Identification of a second inducible DNA – protein interaction in the kappa immunoglobulin enhancer

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ABSTRACT

Control of kappa immunoglobulin light-chain gene expression requires the interaction of tissue specific and developmentally regulated DNA-binding proteins with the kappa gene enhancer. Deletion of enhancer sequences upstream from the NF-kB binding site has been shown to impair enhancer function, implying additional proteins may interact with these sequences. In surveying this region for sites of protein binding, a novel DNA-protein interaction, designated kBF-A, was detected. The binding activity of this factor appears to be specific to activated pre-B cells and correlates with high level induction of kappa transcription in these cells.

INTRODUCTION

The developmental regulation and tissue specific expression of many genes have been shown to involve *cis*-acting enhancer sequences which are thought to function through interactions with specific DNA-binding proteins (1–3). One of the most widely studied mammalian enhancers is associated with the murine kappa immunoglobulin light-chain gene (4–9). This enhancer, which is located within a 200 bp region of the J-C intron, has been shown to contain multiple binding sites for nuclear proteins. Some kappa enhancer binding factors, like kappa gene expression, are tissue specific and developmentally regulated while others are ubiquitously expressed (8–10).

The functional significance of the sequences with which kappa enhancer binding factors interact was first indicated by deletion analysis which showed that progressive deletions into the enhancer caused stepwise reductions in transcription of the kappa gene in plasmacytomas (6). A significant reduction was observed after deletion of a sequence homologous to the core of the SV40 enhancer. This sequence has since been shown to interact with a specific binding factor, NF-kB, and also has been implicated in the control of the kappa gene during B cell development (8,10,11). It now appears that developmental control of the kappa gene is mediated to a large extent by NF-kB since the binding activity of this factor correlates with the induction of kappa gene expression in pre-B cells (3,10,11). Sequences downstream from the NF-kB binding site which are homologous to regions of the

immunoglobulin heavy chain enhancer have also been shown to be important for maximal enhancer function and to interact with nuclear proteins *in vivo* and *in vitro* (1,6,8,12,13,14).

In plasmacytomas, deletions upstream from the NF-kB binding site have been shown to cause stepwise decreases in transcription similar to those observed when downstream sequences containing protein binding sites were deleted from the enhancer (6). In this study we removed sequences upstream of the NF-kB binding site and examined the effects on enhancer function in pre-B cells induced to express the kappa gene. Our results demonstrate this region contains sequences important for maximal enhancer function, and a site for a novel protein-DNA interaction. This binding activity appears to be specific to activated pre-B cells and correlates with increased kappa expression.

MATERIALS AND METHODS

Cell Lines

The characteristics and culture conditions of 70Z/3, 3–1, S194, MPC11, Bal-8 and Hela cells lines have been previously described or referenced (9,15). The Abelson transformed line, 1–8, has pre-B cell characteristics (16). The S107 plasmacytoma cell line actively transcribes the kappa light-chain gene and its nuclear binding activities have been characterized (17,18). Both 1–8 and S107 cell lines were obtained from Dr. R. Perry (Institute for Cancer Research).

Plasmid Constructions, Transfections, and Enzymatic Assays

Plasmid KpCAT was kindly provided by M.L. Atchison and R.P. Perry (described as E⁻CAT in ref. 17). This plasmid contains the V_k21E gene promoter linked to CAT. A 212 bp Dde I-Dde I fragment containing all previously defined sites of protein-DNA interaction in the kappa enhancer was derived from a 512 bp Hinf I-Hinf I enhancer fragment, isolated, and ligated to Hind III linkers. The linkered 212 bp fragment was then ligated to plasmid KpCAT, which had been linearized at the vector Hind III site upstream of the V_k21E promoter, to produce KpCAT-212 (Fig. 1A). A small deletion of the 212 bp Dde I-Dde I fragment was made by cutting this fragment with Hph I, removing 18 bp off the 5' end of this fragment. The resulting 194 bp Hph I-Dde I fragment was ligated to Hind III linkers and then ligated into the

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vector Hind III site of KpCAT to produce KpCAT-194 (Fig. 1A). Both KpCAT-212 and KpCAT-194 have enhancer sequences in the germ-line 5' to 3' orientation.

In transient expression assays, 3–1 pre-B cells were transfected by the DEAE-dextran procedure essentially as described by Grosschedl and Baltimore (19). A total of 4×10^7 cells were washed and resuspended in 2 ml of 25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.3 mM $\text{Na}_2\text{PO}_4 \cdot 7 \text{H}_2\text{O}$, 1 mM MgCl_2 , 1 mM CaCl_2 (pH 7.4) containing 0.5 mg/ml DEAE-dextran (Pharmacia) and 10 μg of plasmid. Cells were incubated in this solution for 30 minutes at 37° C, collected by centrifugation, then resuspended in 10 ml media. From this suspension, 5 ml was added to 20 ml media alone and 5 ml to 20 ml media containing 12.5 $\mu\text{g}/\text{ml}$ bacterial lipopolysaccharide (LPS) (Difco). Final concentration of the LPS was 10 $\mu\text{g}/\text{ml}$. After 24 hours in culture, cells were washed with phosphate-buffered saline, freeze-thawed three times in 0.25 M Tris-HCl (pH 7.5), and heated to 65° C for 5 minutes to inactivate deacetylases. Supernates were collected and assayed for protein according to the method of Bradford (20). CAT activity was assayed by incubating 100 μg of extract in 0.5 M Tris (pH 8.0) with 0.2 μCi of [^{14}C]chloramphenicol and 4 mM acetyl coenzyme A in a final volume of 150 μl for 4 h at 37° C. The degree of acetylation was then determined by thin-layer chromatography, autoradiography, and scintillation counting as described by Gorman et al. (21). Relative CAT activities were determined by normalizing activities of the test plasmids to that of the control plasmid, KpCAT, which has no enhancer elements. Relative CAT activities from at least four independent experiments done with 2 different preparations of the test plasmids were averaged to obtain the data presented. In Figure 1B, relative CAT activities are expressed as a percentage of the relative CAT activity of KpCAT-212 in LPS-stimulated 3–1 cells. Transfections were also performed with 70Z/3 pre-B cells. The transfection efficiency of the 70Z/3 cells was low and this was compensated for in the CAT assay by increasing the concentration of acetyl coenzyme A to 8 mM in the CAT assay and increasing the incubation time to 12 hours.

DNA Probes

Complementary oligonucleotides spanning sites of protein binding were synthesized and are shown in Figure 2B. For use in mobility shift assays, single stranded oligonucleotides were end-labeled with γ -[^{32}P]-ATP and T4 polynucleotide kinase, purified using NENSORB columns (DuPont), then annealed to the complementary strand. A 106 bp Fnu4HI-Hae III fragment used in DNase I footprinting experiments (Fig. 2A) was obtained by digesting the 512 bp Hinf I-Hinf I fragment containing the kappa enhancer with Fnu4HI, dephosphorylating with bacterial alkaline phosphatase, end-labeling with γ -32P-ATP and T4 polynucleotide kinase, digesting with Hae III, then purifying the end-labeled 106 bp fragment from polyacrylamide gels.

Nuclear Extracts

Nuclear extracts were prepared by the method of Dignam and Roeder (22) with the following modifications. Nuclear proteins were extracted with buffer containing 0.4 M NaCl and all buffers contained the protease inhibitors leupeptin (10 $\mu\text{g}/\text{ml}$) and phenylmethanesulfonyl fluoride (0.2 mM). Protein concentration was determined by the method of Bradford (20).

Mobility Shift Assays and DNase I Footprinting

Mobility shift assays and DNase I footprinting were performed essentially as described (15). Mobility shift reaction mixtures typically contained 10 μg nuclear extract, 0.5–1.5 μg polydIdC, and 10,000 cpm (0.1–0.5 ng) end-labeled, duplex oligonucleotide in a final volume of 15 μL . Preparative reactions used in methylation interference and DNase I footprinting assays contained 50–100 μg extract, 5–10 μg polydIdC, and 100,000–200,000 cpm probe.

Methylation Interference Assays

Radiolabeled, duplex oligonucleotide were partially methylated (23) and used in preparative scale mobility shift reactions to determine the methylation interference pattern of kBf-A. Preparative reaction mixtures were loaded on a 4.5% polyacrylamide gels and electrophoresed as described (15). Bound and free oligonucleotides were then purified from the gel, treated with 1 M piperidine for 30 min at 90° C, lyophilized and electrophoresed on 20% acrylamide, 8 M urea gels.

RESULTS

Evidence for an inducible factor binding 5' of NF-kB within the kappa enhancer

The kappa enhancer contains sites of specific protein interaction which have been shown to be important for enhancer function (6,11). Regions upstream of the NF-kB binding site also were shown to be important for maximal enhancer function in plasmacytomas (6). We have examined sequences upstream of the NF-kB binding site within the kappa enhancer for their importance in the induction of a CAT gene associated with the kappa enhancer in pre-B cells. Vectors were constructed which contain a natural kappa promoter with two different fragments from the kappa enhancer, each containing all previously defined sites of enhancer-protein interaction (Fig. 1A), but lacks sequences downstream of the 3' Dde I site (position 4097) which do not appear to play a role in enhancer function (6). Each of these constructs was transfected into 3–1 and 70Z/3 pre-B cells, which were then grown in the presence or absence of bacterial lipopolysaccharide (LPS) and assayed for CAT activity (see Methods). As shown in Figure 1B, removal of the 5' terminal 18 bp of the 212 bp Dde I-Dde I fragment reduces the ability of this DNA to function as an inducible enhancer. CAT activity from the plasmid KpCAT was normalized to 1.0 to determine relative CAT activities. The relative CAT activities of KpCAT-212 and KpCAT-194 in LPS-stimulated 3–1 were 109.6 ± 6.5 and 52.4 ± 5.8 , respectively, while that of KpCAT-212 and KpCAT-194 in unstimulated 3–1 cells were 11.6 ± 1.1 and 8.6 ± 0.8 , respectively (values expressed as mean $\pm$ SEM; $n=4$). These results were consistent in four independent experiments done with two different preparations of each plasmid. Similar data were obtained when 70Z/3 pre-B cells were transfected, although the efficiency of transfection was much lower in these cells (K. Nelms, unpublished data). These experiments indicate that the region between the Dde I site, defining the 5' end of the enhancer fragment in KpCAT-212, and the Hph I site, defining the 5' end of the enhancer fragment in KpCAT-194, contributes to the function of the kappa enhancer as an inducible, *cis*-acting element in pre-B cells.

Because of the decrease in enhancer function associated with the deletion of the 5' terminal region of the 212 bp Dde I-Dde

Figure 1. A. Plasmid constructs used in transient expression assays. KpCAT was obtained from M.L. Atchison and R.P. Perry and has the V_K21E gene promoter linked to the reporter gene, CAT (described as E-CAT in ref. 17). KpCAT-212 and KpCAT-194, which contain kappa enhancer fragments with all previously defined sites of DNA-protein interaction, were constructed as described in Methods. Numbering is according to ref. 34. Restriction sites used to obtain the fragments described in the text are indicated. D, Dde I; Hf, Hinf I; Hp, Hph I. B. Transient expression assays in 3-1 pre-B cells cultured in the presence (open bars) or absence (black bars) of 10 μ g/ml LPS for 24 hours after transfection. Relative activities were determined by normalizing activities to that of the control plasmid KpCAT. Values shown are expressed as a percentage of the KpCAT-212 relative activity in LPS-stimulated 3-1 cells and are the average of four independent experiments done with two different preparations of each plasmid.

I enhancer fragment, it seemed probable that this region may contain a site of DNA-protein interaction which could be defined using mobility shift assays. Complementary oligonucleotides were synthesized which encompass this region to facilitate binding studies (kBF-A, Fig. 2B). Complementary oligonucleotides containing sequences known to interact with NF-kB and octamer binding proteins (NF-A1 and NF-A2) were also synthesized for use in comparative studies (NF-kB and OCTA, Fig. 2B). Interactions of these sequences with specific proteins were examined in nuclear extracts prepared from murine pre-B cell lines not transcribing the kappa light-chain gene, pre-B cells in which kappa transcription had been induced, plasmacytomas, and non-lymphoid cells.

A DNA-binding factor, designated kBF-A, which bound to the oligonucleotide depicted (Fig. 2), was observed in nuclear extracts prepared from the pre-B cell lines 70Z/3, 3-1, and 1-8 only after stimulation with LPS (Fig. 3A). Similarly, inducible enhancer (NF-kB) and octamer (NF-A2) binding factors were

Figure 2. A. DNA probes used in mobility shift, methylation interference and DNase I footprinting assays. Solid bars indicate positions within the kappa enhancer of oligonucleotides used in binding assays. Black boxes indicate protein binding sites as in Figure 1A. Numbering is according to ref. 34. Restriction sites used to obtain fragments described in the text are indicated. D, Dde I; F, Fnu4HI; Hp, Hph I; S, SfaNI; Ha, Hae III. B. Sequences of complementary oligonucleotides used in binding assays. OCTA oligonucleotide sequence was derived from the promoter region of the kappa gene in 70Z/3 pre-B cells. Lines indicate the binding sites of NF-kB and octamer binding proteins.

only found in LPS stimulated pre-B cell nuclear extracts (Fig. 3A). Presence of these factors in stimulated extracts was not due to the use of unequal amounts of extract in the mobility shift reaction mixtures as evidenced by the presence of equal amounts of the ubiquitous octamer binding factor, NF-A1, in each reaction mixture (Fig. 3A). Bands migrating below the indicated specific complexes are due to non-specific binding factors present in all nuclear extracts tested at variable levels. These bands seem to be an artifact of mobility shifts assays performed with duplex oligonucleotides and may be due, in part, to single stranded binding proteins. Also, methylation interference experiments indicate the protein(s) in these complexes do not bind to specific sequences within the duplex oligonucleotides (K. Nelms, unpublished observations).

To test the binding specificity of the kBF-A factor, competitive binding assays were performed. The mobility shift reaction mixtures contained excess non-specific DNA to reduce non-specific binding. To more accurately determine binding specificity, excess cold oligonucleotide containing specific or non-specific sequences was added to mobility shift reaction mixtures at the same time as radiolabeled oligonucleotide. As shown in Figure 3B, when 50-fold molar excess of cold kBF-A duplex oligonucleotide was added to the reaction mixture containing radiolabeled kBF-A probe, kBF-A binding was abolished. However, when an equivalent amount of duplex oligonucleotide containing the NF-kB binding site was added to the reaction, no competition was observed. Analogous results were obtained when NF-kB binding specificity was tested as a control (Fig. 3B). Thus, the binding of kBF-A was found to be inducible in the pre-B cell lines tested and was specific to the sequence of the kBF-A oligonucleotide.

In the pre-B cell line 70Z/3, NF-kB binding activity has been observed within 30 minutes of LPS stimulation (10) and thus

Figure 3. A. Mobility shift assays performed with pre-B cell nuclear extracts and oligonucleotides. Pre-B cells were either uninduced (–) or induced (+) with 15 μ g/ml LPS prior to nuclear extract preparation. NF-kB and octamer mobility shifts are truncated to show shifted bands only. Current was run left to right. Solid black arrows indicate the position of inducible complexes formed with the oligonucleotides shown in Figure 2B. The open arrow indicates the complex formed by the ubiquitous octamer binding protein NF-A1. Asterisks (*) indicate non-specific complexes formed with oligonucleotides. B. Competitive binding assays. Radiolabeled probe was added to binding reactions containing LPS induced 70Z/3 nuclear extract in the absence (–) or presence of 50-fold molar excess of the indicated oligonucleotide competitor. Specific and non-specific complexes are indicated as in Figure 3A. C. Time course of the appearance of kBF-A, NF-kB and octamer binding activity in 70Z/3 pre-B cells after LPS stimulation. Extracts were prepared at the indicated time after LPS stimulation and then assayed by mobility shifts. Black arrows indicate specific, inducible complexes; open arrow indicates ubiquitous NF-A1. Mobility shifts are presented as in Figure 3A. D. Appearance of kappa mRNA in 70Z/3 cells after LPS stimulation. Total cellular RNA was prepared from aliquots of cells used to prepare the nuclear extracts in Fig. 3C and then assayed by Northern analysis. The mature 1.2 kb kappa transcript is indicated.

correlates with the induction of kappa transcription observed in nuclear run-on assays (24,25). To determine when kBF-A binding activity appears after LPS stimulation, 70Z/3 cells were incubated

Figure 4. A. Appearance of kBF-A and NF-kB binding activity after stimulation of 70Z/3 pre-B cells with various modulators of kappa transcription. Nuclear extracts were prepared after cells were stimulated for 4 or 8 hours. The 4 hour LPS mobility shift reactions contained 20 μ g of nuclear extract; all other lanes contained 10 μ g of extract. LPS, 10 μ g/ml bacterial lipopolysaccharide; CYC, 10 μ g/ml cycloheximide; PMA, 50 ng/ml phorbol 12-myristate 13-acetate. B. Presence of kBF-A and NF-kB in nuclear extracts prepared from mature plasmacytomas and non-lymphoid cells. S107, S194 and MPC11 are kappa producing plasmacytoma cell lines. HeLa is a human cervical carcinoma cell line. LPS stimulated 70Z/3 nuclear extracts were used as a positive control. Black arrows indicate specific complexes; asterisks (*) indicate non-specific complexes.

with LPS and nuclear extracts were prepared at various time points within a 24 hour period. kBF-A, NF-kB and NF-A2 binding activities were then compared. The appearance of kappa light-chain transcripts was determined by Northern analysis of total cellular RNA prepared from aliquots of the 70Z/3 cells used for nuclear extract preparation. As shown in Figure 3C, kBF-A binding activity appeared about 4 hours after LPS stimulation and increased throughout the 24 hour period of stimulation, as did NF-A2 binding activity. In contrast, NF-kB binding was first observed after 30 minutes of stimulation and remained relatively constant throughout the stimulation period, in agreement with previous reports (10). The steady-state level of the 1.2 kb kappa light-chain message was also first observed 4 hours after LPS stimulation and increased throughout the 24 hour period (Fig. 3D). Thus, the appearance of kBF-A binding activity directly correlates with detectable levels of kappa mRNA.

Transcription of the kappa locus and NF-kB binding have been shown to be induced by phorbol ester and protein synthesis

Figure 5. A. Methylation interference analysis of kBF-A complex. Partially methylated kBF-A oligonucleotide, with either the coding or non-coding strand end-labeled, was used in preparative binding reactions containing LPS stimulated 70Z/3 nuclear extracts. Bound (B) oligonucleotide was separated from free (F) on 4.5% acrylamide, low-ionic strength gels, isolated, and treated with piperidine as described in Methods. G residues whose methylation interferes with kBF-A binding are indicated by arrows. A/G ladders were prepared according to Maxam and Gilbert (23). **B.** DNase I footprint analysis of the kBF-A complex. A 106 bp Fnu4HI-Hae III fragment labeled at the Fnu4HI site was used in preparative binding reactions containing LPS stimulated 70Z/3 nuclear extracts. Following treatment with DNase I, bound (B) and free (F) fragments were separated on a low ionic strength gel, isolated, and loaded on a sequencing gel along with an A/G ladder of the same fragment (see Methods). The protected sequence is indicated. Numbering is according to ref. 34. **C.** Summary of methylation interference and DNase I footprint analyses. Asterisks (*) indicate G residues which interfered with complex formation. The bar indicates the DNase I protected region on the coding strand.

inhibitors as well as LPS (10,24,25). Thus the effects of these modulators of kappa transcription on the induction of kBF-A binding activity were investigated in 70Z/3 pre-B cells. Cells were incubated for 4 or 8 hours in the presence of 10 μ g/ml LPS, 10 μ g/ml cycloheximide (an inhibitor of translation), 10 μ g/ml LPS and 10 μ g/ml cycloheximide, or 50 ng/ml phorbol 12-myristate 13-acetate (PMA). Binding to the duplex kBF-A oligonucleotide was observed only after 4 and 8 hours of incubation with LPS and after 4 hours of incubation with PMA (Fig. 4A). The 4 hour LPS mobility shift reaction mixtures contained twice as much nuclear extract as the 8 hour reaction mixtures in order to demonstrate the presence of kBF-A in this extract. Extracts prepared from cells incubated with cycloheximide or LPS and cycloheximide had no detectable kBF-A binding activity (Fig. 4A). Thus, protein synthesis appears to be necessary for the expression of kBF-A binding. NF-kB binding was observed in all of the extracts prepared from treated cells except those prepared from cells incubated with PMA for 8 hours (Fig. 4A). These results were not unexpected as they have been observed by others (10).

kBF-A is in stimulated pre-B cells but not mature plasmacytomas

Since kBF-A binding activity seems to correlate with expression of the kappa gene, nuclear extracts prepared from plasmacytomas that actively transcribe the kappa locus were assayed for the presence of the kBF-A binding factor. Also, the presence of this factor in non-lymphoid and T cells was investigated. Surprisingly, no kBF-A binding activity was detected in any of the murine plasmacytomas examined (Fig. 4B), although it has been observed

Figure 6. A. The kBF-A binding site is conserved in the kappa enhancers of different mammalian species. **B.** Spacing between factor binding sites within the kappa enhancer is conserved among mammalian species. Distances indicated are in base pairs. The human kappa enhancer contains two potential NF-kB binding sites in opposite orientations. The rabbit kappa enhancer does not contain a sequence homologous to E3 in the expected region.

at low levels in the human kappa producing plasmacytoma IM-9 (K. Nelms, unpublished data). Conversely, NF-kB binding is detected in most plasmacytoma cell lines which are transcribing the kappa locus, as represented by S194 and MPC11 in Figure 4B. The S107 cell line, however, constitutively expresses the kappa light-chain gene but lacks detectable NF-kB binding activity (Fig. 4B)(17,18). All of the plasmacytomas examined also contained NF-A2 binding activity as well as NF-A1 binding activity, which confirmed the integrity of the extracts (data not shown).

The kBF-A binding activity was not detected in non-lymphoid cells, as represented by the HeLa cell line (Fig. 4B), nor was it detected in the T cell line, Bal-8 (data not shown). HeLa cells treated with PMA have been shown to have NF-kB binding activity (10); however, no kBF-A binding activity was detected in nuclear extracts prepared from PMA treated HeLa cells (data not shown).

kBF-A binds a highly conserved region of the kappa enhancer

Methylation interference and DNase I footprint experiments were performed to determine the specific sequence kBF-A binds within the kappa enhancer. Partially methylated oligonucleotides and nuclear extracts from LPS stimulated 70Z/3 cells were used in methylation interference experiments. Methylation of one G residue on the coding strand and two G residues on the non-coding strand, as indicated in Figure 5A, interfered with complex formation. On both strands, the methylated guanines which interfere with complex formation are flanked by G residues which do not interfere with binding when methylated, thus narrowing the kBF-A binding site to 16 bp within the kBF-A oligonucleotide.

To better define the limits of the binding site, DNase I footprint analysis was performed using a 106 bp Fnu4HI-Hae III fragment containing the sequence of interest (Fig. 2A) and stimulated 70Z/3 nuclear extracts. A region of protection was detected on the coding strand which encompassed the residues protected in

methylation interference experiments (Fig. 5B). This region of protection spans 13 bp (as represented by the bar in Figure 5C) and corresponds to a very highly conserved sequence in the kappa enhancers of various mammalian species (Fig. 6A). A high degree of sequence conservation between species is also observed with the NF-kB binding site and the E motifs within the kappa enhancer (5,8).

DISCUSSION

We have shown that sequences upstream of the NF-kB binding site within the kappa enhancer contribute to the function of this enhancer as an inducible, *cis*-acting element in pre-B cells (Fig. 1). Specifically, deletion of the 5' terminal 18 bp of a 212 bp Dde I-Dde I enhancer fragment reduced the ability of this fragment to function as an inducible enhancer element in pre-B cells, even though the deletion was upstream of all the previously defined sites of enhancer-protein interaction. Thus, we hypothesized that this region may contain additional sites of protein-DNA interaction. It should be noted that deletion of enhancer sequences upstream of the Dde I site at position 3885 also impair kappa enhancer function in pre-B cells, in accordance with observation made in plasmacytomas (K. Nelms, unpublished observations)(6). Further investigation into the role of this region in kappa enhancer function is in progress.

In scanning the 5' region of the 212 bp Dde I-Dde I kappa enhancer fragment for possible protein interactions, a novel binding activity was detected which occurs within the enhancer approximately 50 bp upstream from the NF-kB binding site. We have shown that this binding activity, involving a factor we designate kBF-A, is inducible in pre-B cells and that it binds to a highly conserved sequence of the kappa enhancer. Pre-B cells stimulated by LPS had detectable kBF-A binding activity only after 4 hours of incubation and the binding activity increased throughout a 24 hour period. In contrast, pre-B cells stimulated with PMA had detectable kBF-A binding activity after 4 hours but had lost this activity by 8 hours of incubation. Similar differences between LPS and PMA activation time courses have also been observed with NF-kB (10). Both LPS and PMA are thought to act through protein kinase C (26,27), although the modes of protein kinase C activation may differ (10). The rapid loss of kBF-A and NF-kB binding activity in cells stimulated with PMA, compared to those stimulated with LPS, may reflect a loss of protein kinase C activity known to be associated with prolonged exposure to phorbol esters (28) and may indicate that activated protein kinase C is required for the maintenance of kBF-A binding activity.

Inhibitors of protein synthesis have been shown to activate kappa transcription (24,25). This activation is thought to be mediated by the induction of NF-kB binding (10). Synthesis of a labile protein, IκB, which is normally complexed with NF-kB in the cytoplasm of both lymphoid and non-lymphoid cells, is inhibited by these agents. Loss of IκB allows NF-kB to translocate to the nucleus and bind the kappa enhancer (29). In contrast to NF-kB binding, kBF-A binding was shown to be completely inhibited by cycloheximide. The 4 hour lapse between LPS stimulation of pre-B cells and the appearance of kBF-A binding activity, as well as the inhibition of kBF-A binding activity by cycloheximide, suggest that the kBF-A factor, or factors necessary for kBF-A binding, must be synthesized after pre-B cell stimulation. It has been previously shown that the induction of the kappa promoter binding factor, NF-A2, is also inhibited by cycloheximide (30). Thus, the binding of NF-A2, which is

thought to mediate the tissue specificity of kappa promoters (30), also requires protein synthesis for binding activity.

Although cycloheximide inhibits the synthesis of two inducible factors which interact with kappa control elements, kappa transcription is nevertheless induced by this agent, presumably through the induction of NF-kB binding (24,25). Indeed, the relative CAT activity in stimulated pre-B cells of KpCAT-194, which lacks the kBF-A binding site, consistently exhibits a 50-fold increase in relative CAT activity over the KpCAT construct, which lacks enhancer elements. If kappa transcription is induced in the absence of NF-A2 and kBF-A, what role do these factors play in the early regulation of transcription? kBF-A and NF-A2 binding occurs concomitantly 4 hours after LPS stimulation in 70Z/3 pre-B cells (Fig. 3C) and correlates with an increase in the transcription rate of the kappa gene that has been observed in stimulated pre-B cells. After stimulation of 70Z/3 pre-B cells with LPS, transcription of the kappa gene has been shown to increase 5- to 10-fold within 4 hours and to 25- to 50-fold over the basal level between 4 and 24 hours of stimulation (24). The increase in the rate of transcription from the 4 to 24 hour level occurs even though maximal NF-kB binding occurs within the first 30 minutes of LPS stimulation. Thus, the binding of additional factors, such as NF-A2 and kBF-A, may be necessary for the increase in the rate of transcription between 4 and 24 hours of stimulation. This is supported by the fact that the vector construct containing the kBF-A binding site (KpCAT-212) is significantly more responsive to 24 hour LPS inductions than the vector construct containing enhancer sequences but lacking this binding site (KpCAT-194). The toxicity of prolonged exposure to protein synthesis inhibitors such as cycloheximide makes it difficult in these studies to determine whether or not kappa transcription increases to maximal levels in the absence of kBF-A and NF-A2, although the CAT assay results strongly suggest it does not.

The observation that DNA-protein interactions are occurring simultaneously at the kappa promoter and enhancer in stimulated pre-B cells may be important in that these dual activities may represent an important aspect of promoter-enhancer interplay. This situation is analogous to immunoglobulin heavy chain genes which contain functional octamer motifs in the promoter and enhancer (31). It is interesting to note that sequence bound by kBF-A resembles the octamer motif in base composition (kBF-A: 5'TTTTCGTT 3'; octamer: 5'ATTTCAT 3'), although duplex oligonucleotides containing the octamer motif do not compete for kBF-A binding as efficiently as duplex oligonucleotides containing the kBF-A binding sequence (K. Nelms, unpublished data).

If inducible factors are important for the maintenance of a transcriptionally active state, one would expect to find these factors in mature cells which transcribe the kappa locus. NF-kB and NF-A2 binding activities are found in most plasmacytomas that actively transcribe the kappa locus; however, NF-kB is not found in the kappa-producing plasmacytoma S107 (Fig. 4B)(8,9,17,18). Thus, it has been hypothesized that the inducible factor NF-kB is necessary for the establishment of transcription at the kappa locus but becomes unnecessary following reorganization of the chromatin into a transcriptionally active conformation (18). This hypothesis could also be used to explain our results with the kBF-A factor. The fact that kBF-A is found in pre-B cells which have been induced to transcribe the kappa gene but was not detected in several murine kappa-producing plasmacytomas suggests that this binding activity may be important for the establishment of maximal transcription at the

kappa locus, but is not needed for constitutive kappa transcription in mature cells. Alternatively, the binding affinity of kBF-A may be lower in plasmacytomas and thus may not be detected in these nuclear extracts.

It is interesting to note that the distances between binding sites in the kappa enhancer seem to be conserved among species, as are the binding sites (Fig. 6B). The distance between NF-kE1 and NF-kE2, NF-kE2 and NF-kE3, as well as NF-kB and kBF-A is approximately 50 bp. The conservation of the spacing between factor binding sites may indicate that a specific spatial arrangement of enhancer binding proteins is important for complete enhancer function since it has been shown that stereospecific alignment of proteins is important for promoter and enhancer function (32,33).

Elimination of the NF-kB binding site within the kappa enhancer has been shown to eliminate the inducibility of genes associated with this enhancer in a pre-B cell line (11), suggesting that NF-kB binding is a catalyst for the induction of kappa transcription and is critical for maximal enhancer strength. However, since deletion analyses indicate that other factors appear to be required for maximal kappa enhancer function, our observation that a second inducible factor interacts with the kappa enhancer suggests that more than one developmentally regulated binding factor may be important for complete enhancer function during B cell ontogeny.

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