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Identification of a novel gene regulated by c-Myc in the WEHI-231 B cell line



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# IDENTIFICATION OF A NOVEL GENE REGULATED BY C-MYC IN THE WEHI-231 B CELL LINE

by

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ABSTRACT

The c-Myc protein is a proto-oncogenic transcription factor which has been implicated as playing a role in programmed cell death by apoptosis in certain cell systems. C-Myc may play a role in the antigen receptor-induced apoptosis seen in the WEHI-231 immature B cell line. Antigen receptor stimulation of WEHI-231 cells induces transient elevation in c-Myc expression. Furthermore, activation of exogenous c-Myc using a regulatable c-Myc/ estrogen receptor chimera (mycER) is able to trigger apoptosis in transfected WEHI-231 cells independently of antigen receptor stimulation. Few genes regulated by the c-Myc transcription factor have been identified and their biological relevance has not been well established. I have used the differential display PCR technique to successfully identify and clone part of a novel gene induced by the mycER chimera in WEHI-231 cells. Further studies on this gene may help elucidate the mechanisms of c-Myc action and the role of c-Myc in WEHI-231 antigen receptor-induced apoptosis.

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# INTRODUCTION

## c-Myc: a transcription factor

The product of the c-*myc* proto-oncogene is a short-lived, putative transcription factor which has been implicated as playing an important role in the control of cellular proliferation. There is considerable evidence that c-Myc functions as a transcription factor. C-Myc contains a leucine zipper motif, a helix-loop-helix domain, and a basic domain which have been found in previously identified transcription factors (1). The leucine zipper motif (2) and the helix-loop-helix domain direct specific protein-protein interactions (3). The basic domain makes direct sequence-specific interactions with DNA in a number of transcription factors (4). The contiguous basic/helix-loop-helix/leucine zipper domains found in c-Myc now define a group of closely related transcription factors.

The c-Myc protein does not form stable homodimers (5); however, it forms stable heterodimers with another protein, Max, which is also a basic/helix-loop-helix/leucine zipper protein (6, 7). In contrast to c-Myc, Max is a relatively abundant protein with a long half-life whose expression is generally not regulated by growth factors (7-9). Max also forms homodimers and interacts with at least two other basic/helix-loop-helix/leucine zipper proteins, Mxi1 (10) and Mad (11). The c-Myc/Max heterodimer binds to DNA *in vitro* in a sequence-specific manner (6, 7, 12-17).

Both c-Myc/Max and Max/Max dimers bind most avidly to DNA sequences containing a

central CACGTG core (5, 18-21), but induce opposite bends in the DNA (22). It is generally thought that Myc/Max and Max/Max complexes within cells compete for binding to similar DNA sequences and, respectively, activate and repress transcription. The equilibrium between heterodimeric and homodimeric complexes of Max, therefore, would determine whether a target gene is activated or suppressed (8, 21). Experiments using heterologous promoters with c-Myc binding sequences have confirmed that c-Myc has transcriptional transactivator ability (13, 15, 16, 23-27) whereas Max can repress transcription (15, 24, 26). Transactivation by c-Myc in yeast requires Max (13) and functional Max has been shown to be required for c-Myc-mediated transformation, cell cycle progression, and apoptosis (28, 29), supporting the conclusion that c-Myc functions as a Myc/Max heterodimer.

c-Myc and cellular proliferation

The c-Myc protein has been implicated as playing an important role in the control of cellular proliferation. Activation of c-*myc* expression by translocation to immunoglobulin enhancer sequences is responsible for Burkitt's lymphoma (30), and c-*myc* activation has also been described in many other human cancers (31). In normal cells, c-*myc* is known to be one of the immediate early response genes which are rapidly induced upon mitogenic stimulation, suggesting a role in mediating the transition from quiescence to proliferation.

The downregulation of c-*myc* expression seen after withdrawal of serum or specific growth factors is associated with accumulation of cells in the $G_0/G_1$ phase of the cell cycle (32, 33). Forced expression of c-*myc* in growth factor-deprived cells has been shown to be sufficient to overcome cell cycle arrest and drive cells into S phase. This has been shown with forced c-*myc* expression in IL-3-deprived myeloid progenitor cells (34) and with hormone-mediated activation of a c-Myc/estrogen receptor chimeric protein (mycER) in serum-deprived fibroblasts (35). Antisense oligonucleotides against c-*myc* also block entry of cells into S phase (36, 37). All of this considerable evidence supports the role of c-*myc* as a positive regulator of cell growth whose activation may confer a growth advantage upon a tumor cell.

c-Myc and apoptosis

In apparent contradiction to its role as a positive growth regulator and oncogene, c-*myc* has more recently been shown to also be capable of inducing apoptotic cell death. Enforced c-*myc* expression upon withdrawal of IL-3 from an IL-3-dependent myeloid progenitor cell line continues to drive cells into S phase and accelerates cell death by the morphological characteristics of apoptosis (34). Activation of the regulatable mycER chimeric protein or constitutive c-*myc* expression in serum-deprived Rat-1 fibroblasts or rat embryo fibroblasts similarly triggered apoptotic cell death (35). Heat shock-induced c-*myc* expression under

the control of heat shock promoter sequences in Chinese hamster ovary fibroblasts also induced apoptosis (38). Interestingly, antisense oligonucleotides against c-*myc* blocked the T cell receptor-mediated apoptosis seen in certain T lymphocyte hybridomas (39), and this T cell receptor-mediated apoptosis has been shown to require the functional heterodimerization of the c-*myc* protein product with its partner, Max (40). A model for explaining the role of c-*myc* in apoptosis may be that expression of c-*myc* in the absence of a survival signal (provided by serum or inactivated by antigen receptor stimulation) induces cells to undergo apoptosis (41). This may be a protective mechanism to remove cells proliferating under conditions which are not consistent with physiological proliferation.

Apoptosis in the WEHI-231 B lymphoma cell line

Work done in our laboratory suggests that c-Myc also plays a role in the antigen receptor-induced apoptosis seen in the WEHI-231 B cell line. The WEHI-231 cell line is a model for immature B cells, phenotypically characterized by being surface IgM+, IgD-, $F_c$ receptor-low, and MHC class II-low. Crosslinking of the membrane-bound immunoglobulin molecules that serve as antigen receptors on these cells initiates programmed cell death by apoptosis (42, 43). This antigen receptor-induced apoptotic response may be a mechanism by which self-reactive immature B cells are deleted upon

contact with self-antigens, thereby contributing to immunological tolerance (44). In contrast, stimulation of antigen receptors of mature B cells usually promotes cellular proliferation and differentiation to antibody-secreting plasma cells. Crosslinking of the antigen receptor in B cells leads to increased protein tyrosine phosphorylation (45-48) and the induction of several second messenger signaling pathways (49-54). However, differences in signaling to account for the variation in responses between immature and mature B cells have not been found.

Consistent with its role as an immediate early response gene, c-*myc* is strongly induced in the WEHI-231 cell line following antigen receptor stimulation (55, 56). Stimulation of the receptor produces an initial transient increase in c-*myc* protein and mRNA levels followed by a decline to levels that are below baseline but still detectable (57). C-*myc* levels have already risen and fallen before the cells become committed to undergo apoptosis. Similar to what has been seen in T cell hybridomas, addition of oligonucleotides with a sequence complementary to the first five codons of the c-*myc* gene significantly protected these cells from apoptosis induced by antigen receptor stimulation (57). There is some question, however, about the specificity of this antisense effect. Recent reports suggest that certain bacterial DNA sequence motifs may directly activate B cells (58). The c-*myc* antisense oligonucleotide potentially contains such a motif and the survival of the WEHI-231 cells may be due to stimulation by this motif rather than

inhibition of c-*myc* expression.

Evidence for a possible role for c-*myc* in WEHI-231 apoptosis also comes from another approach using the mycER chimeric protein which contains the ligand-binding domain of the human estrogen receptor fused to the 3' end of the entire human c-Myc protein (Figure 1) (59). This chimeric protein seems to constitutively localize to the cell nucleus (59). Upon addition of estrogen receptor ligands, such as estradiol or hydroxytamoxifen, c-Myc activity is induced. Estradiol-dependent induction of c-Myc activity is dependent on the chimera containing a functional c-Myc molecule, suggesting that activity of the chimera is a result of c-Myc activation and not due to any intrinsic ability of the ligand-binding domain of the estrogen receptor to activate transcription (59). Activation of the mycER chimeric protein in transfected WEHI-231 cells induced apoptosis independently of antigen receptor stimulation. This is similar to apoptosis seen in Rat-1 fibroblasts upon activation of transfected mycER under conditions of serum starvation, except that serum starvation was unnecessary in WEHI-231 cells. In the WEHI-231/mycER cells, concurrent antigen receptor stimulation and estradiol activation of mycER induced more rapid cell death than either stimulation alone (57). Interestingly, in contrast to what is seen after antigen receptor stimulation, mycER induces apoptosis from different stages of the cell cycle without first causing cell cycle arrest in $G_1$ (57). The above data suggest that c-Myc activity is able to induce apoptosis in WEHI-231 cells, but the exact role of c-Myc in WEHI-231 antigen

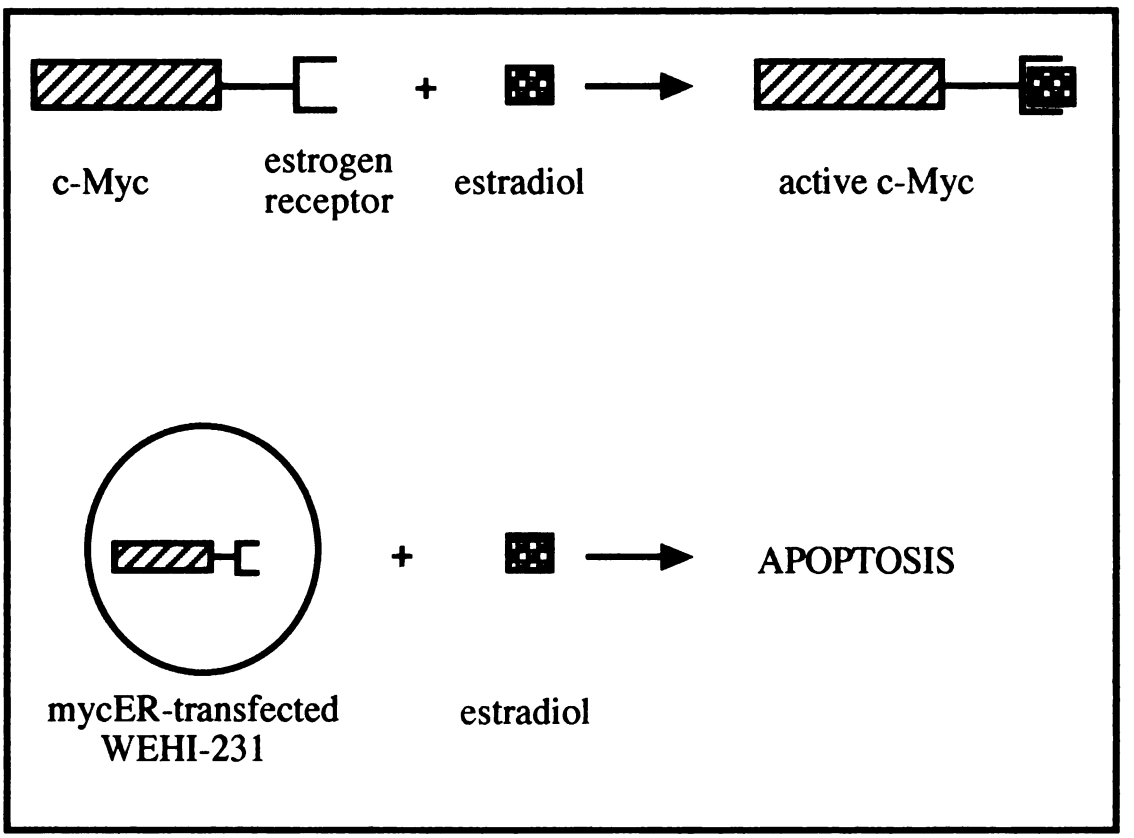


**Figure 1. The mycER chimera.** As shown at the top of this figure, the mycER chimera consists of the ligand-binding portion of the estrogen receptor fused to the 3' end of the entire human c-Myc protein. Binding of estradiol by the estrogen receptor portion of the chimera results in activation of the c-Myc portion of the chimera. As shown at the bottom of this figure, activation of the mycER chimera in WEHI-231 cells results in apoptosis of the cells.

receptor-mediated apoptosis has not been elucidated.

As c-*myc* is believed to act by stimulating transcription, the mycER-induced apoptosis described above is presumably triggered by induction of one or more unidentified genes. Several potential target genes of c-*myc* have been identified (60), but the biological relevance of most is unclear. Perhaps the most convincing identified target gene is ornithine decarboxylase (ODC) which has been shown to be a mediator of c-Myc-induced apoptosis. However, ODC is not essential for c-Myc-induced apoptosis, suggesting that there are other effectors of c-Myc as well (61).

Since relatively little is known about c-Myc-regulated genes, any target genes of c-Myc are of interest given c-Myc's important role in control of cellular proliferation and apoptosis. Identification of c-Myc-regulated genes in the WEHI-231 system may shed light on c-Myc's role in apoptosis in B cells.

In order to identify genes regulated by c-Myc and further elucidate the role of c-Myc in WEHI-231 apoptosis, I have used the WEH-231/mycER system and the differential display PCR technique (62). Using the mycER system allows for the potential isolation of c-Myc-regulated genes while excluding the multitude of other genes that would be regulated by antigen receptor stimulation. As described in the Results section, differential display PCR uses reverse transcription and the polymerase chain reaction to identify genes which are differentially expressed between different cell populations. Using this technique, I have

successfully identified and cloned part of a novel gene whose expression was induced in WEHI-231/mycER cells after activation of mycER by addition of estradiol.

## MATERIALS AND METHODS

**Cell Lines.** The immature IgM+ murine B cell lines, WEHI-231 and CH31.LX; the IgG+ murine secondary B cell line, 2PK3; the mature IgM+ murine B cell line, BAL17; and the surface Ig- murine pre-B cell line, 70Z/3, were all grown at 37°C in a 5% $CO_2$ atmosphere in RPMI 1640 medium (Whittaker MA Bioproducts) supplemented with 10% heat-inactivated fetal bovine serum, 1mM sodium pyruvate, 2 mM glutamine, and 50 mM 2-mercaptoethanol. MycER-transfected WEHI-231 cells obtained from Paul Mittelstadt (DeFranco lab, UCSF) were maintained in the above medium containing 2 mg/ml G418 (Gibco-BRL). All cell lines were grown in logarithmic phase at densities of less than 5 x $10^5$ cells/mL.

**RNA Preparation.** WEHI-231/mycER cells were harvested at concentrations between 1 x $10^5$ and 4 x $10^5$ cells/mL after stimulation with 1μM estradiol, stimulation with 0.1 μM hydroxytamoxifen, or mock stimulation with 0.1% ethanol. All other B cell lines were harvested at similar concentrations after stimulation with 10 μg/mL of goat anti-mouse IgM μ-chain(WEHI-231, CH31.LX, and BAL17 cell lines), goat anti-mouse IgG γ-chain

(2PK3 cell line), or lipopolysaccharide (70Z/3 cell line). Cells were washed twice with ice-cold PBS before lysis. RNA used for differential display PCR was prepared by precipitation through cesium chloride as described (63) and subsequent treatment with 4 units DNase (Promega) in a volume of 50 microliters Tris-EDTA (10mM Tris, 1 mM EDTA) containing 10 mM $MgCl_2$, 1mM DTT, and 40 units RNasin (Promega). All other RNA was prepared by the acid-phenol method as described (64) with the exception that only a single isopropanol precipitation was performed.

**Primer Synthesis.** The primers used for differential display PCR were synthesized by Lois Koren (Hooper Foundation, UCSF) using an Expedite nucleic acid synthesis system (Millipore). The following 14-mer deoxyoligonucleotides were synthesized for use as 3' primers: $T_{12}MA$, $T_{12}MC$, $T_{12}MG$, and $T_{12}MT$ where M denotes a mixture of A, C, and G. Ten-mer deoxyoligonucleotides of arbitrary sequence were used as 5' primers. The identification of the novel gene reported in this paper was accomplished using a 5' primer of the following sequence: 5' CTTTCTACCC 3'.

**Differential Display PCR.** Differential display PCR (DDPCR) was performed essentially as described previously (62, 65) with minor modifications as noted. First, reverse transcription was performed on RNA from either WEHI-231/mycER cells

stimulated with estradiol for two hours or mock-stimulated WEHI-231/mycER cells. 0.2 μg RNA in a 19 μL volume containing 1x RT buffer (NEB), 20 μM dNTP, and 2.5μM 3' primer was denatured at 65 °C for 5 minutes in a thermal cycler (Perkin-Elmer). One μL MMuLV reverse transcriptase (NEB) was then added and the RNA was reverse transcribed at 37 °C for one hour in the thermal cycler.

Next, 2 μL of the RT reaction was used for PCR in a 20 μL final volume containing 1x PCR buffer (Perkin-Elmer), 2 μM dNTP, 5 μCi $^{33}$P-dATP (Amersham, 10 μCi/μL, 3000 Ci/mmol), 0.5 μM 5' primer, 2.5 μM 3' primer, and 1:100 dilution of AmpliTaq polymerase (Perkin-Elmer). Samples were covered with mineral oil and PCR was done in a thermal cycler for 40 cycles of 94 °C for 30 seconds, 40 °C for 2 minutes, and 72 °C for 30 seconds. The final cycle was followed by extension at 72 °C for 7 minutes before storing samples at 4 °C.

For analysis of the PCR products, 4 μL of each PCR sample was mixed with 2 μL of loading dye (formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue) and electrophoresed through a 5% acrylamide/urea gel (Gibco-BRL sequencing gel plates and apparatus) at 60 watts in 0.5x TBE running buffer for approximately 2 hours, until the xylene cyanol dye front was near the bottom of the gel. In order to insure reproducibility of the banding patterns, multiple independently reverse transcribed and PCR'd samples from separately harvested RNA preparations were run side by side on the gels. Gels were

dried and exposed to film.

**Cloning of DDPCR products.** An induced band identified by DDPCR was cut out of the gel and eluted by soaking the gel slice in 100 μL Tris-EDTA for ten minutes, boiling for fifteen minutes, and precipitating the DNA from the supernatant using 0.3M sodium acetate, 2.5 volumes ethanol, and 5 μL of 10 mg/mL glycogen as a carrier. The precipitated DNA was resuspended in 10 μL Tris-EDTA and 4 μL was used for reamplification by PCR using the same primer and amplification conditions as above. The reamplified product was then cloned into the TA cloning vector using the manufacturer's instructions (Invitrogen). The plasmid DNAs from thirty-six transformant colonies containing potential inserts were isolated and the presence of insert was confirmed by restriction enzyme digestion and agarose gel electrophoresis. The DNA from 12 out of the 36 colonies was partially sequenced and the predominant species (5/12) was used for further studies.

**Ribonuclease Protection Assays.** In order to assess expression of the mycER-induced gene, a ribonuclease protection assay was used. The radioactively labeled cDNA probe was made by *in vitro* transcription of the insert in the TA cloning vector using T7 RNA polymerase (Promega). 0.5-1 μg of linearized template (TA vector with insert) and 1

μL T7 RNA polymerase were incubated at 37 °C for one hour in a final volume of 20 μL containing 1x Transcription Optimized buffer (Promega; 40 mM Tris-HCl, 6mM $MgCl_2$, 2 mM spermidine, 10mM NaCl), 0.01M DTT (Promega), 0.5 mM each of rATP, rCTP, and rGTP, 0.075 mM rUTP, 50 μCi $^{32}$P-α-UTP (Amersham, 20 μCi/μL, 800 Ci/mmol), and 40 units RNasin ribonuclease inhibitor (Promega). The DNA template was then removed from the transcription reaction by treatment with 2 units DNase (Promega) at 37 °C for fifteen minutes. Probes to detect murine c-*myc* and GAPDH were similarly prepared using c-*myc* and GAPDH templates in pBS(-) vector (obtained from Gene Yee, Bishop lab, UCSF) with the exceptions that only 0.025 mM rUTP was used and a T3 polymerase (Promega) was used for transcription of the c-*myc* probe. Twenty μL of gel loading buffer (Ambion; 80% formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was then added to the reaction and the probe was electrophoresed through a 5% denaturing acrylamide gel, cut out, and eluted in gel elution buffer (Ambion; 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS). In order to detect mRNA corresponding to the gene of interest, approximately 2 x $10^5$ cpm of gel-purified probe (confirmed to be at least a two-fold excess of the amount needed to detect all of the corresponding cellular mRNA, data not shown) was then hybridized overnight in "Solution A" hybridization buffer (Ambion) with 10 μg total cellular RNA, RNase-treated to remove unhybridized RNA, precipitated, and electrophoresed through a 5% denaturing acrylamide gel as described in the RPAII kit

instructions (Ambion). The gel was then dried, exposed to a PhosphorImager screen (Molecular Dynamics), and appropriately sized RNA fragments protected by the probes were visualized using Phosphorimager software from Molecular Dynamics.

**cDNA Library Screening and Sequencing.** An unstimulated WEHI-231 cDNA library in λzapII (Stratagene) prepared by Jim Richards (DeFranco lab, UCSF) was screened according to the manufacturer's instructions. The probe used for the library screening was made by radioactively labeling the TA clone insert identified by differential display. Forty-five ng of gel-purified insert was labeled using random primer synthesis by the Klenow fragment of DNA polymerase in a 33 μl volume containing 1x hexanucleotide buffer (Boehringer-Mannheim Biochemicals), 83 μCi $\alpha$-$^{32}$P-dCTP (Amersham, 10 μCi/μL, 3000 Ci/mmol), and 5 μL 0.5 mM each of dATP/GTP/TTP. One positive clone from the library screen was obtained. This clone was excised and sequenced by the dideoxynucleotide method using Sequenase reagents (USB) as recommended by the manufacturer.

**Northern Blot Analysis.** PolyA-containing RNA was isolated from unstimulated and estradiol-stimulated (4 hours) WEHI-231/mycER cells using the Micro FastTrack mRNA isolation kit (Invitrogen). The RNA was resolved by electrophoresis through 1.2%

agarose gels containing 2 M formaldehyde buffered with 5 mM Hepes (pH 7.0). After electrophoresis, the mRNAs were transferred to Genescreen membranes (NEN Research Products) and crosslinked to the membranes by UV irradiation by a Stratalinker 1800 (Stratagene). The resulting membranes were incubated for 2-3 hours in buffer containing 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 125 mg/mL denatured salmon sperm DNA. The membranes were then hybridized overnight in the same buffer with random oligonucleotide-primed $^{32}$P-labeled cDNA probes made from the 1.7 kb clone isolated by cDNA library screening. The probed membranes were washed in 0.1x SSC and 1% SDS at 65 $^{o}$C to remove nonspecific binding of the probe before autoradiography.

## RESULTS

### Identification of a c-Myc-induced gene by differential display PCR

Differential display PCR is a technique that uses reverse transcription and the polymerase chain reaction to identify genes which are differentially expressed between two or more different cell populations. I used this technique in mycER-transfected WEHI-231 cells to search for genes whose expression was induced or repressed upon activation of mycER activity by the addition of estradiol.

Differential display PCR uses one of four polyT-based 3' deoxyoligonucleotide primers to prime reverse transcription of polyadenylated mRNA in total RNA obtained from the

cells of interest. The 3' primers contain twelve T's, followed by a mixture of A, C, and G at the penultimate base. The last base consists of either A, C, G, or T and results in each primer theoretically allowing the reverse transcription of one-quarter of the total mRNA population. By performing separate reverse transcription reactions with each of the 3' primers, all of the mRNA species from the cell population should be reverse transcribed.

The reverse transcribed cDNA products are then subjected to PCR in the presence of radioactively labeled nucleotides. The same 3' primer used for reverse transcription is used for PCR. A ten-base long deoxyoligonucleotide of arbitrary sequence is used as the 5' primer. This arbitrary 10-mer should anneal to complementary sequences on many different cDNA species at various distances from the 3' primer depending on the distance of the sequence from the polyA tail on a particular cDNA species. Thus, each RT-PCR reaction with a particular 3'-5' primer pair will result in the amplification of multiple partial cDNA species of varying size which can then be separated on a polyacrylamide sequencing gel. Differences in the banding pattern between the stimulated and unstimulated cells with a given primer pair may signify differential mRNA expression between the two cell populations. Since reproducibility of differences in the banding pattern is a key to isolating cDNAs that exhibit true changes in expression, multiple independently run reverse transcription and PCR reactions on separately harvested RNA were electrophoresed side by side through the gels. Similar to the 3' primers, each particular 5' primer will anneal to and

amplify only a subset of cellular mRNAs. By running multiple experiments using different 3'-5' primer pairs, theoretically one should eventually be able to detect cDNA fragments corresponding to the complete cellular mRNA population.

I have used the differential display PCR procedure with approximately thirty 3'-5' primer pairs to look at differences in gene expression between WEHI-231/mycER cells stimulated with estradiol for two hours and WEHI-231/mycER cells that were unstimulated. The two hour time point was felt to be early enough to be able to detect genes directly regulated by c-Myc while allowing enough time for the mycER chimera to be activated and initiate transcription. Only one of the thirty primer pairs (3' primer: $T_{12}MC$, 5' primer: CTTTCTACCC) identified a differentially expressed band. As shown in Figure 2, a band of approximately 400 base pairs was consistently induced in these cells by mycER activation. Each lane includes PCR products obtained using RNA from a separately stimulated and harvested cell population on which separate reverse transcription and PCR reactions were done using the mentioned primer pair. At least two other similar gels using separately run reactions with the same primer pair confirmed these results showing a 400 base pair band which is faintly present under unstimulated conditions and is significantly induced with estradiol stimulation. This PCR fragment was reamplified and cloned into the TA vector. The cDNA species corresponding to this gene identified by differential display PCR will be referred to as MIG-1.

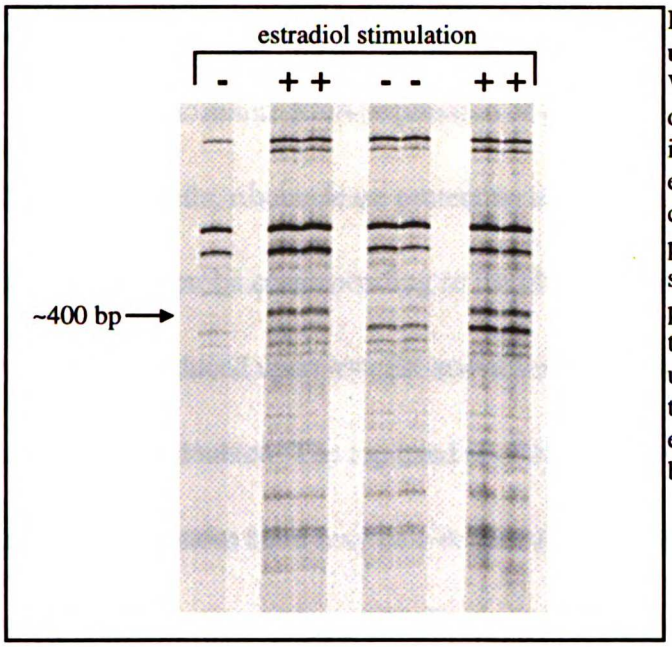


**Figure 2. Differential display PCR of unstimulated and estradiol-stimulated WEHI-231/mycER cells.** Differential display PCR was performed on RNA isolated from unstimulated (-) and two hour estradiol-stimulated (+) WEHI-231/mycER cells. Each lane includes PCR reaction products obtained using RNA from a separately stimulated and harvested cell population on which separate reverse transcription and PCR reactions were done using the 3' and 5' primers mentioned in the text. The arrow denotes a consistently estradiol-inducible band approximately 400 bp in size.

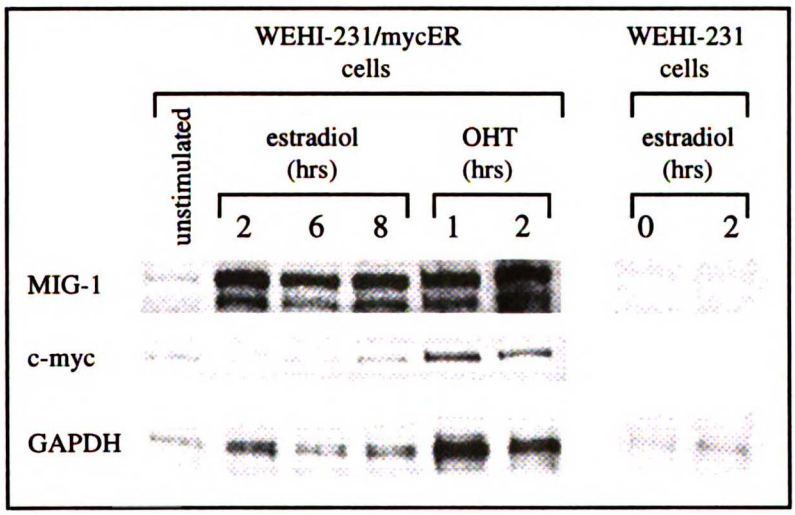


**Figure 3. Induction of MIG-1 expression by mycER**. A ribonuclease protection assay was done on RNA fromWEHI-231/mycER cells (lanes 1-6) using a MIG-1 probe, a c-myc probe, and a GAPDH probe. As shown, MIG-1 was induced by estradiol and hydroxytamoxifen (OHT) stimulation. The MIG-1 induction pattern upon estradiol stimulation has been confirmed by three separate experiments. A separate ribonuclease protection assay experiment was done on RNA from untransfected WEHI-231 cells using MIG-1 and GAPDH probes (lanes 7-8). MIG-1 was not induced by estradiol in untransfected WEHI-231 cells. Note: Exposure times for MIG-1 were longer than for c-myc and GAPDH.

Confirmation of induction by ribonuclease protection assay

In order to examine RNA expression of MIG-1 in stimulated and unstimulated WEHI-231/mycER cells, ribonuclease protection assays were performed using a radioactively labeled RNA probe corresponding to the cloned PCR fragment. As shown in Figure 3, MIG-1 was induced upon mycER activation as expected. The protected MIG-1 species appeared as a doublet. The top band is of the expected size for a fragment protected by the probe. The bottom band may be a degradation product of the top band or it may represent an alternatively spliced form of MIG-1. An alternatively spliced form may have mismatched bases when annealing to the probe, resulting in cleavage by RNase to the lower molecular weight fragment. Probing of RNA from cells stimulated for varying amounts of time with estradiol showed that induction of this RNA species occured within thirty minutes of stimulation (data not shown), peaked by one hour after stimulation, and maintained peak levels for at least eight hours after stimulation. The induction after two hours in three separate experiments, as quantitated by PhosphorImager software and corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels (GAPDH is a "housekeeping" gene whose expression levels are assumed to remain relatively constant which allows it to be used to compare RNA content between different samples), was estimated to be 3.2 fold, 6.2 fold, and 8.0 fold. These three estimates give a mean induction of 5.8 fold with a standard deviation of 2.4 and a standard error of the mean of

1.4. Note that probing of endogenous murine c-*myc* shows that mycER activation by addition of estradiol results in a decline in endogenous c-*myc* levels, presumably as a result of a negative feedback mechanism controlling the amount of c-*myc* expressed.

Ribonuclease protection assays also showed that the identified gene was induced in WEHI-231/mycER cells after stimulation with hydroxytamoxifen (Figure 3). Hydroxytamoxifen is an estrogen receptor antagonist which has been shown to activate c-Myc activity of the mycER chimera while inhibiting the transactivation activity inherent in the estrogen receptor portion of the chimera (66). Thus, these results showing induction of the gene by hydroxytamoxifen suggest that the gene is induced as a result of the mycER chimera's c-Myc activity.

The identified gene was present at low levels but was not induced by estradiol treatment in non-transfected WEHI-231 cells (Figure 3). These results confirm that the gene is induced by the activation of the mycER chimera and not by effects of estradiol on the WEHI-231 cells.

Cloning of a partial cDNA for MIG-1

An unstimulated WEHI-231 cDNA library was screened using as a probe the partial cDNA fragment obtained by differential display PCR. It was felt that the unstimulated library could be used since a low basal level of MIG-1 in unstimulated cells was suggested

by the ribonuclease protection assays. One plasmid containing an approximately 1.7 kilobase cDNA was isolated from the library and this cDNA clone was sequenced (Figure 4). Comparison of the sequence to reported sequences in Genbank using GCG software (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711) showed part of it to be almost identical to a 412 bp expressed sequence tag known as musf034a which was isolated from mouse testis in an effort to identify meiosis-related proteins (67). The isolated cDNA appears to be a partial fragment at the 3' end with no open reading frame.

The absence of a clear open reading frame in the cDNA clone suggested that the full length mRNA is substantially longer than 1.7 kilobases. To address this issue, the 1.7 kilobase cDNA fragment was used as a radioactively labeled probe for Northern blot analysis of WEHI-231/mycER RNA. The Northern blot suggested a full length species of approximately four kilobases (Figure 5). Thus, the full length mRNA has approximately another 2.3 kilobases that has not yet been cloned. This could encode a protein of up to 84 kilodaltons.

Analysis of MIG-1 expression and induction in other cell lines

In order to examine in more detail the expression and regulation of MIG-1, ribonuclease protection assays were done with RNA from several other cell lines. Antigen receptor

**Figure 4. Sequence of 1678 bp cDNA clone of MIG-1 isolated from WEHI-231 cDNA library.**

```
        10          20          30          40          50          60
ATTTACCTTT TTTTTTTTTT TTCCAAAAAA TTTTATTGGG GGAAACTACA AAACATTTAC
TAAATGGAAA AAAAAAAAAA AAGGTTTTTT AAAATAACCC CCTTTGATGT TTTGTAAATG

        70          80          90          100         110         120
AGTACAATGT TTACAGTCAC AATTTGTAGT GAACTGATTC CCCAAAATAT ATTACAACTC
TCATGTTACA AATGTCAGTG TTAAACATCA CTTGACTAAG GGGTTTTATA TAATGTTGAG

        130         140         150         160         170         180
AAGTTGACTT AATCTTGTTA CATTCAAAAA CCTACTTCTG TCAAAGTAGT CCAGAGTGCA
TTCAACTGAA TTAGAACAAT GTAAGTTTTT GGATGAAGAC AGTTTCATCA GGTCTCACGT

        190         200         210         220         230         240
CACGCGGTGC TCCAACTGTA CCTACATACA AACTAAACAA CTGCTCATTT ATCTGCCATC
GTGCGCCACG AGGTTGACAT GGATGTATGT TTGATTTGTT GACGAGTAAA TAGACGGTAG

        250         260         270         280         290         300
CAGGAAAGGC GGAGACATTC CTGCCTCTTT ACATTGAAAA ATAATAGTAC AAGTTTTTGG
GTCCTTTCCG CCTCTGTAAG GACGGAGAAA TGTAACTTTT TATTATCATG TTCAAAAACC

        310         320         330         340         350         360
ACTGTCATTG AACAAGGCAT ATTCATGTAC CACCAACATT TCAGCGCTAA GATGTGGCCT
TGACAGTAAC TTGTTCCGTA TAAGTACATG GTGGTTGTAA AGTCGCGATT CTACACCGGA

        370         380         390         400         410         420
CTTCACTTCA TAGCTATAGT AAAAAAAAAG TGGTAAAAAA TCTTTTCTTT TTGTGCAGGT
GAAGTGAAGT ATCGATATCA TTTTTTTTTC ACCATTTTTT AGAAAAGAAA AACACGTCCA

        430         440         450         460         470         480
GAACCCGTCC GACATTCAAA TTCTCAAGCA CGAATGAAAT ACTTATCTGG TTGAGGAAGA
CTTGGGCAGG CTGTAAGTTT AAGAGTTCGT GCTTACTTTA TGAATAGACC AACTCCTTCT

        490         500         510         520         530         540
TTTAAGGCAA GTTCGGGCCC TATGAAGGCA CTGAGACCAT TTCCCCCATT ATTGCTACAT
AAATTCCGTT CAAGCCCGGG ATACTTCCGT GACTCTGGTA AAGGGGGTAA TAACGATGTA

        550         560         570         580         590         600
GTCTCTATAA TCAATGTCAC AGTAGAGCTG GTGGTTTATA AAGCTGGATG TAGAGAAGCA
CAGAGATATT AGTTACAGTG TCATCTCGAC CACCAAATAT TTCGACCTAC ATCTCTTCGT

        610         620         630         640         650         660
AGCCACACGT GAGCCTGACT GCAGTCTCAC CTTTCTCTCT TTAAAGTTGG ATGGGCTACA
TCGGTGTGCA CTCGGACTGA CGTCAGAGTG GAAAGAGAGA AATTTCAACC TACCCGATGT

        670         680         690         700         710         720
ACCATTATAC ATTCTAAGAA AACCTCAAAA TGTTGGTCAA ACCACTTCCA CAGCATCAAG
TGGTAATATG TAAGATTCTT TTGGAGTTTT ACAACCAGTT TGGTGAAGGT GTCGTAGTTC

        730         740         750         760         770         780
ATTGCATGGT TTCTTCATGC GATCTTTCGA AATTTACGTA AACAAGGAAA GAAATTAATG
TAACGTACCA AAGAAGTACG CTAGAAAGCT TTAAATGCAT TTGTTCCTTT CTTTAATTAC

        790         800         810         820         830         840
AAATAAATAT TACATACAAT CTCTTAAATT AAGATTTTTC TTACTCATTT ACAATAAAAT
TTTATTTATA ATGTATGTTA GAGAATTTAA TTCTAAAAAG AATGAGTAAA TGTTATTTTA
```

**(Figure 4 continued)**

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          850         860         870         880         890         900
AACCAAGTGA AGTTACAAAA GGCATATATT ACTGTGAAAA GAACACACTC CACATTTTGC
TTGGTTCACT TCAATGTTTT CCGTATATAA TGACACTTTT CTTGTGTGAG GTGTAAAACG

          910         920         930         940         950         960
CGATTAATAA TGGCAATTAT TATTTAACAT AATAAAAGAA TATATATCTA TTGCTTTCAT
GCTAATTATT ACCGTTAATA ATAAATTGTA TTATTTTCTT ATATATAGAT AACGAAAGTA

          970         980         990        1000        1010        1020
CATACTCGAT AAATACAGTA TGGACAACAT CCTCAATGTA TACTTTTCAC AAGATAATAA
GTATGAGCTA TTTATGTCAT ACCTGTTGTA GGAGTTACAT ATGAAAAGTG TTCTATTATT

         1030        1040        1050        1060        1070        1080
ATAAGTTAAA TAGTTTTTCA TATTGAGTTG TGGTGCAGTG GTGCGGATCA ACTCAGAACA
TATTCAATTT ATCAAAAAGT ATAACTCAAC ACCACGTCAC CACGCCTAGT TGAGTCTTGT

         1090        1100        1110        1120        1130        1140
ACTAAAAATG AAGCACTTAT TTCCCAACAA CTATGAACTG AGCTCTGCCC ATAGCTTCCA
TGATTTTTAC TTCGTGAATA AAGGGTTGTT GATACTTGAC TCGAGACGGG TATCGAAGGT

         1150        1160        1170        1180        1190        1200
GAGAGCAGAG GAAATGGTTC TAAGCTAAAC ACCCACACTA CGTGGTGGCA ATGAGCCCGT
CTCTCGTCTC CTTTACCAAG ATTCGATTTG TGGGTGTGAT GCACCACCGT TACTCGGGCA

         1210        1220        1230        1240        1250        1260
TATCAAACTT TGGAATGCAA GGGCTTCCCC ACCCCCAGAC ATACAGAACT TTGTCTAAAA
ATAGTTTGAA ACCTTACGTT CCCGAAGGGG TGGGGGTCTG TATGTCTTGA AACAGATTTT

         1270        1280        1290        1300        1310        1320
AACAAAAACA AGGTAAACAA CCACCACAAA GGAGGCTGAG AGCGAGCGAC TCGAATCAAG
TTGTTTTTGT TCCATTTGTT GGTGGTGTTT CCTCCGACTC TCGCTCGCTG AGCTTAGTTC

         1330        1340        1350        1360        1370        1380
GCATGTTCCT GGCATTATTT TGAGTTCTAG AAGCTTATAG TTTAGGGAGG TGGCAGCCGG
CGTACAAGGA CCGTAATAAA ACTCAAGATC TTCGAATATC AAATCCCTCC ACCGTCGGCC

         1390        1400        1410        1420        1430        1440
CTGGCTCACA CTGGTCTTGG AACCCCTTCC AAGAGTTCTC ACATGTGGAC TGTTTCTAAG
GACCGAGTGT GACCAGAACC TTGGGGAAGG TTCTCAAGAG TGTACACCTG ACAAAGATTC

         1450        1460        1470        1480        1490        1500
GCTTTCGAGT GAAAGAAGAG ATGGAATGGT GGTTGGTAGC TGCTGGTGCT GGGNTGATTT
CGAAAGCTCA CTTTCTTCTC TACCTTACCA CCAACCATCG ACGACCACGA CCCNACTAAA

         1510        1520        1530        1540        1550        1560
TTTTGTTGTA AAACTAGAGT TTCAAACTCA TCTGAGACCT AAGTGTTTGG TATCTTTTGT
AAAACAACAT TTTGATCTCA AAGTTTGAGT AGACTCTGGA TTCACAAACC ATAGAAAACA

         1570        1580        1590        1600        1610        1620
TCTTTCCGCA TCACAACCGC CCTGTTGTGA ATATTTTGTT CATCCATACG AAAAAGTTCG
AGAAAGGCGT AGTGTTGGCG GGACAACACT TATAAAACAA GTAGGTATGC TTTTTCAAGC

         1630        1640        1650        1660        1670
TTGGCTTTTA TCAACAAGAA CGAATCTTTA CAATAATAAT AATAATAAAA ATAATAAT-polyA
AACCGAAAAT AGTTGTTCTT GCTTAGAAAT GTTATTATTA TTATTATTTT TATTATTA-polyT
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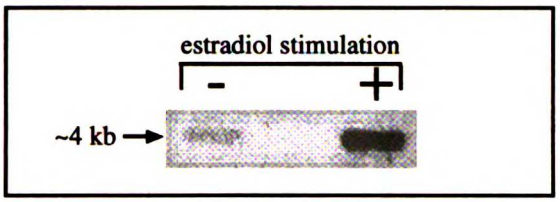


**Figure 5. Northern blot analysis of MIG-1 mRNA expression.** Using the ~1.7 kb cDNA fragment isolated from a WEHI-231 cDNA library, WEHI-231/mycER RNA was probed and showed an estradiol-induced species of approximately 4 kb.

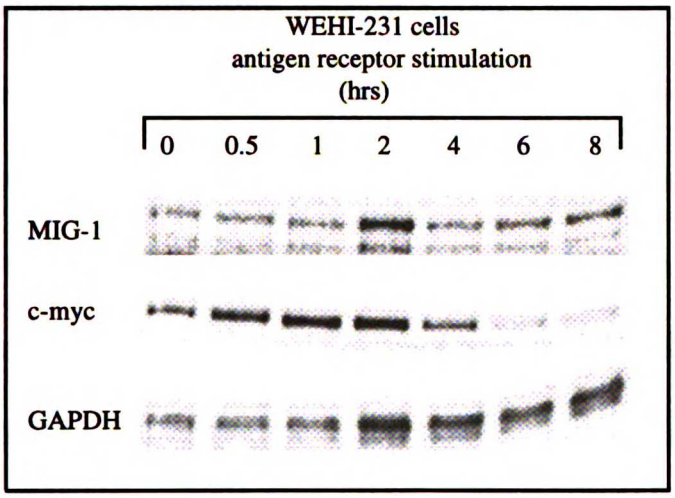


**Figure 6. Lack of induction of MIG-1 in antigen receptor-stimulated WEHI-231 cells.** The ribonuclease protection assay was done with RNA from antigen receptor-stimulated WEHI-231 cells using MIG-1, c-myc, and GAPDH probes. When GAPDH was used to correct for variations in RNA content between samples, there was essentially no induction of the MIG-1 species upon antigen receptor stimulation. Note the induction and subsequent inhibition of c-myc expression, as previously reported.

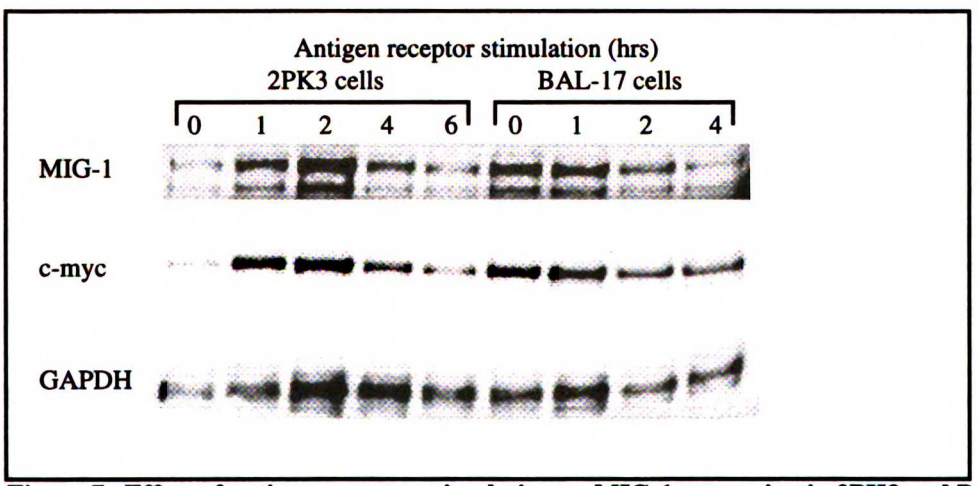


**Figure 7. Effect of antigen receptor stimulation on MIG-1 expression in 2PK3 and BAL-17 cells.** A ribonuclease protection assay was done on RNA from antigen receptor-stimulated 2PK3 and BAL-17 cells using MIG-1, c-myc, and GAPDH probes. After correcting for RNA content using GAPDH, 2PK3 cells showed a peak induction of MIG-1 by 2 hours with a subsequent decline. BAL-17 cells showed relatively high basal levels of MIG-1 which declined upon stimulation. Note that the MIG-1 expression pattern corresponded with the c-myc expression pattern in these two cell lines. The BAL-17 results have been confirmed by duplicate experiments. The 2PK3 results reflect only one experiment.

stimulation of WEHI-231 cells failed to significantly induce expression of this gene, although low levels of basal expression appeared to be present (Figure 6). However, antigen receptor stimulation of the mature, IgG+ B cell line, 2PK3, which does not undergo apoptosis, showed a two- to three-fold corrected induction of the gene by two hours after stimulation which then quickly returned back to basal levels (Figure 7). The mature, IgM+ B cell line, BAL17, showed relatively higher basal expression of MIG-1, and this expression declined two hours after antigen receptor stimulation and showed significantly further decrease by four hours after stimulation (Figure 7). As can be seen in the figures, the expression pattern for MIG-1 follows that of endogenous c-Myc in the 2PK3 and BAL-17 cells. The BAL-17 results have been confirmed by experiments done in duplicate. The 2PK3 results reflect only one experiment.

Preliminary results suggest that the MIG-1 species is induced in WEHI-231/mycER cells stimulated with estradiol in the presence of the protein synthesis inhibitor, cycloheximide (data not shown). This suggests that new protein synthesis is not needed for induction of the gene, which would be consistent with the gene being directly activated by c-Myc. Preliminary results also suggest that there is no significant induction of the MIG-1 species upon apoptosis-inducing antigen receptor stimulation of the CH31.LX cell line, upon antigen receptor stimulation of splenic B cells, or upon lipopolysaccharide stimulation of the 70Z/3 cell line (data not shown). The CH31.LX cells show very low

basal expression of the gene, whereas splenic B cells and 70Z/3 cells show significant basal levels of expression. All of these experiments need to be repeated to confirm the above results.

## DISCUSSION

I have identified a novel gene, MIG-1, regulated by c-Myc in certain B lymphocyte cell lines. The close association of expression of this novel gene with c-*myc* expression and preliminary data from experiments in which mRNA induction was observed in the presence of a protein synthesis inhibitor suggests that this gene may be directly regulated by c-Myc. Only a portion of this gene has been cloned to date. MIG-1 shows a high degree of homology to a reported expressed sequence tag identified as musf034a. The obtained cDNA clone of MIG-1 does not contain an open reading frame, so further work needs to be done to obtain a full length clone and determine the sequence of the coding region.

After trying the differential display technique with approximately thirty primer pairs, only one differentially expressed gene was found in the WEHI-231/mycER system. It is likely that the cellular mRNA population has not been exhaustively examined by these experiments, so by continuing the differential display search with additional primer pairs or at different times after activation of mycER perhaps other differentially expressed genes can be found. Nonetheless, it appears that relatively few genes are regulated by c-Myc in the

WEHI-231/mycER system.

One explanation for this finding may be that the differential display system was not optimized to amplify many different cellular mRNA and that a relatively small number of cellular mRNAs were sampled. The best experiments with a primer pair resulted in thirty to fifty bands on the gel; if each band represents one mRNA species then only approximately 900 to 1500 mRNAs would have been sampled, representing perhaps one-tenth of the number of mRNAs expressed at one copy per cell or more. In this case, it would require experiments with at least two hundred separate primer pairs to cover the approximately ten thousand expressed mRNA species in a cell. Another possible explanation is that endogenous c-Myc present in the proliferating WEHI-231 cell line is interfering with our ability to detect differentially expressed genes since differential display is best at detecting quantitative differences where a species goes from minimal or no expression to significant expression. The only genes detected may be those that are not induced by low c-Myc activity but only by the higher c-Myc transcriptional activity that occurred upon induction of mycER. Given the dramatic biologic effect seen after mycER activation, one would expect significant new gene expression, but it is conceivable that differences in level of expression rather than new expression of genes accounts for the cellular response. Perhaps trying the differential display experiments in a mycER system where endongenous c-Myc is not expressed (i.e. quiescent fibroblasts) would enhance the

ability to detect mycER-regulated genes. A final possibility is that relatively few genes are truly being regulated by mycER in this system and are sufficient to account for the dramatic apoptotic response.

Interestingly, the identified gene, MIG-1, is induced by mycER in mycER-transfected WEHI-231 cells, but not by apoptosis-inducing antigen receptor stimulation. Since antigen receptor stimulation does induce a transient increase in c-Myc level, one would expect this stimulation to induce a c-Myc-induced gene. It is possible that the induction of the gene seen upon mycER activation reflects a significant overexpression of c-Myc activity and that antigen receptor stimulation does not elicit a strong enough increase in c-Myc activity to induce expression of the gene. It is hard to compare the activity of c-Myc between the mycER system and the endogenous c-Myc system. The mycER chimera contains human c-Myc and requires detection by anti-human myc antibody. Thus, it is difficult to compare protein levels with endogenous murine c-Myc which must be detected by an anti-mouse Myc antibody. Also, it is unknown what fraction of the expressed mycER protein is activated upon addition of ligand. Alternatively, it is possible that the antigen receptor stimulation induces separate signaling events which inhibit induction of MIG-1 by c-Myc.

In any case, the absence of induction upon antigen receptor stimulation suggests that the identified gene is not a necessary part of the apoptotic pathway. This is also reflected by the absence of its induction by antigen receptor stimulation in CH31.LX cells, which do

undergo apoptosis. Induction of a c-Myc-induced gene by antigen receptor stimulation would have lended support to the hypothesis suggested by the previously presented antisense data that antigen receptor-induced apoptosis acts by increasing c-Myc activity. The absence of antigen receptor stimulated-induction of a c-Myc-induced gene suggests that antigen receptor stimulation and mycER may work via different pathways to induce apoptosis (a possibility suggested by the observation that antigen receptor stimulation results in cell cycle block before apoptosis while mycER induces apoptosis from all stages of the cell cycle), or that antigen receptor stimulation may induce a distinct signaling event that collaborates with a minimally required basal level of c-Myc to induce apoptosis.

Evidence for regulation of MIG-1 by endogenous c-Myc is provided in the 2PK3 and BAL17 cell lines. Antigen receptor stimulation in the 2PK3 cell line caused a transient increase in MIG-1 expression which correlated with a transient increase in endogenous c-*myc* RNA levels. In the BAL17 cell line, antigen receptor stimulation resulted in a decrease in MIG-1 expression which correlated with a decrease in c-*myc* RNA levels. These results suggest a possible role for MIG-1 in antigen receptor-mediated regulation of B cells.

It is difficult to speculate at this time what the function of this c-Myc-regulated gene might be given the seemingly arbitrary presence of regulation in some c-Myc systems but not in others. Obtaining a full length cDNA clone with an open reading frame will allow further speculation as to its function based on sequence homologies. Further experiments

pursuing tissue expression patterns, expression vector effects, and MIG-1 knock-out mice may provide additional hints regarding the potentially exciting role of this novel c-Myc-regulated gene.

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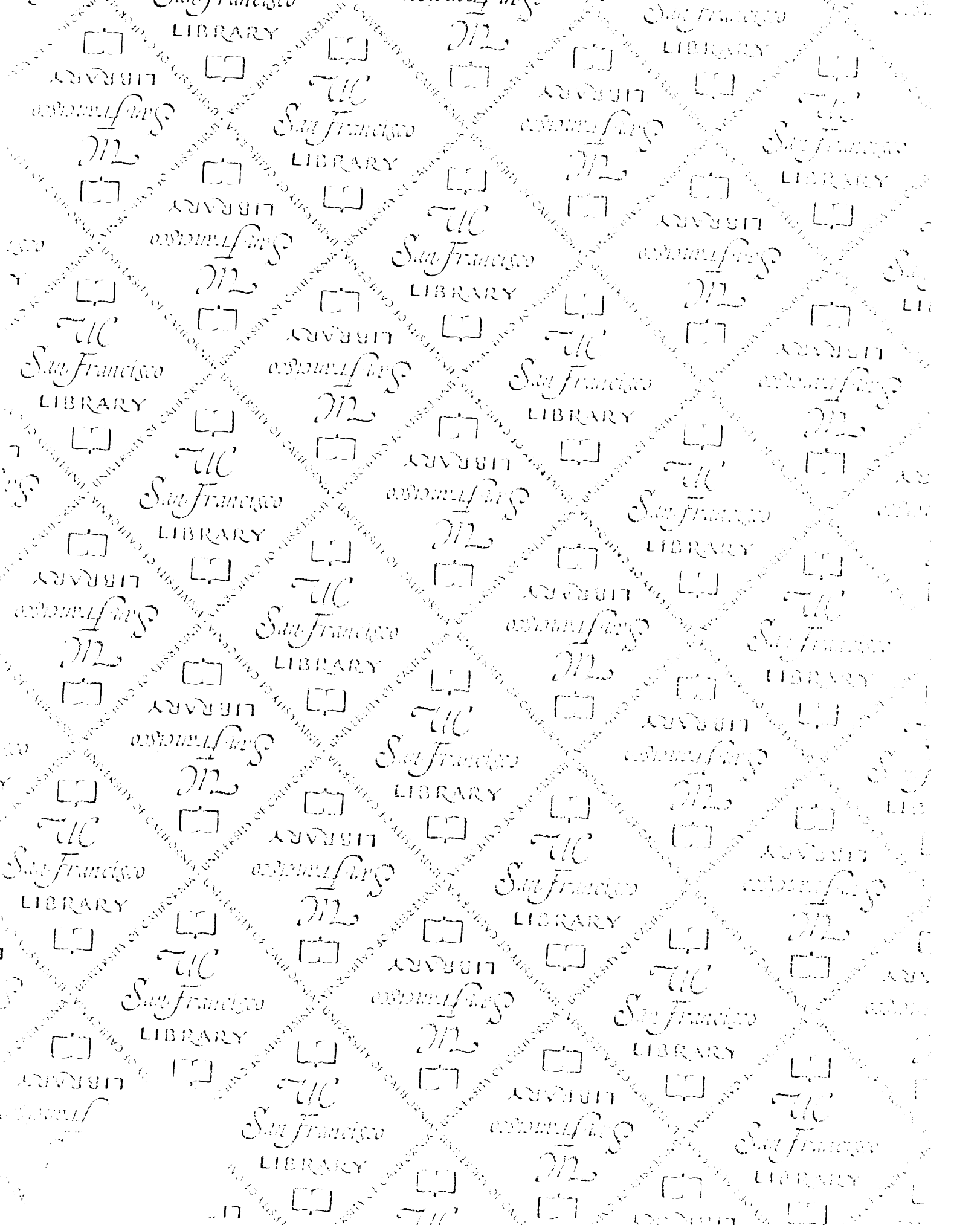
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