

Role of Rel-related Factors in Control of *c-myc* Gene Transcription in Receptor-mediated Apoptosis of the Murine B Cell WEHI 231 Line

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Summary

Treatment of immature murine B lymphocytes with an antiserum against their surface immunoglobulin (sIg)M results in cell death via apoptosis. The WEHI 231 B cell line (IgM, kappa) has been used extensively as a model for this anti-Ig receptor-mediated apoptosis. Anti-sIg treatment of WEHI 231 cells causes an early, transient increase in the levels of *c-myc* messenger RNA and gene transcription, followed by a rapid decline below control values. Given the evidence for a role of the *c-myc* gene in promoting apoptosis, we have characterized the nature and kinetics of changes in the binding of Rel-related factors, which modulate *c-myc* promoter activity. In exponentially growing WEHI 231 cells, multiple Rel-related binding activities were detectable. The major binding species was identified as p50/c-Rel heterodimers; only minor amounts of nuclear factor κ B (NF- κ B) (p50/p65) were detectable. Cotransfection of an inhibitor of NF- κ B (κ B)- α expression vector reduced *c-myc*-promoter/upstream/exon1-CAT reporter construct activity, indicating the role of Rel factor binding in *c-myc* basal expression in these cells. Treatment with anti-sIg resulted in a rapid transient increase in the rate of *c-myc* gene transcription and in the binding of Rel factors. At later times, formation of p50 homodimer complexes occurred. In cotransfection analysis, p65 and c-Rel expression potently and modestly transactivated the *c-myc* promoter, respectively, whereas, overexpression of the p50 subunit caused a significant drop in its activity. The role of activation of Rel-family binding was demonstrated directly upon addition of the antioxidant pyrrolidinedithiocarbamate, which inhibited the anti-sIg-mediated activation of the endogenous *c-myc* gene. Similarly, induction after anti-sIg treatment of a transfected *c-myc* promoter was abrogated upon cotransfection of an κ B- α expression vector. These results implicate the Rel-family in Ig receptor-mediated signals controlling the activation of *c-myc* gene transcription in WEHI 231 cells, and suggest a role for this family in apoptosis of this line, which is mediated through a *c-myc* signaling pathway.

Nuclear factor κ B (NF- κ B)¹ elements mediate control of a number of important growth regulatory genes (1, 2). We identified two nuclear factor (NF)- κ B elements within the murine *c-myc* oncogene. One site, termed the URE, was located 1101–1081 bp upstream of the P1 promoter (3), and the second site (IRE) was localized within exon 1 at +440 to +459 bp (4). NF- κ B is expressed constitutively in nuclei of mature B lymphocytes (5) and can be induced upon differentiation of pre-B to B cells (6). In non-B cells, NF- κ B activity

can be induced by a variety of agents, including IL-1, TNF- α , and phorbol ester, as well as upon infection with several viruses, such as HTLV-1 and HIV-1 (1, 2). We demonstrated that activation of NF- κ B-like factors, upon IL-1 treatment of human fibroblasts or expression of the *tax* gene product of the HTLV-1 virus in T cells, can induce transcription of the *c-myc* oncogene (7, 8).

NF- κ B is now known to be a family of dimeric transcription factors with subunits that contain an amino terminal stretch of \sim 300 amino acids that share homology with the v-Rel oncogene (9–12). Classical NF- κ B is composed of a p50 and a p65 subunit. The p65 subunit contains a more potent transactivation domain than the p50 subunit. However, it binds less avidly (1, 2, 13–15). Using transient transfection in NIH 3T3 fibroblasts, we have recently demonstrated that expression of p65 alone, or of p65, in combination with p50, potently transactivates the murine *c-myc* promoter,

¹ Abbreviations used in this paper: CAT, chloramphenicol acetyl-transferase; dm, double mutant; κ B, inhibitor of NF- κ B; NF- κ B, nuclear factor κ B; PDTC, pyrrolidinedithiocarbamate; sIg, surface Ig; URE, upstream regulatory element.

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whereas p50 and *v-rel* failed to transactivate, and murine *c-rel* transactivated only weakly (16). In addition, other Rel-related subunits have been identified, including RelB and p52 (17–20).

Treatment of immature IgM-bearing B lymphocytes with an antibody against their expressed surface Ig (sIg)M resulted in cell death via apoptosis (21). The WEHI 231 lymphoma cell line (IgM, kappa) had been characterized as an immature B cell on the basis of surface markers and biological properties (22, 23). Treatment of WEHI 231 cells for 18 h with an antibody against its sIgM resulted in formation of an oligosomal DNA pattern, characteristic of apoptotic fragmentation of chromatin within nuclei (24, 25). DNA synthesis ceased within 24–36 h (22, 23, 26). This anti-sIg treatment also resulted in dramatic changes in expression of the *c-myc* oncogene that preceded DNA degradation. A 5- to 15-fold increase in *c-myc* RNA and protein levels between the first and second hours was followed by a rapid decline; by 8 h of treatment, the levels were well below control values (26–28). A major site of control of *c-myc* expression occurred at the level of gene transcription (27). Several groups have now demonstrated that overexpression or inappropriate time of expression of the *c-myc* gene promotes apoptosis in myeloid, T, and fibroblast cells (29–31). Using antisense oligonucleotides, Fischer et al. (32) have recently confirmed a role for *c-myc* expression in induction of apoptosis in the WEHI 231 line. Given our evidence of the role of Rel-related factors in the control of transcription of the *c-myc* gene, we sought to determine the effects of anti-sIg treatment on Rel factor expression. Previously, we demonstrated that exponentially growing WEHI 231 cells expressed a number of Rel species, and that binding was significantly reduced following 24 h of anti-Ig treatment (3). Here, we demonstrate that the predominant binding activity in WEHI 231 cells is p50/c-Rel. Anti-sIg receptor treatment results in an early increase of binding, including classical NF- κ B p50/p65, that is followed by a decrease in overall binding and subsequent appearance of p50 homodimers. Transfection analysis indicates these changes in Rel-related factor binding mediates activation and subsequent inhibition of *c-myc* gene transcription in this B cell line.

Materials and Methods

Culture and Treatment Conditions. WEHI 231 cells were maintained in DMEM, supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 0.35% glucose, 0.058% glutamine, nonessential amino acids (Gibco), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M β -mercaptoethanol, as described previously (26). Before antibody treatment, cells were diluted to a density of $3\text{--}4 \times 10^5$ cells/ml with fresh warm media and allowed to incubate for a minimum of 4–5 h. Cell cultures were incubated with 5.2 μ g/ml anti- μ antibody (Cappel Laboratories, Cochranville, PA) for 1–18 h. Where indicated, cells were pretreated before antibody addition for 1 h with 0.01–2 mM pyrrolidinedithiocarbamate (PDTTC) to inhibit NF- κ B activation (33). The 70Z/3 pre-B lymphoma cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 50 μ M 2-mercapto-

ethanol. Cells were induced to differentiate with 50 nM PMA treatment for 1 h.

Transfection Analysis. Exponentially growing WEHI 231 cells were washed once with medium and resuspended in DMEM supplemented with 20% FCS at a concentration of 20×10^6 cells/ml. Cells (250 μ l) were preincubated on ice for 10 min with DNA (up to 40 μ g). Cells were transfected by electroporation at 240 V and 960 μ F, using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA). After incubation on ice for 5 min, the cell suspension was mixed with 1.75 ml of complete medium and incubated for 10 min at room temperature. The suspensions were transferred to petri dishes and incubated at 37°C for 24 h. Cells were harvested, and the resulting extracts were normalized for total protein content by using the Bio-Rad protein quantitation kit. Equal amounts of lysates were incubated in duplicate in 2.5 μ Ci [3 H]-acetyl coenzyme A (New England Nuclear, Boston, MA; 200 mCi/mmol), 50 μ M acetyl coenzyme A, and 1.6 mM chloramphenicol for 1–2 h, and the acetylated forms were extracted with ethyl acetate and assayed by liquid scintillation counting. Standard deviation was obtained using the Student's *t* test. The p1.6 Bgl chloramphenicol acetyl-transferase (CAT) construct contains –1141 to +513 bp of the murine *c-myc* gene, containing promoter/upstream/exon 1 sequences, including both the URE and IRE (8). The double mutant p1.6 Bgl construct (dm p1.6 Bgl), prepared by the double primer site-directed mutagenesis method, contains two G to C transversions in both the URE and IRE elements, which prevents Rel factor binding and transactivation (8, 16). Vectors expressing the p50 and p65 subunits of NF- κ B and inhibitor of NF- κ B (I κ B)- α and murine c-Rel were obtained from U. Siebenlist (National Cancer Institute [NCI], Bethesda, MD), and J. Pierce (Brandeis University, Waltham, MA), respectively (16, 18).

Nuclear Run-on Analysis. Nuclei were isolated from control and antibody-treated WEHI 231 cells, as described previously (27). Radiolabeled RNA was analyzed with single stranded DNA of the BamHI to HindIII fragment encoding exons 2 and 3 of the *c-myc* gene (*myc*) cloned into the M13 vector to detect mRNA synthesis; β -2 microglobulin cDNA, or M13 vector DNA, as described previously (27).

Electrophoretic Mobility Shift Analysis. Crude nuclear extracts were prepared by the Strauss and Varshavsky method (34). The fragment of DNA spanning Bgl II to Acc I, including base pairs –1139 to –921 relative to the P1 promoter of the murine *c-myc* gene, was subcloned into pUC 19. This DNA, termed fragment A previously (3), includes the NF- κ B upstream regulatory element (URE). DNA fragments were endlabeled with the large fragment of *E. coli* DNA polymerase I (New England Biolabs Inc., Beverly, MA) and [α - 32 P]dNTPs (New England Nuclear). The electrophoretic mobility shift assay was performed as follows: each 32 P-labeled fragment or double-stranded oligonucleotide (25,000 cpm; \sim 2 ng) and 5 μ g of nuclear extract were mixed in 70 mM NaCl/10 mM Hepes, pH 7.5/1 mM EDTA/1mM dithiothreitol/0.1% Triton X-100/4% (vol/vol) glycerol/5 μ g of poly(dI-dC)-poly(dI-dC) copolymer, in a final vol of 25 μ l. This mixture was incubated for 30 min at 22°C. Competition with cold URE oligonucleotide demonstrated that the binding was mediated by this NF- κ B element (3). Alternatively, a double stranded oligonucleotide of the κ B site from the kappa light chain enhancer was used (35). For supershift experiments, antibody preparations were added and the mixture incubated as indicated in the appropriate legend. Antibody preparations against p50 and c-Rel homodimers and p65 were purchased from Santa Cruz; antibodies against p50 and c-Rel were kindly supplied by R. Sen (Brandeis University) and N. Rice (NCI), respectively. Purified I κ B- α was generously provided by U. Siebenlist (NCI).

Gels were electrophoresed at 11 V/cm in a 4% or 4.5% polyacrylamide gel with TAE running buffer (6.7 mM Tris-HCl [pH 7.5], 3.3 mM sodium acetate, and 1 mM EDTA), dried, and subjected to autoradiography.

RNA Analysis. Cytoplasmic RNA was isolated and samples (15 μ g) subjected to Northern blot analysis, as described previously (26).

Results

WEHI 231 Cells Express Multiple Rel-related Factors. We first sought to characterize the nature of Rel-related factors binding to *c-myc* NF- κ B elements, using the URE NF- κ B element. Mobility shift analysis was performed with the 221 bp BglII-AccI fragment that spans -1139 to -921 bp relative to the P1 promoter (Fig. 1). The profiles obtained with nuclear extracts from exponentially growing WEHI 231 cells are similar to those seen previously (3). Copper phenanthroline footprinting and competition analysis has shown that formation of the complexes in bands numbered one to four and band six are due to interaction with the URE element (3). When the profile of complexes obtained with nuclear extracts from WEHI 231 cells was compared to that from 70Z/3 pre-B cells induced to differentiate to B cells with LPS treatment for 1 h, a complex that comigrated with band three was detected (3). Since it has recently been shown that this

treatment causes the 70Z/3 line to express predominantly classical NF- κ B (36), we sought to confirm that band three represents binding of classical NF- κ B. The effects of addition of antibodies against the p65 and p50 subunits were tested (Fig. 1, A and B). Addition of a p65 antibody to binding reactions with a WEHI 231 nuclear extract resulted in a selective reduction in formation of band three (Fig. 1 A). For the p50 subunit, an antibody preparation that recognizes p50 subunits in either homodimer or heterodimer complexes was employed (Fig. 1 B). Addition of this antibody clearly prevented formation of bands two, three, and four. Inhibition of formation of band one is seen on a darker exposure (data not shown). These results indicate that band three, which is only a minor complex in WEHI 231 cells, represents binding of classical NF- κ B, a heterodimer of p50 and p65. Furthermore, most of the Rel-related complexes in exponentially growing WEHI 231 that bind to the NF- κ B *c-myc* element contain a p50 subunit.

To determine whether any of the complexes contained c-Rel, an antibody preparation that reacts with c-Rel was used. Addition of this antibody clearly prevented formation of band two, the major complex observed with nuclear extracts of WEHI 231 cells (Fig. 1 C). Thus, band two appears to represent a heterodimer of p50/c-Rel. This finding is consistent with the faster mobility of complexes formed with the URE

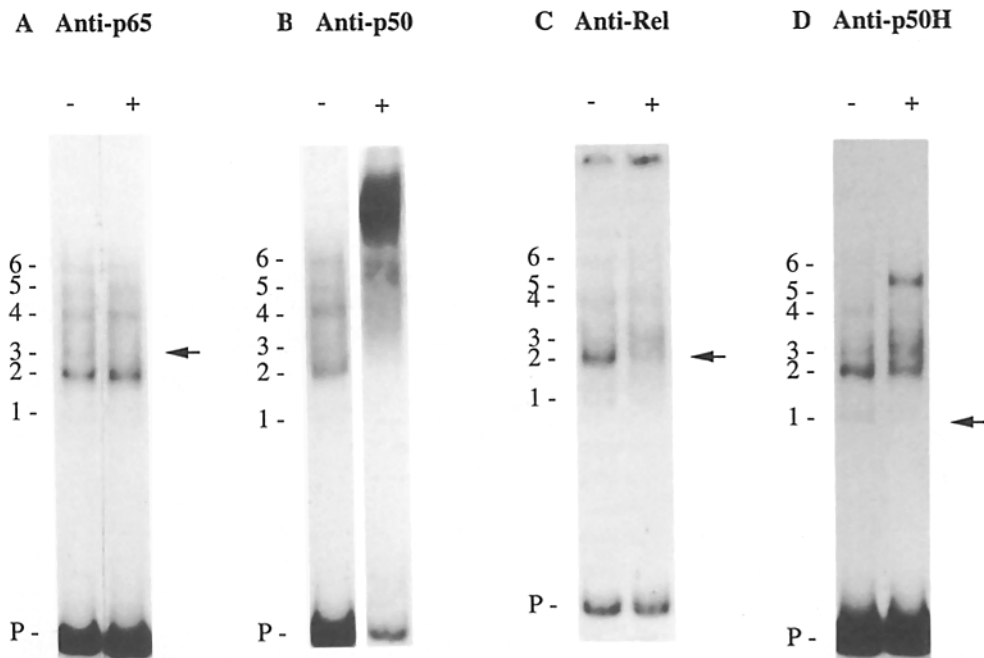


Figure 1. WEHI 231 cells express multiple Rel-related factors. Crude nuclear extracts were prepared by the method of Strauss and Varshavsky (34) from WEHI 231 cells or from 70Z/3 cells treated with 12-O-tetradecanoyl phorbol-13-acetate for 1 h, as described in Materials and Methods. The 221 bp BglII to AccI DNA fragment, which spans -1139 to -921 bp and includes the URE NF- κ B upstream regulatory element, was end-labeled and used in binding reactions. Complexes were resolved by electrophoresis at 11 V/cm in a 4% polyacrylamide gel with TAE running buffer. Position of the resolved bands that interact with the URE element have been numbered 1-6, consistent with the previous nomenclature (3). (A) Effects of an anti-p65 antibody on complex formation. WEHI 231 nuclear extract was incubated for 30 min at room temperature and then 1 h at 4°C in the presence of 0.5 μ g anti-p65 antibody, and the

resulting complexes were resolved as above. (-) Absence of antibody; (+) presence of antibody. (B) Effects of an anti-p50 antibody preparation. Extracts from exponentially growing WEHI 231 cells were similarly incubated with the normal rabbit serum pre-bleed (-), or with an antibody that recognizes p50 heterodimers (+). (C) Effects of anti-c-Rel antibody preparations. Extracts from WEHI 231 cells were incubated for 30 min at room temperature and then 16 h at 4°C in the presence of an antibody that recognizes c-Rel protein. (-) Absence of antibody; (+) presence of antibody. (D) Effects of an antibody that preferentially recognizes p50 homodimers (p50H). Nuclear extracts from WEHI 231 cells were incubated for 30 min at room temperature and then 1 h at 4°C with 1 μ g antibody preparation against p50 homodimers. The dark band that appears in the region between complexes five and six appears due to nonspecific binding of a factor in the antibody preparation to the DNA probe, since it was observed in the absence of added extract. (-) Absence of antibody; (+) presence of antibody.

oligonucleotide and extracts from COS cells expressing p50/c-Rel compared with those expressing p50/p65 (16).

Since p50 appears involved in mediating much of the binding to the *c-myc* elements, an antibody (Santa Cruz) was used that has been found to recognize p50 homodimers more effectively than p50 in heterodimer complexes (Fig. 1 D). Addition of this antibody to a binding reaction preferentially prevented formation of band one. Antibodies against RelB and p52 had little consistent effect on complex mobility (data not shown). Thus, the nature of the p50 binding partner of band four remains to be determined. In summary, bands one, two, and three represent p50 homodimers, and p50/c-Rel and p50/p65 heterodimers, respectively.

The *c-myc* Promoter in Exponentially Growing WEHI 231 Cells Is Transactivated by Rel-related Factor Expression. Since we have demonstrated that Rel-related factors can effectively transactivate the *c-myc* promoter (7, 8, 16), we tested whether their expression in WEHI 231 cells mediated transactivation of *c-myc* promoter transcription. Transfection analysis was performed with murine *c-myc* promoter/upstream/exon1-CAT p1.6 Bgl constructs that contained either wild-type or mutant versions of the URE and IRE NF- κ B elements (8). After electroporation into exponentially growing WEHI 231 cells, the activity of wild type p1.6 Bgl was found to be ~threefold higher than that of the double mutant (dm p1.6 Bgl), suggesting that part of the promoter activity was due to binding of Rel-related factors (Fig. 2). Since I κ B- α has been found to bind to both the p65 and c-Rel subunits and prevent transactivation, a cotransfection experiment was performed using an I κ B- α expression vector. Cotransfection of a vector expressing I κ B- α reduced the activity of wild-type p1.6 Bgl,

in a dose-dependent fashion, to that of the double mutant p1.6 Bgl (Fig. 2). As expected, the activity of dm p1.6 Bgl was essentially unaffected by expression of I κ B- α . These results indicate that Rel-factor binding plays a significant role in transcription of the *c-myc* promoter in exponentially growing WEHI 231 cells.

Anti-sIg Treatment Induces Rel-related Factor Binding. We next sought to determine the effects of anti-sIg treatment on binding of Rel-related factors and to correlate any changes with the effects of this treatment on the rate of *c-myc* gene transcription. Nuclei were isolated from WEHI 231 cells in exponential growth and, after 1, 2, 4, 8, 12, and 18 h of treatment with anti-sIg, used in run-on and mobility shift analyses (Fig. 3). Transcription of *c-myc* RNA increased dramatically by 1 h after anti-sIg treatment, remained elevated at 2 h, and decreased to levels somewhat below those seen in exponentially growing cells within 4 h (Fig. 3 A). Between 4 and 8 h, the rate of transcription dropped well below control values and remained depressed throughout the remainder of the time course.

Treatment with anti-sIg for 1 h caused a very significant increase in binding to the 221 bp fragment. In particular, a transient increase in intensity of formation of complexes in the regions between bands two and five was noted (Fig. 3 B). Specifically, an increase was noted in bands two and three; these are better seen in the duplicate experiment shown in Fig. 3 C. By 4 h, the levels of the all of the complexes, except for band two, were somewhat below those seen in extracts from control cells. Between 8 and 12 h of treatment, an increase in formation of band one was seen. To estimate the extent of the increase in binding after 1 h of anti-sIg treatment, mobility shift analysis was performed with an oligonucleotide containing a single NF- κ B element, under very low resolving conditions, to yield a single band; an approximate twofold increase in binding was observed (data not shown). Thus, an increase in overall binding of Rel-related factors parallels the early increase in the rate of *c-myc* gene transcription after anti-sIg treatment, while the decrease in the rate of transcription is paralleled by an initial drop in overall binding and a later increase in appearance of p50 homodimers.

Expression of p65 and c-Rel Activates the *c-myc* Promoter Transcription. To test whether activation of Rel-related factors upon anti-sIg treatment can significantly enhance transcription of the *c-myc* promoter, cotransfection analysis was performed using the p1.6 Bgl constructs and vectors expressing either the p65 or murine c-Rel subunits. Increasing amounts of p65 expression vector stimulated activity of wild-type p1.6 Bgl up to sixfold, while higher doses displayed somewhat reduced activation (Fig. 4), as seen previously in NIH 3T3 cells (16). The mutant p1.6 Bgl was unaffected by p65 expression, indicating that the activation is due to binding to the NF- κ B elements, as expected. Expression of murine c-Rel was able to modestly induce activity of the *c-myc* promoter; an increase of 1.8-fold was noted with wild-type p1.6 Bgl (Fig. 5). These results are similar to those seen upon cotransfection into NIH 3T3 cells, which express only minimal constitutive binding levels of Rel-related factors. Thus activation of Rel-related factors upon anti-sIg treatment can significantly

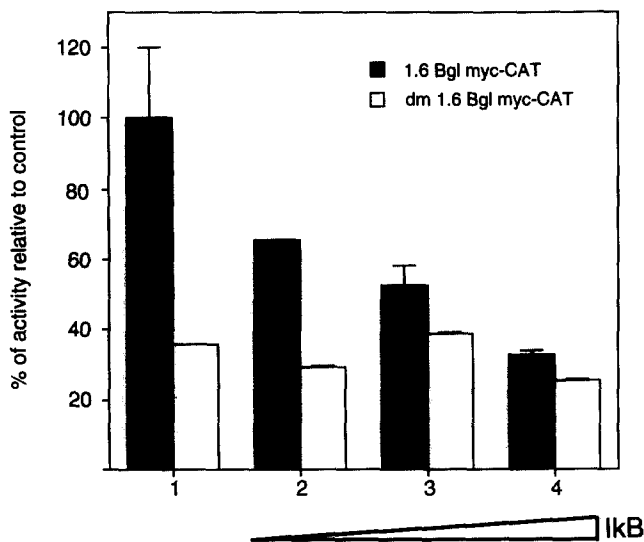


Figure 2. Mutation of the NF- κ B sites and expression of I κ B- α inhibits *c-myc* promoter activity. Exponentially growing WEHI 231 cells were electroporated in duplicate with 30 μ g p1.6 Bgl (filled bars), or with dm p1.6 Bgl (open bars), in the absence (bars 1) or presence of 1, 5, 10 μ g of an I κ B- α expression vector (bars 2-4, respectively), and enough pUC19 DNA to make up a final total amount of 40 μ g DNA. Extracts were assayed for CAT activity and the data presented relative to the p1.6 Bgl alone.

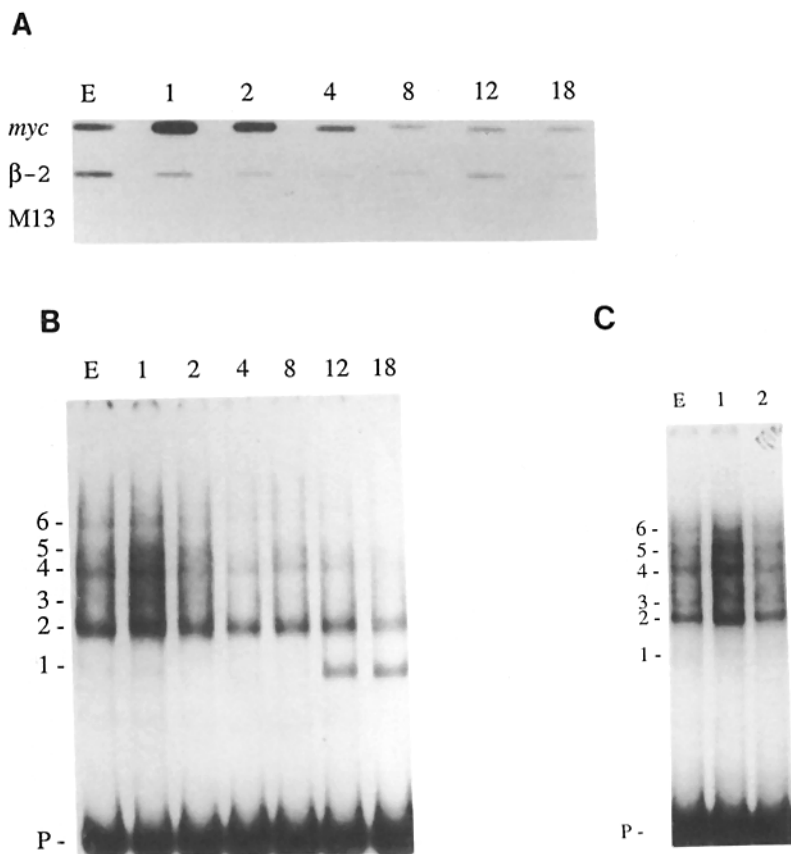


Figure 3. Anti-Ig treatment of WEHI 231 cells transiently activates *c-myc* gene transcription and Rel-related binding. (A) Nuclear run-on analysis. Nuclei were isolated from WEHI 231 cells at the indicated time points and the radiolabeled RNA products analyzed with single-stranded DNA of the BamHI to HindIII fragment encoding exons two and three of the *c-myc* gene (*myc*) cloned into the M13 vector to detect mRNA synthesis; β -2 microglobulin cDNA and M13 vector DNA. A slight overloading of the label was apparent at the 12 h time point. (B) Mobility shift analysis. Extracts were prepared from WEHI 231 cell nuclei, isolated above in A, and used in mobility shift analysis as described in the legend to Fig. 1. (C) Mobility shift analysis of the early effects of anti-Ig treatment. Nuclear extracts were prepared from WEHI 231 cells isolated at the indicated time points and equal amounts of protein used in mobility shift analysis, as described above.

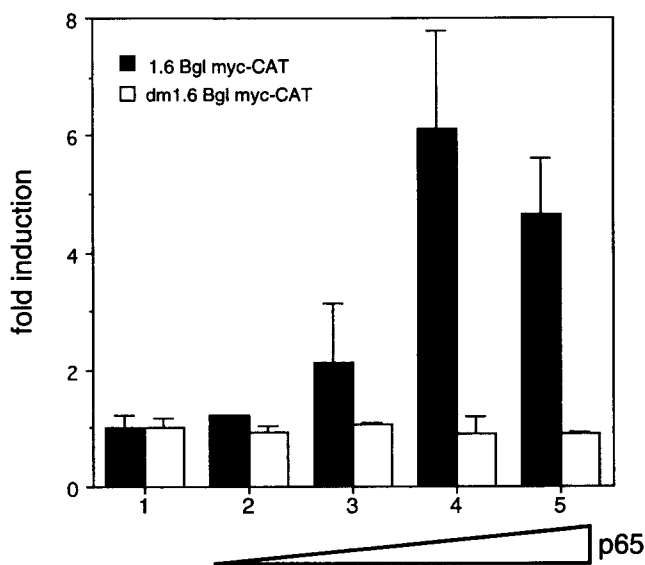


Figure 4. Expression of p65 potently enhances *c-myc* promoter activity. Exponentially growing WEHI 231 cells were electroporated in duplicate with 10 μ g p1.6 Bgl (filled bars) or dm p1.6 Bgl (open bars) in the absence (bars 1) or presence of increasing levels of a p65 expression vector (bars 2-5 contained 1, 5, 10, and 20 μ g, respectively) and enough pUC19 DNA to make a final amount of 40 μ g DNA. Extracts were assayed for CAT activity, as above in Fig. 3. The data are presented relative to the value obtained in the absence of p65 expression vector for both the wild-type and dm p1.6 Bgl constructs.

enhance transcription of the *c-myc* promoter in WEHI 231 cells.

Expression of p50 Represses c-myc Promoter Transcription in WEHI 231 Cells. Expression of the p50 subunit of NF- κ B, which can bind effectively as a homodimer to many NF- κ B elements, including the URE (16), has been found to transactivate in vivo only rarely (16). To determine whether expression of p50 is able to affect the activity of the *c-myc* promoter in WEHI 231 cells, a cotransfection experiment was performed (Fig. 6). Expression of p50 caused a dose-dependent decrease in activity of the p1.6 Bgl, in contrast to the results we obtained in NIH 3T3 cells where it had little effect on the activity of p1.6 Bgl (16). This result suggests that overexpression of p50 in WEHI 231 cells, which can compete for binding of more active Rel-related factors, decreases the activity of the *c-myc* promoter. This suggests that the appearance of p50 homodimers between 8 and 12 h after anti-Ig treatment can negatively affect *c-myc* expression.

Inhibition of Rel-related Factor Activation Prevents Induction of c-myc RNA Levels. Schreck, Baeuerle, and coworkers (33) have shown that the antioxidant PDTC selectively inhibits activation of Rel-related factors. To measure the effects of PDTC addition, WEHI 231 cells were incubated for 1 h in media containing increasing concentrations of PDTC, ranging from 0.01-1.0 mM, and then treated for 1 h with anti-Ig. Mobility shift analysis indicated that pretreatment with PDTC prevented Rel-related factor induction in a dose-dependent

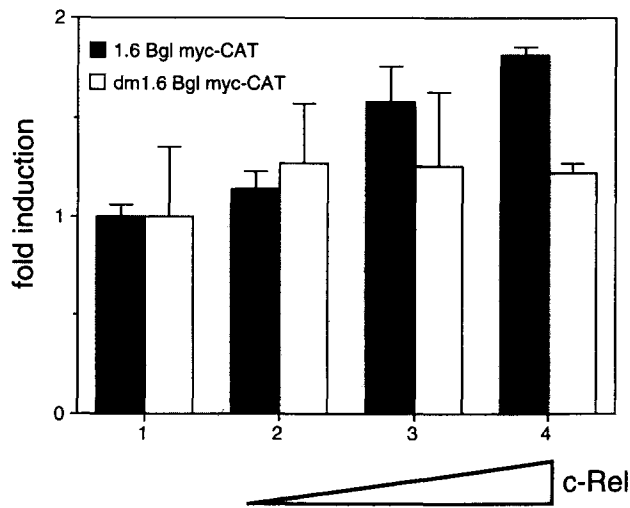


Figure 5. Expression of c-Rel enhances c-myc promoter activity. Exponentially growing WEHI 231 cells were electroporated in duplicate with 10 μg p1.6 Bgl (filled bars) or dm p1.6 Bgl (open bars) in the absence (bars 1) or presence of 5, 10, and 20 μg (bars 2–4, respectively) of a murine c-Rel expression vector. The data are presented as above in Fig. 4.

fashion (data not shown) similar to reports by Schreck et al. (33). Therefore, a concentration of 0.2 mM was chosen. To test the effects of PDTC on induction of c-myc mRNA levels after anti-Ig treatment, cytoplasmic RNA was isolated from WEHI 231 cells in exponential growth, or 1 h after anti-Ig treatment, in the absence or presence of preincubation with 0.2 mM PDTC for 1 h (anti-Ig: – or 1+1h PDTC, respectively). As seen in the resulting Northern blot (Fig. 7), anti-Ig treatment of WEHI 231 caused a significant increase in c-myc mRNA levels, as we have reported previously (26, 27).

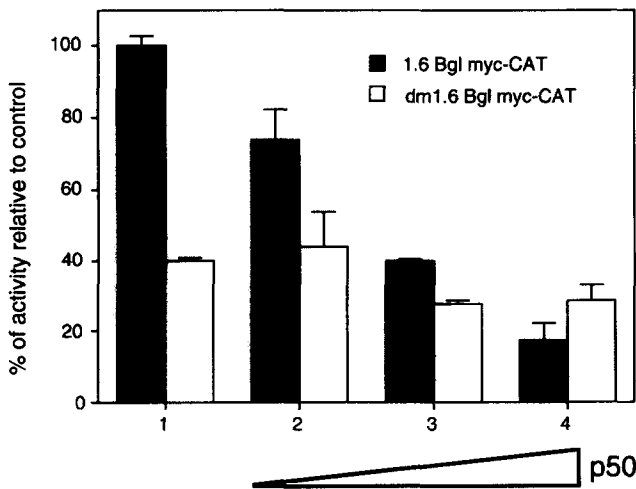


Figure 6. Expression of p50 inhibits c-myc promoter activity. Exponentially growing WEHI 231 cells were electroporated in duplicate with 30 μg p1.6 Bgl (filled bars) or dm p1.6 Bgl (open bars) in the absence (bars 1) or presence of 1, 5, and 10 μg (bars 2–4, respectively) of a p50 expression vector, and analyzed as above in Fig. 2.

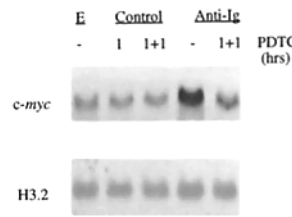


Figure 7. The antioxidant PDTC blocks activation of Rel-binding and induction of c-myc mRNA levels after anti-Ig treatment. Cytoplasmic RNA was isolated from cells in exponential growth (E) or cells pre-treated with 0.2 mM PDTC for 1 h and then incubated in the presence of anti-Ig for an additional 1 h (anti-Ig, 1+1h) or left in the absence of additional treatment (control, 1+1h). Samples (15 μg) were subjected to Northern blot analysis, using the murine c-myc cDNA clone pM-c-myc54 (44) or histone H3.2 probe (37).

Preincubation with PDTC inhibited the transient induction of c-myc mRNA levels. To confirm the specificity of the effects on c-myc expression and to evaluate the early effects of PDTC on cell growth, the Northern blot was probed for histone H3.2 mRNA, an S-phased expressed RNA (37). No changes in expression of this mRNA resulted from the PDTC treatment. Furthermore, there were no significant differences in the major proteins produced when these cytoplasmic RNA preparations were used to prime a rabbit reticulocyte cell-free system (data not shown). Thus PDTC, a selective inhibitor of Rel-related factor activation, blocks the increase in c-myc gene expression in WEHI 231 cells induced by anti-sIg treatment, which is consistent with the transfection analysis.

Mutation or IκB Expression Prevents Activation of the c-myc Promoter after Anti-sIg Treatment. As an additional test of the role of Rel-factor activation in the induction of c-myc gene transcription, we tested the effects of mutation and of cotransfection of the IκB-α vector on the ability of anti-sIg treatment to activate p1.6 Bgl. WEHI 231 cells were analyzed 6 h after anti-sIg treatment to allow for protein translation of induced CAT RNA (Fig. 8). As seen above, the activity of the wild-type p1.6 Bgl was higher than that of dm p1.6 Bgl. A 2.5-fold induction was seen with the wild-type p1.6 Bgl construct. In contrast, anti-sIg treatment failed to induce the double mutant. Furthermore, coexpression of IκB-α blocked the activation of p1.6 Bgl mediated by anti-sIg, but had essentially no effect on dm p1.6 Bgl. These results indicate that the induction of Rel-related factors after anti-sIg treatment plays a role in the activation of c-myc promoter transcription in WEHI 231 cells.

Discussion

Here we have demonstrated that the murine B cell lymphoma line WEHI 231 (IgM, kappa) expresses multiple nuclear Rel-related complexes that bind to the NF-κB elements and regulate transactivation of the c-myc gene. Furthermore, the changes in expression of this family of factors after anti-sIg treatment plays a role in the transient increase and subsequent decrease in c-myc gene transcription, which precedes apoptosis of these cells. In exponentially growing WEHI 231 cells, the predominant nuclear complex binding to the c-myc URE NF-κB element was p50/c-Rel heterodimer, and the

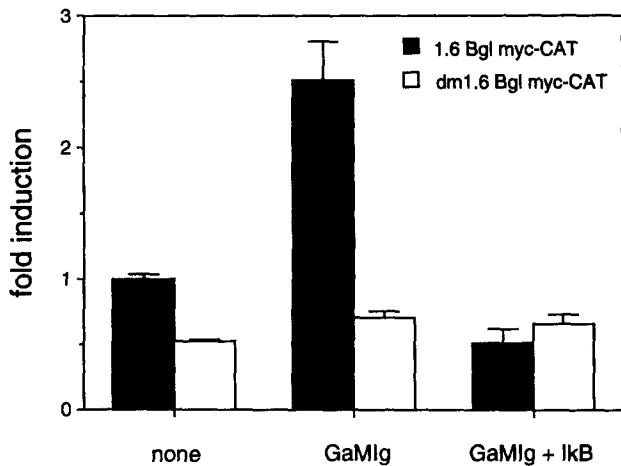


Figure 8. Mutation of the NF- κ B elements or expression of I κ B- α inhibits the anti-sIg-mediated induction of *c-myc* promoter transcription. WEHI 231 cells were electroporated in duplicate with 10 μ g p1.6 Bgl (filled bar) or dm p1.6 Bgl (open bar) in the absence (bars 1 and 2) or presence of 10 μ g I κ B- α expression vector (bars 3) in the presence of enough pUC19 DNA to make a final amount of 40 μ g DNA. After 20 min at room temperature, cells in bars 2 and 3 were treated with anti- μ H chain, and extracts were prepared after 6 h and assayed as above in Fig. 2.

level of classical NF- κ B binding was very low. Comigrating complexes of similar relative intensities were seen with the DNA fragment containing the second *c-myc* NF- κ B element, the IRE (data not shown; reference 4). The binding of these Rel-related factors appeared to functionally transactivate *c-myc* expression, since expression of the inhibitor protein I κ B- α reduced the activity of a transfected *c-myc* promoter significantly, as did site-directed mutations in the two NF- κ B elements. Treatment of WEHI 231 cells with anti-sIg resulted in a transient increase in binding of c-Rel factors that was followed by a decline in binding and a subsequent appearance of p50 homodimers. Cotransfection analysis indicated that the early activation of p65 and c-Rel-containing complexes would result in a transient peak in *c-myc* expression, while induction of p50 would inhibit transcription of this gene. This was further indicated by pretreatment of WEHI 231 cells with the antioxidant PDTC, which prevented induction of Rel-factor binding and blocked the increase in *c-myc* mRNA levels that normally follow anti-sIg treatment, and by the ability of mutations in the URE and IRE sites and of expression of I κ B- α to prevent the anti-sIg-mediated activation of *c-myc* promoter transcription. Taken together, these findings indicate that Rel family expression contributes to the constitutive level of *c-myc* gene expression in WEHI 231 B cells, as well as to the changes in expression of this gene induced by anti-sIg treatment of this line.

Since WEHI 231 cells express the κ light chain gene, we were surprised to determine that the predominant species was p50/c-Rel. Similar results have recently been reported by Verma, Baltimore, Rice and their co-workers (36, 38, 39). These workers have presented evidence that Rel-family expression proceeds in two stages during pre-B cell B cell development. They have shown that in pre-B cells induced to

differentiation to B cells (e.g., 70Z/3 line), activation of p50/p65 occurs (36, 38). Whereas in early B cells, p50/c-Rel was predominant. Thus, a switch that is presumably developmentally regulated has apparently occurred. Thus, our finding that p50/c-Rel is the predominant complex binding to the *c-myc* URE in WEHI 231 is consistent with these other reports. Furthermore, binding of p50/p65 to the URE in exponentially growing WEHI 231 cells was very minimal. Our ability to detect this complex probably reflects the innate ability of the URE sequence to bind classical NF- κ B, as shown by Rosen and co-workers (40). Anti-sIg treatment resulted in a significant induction of Rel-factor binding that activates transcription of the *c-myc* gene. While induction of *c-myc* does not lead to proliferation of the WEHI 231 cells, a similar induction of Rel binding has been reported previously in normal splenic B lymphocytes after treatment with anti-Ig (35). In this case, the B cells are induced to proliferate. The work presented here suggests that anti-Ig receptor-mediated activation of Rel-factor binding may play a role in induction of proliferation of normal B lymphocytes.

The changes in binding of Rel-related factors also appears to play a role in the decrease in the rate of *c-myc* gene transcription. To date, the Rel-family has not been associated with direct negative effects on transcription in mammalian cells. The initial drop in *c-myc* gene transcription can be related in part to a loss of binding. Between 8 and 12 h, a significant increase in binding of p50 homodimers (*band* 1) was noted. This increase could be due to a selective decline in expression of the other subunits (e.g., c-Rel and p65) or a decrease in expression of the inhibitor protein Bcl-3, which has been found to selectively complex with p50 subunits and prevents their binding to DNA (41). We have found that homodimers of p50 fail to transactivate the *c-myc* promoter in NIH 3T3 cells, which express only low levels of Rel-related factors (16). Thus, in WEHI 231 cells, the observed inhibition by overexpression of p50 is most likely due to competition for binding of active factors, such as classical NF- κ B with p50, which lacks a transactivation domain.

The *c-myc* gene has been implicated in programmed cell death. Overexpression of *c-myc* accelerates apoptosis after IL-3 deprivation of the 32D IL-3-dependent myeloid cell line and upon growth arrest, either by serum deprivation, isoleucine deprivation, or thymidine block of 3T3 fibroblasts (29, 31). Addition of an antisense *c-myc* oligonucleotide inhibits receptor-mediated apoptosis in immature T cells and some T cell hybridomas (30, 42). More recently, the anti-sIg-induced apoptosis of WEHI 231 cells has been shown to be similarly blocked by addition of an antisense *c-myc* oligonucleotide, although the disappearance of the *c-myc* protein was not apparent (reference 32, our unpublished observations). Our results suggest a possible role for NF- κ B-like factor expression in the receptor-mediated activation of the pathway leading to programmed cell death in WEHI 231 B cells. Interestingly, high levels of c-Rel expression have been associated with programmed cell death in the developing avian embryo and in bone marrow cells in vitro (43). Experiments are in progress to test the role of Rel-family expression in induction of apoptosis in B cell models of tolerance.

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