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GRANT NUMBER DAMD17-94-J-4102

TITLE: Regulation and Mechanism of Action of the c-Myc Proto-
Oncogene in Human Breast Cancer

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REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

19990811 132

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1998	3. REPORT TYPE AND DATES COVERED Final (1 Oct 94 - 30 Sep 98)		
4. TITLE AND SUBTITLE Regulation and Mechanism of Action of the c-Myc Proto-Oncogene in Human Breast Cancer		5. FUNDING NUMBERS DAMD17-94-J-4102		
6. AUTHOR(S) Dr. Kathryn Calame				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Columbia University New York, New York 10032		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, MD 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE		
13. ABSTRACT (Maximum 200) c-Myc is one of the proto-oncogenes which is known to play a role in breast cancer. c-Myc is elevated in both hormone-dependent and hormone-independent breast cancers and the causes of dysregulation appear to differ. In this work, we have focused on three different aspects of c-Myc regulation and mechanism of action. First, we have shown that one mechanism by which c-Myc acts is to associate with the transcription regulator YY1 and inhibit its ability to regulate transcription. Our data suggest that this mechanism is particularly important in situations in which c-Myc levels are elevated. Second, we have identified a new repressor of c-myc transcription. There is strong evidence that this protein, Blimp-1, is important during terminal differentiation of B lymphocytes; however, we also present evidence suggesting that it may be involved in terminal differentiation of many other lineages. As a repressor of c-myc transcription, Blimp-1 is a good candidate for a tumor suppressor. Finally, we have studied the mechanisms responsible for estrogen-dependent induction of c-Myc in hormone-dependent breast cancer cell lines. Our data show that estrogen increases both the initiation and processivity of c-myc transcription.				
14. SUBJECT TERMS c-Myc Proto-Oncogene, Transcription, Estrogen Receptor, Yin-Yang-1, Growth Control, Cell Transformation, Breast Cancer			15. NUMBER OF PAGES 43	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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Anthony Calame 10/26/98
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INTRODUCTION

c-Myc Is a Growth Regulation and Can Be a Potent Oncogene.

The *c-myc* proto-oncogene encodes a ubiquitously expressed nuclear phosphoprotein (Evans and Littlewood, 1993; Luscher et al., 1989; Marcu et al., 1992). In normal cells c-Myc is required for cell proliferation, is repressed when cells become post-mitotic and terminally differentiated and is important for apoptosis in several physiological settings. Consistent with its role in normal growth regulation, levels of c-Myc have been shown to be elevated in many malignant tumors, including estrogen-dependent and estrogen-independent breast cancers (Ali et al., 1988; Mariani-Costantini et al., 1988).

c-Myc functions as a transcriptional regulator. However, despite clear evidence that c-Myc is important in the control of cellular proliferation, differentiation, apoptosis and transformation the molecular mechanisms by which c-Myc functions are poorly understood. c-Myc has DNA-binding, dimerization and transactivations domains common to other transcriptional activators. Upon dimerization with with partner, Max, c-Myc/Max heterodimers can activate the *Cdc25A* (Galaktionov et al., 1996), *ECA39*, α -prothymosin (Eilers et al., 1991), and ornithine decarboxylase genes. However the number of known c-Myc/Max-regulated genes remains small, suggesting that important facets of c-Myc action remain uncharacterized. In addition, some mutants of c-Myc are defective in transformation ability but not in transcriptional activation ability suggesting that functions other than transcriptional activation may be important for c-Myc function (Li et al., 1994).

c-Myc can also suppress the expression of specific genes including the major histocompatibility complex class I antigens HLA-A2 and HLA-C, cyclin D1 (Philipp et al., 1994), integrin LFA1, adhesion receptor NCAM and transcription factor C/EBP α (Li et al., 1994). c-Myc also represses its own transcription. No c-Myc/Max binding sites have been identified in the regulatory regions of these genes and the mechanism(s) by which Myc suppresses their transcription is poorly understood. The work reported here has described a novel activity of c-Myc which involves its inhibition of the activity of another transcription protein, YY1.

One Mechanism for c-Myc Activity May Involve Association With YY1

We have previously shown that c-Myc can physically associate *in vitro* and in the yeast two-hybrid system with transcription protein YY1 (Shrivastava et al., 1993). YY1 is a ubiquitously expressed zinc finger transcription factor which functions as a transcriptional repressor, activator or initiator, depending upon the context of its binding site (Shi et al., 1996). YY1 binding sites are widely distributed in many cellular and viral promoters (Shrivastava and Calame, 1994). Association with c-Myc inhibits the transcriptional activating and repressing abilities of YY1. We have previously suggested that c-Myc may regulate transcription of YY1-dependent genes.

Since regulation of YY1 activity could provide an additional mechanism for c-Myc-dependent transcription regulation, we have investigated the physiological relevance and biochemical consequences of the YY1/c-Myc association. In this work we have confirmed the physiological relevance of the c-Myc-YY1 association using coimmunoprecipitations.

We have also explored the mechanism by which c-Myc inhibits YY1 activity. Three models were initially considered to explain how c-Myc might alter YY1 activity: i) Myc might inhibit the ability of YY1 to bind DNA, ii) Myc might inhibit the ability of YY1 to associate with other transcription proteins or iii) a Myc/Max dimer might associate with YY1 and sequester YY1 to Myc sites on DNA. Our previous data ruled out the possibility that ternary

complexes of YY/c-Myc/Max sequestered YY1 by binding to Myc/Max sites. Therefore, we tested two other models.

Blimp-1 and Repression of *c-Myc* Transcription During Terminal Differentiation of B Lymphocytes

Previously we identified a protein binding at -290 bp from P1 in the *c-myc* promoter which was restricted to plasmacytoma cell lines and, based on limited tissue distribution, suggested that it might be a negative regulator of *c-myc* transcription (Kakkis and Calame, 1987; Kakkis et al., 1988; Kakkis et al., 1989). In Burkitt lymphoma and murine plasmacytomas (PCs), the translocated *c-myc* allele is aberrantly expressed but the normal allele is transcriptionally silent (Marcu et al., 1992). We suggested that the putative negative regulator might repress the normal, transcriptionally silent allele. Subsequently, the *c-myc* -290bp site was deleted by site-directed mutagenesis and wildtype and mutant promoters were stably transfected into different cell lines. In fibroblasts and early B cells, where the -290 bp protein is absent, mutation of the site did not alter *c-myc* promoter activity; however, in plasmacytomas, where the protein is present, deletion of the site resulted in a significant (30X) increase in *c-myc* promoter activity. In work reported here we have identified this repressor of *c-myc* transcription as the zinc finger protein Blimp-1.

Blimp-1 (B lymphocyte induced maturation protein) was identified in a subtractive screen as a protein which is induced upon stimulation of the BCL1 B-cell lymphoma line with IL-2 + IL-5 (1), a treatment which induces terminal differentiation. Blimp-1 mRNA was detected by Northern analyses in that study only in spleen, B-cell lymphomas and plasmacytomas (Turner et al., 1994), a pattern consistent with a role in terminal B-cell differentiation. Ectopic expression of Blimp-1 in BCL1 cells can drive B-cell terminal differentiation as evidenced by J-chain transcription, immunoglobulin secretion and expression of Syndecan on the cell surface. Sequence analysis of Blimp-1 cDNA revealed that the protein contained five zinc fingers, strongly suggesting that it was a DNA-binding protein, possibly a transcription factor (Turner et al., 1994). Such a function would be very consistent with its role as a master regulator of terminal differentiation because one might imagine that Blimp-1 would control the expression of an array of genes which would be sufficient to initiate terminal differentiation. However, the initial report did not identify a DNA binding sequence for Blimp-1 or any genes which were direct targets of Blimp-1.

The role of Blimp-1 in B-cell development has been studied further. Blimp-1 expression in B cells was found to correlate with Ig secretion and it was suggested that Blimp-1 may play a role in the B-cells' choice between a memory or plasma cell fate. The polyclonal mitogen LPS induces Ig secretion, Blimp-1 expression and B-cell differentiation of splenic B cells (Schliephake and Schimpl, 1996). IL-5 and the IL-5 receptor appear to be important for inducing Blimp-1 in B cells (Turner et al., 1994) and ectopic expression of the early B cell transcription protein BSAP blocks B cell differentiation, Ig secretion and Blimp-1 expression (Usui et al., 1997). Although Blimp-1 appears to activate J-chain transcription, the effect may be indirect since no Blimp-1 binding site or response element has been found in the J chain gene (M. Koshland, pers. communication).

Only a few "master regulators" which are capable of initiating an entire developmental program have been identified in mammalian cells. Blimp-1 is the first of these "master regulators" shown to regulate the *c-myc* gene. This finding is consistent with previous studies showing that expression of *c-myc* blocks terminal differentiation and with the identification of Blimp-1 as a protein that causes terminal B-cell differentiation. Our demonstration that Blimp-1 represses *c-myc* as part of a program of terminal differentiation makes it possible, for the first time, to study an aspect of *c-myc* regulation which is directly

associated with a developmental decision to halt proliferation and to proceed with terminal differentiation.

In addition to studying the role of Blimp-1 in B cell differentiation, we have addressed three further questions. First, is Blimp-1 expressed outside of the B-cell lineage? The original report of Blimp-1 characterized it as a B-cell specific protein. However, we show that it is expressed in many non-B cell and non-hematopoietic lineages. Secondly, we have studied the mechanism by which Blimp-1 represses transcription. Finally, we have asked if ectopic expression of Blimp-1 might alter the growth phenotype of breast cancer cell lines or other transformed lines where the transformed phenotype depends on the presence of c-Myc.

Expression of the *c-Myc* Proto-oncogene Is Elevated in Many Breast Tumors.

Elevated levels of c-Myc have been found in both estrogen-dependent and estrogen-independent breast tumors (Ali et al., 1988; Mariani-Costantini et al., 1988). In cell lines derived from estrogen-dependent breast tumors, estrogen induces *c-myc* mRNA by a poorly understood mechanism (Musgrove et al., 1993; Shiu et al., 1993; Sutherland et al., 1992). In many hormone-independent breast tumors, the *c-myc* gene is amplified, resulting in increased mRNA and protein. However, increased expression of *c-myc* mRNA and protein has also been observed in hormone-independent breast tumors where the gene is not amplified.

Approximately 40% of breast cancer patients have tumors which are estrogen-dependent for growth; however, after anti-estrogen therapy most tumors become estrogen-independent (Shiu et al., 1993). We are particularly interested in understanding the mechanisms which mediate estrogen-inducible *c-myc* expression in estrogen-dependent breast cancer cell lines because this should help us understand important regulatory mechanisms in early breast tumors.

c-Myc Is Necessary for Growth of Breast Cancer Cell Lines and Elevated c-Myc Is One Causal Step in Malignant Transformation of Breast Tissue.

Antisense *c-myc* oligonucleotides have been shown to inhibit *c-myc* expression and growth of both estrogen-dependent and estrogen-independent breast cancer cell lines (Watson et al., 1991). This directly establishes that c-Myc protein is required for growth of breast cancer cell lines. A similar requirement for c-Myc function has been demonstrated in other transformed cell lines (Sawyers et al., 1992).

Although over-expression of c-Myc alone does not cause malignant transformation of mammary epithelial cells, the elevated levels found in many breast tumors suggest it can be one causal step in transformation (Ali et al., 1988). This possibility is corroborated by the demonstration that inhibiting *c-myc* expression in breast cancer cell lines inhibits their growth (Dubik and Shiu, 1988). It is also corroborated by experiments using mice. Expression of a *c-myc* transgene driven by the murine mammary tumor virus long terminal repeat caused a dramatic increase in murine mammary tumors (Leder et al., 1986) and expression of the *v-myc* gene caused preneoplastic growth in primary mammary epithelial cells transplanted into mammary fat pads (Edwards et al., 1988).

The Estrogen-dependent Expression of *c-Myc* Has Been Studied in Hormone-Dependent Breast Cancer Cell Lines.

c-Myc mRNA increases rapidly (within one hour) after estrogen stimulation of human breast cancer cells lines which are hormone-dependent. The extent of mRNA induction is 10-12 fold (Dubik and Shiu, 1988). The induction occurs without new protein synthesis and transcripts from both promoter P1 and promoter P2 are increased. Dubik et al demonstrated a rapid increase in transcription initiation by a nuclear run-on transcription assay; however, they used only one, double-stranded probe which precluded any assessment of polymerase pausing or premature termination within exon one and did not distinguish sense and anti-sense transcripts (Dubik and Shiu, 1988). Another study, using a similar probe, in which later times were analyzed failed to show that estrogen increased transcriptional initiation (Santos et al., 1988). Thus, it seems likely that estrogen affects either initiation or processivity of *c-myc* transcription but additional data are needed to confirm and clarify this important point. In this work we have re-investigated this question.

In other genes estrogen induction of transcription initiation has been well-documented (Lucas and Granner, 1992). In these cases, estrogen binds the estrogen receptor (ER), a zinc finger protein which binds to DNA sequences called estrogen response elements (EREs). Binding of ER to EREs confers altered chromatin conformation and increases transcription initiation. Depending on the context, ER sometimes requires interaction with other proteins to mediate its transcriptional effects (Philips et al., 1993).

Estrogen response elements (EREs) have been sought in the *c-myc* gene but a consensus ERE is not evident. In one study *c-myc*- promoter-dependent reporters were either transfected into MCF-7 cells or cotransfected into HeLa cells with an estrogen receptor expression vector (Dubik and Shiu, 1992). 116 bp surrounding *c-myc* promoter P2 were sufficient for estrogen responsiveness of the reporter in HeLa cells but transcription of the reporter was not inhibited by the antiestrogen tamoxifen even though tamoxifen inhibits transcription of the endogenous *c-myc* gene in MCF-7 cells. Tamoxifen is known to compete with estrogen for binding to one of the activation domains of the estrogen receptor (ER) (Berry et al., 1990) and estrogen-dependent effects mediated by the ER should be inhibited by tamoxifen; therefore the transfection study may not adequately reflect normal estrogen activation of *c-myc*. Neither the 116 bp region identified in this study, nor a larger region surrounding the human *c-myc* gene contains a consensus ERE sequence (Merrell and Calame, unpubl.). Thus, the mechanism by which estrogen induces *c-myc* expression in human breast cancer cells is not well-understood and may be complex or unusual compared to other estrogen-inducible genes. In this work, we have investigated this important regulatory event.

BODY

Experimental Methods

Plasmid construction. Full length YY1 cDNA, 1.8 kbp, was excised from pGEM-4Z-YY1 (Hariharan et al., 1991) by Nco I digestion, end-filling and BamH I digestion, and inserted into Bam HI and Sma I restriction sites of pCGN. The His-YY1 plasmid was constructed by ligating Kpn I fragment from pCGN-YY1 into the pQE32 vector (Qiagen). Gst-Myc was constructed by ligating 600 bp PCR fragment of *c-Myc*, corresponding to 259-439 amino acids, into the Bam HI and Sma I sites of pGEX-3X vector. The PCR fragment was generated by using oligos that put Bam HI site at 5' end.

Purification of proteins. His-YY1 was expressed by IPTG induction of bacteria containing the YY1 expression plasmid. Protein was purified by binding to a Ni-NTA resin (as described in Qiagen protocol). Gst-fusion proteins were expressed and purified on glutathione agarose as described (Artandi and Calame, 1993).

GST assays GST assays were done as described (Artandi and Calame, 1993). Association assays were done in buffer that had final conditions of 50 mM NaCl, 7 mM CaCl₂, 10 mg/ml BSA, 5 mM DTT, 1 mM PMSF, 20 mg/ml of aprotinin, leupeptin and pepstatin.

Antiserum preparation. Murine c-Myc antiserum was generated by injecting bovine albumin serum (BSA) coupled to a synthetic peptide representing the C-terminal 13 amino acids of murine c-Myc into rabbits.

Co-immunoprecipitation. 2x10⁷ NIH 3T3, MEL or M12 cells were washed, resuspended in buffer X (50 mM Tris pH 7.5, 50 mM NaCl, 7 mM CaCl₂, 10 mM EDTA, 5 mM DTT, 0.5% NP40, 1 mM PMSF, 20 mg/ml of pepstatin, leupeptin and aprotinin), sonicated on ice and centrifuged for 30 minutes at 13000 rpm to obtain lysates used for coimmunoprecipitation. Lysate was precleared by incubating 20 minutes with protein A-sepharose beads in buffer X. Lysate was kept on ice for 1 hr, incubated with anti-c-Myc antisera for 2 hrs, and then protein A-sepharose beads for 4 hrs. Proteins bound to beads were resolved by SDS-PAGE and visualized by immunoblotting with anti-c-Myc polyclonal antiserum and anti-YY1 monoclonal antibody. One tenth of each immunoprecipitate was used for the c-Myc blots and 9/10 for the YY1 blots.

BCL₁ cell differentiation and analysis. BCL₁ cells in culture were treated with IL2 + IL5 and whole cell extracts were prepared at various times. Ten µg of each sample was electrophoresed on SDS-polyacrylamide gels (8%), transferred to a nitrocellulose membrane and immunoblotted with polyclonal antiserum raised against the C-terminus of murine c-Myc. The bands were quantitated and the amounts relative to that prior to treatment (0 hr.) were calculated. RNA was also prepared and analyzed by Northern blotting using a *blimp-1* cDNA probe and a control *β-actin* probe.

Ectopic expression of Blimp-1 in 18-81 cells. A pBJ-neo plasmid containing either antisense (Blimp AS) or sense (Blimp S) *blimp-1* cDNA was cotransfected into 18-81 pre-B cells with the pSV₂ c-Myc expression vector or a pSV₂ control vector. Cells were diluted into 96 well plates, cultured with 800 mg/ml G418 and resistant colonies were counted 10 days later. For inducible expression, the *blimp-1* cDNA was cloned into a plasmid under the control of the sheep metallothionein promoter and stably transfected into 18-81 pre-B cells using selection in G418.

Northern Analyses Total RNA was prepared by lysing cells with 4 M guanidium thiocyanate, 25mM sodium citrate, 0.5% n-lauryl sarcosine, 100mM mecaptoethanol. Lysates were pelleted through a 5.7 M CsCl cushion by centrifuging for 12 hr at 36,000 rpm in a SW50 rotor. For northern blots, 40 µg of total RNA was resuspended in 1X formaldehyde gel-buffer (0.1M MOPS pH 7.0, 40 mM sodium acetate, 5mM EDTA), 17.5% formaldehyde, 50% formamide, 1X gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and separated on a formaldehyde-agarose gel (1% agarose, 1X formaldehyde gel buffer, 2.2 M formaldehyde). RNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) by capillary action using 20X SSC (3M NaCl, 0.3M Sodium Citrate). RNA was fixed to the membrane by baking for 2 hr at 80°C and

hybridized with radiolabeled probes at 65°C in 1.5X SSPE (0.23 M NaCl, 1.5×10^{-2} M NaH_2PO_4 , 1.5×10^{-3} M EDTA), 10% Polyethelene glycol, 7% SDS and 0.1 mg/ml salmon sperm DNA for at least 12 hours. Filters were washed once at 22°C in 2X SSC, 0.1% SDS for 15 min, once at 65°C in 1X SSC for 15 min, 0.1% SDS and once at 65°C in 0.3X SSC, 0.1% SDS for 15 min. Filters were stripped by washing at 100°C in 0.1% SDS. Radiolabeled probes with a specific activity of $> 2 \times 10^8$ were generated by random priming gel isolated DNA fragments according to the manufacturer's specifications (United States Biochemical, Cleveland, OH).

Electrophoretic Mobility Shift Assays. These assays were performed as described previously (Peterson et al., 1986) using double-stranded oligonucleotides corresponding to the murine *c-myc* PRF site at -290 as probe. Super-shifts were performed as described previously having wt sequence or a mutation at the PRF site.

Cell Culture. HL-60 promyelocytic and U937 promonocytic cell lines were cultured in RPMI supplemented with 10% fetal calf serum. To initiate macrophage differentiation, HL-60 or U937 cells were supplemented with phorbol myristic acid (PMA). Using flow cytometry we monitored Mac-1(CD11b), ICAM and Class II MHC, which are expressed during macrophage differentiation. Differentiation was also monitored by staining for nonspecific and specific esterase activity (Yam et al., 1971). Granulocytic differentiation of HL-60 cells was initiated by culturing the cells in DMSO. It was monitored by NBT (Nitroblue Tetrazolium) which is converted to insoluble intracellular blue formazan by superoxide (Collins et al., 1979). MCF-7 cells were grown under + estrogen conditions in IMDM supplemented with 10 μ g/ml of insulin and 10% fetal bovine serum (FBS). To estrogen-deplete the cells, plates were washed once with PBS at 70% confluency and growth-arrested using phenol red-free IMDM supplemented with 10% charcoal stripped FBS for 48 hours.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay. 18-81 cells (2×10^3) were centrifuges to slides. Cells were treated with proteinase K, fixed with 4% paraformaldehyde at room temperature for 10 min., washed with PBS and TdT buffer (0.5 M cacodylate, 1mM CoCl_2 , 0.5mM DTT, 0.05% BSA and 0.15M NaCl). 300 μ l of TdT labeling mixture (20U TdT, 0.5 μ l biotin-16-dUTP in 100 μ l of TdT buffer) was added to each slide and incubated for 1 hr. at 37°C. The slides were washed and blocked with 2% BSA, washed further and then developed with 300 μ l developing buffer (45 μ l 4-nitro blue tetrazolium chloride, 35 μ l phosphate and 24 μ l levamisol) was added and incubated for 15 min. at room temperature. Finally the slides were dehydrated (1x 70% ethanol, 2x 95% ethanol, 2x 100% ethanol, 2x xylene) and mounted with histoclear.

Non-radioactive In Situ Hybridization to detect Blimp-1 mRNA. These assays were carried out using established protocols (Wilkinson, 1992; Holtke et al., 1992; Schaeren-Weimers and Gerfin-Moser, 1993). The DIG-labeled RNA probe is synthesized from linearized DNA templates in both anti-sense and sense (as control) orientations. Paraffin-embedded tissue sections on microscope slides are pre-treated with a series of dewaxing, rehydration, permeabilization, fixation, and prehybridization before hybridized with the DIG-probes. Hybridized DIG-probes are subsequently recognized and bound by Anti-DIG antibody. The alkaline phosphatase conjugated to the anti-DIG antibody couples a redox reaction of BCIP and NBT which deposits a purple-blue colored precipitate specifically in the cells expressing the target mRNAs.

Nuclear runon assay. MCF-7 or T47D cells were grown under + estrogen conditions in IMDM supplemented with 10 μ g/ml of insulin and 10% FBS. To estrogen-deplete the cells, plates were washed once with PBS at 70% confluency and growth-arrested using phenol red-free IMDM supplemented with 10% charcoal stripped FBS for 48 hours. Transcriptionally active nuclei were isolated by trypsinizing 2x10⁸ MCF-7 cells, washing them twice in PBS, and lysing them in NP-40 lysis buffer. Lysed cells were incubated for 5 minutes on ice, the nuclei pelleted by spinning at 200xg in a Beckman Model T-J6 centrifuge and washed once in NP-40 lysis buffer. Nuclear run-on transcription was performed by adding 40 μ l of 10 mCi/ml ⁻³²P]UTP and incubating 45 minutes at 30°C with shaking. To digest DNA and protein, RNase-free DNase I and proteinase K were added to the transcription reaction and incubated for 5 minutes at 30°C and 30 minutes at 42°C, respectively. RNA was extracted with an equal volume of 25:24:1 buffered phenol/chloroform/isoamyl alcohol and TCA-precipitated in the presence of tRNA carrier. The precipitate was filtered onto a 0.45 μ m Millipore HA filter, washed, and treated again with DNase I and proteinase K. RNA was eluted from the filter by heating to 65°C for 10 minutes and extracted once more with 25:24:1 phenol/chloroform/isoamyl alcohol, then ethanol precipitated and resuspended in TES/NaCl solution. Hybridization of RNA to cDNA immobilized on a nitrocellulose membrane strip was performed in a scintillation vial for 36 hours at 65°C in a total volume of 1 ml with a probe activity of 10⁷ cpm/ml. Washes were carried out in 2x SSC at 65°C followed by treatment of the filter with RNase A to remove single stranded overhanging ends.

DNase I hypersensitive site mapping. 5x10⁷ MCF-7 cells were trypsinized and washed twice with ice cold PBS, then lysed in 3 ml of cold RSB (10 mM Tris pH 7.9, 10 mM NaCl, 5 mM MgCl₂) by adding NP-40 to a final concentration of 0.35%. Lysis was allowed to proceed for 5 minutes on ice and checked for completeness under the microscope; if nuclei did not appear free of membranous material, they were gently pipeted up and down and left in lysis solution on ice for another 5 minutes. Nuclei were pelleted by spinning at 200xg in a Beckman Model T-J6 tabletop centrifuge, resuspended in 2 ml of cold KPS (85 mM KCl, 5 mM Pipes pH 7.5, 5.5% sucrose, 0.5 mM spermidine), and aliquots of 200 μ l pipeted into eppendorf tubes for subsequent DNase I digestions. Nuclei were pelleted by spinning at 200xg in an eppendorf microfuge and resuspended in cold DNase I digestion buffer (KPS supplemented with 3 mM MgCl₂ and 0.5 mM CaCl₂) at approximately 5x10⁷ nuclei/ml. DNase digestions were carried out at room temperature for four minutes at DNase I concentrations of 0 - 100 U/ml and stopped by adding four volumes of STOP buffer (12.5 mM EDTA, 0.3125% SDS) and Proteinase K to 50 mg/ml. DNA was incubated overnight at 37°C and purified by two phenol/sevag extractions followed by two sevag extractions and ethanol precipitation. DNA pellets were washed in 70% ethanol and resuspended in 100 μ l of TE, and DNA concentrations in each tube calculated from O.D. readings at 260nm. 30 μ g of DNA from each tube were digested to completion with a suitable restriction enzyme and hypersensitive sites determined using a standard Southern Blot protocol and a probe hybridizing to either the 5' or 3' end of the restriction fragment of choice.

Results and Discussion

Association with c-Myc Does Not Inhibit Binding of YY1 to DNA.

We know that c-Myc associates directly with YY1 and inhibits its ability to activate or repress transcription (Shrivastava et al., 1993). One mechanism for how c-Myc inhibits YY1 activity is that it might block YY1 binding to DNA. To test this possibility, increasing amounts of GST-c-Myc (259-439 aa) or GST were added with YY1 in binding reactions and assayed by electrophoretic mobility shift assay (EMSA) using a probe containing a YY1

binding site (Fig. 1). In addition to the YY1-DNA complexes, a lower mobility complex specific to GST-c-Myc (lanes 1-3) but not GST (lanes 5-7) was also observed. When YY1 was omitted no complexes were observed (lane 4). The complexes were competed by excess YY1 binding sites (lane 10) and not by excess c-Myc binding sites (lane 9). Thus, the low mobility complex corresponds to YY1/c-Myc bound to YY1 sites. We therefore conclude that association with c-Myc does not block the ability of YY1 to bind DNA although our data do not allow us to determine if association with c-Myc alters the affinity of YY1 for its binding site.

Association of YY1 with TBP and TFII-B Requires the Same Region of YY1 Which Is Required for Association with c-Myc.

Since association with c-Myc does not inhibit the ability of YY1 to bind DNA, we hypothesized that c-Myc blocks YY1 action by inhibiting functionally important protein-protein associations between YY1 and components of the basal transcription machinery or other transcriptional proteins. YY1 is known to associate with two components of the basal transcription machinery, TBP and TFII-B (A. Berrier and K. Calame, unpublished and (Usheva and Shenk, 1994)).

We wished to test the possibility that association with c-Myc abrogated the ability of YY1 to bind DNA. An electrophoretic mobility shift assay (EMSA) using the YY1 site from the immunoglobulin heavy chain enhancer μ E1 site) We determined which portion of YY1 was required for association with TBP and TFII-B using a GST fusion protein assay. Both TBP and TFII-B associated with full-length GST-YY1 but not GST alone (Fig. 2 lanes 2 and 7). C-terminal truncations of YY1 were then tested. GST-YY1 (1-343) still associates with TBP and TFII-B (Fig. 2 lanes 3 and 8), demonstrating that amino acids 344-414, which include three of the four zinc finger domains, are not required for the association. However, GST-YY1 (1-201) fails to associate with either TBP or TFII-B (lane 4 and 9), demonstrating that YY1 amino acids 201-343 are required for association with TBP and TFII-B. GST-YY1 (201-343) associates with TBP but not with TFII-B (lanes 5, 10), demonstrating that amino acids 201-343 are sufficient for association with TBP but are not sufficient for association with TFII-B. We have previously shown that YY1 amino acids 201-343 are necessary but not sufficient for association with c-Myc (Shrivastava et al., 1993) and data not shown) Thus we conclude that the same region of YY1, amino acids 201-343, is required for association with c-Myc, TBP and TFII-B.

YY1 Associates with c-Myc in Mammalian Cells.

We previously showed that c-Myc and YY1 associated in yeast when they were ectopically expressed; however, we wished to determine if physiological levels of the two proteins allowed their *in vivo* association. To develop an immunoprecipitation assay, crude cellular lysates from Daudi cells and 293T cells stably transfected with a CMV-YY1 expression vector were mixed. After incubation, c-Myc was immunoprecipitated from the mixture using conditions which allowed association of c-Myc and YY1 as judged by the *in vitro* GST fusion protein assay (data not shown). Analysis of the immunoprecipitates on immunoblots developed with a monoclonal antibody to YY1 (IG3a, a gift from Dr. T. Shenk) revealed a YY1 band. Specificity was demonstrated by blocking with the 13 amino acid c-Myc peptide used to elicit the c-Myc antiserum. Thus, our conditions immunoprecipitate c-Myc and YY1 which associate in lysates of mammalian cells.

Subsequently, we coimmunoprecipitated endogenous c-Myc and YY1 from M12, a murine B-cell lymphoma. Polyclonal antiserum raised to the C-terminal 13 amino acids of murine c-Myc was used for immunoprecipitation of M12 lysates and the immunoprecipitate was analyzed by immunoblotting. Fig. 3A lanes 1-2 show that α -c-Myc but not pre-immune

serum immunoprecipitated c-Myc. Fig. 3B shows that YY1 was co-immunoprecipitated by anti-c-Myc (lane 5) but not by pre-immune serum (lane 4). The specificity of the YY1 band was established by developing parallel lanes of the blot with an isotype matched control monoclonal antibody which did not show a YY1 band (Fig. 3C). Since M12 cells express YY1 and c-Myc only from the endogenous genes, these results show that physiological levels of the two proteins allow their association *in vivo*.

The Amount of YY1 Associated with c-Myc Varies When c-Myc Levels Change.

We reasoned that changes in c-Myc protein levels might regulate the ratio of free YY1 to c-Myc-associated YY1 in situations where overall YY1 levels remained unchanged. To test this hypothesis we coimmunoprecipitated YY1 associated with c-Myc from 3T3 cells in which c-Myc is induced in response to serum stimulation and from murine erythroleukemia (MEL) cells where c-Myc levels decrease upon differentiation in response to DMSO. c-Myc, YY1 and YY1 associated with c-Myc were determined before and after the treatments to alter c-Myc levels. Fig. 4A shows that when quiescent 3T3 cells were treated with serum for two hours, c-Myc was strongly induced but YY1 levels did not change during this time (Fig. 4B). Measured by co-immunoprecipitation, the amount of YY1 associated with c-Myc also increased from undetectable to detectable amounts (Fig. 1C). Thus mitogenic stimulation induces c-Myc, resulting in association between YY1 and c-Myc and thus decreasing the amount of free YY1 available to regulate YY1-dependent genes. Similarly, when MEL cells were stimulated to differentiate in response to DMSO, c-Myc levels decreased 75% and YY1 associated with c-Myc decreased 62% although YY1 levels did not change (data not shown). These results support a model in which changes in c-Myc levels modulate the availability of active YY1.

YY1 Transgenic Mice.

If part of c-Myc's biological activity is due to its ability to inhibit YY1 activity, one would predict that ectopic expression of YY1 might have effects similar to partial repression of *c-myc*. This might include slow growth of particular cell lineages or failure of certain cells to undergo apoptosis. In addition, ectopic expression of YY1 is likely to have additional effects which are not usually or ever affected by c-Myc levels. To address the importance of c-Myc in regulating YY1 as well as the overall importance of YY1, we wished to compare the effect of wt YY1 and mutant forms YY1 when expressed as transgenes in mice. Since a completely c-Myc deficient mouse dies during embryonic development, our strategy was to target transgene expression to particular tissues which are dispensable for mouse development. Since lymphoid cells fulfill this criterion, in our initial experiments YY1 transgene expression was targeted to B and T lymphocytes by using the IgH E_h enhancer. We analyzed 15 founders in which transgene copy number varied from 1 to >10. When B cells from spleen and bone marrow of these mice was analyzed for transgene expression, we were unable to detect elevated levels of YY1 mRNA. Furthermore, analysis of the number and developmental stages of B cells present in both spleen and bone marrow showed no difference between transgenic animals and controls, consistent with the lack of transgene expression. We do not understand why the YY1 transgene was not expressed and are not pursuing these studies further.

Blimp-1/PRDI-BFI Encodes PRF Activity.

In previous work, we identified a site in the *c-myc* promoter which appeared to be recognized by a plasmacytoma-specific repressor factor which we dubbed PRF (Kakkis and

Calame, 1987; Kakkis et al., 1988; Kakkis et al., 1989). We noticed that the PRF binding site at -290bp in the *c-myc* gene showed strong homology to interferon (IFN) response elements in IFN stimulated gene factor binding sites (ISGF) and to the PRD-1 element of the β IFN gene promoter. A comparison of the PRF site with a consensus ISGF site and the PRD1 site shows that they are strikingly similar:

T C A A A G A A A A A G G	ISGF consensus
* * * * * * *	
A G A A A G G G A A A G G A	PRF
* * * * * * * *	
G A G A A G T G A A A G T G	PRDI

(* indicates homology between PRF and ISGF, top, or PRD1, bottom)

Oligonucleotides corresponding to the ISGF and PRDI sites compete very efficiently for PRF binding on the *c-myc* promoter; PRD1 oligos compete approximately 10X better than ISGF oligos (Wong and Calame, data not shown). We therefore obtained from Dr. Mark Davis (Stanford) an expression plasmid for Blimp-1 and antiserum to Blimp-1 (Turner et al., 1994). The expression plasmid was transfected it into 293T cells. When nuclear extracts from transfected and control cells were analysed by electrophoretic mobility shift assay (EMSA) for binding to the *c-myc* PRF site, the transfected extracts had one retarded band which was not present in the control cells (Fig. 5 lane 7 vs. lane 7). This band had identical mobility to the PRF band from plasmacytoma P3X cells (lane 6) and its specificity for the PRF site was demonstrated by competition with PRF oligonucleotides (lane 10) but not by non-specific (N.S.) oligonucleotides (lane 11). These data strongly suggested that the PRF activity in P3X cells corresponded to Blimp-1. This hypothesis was confirmed in supershift experiments where Blimp-1 antiserum (lane 12), but not preimmune antiserum (lane 13), ablated the PRF complex from P3X cells. Therefore, we conclude that the binding activity we identified as PRF is encoded by the Blimp-1/PRDI-BFI gene.

PRDI-BFI was identified as a virus-inducible 88 kDa zinc finger protein which represses the β IFN gene promoter (Keller and Maniatis, 1991). It is identical to Blimp-1 (Huang, 1994). However, Blimp-1 was identified as a plasma-cell specific protein which activates the late differentiation program of B cells, including induction of J chain expression (Turner et al., 1994). Therefore, this five zinc finger protein appears to be capable of transcriptional activation and transcriptional repression in different gene contexts. It is interesting that this activity is similar to another zinc finger protein, YY1, which binds near Blimp-1 in the *c-myc* promoter.

Blimp-1 Represses the *c-Myc* Promoter in B Cells but Activates in Fibroblasts.

Cotransfections were performed using a luciferase reporter dependent upon the wild type *c-myc* promoter (BBLUC) a *c-myc* promoter with a site-directed mutation of the Blimp-1 (PRF) site. These reporters were tested with Blimp-1 or control (Blimp-1 antisense) expression plasmids. In 18-81 preB cells, Blimp-1 repressed *c-myc*-dependent transcription approximately 67%, and repression depended upon the PRF site since the PRF mutant promoter was not repressed (Fig. 6A). Interestingly, in 3T3 fibroblasts, Blimp-1 activated the *c-myc* promoter approximately 3 fold in a binding site-dependent manner (Fig. 6B).

We do not currently know why Blimp-1 has different activity on the *c-myc* promoter in different lineages of cells. However, we have noticed that immunoblots show that Blimp-1 in extracts from B cells has a slightly slower mobility than Blimp-1 from fibroblasts. We are

investigating whether this difference reflects a B-cell specific phosphorylation state and whether this might be important for Blimp-1 activity.

Blimp-1 Represses the Endogenous *c-Myc* gene in BCL1 Cells.

BCL₁ is a mature B-cell line which, upon stimulation with IL2 + IL5, differentiates into a plasma cell-like state accompanied by Ig secretion and morphological changes. Blimp-1 is induced early in BCL1 cell differentiation. Based on the ability of transfected Blimp-1 to repress the *c-myc* promoter, we predicted that differentiation-dependent induction of Blimp-1 in BCL₁ cells would cause a decrease in endogenous *c-Myc*. Seventy-two hours after BCL₁ cells were treated with IL2 + IL5, differentiation was verified by increased immunoglobulin secretion and changes in cell size as indicated by changes in forward vs. orthogonal scatter (not shown). *c-Myc* levels during this period were assessed by immunoblot (data not shown). Following a transient increase, *cMyc* levels decreased approximately four fold between 1 and 2 hrs of IL2 + IL5 stimulation and remained low for 24 hours. Northern analyses showed that 1 hour after stimulation, *Blimp-1* mRNA increased approximately 5 fold, as previously reported. These data are consistent with the notion that Blimp-1 represses endogenous *c-myc* transcription, leading to decreased *c-Myc* protein levels.

To further verify the idea that Blimp-1 repressed *c-myc* transcription during terminal differentiation of BCL1 cells, we established stable transfectants of BCL1 cells in which Blimp-1 expression was regulated by a metallothionein promoter and could be induced by treatment with cadmium. Cadmium treatment of Blimp-1 lines, but not of mock transfectants, caused a decrease in *c-Myc* levels, measured by immunoblot (Fig. 7A), and differentiation, measured by surface expression of Syndecan (Fig. 7B). These data confirm the transient experiments of Davis et al, showing that Blimp-1 is sufficient to program BCL1 cells for terminal differentiation and they also confirm that Blimp-1 represses *c-Myc* expression.

Constitutive Expression of Blimp-1 in BCL1 Cells Blocks Cytokine-dependent Differentiation.

Our data showed that *c-myc* is a target of Blimp-1. It is reasonable to imagine that repression of *c-myc* is required for terminal differentiation since *c-Myc* is associated with cell division and terminally differentiated BCL1 cells stop dividing. To test this possibility experimentally, we made stable transformants of BCL1 cells which harbored *c-myc* cDNA expressed from a constitutive promoter. Immunoblots confirmed expression of *c-Myc* from the transfected gene (data not shown). These transformants and control lines were treated with IL-2+IL-5 to determine their ability to differentiate. As measured by surface expression of Syndecan and by secretion of immunoglobulin, constitutive expression of *c-Myc* blocked the ability of *c-Myc* transfectants, but not controls, to differentiate (Fig. 8). These data show that repression of *c-myc* expression is required for differentiation of BCL1 cells. Therefore, *c-myc* is a critical target of Blimp-1.

Constitutive Expression of Blimp-1 Suppresses Growth of 18-81 Cells and This Effect Is Overcome by Expression of *c-Myc*.

We also wished to determine the effect of Blimp-1 expression in B cells representing an earlier developmental stage. We tried to transfect stably 18-81 pre-B cells with a Blimp-1 expression plasmid containing the neo^R gene. However, few G418-resistant clones were

obtained, demonstrating that over-expression of Blimp-1 suppressed cell growth as measured by neo^R colony formation (not shown). This is consistent with the findings of others who have also experienced difficulty in obtaining Blimp-1 over-expressing cell lines.

Since Blimp-1 can repress *c-myc* transcription, we speculated that Blimp-1-dependent suppression of growth might be caused directly by the decreased abundance of c-Myc. Therefore, we tested whether ectopic expression of c-Myc could reverse Blimp-1-dependent growth suppression. 1881 cells were transfected with combinations of the pBJ1-neo Blimp-1 expression plasmid or the same plasmid with Blimp-1 in antisense orientation and the pSV2*c-myc* expression plasmid or a control pSV2 plasmid. After 10 days of culture, the number of G418 resistant colonies was determined (Fig. 9). The Blimp-1 antisense plasmid provided a control to establish transfection efficiency and functionality of the neo^R gene. As before, few colonies were obtained with the Blimp-1 expression plasmid. Upon immunoblot analysis using antiserum to Blimp-1, 16/16 clones failed to express Blimp-1 (not shown). However, co-expression of c-Myc partially reversed Blimp-1 suppression of growth. Compared to control antisense Blimp-1 and *c-myc* plasmids, transfections with Blimp-1 sense and *c-myc* plasmids gave 17-65% the number of control colonies, depending on the ratio of *c-myc* to Blimp-1 plasmid transfected (Fig. 4B). Northern blot analyses confirmed that, unlike the colonies from transfection with the Blimp-1 expression plasmid alone, 4/5 of these colonies expressed Blimp-1 (not shown). This demonstrates that ectopic c-Myc blocks the growth suppressing effect of high Blimp-1 expression. The simplest interpretation of these results is that repression of *c-myc* transcription by over-expressed Blimp-1 is directly responsible for the failure of Blimp-1 over-expressing cells to grow.

Induction of Ectopic Blimp-1 in 18-81 Cells Leads to Apoptosis.

We wished to test more directly the ability of Blimp-1 to repress transcription of the endogenous *c-myc* gene in 18-81 cells and to learn how ectopic expression of Blimp-1 caused the cells to stop growing. Therefore, 18-81 cells were stably transfected with an inducible Blimp-1 expression plasmid in which the expression of Blimp-1 was dependent upon the sheep metallothionein promoter (MT) and could be regulated by cadmium in the culture medium. Northern analyses confirm that treatment with increasing concentrations of Cd results in increased Blimp-1 mRNA and immunoblots show that c-Myc levels decrease significantly following Cd treatment (data not shown). Growth of cells harboring MT-Blimp-1 is slow compared to MT control cells when Cd is present (data not shown). Analysis of genomic DNA from MT-Blimp-1 cells in the presence and absence of Cd also reveals that the dying cells are undergoing apoptosis as evidenced by characteristic DNA fragmentation (not shown). In addition, TUNEL assays confirmed that Blimp-1 expression caused apoptotic death in 18-81 cells (Fig. 10).

Thus, in the 18-81 cell model, Blimp-1-dependent reduction of c-Myc causes programmed cell death. The reason for this is not understood at present. This observation could represent a physiologically relevant control to guard developing B cells against premature differentiation. Alternatively, since 18-81 cells are transformed by Abelson murine leukemia virus, which blocks further differentiation by inhibiting induction of RAG1 and RAG2 and activation of NF- κ B, apoptosis may result from the inability of these cells to continue differentiation.

Blimp-1 and YY1 Regulate *c-Myc* Transcription Independently.

In the *c-myc* and β -casein promoters, there are YY1 sites which are located near Blimp-1 sites. We wished to test the possibility that in promoters where their binding sites are

located near each other, YY1 and Blimp-1 may interact physically and/or functionally. We performed cotransfection studies using reporters driven by wild type and mutant forms of the *c-myc* promoter and expression plasmids for YY1 and Blimp-1. As shown in Fig. 11, we confirmed that YY1 activates and Blimp-1 represses the *c-myc* promoter in 18-81 cells; when both are present, the activities cancel one another (Fig. 11A). Blimp-1 represses equally well on a wt promoter or a promoter lacking a YY1 site (Fig. 11B) and YY1 activates equally well on a wt promoter or a promoter lacking a Blimp-1 site (Fig. 11C). Thus, we conclude that YY1 and Blimp-1 function independently on the *c-myc* promoter.

Blimp-1 Is Expressed in Lineages Outside the B Lymphocyte Lineage and Its Presence Is Associated with Terminal Differentiation.

Since all terminally differentiated cells stop dividing, and therefore presumably need to shut off their expression of *c-myc*, we wondered if Blimp-1, a repressor of *c-myc* transcription during terminal differentiation of B cells, might play a similar role in cells outside of the B lymphocyte lineage. Therefore, we tested the hypothesis that Blimp-1 is induced upon terminal differentiation of other, non-B, cell lineages. Our initial experiments have utilized two well-established systems for terminal differentiation of cultured cells: i) HL-60, a multi-potential promyelocytic line which differentiates into macrophage-like cells in response to PMA and into granulocyte-like cells in response to DMSO and ii) U937, a promonoblastic line which differentiates into macrophages in response to PMA. We observe clear and striking induction of Blimp-1 mRNA which accompanies all three differentiation programs studied--differentiation of U937 into macrophages, of HL-60 into macrophages and of HL-60 into granulocytes. Northern blots of total RNA prepared at intervals during the differentiation process were probed first with Blimp-1 cDNA, then stripped and probed with *c-myc* cDNA and finally stripped and probed with GAPDH cDNA. Fig. 12A shows a Northern blot of total RNA from HL-60 cells following activation with PMA. After 72 hrs. macrophage differentiation was complete as judged by altered cell morphology and increased expression of Mac-1 and ICAM. During differentiation Blimp-1 mRNA was induced, showing one peak early and one peak late in the differentiation process. *c-Myc* mRNA, consistent with previous reports (15), decreased after an early increase. Although the precise patterns vary somewhat, we also observed similar patterns of increases in Blimp-1 mRNA and decreases in *c-myc* mRNA during differentiation of HL-60 cells to granulocytes in response to DMSO (Fig. 12B) and of U937 cells to macrophages in response to PMA. The data were quantitated by PhosphorImager and normalized for loading differences using GAPDH (Fig. 12B). In each case, a decrease in *c-myc* mRNA correlates with a rapid increase in Blimp-1, consistent with the idea that Blimp-1 represses *c-myc* transcription in these cells. We do not yet know the significance of the prominent second increase in Blimp-1 mRNA.

Blimp-1 mRNA Is Present in Non-Lymphoid Tissues.

The finding that Blimp-1 mRNA is induced upon terminal differentiation of two promyelocytic cell lines in culture prompted us to probe more carefully the possibility that Blimp-1 mRNA may be expressed in some/many non-lymphoid adult tissues, especially tissues which contain terminally differentiated cells. Our initial analyses have been Northern blots. As shown in Fig. 13, Blimp-1 mRNA is significantly lower in all normal tissues than in plasmacytoma lines such as P3X. Nevertheless, we find expression of Blimp-1 mRNA in many tissues. Highest levels are observed in lung and ovary. Heart, brain, skeletal muscle, small intestine, kidney and spleen also express Blimp-1 mRNA. Three sizes of Blimp-1 mRNA, 5.7, 4.0 and 3.4 kb are present in P3X cells; varying relative amounts of these three isoforms occur in different tissues. At present we do not know if these isoforms encode

functionally different forms of the Blimp-1 protein; our cDNA is derived from the largest mRNA. Blimp-1 mRNA was undetectable in liver and thymus, as well as in 18-81 preB cells. These results clearly show that Blimp-1 expression is not limited to the B lymphocyte lineage but occurs in multiple tissues.

We wish to understand which cells within these tissues express Blimp-1. Therefore, in-situ hybridization studies to detect Blimp-1 mRNA using digoxigenin-labeled probes has been initiated. We see specific expression in several tissues, including kidney, brain, testes, and ovary. In kidney, Blimp-1 is expressed in podocytes in the glomeruli and in distal and collecting tubule cells (Fig. 14). In brain, Blimp-1 is expressed in oligodendrocytes (Fig. 15). Further studies on these and other adult tissues, including mammary tissues, are underway. In addition, we will perform the same studies on staged embryos. Although these studies are far from complete, the current results clearly show that Blimp-1 is expressed in many lineages of cells and support the notion that Blimp-1 expression is associated with specialized, post-mitotic cells.

Estrogen Increases Both Initiation and Processivity of *c-Myc* Transcription in Hormone-Dependent Mammary Tumor Cells.

Steady-state *c-myc* RNA is known to be regulated at multiple levels including transcription initiation, polymerase pausing, polymerase processivity and transcript stability. We have used a sensitive runon transcription assay to ask how estrogen stimulation affects transcription initiation, polymerase pausing and polymerase processivity in T47D breast cancer cells. Single-stranded DNA representing three regions of the *c-myc* gene were used to probe RNA transcribed by isolated nuclei *in vitro*.

Transcription was carried out under both low salt and high salt conditions with nuclei from cells before and after estrogen stimulation. In high salt conditions, all polymerase molecules which are recruited to the promoter are allowed to proceed; however, in low salt conditions, polymerases which are paused *in vivo* do not proceed during the *in vitro* reaction. Comparison of polymerase density corresponding to different portions of the gene, provides a measure of transcription processivity. Table I summarizes the current results of these studies; we are continuing to collect data. Three conclusions can, tentatively be made. Transcription initiation, considering either all polymerase molecules (high salt) or only considering non-paused polymerase molecules (low salt), is approximately 2.5-3.5 fold higher following estrogen treatment of T47D cells. The processivity of transcription is 3-4 fold greater following estrogen treatment in T47D cells. Thus, the net result of estrogen-dependent effects on transcription initiation and polymerase processivity amount to approximately 9-11 fold in T47D, which is sufficient to explain the observed increase in steady-state mRNA levels following estrogen treatment. In MCF7, the results are still partial, but the data indicate that the estrogen effect on transcription initiation is observed but an estrogen effect on processivity is not seen. We are particularly intrigued with the estrogen-dependent increase in polymerase processivity in T47D cells, as this effect has not been previously observed and may be important for understanding the complex effect of estrogen in mammary cells.

Identification of Estrogen-dependent DNase I Hypersensitive Sites Within the *c-Myc* Gene.

Having determined that estrogen affects initiation of *c-myc* transcription, we wish to study the mechanism responsible for this effect. As discussed above, there are no consensus EREs within approximately 2 kb 5' or 5 kb. 3' of the major transcription initiation sites at P1 and P2. Thus, the estrogen-dependent effect may be mediated by a non-consensus ERE or site unrelated to an ERE which binds ER in combination with another protein. In order to search the entire *c-myc*

gene, both 5' and 3' of the coding region, for site(s) which might be bind activators in an estrogen-dependent manner, we have utilized DNase I hypersensitive site mapping. Fig. 16A shows a map of the *c-myc* gene and indicates the end-labeling probes which have been used for these studies. These studies have searched more than 45 kb 5' of the *c-myc* region, including 6.5 kb 5' and ~35 kb 3' of the coding exons. Note that the 3' region includes the region recently identified as a 3' transcriptional enhancer element (Mautner et al., 1995).

Fig. 16 B shows a typical blot from this series of experiments. In addition to the full-length fragment, smaller are seen, indicating DNase I hypersensitivity sites. When chromatin preparations from MCF-7 cells grown in the absence and presence of estradiol are compared, it is clear that two hypersensitive sites, indicated by arrows, are present in cells grown in estradiol but not in cells grown in the absence of estradiol. Fig. 16C summarizes the combined results of multiple experiments using a variety of probes. The results show that in MCF-7 cells we observe most of the hypersensitive sites in the vicinity of the three exons which have been reported by others in different cells. Many of the hypersensitive sites were present regardless of whether the MCF-7 cells were quiescent, in the absence of estrogen, or were growing in the presence of estrogen. However, significant differences were observed. Three regions of hypersensitivity were present in the absence of estrogen and disappeared upon treatment with estrogen. The significance of these sites is not clear although in theory they could represent the binding site of a repressor which cannot bind in the presence of estrogen. Four hypersensitive sites appeared when the cells were grown in estrogen. These could correspond to the binding sites for an estrogen-dependent activator. Two correspond to the P2 and P3 promoters; the remaining two are located 3' of the gene in the region encompassed by the enhancer. The two 3' sites have been multimerized and cloned upstream of a minimal promoter. Upon transfection into MCF7 cells, we did not observe any effect of the sites on transcription. Thus, at the present time we have no direct evidence that these regions act as estrogen response elements. Further studies are in progress to explore the function of the estrogen-dependent hypersensitive sites in more detail.

CONCLUSIONS

In the work funded by this grant, we have explored how the powerful proto-oncogene *c-myc* is regulated and also studied a novel aspect of its activity. Dysregulated expression, resulting in elevated c-Myc levels, is known to be one functionally important event in breast cancers, as well as in many other forms of cancer. Furthermore, c-Myc is critical for growth and differentiation of normal cells. Therefore, the significance of these studies is clear. Our major conclusions from each facet of the work are summarized below.

A Novel Aspect of c-Myc's Mechanism of Action Involves Association with the Transcriptional Regulator YY1.

We had previously shown that YY1 and c-Myc associate in the yeast two hybrid system and in *in vitro* binding assays. We had also shown that c-Myc inhibits both the transcriptional activating and repressing activities of YY1. In this work we have established the physiological significance of the YY1-c-Myc association by demonstrating that the endogenous proteins associate *in vivo*. Further, we have shown that physiological changes in c-Myc are sufficient to alter the amount of YY1 associated with c-Myc. Thus, it seems likely that physiological changes in c-Myc levels affect the amount of YY1 free to regulate YY1-dependent genes. The growing list of YY1-dependent genes currently includes 17 cellular genes and 8 viral genes, including ubiquitously expressed genes, tissue specific genes and proto-oncogenes *c-fos* and *c-myc*. YY1 binding sites are also found in proto-oncogene N-ras

and the cell cycle regulated E2F1 promoter. Clearly, altered expression of YY1-dependent genes could have important effects on cell growth and, thus, could be responsible for some effects of c-Myc. The most dramatic changes in c-Myc levels are associated with tumors where *c-myc* gene expression is deregulated. Both estrogen-dependent and estrogen-independent breast cancers are known to have elevated levels of c-Myc. Our data suggest that abnormal elevation of c-Myc in tumors is likely to cause a significant change in expression of YY1-dependent genes.

We also studied the mechanism by which c-Myc inhibits YY1 activity. c-Myc is likely to block the transcriptional activation and/or repression activity of YY1 by interfering with functionally important YY1-TF-IIB and/or -TBP associations. We favor the following model: when a YY1/c-Myc complex binds an upstream YY1 site, normal YY1 contacts with TBP and TF-IIB are blocked and TBP and TF-IIB may be spatially displaced in the preinitiation complex. In addition, TBP may associate with c-Myc in the c-Myc-YY1 complex. This association would stabilize an altered arrangement of proteins and facilitate the ability of c-Myc to inhibit YY1 function.

Blimp-1 Is a Repressor of *c-Myc* Transcription and a Protein which Appears To Be Involved in Terminal Differentiation of Many Lineages.

Blimp-1 has been shown to be a "master regulator" of terminal B-cell differentiation. In work funded by this grant we have shown that the *c-myc* gene is a target of Blimp-1-dependent transcriptional repression. Further, we have shown that repression of *c-myc* is required for terminal B cell differentiation. In addition, we have shown that Blimp-1 is induced upon terminal differentiation of monocytic cells and is expressed in several other tissues in specialized, post-mitotic cells. The data suggest that Blimp-1-dependent repression of *c-myc* is critical for differentiation of many tissues. Furthermore, these data also suggest that Blimp-1 may function as a tumor suppressor.

Estrogen-dependent Induction of c-Myc mRNA in Hormone-dependent Breast Cancer Cells Is Mediated by a Combination of Two Mechanisms: Increased Transcription Initiation and Increased Polymerase processivity. Our data show that while estrogen increases transcription initiation of the *c-myc* gene about two fold, it has a bigger effect on the processivity of transcription through the *c-myc* gene. This is the first situation where estrogen has been shown to affect polymerase processivity and suggests a new and functionally important mechanism by which estrogen causes uncontrolled growth of breast cancers.

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APPENDICES

Table I

cell line	condition	volume +E2	volume -E2	percent +E2	percent -E2	ratio +/-
T47D	high salt	NS: 9646 RS: 5034 SA: 1439	NS: 2779 RS: 1010 SA: 159	100 52.2 14.9	100 36.3 5.7	3.47 4.99 9.05
T47D	low salt	NS: 57.8 RS: 47.5 SA: 39.1	NS: 24 RS: 6.6 SA: 3.5	100 82.2 67.6	100 27.5 14.6	2.41 7.2 11.17
T47D	low salt	NS: 20.4 RS: 17.7 SA: 10.8		100 86.8 52.9		
MCF-7	high salt	NS: 3863 RS: 632 SA: 310	NS: 456 RS: 80 SA: 33	100 16.4 8	100 17.5 7.2	8.47 7.91 9.41
MCF-7	high salt		NS: 152.8 RS: 29.8 SA: 22.3		100 19.5 14.6	
MCF-7	high salt		NS: 244.5 RS: 30.9 SA: 6.8		100 12.6 2.8	
MCF-7	low salt	NS: 70.3 RS: 25.5 SA: 18.5	NS: 32.2 RS: 12.7 SA: 12.3	100 36.2 26.2	100 39.5 38.1	2.18 2 1.5

Figure 1

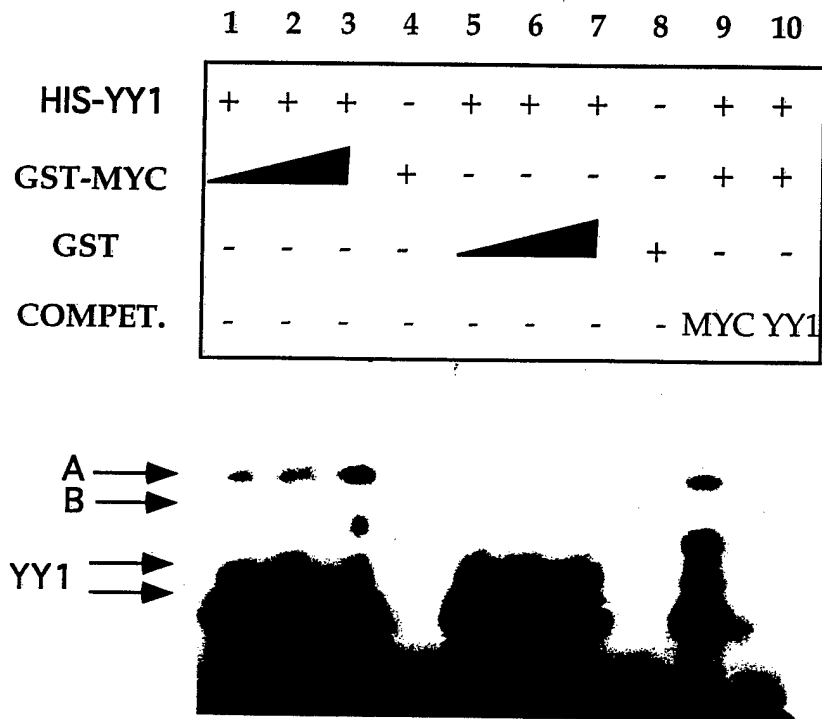


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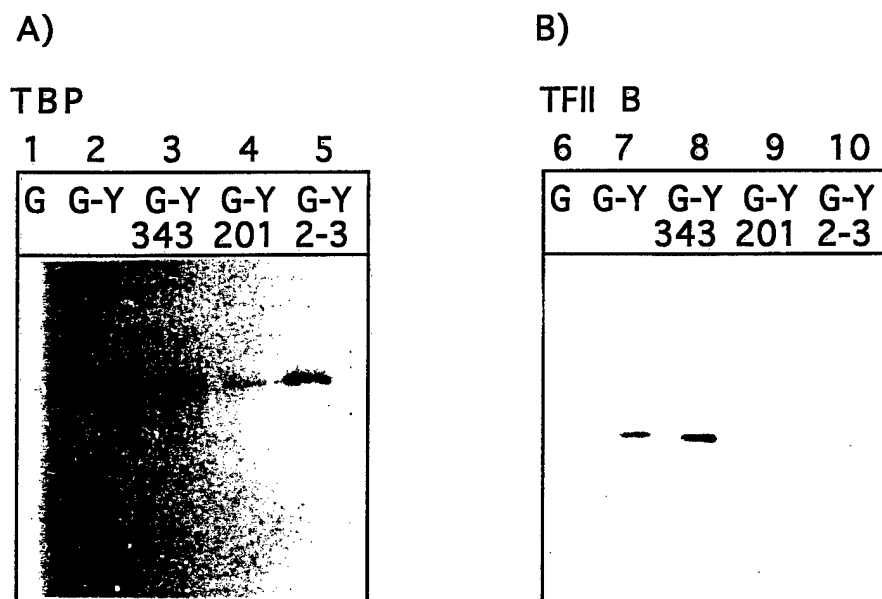


Figure 3

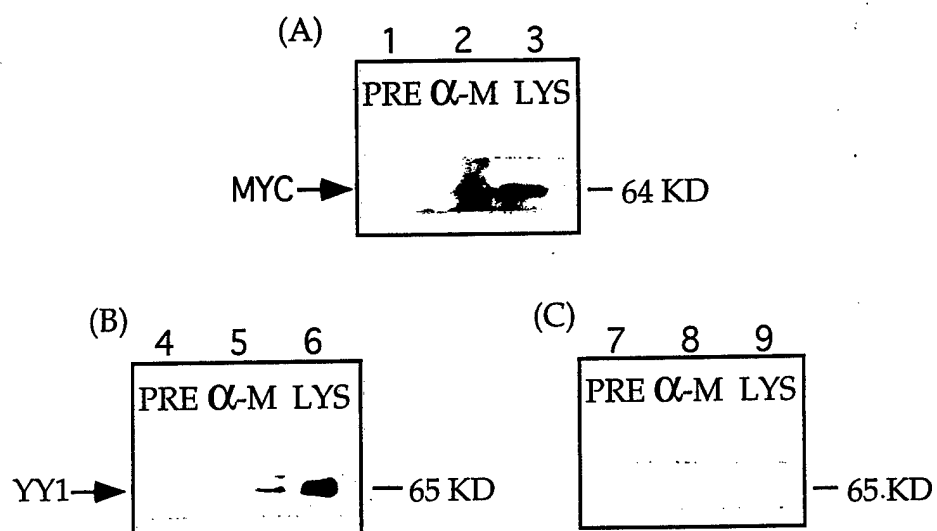


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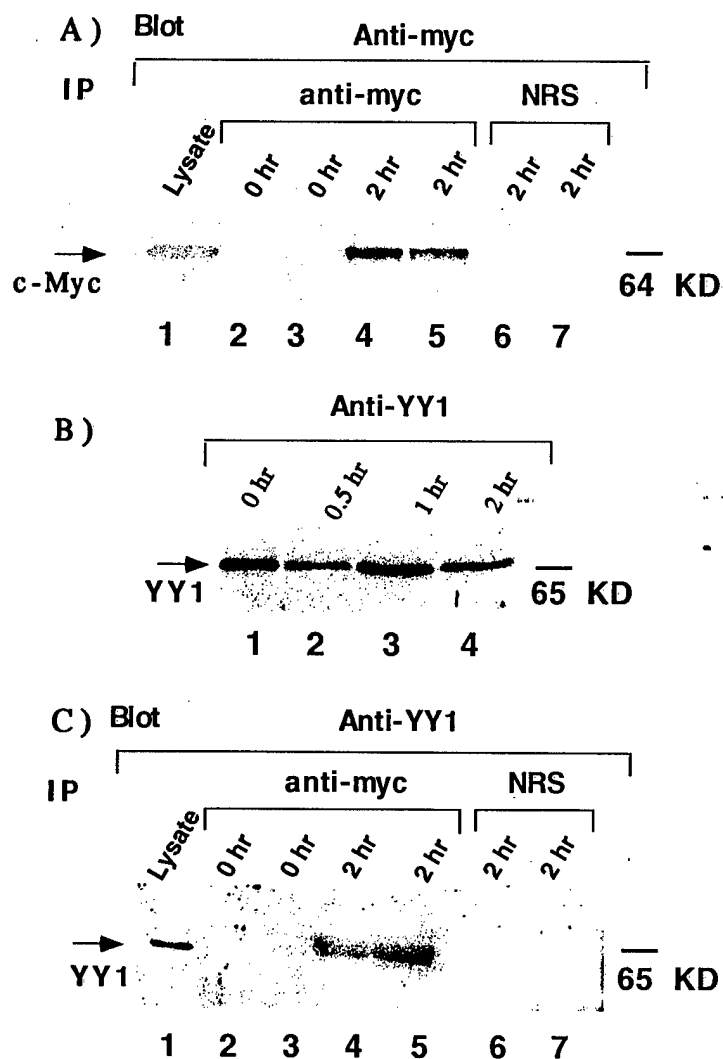


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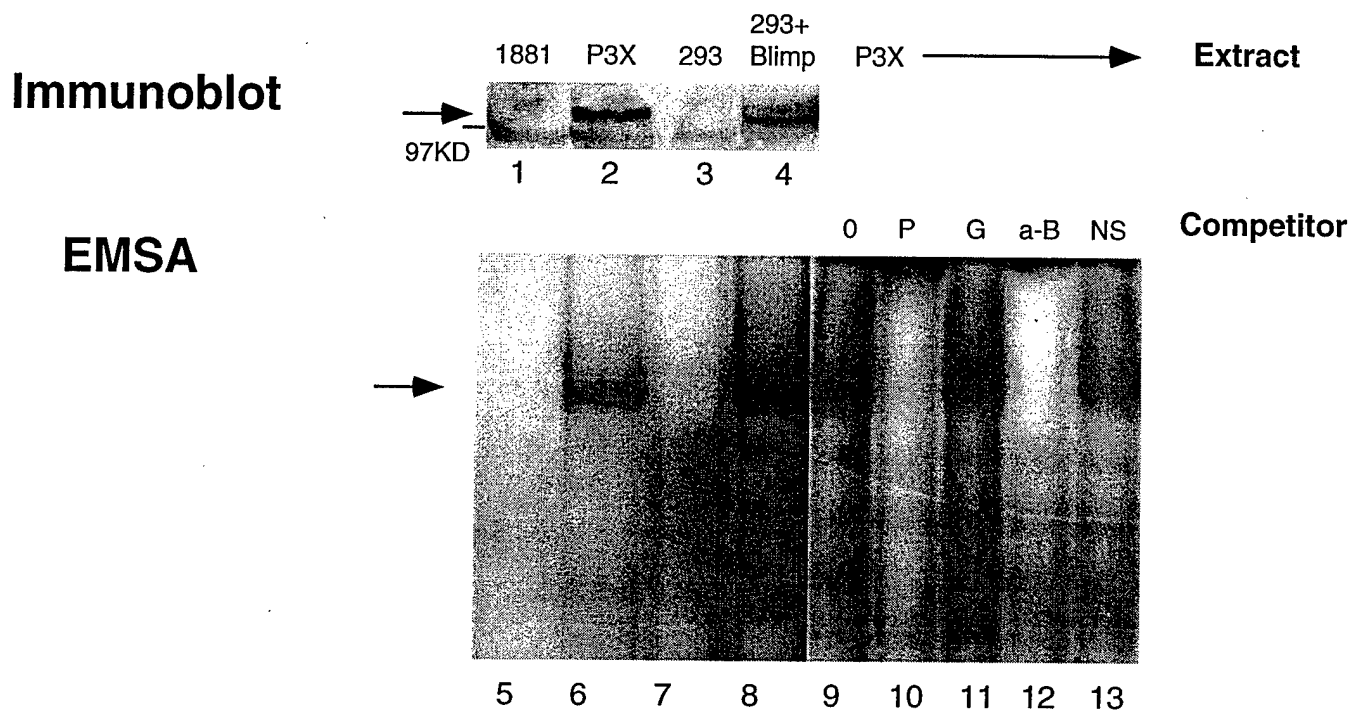


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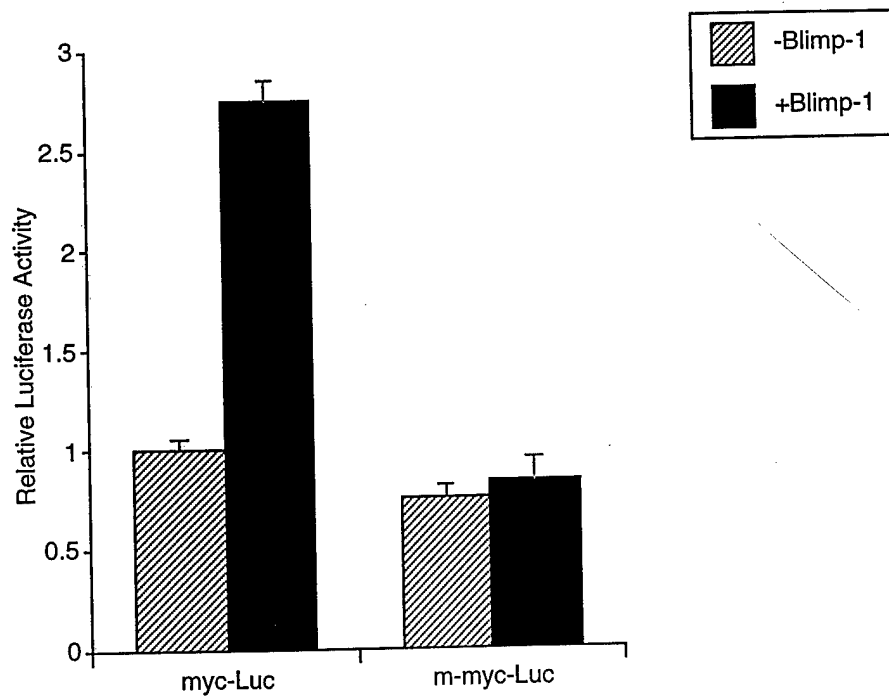
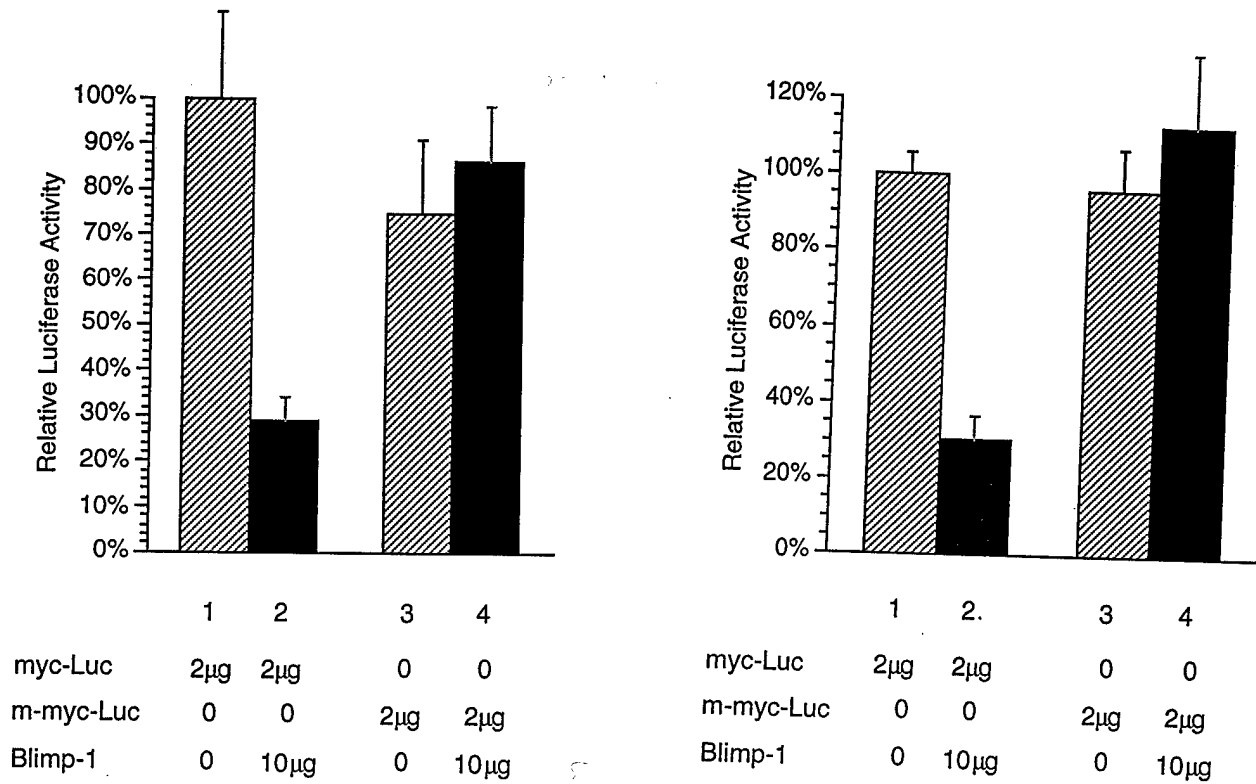
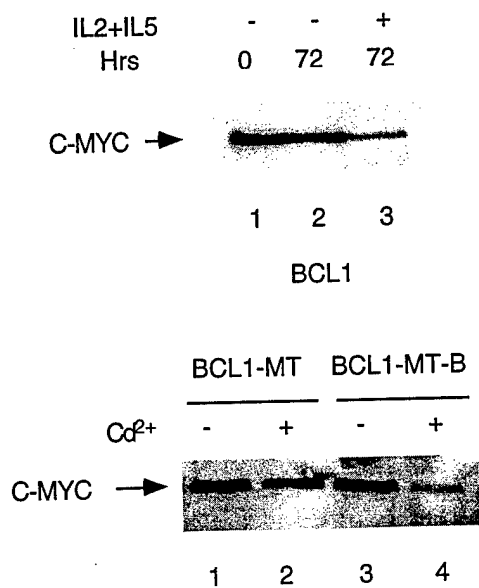


Figure 7

A.



B.

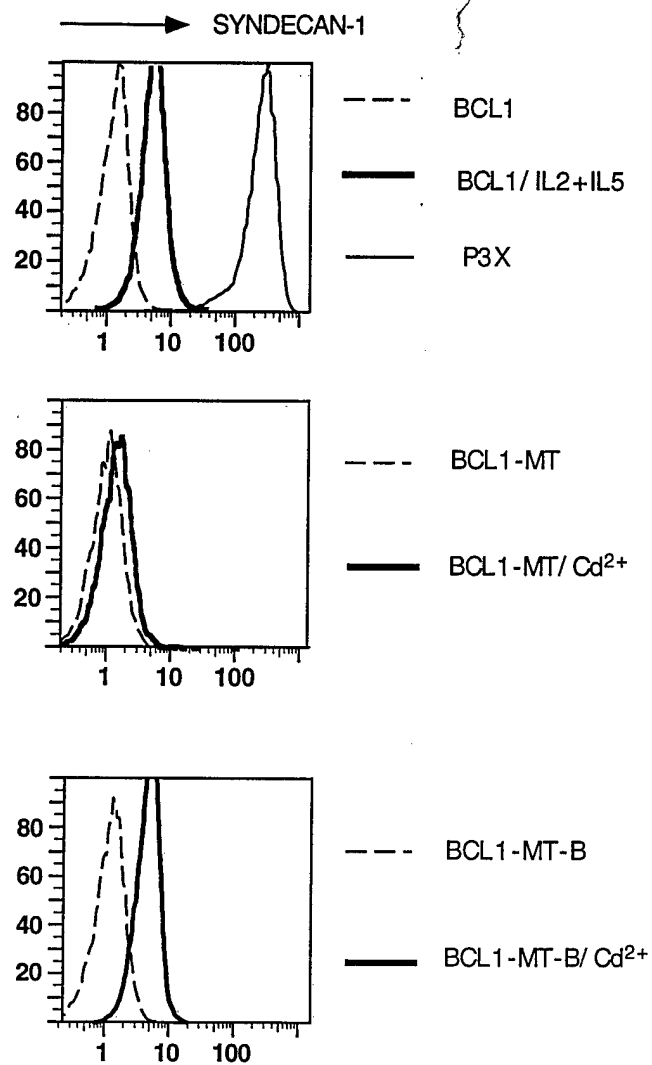


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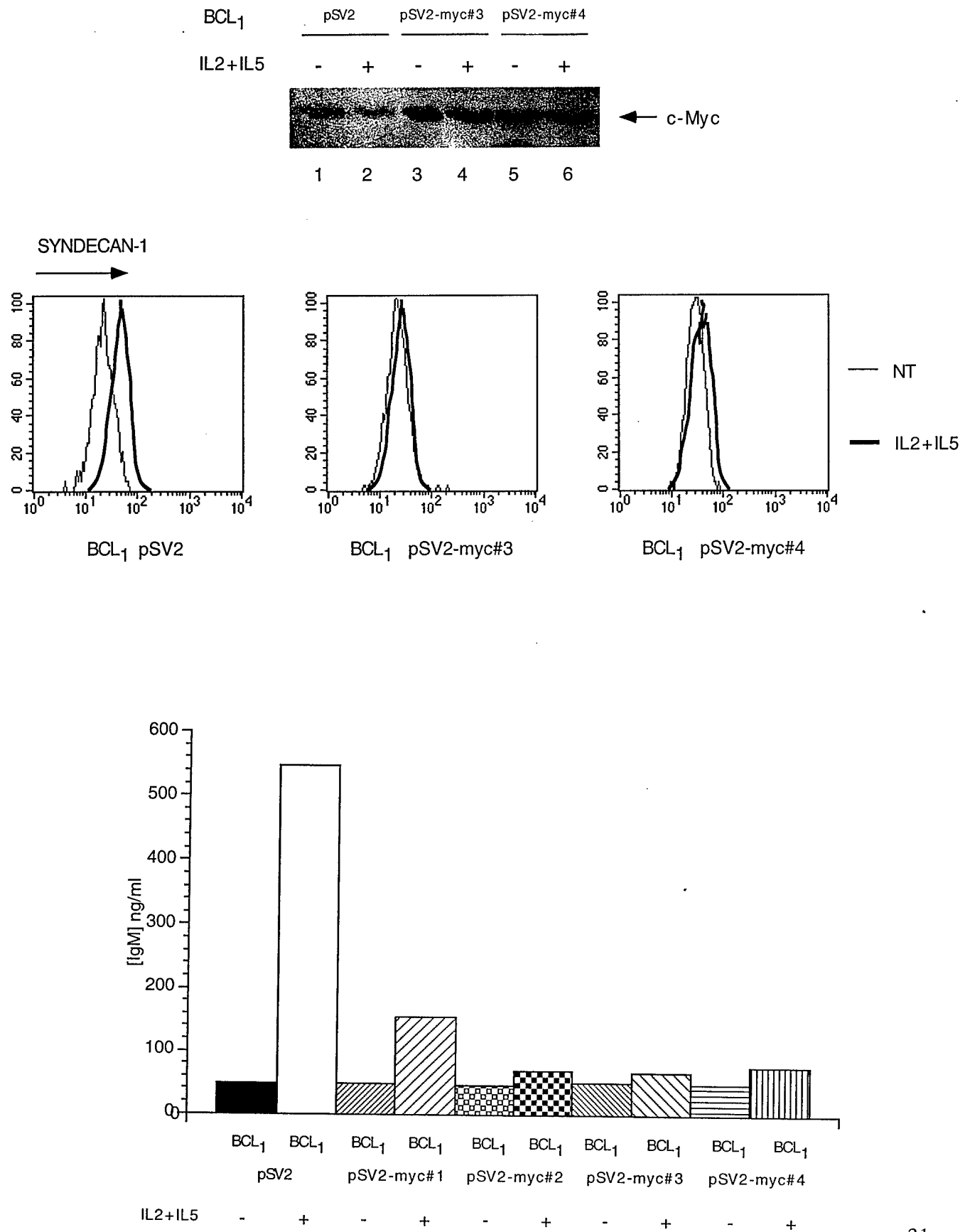


Figure 9

A

Blimp AS	Blimp S	pSV2	pSV2 c-Myc	G418 RESISTANT COLONIES
5	0	10	0	1033 +/- 46
0	5	10	0	34 +/- 7
2	0	10	0	397 +/- 19
0	2	10	0	7 +/- 1
5	0	0	10	2011 +/- 153
0	5	0	10	347 +/- 73
2	0	0	10	599 +/- 24
0	2	0	10	340 +/- 11
2	0	0	20	684 +/- 19
0	2	0	20	446 +/- 30

B

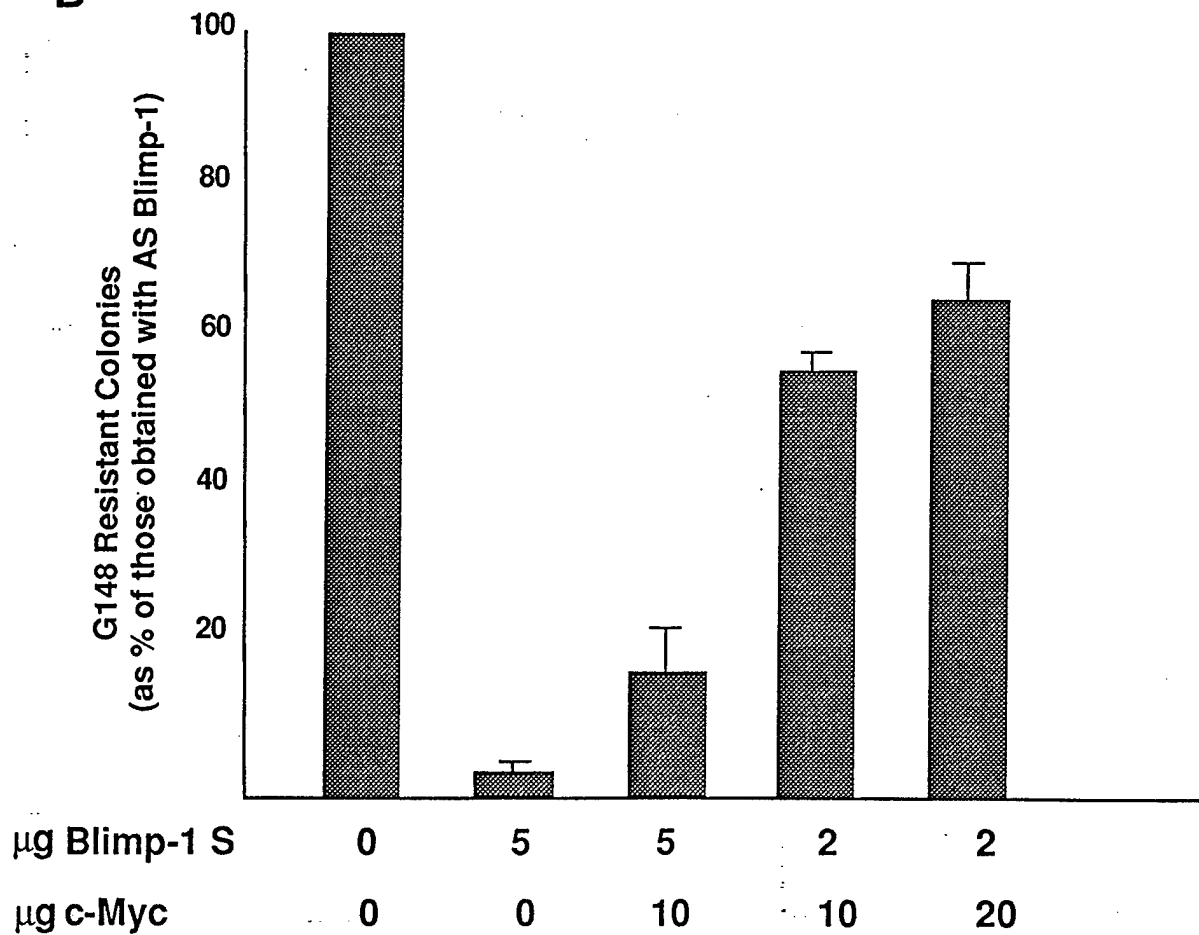


Figure 10

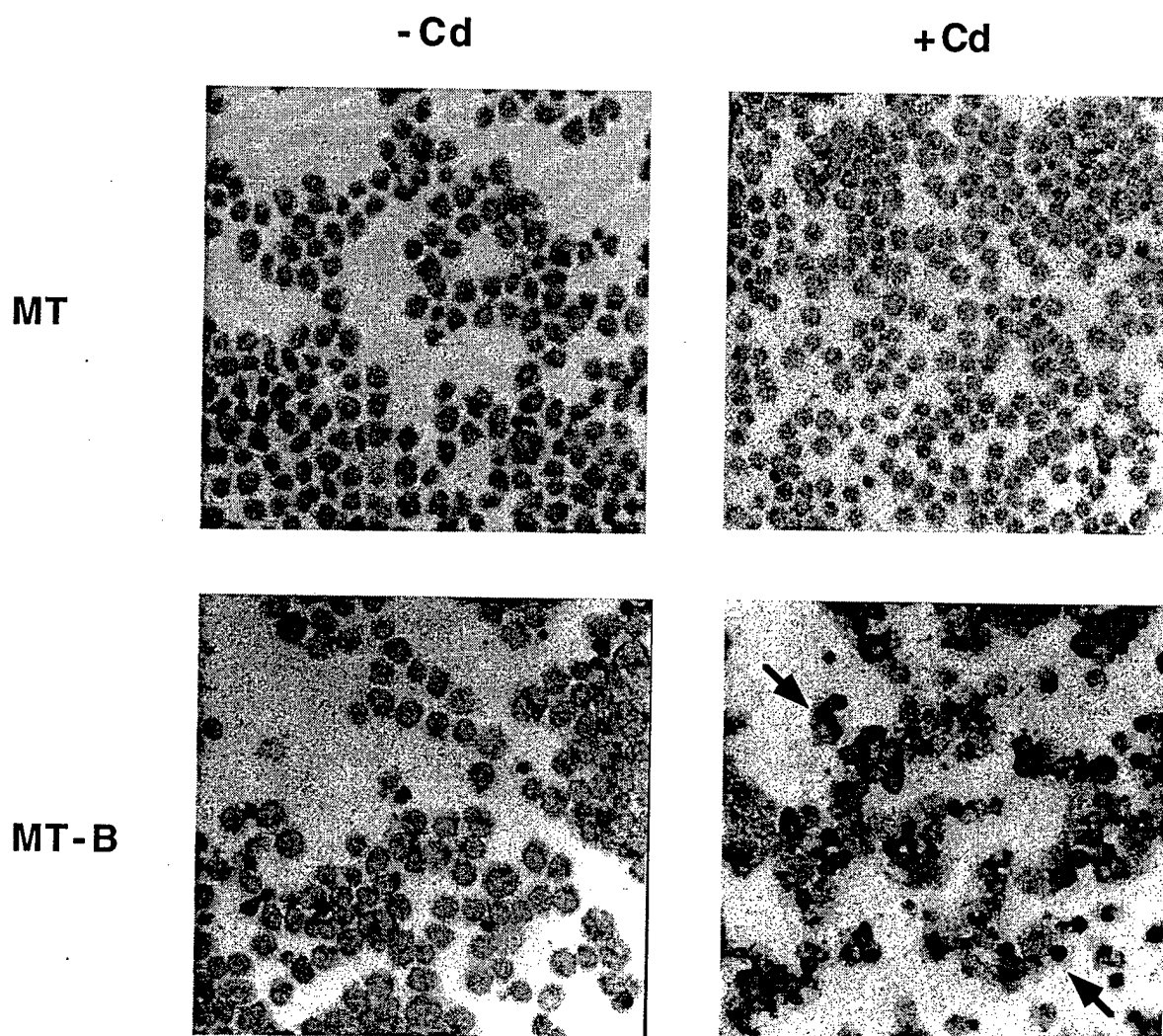


Figure 11

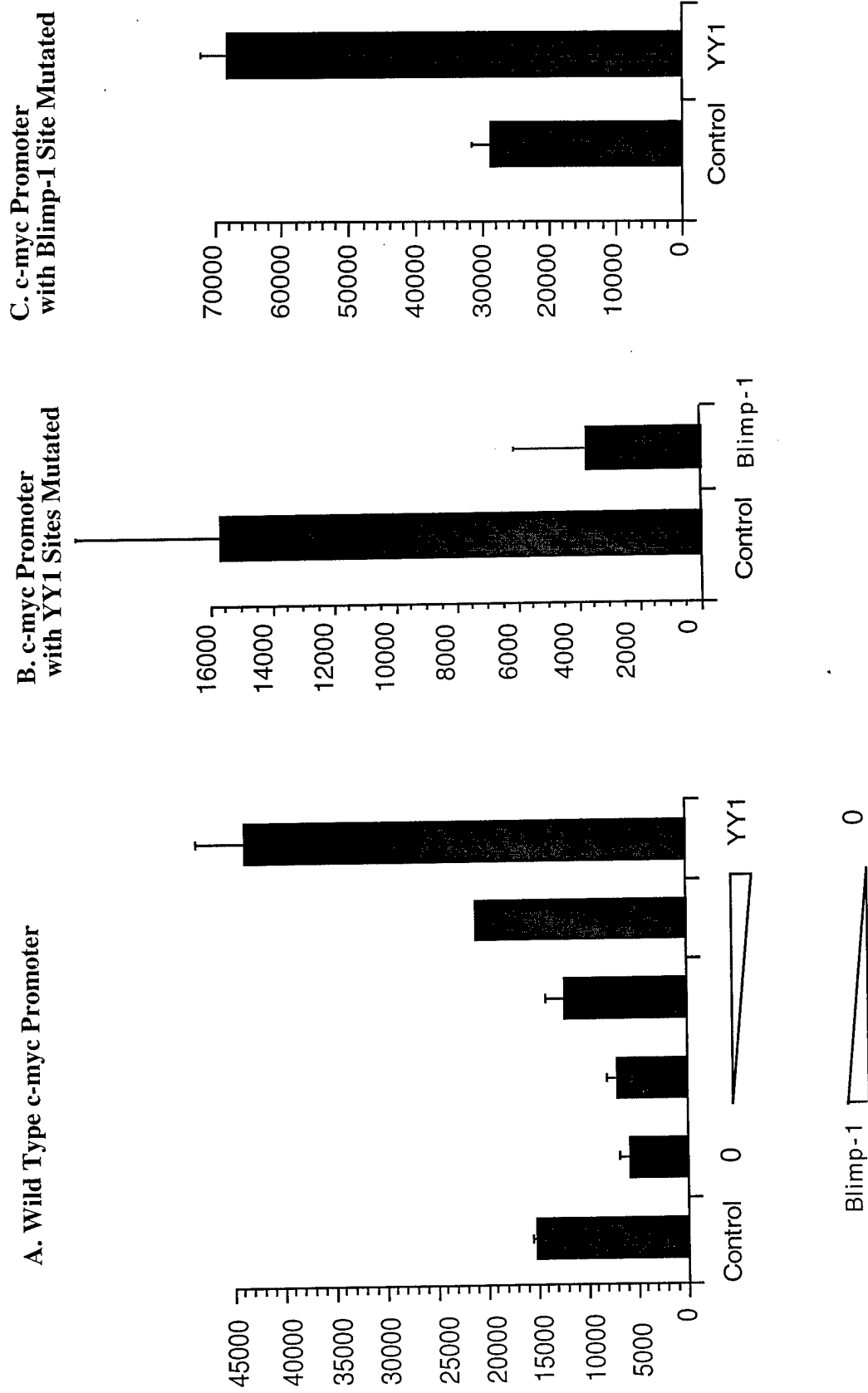


Figure 12

A. HL-60 Cells Treated with PMA

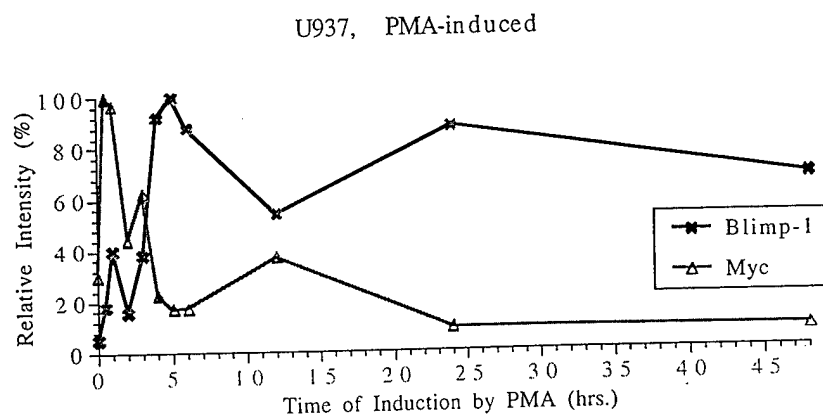
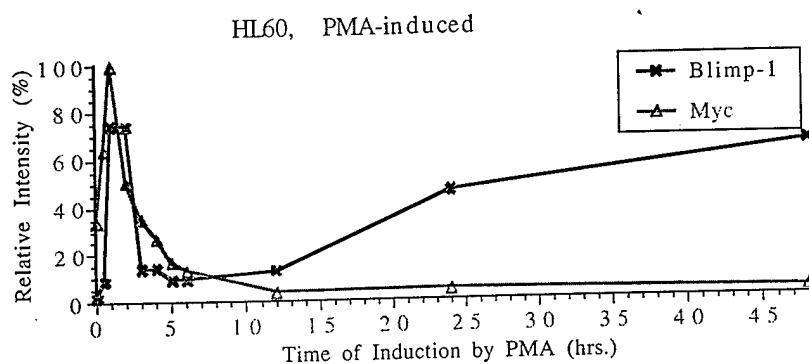
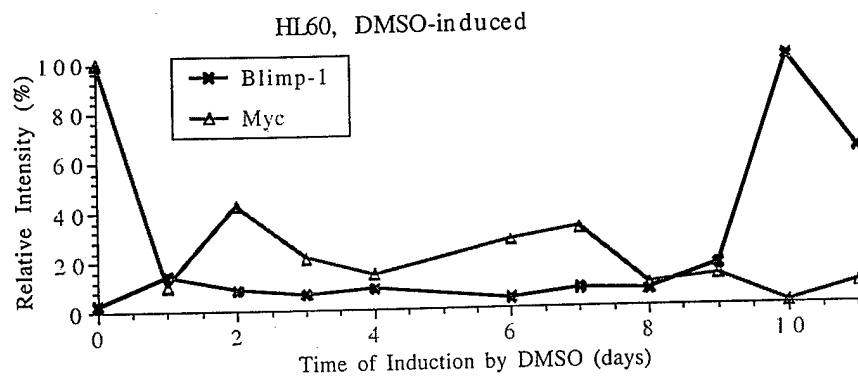
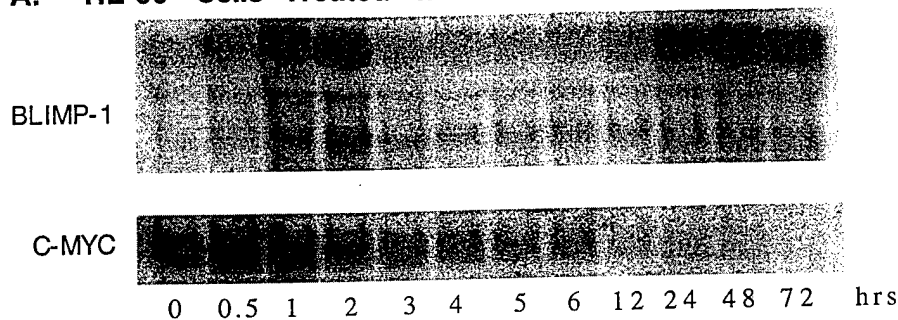


Figure 13

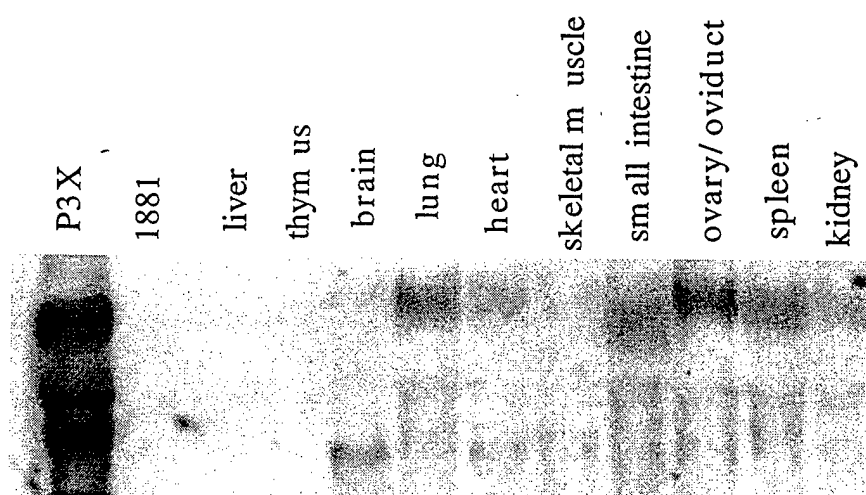


Figure 14

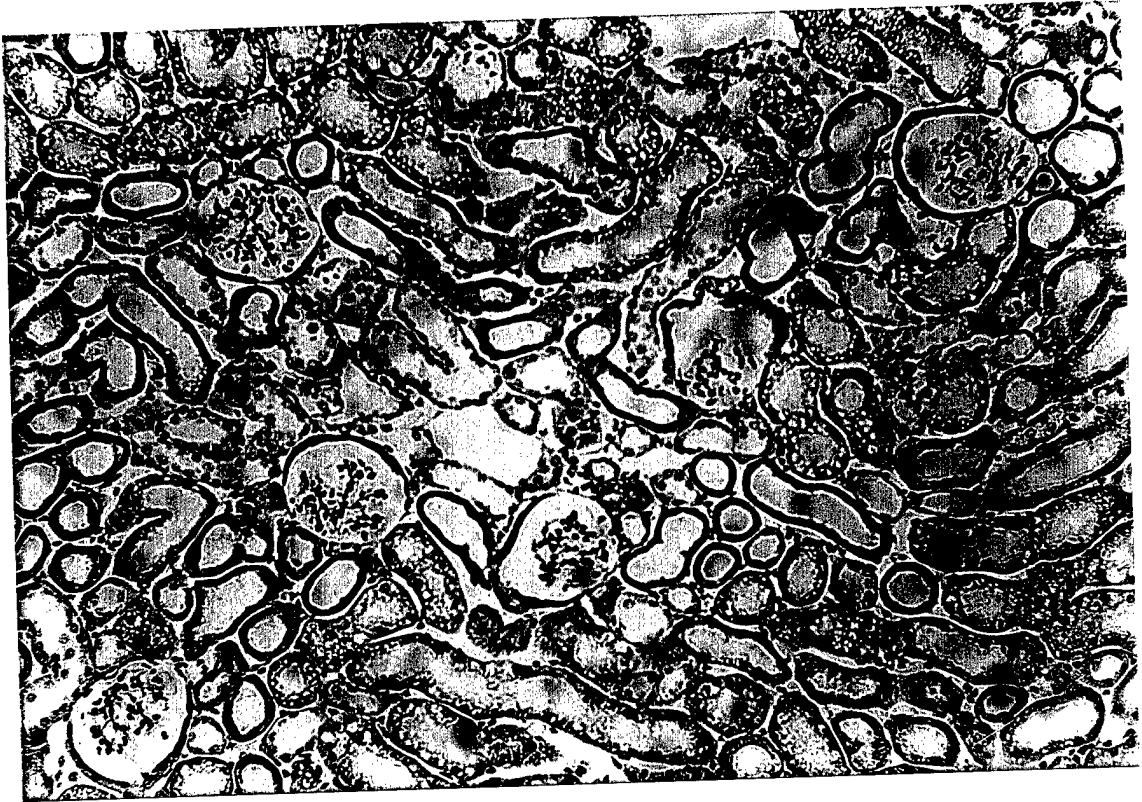


Figure 15

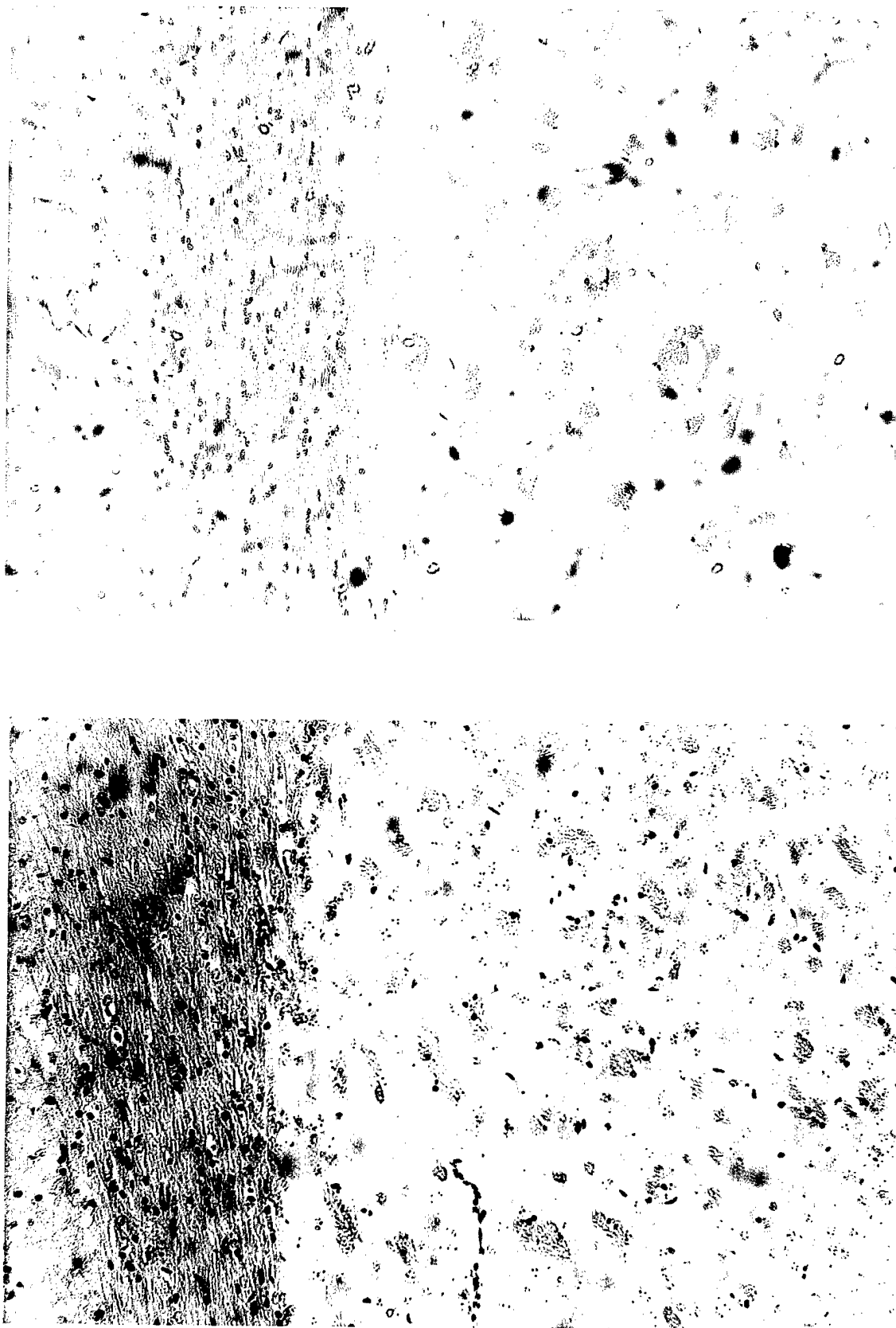


Figure 16

End-labeling Probes Used for Hypersensitive Site Mapping

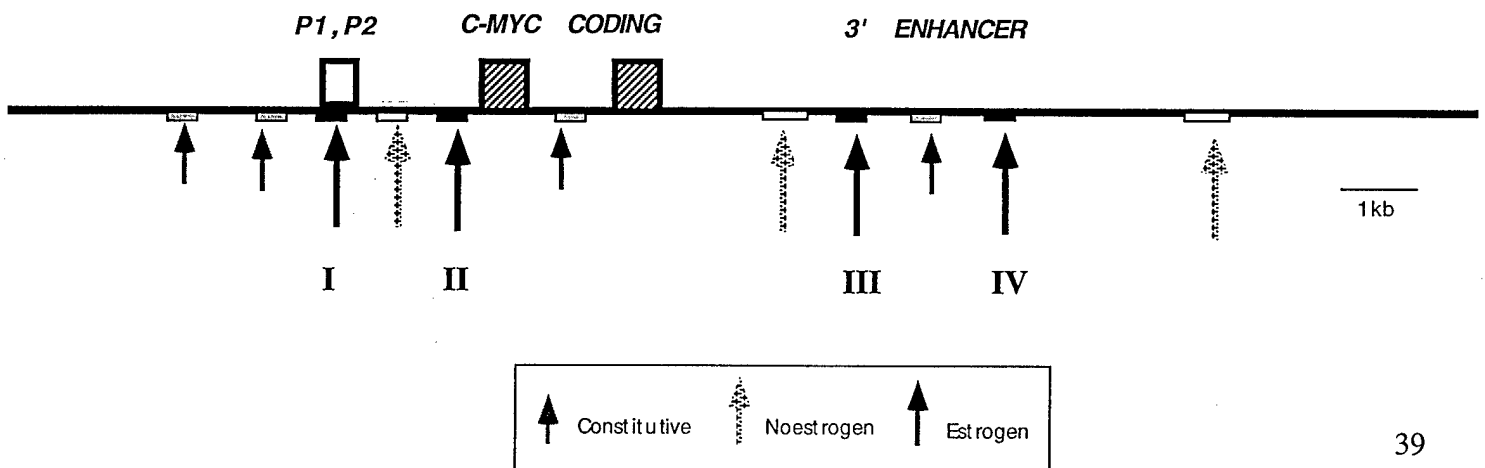
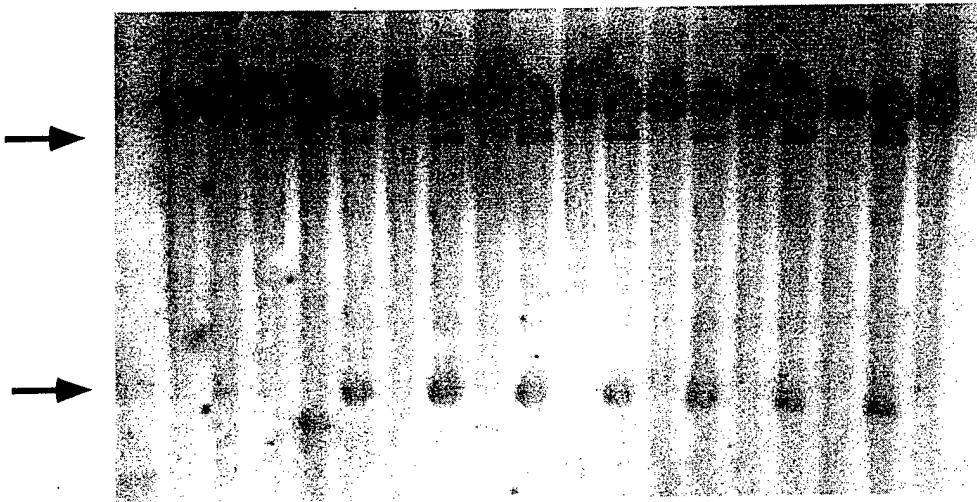
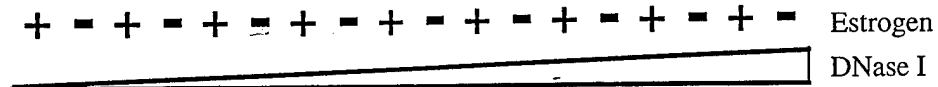
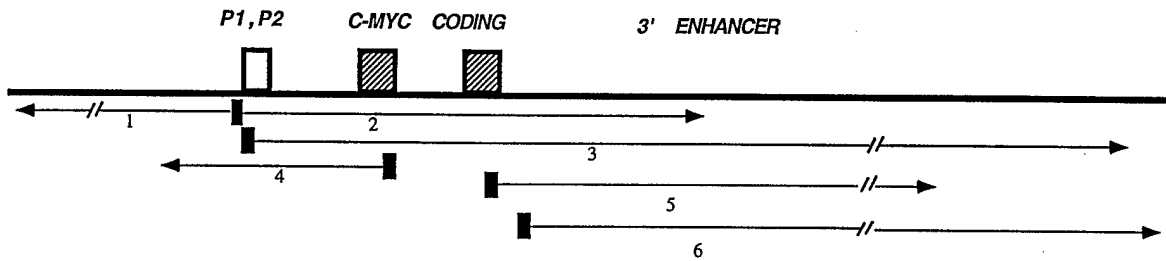


TABLE LEGEND

Summary of results from runon transcription in isolated nuclei from MCF7 and T47D cells. Runon transcription was carried out under two conditions: in high salt conditions, all polymerases which have been recruited to the c-myc gene in vivo are allowed to transcribe while under low salt conditions, which more nearly resemble in vivo conditions, only polymerases which are not paused in vivo transcribe in vitro. The three single-stranded probes are designed to reflect processivity of transcription through the c-myc gene. NS is +47-+169 3' to P2; RS is +169-+349 3' to P2 and SA is +775-+911 3' to P2. Polymerases which transcribe the "SA" region have passed the known transcription termination sites in the c-myc gene. The numbers reflect the amount of labeled RNA hybridized to each probe after correction for cell number and probe length. +E and -E indicate cells grown in estrogen or depleted of estrogen.

FIGURE LEGENDS

Figure 1. c-Myc/YY1 complex binds to YY1 DNA binding site. EMSA using an μ E1 double-stranded oligonucleotide probe and purified bacterially expressed His-YY1, GST-Myc and control GST. Competitors were unlabeled double-stranded oligonucleotides corresponding to the μ E1 site (YY1) or consensus Myc site (Myc) and were added in 100 fold excess.

Figure 2. The same region of YY1 that associates with c-Myc also associates with TBP and TFIIB. GST binding assays of ^{35} S Met-labeled TBP (A) and TFIIB (B) to GST (G), GST-YY1 (G-Y), GST-YY1(amino acids 1-343) (G-Y 343), GST-YY1 1-201 (G-Y 201) and GST-YY1 201-343 (G-Y 2-3). All GST fusion proteins were present at similar levels as judged by Coomassie stained SDS-PAGE (data not shown).

Figure 3. c-Myc associates with endogenous YY1 in M12 cells. c-Myc was immunoprecipitated from M12 cell lysate using preimmune serum (Pre) or polyclonal antiserum raised against the C-terminal 13 amino acids of mouse c-Myc (α -M); untreated lysate (Lys) was also analyzed. A. 1/10th of each immunoprecipitate and untreated lysate were analyzed using polyclonal antiserum raised against the C-terminal 13 amino acids of mouse c-Myc. B. 9/10 of each immunoprecipitate was analyzed using YY1 Mab. C. Lanes identical to those in B were analyzed using an isotype matched control Mab.

Figure 4. YY1/c-Myc complexes increase when 3T3 cells are stimulated with serum. A) Immunoblot developed with α -c-Myc antiserum. Lane 1, lysate from serum stimulated cells; lanes 2-3, immunoprecipitates using α -c-Myc antiserum from serum starved cells; lanes 4-5 immunoprecipitates using α -c-Myc antiserum from cells 2 hrs. after adding serum; lanes 6-7 immunoprecipitates from serum-treated cells using pre-immune serum. B) Immunoblot of cell lysates before and after serum stimulation developed with α YY1 antiserum. C) Immunoblot developed with α -YY1 antiserum. Lanes are identical to those in A.

Figure 5. EMSA analysis showing that PRF complex contains Blimp-1. Crude nuclear extracts from plasmacytoma P3X, 293T cells and 293T cells transfected with a Blimp-1 expression plasmid were analyzed by immunoblot for Blimp-1 and used for EMSA with a double-stranded PRF oligonucleotide probe. Double-stranded oligonucleotide competitors which contained the PRF sequence (PRF) or non-specific sequence (N.S.) were added in 50X molar excess. Crude nuclear extracts from P3X plasmacytoma were used in an EMSA to which either buffer, anti-Blimp-1 or pre-immune serum was added.

Figure 6. Effect of Blimp-1 expression on the c-myc promoter. A. A luciferase reporter dependent upon a portion of the murine c-myc promoter (BBLuc) or the same promoter with a site-directed mutation in the -290 PRF site (mPRFBBLuc) was cotransfected into 18-81 (left panel) or 300-18 (right panel) pre-B cells with expression plasmids for Blimp-1 (pBDP1-F) or and antisense control (pBDP1-B). The results show the average and standard deviation of at least three independent transfections. B. Cotransfections into 3T3 fibroblasts using the same plasmids described in A.

Figure 7. Ectopic expression of Blimp-1 in BCL1 mature B cells represses endogenous c-Myc levels and drives terminal differentiation. A. (Top) BCL1 cells were treated with or without IL2+IL5 for 72 hrs, harvested and whole cell extracts were used for immunoblotting with anti-murine c-myc antiserum. (Bottom) BCL1 cells wither transfected by metallothionein-Blimp (MT-B) or metallothionein (MT) promoter control plasmids were grown without or with cadmium for 72 hrs., harvested and whole cell extracts were used for immunoblotting to detect c-Myc levels. B. BCL1 cells transfected by MT-B or MT plasmids were grown without or with cadmium for 72 hrs. and anlyzed for the expression of Syndecan-1 by flow cytometry.

Figure 8. Constitutive expression of c-Myc in BCL1 cells blocks cytokine-drives terminal differentiation. BCL1 cells were stably transfected with a constitutive c-Myc expression plasmid or promoter control plasmid; they were were treated with IL2+IL5 for 72 hours. A. Immunoblot of whole cell lysates to detect c-Myc from control transfectants and two c-Myc expressing clones. B. BCL1 transfectants were analyzed for surface expression of Syndecan-1 by flow cytometry. C. 10^5 cells from different transfectants were treated with cytokines and IgM levels in the culture medium were measured by ELISA.

Figure 9. c-Myc blocks the growth suppression effect of high Blimp-1 expression. A. A pBJ-neo plasmid containing either antisense (Blimp AS) or sense (Blimp S) *blimp-1* cDNA was cotransfected into 18-81 pre-B cells with the pSV₂ c-Myc expression vector or a pSV₂ control vector. The amount (μ g) of each plasmid used is shown on the left. Cells were diluted into 96 well plates, cultured with 800 mg/ml G418 and resistant colonies were counted 10 days later. Results show the average of 3 independent experiments. (B) A graphic representation of the data in A. "0" for Blimp-1 indicates 5 μ g antisense Blimp-1 plasmid and "0" for c-Myc indicates 10 μ g pSV2 plasmid.

Figure 10. Detection of Blimp-1 induced apoptosis in 18-81 transfectants by TUNEL staining. 18-81 cells, either transfected with MT-B or MT control plasmid were grown without or with cadmium for 36 hrs, centrifuges onto slides and fixed with 4% paraformaldehyde. The TUNEL staining was done as described in Methods. Arrows indicate apoptotic cells.

Figure 11. YY1 and Blimp-1 regulate the c-myc promoter independently. One μ g of a luciferase reporter fused with either a Wild Type or YY-1 sites mutated or Blimp-1 site-mutated c-myc pomoter was cotransfected with 10 μ g of either Blimp-1 expression vector or YY1 expression vector or mix of Blimp-1 and YY1 at different ratios into 18-81 pre-B cells. Luciferase activity was then measured after transfection. A. Blimp-1 and YY1 have an additive effect on the wild-type c-myc promoter. B. Blimp-1 represses a c-myc promoter in which the YY-1 sites are mutated. C. YY1 activates a c-myc promoter in which the Blimp-1 site is mutated.

Figure 12. Blimp-1 mRNA is induced when U937 and HL60 cells differentiate. A. Total RNA was prepared from HL60 cells at the times indicated following treatment with PMA. The top panel shows a Northern blot with 20 µg /lane of total RNA probed with Blimp-1 cDNA; the lower panel shows the same blot after stripping and reprobing with *c-myc* cDNA. B. Graphic Representation of Blimp-1 and *c-myc* mRNA Levels During Differentiation of Promyelocytic Cell Lines. The data were derived from Northern blots which were hybridized sequentially with probes corresponding to Blimp-1, *c-myc* and GAPDH cDNAs. The bands were quantitated using a PhosphorImager and normalized to GAPDH. They are expressed relative to the highest level, taken as 100%.

Figure 13. Expression of Blimp-1 mRNA in Adult Murine Tissues. Total RNA (20 µg) from adult tissues was analyzed by Northern blotting using a Blimp-1 probe and compared to the levels in an equivalent amount of RNA from plasmacytoma P3X.

Figure 14. Specific expression of Blimp-1 mRNA in adult murine kidney. Digoxigenin-labeled probes for sense (upper) and antisense (lower) Blimp-1 were hybridized to sections from adult mouse kidney. Positive expression is indicated by blue color from alkaline phosphatase-linked antibody. Specific staining is observed in the podocytes, distal and collecting tubules, but not in the proximal tubules.

Figure 15. Specific expression of Blimp-1 mRNA in adult murine forebrain. Digoxigenin-labeled probes for sense (upper) and antisense (lower) Blimp-1 were hybridized to sections from adult mouse forebrain. Positive expression is indicated by blue color from alkaline phosphatase-linked antibody. Specific staining is observed in the oligodendrocytes.

Figure 16. Identification of estrogen-regulated DNase I hypersensitive sites in the *c-myc* gene. A. Strategy for mapping hypersensitive sites within the *c-myc* gene. The top line represents the genomic region containing the *c-myc* gene and its surrounding regions. The open box indicates the 5' untranslated exon which contains the two major transcription initiation sites, P1 and P2. The hatched boxes indicate the coding exons. The solid boxes beneath the gene indicate end-labeling probes which were used with restriction digests which allowed hypersensitive sites within the regions indicated by the arrows extending from the boxes to be mapped. Each region of the *c-myc* gene was scanned using more than one digest. B. Representative blot showing DNase I hypersensitive sites using a *ScaI* digest (# 2 in A). C. Summary of DNase I hypersensitive sites found in the *c-myc* gene in MCF-7 cells. The *c-myc* genomic region is represented as described in A. DNase I hypersensitive sites which appeared constitutively, only in the absence of estrogen or only in the presence of estrogen are indicated by arrows. The estrogen-induced hypersensitive sites are also indicated by roman numerals.

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- Zou, X., Lin, Y., Rudchentko, S. and Calame, K (1997) "Positive and Negative Transcriptional Regulation of *c-Myc*" in Current Topics in Microbiology and Immunology (M. Potter and F. Melchers, eds) 1997.

PERSONNEL SUPPORTED BY THIS GRANT

Adlam, Matthew
Almanzar, Yinesqui
Angelin-Duclos, Cristina
Calame, Kathryn
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Duncan, David
Goris, Hose
Hernandez, Yamille
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Xu, Yuming
Yu, Jin
Zhang, Qing