Lymphoma Models for B Cell Activation and Tolerance. X. Anti-μ-mediated Growth Arrest and Apoptosis of Murine B Cell Lymphomas Is Prevented by the Stabilization of myc

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Summary

Treatment of the WEHI-2131 or CH31 B cell lymphomas with anti-μ or transforming growth factor (TGF)-β leads to growth inhibition and subsequent cell death via apoptosis. Since anti-μ stimulates a transient increase in c-*myc* and c-*fos* transcription in these lymphomas, we examined the role of these proteins in growth regulation using antisense oligonucleotides. Herein, we demonstrate that antisense oligonucleotides for c-*myc* prevent both anti-μ- and TGF-β-mediated growth inhibition in the CH31 and WEHI-231 B cell lymphomas, whereas antisense c-*fos* has no effect. Furthermore, antisense c-*myc* promotes the appearance of phosphorylated retinoblastoma protein in the presence of anti-μ and prevents the progression to apoptosis as measured by propidium iodide staining. Northern and Western analyses show that c-*myc* message and the levels of multiple myc proteins were maintained in the presence of antisense c-*myc*, results indicating that myc species are critical for the continuation of proliferation and the prevention of apoptosis. These data implicate c-*myc* in the negative signaling pathway of both TGF-β and anti-μ.

Cross-linking of membrane IgM receptors on a subset of murine B cell lymphomas, or addition of TGF-β to the same cells, can lead to increased transcription of the early response genes, cell cycle arrest in late G_1, and eventual apoptosis (1–5). For example, anti-μ treatment of these cells causes c-*myc* messenger RNA to increase within the first hour, but message levels for this oncogene then decrease to below baseline levels at 4–8 h and completely disappear by 24 h in unsynchronized cells (4). Membrane IgM cross-linking on the WEHI-231 cell line also has been reported to cause a transient increase in c-*fos* transcription (5). In these inhibited cell lines, the *retinoblastoma* gene product (pRB)[1] is found in the hypophosphorylated, active form within 12 h in unsynchronized cells (6, 7, and Joseph, L., and D. W. Scott, manuscript submitted for publication). Despite extensive studies, the role(s) of these genes in regulating cell cycle progression and apoptosis in murine B lymphoma lines remains unresolved. Our work starts to elucidate the link between early response genes (c-*myc*, c-*fos*) and later responses (pRB phosphorylation) in these B cell lymphomas.

pRB, an anti-oncogene that regulates cell cycle progression, is differentially phosphorylated throughout the cell cycle (8–10). Indeed, the state of phosphorylation of pRB has been linked to anti-μ- and TGF-β-induced cell cycle arrest (6, 8–10). Thus, pRB becomes phosphorylated during G_1 and remains in that state until it is dephosphorylated at G_2/M; the hypophosphorylated form of the pRB protein is associated with the growth arrested phenotype of WEHI-231 B lymphoma cells (6).

The involvement of the c-*myc* gene in cell growth regulation has been well documented in several systems. For example, myc has also been implicated in cell cycle progression; thus, antisense oligonucleotides to c-*myc* have been shown to block cell cycle progression into S phase, but not egress from G_0 to G_1 in human T cells (11). A role of c-*myc* has been postulated in the development of Burkitt's lymphoma since translocation of c-*myc* places this gene under control of the immunoglobulin enhancer. Upregulated and dysregulated expression of c-*myc* from this strong enhancer is thought to be a prime factor in oncogenesis and uncontrolled growth of these tumors (12). Similarly, dysregulated expression of the c-*myc* from the immunoglobulin μ or κ enhancer results in fatal lymphoma in myc transgenic mice (13). Insertion of viral sequences, generally long terminal repeat sequences, 5′

G. Fischer and S. C. Kent contributed equally to this manuscript.

[1]*Abbreviation used in this paper*: pRB, retinoblastoma protein.

of c-*myc* has been implicated in greatly upregulating and dysregulating expression of c-*myc* and resulting in leukemia or lymphoma (14, 15). Thus, c-*myc* expression and regulation are crucial elements in cell cycle progression, oncogenesis, and proliferation control.

Recent data by Evan et al. (16) and Bissonette et al. (17) suggest that overexpression of myc protein(s) at critical cell cycle barriers could lead to apoptosis and cell death. In addition to results with mitogen-activated T cells (11), antisense oligonucleotides against the c-*myc* gene have been used to block cell cycle progression or apoptosis in several model systems, including T cell hybridomas (18), human breast cancer (19), smooth muscle cells (20), and keratinocytes (8). In all of these cells, antisense treatment was shown to lead to a loss of myc protein and to cause either cell cycle arrest or the prevention of apoptosis.

Based on the hypothesis that increased myc expression might target B lymphoma cells for apoptosis, we utilized antisense oligonucleotides for c-*myc* in order to block the increase in myc induced by anti-μ. In the present studies, we demonstrate that antisense c-*myc* oligonucleotides, but not antisense for c-*fos*, protected against anti-μ– (or TGF-β–) induced apoptosis in B lymphoma cells. However, antisense c-*myc* surprisingly acted by protecting against the loss of certain species of myc protein and not by decreasing the expressed levels of the oncogene product. Our results implicate a critical role for the stabilization of myc protein in modulating growth arrest by both of these reagents, and allowing continued cell cycle progression.

Materials and Methods

Cells and Antibodies. CH31 and WEHI-231 are both sIgM$^+$ murine B cell lymphomas that have been extensively characterized (1, 3–5). They were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg, MD), supplemented with 5% FBS (Hyclone Laboratories, Logan, UT), 2-ME, L-glutamine, penicillin, streptomycin, MEM nonessential amino acids, and sodium pyruvate as previously described (GIBCO BRL) (1, 21). Rabbit polyclonal anti-IgM (anti-μ) was affinity purified on an IgM, λ (MOPC104E) column by standard methods and used at 0.1–10 μg/ml for growth inhibition. TGF-β was obtained from R & D Systems, Inc. (Minneapolis, MN) and used at 1–10 ng/ml.

Antisense Oligonucleotides. Phosphorothioate oligonucleotides were designed against the translational start sites of the respective genes. For c-*myc*, the oligonucleotide was designed against the translational start site in exon 2. The nonsense sequence was derived by randomizing the antisense sequence. These oligonucleotides were purchased from the Regional DNA Synthesis Lab at the University of Calgary (Calgary, Alberta, Canada). The sequences are as follows:

murine antisense c-*myc* 5′ GAAGTTCACGTTGAGGGGCAT 3′

murine nonsense c-*myc* 5′ ATCTGGTGAGGGCAAGCTATG 3′

murine antisense c-*fos* 5′ GTTGAAACCCGAGAACATCAT 3′

Centrifugal Elutriation. The method of centrifugal elutriation has been reported previously (21). Briefly, 5 × 10^8 exponentially growing CH31 cells were loaded into the separation chamber at rotor speed of 3,250 rpm and a flow rate of 30 ml/min. After loading the samples the rotor speed was decreased in increments to 2,770 rpm, with two 40-ml fractions collected at each increment. The cell number and size distribution were measured from each fraction with a Channelyzer (Coulter Corp., Hialeah, FL) system to verify the purity and size of each fraction. The cell cycle stage of each fraction was verified by flow cytometry analysis of propidium iodide stained cells to stain for DNA content.

[^{3}H]Thymidine Incorporation Assay and Data Presentation. 100 μl of lymphoma cells (2 × 10^5/ml) were placed in 96-well plates and incubated 24 or 48 h with varying concentrations of anti-μ or TGF-β and antisense DNA. 1 μCi of [^{3}H]thymidine (Amersham Life Sciences, Arlington Heights, IL) was added to each well and the cells were harvested 4–6 h later on a 96-well plate cell harvester; thymidine incorporation was measured on a 96 direct beta counter (both from Packard Instrument Co., Meriden, CT). All data are presented as percent control thymidine incorporation, using wells containing no anti-μ as the control for each oligonucleotide treatment group. Addition of antisense or nonsense oligonucleotides did not significantly change the levels of thymidine incorporation in these controls by >10–20%. Typically, thymidine incorporation in control wells was >10^5 per sample and errors were <10% (and not shown).

Propidium Iodide Staining for Apoptotic Nuclei. Cells were resuspended in 1 ml 100% ethanol, placed at 4°C overnight, washed, and resuspended in 1 ml PBS containing 10 μg/ml RNase; cells were then incubated at 37°C for 0.5 h, after which propidium iodide (50 μg/ml; both RNase and propidium iodide were from Sigma Chemical Co., St. Louis, MO) was added. 10 μl of each cell suspension was then placed on slides and apoptotic bodies were visualized by fluorescence microscopy and recorded for the presence or absence of fragmented nuclei (22).

Northern Analysis. Total cellular RNA was extracted from exponentially growing WEHI-231 or CH31 cells by the method of Huang and High (23). Cells were lysed in 2% SDS, 200 mM Tris-HCl, pH 7.5, and 1 mM EDTA, on ice for 20 min. Protein and genomic DNA were precipitated with potassium acetate (4.4 M with 2 M acetic acid) and pelleted by centrifugation. The supernatant was extracted twice with chloroform/isoamyl alcohol (24:1). RNA was precipitated with cold isopropanol, centrifuged and the pellet then washed with cold absolute ethanol. RNA was resuspended in diethylpyrocarbonate (DEPC)-treated (Aldrich Chemical Co., Milwaukee, WI) water with 40 U RNAsin (Promega, Madison, WI) and stored at −70°C until use.

10 μg of total RNA was loaded onto formaldehyde gels with 1.26 μM ethidium bromide (Sigma Chemical Co.) and run at 300 V/h. Equal loading of lanes was confirmed by comparison of 28S and 18S ribosomal RNA bands. RNA was transferred to nylon membranes (Hybond N$^+$; Amersham Life Sciences) by capillary blot. After fixing and prehybridization, membranes were probed with a ^{32}P-labeled 1-kb fragment from rat c-*myc* exon 3, which cross-hybridizes with murine c-*myc*. After washing, membranes were exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for 7 d. Blots were stripped and reprobed with a labeled 1.5-kb fragment of murine β-*actin*. Membranes probed with β-*actin* were exposed to film for 1 d. Autoradiographs of c-*myc* and β-*actin* message levels were quantitated by scanning laser densitometry (Ultroscan XL; LKB Bromma, Gaithersburg, MD).

Western Blotting for the pRB protein. Cells were prepared and lysed as described previously (6). Briefly, lymphoma cells were lysed with SDS stop buffer containing 2-ME, and the extract was boiled, electrophoresed, and transferred to nitrocellulose. Western blots

were probed using the anti-human pRB monoclonal, Mh-rb-02 (PharMingen, San Diego, CA), which detects pRB in WEHI-231 cells (6), followed by goat anti-mouse IgG coupled to alkaline phosphatase (Fisher Scientific Co., Pittsburgh, PA). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; both from Sigma Chemical Co.) were used for visualization of the bands.

Western Blotting for the myc Protein. 10^6 cells were treated as indicated, washed twice in cold PBS, and lysed in RIPA buffer (50 mM Tris, pH 8.0, 0.1% SDS, 0.5% deoxycholate, 1% NP40, 150 mM NaCl) in the presence of 10 μg/ml each of leupeptin, aprotinin, and AEBSF (Calbiochem, La Jolla, CA). After boiling, the cell lysates were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were blocked with 10% goat serum (Sigma Chemical Co.) and 1% Nonfat dry milk in Tris-buffered saline with 0.5% Tween-20 at room temperature for 2 h. Protein was visualized by incubation with rabbit anti-mouse myc (Upstate Biotechnology, Inc., Lake Placid, NY) at 1 μg/ml for 2 h at room temperature. After washing, blots were incubated with goat anti-rabbit IgG (1 μg/ml; Fisher Scientific Co.) conjugated to alkaline phosphatase. NBT and BCIP were used for visualization of the bands.

Results

Antisense c-myc Oligonucleotides Prevent Cell Cycle Arrest by Anti-μ. Treatment of murine B cell lymphomas with anti-μ leads to an increase in c-*myc* transcription within 30 min (4). We hypothesized that expression of myc protein could lead to growth arrest and apoptosis, as described recently in two models, including activation-induced apoptosis (16, 18). Initially, we cultured WEHI-231 and CH31 cells with phosphorothioate-modified c-*myc* antisense oligonucleotides to determine the role of myc protein in the continued growth of these cell lines. Surprisingly, both cell lines divided normally in the presence of up to 10 μM of antisense c-*myc* oligonucleotides (Fischer, G., and D. W. Scott, data not shown), thus allowing us to test the effects of these oligonucleotides on external signals and cell cycle progression.

We next treated the WEHI-231 and CH31 lymphomas with increasing amounts of anti-μ and simultaneously added oligonucleotides for c-*myc* antisense sequences or added nonsense oligonucleotides of the same base composition as a control. The data in Fig. 1 demonstrate that antisense oligonucleotides for c-*myc* virtually eliminated the growth inhibition by anti-μ of CH31 (Fig. 1 *A*) or WEHI-231 (Fig. 1 *B*) lymphomas, as measured by thymidine incorporation; nonsense oligonucleotides had a minimal effect, which was observed with all *S*-oligonucleotides. Growth inhibition was significantly prevented by 0.5 μM antisense c-*myc*, and the effects of anti-μ were completely reversed at 1 μM antisense c-*myc*.

c-fos Antisense Oligonucleotides Do Not Reverse the Effects of Anti-μ. It has previously been reported that anti-μ not only induces an early increase in c-*myc* transcription, but also elicits an transient rise in c-*fos* message levels in WEHI-231 cells (5). To examine the specificity of the antisense treatment for c-*myc*, we next tested whether antisense for c-*fos* had any effect on growth inhibition by anti-μ. The data in Fig. 2 demonstrate that antisense for c-*fos* did not modulate cell cycle arrest, whereas antisense c-*myc* reproducibly prevented anti-μ inhibition of growth. The minimal effects of unrelated con-

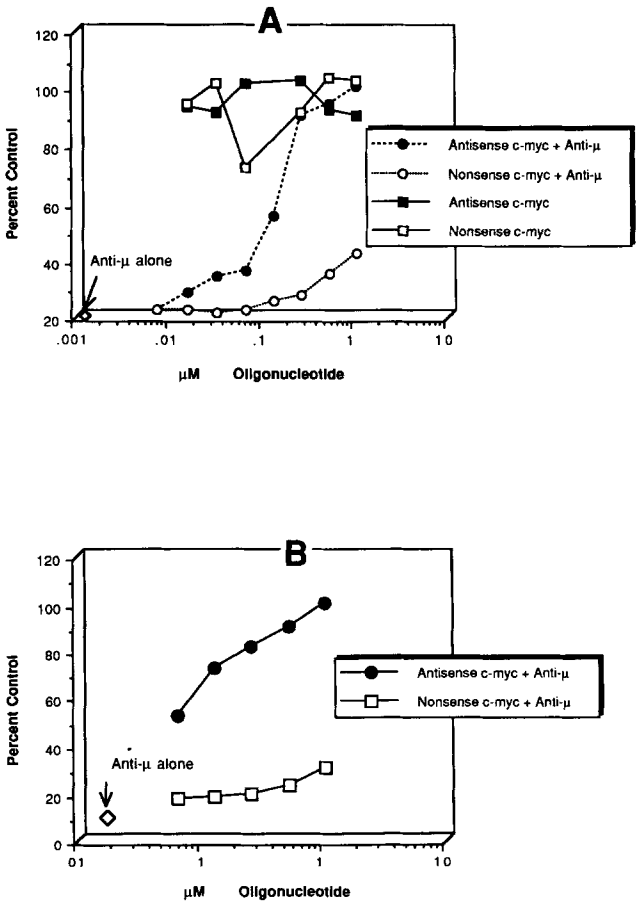

Figure 1. Effect of antisense c-*myc* on anti-μ–mediated growth inhibition of CH31 (*A*) and WEHI-231 (*B*) B lymphoma cells. Increasing concentrations of *S*-oligonucleotides were added to the cultures in the presence of 1 μg/ml anti-μ or no antibody. [^{3}H]Thymidine ([^{3}H]TdR) was added at 20 h, and cells were harvested 6 h later. Nonsense oligonucleotides were added at equivalent concentrations as controls and had minimal effects on growth inhibition, whereas antisense c-*myc* protected at >0.5 μM. The data are expressed as a percent of the untreated control for each condition. One of three similar experiments.

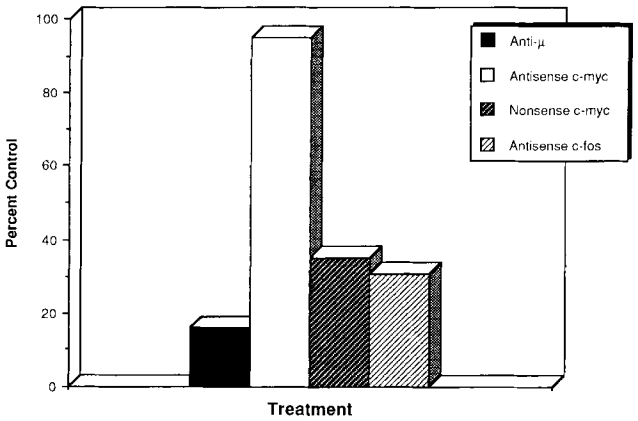

Figure 2. Specificity of antisense c-*myc* effect on growth inhibition. CH31 cells were treated with anti-μ in the presence of antisense c-*myc* or antisense c-*fos*, as well as control oligonucleotides (all at 10 μM), and harvested at 24 h as in Fig. 1. Only antisense c-*myc* was able to protect against growth inhibition by anti-μ. The data are expressed as a percentage of the untreated control.

trol oligonucleotides on [^{3}H]thymidine incorporation were nonspecific and have been observed with nonsense c-*myc* and irrelevant phosphorothioate oligonucleotides, as well (Fisher, G., and D. W. Scott, unpublished data).

Effect of Antisense c-myc on TGF-β–mediated Growth Arrest. TGF-β treatment of these B cell lymphomas leads to G_1/S blockade and subsequent cell death, similar to anti-μ (6). However, TGF-β addition leads to a decrease in c-*myc* transcription in both lymphomas and epithelial cells (Kent, S. C., and D. W. Scott, unpublished data and reference 8) and eventual growth arrest. In fact, CAT assays with c-*myc* P1 and P2 promoters demonstrate that TGF-β downregulates c-*myc* transcription, whereas anti-μ upregulates c-*myc* via these promoters (Kent, S. C., and D. W. Scott, manuscript in preparation). To further examine whether increased c-*myc* transcription is required for growth inhibition, we determined whether antisense c-*myc* prevented the effects of TGF-β on these lymphomas. When antisense c-*myc* was added simultaneously with TGF-β to B lymphomas, growth arrest was also prevented (Fig. 3), although the effect was not as dramatic as the protection against anti-μ–mediated inhibition in all experiments. This suggests that the myc protein is an important component of both signaling pathways, but the transient increase in c-*myc* transcription is not necessary for growth inhibition.

Kinetics of Antisense c-myc Reversal of Growth Inhibition. Anti-μ–mediated negative signaling occurs at a critical point in early G_1 (21). To establish the time at which myc acts in lymphoma cell cycle control, early G_1 cells were collected by centrifugal elutriation, placed in culture with anti-μ, and antisense c-*myc* added at various times. Fig. 4 demonstrates that simultaneous addition of antisense c-*myc* (or at 2 h after the initiation of the incubation) with anti-μ allowed these cells to progress normally through the cell cycle. However, addition of antisense c-*myc* 4 or 6 h after the anti-μ did not prevent cell cycle arrest. Nonsense sequences had minimal effect

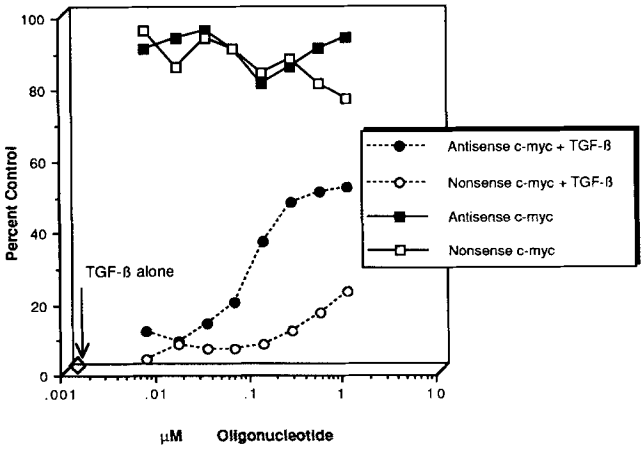

Figure 3. Antisense c-*myc* blocks TGF-β–mediated growth inhibition. CH31 B lymphoma cells were treated with 1 ng/ml TGF-β in the presence of antisense or nonsense c-*myc* as in Fig. 1. One of two similar experiments.

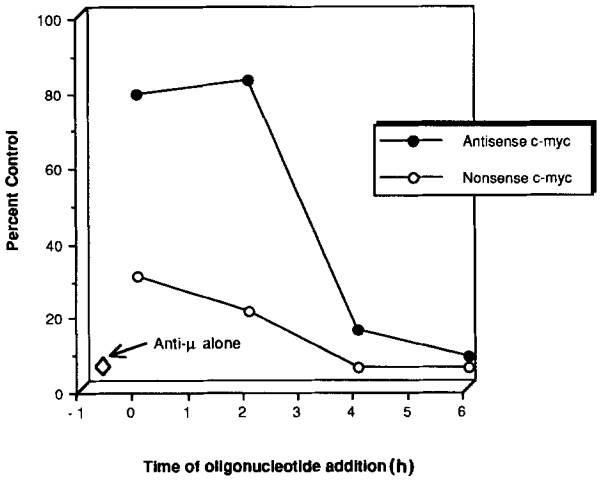

Figure 4. Requirement for c-*myc* antisense before mid G_1 in order for growth inhibition to be prevented. CH31 B lymphoma cells were separated by centrifugal elutriation, and early G_1 cells placed in culture ± 0.4 μg/ml anti-μ. 2 μM antisense or nonsense c-*myc* was added at indicated times. 20 h after addition of anti-μ, cells were pulsed with [^{3}H]TdR, and harvested at 26 h. Data are from one of two similar experiments.

when added at any time. These data suggest that myc is playing a critical role in cell cycle progression/arrest beginning in early to mid-G_1. Since pRB phosphorylation begins at this point in the cell cycle, we next examined the effect of antisense c-*myc* on this process.

pRB Is Hyperphosphorylated in Antisense c-myc-treated Lymphomas. We and others (6, 7) have demonstrated that anti-μ or TGF-β addition leads to the production of underphosphorylated pRB, which is the active growth suppressive form of this anti-oncogene. The data in Fig. 5 demonstrate that lymphomas treated with anti-μ or TGF-β and antisense c-*myc* possess pRB in the hyperphosphorylated form, whereas control cells treated with anti-μ or TGF-β alone contain the active, underphosphorylated form of pRB. Thus, an event initiated by antisense c-*myc* addition leads to a prevention of cell cycle arrest by promoting the phosphorylation of pRB.

Antisense c-myc Prevents Apoptosis. Anti-μ and TGF-β growth arrested cells begin apoptosis and die within 24–48 h (6, 21, 22) of treatment. The data in Fig. 6 demonstrate

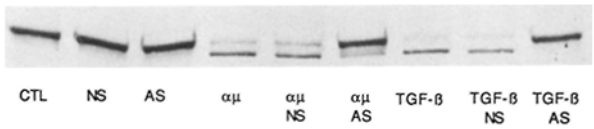

Figure 5. Antisense c-*myc* prevents the anti-μ– (1 μg/ml) and TGF-β– (1 ng/ml) mediated decrease in hyperphosphorylated pRB. The state of phosphorylation of pRB in anti-μ– or TGF-β–treated WEHI-231 cells (in the presence of antisense c-*myc*) was determined by Western blotting (6×10^6 cells/lane). Cells were treated for 24 h with the indicated reagents and then prepared for anti-pRB Western blotting as described in reference 6. (*CTL*, untreated cells; *AS*, antisense c-*myc*; *NS*, nonsense c-*myc*; *αμ*, anti-IgM.)

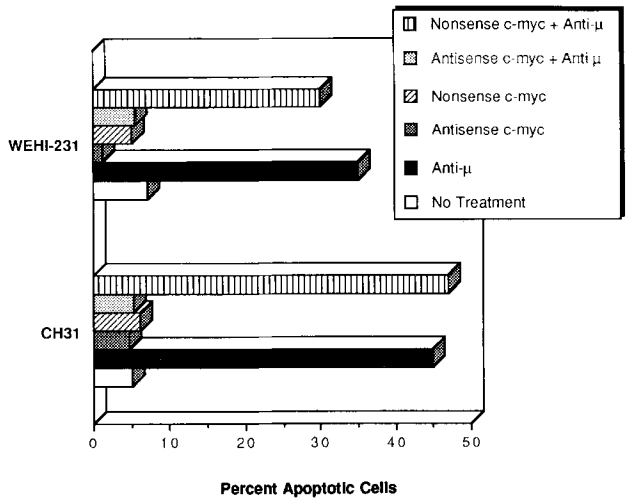

Figure 6. Antisense c-*myc* blocks apoptosis induced by anti-μ. Cells were treated as with anti-μ (0.4 μg/ml) ± antisense (2 μM) or nonsense (2 μM) c-*myc* as in Fig. 5, but were then labeled with propidium iodide for detection detection of apoptotic bodies. At least 200 cells were counted for each sample.

that antisense c-*myc* also prevents apoptosis as measured by propidium iodide staining. For example, with the CH31 B lymphoma, 45% of cells treated with only anti-μ contained apoptotic bodies, but <5% of cells treated with both antisense and anti-μ were undergoing programmed cell death. Very few apoptotic bodies were seen in the untreated (5.1%), nonsense (6.1%), and antisense only treated (5.3%) cells. These data were confirmed by gel electrophoresis of genomic DNA (data not shown) in that cells exposed to anti-μ only show typical laddering of DNA, whereas in cells treated with anti-μ and antisense c-*myc* there was no DNA degradation, as reported for T cell hybridomas (18). Therefore, treatment with anti-μ and antisense c-*myc* inhibited the formation of apoptotic bodies normally induced by anti-μ treatment alone.

Effects of Antisense c-myc on c-myc Message and Protein Levels. To determine whether the antisense oligonucleotides were acting at the level of transcription or translation, we isolated RNA from cells treated with anti-μ or TGF-β with or without antisense or nonsense c-*myc*. The data in Fig. 7 *A* reflect Northern blots in which ^{32}P random-labeled rat c-*myc* exon 3 was used to probe whole cell RNA blots obtained at 20 h. The relative abundance of c-*myc* mRNA is presented relative to actin message in Fig. 7 *B* for time points between 30 min and 20 h. This amount of c-*myc* mRNA increased at 0.5 h (~ a 2.5-fold increase) after treatment with anti-μ, and had decreased to below background levels at 1 h, as previously reported for WEHI-231 (4). Interestingly, antisense c-*myc* alone and antisense c-*myc* plus anti-μ led to an increased amount of c-*myc* message at 2, 4, 6, and 20 h in CH31 lymphoma cells; by 20 h, c-*myc* message levels were significantly decreased with anti-μ, but were at or above baseline levels in the cells treated with antisense plus or minus anti-μ in both CH31 (Fig. 7 *B*) and WEHI-231 (data not shown). TGF-β also lead to a depletion of c-*myc* message at

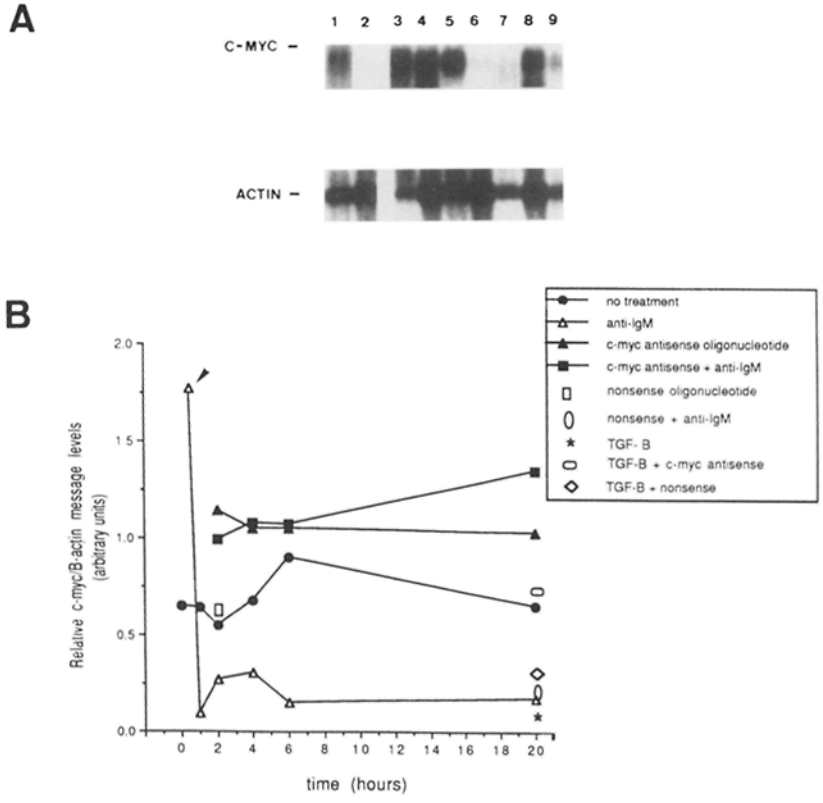

Figure 7. Effect of antisense c-*myc* oligonucleotide on c-*myc* mRNA levels at various times after treatment with anti-μ or TGF-β. (*A*) Northern blot analysis of total cellular RNA (10 μg/lane) of CH31 cells incubated for 20 h with medium alone (lane *1*); 3 μg/ml affinity purified rabbit anti-μ (lane *2*); 2 μM antisense c-*myc* plus 3 μg/ml anti-μ (lane *3*); 2 μM antisense c-*myc* alone (lane *4*); 2 μM nonsense c-*myc* (lane *5*); 2 μM nonsense c-*myc* plus 3 μg/ml anti-μ (lane *6*); 10 ng/ml TGF-β (lane *7*); 10 ng/ml TGF-β plus 2 μM antisense c-*myc* (lane *8*); 10 ng/ml TGF-β plus 2 μM nonsense c-*myc* (lane *9*). One of three representative experiments is shown. (*B*) Northern blot analysis of total cellular RNA from CH31 cells treated as in Fig. 7 *A* for various times and probed for c-*myc* and β-*actin*. Autoradiographs were scanned by laser densitometry and presented as a ratio of c-*myc* to β-*actin* message. One of three representative experiments is shown. The arrow indicates data obtained at 30 min after addition of anti-μ alone.

20 h (Fig. 7 *A*); antisense c-*myc* treatment in addition to TGF-β lead to the presence of c-*myc* mRNA at 20 h. Antisense c-*myc* did not upregulate c-*fos* or *egr-1* mRNA (Kent, S. C., unpublished data). Thus, antisense c-*myc* appears to stabilize c-*myc* message levels, presumably allowing translation of new myc protein and continued progression through the cell cycle (see below).

To establish whether the changes in c-*myc* RNA correlated with myc protein levels, we used Western blotting to examine lysates of cells treated with anti-μ (or TGF-β) with or without antisense c-*myc*. At least two myc species have been described in erythroleukemia cells by Spotts and Hann (24). We not only found these species, but also observed that the presence of anti-μ or TGF-β led to the disappearance of these myc proteins (Fig. 8). In cells treated with antisense c-*myc* or antisense c-*myc* plus anti-μ/TGF-β, normal levels of myc proteins were detected at all times. Within 30 min of the addition of anti-μ, a new species, postulated to be a myc degradation product (24) appeared; at this time the original (62–67 kD) myc proteins were still present. Cyclohexamide treatment was used to show that observed protein species turned over rapidly. No similar c-*myc* mRNA species was detected that would account for the appearance of the lower molecular weight protein species (data not shown).

In CH31 cells treated for 5 h with anti-μ, there were significantly lower amounts of all myc protein species present. TGF-β treatment alone caused a gradual loss of myc protein, with levels comparable with the anti-μ treatment alone being reached by 5 h. Again, antisense c-*myc* maintained the presence of these myc species. These results are consistent with the c-*myc* message levels, in that the increased message levels appear to correlate with the continued presence of the myc protein. We postulate that this c-*myc* antisense oligonucleotide is not acting by blocking translation, but rather is stabilizing the c-*myc* message, and allowing continued translation of new myc protein.

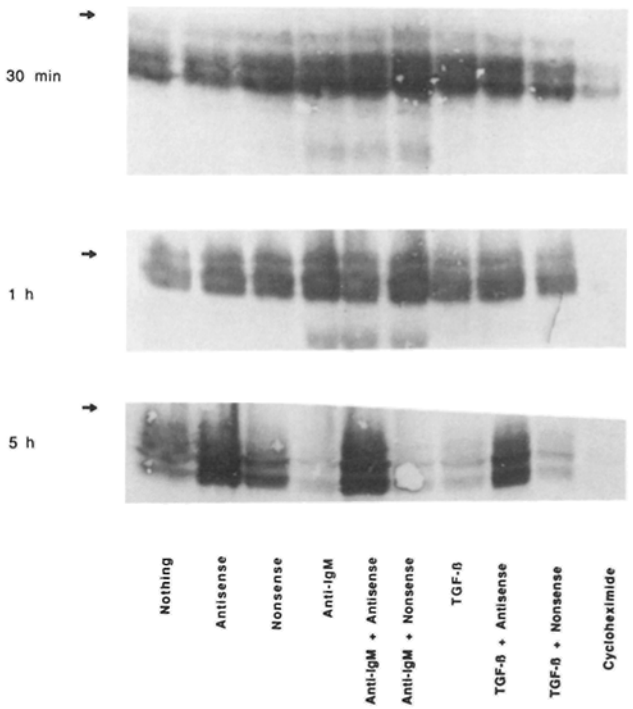

Figure 8. Effect of antisense c-*myc* on c-myc protein levels at various times after addition of anti-μ (1 μg/ml) or TGF-β (1 ng/ml). Protein was isolated from samples treated as in Fig. 7. By 5 h, the higher molecular weight species of myc protein are decreased in lanes treated with anti-μ or TGF-β in the absence of antisense c-*myc*. Cyclohexamide was used at 1 μg/ml to block protein synthesis and to verify rapid turnover of detected proteins. The arrows indicate the location of the 69-kD molecular mass marker.

Discussion

Our data demonstrate that antisense oligonucleotides for c-*myc*, but not for c-*fos*, are able to prevent cell cycle arrest and apoptosis induced by both anti-μ and TGF-β. Since both anti-μ and TGF-β lead eventually to a loss of c-*myc* message in these cells, despite an initial increase with the former treatment, we suggest that myc dysregulation is the common denominator for growth arrest and apoptosis in these cells. Surprisingly, however, antisense c-*myc* did not protect against apoptosis by interfering with myc translation. In fact, we found that antisense c-*myc*, even in the presence of anti-μ or TGF-β, appeared to stabilize the c-*myc* message and allowed for continued translation of myc protein, normal phosphorylation of pRB, and unimpaired cell cycle progression. This is a novel function of an antisense oligonucleotide molecule.

These results also indicate that stabilization of myc must occur at least 2 h after the addition of anti-μ since addition of antisense c-*myc* 4 or 6 h after anti-μ could not prevent cell cycle arrest in synchronized early G_1 cells. This implies that the irreversible dysregulation of c-*myc* message and myc protein committing the cell to programmed cell death occurs quickly after surface IgM ligation. Indeed, in unsynchronized CH31 cells, significant modulation of c-*myc* message begins within 1 h of anti-μ stimulation (Fig. 7; references 4, 7), but loss of myc protein is not apparent until 5 h. While experiments are in progress to determine whether this occurs in both G_1 and in S phase cells treated with anti-μ, these data imply that myc protein is necessary for G_1 progression to continue in these B cell lymphomas.

The phosphorylation state of the *retinoblastoma* gene product has been implicated in cell cycle control; indeed, pRB must be phosphorylated in mid- to late G_1 in order for cells to progress to the S phase (9, 10). We and others have shown that treatment with anti-μ (or TGF-β) affects the state of phosphorylation of pRB (reference 6; Fig. 5) in murine B cell lymphomas. Although modulation of the myc protein in unsynchronized murine B cell lymphomas occurs within 5 h of surface IgM ligation, it is interesting that significant pRB hypophosphorylation is not detected until 10–12 h after anti-μ or TGF-β addition (Joseph, L., and D. W. Scott, manuscript submitted for publication). This suggests that myc changes precede, and may be required for, phosphorylative effects on pRB, although it will be critical to establish the kinetics of these changes in early G_1 cells.

Recent data also suggest that myc and pRB may directly interact (25) and can have opposing effects on cell cycle progression. Indeed, transiently expressed pRB was found to suppress c-*myc* transcription (26). Moreover, microinjection of pRB into Saos-2 cells suppressed entry into S phase, while co-injection of pRB and myc protein allowed ~50% of the cells to progress into the cell cycle (27). Thus, pRB and myc may act together to regulate cell cycle control.

The ability of antisense c-*myc* to prevent anti-μ– and TGF-β–mediated growth arrest, and our previous elucidation of the initial second messengers induced by anti-μ (28), suggest the convergence of the signaling pathways at the myc protein. Moreover, this result suggests that increases in myc protein and message levels per se do not commit these lymphomas to an apoptotic pathway. The appearance of the phosphorylated form of pRB in cells treated with antisense c-*myc* and either anti-μ or TGF-β implicates myc in the process of aiding pRB phosphorylation, leading to cell cycle progression and avoidance of cell death. Since phosphorylation of pRB requires that critical proteins bind to the pocket region of the RB gene product, we propose that the myc protein, either directly or indirectly, affects one of these pocket proteins to aid association and subsequent phosphorylation of the RB gene product (29). Alternatively, a shift in the interaction of myc with myn (30) may affect the ability of myc to *trans*-activate and alter the expression levels of other, yet unidentified, genes, including cyclin–*cdk* complexes. Attempts to identify the pRB and myc interacting proteins are underway in this and other laboratories.

Antisense c-*myc* can be acting through several mechanisms. It is known that a block to transcriptional elongation downregulates c-*myc* mRNA steady state levels (31); antisense oligonucleotides may interfere with normal transcriptional attenuation. A second possible mode of c-*myc* antisense action is by direct interaction with the myc protein, causing increased apparent protein stability. We consider these possibilities to be unlikely. While no attempt has been made here to examine the mechanism of antisense oligonucleotide action, we favor instead the hypothesis that specific stabilization of c-*myc* mRNA may occur, allowing increased translation. This is supported by the fact that elevated levels of c-*myc* mRNA were found when antisense oligonucleotides were added to B lymphoma cells, regardless of other treatment (Fig. 7). Efforts are underway to elucidate the exact mechanism of antisense c-*myc* action.

Our system, therefore, begins to elucidate the link between myc and pRB, a possible effect of one on the other, and the control that myc has on cell cycle progression in lymphoid cells. These experiments suggest that c-*myc* may play a different role in B lymphoma cells than in other differentiated cells. In T cell hybridomas, TGF-β does not induce cell cycle blockade, but it does inhibit transcription of c-*myc* and activation-induced apoptosis (Green, D. R., personal communication). In contrast, in our B cell lymphomas, TGF-β is a strong negative regulator of growth. Moreover, DNA fragmentation, a distinct sign of activation-induced apoptosis, can be seen within 4–6 h in anti-CD3–treated T cell hybridomas (Green, D. R., personal communication), whereas in anti-μ–treated B lymphomas it is seen between 12 and 24 h (data not shown). Shi et al. (18) have shown that antisense c-*myc* blocks translation of myc protein in their system and prevents apoptosis induced by anti-CD3 activation. In further contrast to results with T cell hybridomas (18), we do not see a loss of the myc protein at 24 h with antisense c-*myc* treatment. We suggest, instead, that critical *myc* mRNA or myc protein species are stabilized by antisense oligonucleotides in these lymphomas, thus allowing continued progression through the cell cycle, and preventing apoptosis.

It is tempting to speculate on the importance of the balance of the myc protein and its relation to other anti-oncogenes and the cyclin complexes. It is interesting to note that recent data (32, 33) suggest that the p53 growth suppressor protein may be necessary for certain forms of apoptosis. However, in preliminary experiments, we have found that p53 protein levels in CH31 B lymphomas were not affected by treatment with either anti-μ or TGF-β with and without antisense c-*myc* (Fischer, G., and D. W. Scott, data not shown). The role of myc and other oncogenes and anti-oncogenes in preventing and promoting apoptosis is under investigation.

We thank Deirdre Nelson, who performed the initial experiment, and Lenny Dragone for a review of the manuscript.

These studies were supported by United States Public Health Service grants, CA-55644 (D. W. Scott) and AI-31591 (D. R. Green), and American Cancer Society grant IM-495 (D. W. Scott). This is publication 87 of the Immunology Division, University of Rochester Cancer Center.

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Received for publication 20 May 1993 and in revised form 22 September 1993.

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