Coordinate Transcription and V(D)J Recombination of the Kappa Immunoglobulin Light-Chain Locus: NF- κ B-Dependent and -Independent Pathways of Activation

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To further elucidate the potential role of mitogens and cytokines in regulation of the kappa immunoglobulin light-chain locus, we have characterized the activation of transcription factor binding, kappa germ line transcription, DNase I hypersensitivity, and V κ -to-J κ recombination upon induction of model pre-B-cell lines. We find that both lipopolysaccharide (LPS) and gamma interferon (IFN- γ) are capable of activating germ line transcription, DNase I hypersensitivity, and recombination of the kappa locus. We also find that transforming growth factor β is capable of completely inhibiting LPS activation of transcription and recombination but has no apparent effect on activation of transcription factor binding, including activation of NF- κ B. To address the functional role of NF- κ B in LPS and IFN- γ induction of these events, we blocked the nuclear translocation of NF- κ B by overexpression of a dominant negative mutant of I κ B- α (I κ B Δ N). Overexpression of the I κ B Δ N protein results in an inhibition of LPS but not IFN- γ activation of germ line transcription, DNase I hypersensitivity, and V κ -to-J κ recombination. Our results demonstrate that activation of NF- κ B is necessary but not sufficient for LPS activation of transcription and recombination at kappa. These results also suggest that NF- κ B is not required for IFN- γ activation of transcription or recombination. These results are important in establishing that there are multiple independent pathways of activation of both transcription and recombination.

It is becoming increasingly clear that the tissue-specific and developmentally regulated transcription and recombination of immunoglobulin (Ig) gene segments represent overlapping points of control during B-cell development. The concomitant activation of transcription and recombination of all Ig loci has led to the suggestion that transcription of unrearranged Ig genes plays a key role in regulating recombination (23, 31, 37, 44–46, 55, 60, 61). A number of observations have provided strong support for this hypothesis. First, while a common V(D)J recombinase is expressed in both B cells and T cells, functional recombination of Ig genes is restricted to B cells, where they are also transcriptionally active (62). While ectopic expression of the recombinase activating genes *RAG-1* and *RAG-2* has been shown to confer V(D)J recombinase activity to integrated and extrachromosomal substrates in nonlymphoid cells, the endogenous Ig loci, which are transcriptionally silent in these cells, do not undergo recombination (19, 25, 33). In addition, it has been demonstrated previously that induction of germ line transcription of the unrearranged kappa Ig light-chain and heavy-chain loci is accompanied by an increased frequency of gene rearrangement (44, 45). More direct evidence that *cis*-acting transcriptional regulatory elements are involved in recombination has been demonstrated by integration of recombination substrates by using transfection approaches (5, 6, 11, 24, 35), transgenic approaches (15–17, 20, 21), and targeted knockouts of endogenous sequences (18, 48, 54, 59).

While there is strong evidence that *cis*-acting transcriptional

regulatory regions within the kappa Ig light-chain locus play a role in recombination, the mechanism by which they regulate recombination and the *trans*-acting factors involved have yet to be elucidated. The observation that cells are able to regulate the ability of the endogenous kappa locus to serve as a substrate for the V(D)J recombinase has led to the suggestion that transcription may target the locus by providing accessibility of the locus to the recombinase (see reviews in references 32, 39, and 47). The mechanism by which transcription may mediate accessibility is not clear; however, changes in local chromatin structure within the kappa locus have been shown to occur upon transcriptional activation (38). The accessibility model suggests that those *cis*-acting transcriptional regulatory regions and *trans*-acting factors which regulate germ line transcription of the unrearranged kappa locus play an important role in mediating accessibility of the locus during early B-cell development.

Multiple *cis*-acting transcriptional regulatory elements and *trans*-acting factors that mediate the developmental regulation of the kappa locus have been identified (51). These include a tissue-specific promoter element associated with each variable (V)-region gene segment (58), as well as germ line promoter elements that initiate transcription of the unrearranged locus (55) (Fig. 1). Two tissue-specific transcriptional enhancers contribute to the developmental regulation of expression: one between the joining (J)- and constant (C)-region exons referred to as the intron enhancer (40), and another located 9 kb downstream of the C-region exon (3'KE) (27). Each of these elements plays a role in the developmental regulation of kappa transcription, and many have been implicated in a potential role in regulation of V κ -to-J κ recombination (13, 18, 22, 59).

Induction of kappa transcription by cytokines and mitogens, including lipopolysaccharide (LPS), in Abelson virus-transformed pre-B-cell lines has provided an important model sys-

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FIG. 1. Genomic organization of the kappa locus and quantitative detection of kappa transcription and recombination. (A) The kappa locus consists of hundreds of V-region gene segments located ~23 kb upstream of four functional J-gene segments (numbered 1 through 4) and a single C-region gene segment. Multiple promoter elements, including two germ line promoters located upstream of the J-gene segments and a promoter associated with each of the V-gene segments, are indicated by triangles. The transcriptional enhancer located within the intron between the J- and C-gene exons (E_i) includes a number of important transcription factor binding sites, some of which are indicated in boxes. Transcription from the germ line promoters results in two transcripts, indicated by dashed lines, which are detected by RT-PCR amplification with primers A, B, and C as shown. Detection of the products of recombination by PCR amplification from genomic DNA templates with oligonucleotide primers E and F results in PCR products ranging in size from 2,602 to 1,279 bp as indicated, depending on the J-gene segment involved in the rearrangement event. Amplification with oligonucleotide primers G and H is included in the PCR amplification as an internal control for template loading and amplification efficiency. Products of recombination amplified by PCR are detected with a J-region-C-region-specific probe (probe B). Transcription from rearranged V regions, indicated by the dashed line, is detected by RT-PCR amplification with oligonucleotide primers D and B. DNase I hypersensitivity at E_i is detected by genomic Southern blotting with probe A. (B) Quantitative detection of kappa germ line transcripts by RT-PCR. Increasing volumes of cDNA from LPS-induced pre-B cells were amplified by PCR with radioactively labeled oligonucleotide primers specific for the kappa 5' germ line transcript and PBGD as an internal control. Products were subsequently detected and quantitated by PhosphorImager analysis. (C) Quantitation of RT-PCR products versus starting cDNA concentration. (D) Quantitative detection of kappa recombination by PCR. Increasing quantities of genomic DNA templates containing V_K-to-J_K rearrangements (LPS-induced pre-B cells) were amplified by PCR with oligonucleotides specific for kappa. PCR products were Southern blotted and subsequently detected and quantitated by PhosphorImager analysis. The five products detected by Southern blot analysis are indicated at the right. (E) Quantitation of PCR products detected versus starting DNA template concentration.

tem for studying the developmental activation of the kappa locus (32). The functional analysis of sequences that contribute to the developmental regulation of kappa has led to the identification of a number of important LPS-inducible transcription factors, including NF- κ B (47), Oct-2 (52), KBF-A (30), and AP-1 (41). While NF- κ B is critical for LPS-mediated activation

of kappa transcription, it may not be required for gamma interferon (IFN- γ) activation. It has been suggested that IFN- γ can induce transcription in an NF- κ B-independent pathway and that IFN- γ induction, unlike LPS induction, is not inhibited by transforming growth factor β (TGF- β) (8). Therefore, while Brockman et al. have shown that LPS activation is

NF- κ B dependent (9), different transcriptional activators may mediate kappa transcription through distinct pathways involving the activation of different complements of transcription factors. The importance of cytokine-induced transcription factors in mediating V κ -to-J κ recombination has not been addressed in these model systems.

We have made use of several model pre-B-cell lines that show both inducible expression of germ line transcription and recombination of the endogenous kappa locus. The clonal response of these cells to a number of mitogens and cytokines provides an opportunity to examine the link between activation pathways of transcription and recombination. In this study, we correlate mitogen and cytokine activation of transcription factors, locus accessibility, and V κ -to-J κ recombination. Moreover, we have specifically suppressed NF- κ B activation by overexpression of a dominant negative mutant of I κ B- α and have shown distinct effects on LPS- and IFN- γ -induced transcription and recombination. Our results demonstrate that NF- κ B is necessary but not sufficient for LPS-induced recombination and that NF- κ B is not required for IFN- γ induction of either transcription or recombination.

MATERIALS AND METHODS

Cell culture. Cells were cultured in RPMI 1640 (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal calf serum, 50 U each of penicillin and streptomycin per ml, 2 mM glutamine, and 50 μ M β -mercaptoethanol. Cells were induced with LPS (Difco, Detroit, Mich.) at 1 μ g/ml, IFN- γ (R+D, Minneapolis, Minn.) at 50 U/ml, interleukin-1 α (IL-1 α ; Immunex) at 50 U/ml, IL-4 (Immunex) at 100 U/ml, IL-7 (R+D) at 10 ng/ml, tumor necrosis factor α (TNF- α ; R+D) at 0.5 ng/ml, and TGF β -1 (R+D) at 1 ng/ml. Cells were counted, washed, and induced at 0.5×10^6 cells/ml on a daily basis for up to 7 days. Kappa recombination was assayed after 7 days of induction. Transcription and transcription factor binding were assayed after 3 days of induction.

RNA extraction and reverse transcription-PCR (RT-PCR). Total RNA was isolated by extraction with TriZol (BRL) according to the manufacturer's instructions. The RNA was quantitated spectrophotometrically, and 1 μ g was reverse transcribed with SuperScript II reverse transcriptase (BRL) at 42°C for 1 h with 300 ng of oligo(dT) (Pharmacia, Piscataway, N.J.) in a 20- μ l reaction mixture according to the manufacturer's recommendations. For amplification of *RAG-1*, cDNA was primed with an oligonucleotide specific for *RAG-1* (*RAG-1*[RT]; see below). The cDNA was subsequently diluted by the addition of an equal volume of distilled H₂O, and 2 μ l was amplified with oligonucleotides specific for the kappa germ line transcripts, V-region transcripts, *RAG-1* or *RAG-2*, and porphobilinogen deaminase (PBGD) as an internal control in the same reaction (see below for oligonucleotide sequences). The amplification reaction mixtures contained 200 nM each deoxynucleoside triphosphate, 200 nM each oligonucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 3.0 mM MgCl₂, and 0.125 U of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, Conn.). The oligonucleotides were end labeled with polynucleotide kinase (New England Biolabs, Beverly, Mass.) in the presence of 5 μ l (50 μ Ci) of [γ -³²P]dATP (specific activity, >3,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) in a total volume of 25 μ l prior to PCR amplification. The reactions were cycled 20 to 30 times at 94°C for 60 s, 65°C for 60 s, and 72°C for 30 s in a DNA Thermalcycler (Perkin-Elmer/Cetus). Ten-microliter aliquots of the samples were electrophoresed through a 5% polyacrylamide gel. The samples were subsequently visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis. The oligonucleotides used for amplification are complementary to regions within different exons of the genes so that amplification of cDNA templates can be distinguished from amplification of genomic DNA templates. The sequences of the oligonucleotides used for amplification are as follows:

5' kappa germ line transcript 5'-cca cat gcc ttt ctt cag gga caa gtc gga-3'
5'-gtt atg tgc ttc ata ctc gtc ctt ggt caa c-3'

3' kappa germ line transcript 5'-gag ggg gtt aag ctt tgc cct acc cac-3'
5'-gtt atg tgc ttc ata ctc gtc ctt ggt caa c-3'

PBGD 5'-tgt ccc ggt aac ggc ggc ggc gcc aca ac-3'
5'-gcc acc aca gtc tgc gtc gtc atg cga gc-3'

RAG-1[RT] 5'-gtc tct tcc tct tga gtc ccc gat ggg cag t-3'

RAG-1 5'-cca agc tgc aga cat tct agc act ctg gcc g-3'
5'-caa cat ctg cct tca cgt cga tcc-3'

RAG-2 5'-gag atc cac aag cag gaa gta ctc ata c-3'
5'-ggg tca ggg aca tct cct act aag tct ttc tc-3'

Nuclear extract preparation. Nuclear extracts were prepared essentially as described previously (12) except that the cells were lysed by detergent instead of Dounce homogenization. Briefly, the cells were washed in cold 1 $\times$ phosphate-buffered saline and resuspended in 5 pellet volumes of buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.1% Nonidet P-40). The cells were incubated on ice for 2 min to lyse the cells. The nuclei were pelleted by centrifugation at 9,000 $\times$ g for 30 s at 4°C. The supernatant was removed, and the pellet was resuspended in 2 pellet volumes of extraction buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM DTT). Nuclei were rocked at 4°C for 30 min and then centrifuged at 12,000 $\times$ g for 20 min to remove insoluble debris. The supernatant (nuclear extract) was then dialyzed against 20 mM HEPES (pH 7.9)–20% glycerol–0.1 M NaCl–0.2 mM EDTA–0.2 mM PMSF–1.0 mM DTT and stored at –70°C. Protein concentration was determined by a Bradford assay with Protein Assay Dye Reagent (Bio-Rad, Hercules, Calif.) according to the manufacturer's recommendations.

Electrophoretic mobility shift assay (EMSA). DNA binding reaction mixtures consisted of 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1.5% glycerol, 0.5 to 3.0 μ g of poly(dI-dC) (Sigma), 5,000 to 15,000 cpm of ³²P-labeled double-stranded oligonucleotide, and 10 μ g of nuclear extract in a total volume of 20 μ l. The binding reaction mixtures were incubated at room temperature for 30 min prior to electrophoresis on 4.5% polyacrylamide–0.5 $\times$ Tris-borate-EDTA–5% glycerol gels at 175 V. Specific competitors (25- to 200-fold molar excess) were added to ensure specificity. The images were acquired with a PhosphorImager (Molecular Dynamics). The sequences of the oligonucleotide probes (upper strand shown) are as follows: Oct, 5'-ctg tat ctt ggc att tgc ata tta cat ttt cag-3'; PBK, 5'-caa aaa tat gac taa taa ttt agc aca-3'; NF- κ B, 5'-tct caa cag agg gga ctt tcc gac agg cca tct g-3'; κ E2, 5'-tcc tcc gac gca ggt gcc cca gat tac a-3'; KA, 5'-aag aac tct gat ttt tta cta cct ctg-3'; κ E3, 5'-cta aaa att gtc cca tgt ggt tac aca cc-3'; and KI/KII, 5'-aag cgc ttc cac gca tgc ttg gag agg ggg-3'.

Isolation of genomic DNA. Cells ($\sim 10^7$) were harvested by centrifugation and lysed in 3 ml of lysis solution (10 mM Tris-HCl [pH 8.0], 25 mM EDTA, 0.5% sodium dodecyl sulfate, 2 μ g of RNase A per ml) and then incubated at 37°C for 30 min. Proteins were precipitated by addition of 1 ml of 5 M ammonium acetate at 37°C followed by vigorous vortexing for 20 s. The precipitate was pelleted by centrifugation at room temperature for 20 min at 3,000 rpm in a clinical centrifuge. The supernatant was transferred to a fresh tube, and the DNA was precipitated by the addition of 0.5 volume of isopropanol. The DNA was pelleted by centrifugation at 3,000 rpm, washed once with 70% ethanol, and rocked overnight in 250 μ l TE (10 mM Tris [pH 8.0], 0.5 mM EDTA) at 4°C to dissolve. The DNA was quantitated by Warburg software with a DU-52 spectrophotometer (Beckman Instruments).

V(D)J recombination assay. Genomic DNA isolated from cells grown in the presence or absence of cytokines served as a template for PCR amplification of products of V(D)J recombination at the kappa locus. Amplification reaction mixtures consisted of 0.5 μ g of genomic DNA, 200 nM each deoxynucleoside triphosphate, 200 nM each oligonucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 3.0 mM MgCl₂, and 0.250 U of *Taq* polymerase (Perkin-Elmer/Cetus) in a 50- μ l volume. Reactions were cycled as follows: 4 cycles of 97°C for 45 s, 65°C for 1 min, and 72°C for 2.5 min; and 21 cycles of 94°C for 45 s, 65°C for 1 min, and 72°C for 2.5 min. PCR products (15 μ l) were size fractionated on a 1.0% agarose gel and then blotted to a Zetabind membrane (Cuno) with 18 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1.0 M ammonium acetate (29). The DNA was UV cross-linked to the membrane with 1,200 mJ in a Stratelinker (Stratagene). The membrane was prehybridized for 1 h according to the manufacturer's recommendations. The membrane was subsequently hybridized for 18 to 24 h in hybridization buffer containing 200 ng of pSPECK probe (55) radioactively labeled by nick translation (BRL). The pSPECK plasmid is a 9.0-kb *EcoRI*–*Bam*HI clone of the germ line kappa locus including the J- and C-region gene segments. The membranes were washed with 0.1 $\times$ SSC–1.0% sodium dodecyl sulfate at 65°C for 30 min. The images were acquired and quantitated with a PhosphorImager (Molecular Dynamics). The oligonucleotides used for amplification (Fig. 1) are as follows: E, 5'-gtc cct gcc agg ttt agt ggc agt ggr tcw rgg ac-3' (where y = c or t, r = a or g, and w = t or a); F, 5'-tta gtg gct ctg ttc cta tca ctg tct cct cag g-3'; G, 5'-cca agg acg agt atg aac gac ata aca gct ata c-3'; and H, 5'-gtg taa tct cac ggt ata gag gtc ctt gaa g-3'.

Stable transfection of cells. Cells were harvested by centrifugation and resuspended at a concentration of 3×10^7 cells/ml in 400 μ l of complete RPMI 1640 containing 10% fetal calf serum, penicillin, streptomycin, glutamine, and 50 μ M β -mercaptoethanol. Linearized DNA containing selective markers (1 μ g of pZeoSV [Invitrogen] cut with *EcoRI* or 1 μ g of pGem.Neo.1.3 cut with *NorI*) was mixed with a 10-fold molar excess of linearized expression vector or reporter vector. Expression vectors included the *RAG-1* expression vector, PepB/DR1, which was linearized with *SalI*. The *RAG-2* expression vector, PepB/DR2, was linearized with *NorI*. The I κ B Δ N expression vector, PepB/DN, was linearized with *NorI*. All PepB expression vectors are driven by the heavy-chain intron enhancer. The NF- κ B reporter vector, 2xKB.Pk.luc, containing a multimerized KB binding site and a kappa V-region promoter, was linearized with *Bam*HI.

FIG. 2. (A) RT-PCR detection of kappa germ line transcription in 38B9 pre-B cells cultured in the presence of LPS and cytokines. Cells were cultured in the presence of LPS and cytokines as indicated for 24 h. RNA was subsequently isolated, and germ line transcription was assayed by RT-PCR. (B) PCR detection of V κ -to-J κ recombination in 38B9 pre-B cells. Cells were cultured in the presence of LPS and cytokines as indicated for 7 days. Genomic DNA was isolated and used as templates for PCR amplification of products of recombination.

DNA was added to the cells and transferred to a 0.4-cm-gap-width electrocuvette. Cells were electroporated at 300 V and 960 μ F in a GenePulser (Bio-Rad). Cells were allowed to recover for 20 min at room temperature and then transferred to 20 ml of complete RPMI 1640 and incubated for 24 to 48 h at 37°C and 7% CO₂ before selection in Geneticin (800 μ g/ml; BRL) or Zeocin (500 μ g/ml; Invitrogen). Fresh selective medium was added every 3 to 5 days for 10 to 14 days, at which time the antibiotic-resistant clones were selected and expanded.

Luciferase assay. Luciferase activity in cells stably transfected with the NF- κ B reporter vector (2xKB.Pk.luc) was assayed as instructed by the manufacturer of the luciferase assay kit (Promega, Madison, Wis.). Briefly, 5×10^6 cells were harvested by centrifugation and lysed in 100 μ l of cell lysis buffer. Cells were lysed by incubation at room temperature for 10 min, and the cellular debris was pelleted by centrifugation at maximum speed in a microcentrifuge. Twenty microliters of the supernatant was assayed for luciferase activity with the luciferase substrate in a Berthold 9501 Luminometer for 60 s. Relative light units are reported.

Genomic Southern blotting. Ten micrograms of genomic DNA isolated by the procedure described above was digested with restriction endonucleases *Eco*RI and *Bam*HI as recommended by the manufacturer (New England Biolabs). The DNA was size fractionated on a 20-cm 0.7% agarose gel in 1 $\times$ Tris-borate-EDTA. The DNA was then blotted for 2 h as described above for the V(D)J recombination assay. The membrane was treated as described above and hybridized as indicated in the figure legends with either the pSPECK probe described above or 25 ng of a 548-bp PCR product containing C κ which was gel purified and radioactively labeled by random prime labeling (BRL). The oligonucleotides used for amplification of the probe were C5'P (5'-gtc tcg agc atg gaa ctg aaa aga atg tag ttt c-3') and CRT2 (5'-gtt atg tcg ttc ata ctc gtc ctt ggt caa c-3').

DNase I hypersensitivity assay. Hypersensitivity assays were performed essentially as described previously (38) except that nuclei were isolated by lysis of the cells in Nonidet P-40. Briefly, cells were cultured in the presence or absence of cytokines for 24 h as described above. Approximately 1.5×10^8 cells were harvested by centrifugation at 1,000 rpm at 4°C for 10 min in a clinical centrifuge. The cells were then washed once in 10 ml of ice-cold 1 $\times$ phosphate-buffered saline and resuspended in 1 ml of buffer A (0.25 M sucrose, 10 mM Tris-HCl [pH 8.0], 10 mM MgCl₂) containing 0.2% Nonidet P-40 (Sigma). The cells were lysed for 2 min on ice. The nuclei were pelleted by centrifugation at 4°C for 30 s at 4,000 rpm in a microcentrifuge. The nuclei were washed three times in buffer A, resuspended in 600 μ l of buffer A, and then aliquoted (100 μ l each) into 15-ml screw-cap tubes. The cells were incubated for exactly 1 min at 37°C before

addition of 100 μ l of buffer A at room temperature containing DNase I (Worthington) at 0, 4.0, 8.0, 12.0, 20.0, or 30.0 μ g/ml to give 0, 2.0, 4.0, 6.0, 10.0, or 15.0 μ g/ml (final concentration). The nuclei were incubated for exactly 6 min at 37°C before addition of 3 ml of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) to stop the reaction and lyse the nuclei. Genomic DNA was isolated as described above for isolation of genomic DNA and analyzed by Southern blotting as described above.

Digital image analysis. All images were acquired with a model 445SI PhosphorImager (Molecular Dynamics). Visualization and quantitation of images were performed with IPLab Gel software, 1.5 (Signal Analytics, Vienna, Va.).

RESULTS

To address the role of germ line transcription and transcription factor activation in the induction of V κ -to-J κ recombination at the kappa Ig light-chain locus, we have developed a number of pre-B-cell lines as model systems. These cell lines provide a range of responses to various activators and inhibitors of kappa regulation and include two Abelson virus-transformed pre-B-cell lines, 38B9 and 3-1, and a chemically induced late pre-B-cell line, 70Z/3. We found that the 70Z/3 cell line was V(D)J recombination incompetent, presumably due to the downregulation of *RAG-1* and *RAG-2*. As shown below, the stable expression of *RAG-1* and *RAG-2* in this cell line results in the inducible reactivation of V κ -to-J κ recombination. We have used these cell lines to examine a variety of both positive and negative transcriptional regulators for effects on germ line transcription, transcription factor binding, DNase I hypersensitivity, and V κ -to-J κ recombination. In all cases, we have focused on the endogenous kappa locus.

The magnitude of transcriptional activation of the kappa locus is directly proportional to the induction of recombination. We cultured the Abelson virus-transformed mouse pre-

FIG. 3. RT-PCR detection of *RAG-1* and *RAG-2* expression in cells cultured in the presence of cytokines. RNA was isolated from 38B9 pre-B cells cultured in the presence of LPS and cytokines as indicated for 3 days. *RAG-1* and *RAG-2* expression was assayed by RT-PCR amplification. Coamplification of PBGD by RT-PCR was included as an internal control for template loading.

B-cell line, 38B9, in the presence of LPS and a number of cytokines shown previously to regulate kappa expression or transcription factor binding at kappa (6, 8, 26, 28, 30, 34, 36, 38a, 39, 41, 45, 47, 50, 52). Induction of kappa germ line transcription and recombination was assayed by quantitative RT-PCR and PCR within the linear range of the assays (Fig. 1B to E). As shown in Fig. 2, we detect a significant induction of both germ line transcription (Fig. 2A and B) and V_{κ} -to-J κ recombination (Fig. 2C and D) in the 38B9 pre-B cells upon induction with both LPS and IFN- γ . Furthermore, we find that the magnitude of the transcriptional induction is proportional to the induction of recombination. Other cytokines, including IL-1 α , IL-4, IL-7, TGF- β , and TNF- α , had no significant effect on either transcription or recombination in this cell line.

Previous results have suggested that within a given cell line, the V(D)J recombinase activity correlates well with the expression of *RAG-1* and *RAG-2* over a broad range (1, 26). It was possible that induction of *RAG-1* or *RAG-2* by LPS and IFN- γ accounted for the observed induction of recombination. To address this issue, we assayed *RAG-1* and *RAG-2* expression by RT-PCR in 38B9 pre-B cells cultured in the presence and absence of cytokines and LPS. As shown in Fig. 3, we find no significant difference in the expression of *RAG-1* or *RAG-2* in cells cultured in the presence of LPS or IFN- γ in comparison to control cells cultured in the absence of LPS or IFN- γ . Therefore, differences in the levels of RAG expression in the presence of LPS and IFN- γ do not account for the observed induction of kappa recombination.

LPS and IFN- γ stimulation results in the induction of distinct transcription factor binding activities. Previously published results suggest that IRF-2 constitutively binds to the kappa interferon-stimulated response element located within the intron enhancer (previously identified as the KA site) and that IFN- γ stimulation results in the induction of IRF-1 binding activity to this site (10). However, these studies also suggest that IRF-1 and IRF-2 are not sufficient for maximal IFN- γ activation. To further address this issue and to determine the complement of transcription factors activated by LPS and cytokines in the 38B9 pre-B cells, we performed gel shift analysis of transcription factor binding to the KB, octamer, κ E3, κ E2, KA, and AP-1 sites, all of which have been shown previously to bind transcription factors involved in regulation of kappa. We also assayed KLP protein binding to the KI/KII site (57) located upstream of the J-gene segments, as this site is involved in activation of recombination at kappa (13). As shown in Fig. 4, LPS results in activation of AP-1, Oct-2, NF- κ B, and KBF-A

binding activity and has no apparent effect on the constitutive expression of Oct-1, TFE3, or KLP. IFN- γ results in a very small but detectable induction of NF- κ B binding activity, as well as induction of a novel complex binding to the KA site which, based on size, appears to be distinct from the IRF-1- and IRF-2-bound complexes described previously (10). IFN- γ stimulation also resulted in the induction of binding activity to the KI/KII and AP-1 sites but did not induce Oct-2 or KBF-A binding activity, suggesting that the latter two factors are not required for germ line transcription or recombination in 38B9 cells. IFN- γ stimulation has no apparent effect on the constitutive binding of Oct-1 or TFE3. Interestingly, induction with LPS and IFN- γ together results in the inhibition of Oct-1 binding activity (Fig. 4B), suggesting that Oct-1 is not required for activation of either transcription or recombination in 38B9 cells. We also find that LPS and IFN- γ result in consistent changes in the complexes bound to the κ E2 site (Fig. 4D), although we did not investigate this further. LPS, IFN- γ , and IL-7 also mediated changes in the complexes bound to the kappa AP-1 site (Fig. 4G), although the complex induced by LPS and IFN- γ are apparently different based on the difference in gel mobility. The unique complement of transcription factors induced by LPS and IFN- γ suggested to us that activation of recombination may occur through distinct mechanisms.

TGF- β inhibition of kappa transcription correlates with inhibition of recombination but has no effect on transcription factor binding to the kappa intron enhancer or promoter. We have found that the 3-1 pre-B cells also show LPS-inducible germ line transcription and V_{κ} -to-J κ recombination; however, we found that the 3-1 cells, unlike the 38B9 cells (Fig. 2), were

FIG. 4. EMSA of transcription factor binding upon cytokine induction. Nuclear extracts were prepared from cells cultured in the presence of LPS and cytokines as indicated for 7 days. The presence of transcription factor binding activity was then assayed by incubation of nuclear extract with DNA probes as indicated.

FIG. 5. TGF- β inhibits kappa germ line transcription and recombination. (A) RT-PCR detection of kappa 5' germ line transcription in pre-B cells cultured in the presence of cytokines as indicated for 24 h. (B) PCR detection of V κ -to-J κ recombination of the kappa locus in pre-B cells cultured in the presence of cytokines as indicated for 7 days. (C) EMSA analysis of transcription factor binding to the κ B and octamer sites upon cytokine induction for 3 days.

sensitive to TGF- β . To further address the role of transcription and transcription factor activation in targeting the kappa locus for recombination, we cultured the 3-1 pre-B cells in the presence and absence of both LPS and TGF- β . TGF- β significantly inhibited the LPS induction of both germ line transcription (Fig. 5A) and recombination (Fig. 5B) in this cell line. Interestingly, TGF- β had no apparent effect on transcription factor binding to the octamer, NF- κ B, AP-1, KI/KII, κ E2, and κ E3 sites (Fig. 5C and data not shown). This result is in contrast to previously published results which suggested that TGF- β may inhibit transcriptional activation of kappa by inhibition of the transcriptional activation of Oct-2 (28). However, despite the significant suppression of transcription and recombination, binding of key transcription factors (e.g., NF- κ B) was not affected, suggesting that binding is not sufficient for either activity. Alternatively, TGF- β may induce a specific inhibitor of kappa transcription and recombination.

Ectopic expression of *RAG-1* and *RAG-2* in the 70Z/3 cell line results in LPS- and IFN- γ -inducible transcription and recombination of the endogenous kappa locus. Previously published results suggested that IFN- γ activation of kappa transcription is NF- κ B independent and not inhibited by TGF- β (8). However, the effect of TGF- β on IFN- γ induction of recombination has not been addressed. While we found that TGF- β inhibited LPS induction of transcription and recombination in the 3-1 pre-B cells, we found that these cells were not IFN- γ inducible (data not shown). Similarly, the 38B9 cells were IFN- γ inducible but insensitive to TGF- β (Fig. 2). Therefore, the effect of TGF- β on IFN- γ could not be addressed with either the 38B9 or 3-1 cell line because neither is sensitive to both cytokines. To address the ability of TGF- β to inhibit

IFN- γ induction of recombination, we used the late pre-B-cell line 70Z/3. This cell line contains a single functionally recombined allele and one allele in the germ line configuration (VJ1 κ^+ / κ^0) and is sensitive to both IFN- γ and TGF- β .

We found that the 70Z/3 cell line is V(D)J recombination incompetent, presumably due to the downregulation of both *RAG-1* and *RAG-2* (data not shown). Therefore, we ectopically expressed both *RAG-1* and *RAG-2* in these cells by stable integration of constitutively expressed *RAG-1* and *RAG-2* expression vectors. As shown in Fig. 6, induction of cells expressing *RAG-1* and *RAG-2* (70Z.RAG) with LPS and IFN- γ results in the induction of transcription and recombination, similar to the results described previously with the Abelson virus-transformed cell lines (Fig. 2 and 5). It should be noted that LPS induction of multiple independently derived clones expressing both *RAG-1* and *RAG-2* resulted in activation of recombination of the endogenous kappa locus (data not shown). Induction with IL-1 results in a detectable induction of transcription, recombination, and NF- κ B binding activity (Fig. 7 and data not shown); however, we did not pursue this further given the small magnitude of induction of recombination. These results demonstrate that ectopic expression of *RAG-1* and *RAG-2* in 70Z/3 cells is sufficient to establish recombination competency of the endogenous kappa locus and establishes another model system for studying both transcription and recombination.

Consistent with the results from the Abelson virus-transformed cells, we find that TGF- β inhibits LPS activation of transcription and recombination in these cells (Fig. 6). We also find that TGF- β has no apparent effect on IFN- γ activation of

FIG. 6. The 70Z/3 cell line stably transfected with *RAG-1* and *RAG-2* is V(D)J recombination competent and inhibited by TGF- β . (A) RT-PCR detection of V-region transcription and 3' germ line transcription in 70Z.RAG pre-B cells cultured in the presence of cytokines as indicated for 24 h. (B) PCR detection and quantitation of recombination in 70Z.RAG pre-B cells cultured in the presence of cytokines as indicated for 7 days.

FIG. 7. LPS and IL-1, but not IFN- γ , induce NF- κ B-dependent reporter gene activity. The 70Z.RAG cell line was stably transfected with an NF- κ B-dependent luciferase expression vector and subsequently cultured in the presence of LPS, IFN- γ , and IL-1 for 24 h. Cells were harvested and luciferase activity was assayed as described in Materials and Methods. RLU, relative luciferase units.

transcription but significantly suppresses IFN- γ activation of recombination (Fig. 6). Our results suggest that while TGF- β can inhibit transcription and recombination at kappa, it also has effects on recombination that are independent of any apparent effect on transcription.

Functional activation of NF- κ B by LPS but not IFN- γ .

Given the significant differences in the levels of NF- κ B induction by LPS and IFN- γ in both 38B9 and 70Z/3 cells (Fig. 4 and data not shown), we determined the functional significance of NF- κ B induction by both LPS and IFN- γ by measuring luciferase activity in cells stably transfected with an NF- κ B-dependent luciferase reporter vector. As shown in Fig. 7, we found a significant amount of luciferase activity upon induction with either LPS or IL-1 in multiple independent clones, which was consistent with the NF- κ B binding activity as assayed by EMSA (data not shown). However, we found no significant induction of luciferase activity by IFN- γ in any of the clones. These results suggest that IFN- γ induction does not lead to NF- κ B-dependent expression and that activation of transcription and recombination by IFN- γ may be NF- κ B independent.

Overexpression of dominant negative I κ B- α inhibits LPS but not IFN- γ activation of transcription and recombination. NF- κ B is normally sequestered in the cytoplasm of unstimulated cells by I κ B- α (reviewed in references 3 and 56). Upon LPS stimulation, I κ B- α is phosphorylated, resulting in the subsequent ubiquitination and degradation of I κ B- α and translocation of NF- κ B to the nucleus. While it has recently been shown that overexpression of a dominant negative mutant of I κ B α (I κ B Δ N) results in the inhibition of LPS-induced transcription and recombination (9), the effect of I κ B Δ N expression on IFN- γ activation of kappa has not been addressed. To further address the functional importance of NF- κ B activation in LPS and IFN- γ induction of transcription and recombination, we stably transfected both the 38B9 and 70Z.RAG cell lines with the I κ B Δ N mutant. As expected, expression of the I κ B Δ N protein resulted in a marked inhibition of LPS induction of NF- κ B, kappa germ line transcription, and recombination

in the 70Z.RAG cell line (Fig. 8, 70Z. Δ N2 clone). However, this same clone demonstrated IFN- γ -inducible transcription and recombination comparable to that of the parent cell line. These results further confirm that although the activation of transcription and recombination by LPS is dependent on activation of NF- κ B, activation by IFN- γ is not dependent on NF- κ B.

LPS and IFN- γ result in DNase I hypersensitivity at kappa which is blocked by I κ B Δ N but not TGF- β . The accessibility model suggests that the transcriptional activation of a locus targets the locus for recombination due to changes in the local chromatin structure which provides accessibility to the V(D)J recombinase. In fact, it has been shown previously that LPS induces changes in the local chromatin structure of the kappa intron enhancer as measured by DNase I hypersensitivity (38). Because IFN- γ appears to induce a complement of transcription factors that is distinct from that induced by LPS, including differences in NF- κ B activation, we wanted to determine whether IFN- γ could also induce hypersensitivity within the intron enhancer. To test this, we assayed the induction of hypersensitivity within the intron enhancer upon LPS and IFN- γ stimulation. As shown in Fig. 9A, we find that both LPS and IFN- γ induce hypersensitivity at the kappa intron enhancer. Thus, although LPS and IFN- γ show significant differences in the complement of transcription factors induced upon stimulation, they show comparable induction of DNase I hypersensitivity at the intron enhancer, suggesting that induction of hypersensitivity by LPS and IFN- γ is mediated by distinct factors.

We also hypothesized that the TGF- β inhibition of recombination may be due to an inhibition of induced accessibility, preventing the locus from being targeted by the V(D)J recombinase. To test this hypothesis, we assayed DNase I hypersensitivity upon stimulation with LPS alone or LPS and TGF- β . We find that although TGF- β significantly suppresses tran-

FIG. 8. Overexpression of dominant negative I κ B α (I κ B Δ N) inhibits LPS but not IFN- γ induction of germ line transcription and recombination. The 70Z.RAG cell line was stably transfected with an I κ B Δ N expression vector, resulting in clones expressing high (70Z. Δ N2) and low (70Z. Δ N3) levels of I κ B Δ N. As a control, the 70Z.RAG cell line was also transfected with the selectable marker in the absence of I κ B Δ N expression vector, giving rise to the 70Z.NEO cell line. (A) Nuclear extracts from cells cultured in the presence of LPS and IFN- γ were assayed for NF- κ B binding activity after 24 h of induction. (B) RT-PCR amplification of 3' germ line transcription in cells cultured in the presence of LPS and IFN- γ for 24 h. (C) PCR detection of recombination in cells cultured in the presence of LPS and IFN- γ for 7 days.

FIG. 9. Effects of LPS, IFN- γ , TGF- β , and I κ B Δ N on kappa accessibility as measured by DNase I hypersensitivity at the intron enhancer. (A) 38B9 pre-B cells were cultured in the presence of LPS and IFN- γ as indicated, and DNase I hypersensitivity was assayed as described in Materials and Methods. The +LPS blot was probed with pSPECK (see Materials and Methods) while the +IFN- γ blot was probed with a C-region-specific PCR product (see Materials and Methods). (B) 3-1 pre-B cells cultured in the presence of LPS alone or LPS and TGF- β . Membranes were probed with a C-region-specific PCR product. (C) 38B9 pre-B cells stably transfected with I κ B Δ N expression vector [Δ N(+)] or selectable marker in the absence of I κ B Δ N expression vector [Δ N(-)] cultured in the presence and absence of LPS. The Δ N(-) blot was probed with pSPECK, while the Δ N(+) blot was probed with a C-region-specific PCR product. (D) 70Z.RAG pre-B cells stably transfected with I κ B Δ N expression vector (70Z. Δ N2) cultured in the presence and absence of IFN- γ . Blots were probed with the C-region-specific PCR product.

scription and recombination, it has no apparent effect on the LPS-induced hypersensitivity at the kappa locus (Fig. 9B), suggesting that differences in accessibility do not account for the TGF- β inhibition of recombination.

To further address the role of NF- κ B in activation of accessibility of the kappa locus, we assayed DNase I hypersensitivity in cells which overexpress the I κ B Δ N inhibitor of NF- κ B. As shown in Fig. 9C and D, we find that overexpression of I κ B Δ N completely inhibits LPS-induced hypersensitivity at the intron enhancer but has no apparent effect on IFN- γ -induced hyper-

sensitivity. This result correlates very well with the effect of I κ B Δ N on LPS- and IFN- γ -induced kappa transcription and recombination (Fig. 8). These results suggest that overexpression of I κ B Δ N can inhibit LPS but not IFN- γ induction of accessibility. In addition, it appears that NF- κ B binding is not required for IFN- γ -induced DNase I hypersensitivity within the intron enhancer. The differential block in accessibility by I κ B Δ N may reflect important differences in the mechanism by which LPS and IFN- γ mediate accessibility of the kappa locus.

DISCUSSION

We have established a number of cell lines as model systems for studying the activation of transcription and recombination of the kappa Ig light-chain locus *in vitro*. These cells provide monoclonal responses to various cytokines, which allows the investigation of the potential *in vitro* role of cytokines, transcription, transcription factors, and signaling pathways involved specifically in regulation of recombination. These cell lines show distinct responses to mitogens and various cytokines, which we exploited to determine effectors that link kappa transcription and recombination. The ability to manipulate the expression of target genes in these cells is also an important consideration, as it allows opportunities to investigate the mechanism by which transcription and recombination are coordinately regulated.

Our focus in this paper has been on the kappa intron enhancer because of its importance in activation of kappa germ line transcription in pre-B cells (14, 39). In addition, the inducible accessibility of the intron enhancer as measured by DNase I hypersensitivity (38) may represent some of the earliest events involved in providing accessibility of the locus to the V(D)J recombinase. There are also a number of inducible transcription factors that bind to the intron enhancer or adjacent sequences, including AP-1, Oct-2, KBF-A, and NF- κ B (30, 41, 47, 52). It has also been shown previously that the intron enhancer is capable of targeting recombination of substrates in transgenic mice (15, 20). As shown previously, the binding of NF- κ B upon LPS stimulation is especially important in mediating transcription and recombination at kappa, as evidenced by overexpression of the I κ B Δ N protein (9).

Using cell lines as model systems, we have extended the previous analysis of kappa transcriptional activators to include other transcriptional regulators, including IFN- γ and IL-1 α . We find that not only LPS but also other transcriptional activators are capable of activating kappa recombination and that there is a quantitative correlation between transcription and recombination. These results are significant for a number of reasons. First, the observation that both LPS and IFN- γ activate transcription and recombination, yet activate distinct complements of transcription factors, suggests that there may be multiple independent pathways by which cells can potentially regulate activation of the kappa locus. Although the LPS induction of transcription and recombination has been reported previously (9, 45), this is the first report of a naturally occurring cytokine which is able to induce kappa recombination. While we have shown that NF- κ B is required for LPS activation of kappa transcription and recombination, our analysis of IFN- γ activation suggests that there may be no concordance of transcription or recombination with activation of any single transcription factor. However, it is formally possible that both LPS and IFN- γ induce an as yet unidentified factor that is essential for both LPS and IFN- γ inductions.

We extended this experimental approach to include inhibitors of transcription. The analysis of transcription factor binding upon TGF- β inhibition of transcription and recombination suggests that intron enhancer occupancy by transcription factors, including NF- κ B, Oct-1, Oct-2, E47, and TFE3, is sufficient for locus accessibility, as measured by DNase I hypersensitivity, but is not sufficient to target the locus for recombination. These results suggest that transcription *per se* may be required to target the locus for recombination. Although we find that TGF- β has no apparent effect on transcription factor activation, perhaps TGF- β inhibits other, as yet unidentified transcription factors critical for kappa induction; alternatively, TGF- β may inhibit posttranslational modifica-

tions which are required for transcriptional activation (7). In support of this hypothesis, we find that the LPS induction of luciferase activity in the 70Z.KB.LUC cells (Fig. 7) is significantly inhibited by TGF- β (data not shown).

We have also extended the previous analysis of the effects of I κ B Δ N to include its effects on IFN- γ activation of transcription, recombination, and DNase I hypersensitivity. Consistent with previous results, we find that overexpression of I κ B Δ N inhibits the LPS activation of transcription and recombination in multiple independent cell lines, including 70Z.RAG (9). We also find that overexpression of I κ B Δ N inhibits the LPS activation of DNase I hypersensitivity within the kappa intron enhancer. However, in contrast to the inhibition of LPS-mediated activation of kappa in these cells, we find that overexpression of I κ B Δ N does not inhibit the IFN- γ activation of transcription, recombination, or DNase I hypersensitivity. This is consistent with our finding that LPS but not IFN- γ stimulation results in activation of NF- κ B-dependent reporter gene expression. There are also previous reports suggesting that IFN- γ activation of transcription is NF- κ B independent (8). This is the first report of NF- κ B-dependent and -independent pathways by which cells can activate transcription and recombination.

This conclusion is supported by the analysis of mice containing knockouts of either the p50 or p65 subunit of NF- κ B (4, 49, 50). Although p50-deficient mice have a number of immunological deficiencies, they are developmentally normal and contain mature B lymphocytes. The p65-deficient mice are not viable; however, transfer of hematopoietic stem cells from p65-deficient mice into irradiated hosts results in regeneration of the lymphoid compartment (2). These results suggest that neither p50 nor p65 is required for generation of mature B cells. Therefore, while NF- κ B may be important in B-cell activation and normal development, neither p50 nor p65 is required for V(D)J recombination of either heavy- or light-chain Ig. This does not preclude the possibility that NF- κ B normally contributes significantly to the activation of recombination, but it suggests that cells have alternative NF- κ B independent pathways *in vivo*, perhaps mediated by IFN- γ activation.

Interestingly, targeted deletions of either the kappa intron or 3' enhancer results in a decreased efficiency of recombination at kappa (18, 59). These results suggest that while both enhancers contribute to activation of kappa recombination, neither enhancer is strictly required. The effect of enhancer deletion on activation of germ line transcription has not been addressed; however, these results may suggest that there are additional transcriptional regulatory regions in kappa that are sufficient to activate early transcription of the locus. Regulation of such regions could be mediated by LPS, IFN- γ , or other, as yet unidentified responses.

We find that expression of *RAG-1* and *RAG-2* in the 70Z/3 cells results in the reactivation of recombination of the endogenous kappa locus. While ectopic expression of *RAG-1* and *RAG-2* in lymphoid and nonlymphoid cell lines results in V(D)J recombination of integrated and extrachromosomal recombination substrates (35, 42), this is the first report of inducible reactivation of recombination of the endogenous kappa locus in a cell with a functionally rearranged allele. Importantly, recombination in the 70Z/3 cells stably transfected with *RAG* is still dependent on transcriptional activation. Thus, our results are consistent with the notion that ectopic expression of *RAG* genes in fibroblasts is unable to induce recombination of the endogenous kappa locus, presumably due to the lack of transcriptional activation. Moreover, the results support the hypothesis that germ line transcription and *RAG* expression are sufficient to target the endogenous locus

for recombination and that downregulation of recombinase activity may be mediated solely by downregulation of RAG expression. There do not appear to be additional mechanisms by which cells maintain allelic exclusion, at least at this late pre-B-cell stage.

Even the productively rearranged allele in 70Z.RAG can undergo secondary recombinations. We have found a number of subclones with replacements of VJ1 (data not shown). Since transcriptional activators induce the V-region promoter, these observations suggest that the induction of rearrangement is not strictly dependent on the germ line promoter or sequences upstream of the J1 gene segment. They further suggest that the apparent contribution of the KI/KII sequence element (13) is only in the context of a germ line allele.

It is becoming increasingly clear that LPS-mediated activation of kappa transcription, recombination, and accessibility are NF- κ B dependent and that NF- κ B has other pleiotropic effects on transcription factor activation, including Oct-2 (data not shown). However, little is known about the molecular mechanism of IFN- γ activation. We find that IFN- γ stimulation results in the inducible binding of a number of unidentified factors detected by mobility shift assays. We have also observed the apparent induction of IRF-1 to the KA site (data not shown), consistent with previous reports. However, we find that the induction of IRF-1 is minor and considerably more difficult to detect than the higher-molecular-weight complex shown in Fig. 4E. We find that IFN- γ does not induce Oct-2 and that overexpression of Oct-2 is not sufficient to activate kappa transcription or recombination (data not shown). These results suggest that Oct-2 is neither necessary nor sufficient for activation of kappa. While we have demonstrated that NF- κ B is not required for IFN- γ activation, the factors important in mediating IFN- γ activation of transcription, recombination, and accessibility have yet to be elucidated.

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