Members of the Nuclear Factor κB Family Transactivate the Murine c-*myb* Gene*

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Expression of the c-*myb* proto-oncogene is primarily detected in normal tissue and tumor cell lines of immature hematopoietic origin, and the down-regulation of c-*myb* expression is associated with hematopoietic maturation. Cell lines that represent mature, differentiated hematopoietic cell types contain 10–100-fold less c-*myb* mRNA than immature hematopoietic cell types. Differences in steady-state c-*myb* mRNA levels appear to be primarily maintained by a conditional block to transcription elongation that occurs in the first intron of the gene. The block to transcription elongation has been mapped, using nuclear run-on analysis, to a region of DNA sequence that is highly conserved between mouse and man. Two sets of DNA-protein interactions, flanking the site of the block to transcription elongation, were detected that exhibited DNA-binding activities that strongly correlated with low steady-state c-*myb* mRNA levels. Several criteria demonstrated that members of the nuclear factor κB (NF-κB) family of transcription factors were involved in the DNA-protein interactions identified in these two sets. Surprisingly, cotransfection experiments demonstrated that coexpression of members of the NF-κB family, specifically p50 with p65 and p65 with c-Rel, transactivated a c-*myb*/chloramphenicol acetyltransferase reporter construct that contained 5'-flanking sequences, exon I, intron I, and exon II of the c-*myb* gene. Transactivation by these heterodimer combinations was dependent on regions of the c-*myb* first intron containing the NF-κB-binding sites. These findings suggest that NF-κB family members may be involved in either modifying the efficiency of transcription attenuation or acting as an enhancer-like activity to increase transcription initiation. Thus, the regulation of c-*myb* transcription may be quite complex, and members of the NF-κB family likely play an important role in this regulation.

The c-*myb* proto-oncogene was first identified as the cellular homologue of the transforming gene in avian myeloblastosis virus and encodes a 78-kDa transcription factor that binds to the consensus DNA sequence (T/C)AAC(T/G)G (1, 2). The c-*myb* gene product has been shown to function as a transcription activator and appears to be involved in the regulation of a number of cellular genes associated with proliferation as well as genes that are expressed in a lineage-specific fashion (3–8). Expression of c-*myb* is detected almost exclusively in hematopoietic tissue, although c-*myb* mRNA expression has been reported in primary chicken embryo fibroblasts (9), smooth muscle cells (10), and several nonhematopoietic human tumors (11–13). The down-regulation of c-*myb* expression is associated with hematopoietic maturation, and in each hematopoietic lineage examined (erythroid, myeloid, and lymphoid), expression of c-*myb* mRNA and protein is highest in immature normal tissue and tumor cell lines (9, 14–18). Several pieces of evidence suggest that the c-*myb* gene product plays an important role during hematopoietic maturation. First, c-*myb* antisense oligonucleotides have been shown to inhibit both erythroid and myeloid colony formation *in vitro* (19). Second, murine erythroleukemia cells stably transfected with either constitutively expressed or inducible c-*myb* expression vectors were blocked in their ability to terminally differentiate in response to chemical inducing agents (20, 21). Similar transfection experiments using a constitutively expressed c-*myb* transgene have demonstrated that Myb will block the differentiation of myeloid leukemic cells (22). Most recently, Mucenski *et al.* (23) reported that transgenic mice lacking a functional c-*myb* gene developed normally to day 14, after which they died with severely disrupted patterns of erythroid and myeloid development.

Although expression of c-*myb* mRNA has been extensively studied, relatively little is known about the regulation of c-*myb* transcription. The c-*myb* promoter is constitutively transcribed even in cell lines where there is no detectable c-*myb* mRNA, and no transcriptional control elements have been mapped within the promoter region in the murine gene (24, 25). Recently, cotransfection studies have demonstrated that c-Jun, JunD, and c-Myb can transactivate transcription from a human c-*myb* promoter/reporter construct (6, 26). However, similar experiments have not been carried out with the murine c-*myb* promoter. In murine B lymphoid tumors, steady-state c-*myb* mRNA is detected at 10–100-fold lower levels in B cell lymphoma and plasmacytoma cell lines than in pre-B cell lymphoma cell lines (14). Maintenance of differential steady-state c-*myb* mRNA levels in these cell lines is primarily attributed to a block to transcription elongation (attenuation) that takes place in the first intron of the gene (24, 27, 28). In addition, the block to transcription elongation is involved in the down-regulation of c-*myb* mRNA that occurs during chemically induced terminal differentiation of murine erythroleukemia cells (29) and human HL-60 cells (30). The conditional block to transcription elongation is detected in cell lines that express both high and low levels of c-*myb* mRNA, and it is changes in the efficiency of the block that appear to be regulated (24, 27–29). In murine B lymphoid tumors, a major DNase I-hypersensitive site (site IV) has been mapped near the transcription block and
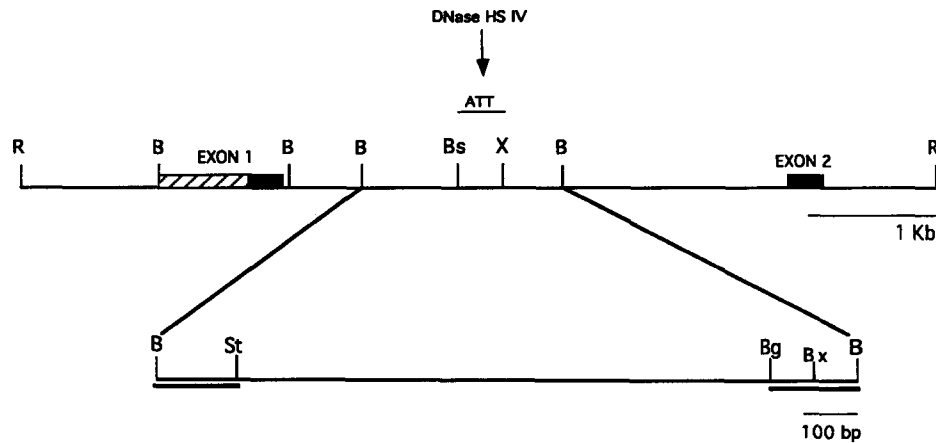
FIG. 1. **Schematic representation of the 7.6-kb murine c-myb EcoRI genomic restriction fragment containing 5'-flanking sequences/exon I (5'-untranslated sequences denoted by the hatched box and coding sequences denoted by the filled boxes), intron I, exon II, and part of intron II.** The region containing the block to transcription elongation is designated *ATT*. The differentially detected DNase I-hypersensitive site (site IV; *DNase HS IV*), which was more prominent in the A20.2J B cell lymphoma than in the 70Z/3.12 pre-B cell lymphoma, is indicated by the *arrow* (14). The lower portion of the diagram shows the 1.5-kb *Bam*HI fragment from intron I including the two restriction fragments (*underlined*) used as probes to characterize DNA-protein interactions by EMSA in this study. Sites of restriction enzyme digestion are indicated as follows: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*E2; Bx, *Bst*XI; R, *Eco*RI; St, *Sty*I; X, *Xba*I.

is more readily detected as the efficiency of attenuation increases, suggesting that changes in higher order chromatin structure accompany changes in attenuation (14). DNA-protein interactions have been identified in the first intron of the gene (near the site of attenuation) that have been suggested to play a role in the transcription block, and the presence of several of these factors correlated with high levels of c-*myb* mRNA expression (31). However, the identity of these proteins is not known, and as yet, there is no direct evidence that these proteins are involved in the regulation of c-*myb* transcription.

Nuclear factor κB (NF-κB)[1] was first characterized as a heterodimeric protein complex that bound a regulatory site within the immunoglobulin κ-light chain enhancer (32). NF-κB is now known to regulate a wide range of cellular and viral genes by binding to the consensus DNA sequence GGGRN-NYYCC (33–35). NF-κB-like DNA-binding activity is constitutive in mature B cells, and an inactive form, bound to the specific inhibitor IκB, exists in the cytoplasm of most cell lines (35). The release of an active NF-κB DNA-binding complex is induced by multiple signal transduction pathways that involve dissociation from IκB (36). A family of NF-κB-related proteins has now been identified, each member of which has different DNA-binding specificities and transactivation properties (37). The result of such a diverse family is a plethora of functional combinations that can affect the transcription rates of NF-κB-regulated genes differently depending on the specific sequence of the NF-κB site, the promoter context of the site, and the presence of additional, heterologous DNA-binding factors in the transactivating complex (38).

We have identified two NF-κB-like binding sites in the first intron of the murine c-*myb* gene that flank the site of attenuation. Protein complexes that bind to these sequences were readily detected in cell lines that contained small amounts of c-*myb* mRNA, but not in cell lines that contained abundant c-*myb* mRNA. The protein-binding sequence is similar to the NF-κB consensus sequence, and an oligonucleotide that includes an NF-κB site from the immunoglobulin κ-light chain enhancer specifically competes for binding. Electrophoretic mobility shift assays (EMSA) demonstrate that the p50 subunit of

NF-κB as well as Rel-related proteins can bind to these sequences and that an NF-κB-like DNA-binding activity that interacts with these sequences is induced by LPS in 70Z/3.12 cells. In addition, the specific inhibitor IκB-α/MAD-3 was able to inhibit the LPS-inducible DNA-binding activity. In cotransfection studies, members of the NF-κB family of transcription factors were able to transactivate a c-*myb*/CAT reporter construct dependent on regions containing the two NF-κB sites in the first intron of the c-*myb* gene. These results provide strong evidence that DNA sequences in the murine c-*myb* first intron are involved in the regulation of c-*myb* transcription, and this, in part, is mediated by members of the NF-κB family.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts—Murine cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol at 5% CO_2 in a humidified chamber. In some experiments, 70Z/3.12 murine pre-B cell lymphoma cells were stimulated with 10 μg/ml *Escherichia coli* LPS for 2 h. HeLa cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum. Crude nuclear extracts used in Figs. 3 and 4 were prepared as described by Dignam *et al.* (39). All other extracts were prepared using a modified Dignam protocol (40).

Plasmid Constructs and Oligonucleotide Synthesis—Murine c-*myb* intron I DNA fragments to be used as probes in EMSA were derived from the plasmid p*myb*13 (27), which contains a 7.6-kb murine c-*myb* *Eco*RI genomic DNA fragment (see Fig. 1), and were subcloned into pGEM-3Z (Promega). DNA fragments used in EMSA were isolated following restriction digestion with appropriate enzymes by electrophoresis on 1% agarose gels, excised, and purified by electroelution. Oligonucleotides were synthesized and high pressure liquid chromatography-purified by the Protein and Nucleic Acid Research Facility at the University of Virginia. The DNA sequences of the sense strand of the double-stranded oligonucleotides used in this study are as follows: c-*myb*, GCATGCTCTGGAAAGTACCTTAAAGATAGA; NF-κB, CAGAG-GGGACTTTCCGAGAGGC; and TA-2, TCGCAGAAGCCACATCCTCT-GGAAAGAAGA. The NF-κB-binding sequence was derived from the murine immunoglobulin κ-locus enhancer region. The TA-2 (Ets-1)-binding sequence was from the murine T cell receptor α-chain enhancer.

The c-*myb* promoter/CAT reporter construct (see Fig. 8A), which included the murine c-*myb* 5'-flanking/exon I region, intron I, and part of exon II fused to a CAT reporter gene, was assembled in pGEM-7Z(f−) (Promega). A 340-bp *Bcl*I/*Bam*HI fragment from SV40, containing polyadenylation sequences, was cloned 3' of a 734-bp *Hin*dIII/*Bam*HI fragment from pSV₂CAT (41) that contains the CAT coding sequences. The 6.2-kb c-*myb* fragment (see Fig. 8A) was inserted at the *Eco*RI site in the pGEM-7Z(f−) polylinker upstream of the CAT gene. The 6.2-kb fragment was isolated by introducing a deletion into exon II, from the 3'-end of the 7.6-kb c-*myb* genomic fragment shown in Fig. 1, such that

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[1] The abbreviations used are: NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay(s); LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s); RRBE, Rel-related protein-binding element; Ab, antibody.

the *Eco*RI site was maintained. The resulting 6.2-kb *Eco*RI DNA fragment was then cloned into the pGEM-7Z(f−)/CAT construct at the *Eco*RI site. The ATG translation start codon in the first exon of c-*myb* was deleted by mung bean nuclease digestion to reduce aberrant translation. The *d*RRBE/CAT reporter construct (see Fig. 8A) was built by replacing the 1.5-kb *Bam*HI intron I fragment of c-*myb* with an internal 1.2-kb *Sty*I/*Bst*XI fragment, which resulted in the deletion of the two RRBE sequences. To do this, the c-*myb*/CAT reporter construct was partially digested with *Bam*HI religated, and a ligation product was obtained that had the 1.5-kb *Bam*HI fragment deleted. This plasmid (*d*1.5Bam/CAT) was partially digested with *Bam*HI, and the linearized vector was isolated electrophoretically. The 1.2-kb *Sty*I/*Bst*XI fragment was derived from a subclone of the 1.5-kb *Bam*HI fragment by restriction digestion with *Bst*XI and a partial digest of *Sty*I, blunted with Klenow fragment, isolated by gel purification, and subcloned into *d*1.5Bam/CAT. The *d*RRBE/CAT reporter construct was then identified and characterized by restriction mapping of the ligation products.

Nuclear Run-on Assay—Nuclei from exponentially growing 70Z/3.12 murine pre-B cell lymphoma and A20/2J B cell lymphoma cells were prepared in isosmotic buffer containing 0.5% Nonidet P-40, followed by sucrose gradient centrifugation as described previously in detail (28, 42). Preparation of nitrocellulose slot blots with single-stranded DNA targets, nuclear run-on transcription, isolation and hybridization of [α-^{32}P]UTP-labeled nascent RNA probes, and filter wash conditions were carried out as described previously by Groudine *et al.* (43, 44) and as modified elsewhere (27, 28). Nitrocellulose filters were exposed to Kodak XAR-5 film with a Cronex Lightning II Plus enhancer screen at −80 °C for 5–7 days.

Murine c-*myb* exon I, intron I, and exon II DNA fragments used as targets in Fig. 2 (*A* and *B*) were cloned into M13mp10 or M13mp11 and used as single-stranded DNA. The target listed in Fig. 2A as cDNA is a 1.1-kb murine c-*myb* cDNA fragment that contains coding sequences 3′ of exon II (45) cloned into M13mp10 and used as single-stranded DNA. Single-stranded M13mp10 was used to monitor background hybridization, and a 1.2-kb *Pst*I fragment containing cDNA sequence from the avian glyceraldehyde-3-phosphate dehydrogenase mRNA (46) and cloned into pGEM-3Z was used as a positive control for RNA polymerase II transcription.

Electrophoretic Mobility Shift Assay—Restriction fragments to be used for EMSA were isolated electrophoretically, end-labeled using the large fragment of *E. coli* DNA polymerase I (Life Technologies, Inc.) with [α-^{32}P]NTPs (Amersham Corp.), and purified on Sephadex G-50 spun columns. Oligonucleotides were end-labeled using T4 polynucleotide kinase (Life Technologies, Inc.) with [γ-^{32}P]ATP (Amersham Corp.) and purified on Nen-Sorb columns (DuPont NEN) per the manufacturer's instructions. The EMSA experiments were carried out as described previously by Singh *et al.* (47). 10,000 cpm of labeled probe (approximately 5–25 fmol) were used per EMSA reaction in binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol). DNA-protein complexes were resolved on 4% native polyacrylamide gels at 11 V/cm in 25 mM Tris base, 25 mM boric acid, and 0.5 mM EDTA. The gels were dried and exposed to Kodak XAR-5 film with a Cronex Lightning II Plus enhancer screen at −80 °C. For competition, supershift, and IκB experiments, competitor DNAs, antibodies, or inhibitors were added prior to the addition of labeled probe for 10 min at 22 °C. Two polyclonal rabbit antisera made against human p50 (Ab2 and Ab3) were the gift of Dr. A. Israel (Institut Pasteur, Paris). Polyclonal rabbit antisera made against v-Rel and a human c-Rel peptide were the gift of Dr. N. Rice (Frederick Cancer Research and Development Center, Frederick, MD). IκB-α/MAD-3 protein was purified as described by Haskill *et al.* (48).

DNase I Footprinting—The *Bgl*II/*Bam*HI fragment, derived from the murine c-*myb* first intron, was end-labeled using T4 polynucleotide kinase (Life Technologies, Inc.) and gel-purified on a 6% polyacrylamide gel. The labeled fragment was eluted overnight in 0.5 M ammonium acetate and 1 mM EDTA at room temperature. EMSA binding reactions were scaled up 5-fold and digested with 0.1 units of DNase I (Worthington) for 90 s at room temperature. The reaction was stopped by the addition of EDTA to a 140 mM final concentration and subjected to electrophoresis on native 4% polyacrylamide gels as described above. DNA-protein complexes were visualized by autoradiography of the wet gel. Free and bound fragments were excised from the gel and electroeluted onto DE81-cellulose paper (Whatman). The DNA fragments were eluted from the filter paper by incubation in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 M NaCl at 65 °C for 30 min. Equal numbers of counts/minute of free and bound DNA fragments were fractionated electrophoretically on an 8 M urea, 6% polyacrylamide gel in parallel with a Maxam-Gilbert G reaction of undigested probe for determination

of the binding sequence. Gels were dried and exposed to Kodak XAR-5 film with a Cronex Lightning II Plus enhancer screen at −80 °C

Transient Transfections and Assays for CAT Activity—Transient transfections into the EL4 murine thymoma cell line were carried out using DEAE-dextran (49). Transfected cells were harvested 48 h post-transfection, washed in ice-cold phosphate-buffered saline, and lysed by three cycles of freezing and thawing. CAT assays were performed as described by Gorman *et al.* (41). The results were quantified using a Molecular Dynamics PhosphorImager and normalized based on protein content of cell extracts as determined using a Bradford protein assay (Bio-Rad).

RESULTS

The Block to Transcription Elongation at the Murine c-myb Locus Maps to a Highly Conserved Region of the First Intron— Our group (27, 50) as well as others (24, 29) have demonstrated that differential expression of steady-state c-*myb* mRNA is mediated at the level of transcription elongation. For example, the approximately 20-fold difference in steady-state c-*myb* mRNA levels between the 70Z/3.12 murine pre-B cell lymphoma and the A20/2J murine B cell lymphoma is maintained by a block to transcription elongation that occurs in the first intron of the murine c-*myb* gene (see Refs. 27 and 28 and Fig. 2A). To more accurately map the site of attenuation, [α-^{32}P]UTP-labeled nascent RNA transcripts isolated from A20/2J murine B cell lymphoma nuclei were hybridized to two overlapping sets of single-stranded M13 clones containing fragments of murine c-*myb* exon I, intron I, and exon II. As shown in Fig. 2B, we readily detected hybridization of labeled nascent transcripts to targets a–d, but not to targets e and f, demonstrating that the block to transcription elongation occurs within or just 3′ of the 750-bp *Bst*EII/*Bam*HI fragment contained in target d. Targets c′ and d′ subdivide target d into two fragments. Hybridization of nascent c-*myb* transcripts was detected with target c′, but not d′. Thus, the block to transcription elongation must occur either within or just 3′ of the 300-bp *Bst*EII/*Xba*I region of the murine c-*myb* first intron.

Recently, the block to transcription elongation that occurs in the first intron of the human c-*myb* gene has been suggested to take place within a region of the c-*myb* first intron that is highly conserved between mice and humans (51–53). However, data mapping the transcription block to this region have not been presented. We used a dot matrix program to align the murine and human c-*myb* intron I DNA sequences (data not shown) and identified a highly conserved region of DNA that included the site of attenuation as defined by nuclear run-on assay (Fig. 2, *B* and *C*). The DNA sequence similarity in the conserved region is approximately 77% over the entire region and 78% across the 300-bp *Bst*EII/*Xba*I fragment. This degree of sequence conservation is quite high for intron DNA sequences and strongly suggests that this region is involved in regulating c-*myb* transcription. The DNA sequence similarity breaks down on either side of this region, although other less extensive regions of similarity have been identified when comparing the murine and human c-*myb* intron I DNA sequences (51, 53).

Identification and Characterization of a Differentially Detected DNA-Protein Interaction in the First Intron of the Murine c-myb Gene—To begin to understand the regulation of c-*myb* transcription, DNA-protein interactions were characterized in the highly conserved region of the first intron using EMSA. Crude nuclear extracts were prepared from exponentially growing murine hematopoietic cell lines and tested for DNA-binding activity with radiolabeled probes derived from the first intron. As shown in Fig. 3A, a set of DNA-protein interactions was detected using a 155-bp *Bgl*II/*Bam*HI fragment (see Fig. 1) that exhibited DNA-binding activity correlating with low levels of c-*myb* mRNA expression. Upon incubation with the ^{32}P-

labeled *Bgl*II/*Bam*HI fragment, two major DNA-protein complexes were detected, marked as complexes 1 and 2 (in some experiments, complex 1 resolves into a doublet), with nuclear extracts from the WEHI-231 immature B cell lymphoma (Fig. 3A, *lanes 6* and *7*) and the S194 plasmacytoma (*lanes 8* and *9*). In contrast, the C19 erythroleukemia line and the 70Z/3.12 pre-B cell lymphoma contained little or no DNA-binding activity that was detected with this DNA fragment (Fig. 3A, *lanes 2–5*). Seven additional cell lines (murine erythroleukemia F-MEL; pre-B lymphomas Haftl1 and LS8T.2; B cell lymphomas A20/1.11, 2PK3, and BCL$_1$; and plasmacytoma MOPC 11) were examined for DNA-binding activity, and in each case, the mature hematopoietic lines contained high levels of DNA-binding activity, while the immature cell lines did not (data not shown). The formation of complexes 1 and 2 was lost in cold competition experiments in which an unlabeled homologous DNA fragment was used as competitor (Fig. 3B, *lanes 4–6*). In contrast, complex 1 was not competed, and complex 2 was only weakly competed when a nonhomologous *Bgl*I/*Bst*EII DNA fragment from the first exon of the murine c-myb gene was used as unlabeled competitor (Fig. 3B, *lanes 7* and *8*), demonstrating specificity of the two major interactions. Minor DNA-protein interactions (denoted by the *tildes* in Fig. 3B) appear to be nonspecific interactions since they were not effectively competed by a homologous fragment. Thus, a set of DNA-protein interactions was detected whose binding activities correlated with efficient attenuation, and the protein-binding site(s) mapped in the region of conserved DNA sequence near the site of attenuation.

To further characterize the DNA-binding site(s) on the *Bgl*II/*Bam*HI fragment, DNase I footprinting was performed. End-labeled *Bgl*II/*Bam*HI probe was incubated with nuclear extract from the plasmacytoma S194 and subjected to limited DNase I digestion. The free and bound species (corresponding to the slower mobility complex denoted by the *asterisk* in Fig. 3B) were identified by autoradiography and excised from the gel. DNase I digestion products were resolved on an 8 M urea, 6% polyacrylamide gel in parallel with a Maxam-Gilbert G reaction. As shown in Fig. 4A, the sequence GGAAAGTACC was protected in the bound lane. This sequence was identical to the RRBE recently identified in the promoter of the human urokinase gene and is homologous to the NF-κB consensus sequence (54). The c-myb RRBE protein-binding sequence is conserved in the human c-myb intron (see Fig. 2C). We will refer to this sequence as the c-myb RRBE throughout this manuscript.

To determine if the c-myb RRBE sequence is involved in the DNA-protein interaction detected on the parental *Bgl*II/*Bam*HI fragment, a double-stranded oligonucleotide corresponding to the c-myb RRBE protein-binding sequence was synthesized and used to compete for binding to the radiolabeled *Bgl*II/*Bam*HI fragment. As shown in Fig. 4B, this oligonucleotide was able to completely inhibit the formation of complexes 1 and 2 on the *Bgl*II/*Bam*HI fragment (*lanes 2–5*). An irrelevant oligonucleotide containing a protein-binding sequence for the octamer-binding proteins failed to compete for binding to the c-myb RRBE (Fig. 4B, *lane 6*). DNA-protein complexes that remain in *lanes 3–5* are the nonspecific DNA-protein interactions that were also detected in Fig. 3B. As with the *Bgl*II/*Bam*HI fragment, detection of DNA-binding activity correlated with efficient attenuation (data not shown). Interestingly, a similar binding sequence (GGAAAGTGCT) is located 1.2 kb upstream of the *Bgl*II/*Bam*HI fragment in a *Bam*HI/*Sty*I fragment (Fig. 1). This *Bam*HI/*Sty*I fragment, containing the c-myb RRBE-like sequence, demonstrated the same pattern of DNA-protein interactions as the *Bgl*II/*Bam*HI fragment, and the c-myb RRBE oligonucleotide was able to compete for binding to the
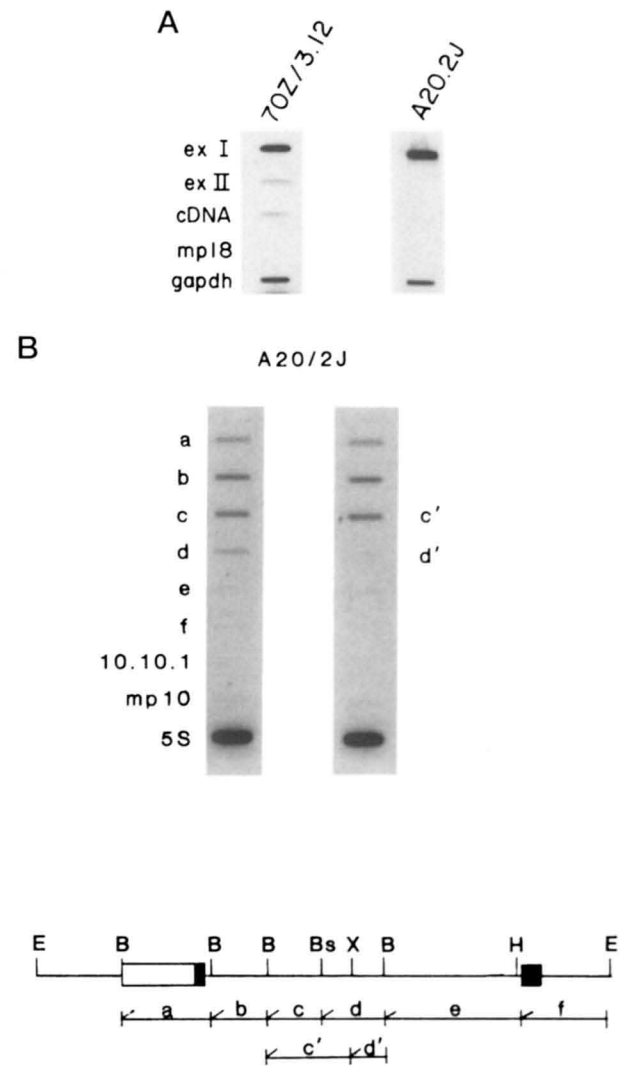

FIG. 2. **The site of c-myb transcription attenuation in murine B lymphoid tumors maps to a highly conserved region in the c-myb first intron.** A, differential levels of steady-state c-myb mRNA are maintained by a block to transcription elongation. [α-^{32}P]UTP-labeled nascent RNA was isolated from approximately 5×10^7 70Z/3.12 pre-B cell lymphoma or A20/2J B cell lymphoma nuclei and hybridized to nitrocellulose filters containing single-stranded target DNA as described under "Materials and Methods." The target-labeled cDNA is a 1.1-kb murine c-myb cDNA fragment that contains coding sequences 3′ of exon (*ex*) II. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, mapping the site of attenuation in the A20/2J B cell lymphoma cells using nuclear run-on assay. ^{32}P-Labeled nascent RNA was isolated after nuclear run-on transcription from A20/2J nuclei. Half of the run-on reaction was hybridized to the filter on the left and half to the filter on the right. Derivation of single-stranded DNA targets is represented schematically beneath the autoradiographs. The target labeled *10.10.1* is a 2.4-kb murine c-myb cDNA containing coding sequences 3′ of exon II and the 3′-untranslated region. See legend to Fig. 1 for definition of restriction enzyme abbreviations. C, alignment of the conserved murine and human c-myb intron I sequences containing the site of attenuation. The murine DNA sequence (*upper strand*) was derived from our previously reported 7.6-kb murine c-myb genomic clone, which contains 5′-flanking/exon I sequences, intron I, exon II, and part of intron II (27). The human DNA sequence (*lower strand*) is from Jacobs *et al.* (51). Comparison of DNA sequences was carried out using DNA sequence alignment programs from the Microgenie package of sequence analysis programs (Beckman Instruments). The murine sequence starts at the *Bst*EII site, which is located 1448 bases 3′ of the exon I/intron I junction, while the human sequence begins 1451 bases 3′ of the exon I/intron I junction. Attenuation at the murine c-myb locus occurs either within or just 3′ of the *Bst*EII/*Xba*I fragment. The conserved c-myb 3′-RRBE (identified in Fig. 4A) is *boxed* and *shaded*.

C

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        Bst E2
1448 : GGTCACCTG.............TTCTGCAGAAAGCTGAAACCTGCAGCAGGAGGAGGGAG  murine
1451 : -----T---tttttttttttttt--T-TTC--G---------TG-----------------  human


1495 : TTAGGAAGGGGTGCTCCCAGGAGGTGCGGCCCAAGCTGGCCGCCCCAAGGT.TCAG...T
1511 : -CG--C---------GGG------C-----A-GGCTCC--------G----c--G-cccC


1550 : CGGGGCGTGGCCGCGCAGGTTCCCTGGGGCTCGCAGGTAGCCCGGAGCGGAGGCGGCAGG
1571 : -------G-----------------A-----.----------A---G---------T--


1610 : GAGGGGCGGGAGCCTTGAACTTGACTCAGGGGGAAGAGAAACCCGGCTGCGGGCTCGGAG
1630 : ------------------C--C----.---------------A-------------


1672 : CCGTTTCTTGC.AGGGCTAGCGTCAACTCGGCAGGGAT..TATTAACCAGGTCAGCGAAA
1688 : -----------a-----G---------------------ta------------------


        Xba 1
1728 : TGACTCTGAACCCCTTTTCTAGAATCAC.GTAGCGGCCAC.AGGCACTTCCTCCGGTCCC
1748 : ---G-T----------....CC-------a----------a------------C-CT--


1786 : CCTCCCTCTTCCCAACCCC.....CCAGTGTTCTTTTTCGTTTCTTTGCACGGTTTGTTT
1805 : ------------T------cagca--TC-T-----------------------------


1840 : CCTTGATAGAATGTAACATTCATAGTTGTACTCTTGTAACCACACCGGCTCTCGCAGGTG
1865 : ------C-----------------------------------------G---A-------


1900 : GAGCAGTCGAGAGCATCTAGCTTGAAATCAGCCTTTGAAAGTCCTTTGACTGTTGGGTCT
1925 : -.-G-------T-----A-C-C----------------A----C-------------


        Bgl II
1960 : GAGATCTTGTC.GAGCAGATAAAGGAAAAGTTATTTC.GGGTCCTTTGTCGAAATTAATC
1984 : -----------a-------------------------a--T-----A--A---------


2019 : ATGCTACCGAGGCGACCAGGTCATTTTAGAGCAAGGAAATGGCATGCAGAGCTCAGA.GC
2044 : -----T-TAG-A----.........----------C----C-------A--T---A-g--


2078 : AAGCC.CCTTGTCAAAGCTGACAG...........................AATCCTGGA
2095 : G----tGGG--G-C-GC---G---ggaaactcaggagctgggcgcgtgagc-GC--C---


        Bgl II
2110 : CTCGCCCAGCTCCCTCTCACAGCCTTTCAAGATCTGCAAGAGTCACACGCCACATTTCAC
2155 : -------T---G-G--.-TGGC--C----G-TC-----GA------T-T----C--G-.-


2170 : CCCAAGACTTTGGCTTTGCACATC..TGCC.CAGGGCTTGCTTTTTAAGAGGCACAGAA.
2213 : --------C--A-T----------tt----t-T-T----AGC------------G---g


2226 : GACA..GTAGAGGCATG.....CCTC▓▓▓▓▓▓▓▓▓▓▓▓TTAAACATAGAATCC.....CCT
2273 : ----tg-C-TG-T----tattaG-G-▓▓▓▓▓▓▓▓▓▓▓▓---G-A----G----aagcaAGC


        Bam H1
2274 : CCCTAGTGTGTAAGATGGGATCC
2333 : --T--T---AA-G-CGA---A-T
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FIG. 2—*continued*

*Bam*HI/*Sty*I fragment as well (data not shown). Thus, two RRBE sequences that flank the site of the transcription block were identified in a region of the c-*myb* first intron.

Members of the NF-κB Family Bind to the c-myb Intron I RRBE Sequence—Since the protected sequences on the *Bgl*II/*Bam*HI fragment were identical to the urokinase RRBE se-
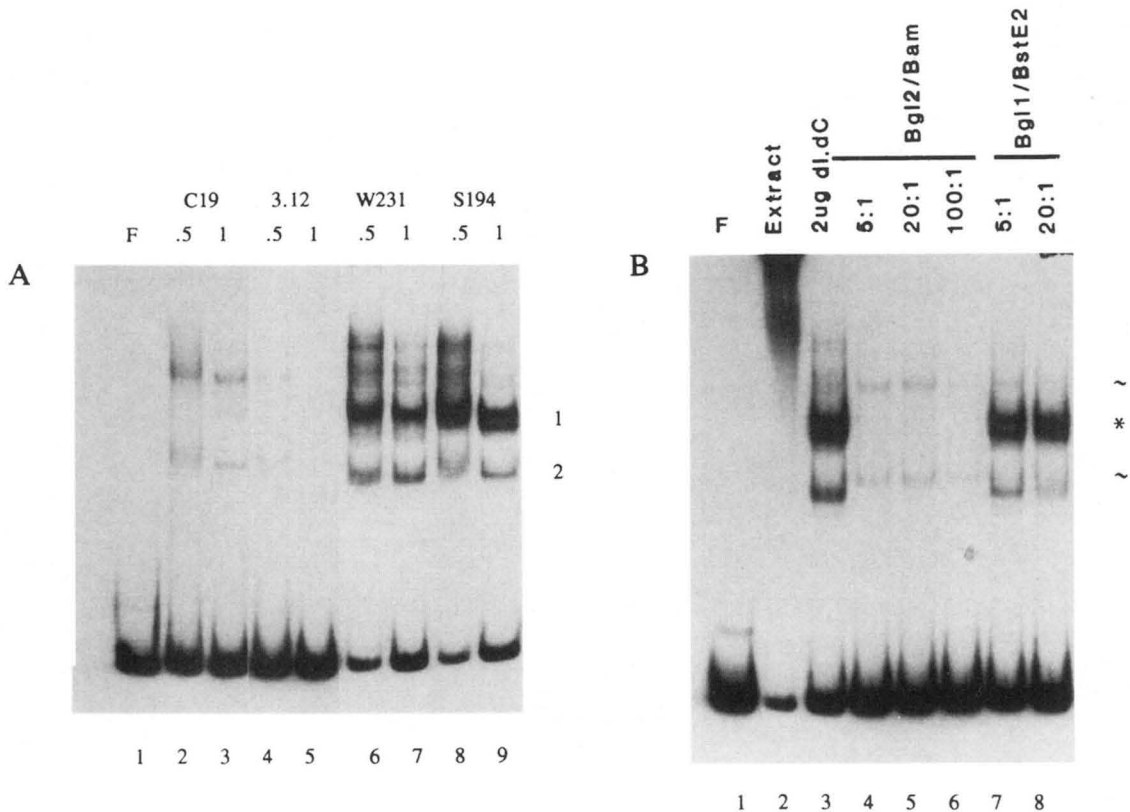
Regulation of c-myb Transcription

FIG. 3. **Detection of DNA-protein interactions on the 155-bp *BglII/Bam*HI intron I fragment.** *A*, DNA-protein interactions detected with the radiolabeled *BglII/Bam*HI fragment by EMSA. *Lane 1* is the free ^{32}P-labeled fragment (*F*). 4 μg of protein from each nuclear extract were incubated with either 0.5 or 1 μg of poly(dI-dC). The cell lines used for the analysis were the murine erythroleukemia cell line C19 (*lanes 2* and *3*), the pre-B cell lymphoma 70Z/3.12 (*lanes 4* and *5*), the B cell lymphoma WEHI-231 (*lanes 6* and *7*), and the plasmacytoma S194 (*lanes 8* and *9*). The major DNA-protein complexes are designated *1* and *2*. *B*, EMSA competition analysis. 4 μg of protein from the plasmacytoma S194 were preincubated with an unlabeled homologous *BglII/Bam*HI fragment at molar ratios of unlabeled to labeled probe of 5:1 (*lane 4*), 20:1 (*lane 5*), and 100:1 (*lane 6*) as well as with an unlabeled nonhomologous c-myb exon I *BglI/Bst*EII fragment at 5:1 (*lane 7*) and 20:1 (*lane 8*). *Lane 1* contains free probe, *lane 2* contains probe incubated with 4 μg of protein from the S194 nuclear extract, and *lane 3* is the same as *lane 2* with the addition of 2 μg of poly(dI-dC) as nonspecific competitor. DNA-protein complexes denoted by the *asterisk* were used for DNase I footprinting analysis in Fig. 4. The minor DNA-protein interactions denoted by the *tildes* are nonspecific interactions that are not competed by the unlabeled homologous *BglII/Bam*HI fragment.

quence and resembled an NF-κB-binding site, further analysis relating to the identity of the DNA-binding factors that bind to the c-myb RRBE *in vitro* was carried out. One characteristic of the NF-κB family is that DNA-binding activity can be induced by treatment of 70Z/3.12 cells with LPS (32). Nuclear extracts were prepared from 70Z/3.12 cells before and after treatment for 2 h with 10 μg/ml LPS, and the ^{32}P-labeled c-myb RRBE oligonucleotide was used for EMSA to detect DNA-binding activity. A strong induction of DNA-binding activity was detected when comparing the lane containing nuclear extract from untreated 70Z/3.12 cells with the lane containing nuclear extract from 2-h LPS-treated 70Z/3.12 cells (Fig. 5, *lanes 2* and *3*, respectively). More important, a double-stranded oligonucleotide containing the NF-κB site from the immunoglobulin κ-locus (GGGACTTTCC) was able to compete for binding, while a control oligonucleotide containing a binding site for the Ets-1 protein (TA-2, CAGAGGATGTG) was not (Fig. 5, *lanes 4* and *5*). As expected, the unlabeled c-myb RRBE oligonucleotide completely inhibited the formation of complexes 1 and 2 on the radiolabeled c-myb oligonucleotide (Fig. 5, *lane 6*). Therefore, LPS-inducible DNA-binding activities that interact with the NF-κB-binding sequence *in vitro* can also interact with the c-myb RRBE.

To determine whether the protein complexes that bind the c-myb RRBE intron sequence include members of the NF-κB family, polyclonal antisera directed against the p50 subunit of the NF-κB complex were used in EMSA (55). However, as the

available antisera made against p50 only bind the human homologue, crude nuclear extracts prepared from HeLa cells were used for these experiments rather than nuclear extracts derived from murine cell lines. As shown in Fig. 6, a similar pattern of DNA-binding complexes is detected in HeLa cell nuclear extracts with both the c-myb RRBE and NF-κB oligonucleotides. However, the signal detected by binding to the c-myb RRBE oligonucleotide is less intense than the signal detected with the NF-κB oligonucleotide, suggesting that human NF-κB family members may bind this sequence less well than murine proteins. The Ab2 antiserum was directed against epitopes on p50 that are available when p50 binds DNA as either a homodimer or a heterodimer. Preincubation of Ab2 with HeLa nuclear extract resulted in the formation of a supershift (denoted by the *arrow*) with the c-myb RRBE probe as well as with the NF-κB probe (Fig. 6, *lanes 3* and *7*). The antiserum also inhibited the formation of complexes 1 and 2 with the NF-κB probe. However, it was difficult to determine whether inhibition occurred with the c-myb RRBE oligonucleotide because complexes 1 and 2 consistently did not resolve well in the presence of serum. Antiserum Ab3 reacts with epitopes present only on the p50 homodimer. With both probes, a supershift was detected (Fig. 6, *lanes 4* and *8*, denoted by the *arrow*) following preincubation of the nuclear extract with Ab3. In addition, this antiserum appeared to stimulate the formation of complex 2 (Fig. 6, *lanes 4* and *8*), suggesting that Ab3 alters the relative concentrations of the different NF-κB sub-

A

G F B

B

FIG. 4. **DNase I footprinting analysis of the DNA-protein interactions detected on the *Bgl*II/*Bam*HI fragment.** *A*, the ^{32}P-end-labeled *Bgl*II/*Bam*HI fragment was incubated with 20 μg of protein from the plasmacytoma S194, subjected to limited digestion with 0.01 units of DNase I, and isolated as described under "Materials and Methods." Free (*F*) and bound (*B*) fragment DNase I digestion products (denoted by the *asterisk* in Fig. 3*B*) were fractionated on an 8 M urea, 6% polyacrylamide gel alongside a Maxam-Gilbert G reaction (*G*) to identify the protected DNA. The sequences protected by the addition of nuclear extract are designated (GGTACTT-TCC) next to the *B* lane. *B*, shown are the results from cold competition analysis using the c-*myb* RRBE oligonucleotide as unlabeled competitor for binding to the ^{32}P-labeled *Bgl*II/*Bam*HI fragment. 10 fmol of the radiolabeled *Bgl*II/*Bam*HI fragment (*lanes 1–6*) were incubated with 4 μg of nuclear extract from the plasmacytoma S194. *Lane 1* corresponds to free ^{32}P-labeled probe (*F*). *Lanes 1–6* contain 2 μg of poly(dI-dC). The unlabeled RRBE oligonucleotide at the unlabeled:labeled molar ratios shown was preincubated with nuclear extract (*NE*) prior to the addition of radiolabeled probe. An oligonucleotide containing the protein-binding sequence for the octamer-binding proteins (*OCTA*; ATTTGCAT) was used at a 20:1 molar ratio as nonhomologous competitor (*lane 6*). Complexes 1 and 2 correspond to complexes 1 and 2 detected in Fig. 3*A*.
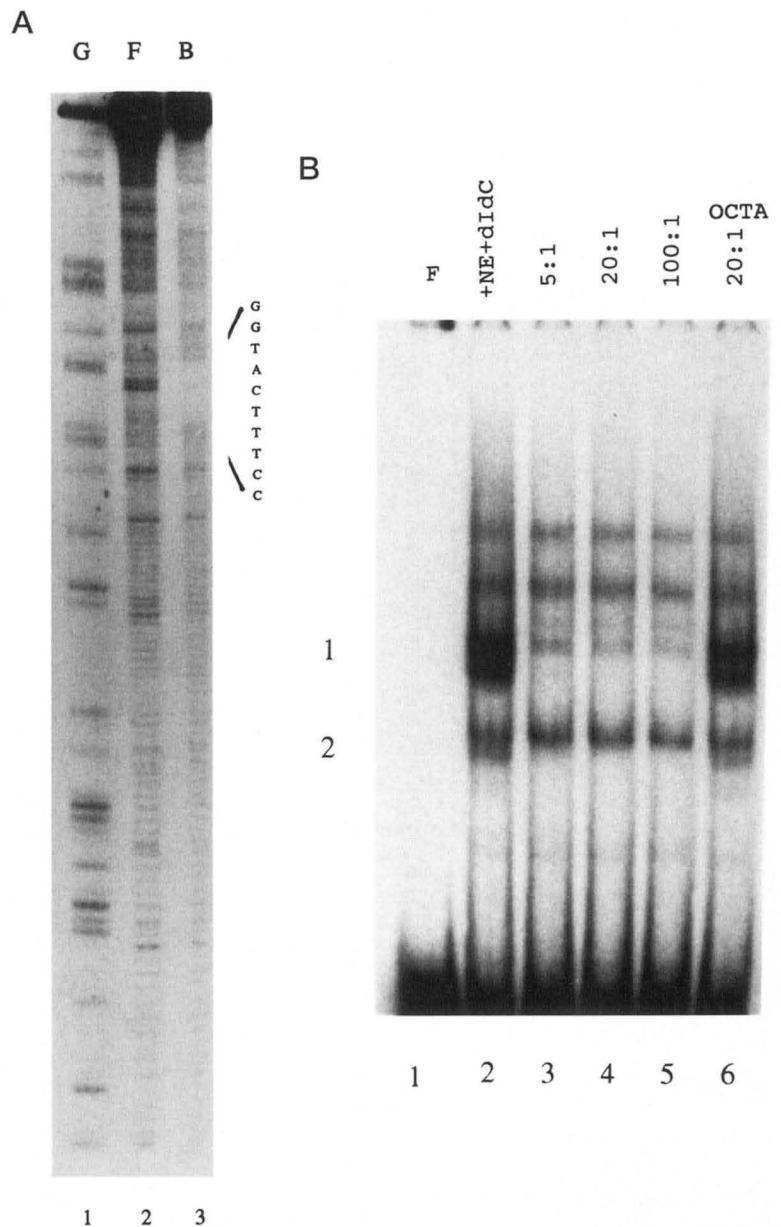
unit combinations. As seen with Ab2, complex 1 was inhibited in the NF-κB lane. No changes in the EMSA patterns were detected using a preimmune serum control (data not shown). Thus, the p50 subunit of NF-κB or proteins antigenically related to it interact with the c-*myb* RRBE *in vitro*.

To determine whether other members of the NF-κB family were able to bind to the c-*myb* RRBE oligonucleotide, polyclonal antisera made against v-Rel and a human c-Rel peptide were used in EMSA (56, 57). Crude nuclear extracts from 70Z/3.12 cells treated for 2 h with 10 μg/ml LPS were preincubated with the two anti-Rel sera in separate experiments prior to addition of ^{32}P-labeled oligonucleotides. Preincubation of the LPS-induced 70Z/3.12 extract with the c-Rel antiserum did not result in a supershift with either probe, but a slight stimulation of complex 2 was detected with both probes (Fig. 7, *lanes 3 and 8*), suggesting that this antiserum alters the available population of DNA-binding complexes. Preincubation with the v-Rel antiserum induced a supershift with both the c-*myb* RRBE and NF-κB oligonucleotides (Fig. 7, *lanes 4 and 9*, denoted by the *arrow*). The formation of a supershift by preincubation of the v-Rel antiserum with nuclear extract demonstrates that c-Rel or a Rel-related protein was detected in protein complexes that

bind the c-*myb* RRBE sequence.

To further examine if the DNA-binding activities detected on the c-*myb* RRBE were related to the NF-κB family, the NF-κB inhibitor protein IκB-α/MAD-3, which is known to inhibit the DNA-binding activity of heterodimers consisting of p49 with p65, p50 with p65, and p50 with c-Rel to the cognate binding site, was used in EMSA (48, 58). Nuclear extracts from LPS-treated 70Z/3.12 cells were preincubated with the IκB-α/MAD-3 protein prior to the addition of the labeled oligonucleotides. IκB was able to inhibit binding of complex 1 and the complex denoted by the *asterisk* in Fig. 7 (*lanes 5 and 10*). A stimulation of complex 2 DNA-binding activity, which is probably due to increased affinity of p50 for DNA when complexed with IκB (59), was also seen as a consequence of the IκB inhibition. In addition, one of the low mobility complexes (Fig. 7, denoted by the *double asterisks*) was not affected by IκB-α/MAD-3, indicating that novel factors or combinations may be involved in binding to the c-*myb* RRBE sequence.

Members of the NF-κB Family Transactivate a c-myb/CAT Reporter Construct—To determine if the two c-*myb* RRBE sequences potentially play a role in the transcriptional regulation of the c-*myb* gene, a CAT reporter construct was built linking a
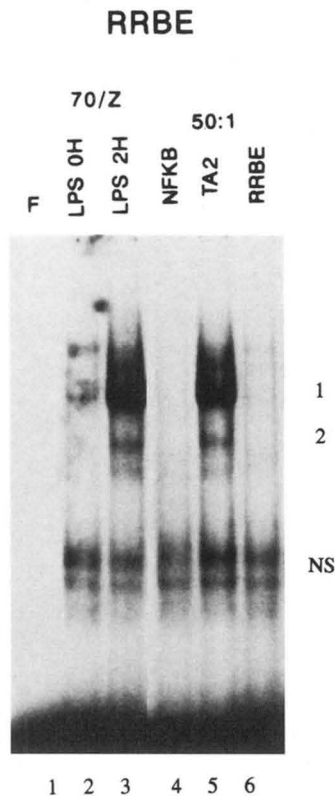
RRBE

FIG. 5. **DNA-binding activity detected with the c-myb RRBE is induced by LPS treatment of 70Z/3.12 cells.** The ^{32}P-end-labeled c-myb RRBE was incubated with 4 µg of nuclear extract from untreated 70Z/3.12 cells (*lane 2*) and from 70Z/3.12 cells treated with 10 µg/ml *E. coli* LPS (*lanes 3–6*). Lanes *4–6* are the results from unlabeled competition analysis of the c-myb RRBE with the following oligonucleotides at a 50:1 molar ratio of unlabeled to labeled probe: an oligonucleotide containing an NF-κB site from the immunoglobulin κ-locus (*NFKB; lane 4*), an oligonucleotide containing an Ets-1-binding site from the T cell receptor α-chain enhancer TA-2 (*TA2; lane 5*), and the c-myb RRBE (*RRBE; lane 6*). Free RRBE (*F*) is in *lane 1*. Complexes *1* and *2* correspond to complexes 1 and 2 detected in Fig. 3A.

6.2-kb murine c-myb genomic fragment (containing 5′-flanking sequences/exon I, intron I, and part of exon II) to the bacterial CAT gene and 3′-polyadenylation sequences from SV40 (Fig. 8A). In addition, a second c-myb/CAT reporter construct was built in which both RRBE sequences were deleted (Fig. 8A, dRRBE/CAT). The c-myb/CAT reporter was cotransfected with expression vectors for the p50, p65, and c-Rel members of the NF-κB family (60) into the murine thymoma EL4, which contains low levels of endogenous NF-κB DNA-binding activity (61). Titration of each homodimer and heterodimer was performed to assess the effects of their expression on the c-myb/CAT reporter construct. Cotransfection of p50, p65, c-Rel, or p50 with c-Rel did not result in altered expression from the c-myb/CAT reporter construct (data not shown). However, co-expression of p50 with p65 and c-myb/CAT resulted in a 3.7-fold increase in CAT activity at 4 µg and a 4.1-fold increase at 6 µg of each expression vector (Fig. 8B, *shaded bars*). Coexpression of p65 with c-Rel resulted in the strongest transactivation of the c-myb/CAT reporter construct: an 8.2-fold increase at 4 µg and a 10-fold increase at 6 µg of each vector (Fig. 8B, *hatched bars*). Since each expression vector alone failed to transactivate the c-myb/CAT reporter construct, these results are not simply due to competition for a negatively acting transcription factor(s) by the NF-κB family member expression vector sequences. To determine whether transactivation of the c-myb/CAT reporter construct by members of the NF-κB family required the two RRBE sequences identified in the first intron,
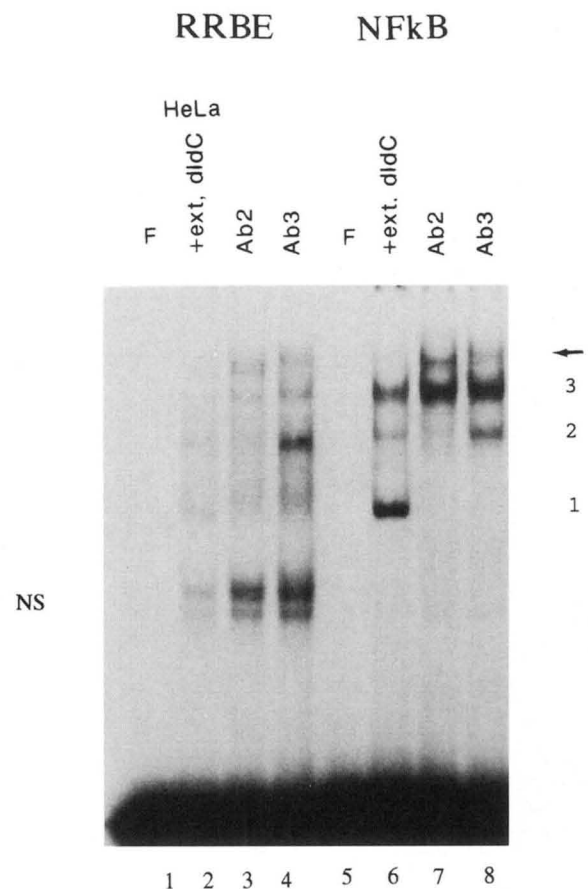
RRBE   NFkB

FIG. 6. **Proteins related to the NF-κB p50 subunit bind to the c-myb RRBE.** Polyclonal antisera directed against the p50 subunit of the NF-κB family (55) were incubated with nuclear extract from HeLa cells prior to the addition of the ^{32}P-end-labeled c-myb RRBE oligonucleotide (*lanes 1–4*) or the murine NF-κB oligonucleotide (*lanes 5–8*). *NS* corresponds to nonspecific DNA binding (data not shown). Free RRBE (*F*) is in *lane 1*. Lanes *2–4* and *6–8* contain 4 µg of protein from HeLa nuclear extracts (*ext*)and 0.5 µg of poly(dI-dC). Antiserum Ab2 (*lanes 3 and 7*) is directed against epitopes accessible on either p50 homodimers or p50-containing heterodimers. Antiserum Ab3 (*lanes 4 and 8*) is directed against epitopes accessible only on p50 when it binds DNA as a homodimer. The *arrow* designates a supershifted ternary DNA-protein-antibody complex. The three major DNA-protein complexes are denoted as complexes *1–3*.

the dRRBE/CAT reporter construct was transfected in parallel experiments with the wild-type c-myb/CAT reporter construct with 4 or 6 µg each of the p50 with p65 or p65 with c-Rel heterodimer combinations. In contrast to results obtained using the c-myb/CAT reporter construct, expression of the dRRBE/CAT reporter construct was unchanged or slightly decreased by cotransfection of p50 and p65: 0.84-fold with 4 µg and 0.77-fold with 6 µg of p50 and p65. Similarly, expression of the dRRBE/CAT reporter was unaffected by cotransfection of p65 and c-Rel: 0.90-fold with 4 µg and 1.1-fold with 6 µg of p65 and c-Rel. Thus, deletion of regions containing the RRBE sequences from the c-myb/CAT reporter construct resulted in the loss of a transactivation response due to coexpression with members of the NF-κB family. These experiments demonstrate that the c-myb RRBE sequences are required for NF-κB family members to transactivate the c-myb/CAT reporter construct and suggest that members of the NF-κB family play a role in the transcriptional regulation of the c-myb gene.

DISCUSSION

We have identified two DNA-protein interactions in the first intron of the murine c-myb gene flanking the region where
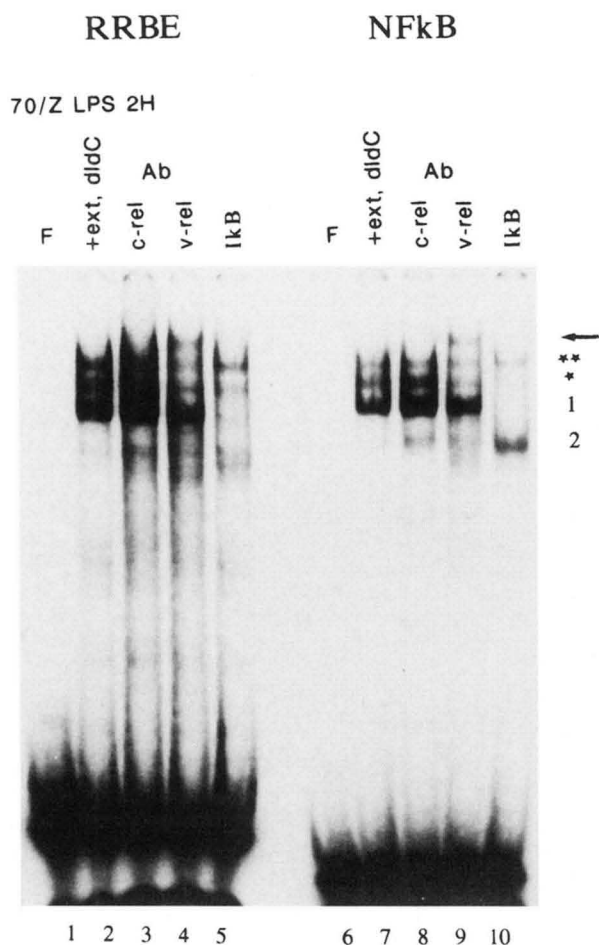
RRBE   NFkB

70/Z LPS 2H

Fig. 7. **Proteins related to the NF-κB c-Rel subunit bind to the c-*myb* RRBE and IκB-α/MAD-3 inhibition of DNA-binding activity detected on the c-*myb* RRBE.** Polyclonal antisera against a human c-Rel peptide (*lanes 3* and *8*) and v-Rel protein (*lanes 4* and *9*) were incubated with nuclear extract from 70Z/3.12 cells treated for 2 h with 10 μg/ml LPS prior to the addition of the ^{32}P-end-labeled c-*myb* RRBE oligonucleotide (*lanes 1–5*) or the murine NF-κB oligonucleotide (*lanes 6–10*). The complex indicted by the *arrow* denotes a supershifted ternary complex of DNA-protein-antibody. *Lanes 1* and *6* contain free oligonucleotide (*F*). *Lanes 2* and *7* contain the ^{32}P-end-labeled oligonucleotide incubated with 4 μg of protein from the 70Z/3.12 LPS-induced nuclear extracts (*ext*) and 0.5 μg of poly(dI-dC). The IκB-α/MAD-3 protein (48) was preincubated with 4 μg of protein from 70Z/3.12 cells treated for 2 h with LPS prior to the addition of the ^{32}P-end-labeled oligonucleotide in *lane 5* (c-*myb* RRBE) and *lane 10* (NF-κB). Presumptive p50 homodimer-DNA complexes are denoted as complex 2. Complex 1 and the complex denoted by the *single asterisk* were inhibited by the IκB-α/MAD-3 protein. The low mobility DNA-protein complex that was not inhibited by IκB-α/MAD-3 is denoted by the *double asterisks*.

transcription attenuation occurs. DNA-binding activities detected with the *Bam*HI/*Sty*I and *Bgl*II/*Bam*HI fragments correlated with low levels of c-*myb* mRNA expression (Fig. 3*A*). The protein-binding sequences detected on the *Bam*HI/*Sty*I and *Bgl*II/*Bam*HI fragments were homologous to the NF-κB cognate binding sequence and identical to the recently described RRBE element in the promoter of the human urokinase gene, which binds members of the NF-κB family (35, 54). We have demonstrated that members of the NF-κB family of transcription factors can bind to the c-*myb* RRBE element *in vitro* by several criteria. (i) DNA-binding activity was induced upon LPS treatment of 70Z/3.12 cells, and an NF-κB oligonucleotide competed for binding to the c-*myb* RRBE; (ii) antisera raised against the p50 subunit of NF-κB as well as the c- and v-Rel proteins supershifted or inhibited several of the DNA-protein complexes that formed on this element; and (iii) IκB-α/MAD-3

inhibited the formation of several of the DNA-protein complexes that were able to form on the c-*myb* RRBE element. However, preincubation of the extracts with either of the Rel antisera did not alter each of the complexes that we detected. Similarly, preincubation of the extracts with IκB-α/MAD-3 resulted in the selective loss of some but not all of these same complexes. Thus, our results suggest that additional factors or modifications to NF-κB family members may be involved in binding to the c-*myb* RRBE. In addition, the DNA-binding patterns detected with the c-*myb* RRBE were more complex than those reported with the RRBE from the human urokinase gene, where only two DNA-binding complexes were detected, suggesting that flanking sequences may be important in determining binding to this element.

At present, the level at which NF-κB acts to increase expression from the c-*myb*/CAT reporter construct is not understood. NF-κB family members may act at the level of transcription initiation to increase transcription from the c-*myb* promoter. Both p65 and c-Rel contain potent transcription activator domains (62–64), and transactivation of the c-*myb*/CAT reporter construct correlated with coexpression of both subunits. The strongest activation of the c-*myb*/CAT reporter construct was obtained with the p65 and c-Rel combination, suggesting that this novel heterodimer may play a role in activating the c-*myb* promoter. Thus, it is of considerable interest to note that Hansen *et al.* (65) have recently isolated a p65 with c-Rel heterodimer that binds and transactivates the urokinase promoter via the RRBE element. Similarly, Oeth *et al.* (66) have identified a κB-related binding site in the tissue factor promoter that mediates LPS-induced transcription of the tissue factor gene in human monocytes. This element binds the p65 with c-Rel heterodimer, but does not bind p50 with p65 heterodimers or p50 homodimers. Alternatively, since c-*myb* RRBE DNA-binding activity correlated with efficient attenuation and the c-*myb* RRBE sites flank the site of attenuation, members of the NF-κB family may regulate c-*myb* mRNA at the level of transcription elongation. In this case, NF-κB binding may alter the elongation competence of polymerase complexes at the promoter, modify the processivity of the elongating transcription complex at the site of attenuation, or alter the conformation of chromatin in the first intron, resulting in the read-through of elongating transcription complexes. Previous work has demonstrated that both the p50 and p65 subunits can induce DNA bending upon binding (67), and the location of the c-*myb* RRBE sequences within the first intron suggests that they may play a role in determining the conformation of the region through binding of NF-κB family members. In this respect, we have previously identified a DNase I-hypersensitive site (site IV) in the A20/2J murine B cell lymphoma cell line that is differentially detected and correlates with efficient attenuation (27). An alteration of the higher order chromatin structure within the intron may make the region more accessible to DNase I, and it is possible that the hypersensitive site is stimulated by RRBE binding.

The ability of NF-κB family members to transactivate a c-*myb*/CAT reporter construct points to a paradox in that we detected binding to the c-*myb* RRBE element in cell lines that contain small amounts of c-*myb* mRNA and that demonstrate efficient attenuation (24, 27–29). Resting lymphocytes contain little, if any, detectable c-*myb* mRNA (68, 69). However, during activation of human peripheral blood T cells (68, 69) or cloned T cell lines (70), c-*myb* mRNA expression is induced during late G_1/early S phase of the cell cycle. It is of particular interest to note that cross-linking of the T cell receptor, which is sufficient to induce G_0 to G_1 transition, does not induce expression of c-*myb* mRNA and that interleukin-2 is required both for G_1 to
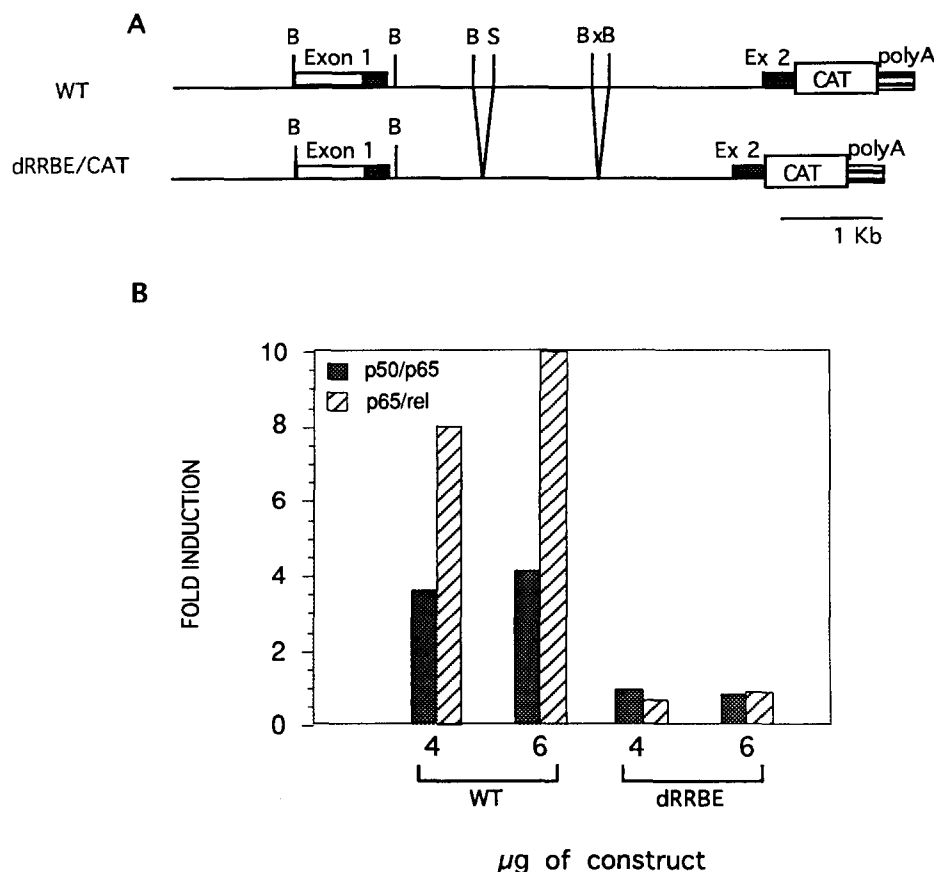
A

B

μg of construct

FIG. 8. *A*, diagrams of the c-*myb*/CAT and *d*RRBE/CAT reporter constructs. The *shaded boxes* in exons I and II denote coding sequences. The ATG start codon in exon I was deleted by mung bean nuclease digestion to reduce aberrant translation from c-*myb* sequences. *CAT* indicates the 734-bp *Hind*III/*Bam*HI fragment from pSV₂CAT encoding the bacterial chloramphenicol acetyltransferase gene. *polyA* denotes the 340-bp *Bcl*I/*Bam*HI fragment from SV40 containing polyadenylation sequences. The two RRBE sequences in the first intron are contained on *Bam*HI/*Sty*I and *Bst*XI/*Bam*HI restriction fragments. The *d*RRBE/CAT reporter construct is identical to the c-*myb*/CAT reporter construct except that the 1.5-kb *Bam*HI fragment from the first intron of c-*myb* was replaced by the internal 1.2-kb *Sty*I/*Bst*XI fragment. This specifically deleted the two c-*myb* RRBE sequences from the c-*myb*/CAT reporter construct (see "Materials and Methods" for details of construction). *WT*, wild-type. See legend to Fig. 1 for definition of restriction enzyme abbreviations. *B*, results from cotransfection experiments that examined transactivation of the c-*myb*/CAT and *d*RRBE/CAT reporter constructs by p50 with p65 and p65 with c-Rel heterodimer combinations. The *x axis* corresponds to the microgram amount of each expression vector cotransfected with 2 μg of the c-*myb*/CAT reporter construct. The *y axis* corresponds to the -fold induction of CAT activity from each vector calculated by comparing the CAT activity resulting from cotransfection of c-*myb*/CAT or *d*RRBE/CAT with the indicated NF-κB family members to the activity resulting from transfection of each construct alone. Results are the average of duplicate transfections and are representative of four independent experiments.

S phase transition and for induction of c-*myb* mRNA expression (69, 70). Similarly, the DNA-binding activity of different members of the NF-κB family is regulated during T cell activation, and p50 with p65 heterodimers rapidly translocate to the nucleus and are able to bind DNA within minutes of T cell receptor cross-linking (68, 71). However, it has been reported that c-Rel is not detected in DNA-binding complexes until G₁ (71). Thus, differential regulation of NF-κB family DNA-binding activity may play a role in regulating c-*myb* expression during lymphocyte activation. Previous studies examining c-*myb* mRNA expression during T lymphocyte activation did not characterize nascent c-*myb* mRNA expression by nuclear run-on assay, and it is not known whether increased detection of steady-state c-*myb* mRNA is due to changes in the rate of transcription initiation or changes in the efficiency of attenuation (69, 70). Changes in the DNA-binding activities of NF-κB family members may act at either level. Alternatively, non-NF-κB DNA-binding activities that act synergistically with NF-κB family members may be induced by interleukin-2 stimulation to increase steady-state c-*myb* mRNA levels. For instance, activation of the β-interferon and interleukin-2 α-receptor promoters involves the interaction of NF-κB with interferon-regulated factor-1 and the serum response factor,

respectively (72, 73). Thus, interaction of NF-κB family members with other transcription factors may be required for NF-κB to transactivate expression of c-*myb* mRNA. Finally, Klug *et al.* (74) have recently demonstrated that transformation of pre-B cells by Abelson leukemia virus inhibits the activated state of NF-κB-Rel complexes, while pre-B cells expanded from normal bone marrow contain active NF-κB complexes. Thus, NF-κB/Rel family members are active and may be involved in regulating c-*myb* expression at several stages of B cell development.

The c-*myb* RRBE sequences in the first intron may have different effects on transcription depending upon which NF-κB subunits occupy the sites. Even though the combination of p65 and c-Rel transactivated the c-*myb*/CAT reporter in cotransfection assays in EL4 cells, c-Rel may have a repressive effect on transcription in other cell types, at different stages of differentiation, or in combination with other NF-κB-binding proteins. For example, decreased expression of c-*myb* mRNA in maturing thymocytes has been inversely correlated with expression of c-*rel* mRNA (75), and c-Rel-like proteins have also been shown to be involved in repression of the interleukin-6 gene in lymphoid cells (76). Thus, it should be noted that Miyamoto *et al.* (77) have reported a shift in the NF-κB subunits that bind

the κB site during B cell differentiation. Changes in the relative subunit concentrations during development may alter the function of the c-*myb* RRBE sites, resulting in increased or decreased c-*myb* expression. Only three members of the NF-κB family were examined in this study, and other NF-κB family members or heterodimer combinations may be the actual factors that regulate c-*myb* transcription. For example, there has been a report of a novel NF-κB DNA-binding activity (NP-TC$_{II}$) that is present in unstimulated as well as stimulated T and B cells, but is not detected in nonhematopoietic cells (61). It will be of considerable interest to determine how members of the NF-κB/Rel family regulate c-*myb* transcription and the consequences of shifts in the relative activity of the NF-κB proteins to c-*myb* expression.

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