, we tested whether overexpression of Pax-5a could increase transcription from a blk minimal promoter fragment driving expression of the CAT reporter gene. Transient co-transfections of the Pax-5a expression construct (pcDNA3/Pax-5a) and the blk reporter construct (−191)blkCAT2θE and the Pax-5a effector construct pcDNA3/Pax-5a into B cell lines, left, cell line M12; right, cell line A20/23. Left and right: lane 1, reporter alone; lane 2, reporter plus effector construct; lane 3, reporter plus negative control effector construct (see "Materials and Methods"). -Fold change in CAT conversion is shown as compared with reporter construct alone ± S.D. (n = 3); transient transfections of the blkCAT reporter construct in S194 clones stably transfected with an Pax-5a expression construct. Results of CAT assays are shown as -fold increase of CAT conversion ± S.D. compared with non-Pax-5a-transfected S194 cells. Lane 1, S194 + blkCAT; lane 2, S194-Tf1 (low Pax-5a) + blkCAT; lane 3, S194-Tf4 (high Pax-5a) + blkCAT.

Next, we tested whether mutations within or in the vicinity of the Pax-5 binding site would affect the activity of the blk promoter in vitro. Earlier work (2) using mobility shift assays had already established that mutations at the 5'-end and center of the Pax-5 binding site (mutA and mutB), but not at the 3'-region (mutC), prevented Pax-5a from binding to the blk promoter in vitro. The location of the mutated blk sequences relative to the Pax-5 binding site is shown in Fig. 2A. The WT (−191)blkCAT2θE or one of three mutated constructs (mutA, mutB, and mutC; see Fig. 2A), were transiently transfected into the highly differentiated, mature B cell line A20/23J (which expresses moderate levels of Pax-5a). Unexpectedly, CAT assays showed that mutations at the 5'-end of the binding site (mutA) led to a significant increase in promoter activity. As expected, mutation of the central region of the Pax-5 site (mutB) significantly reduced promoter activity, whereas mutation of mutC had no significant effect on promoter activity. Results from the CAT assays are shown in Fig. 2B. These data at least partially agreed with the mobility shift assays mentioned above, because both in vivo and in vitro studies pointed toward the importance of the central region (mutB) but not the 3'-region (mutC) of the Pax-5 binding site for interaction with Pax-5a. The observation that mutA causes an increase in blk activity will be discussed in more detail below.

NF-κB/p50 Binds blk Promoter

Fig. 1. Pax-5a overexpression in B cell lines and resulting changes in activity of blkCAT reporter construct. A, acetylated chloramphenicol; U, unacetylated chloramphenicol. A, transient co-transfections of the blkCAT reporter construct (−191)blkCAT2θE and the Pax-5a effector construct pcDNA3/Pax-5a into B cell lines. Left, cell line M12; right, cell line A20/23J. Left and right: lane 1, reporter alone; lane 2, reporter plus effector construct; lane 3, reporter plus negative control effector construct (see "Materials and Methods"). -Fold change in CAT conversion is shown as compared with reporter construct alone ± S.D. (n = 3); transient transfections of the blkCAT reporter construct in S194 clones stably transfected with an Pax-5a expression construct. Results of CAT assays are shown as -fold increase of CAT conversion ± S.D. compared with non-Pax-5a-transfected S194 cells. Lane 1, S194 + blkCAT; lane 2, S194-Tf1 (low Pax-5a) + blkCAT; lane 3, S194-Tf4 (high Pax-5a) + blkCAT. Fig. 1B. Together, the transcription studies indicated that Pax-5a functions as an activator of transcription on the murine blk gene.
Blk levels drop when mature B cells differentiate into plasma cells, and plasma cells have undetectable levels of blk transcripts (1). If Pax-5a is an activator of blk transcription, a decrease of promoter-bound Pax-5a would be expected when mature B cells are stimulated by LPS or anti-IgM. To obtain data on a range of B cell activation stages, we decided to use small resting B cells isolated from mice spleens using Percoll gradients. These cells were activated with LPS and collected at various time points after the LPS addition, and nuclear extracts were prepared. To investigate the levels of Pax-5 proteins in these samples, mobility shift assays were used with a labeled blk DNA fragment F3, which covers nt 270 through 136 of 5'-blk. 

Results of the mobility shift assays are shown in Fig. 3A. The data showed that the amount of Pax-5a interacting with the blk promoter decreased after 48 h of activation by LPS to very low levels at 73 h (Fig. 3A, arrow 2). To distinguish between a true decrease in total Pax-5a levels in the nucleus and a change in the amount of Pax-5a bound to the blk promoter, Western blot analyses were performed on the nuclear extracts. An anti-Pax-5 serum, Ed-1, which recognizes all four isoforms of the Pax-5 gene, was generated for this purpose (see “Materials and Methods”). Results of the Western blots are shown in Fig. 3B. Surprisingly, these data indicated that Pax-5a levels in the nucleus did not change significantly during this 73-h period.

This observation could suggest that blk-bound Pax-5a is replaced by a second transcriptional regulator, which is expressed after LPS induction. In agreement with this hypothesis, we noticed the presence of a new, slower migrating complex in activated cells (Fig. 3A, arrow 3). This complex was present at almost undetectable levels in resting B cell nuclei but became a major complex present on the blk probe after 24–48 h of LPS stimulation. At the 73-h time point, this higher complex was still very prominent, in contrast to the Pax-5a complex. A second induced complex was found to bind very weakly to the blk promoter as well (Fig. 3A; indicated by arrow 4).

A somewhat unexpected finding was that in nuclear extracts prepared from resting B cells, a protein that is likely to represent the alternatively spliced isoform Pax-5d bound to the blk promoter at levels almost identical to Pax-5a (see Fig. 3A, arrow 1). Interestingly, after 24 h of LPS stimulation, amounts of this protein had already dropped to a base-line level. Because no antisera specific to Pax-5d are yet available, we could not determine the exact nature of this protein. Although it was recognized by the anti-Pax-5 antiseraum Ed-1, it could also be a proteolytic fragment derived from isoform Pax-5a. This possibility is still under investigation.

The LPS-inducible complex was studied in more detail using Fig. 2.
NF-κB/p50 Binds blk Promoter

Fig. 3. Detection of Pax-5 proteins present in normal mouse B cells before and after LPS stimulation. A, mobility shift assay using the F3 blk probe (see “Materials and Methods”). Arrow 1, protein that resembles Pax-5d; arrow 2, Pax-5a; arrow 3, the major LPS-inducible complex; arrow 4, a minor LPS-inducible complex. Time points of cell collection (in hours) after LPS induction are indicated at the top. B, Western blot analysis of Pax-5a, using Ed-1 antiserum (see “Materials and Methods”). Arrow 1, a major LPS-inducible complex. Time points of cell collection after LPS induction (in hours) are indicated at the top; the arrow indicates Pax-5a protein.

NF-κB proteins bind their targets either as homodimers or heterodimers. Based on the position of the inducible complex on shift gels (discussed with Fig. 6), as well as its inability to be recognized by p65 (see arrow 2 in Fig. 5), we hypothesize that this complex represents a homodimer of p50. One study showed that the optimal DNA binding site for p50/p50 homodimers is different from that of p50/p65 heterodimers (26). Preferential binding of p50 homodimers on the blk promoter in agreement with the NF-κB site on blk: 9 of 10 nt correspond to a p50/p50 homodimer consensus sequence, whereas only 8 of 10 nt correspond to a p50/p65 consensus site (see Fig. 4A). Because the p50 subunit lacks a transactivating domain, it has been implicated in repressor functions. The increased levels of p50 homodimers accessible to the blk promoter may provide a mechanism to down-regulate blk expression after B cells become activated.

In addition to this p50 homodimer, a faint, slower migrating complex was detected after cell induction (arrow 3). This complex was found to contain p50 but not p65 and could be competed by an oligonucleotide containing the NF-κB binding site. The fact that both inducible complexes represent members of the NF-κB family is perhaps not surprising, considering that such factors are normally induced in B cells by either IgM cross-linking or LPS.

The DNA binding sites for Pax-5 and NF-κB/p50 are very close together and may in fact overlap (Fig. 4A). We wished to investigate whether the two factors bind to the blk promoter together, which would be indicative of a “Docking” function for Pax-5a, similar to the situation on the mb-1 promoter (16). To address this possibility, co-immunoprecipitation experiments were performed using antibodies specific to both Pax-5a and NF-κB/p50. Nuclear extracts from B cell presecretor line BCL1 and pre-B cell line PD36 were incubated with antibody-coated beads overnight, the immunoprecipitated proteins were separated on SDS-polyacrylamide gels, and Western blot analysis was performed. We were unable to demonstrate any association between NF-κB/p50 and Pax-5a in these experiments (data not shown). Some further evidence for a “replacement” but not a “cooperation” mechanism was supported by mobility shift assays in which the addition of excess in vitro translated Pax-5a to nuclear extracts from BCL1 (containing both p50 and Pax-5a protein) resulted in increased levels of bound Pax-5a and a simultaneous decrease of bound p50 (data not shown).

Together, these data suggested that at least in vitro, Pax-5a and NF-κB/p50 compete for binding to the −65 to −45 region of blk rather then binding to the sites together.

To investigate whether the induction of an early B cell line would also result in binding of p50 homodimers to the blk promoter, we used the pre-B cell line 703/Z, which has been shown by others to be inducible by LPS (21). Cells were activated with LPS as described (above), and time points were collected at 0, 4, 48, and 120 h. Relative levels of p50 and Pax-5a from uninduced or induced cells were compared using mobility shift assays. As shown in Fig. 6A (lane 2), reasonably high levels of bound Pax-5a were present in unstimulated 703/Z cells, but no or very low levels of p50 homodimers were detectable. After 4 h of LPS stimulation, a higher molecular weight complex was detected, at a level equal to that of bound Pax-5a (Fig. 6A, lanes 3–5). The mobility shift pattern at 4 h of LPS stayed unchanged for the remainder of the 120 h (not shown). Interestingly, the induced complex appeared to run at a higher position in the gel compared with the p50 homodimer complex that we had previously identified in activated B cells and BCL-1 cells (Fig. 4B, arrow 2). Levels of p50 homodimers were not affected by LPS stimulation. Further analysis showed that the induced complex represents a heterodimer containing both p65 and p50 subunits of NF-κB, which are normally involved in activation of transcription (Fig. 6A, lanes 6 and 7).
Thus, at least in vitro, LPS stimulation of an early B cell line leads to interaction of the p50/p65 with the \textit{blk} promoter, but not p50/p50, whereas in activated mature B cells or presecretor B cell lines, the p50/p50 homodimer binds preferentially. To see whether this distribution reflected patterns present during B cell ontogeny, a number of B cell lines representing different stages of B cell development were analyzed for the presence of p50/p50 homodimers versus p50-containing heterodimer complexes on the \textit{blk} promoter. Using mobility shift assays, we found that early B cell lines, including pro- and pre-B cell lines, have very low levels of promoter-bound p50 homodimers. Mature B cells, presecretors, and plasma cell lines have increasing levels of bound p50 homodimer. Levels of p65/p50 or other heterodimeric NF-\kappa B complexes varied in these lines. Results are shown in Fig. 6B.

Both p50/p65 and p50/Rel run at a similar position in the shifts, making it difficult to distinguish between the two unless anti-p65 antisera were used. To address this question, additional mobility shift assays as well as Western blot analyses were performed on a number of B cell lines including A20/2J, CH12, BCL1, SP2/0, and S194. Results from mobility shift assays (not shown) indicated that the slower migrating complex in these lines contained p50 but no, or only a relatively small amount of, p65. Interestingly, Western blot analysis showed that all tested lines had both p50 and p65 present in

**Fig. 4.** Mutational analysis of \textit{blk} promoter sequence and its effects on binding of Pax-5a and the inducible factor \textit{in vitro}. Competition shift assays using BCL1 nuclear extracts and the F3 \textit{blk} probe. A, schematic overview of the promoter sequences of WT and mutated \textit{blk}, WT CD19, and WT J-chain in the vicinity of the Pax-5 binding sites. Nucleotide positions of the \textit{blk} sequence relative to its first transcription start site are indicated. The alignment with Pax-5 (4, 10) and NF-\kappa B (26) consensus binding sites is indicated. B, results of competition shift assays with a 500-fold molar excess of unlabeled oligonucleotides (see Fig. 4A) as indicated. Arrow 1, Pax-5a; arrow 2, the LPS-inducible complex; arrow 3, a minor, LPS-inducible complex. \textit{ivt}, \textit{in vitro} translated.

**Fig. 5.** Identification of the NF-\kappa B site on the \textit{blk} promoter. Competition shift assays using the \textit{blk} BSAP oligonucleotide as a probe; BCL1 nuclear extracts and specific antisera (1:5 dilution) or control (pre-immune, PI, 1:5) serum as indicated; or a 500-fold molar excess of unlabeled NF-\kappa B oligonucleotides. The arrows represent the same complexes as in Fig. 4B.
the nucleus, illustrating the difference between total levels of these factors present in the nucleus versus the amount bound to the \( \text{blk} \) promoter.

Interestingly, the experiments shown in Fig. 6B suggest that the ratio of bound p50 homodimers to bound Pax-5a strongly increases when B cells progress through their various developmental stages, from very low in pro- and pre-B cells to very high in plasma cell lines. These data agree with the patterns seen in LPS-activated normal B cells and may be indicative of a mechanism that regulates the levels of \( \text{blk} \) transcripts during B cell development.

**Discussion**

In the studies described here, we sought to explore the functionality of the Pax-5 binding site on the \( \text{blk} \) promoter, which was shown in earlier studies to interact with Pax-5a and its alternatively spliced isoform Pax-5d in vitro. Our results indicate that the activity of the \( \text{blk} \) promoter is regulated by the relative level of at least three factors, including Pax-5a, the NF-\( \kappa \)B/p50/p50 homodimer, and the NF-\( \kappa \)B/p50/p65 heterodimer. Whether a fourth factor, Pax-5d, plays a functional role in transcription regulation of \( \text{blk} \) or other \( \text{blk} \) target genes is still under investigation.

**NF-\( \kappa \)B/p50 Homodimers Bind to the \( \text{blk} \) Promoter during Activation of Mature B Cells**—An NF-\( \kappa \)B site was identified on the \( \text{blk} \) promoter, which overlaps with the Pax-5 binding site. The NF-\( \kappa \)B family contains at least five members, including p50, p52, p65, RelB, and c-Rel, which share a conserved Rel (DNA-binding) domain that interacts with NF-\( \kappa \)B sites on its target genes (20, 26). Relatively high levels of p50 homodimers, but not p50 heterodimers, were found to bind to the \( \text{blk} \) promoter during the mature B, activated B, and plasma cell stages, although high levels of p50 heterodimers are reportedly present in the nucleus at these cell stages as well (21). One explanation for this could be that the \( \text{blk} \) sequence has a slightly higher homology with the NF-\( \kappa \)B/p50 homodimer consensus site as compared with the p50/p65 dimer consensus site (26), although the combination of other factors already binding to the \( \text{blk} \) promoter may influence this selective behavior as well. No evidence was found for interaction of the p52 subunit with the \( \text{blk} \) promoter, in agreement with the relatively low levels of this subunit during all B cell stages except the plasma cell stage (21).

The members of the NF-\( \kappa \)B family of transcription factors are important regulators of the immune and inflammatory responses in many cell types, including B cells (19, 27–29). We propose that the NF-\( \kappa \)B p50 homodimer plays an important role in the down-regulation of the \( \text{blk} \) gene when B cells are activated and differentiate into plasma cells. As has been shown by others, p50 proteins contain an intact DNA binding domain but no transactivation domain, suggesting that they act as repressors (22–26).

**Relative Levels of Bound Pax-5 and NF-\( \kappa \)B Dimers Regulate \( \text{blk} \) Expression**—Although a second transcription factor, NF-\( \kappa \)B/p50, binds to a site overlapping the Pax-5 site, no evidence pointed toward collaboration or interaction between Pax-5a and p50, using mobility shift assays and co-immunoprecipitations. In contrast, our experiments suggested that either Pax-5a or p50, can bind to this region on the \( \text{blk} \) promoter at a given time and that the relative concentration of the two fac-
tors in the nucleus influences their binding frequency. NF-κB sites have been found in the vicinity of Pax-5 sites in other Pax-5 target genes. NF-κB/p50 binds to two sites on the 3′-α-enhancer of the Ig heavy chain genes, both the 3′α-E (hs1,2) and 3′α-hs4 sites. The two sites are not immediately adjacent, and whether these situations Pax-5a could prevent p50 from binding or vice versa. In this regard, two interesting studies support the hypothesis that binding of Pax-5a can somehow prevent other factors from binding in its vicinity, as shown for both the Ig heavy chain 3′-α-enhancer (27) and the J-chain promoter. Whether Pax-5a is able to prevent binding of NF-κB factors and is relieved from this function after B cell activation is unclear. In any event, overlapping of two binding sites on the blk promoter allows the B cell to somehow regulate the activities of the involved factors during different stages of B cell differentiation.

**Heterodimers of the NF-κB Complex May Increase blk Promoter Activity—**LPS activation of the pre-B cell line 703Z induces the formation of a p50/p65 heterodimer complex on the blk promoter. This result is in agreement with studies by others who activated 703Z cells using LPS (20). They found that in pre-B cell lines, both p50 and p65, but not p52, RelB, or Rel are induced after short term (4-h) LPS exposure. In addition, these authors report that in mature B cell lines, p50, p65, and Rel are present constitutively, whereas plasmacytomas mostly express the p52 and RelB subunits. Since the p50/p65 complex induced in 703Z cells contains a functional activation domain, this would be expected to lead to increased levels of blk transcripts.

These data provide an explanation for earlier studies (1, 2) that had shown a remarkable variation in the amount of blk transcripts in different B cell lines, which did not correlate with the amount of promotor-bound Pax-5a. Results from the study described here provide an explanation for this variation. Generally, B cell lines that express high levels of blk RNA but possess low levels of Pax-5a have relatively high levels of p50 heterodimers (containing p65 or Rel). Because such heterodimers presumably are strong activators of blk expression, their interaction with the NF-κB site on the blk promoter should result in high levels of blk. This is particularly clear in the mature B cell lines WEHI-231 and BFO3. In contrast, cell lines that express low levels of blk RNA in the presence of relatively high levels of Pax-5a usually have high levels of p50 homodimers but not p50 heterodimers bound to the blk promoter, for example the mature B cell lines B17.10 and the presecretor BCL1. Thus, both Pax-5a and the various NF-κB complexes influence the level of blk expression.

The transfection experiments in mature B cell line A20/2J, although hampered by low activity of the blk promoter, may be indicative of a role for p50 heterodimers in increasing blk levels in mature B cells. This cell line represents a normal resting B cell, with low levels of p50 homodimers and moderate levels of Pax-5a. Mutation of a region on the blk promoter (mutA) that is necessary for binding of Pax-5a, but not p50, resulted in an increase in CAT activity. We hypothesize that, as a result of this mutation, other p50-containing complexes will now be able to bind and replace Pax-5a. Since both p50 and p65 subunits were detected in this line, they may form heterodimers and be responsible for the increase in CAT activity.

The co-transfection experiments in A20/2J also imply that p50-containing heterodimers are stronger activators of blk than Pax-5a. This is interesting in regard to the observation that in pro-B cells of Pax-5 knockout mice, blk levels had not decreased significantly. We speculate that this reflects the redundancy of transactivators that have affinity for the blk promoter. It is possible that only low levels of p50/p65 heterodimers are present in the early B cell progenitors of the Pax-5 null mice, levels are high enough to replace Pax-5a functionally, by binding to the NF-κB site in its absence. To test this hypothesis, it would be interesting to create double knockout mice that lack both Pax-5 and NF-κB/p50; such mice would not be expected to have any detectable levels of blk in their pro-B cells or, alternatively, may have a developmental block prior to that of the pre-B cell stage.

**Functional Significance of the p50 Homodimer—**We did not detect a decrease in Pax-5a levels in nuclei of activated B cells, even after 73 h of LPS stimulation, which may be explained by the fact that B cells do not differentiate into plasma cells until 3–5 days after antigen exposure. It may be important that blk expression is down-regulated early in the B cell response; therefore, it may need to be shut off by p50/p50 and not by Pax-5a. This mechanism may be necessary because only a subset of Pax-5 target genes is down-regulated at this time, and the exact time point of down-regulation may vary among its target genes. Down-regulation is likely to depend on the unique function of each target gene during activation processes. It will be interesting to investigate blk levels in B cells of p50 knockout mice, which reportedly do not respond to LPS stimulation (27). Such mutant mice may have a delayed decrease in blk expression, which may prevent the cells from efficiently proliferating and differentiating into plasma cells. One report in agreement with this hypothesis suggests that blk is a growth inhibitor signaling molecule; the authors show that antisense oligonucleotides to blk prevent growth inhibition and apoptosis of an anti-μ-chain activated B cell lymphoma (30).

Regulation of gene expression through differential usage of NF-κB complexes has been shown to exist in T cells. The interleukin-2 promoter of resting T cells has been found to be associated with the p50 homodimer complex but not with other NF-κB complexes (25, 31). This homodimer complex decreased after full antigenic stimulation of the cells, whereas the amount of NF-κB p50/p65 was sharply increased and could be correlated with increased expression of the interleukin-2 gene. These experiments reveal a physiological function for p50 in the T cell response to antigen in that p50 homodimers appear to inhibit expression of interleukin-2 by NF-κB heterodimers.

In conclusion, our experiments show that the relative levels of Pax-5 and NF-κB proteins provide a mechanism that differentially regulates blk expression during B cell development, activating blk transcription early in development and down-regulating its levels during the final stages of B cell differentiation.

**Acknowledgments**—We thank Dr. W. Sha for NF-κB reagents, Dr. Yi Zhang for mouse spleens, and Ilisa Kaatari and Joan Fujita for excellent technical support.

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