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Interaction of an NF-κB-like factor with a site upstream of the c-*myc* promoter

(transcription regulation/B-cell lymphoma)

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ABSTRACT **The c-*myc* protooncogene has been implicated in control of growth and differentiation of mammalian cells. For instance, growth arrest is often preceded by reduction in c-*myc* mRNA and gene transcription. To elucidate the mechanisms of control of c-*myc* gene transcription, we have begun to characterize the interaction of nuclear factors with the 719-base-pair (bp) c-*myc* regulatory domain, located 1139–421 bp upstream of the P1 start site of the mouse gene. Nuclear extracts from exponentially growing WEHI 231 murine B-lymphoma cells formed multiple complexes in mobility-shift assays. Changes in complex distribution were observed in growth-arrested WEHI 231 cells, and a major site of this interaction mapped to a 21-bp sequence that is similar to the sequences recognized by the NF-κB family of proteins. Binding of NF-κB-like factors was demonstrated by oligonucleotide competition. Induction of complex formation upon 70Z/3 pre-B- to B-cell differentiation, enhancement of binding by GTP, and detergent-induced release of inhibitor protein suggested that NF-κB itself is one member of the family that can bind. Transfection of thymidine kinase–chloramphenicol acetyltransferase constructs containing the 21-bp c-*myc* sequence into Jurkat cells demonstrated increased chloramphenicol acetyltransferase activity upon phorbol ester and phytohemagglutinin treatment. These results suggest the involvement of NF-κB-like factors in the regulation of c-*myc* transcription.**

A major regulatory site for c-*myc* gene expression in the response of cells to growth or differentiation occurs at the level of transcription. Changes in the rate of c-*myc* gene transcription are likely to be mediated by the interaction of specific proteins with regulatory elements near or within the gene. Both positive and negative regulatory elements have been identified for human and murine c-*myc* genes (1–3). Using chloramphenicol acetyltransferase (CAT) reporter gene constructs in transfection assays, Marcu and coworkers (3) have identified a regulatory element located between 1139 and 421 base pairs (bp) upstream of the first transcription start point in the murine c-*myc* gene. Removal of this 719-bp element led to increased CAT expression, suggesting that it includes a site of negative regulation (3). The function of the 719-bp fragment was independent of its position and orientation and was thus termed a "dehancer."

To elucidate the mechanisms involved in the control of c-*myc* transcription, we sought to relate changes in the interaction of nuclear factors with identified regulatory elements to changes in the rate of c-*myc* transcription. The WEHI 231 early B-lymphoma cell line was used as a model system, since a major component of regulation of c-*myc* gene expression in these murine cells occurs at the level of transcription (4). Proliferation of WEHI 231 cells can be arrested within 24–48 hr after incubation with an antiserum against the expressed surface immunoglobulin, such as a goat anti-mouse immunoglobulin preparation (GaMIg) (5, 6). Previously, we demonstrated that a selective 5- to 10-fold decrease in c-*myc* mRNA expression occurs within 24 hr of treatment (6); this drop correlated with a decrease in the transcription of the c-*myc* gene (4). Here we report changes in the complex interaction of proteins with the 719-bp upstream regulatory region that occur during the down-modulation of c-*myc* gene transcription in WEHI 231 cells. The sequences mediating this binding revealed homology to an NF-κB binding motif (7), and the functional nature of this site was demonstrated by using thymidine kinase (TK)–CAT reporter gene constructs.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts. Culture conditions and GaMIg treatment of the WEHI 231 early B-lymphoma cell line are as described (6). 70Z/3 pre-B lymphoma cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 50 μM 2-mercaptoethanol. Jurkat cells were grown in RPMI 1640 medium supplemented with 10% FBS. Stimulation of 70Z/3 cells was carried out with either 10 μg of lipopolysaccharide (LPS) per ml for 2 hr, or 50 nM phorbol 12-myristate 13-acetate (PMA) for 1 hr. Crude nuclear extracts were prepared by the method of Strauss and Varshavsky (8). Cytosolic fraction from 70Z/3 cells was isolated as described by Baeuerle and Baltimore (9) and was activated *in vitro* by using 0.2% sodium deoxycholate/1.2% Nonidet P-40.

Plasmid Constructs and Electrophoretic Mobility-Shift Analysis. The three fragments shown in Fig. 1*A* were end-labeled with the large fragment of *Escherichia coli* DNA polymerase I (New England Biolabs) and [α-^{32}P]dNTPs (ICN). The electrophoretic mobility-shift assay was performed as follows: each ^{32}P-labeled fragment (2 ng) and ≈5 μg of nuclear extract were mixed in 70 mM NaCl/10 mM Hepes, pH 7.5/1 mM EDTA/1 mM dithiothreitol/0.1% Triton X-100/4% (vol/vol) glycerol/5 μg of poly(dI-dC)·poly(dI-dC) copolymer, in a final vol of 25 μl. This mixture was incubated for 30 min at 22°C and electrophoresed at 11 V/cm in a 4% polyacrylamide gel with a gel running buffer consisting of 6.7 mM Tris·HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. Gels were dried and autoradiographed. For competition experiments, competitor DNAs were included in the mixture before addition of labeled fragment A.

Footprint Analysis. For DNase I protection, the noncoding strand of fragment A was labeled and the DNA was incubated with nuclear extract in a binding reaction mixture that had

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Abbreviations: GaMIg, goat anti-mouse immunoglobulin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin; IL-2, interleukin 2; IL-2R, IL-2 receptor; HIV, human immunodeficiency virus.

been scaled up 28-fold. The mixture was digested with 2 or 5 μg of DNase I per ml and was then subjected to electrophoresis as described above. Complexed and uncomplexed bands were visualized by autoradiography, excised from the gel, and electroeluted. Equal dpm of bound and free samples were subjected to electrophoresis in an 8% polyacrylamide/8 M urea sequencing gel. Labeled fragment A DNA, which had been subjected to Maxam–Gilbert sequencing reactions (10), was electrophoresed in parallel to determine the protected sequence. Copper *o*-phenanthroline protection and methylation interference analyses were performed as described by Kuwabara and Sigman (11) and Sen and Baltimore (12), respectively. Products were analyzed as described above.

Transfection and CAT Assay. Oligonucleotides corresponding to site 1 and a mutant site 1 sequence were synthesized with *Bam*HI linkers and cloned into the *Bam*HI site of TK–CAT vector (13). Transient transfections of Jurkat cells were performed according to Lieber *et al.* (14) with 10 μg of DNA per 5×10^6 cells. Twenty-four hours after transfection, cells were treated with 50 nM PMA and 1 μg of phytohemagglutinin (PHA) per ml for an additional 20 hr. The cells were then collected, washed with Puck's saline, and lysed by freezing and thawing in a solution containing 150 mM Tris·HCl (pH 8.0). Cell extracts, normalized for total protein content, were assayed for CAT activity (15).

RESULTS

Electrophoretic Mobility-Shift Analysis of WEHI 231 Nuclear Protein Interactions with the 719-bp Regulatory Element of c-*myc*. To characterize protein–DNA interactions with the 719-bp fragment, the region was subdivided into three domains to provide DNA segments of appropriate size for effective use in electrophoretic mobility-shift assays (Fig. 1*A*). Crude nuclear extracts were prepared from exponentially proliferating WEHI 231 cells and from the same cells treated for 24 hr with GaMIg, at which time the transcription of c-*myc* is diminished. After incubation with extracts from exponentially proliferating cells, each of the three fragments formed multiple complexes of various intensities, which were resolved by gel electrophoresis (Fig. 1*B*, lane E). When nuclear extracts from cells treated with GaMIg for 24 hr (lane 24) were compared to those of exponentially proliferating cells, the most striking change in binding profile was observed with fragment A. In growth-arrested cells, an increase in relative intensity of band 1 with respect to band 2 was seen as well as a general reduction in intensity of the more slowly migrating complexes (bands 3–6) (Fig. 1*B*). Loss of all complexes was observed upon competition with excess unlabeled intact fragment A, whereas B and C had no effect (data not shown), confirming the specificity of binding.

To map the specific site(s) of interaction within this region, fragment A DNA was labeled at either end and subjected to digestion with various restriction endonucleases. The resulting series of subfragments were used independently in mobility-shift assays with nuclear extracts from exponentially proliferating WEHI 231 cells (data not shown). The results indicated that there are at least two distinct binding domains within fragment A, and this was confirmed by competition analysis (data not shown; see Fig. 3). The majority of the complexes (bands 1, 2, 3, 4, and 6) requires factor interaction with sequences between or near the *Hpa* II and *Pfl*MI restriction sites (Fig. 1*A*). Formation of a single complex (band 5) involves the region between *Alu* I and *Hae* III sites. These two regions will be referred to as sites 1 and 2, respectively.

Determination of a Specific Binding Sequence Within Site 1. DNase I protection analysis was performed to identify the sequences within site 1 mediating protein interaction. Labeled fragment A was incubated with nuclear extracts from exponentially growing cultures and subjected to DNase I digestion. Since the vast majority of binding mapped to site 1 within fragment A, all complexes (bands 1–6) were isolated and analyzed. The sequence surrounding the *Hpa* II site and extending toward the *Pfl*MI site was protected (Fig. 2*A*). Specifically, we observed protection of the sequence 5′-AAGTCCGGGTTTTCCCCAACC-3′, which spans from 1101 to 1081 bp upstream of the c-*myc* P1 start site. This finding is consistent with the localization of the major binding site by mobility-shift experiments. Protection of other sequences within the region was not apparent.

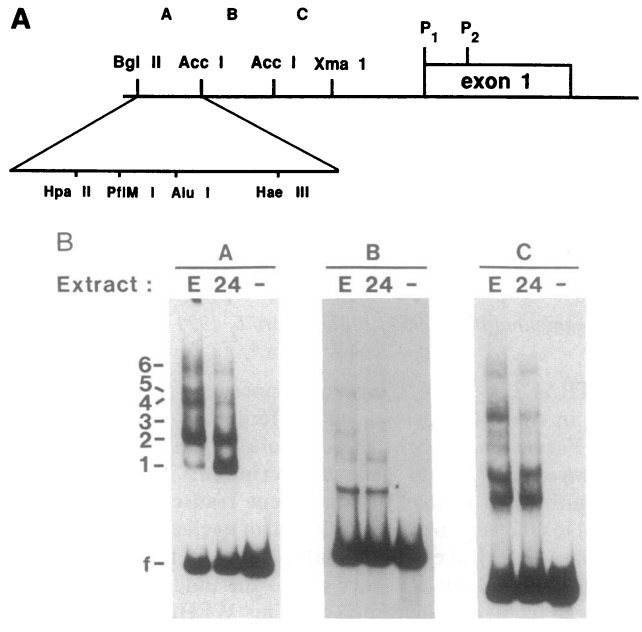

FIG. 1. Binding of WEHI 231 nuclear extracts to the upstream regulatory element. (*A*) Schematic representation of the 719-bp regulatory region. The DNA spanning base pairs 1139–421 upstream of the c-*myc* P1 start site was subcloned to generate three fragments: A, *Bgl* II/*Acc* I (base pairs −1139 to −921); B, *Acc* I/*Acc* I (base pairs −920 to −613); C, *Acc* I/*Xma* I (base pairs −612 to −421). Expanded portion illustrates restriction sites within fragment A used in this study. (*B*) Electrophoretic mobility-shift analysis of labeled fragments A, B, and C using nuclear extracts from exponentially proliferating (lane E) or GaMIg-treated (lane 24) WEHI 231 cells. Lane −, absence of nuclear extract in binding reaction. The six bands observed with fragment A DNA are labeled. f, Unbound fragment.

To footprint the binding of individual complexes, a copper *o*-phenanthroline chemical cleavage protection assay was performed (11). Bands 1 and 2 were mapped to determine whether distinct or overlapping sites within this *Pfl*MI/*Hpa* II sequence are involved. The results presented in Fig. 2*B* indicate that the complexes represented by both band 1 (lane b1) and band 2 (lane b2) protected the same sequence as that seen with DNase I footprinting. (Similar footprint patterns for bands 1 and 2 were obtained with extracts from GaMIg-treated cells; data not shown.)

A double-stranded oligonucleotide containing the protected sequence of site 1 was synthesized (site 1 oligonucleotide) to test whether this sequence directly mediates complex formation. The ability of this sequence to compete for binding was tested (Fig. 3). When a 50-fold molar excess of site 1 oligonucleotide was used in a binding competition experiment with labeled fragment A, formation of labeled bands 1, 2, 3, 4, and 6 was prevented. As expected, formation of band 5, which involves site 2, is resistant to competition. Furthermore, binding of labeled site 1 oligonucleotide to WEHI 231 nuclear extracts displayed formation of multiple complexes (data not shown), indicating that this oligonucleotide contains the major binding site for protein interactions within fragment A.

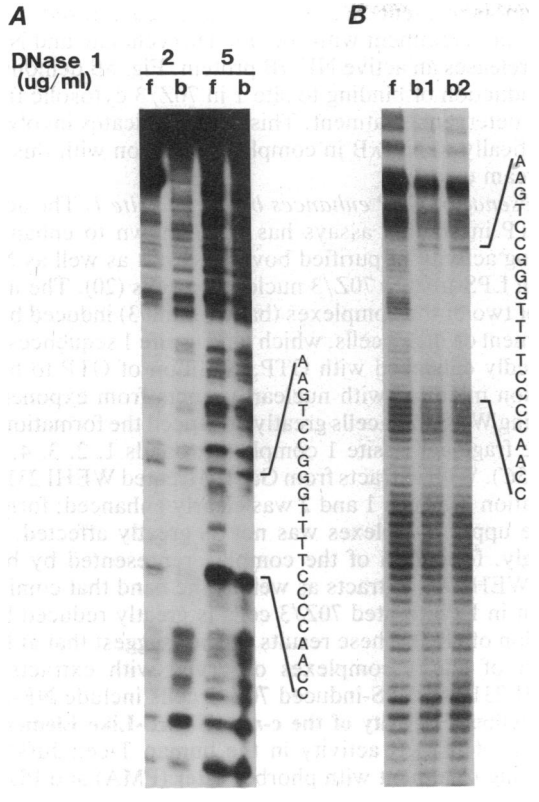

FIG. 2. Footprint analysis defines the major binding site within fragment A. (*A*) DNase I protection analysis. Binding reactions performed with nuclear extracts from exponentially growing WEHI 231 cells were digested with either 2 or 5 μg of DNase I per ml. The unbound fragment is indicated (lanes f). Bound fragment (lanes b) represents all complexes formed with fragment A (bands 1–6). The sequence shown is the protected region and is given for the coding strand. (*B*) Copper *o*-phenanthroline cleavage protection analysis. Free fragment A (lane f) and complexes represented by band 1 (lane b1) and band 2 (lane b2) in the mobility-shift assay were analyzed. The sequence protected from cleavage is shown.

To define the nucleotides directly involved in this protein–DNA interaction, methylation interference experiments were performed with nuclear extracts from exponentially growing

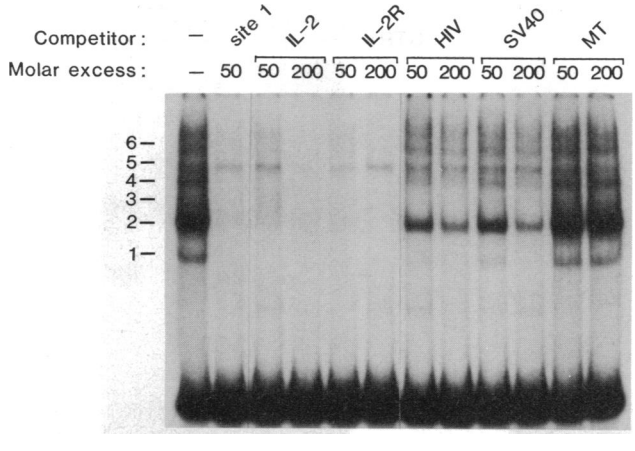

FIG. 3. Competition for binding by NF-κB motifs from different genes. Binding to fragment A DNA of extracts from exponentially growing WEHI 231 cells was analyzed in the absence (lane –) or presence of oligonucleotides that represent NF-κB-like binding sites from different genetic elements. Oligonucleotide sequences containing NF-κB motifs are given in Table 1. The sequence of the metallothionein (MT) oligonucleotide is GAACTGACCGCCCGCG-GCCCGTGTGCAGAG. The competitor oligonucleotides and the amounts used are as indicated. SV40, simian virus 40.

WEHI 231 cells. The major complex represented by band 2 was initially analyzed by using DNA labeled on the noncoding strand of fragment A. Binding can be specifically prevented upon methylation of any of four adenine or guanine residues within the site 1 sequence (Fig. 4, Non-coding, lane b). To determine whether the other complexes give the same interference pattern, similar analysis was performed on complexes represented by band 1 (lanes b1), band 2 (lanes b2), and the remaining lower mobility complexes (lanes b3) using DNA labeled on both the noncoding and coding strands of fragment A. All the complexes gave a methylation interference pattern on the noncoding strand similar to that seen with band 2 (Fig. 4, lanes b and b2 vs. lanes b1 and b3). With fragment A labeled on the coding strand, methylation of either of three guanine residues prevents interaction of proteins with the site 1 sequence with all three complexes. Thus, the complexes observed require contact with 11 bases within the sequence GGGTTTTCCCC and its complementary strand (Fig. 4).

Site 1 Contains an NF-κB-Like Binding Motif. Comparison with the binding sites of known nuclear factors revealed homology of the site 1 binding sequence to sequences mediating binding of NF-κB (GGGGACTTTCC) and members of the NF-κB family (16). Furthermore, the pattern of methylation interference is strikingly similar to that seen with NF-κB (12) and a related family member, H2TF1 (17).

As an initial test of whether an NF-κB-like factor is binding to fragment A, competition analysis was performed with several double-stranded synthetic oligonucleotides representing various NF-κB binding sites (see Table 1). As shown in Fig. 3, competition with a 50-fold molar excess of either the interleukin 2 (IL-2) or IL-2 receptor (IL-2R) NF-κB-like binding motifs effectively prevented the formation of complexes represented by bands 1, 2, 3, 4, and 6, but not band 5, which maps to the site 2 domain. NF-κB binding sequences in simian virus 40 and human immunodeficiency virus (HIV) (which are identical to the site in the κ light-chain gene enhancer) also competed; however, the efficiency of competition varied for the different complexes. Bands 1 and 3 were most sensitive to competition. An oligonucleotide containing an AP2 binding site derived from the metallothionein gene, which does not contain an NF-κB binding site, failed to compete for any binding.

Binding to Site 1 Involves NF-κB. *Analysis of nuclear extracts from 70Z/3 pre-B cells.* Sen and Baltimore (18) have

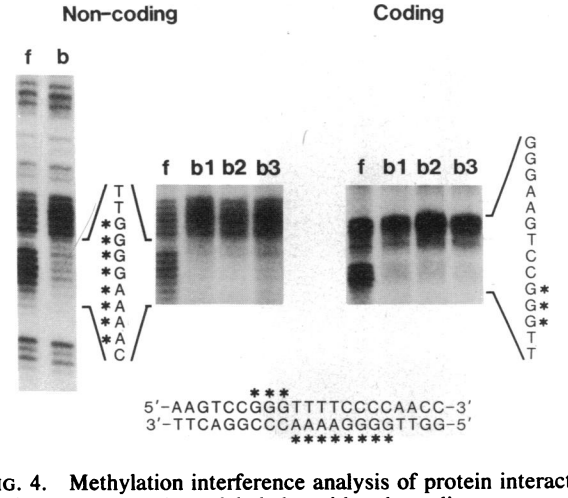

FIG. 4. Methylation interference analysis of protein interaction with site 1. Fragment A was labeled on either the coding or noncoding strand. Unbound (free) fragment A is indicated. Lanes: b, band 2; b1, band 1; b2, band 2; b3, remaining lower mobility complexes (bands 3–6). Asterisks indicate the location of guanine and adenine residues whose methylation specifically inhibits the formation of complexes to site 1.

Table 1. Synthetic oligonucleotide sequences containing NF-κB binding motifs

Gene	Oligonucleotide sequence
Simian virus 40	GTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAG
HIV	GATCCAGGGACTTTCC
IL-2Rα	GATCCGGCAGGGGAATCTCCCT
IL-2	GATCCACAAAGAGGGATTTCACCTACATCC

Sequences involved in NF-κB binding are underlined.

shown that NF-κB activity is induced upon differentiation of 70Z/3 pre-B cells to B cells after treatment with LPS. This induction of binding activity by LPS treatment is considered diagnostic of NF-κB (18, 19). To evaluate whether NF-κB itself can bind to site 1, we tested the binding to fragment A of nuclear extracts from 70Z/3 cells before or after LPS treatment (Fig. 5*A*). In uninduced cells, the major binding detected appeared to comigrate with band 5, although it is not known whether this complex is the same as that observed with WEHI 231 extracts. After treatment with LPS, the presence of three new complexes was observed. The major complex comigrated with the complex represented by band 3 in WEHI 231 extract and the two minor complexes comigrated with bands 1 and 2 (positions indicated in the figure). To demonstrate that these protein complexes are binding to site 1 within fragment A, binding competition with the site 1 oligonucleotide was performed with the LPS-treated 70Z/3 nuclear extracts (Fig. 5*A*). A 100-fold molar excess of site 1 oligonucleotide selectively eliminated the binding represented by the three putative site 1 bands, confirming that these complexes do map to this site. Competition with an oligonucleotide containing the HIV NF-κB motif also eliminated these three bands (Fig. 5*A*), suggesting that one of the proteins involved in formation of these site 1 complexes in both WEHI 231 and induced 70Z/3 cells is likely to be NF-κB or a closely related protein.

Detergent releases binding activity. Baeuerle and Baltimore (9) have demonstrated that in non-B cells, NF-κB protein is present in the cytoplasm complexed with an inhibitor. Treatment with sodium deoxycholate and Nonidet P-40 releases an active NF-κB protein. Fig. 5*B* demonstrates the induction of binding to site 1 in 70Z/3 cytosolic fraction upon detergent treatment. This result indicates involvement specifically of NF-κB in complex formation with this c-*myc* upstream element.

Presence of GTP enhances binding to site 1. The addition of GTP in binding assays has been shown to enhance the binding activity of purified bovine NF-κB as well as NF-κB within LPS-treated 70Z/3 nuclear extracts (20). The intensities of two of the complexes (bands 1 and 3) induced by LPS treatment of 70Z/3 cells, which bind to site 1 sequences, were markedly enhanced with GTP. Addition of GTP to binding reaction mixtures with nuclear extracts from exponentially growing WEHI 231 cells greatly enhanced the formation of all of the fragment A site 1 complexes, bands 1, 2, 3, 4, and 6 (Fig. 5*C*). With extracts from GaMIg-treated WEHI 231 cells, formation of bands 1 and 2 was clearly enhanced; formation of the upper complexes was not as greatly affected. Interestingly, formation of the complex represented by band 5 with WEHI 231 extracts as well as the band that comigrates with it in LPS-treated 70Z/3 cells is greatly reduced by the addition of GTP. These results further suggest that at least a subset of site 1 complexes observed with extracts from WEHI 231 and LPS-induced 70Z/3 cells include NF-κB.

Functional Activity of the c-*myc* NF-κB-Like Element. Induction of NF-κB activity in the human T-cell Jurkat line following treatment with phorbol ester (PMA) and PHA has been demonstrated by several groups (18, 21, 22). To test the functional activity of the c-*myc* NF-κB-like binding site, constructs were prepared by using a TK promoter–CAT clone (13). Two or three copies of the site 1 oligonucleotide were ligated, in either orientation, into the *Bam*HI site upstream of the TK promoter. Constructs containing site 1 oligonucleotide mutated by conversion of the two internal guanine residues to cytosine residues were similarly analyzed (see legend to Fig. 6). This mutated oligonucleotide fails to

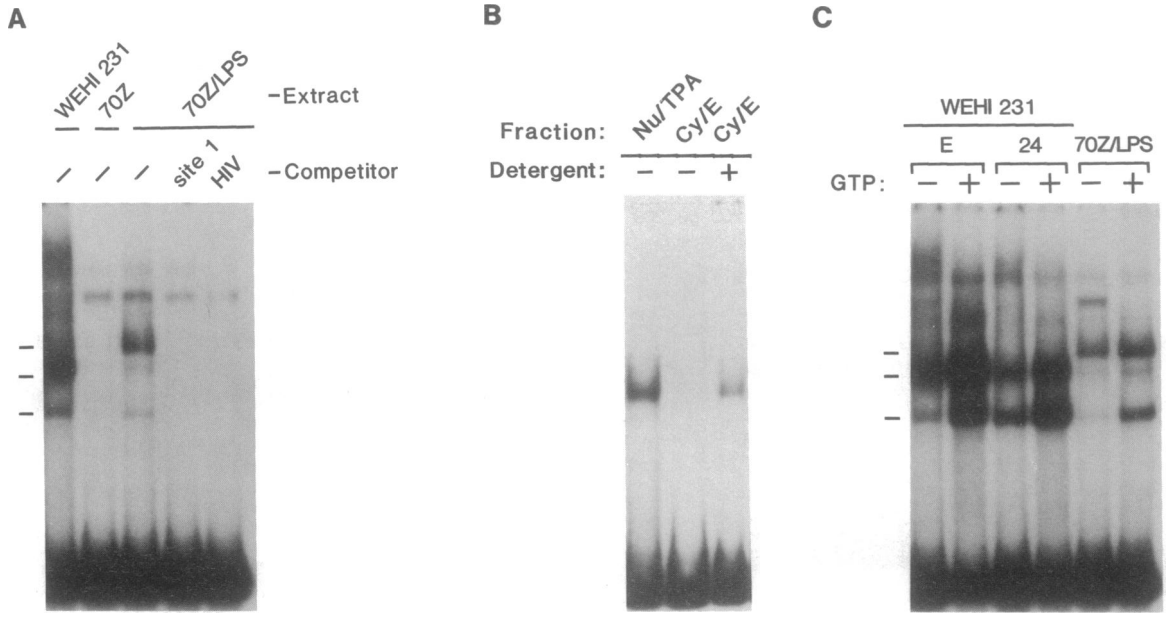

FIG. 5. (*A*) Binding analysis of 70Z/3 and LPS-induced 70Z/3 cell line. Competition binding analysis was performed with extracts from LPS-induced 70Z/3 cells in the presence of 100-fold molar excess site 1 and HIV NF-κB oligonucleotides. Bars indicate the positions of WEHI 231 site 1 bands 1, 2, and 3. (*B*) Cell-free activation of NF-κB precursor in the cytosolic fraction (Cy) by detergent treatment. The cytosolic fraction from exponentially growing (E) 70Z/3 cells was used directly (lane −) or after treatment (lane +) with 0.2% sodium deoxycholate and 1.2% Nonidet P-40. Complexes formed with nuclear extracts from PMA-treated (lane Nu/TPA) 70Z/3 cells are shown for comparison. (*C*) Effects of GTP on the binding of nuclear proteins to fragment A. Binding of nuclear extracts from exponentially growing (lane E) and GaMIg-treated (lane 24) WEHI cells and LPS-induced 70Z/3 cells were analyzed in the absence (lanes −) or presence (lanes +) of 3 mM GTP.

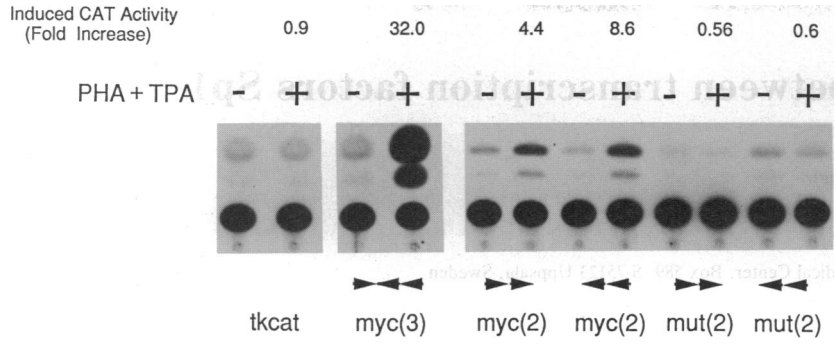

FIG. 6. CAT activity of the c-*myc* NF-κB-like element. TK–CAT constructs containing three copies (myc3) or two copies (myc2) of wild-type and two copies of mutant (mut2) c-*myc* NF-κB-like element as well as TK–CAT without any NF-κB-like site (tkcat) were transfected into Jurkat cells. Cells were incubated in the absence (lanes −) or presence (lanes +) of PMA (TPA) and PHA for 20 hr. Arrowheads correspond to the direction in which the oligonucleotides are cloned. The stimulation ratios for CAT activity are given above. The mutant sequence is 5′-AAGTCCGCCTTTTCCC-CAACC-3′.

form any complex with WEHI 231 nuclear extracts (data not shown). Treatment of Jurkat cells with PMA and PHA resulted in a 4- to 9-fold stimulation in CAT activity with TK–CAT constructs containing two copies of site 1 (Fig. 6) consistent with values obtained for HIV and IL-2R from other laboratories (21–23). Three copies of site 1 resulted in a >30-fold activation of CAT activity. The constructs containing no site 1 or mutated site 1 oligonucleotide did not display stimulation. Thus this site, located upstream of the c-*myc* promoter, can bind NF-κB-like factors and function to modulate transcriptional activity.

DISCUSSION

We have identified a site, located 1101–1081 bp upstream of the c-*myc* P1 promoter, that is recognized by the NF-κB family of proteins. Interaction of nuclear proteins from WEHI 231 cells with this site yields multiple complexes. Involvement of NF-κB itself has been demonstrated based on the observed induction of binding to this site upon differentiation of 70Z/3 pre-B to B cells, the detergent release of binding activity in cytoplasmic extracts, and the GTP enhancement of binding. However, the multiplicity of complexes formed in mobility shift analyses is greater than that observed typically with members of the NF-κB family, including binding to the κ enhancer B site (18). This result implies (*i*) that multiple members of the NF-κB family interact with this site, (*ii*) that modified forms of the factors can bind, and/or (*iii*) that additional proteins are present in the complexes. Identification of the specific components of the different site 1 complexes awaits protein purification.

While NF-κB was originally implicated in control of κ light-chain expression in B cells, this factor and other related family members have been shown to be involved in the activation of a growing number of genes (21–29). Furthermore, such diverse agents as mitogens, phorbol ester, interleukins, viral infection, and cycloheximide have all been observed to induce activity in various cell types. The transfection experiments in Jurkat cells presented here suggest a possible role of this family of factors during induction of c-*myc* expression. Experiments to test the functional significance of this binding site in the regulation of c-*myc* gene transcription remain to be done. Interestingly, the ABPC22 myeloma has recently been shown to have a viral insertion 22 bp downstream of this binding site in the murine c-*myc* gene (J. Shaughnessy and M. Potter, personal communication).

Anti-immunoglobulin-induced growth arrest of WEHI 231 cells correlates with a drop in the rate of c-*myc* gene transcription that is accompanied by several changes in the formation of complexes with site 1. A loss of ability to form band 3 as well as the larger complexes (bands 4–6) and the concomitant increase in ability to form the highest mobility complex (band 1) was observed. While the specific nature of the changes in factors involved is unclear, all of these complexes appear to involve NF-κB-like factors based on the competition analyses presented in Fig. 3. Thus, if altered binding to site 1 has a functional role in the transcriptional down-modulation of c-*myc*, the results with WEHI 231 cells are unusual in that alteration in binding of the NF-κB family of factors is involved in a negative regulatory event.

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