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The biological relevance of the c-Myc/YY1 association has been established by demonstrating that the two proteins associate *in vivo* and showing that the amount of YY1 associated with c-Myc varies when c-Myc levels change. We have also shown that association with c-Myc does not inhibit the ability of YY1 to bind DNA, but appears to alter its ability to associate with basal transcription proteins TBP and TF-IIB. Steady-state c-*myc* RNA in MCF-7 cells was shown to increase approximately 10X in response to estrogen. We expect that at least part this effect depends on transcription because we have identified two estrogen-dependent DNase I hypersensitive sites in the c-*myc* gene. One of these maps to a region containing NF-kB and C/EBP sites. We are exploring how these proteins may mediate a response to estrogen. Finally, we have shown that PRDIBFI/Blimp-1 is a repressor of c-*myc* transcription. We are exploring its mechanism of action and determining how overexpression of the gene may alter the growth phenotype of various breast tumor cell lines.

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Kathryn Calame 10/25/95

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(Note: Since our work has covered a number of separate topics, each topic has been written with a separate introduction, methods, results-discussion and conclusion section; there is one reference section at the end for the entire report followed by figure legends and figures.)

## I. MECHANISM BY WHICH -MYC INHIBITS YY1 ACTIVITY

Ajay Shrivastava and Kathryn Calame

### INTRODUCTION

Our previous work showed that c-Myc and the transcriptional regulator YY1 associate physically and that association with c-Myc inhibits the transcriptional activating and repressing activity of YY1 (1). We wished to explore the mechanism by which c-Myc association inhibits YY1 activity. Three models were initially considered: 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 (1). Therefore, we havetested the other two models.

### METHODS

**Plasmids construction**. Full length YY1 cDNA, 1.8 kbp, was excised from pGEM-4Z-YY1 (2) by Nco I digestion, end-filling and BamH I digestion, and inserted into Bam HI and Sma I restriction sites of pCGN (3). The His-YY1 plasmid was constructed by ligating Kpn I fragement from pCGN-YY1 into the pQE32 vector (Qiagen). Gst-Myc was constucted by ligating 600 bp PCR fragement of c-Myc, corresponding to 259-439 amino acids, into the Bam HI and SmaI sites of pGEX-3X vector (4). 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 (5).

**GST assays** GST assays were done as described (1). 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 aprotonin, leupeptin and pepstatin.

## RESULTS and DISCUSSION

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

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 (μE1 site) (6) was used as a probe for binding of recombinant YY1 and GST-c-Myc purified from bacteria. Increasing amounts of GST-c-Myc (259-439 aa) or GST were added with YY1 in binding reactions (Fig. 1). In additional to the YY1-DNA complexes, a lower mobility complex specific to GST-c-Myc (lanes 1-3) but not GST (lanes 5-7) addition 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, unpublshed and (7)). 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 ((1) 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.

## CONCLUSIONS

The association between YY1 and TFII-B has been shown to be functionally important for YY1 to initiate transcription *in vitro* (7). It is reasonable to assume that associations with TBP and TFII-B are also functionally important for the ability of YY1 bound at upstream sites to activate or repress transcription, although this has not been shown experimentally. Our data show that amino acids 201-343 of YY1, which are required for association with c-Myc (1), are also required for association with both TBP and TF-IIB. Since c-Myc association requires the same YY1 region as TF-IIB and TBP association, it follows that association with 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.

Unfortunately, we have been unable to test this model directly due to the association of TBP and TF-IIB with c-Myc as well as with YY1 (A. Shrivastava and J. Yu, unpublished). However, adenovirus E1A has been shown to compete with c-Myc for association with YY1 (1)and amino acids 201-343 are part of the region of YY1 required for the association E1A protein (8). Therefore, by analogy, c-Myc probably competes with TBP and TF-IIB for association with YY1. It is probably significant that to date, all proteins which associate with YY1 require amino acids 201-343, thus defining this region as a protein association domain of YY1. By associating with YY1 via this domain, c-Myc may block association of YY1 with many functionally important proteins. A related mechanism has been suggested for c-Myc's inhibition of TFII-I activity--i.e. that it blocks association between TFII-I and USF (9)

c-Myc also associates with TBP (10, 11) and with TFII-B (A. Berrier and K. Calame, unpublished) although the functional importance of these interactions is not known. The N-terminal region of c-Myc (amino acids 1-179) is required for association with TBP, distinct from the C-terminal region of c-Myc (amino acids 250-434), required for association with YY1 (1, 10). Thus ternary complexes of TBP, c-Myc and YY1 are theoretically possible. Together these facts suggest 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 could stabilize an altered arrangement of proteins and might facilitate the ability of c-Myc to inhibit YY1 function.

## II. IN VIVO ASSOCIATION OF YY1 AND C-MYC

Ajay Shrivastava, Jin Yu and Kathryn Calame

### INTRODUCTION

The c-*myc* proto-oncogene encodes a ubiqutiously expressed nuclear phosphoprotein (12-14). Despite clear evidence that c-Myc is important in the control of cellular proliferation, differentiation, apoptosis and transformation (13), 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 (13). c-Myc/Max heterodimers can activate the p53 (15), ECA39 (16), α-prothymosin (17), DHFR (18), and ornithine decarboxylase genes (19, 20). 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 (21), suggesting that functions other than transcriptional activation may be important for c-Myc function.

c-Myc can also suppress the expression of specific genes including the major histocompatibility complex class I antigens HLA-A2 and HLA-C (22), cyclin D1 (15) integrin LFA-1 (23), adhesion receptor N-CAM (24) and transcription factor C/EBPα (25). c-Myc also represses its own transcription (26). 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.

We have previously shown that c-Myc can physically associate *in vitro* and in the yeast two-hybrid system with transcription protein YY1 (1). YY1 is a ubiquitiously expressed zinc finger transcription factor (2, 27-30) which functions as a transcriptional repressor, activator or initiator, depending upon the context of its binding site. YY1 binding sites are widely distributed in many cellular and viral promoters (30). Association with c-Myc inhibits the transcriptional activating and repressing abilities of YY1 (1). We have previously suggested that c-Myc may regulate transcription of YY1-dependent genes by modulating YY1 activity (1). Since regulation of YY1 activity could provide an additional mechanism for c-Myc-dependent transcription regulation, we have investigated the physiological relevance of the YY1/c-Myc association.

## METHODS

**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 mouse c-Myc protein into rabbits.

**Co-immunoprecipitation.** $2x10^7$ M12 or MEL 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 aprotanin), sonicated on ice and centrifuged for 30 minutes at 13000 rpm to obtain lysates used for co-immuno precipitation. Lysate was precleared by incubating 20 minutes with protein A-sepharose beads in buffer X. Preclared lysate was kept on ice for 1 hr, incubated α–Myc antisera for 2 hrs, and then protein A-sephrose beads for 4hrs. Proteins bound to beads were resolved by SDS-PAGE and visualised by immunoblotting with α-Myc polyclonal antiserum and α-YY1 antibody.

## RESULTS AND DISCUSSION

**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 (31) 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 used murine erythroleukemia (MEL) cells. When MEL cells differentiate in response to DMSO, c-Myc levels transiently rise and then fall (32). We determined the amounts of c-Myc, YY1 and YY1 associated with c-Myc before and after DMSO-induced differentiation of MEL cells. As judged by benzidine staining for hemoglobin, 30% of the cells had differentiated after 2 days in DMSO and 80% had differentiated after 4 days (data not shown). Fig. 4A lanes 1,3,5 show that c-Myc levels decreased to 49% of initial levels after 2 days and to 25% after 4 days in DMSO as determined by immunoprecipitation. YY1 levels did not change during this time (Fig. 4B). Measured by co-immunoprecipitation, the amount of YY1 associated with c-Myc dropped to 56% after 2 days and to 38% after 4 days (Fig. 4C lanes 1,3,5). Thus physiological changes in c-Myc levels affect the amount of YY1 which is associated with c-Myc in MEL cells, changing the amount of free YY1 available to regulate YY1-dependent genes.

## CONCLUSIONS

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* (30). 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 due to chromosomal translocation, gene amplification or retroviral insertion (13). We suggest that abnormal elevation of c-Myc in tumors is a situation where c-Myc is likely to cause a significant change in expression of YY1-dependent genes. It will be interesting to determine if any genes

which are differentially expressed in tumors with elevated levels of c-Myc lack c-Myc/Max sites and are YY1-dependent.

## III. INDUCTION OF C-MYC BY ESTROGEN IN MCF7 CELLS

Yi Lin and Kathryn Calame

### INTRODUCTION

The c-Myc protein is important for proliferation of normal cells but abnormally high levels of c-Myc are found in many malignant tissues. Elevated levels of c-Myc have been found in both estrogen-dependent and estrogen-independent breast tumors (33-35) We wish to understand the mechanism(s) responsible for estrogen-induction of c-Myc, using human breast tumor cell line MCF-7 as a model system.

### METHODS

**Northerns**

Total RNA was prepared by lysing cells with 4 M guanidium thiocynate, 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 mg 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 Hieghts, 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 X $10^{-2}$M $NaH_2PO_4$, 1.5 X $10^{-3}$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 primering gel isolated DNA fragments according to the manufacturer's specifications (United States Biochemical, Cleveland, OH).

**DNase I Hypersensitive Sites**

DNaseI hypersensitive sites were mapped on the human c-*myc* gene as described previously (36). DNA was digested with XhoI and cDNAs corresponding to sequences located just 5′ and just 3′ of the XhoI site in the first exon were used to probe the blots.

## RESULTS AND DISCUSSION

**Estrogen stimulation causes 10 X increase in c-*myc* steady-state mRNA in MCF-7 cells.**

Previous studies have demonstrated that estrogen treatment causes an increase in steady-state levels of c-*myc* mRNA. We have confirmed that this is true in our hands. As shown in (Fig. 5) an increase of 10 fold in c-*myc* mRNA results 2 hrs. after MCF-7 cells are stimulated with estradiol. Since steady-state levels of mRNA increase, we we are currently performing nuclear runon transcription assays to determine if transcription initiation or transcription elongation are affected by estrogen treatment. The results of these experiments are not yet complete.

**Estrogen stimulation of MCF-7 cells induces two DNase I hypersensitive sites 5′ of the c-*myc* gene.**

The c-*myc* gene does not contain any classical estrogen response elements (EREs) although the gene appears to be regulated by estrogen. Thus we suspect that estrogen acts in an unusual way to activate c-*myc* transcription. Therefore we are using DNaseI hypersensitive site mapping to identify potential transcriptional regulatory regions within the c-*myc* gene which might be responsive to estrogen.

As shown in Fig. 6, two DNase I hypersensitive sites have been observed 5′ of the c-*myc* gene when MCF-7 cells are treated with estrogen. These regions occur approximately 1 and 2 kb 5′ of the P1 transcription start site. Interestingly, the proximal site occurs in the vicinity of sequences corresponding to NF-κB and CREB binding sites. It has recently been shown that NF-κB and CREB sites constitute an estrogen response element in the IL6 gene (37). Therefore, we are initiating functional studies to determine if these sites are also EREs in the c-*myc* gene.

## CONCLUSIONS

Although these experiments are not yet complete, our current results suggest that at least part of the increased steady-state c-*myc* mRNA which appears in MCF-7 cells stimulated with estrogen is caused by increased transcription. It will be necessary to complete our runon transcription assays to confirm this hypothesis and to determine how much of the response is due to transcription initiation. However,

the appearance of two new DNase I hypersensitive sites in the c-*myc* gene is consistent with the transcription initiation model and also suggests that the hypersensitive regions are important in the estrogen response. Further studies in the next year will confirm and extend our current understanding of the two putative estrogen-responsive regions (EREs) we have identified. Subsequently it will be interesting to determine if the ERE sites or factors which bind them are altered in estrogen dependent or independent tumors.

## IV. IDENTIFICATION OF A REPRESSOR OF C-MYC TRANSCRIPTION

Yi Lin, Kwok-kin Wong and Kathryn Calame

### INTRODUCTION

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 (38-40). In Burkitt lymphoma (BL) and murine plasmacytomas (PCs), the translocated c-*myc* allele is aberrantly expressed but the normal allele is transcriptionally silent. We suggested that the putative negative regulator might repress the normal, transcriptionally silent allele (38).

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. Riboprobe analyses showed that deleted constructs had correct transcripts initiating from P2. Thus we conclude that the -290 binding activity is a repressor of c-*myc* transcription and have named it Plasmacytoma Repressor Factor (PRF) (39, 40).

Our current efforts are focused on identification of the PRF gene and characterization of PRF activity. We are particularly interested to determine if over-expression of PRF will alter the growth or transformed properties of breast cancer cells, such as MCF-7, which depend upon c-Myc for their proliferative phenotype.

### METHODS

**Electrophoretic Mobility Shift Assays.** These assays were performed as described previously (38) using double-stranded oligonucleotides corresponding to the

murine c-*myc* PRF site at -290 as probe. Super-shifts were performed as described previously (41) antiserum to Blimp-1 kindly supplied by Dr. M. Davis, Stanford.
**Transient Transfections** were performed using luciferase reporters dependent upon the murine c-*myc* promoter (41) having wt sequence or a mutation at the PRF site (40).

## RESULTS AND DISCUSSION

**Blimp-1/PRDI-BFI encodes PRF activity.**

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 ßIFN gene promoter (42) . 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 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. 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. 7A, lane 7 vs. lane 4). This band had identical mobility to the PRF band from plasmacytoma P3X cells (lane 1) and its specificity for the PRF site was demonstrated by competition with PRF oligonucleotides (lanes 2 and 8( but not by non-specific (N.S.) oligonucleotides (lanes 3 and 9). These data strongly suggested that the PRF activity in P3X cells corresponded to Blimp-1. This hypothesis was confirmed in supershift experiments (Fig. 7B), where Blimp-1 antiserum, but not preimmune antiserum, supershifted 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 βIFN gene promoter. It is identical to Blimp-1. 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. 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) (43) or a c-*myc* promoter with a site-directed mutation of the Blimp-1 (PRF) site (40). 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. 8A). Interestingly, in 3T3 fibroblasts, Blimp-1 activated the c-*myc* promoter approximately 3 fold in a binding site-dependent manner (Fig. 8B) .

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.

## CONCLUSIONS

Blimp-1 has been identified as a repressor of the c-*myc* gene. This exciting finding opens several interesting avenues for further study. First, we are currently designing Blimp-1 expression plasmids which will allow regulated expression of Blimp-1 in different cell lines. We wish to test the effects of Blimp-1 over-expression on the growth and transformed phenotype of MCF-7 breast tumor cells since these are known to depend upon the function of c-Myc (44). In addition, we wish to learn the mechanism by which Blimp-1 represses c-*myc* and how its activity is regulated.

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## FIGURE LEGENDS

**Figure 1.** c-Myc/YY1 complex binds to YY1 DNA binding site. EMSA using an μE1 double-stranded oligonucleotide probe and purified bacterially expressed His-YY1, GST-Myc and control GST. Competitors were unlabled double-stranded oligonucleotides corresponding to the μE1 site (YY1) or consensus Myc site (Myc) and were added in 100 fold excess.

**Figure 2.** GST binding assays of $^{35}S$ Met-labeled TBP (A) and TFII-B (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.** Amounts of YY1 associated with c-Myc depend upon c-Myc levels in MEL cells. Panels A and C show MEL cell lysates immunoprecipitated with preimmune serum (P) or antiserum to murine c-Myc C-terminal peptide (M) following treatment for 0 (lanes 1,2), 2 (lanes 3,4) and 4 (lanes 5,6) days in 1.5% DMSO . (A) Immunoblot of 1/10 of each immunoprecipitate analyzed with α-c-Myc. (B) Immunoblot of MEL cell lysates analyzed with YY1 Mab. (C) Immunoblot of 9/10 of each immunoprecipitate analyzed with α-YY1 Mab.

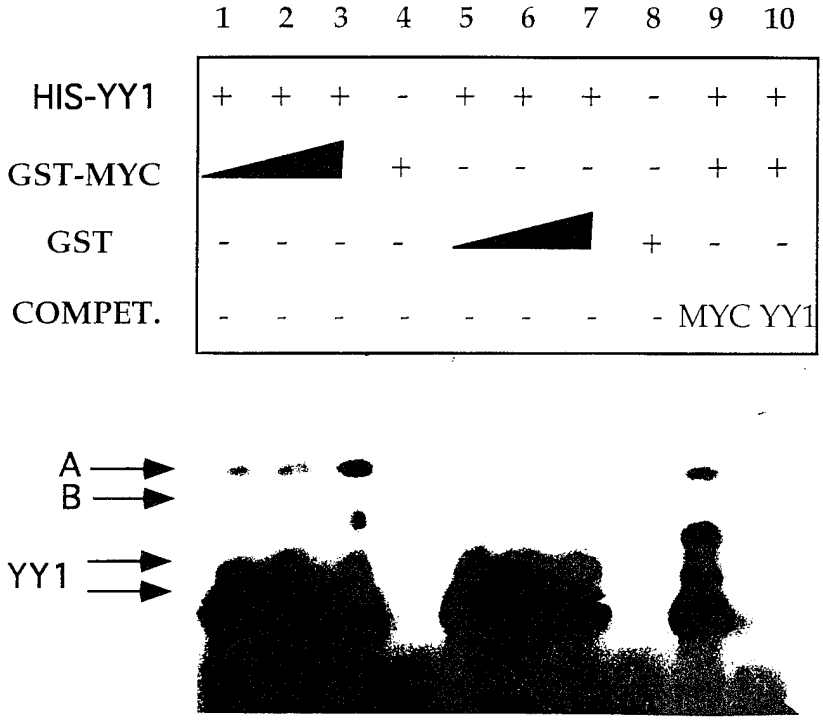
**Figure 5.** Northern blot of c-myc RNA in MCF-7 cells. Total RNA was harvested from MCF-7 cells before and 2, 4 and 24 hrs. following stimulation with estradiol and the blot was probed with c-myc cDNA. The blot was stripped and reprobed with beta actin cDNA to control for RNA loading.

**Figure 6.** DNase I hypersensitive site mapping in the human c-myc gene. Nuclei from estradiol treated (+) and untreated (-) MCF7 cells were incubated with increasing amounts of DNase I prior to DNA purification. Equal amounts of DNA were then digested with XhoI and subjected to Southern analysis with the probe indicated by a solid bar.

**Figure 7.** EMSA analysis showing that PRF complex contains PRDI/BFI/Blimp-1. A. Crude nuclear extracts from plasmacytoma P3X, 293T cells and 293T cells transfected with a Blimp-1 expression plasmid were 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. B. Crude nuclear extracts from P3X plasmacytoma were used in an EMSA to which either buffer (lane 1), anti-Blimp-1 (lane 2) or pre-immune (lane 3) serum was added.

**Figure 8.** Effect of PRDI/BFI/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 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 1

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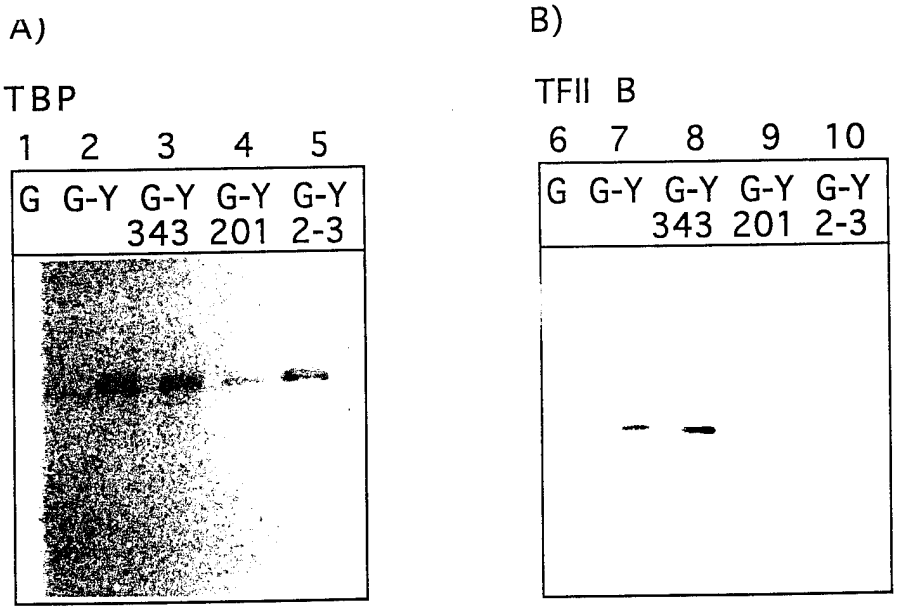

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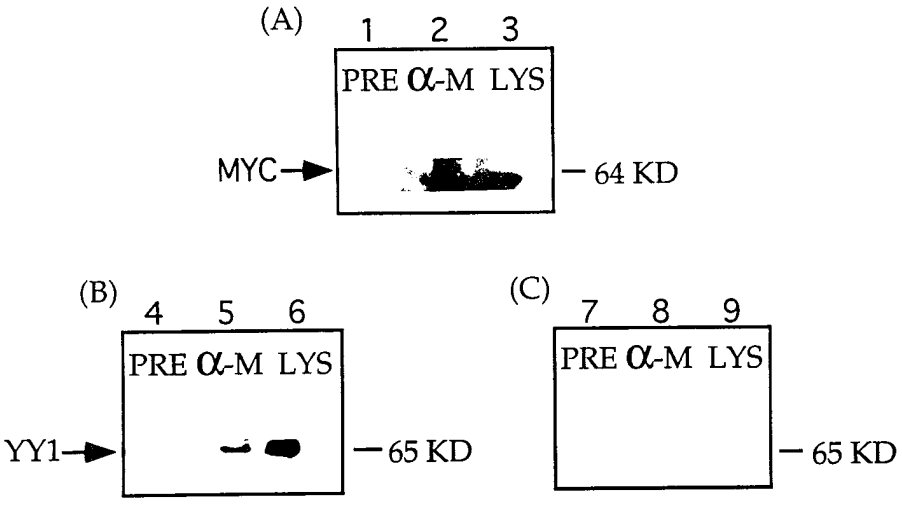
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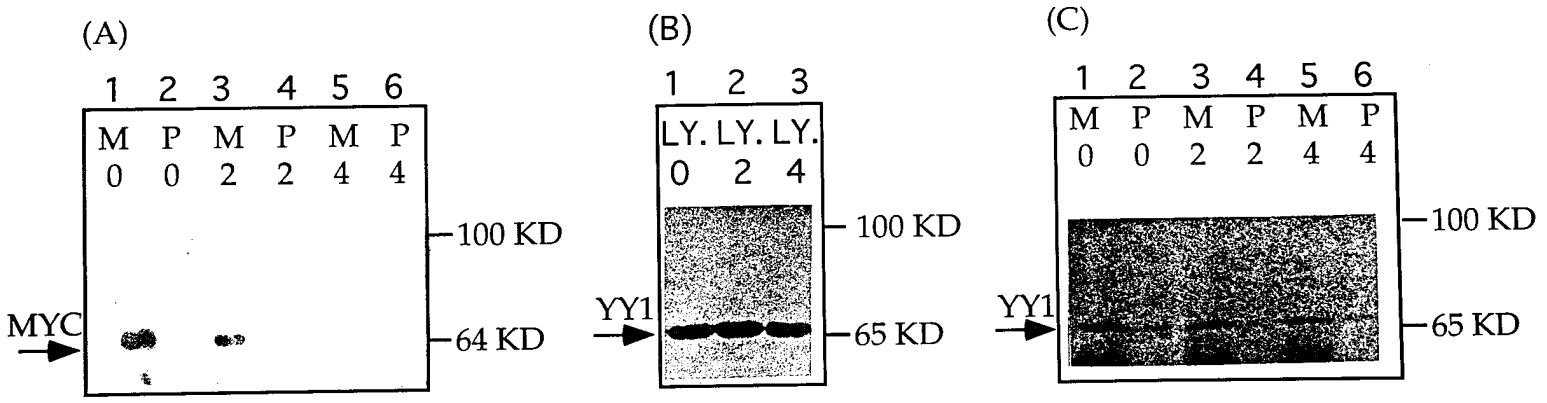
PRE α-M LYS
MYC
64 KD
(B)
4 5 6
PRE α-M LYS
YY1
65 KD
(C)
7 8 9
PRE α-M LYS
65 KD

Figure 4


(A)
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M P M P M P
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100 KD
MYC
64 KD
(B)
1 2 3
LY. LY. LY.
0 2 4
100 KD
YY1
65 KD
(C)
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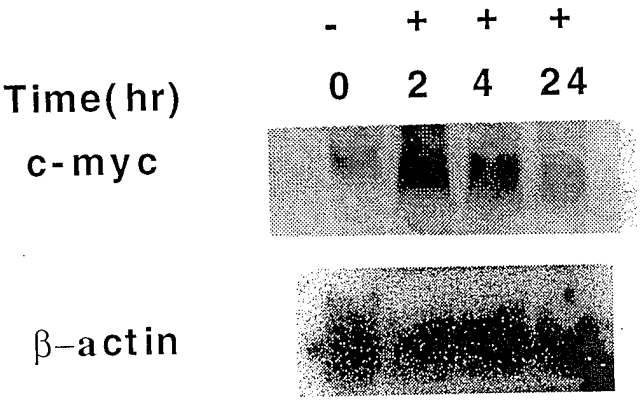
YY1
65 KD

Figure 5

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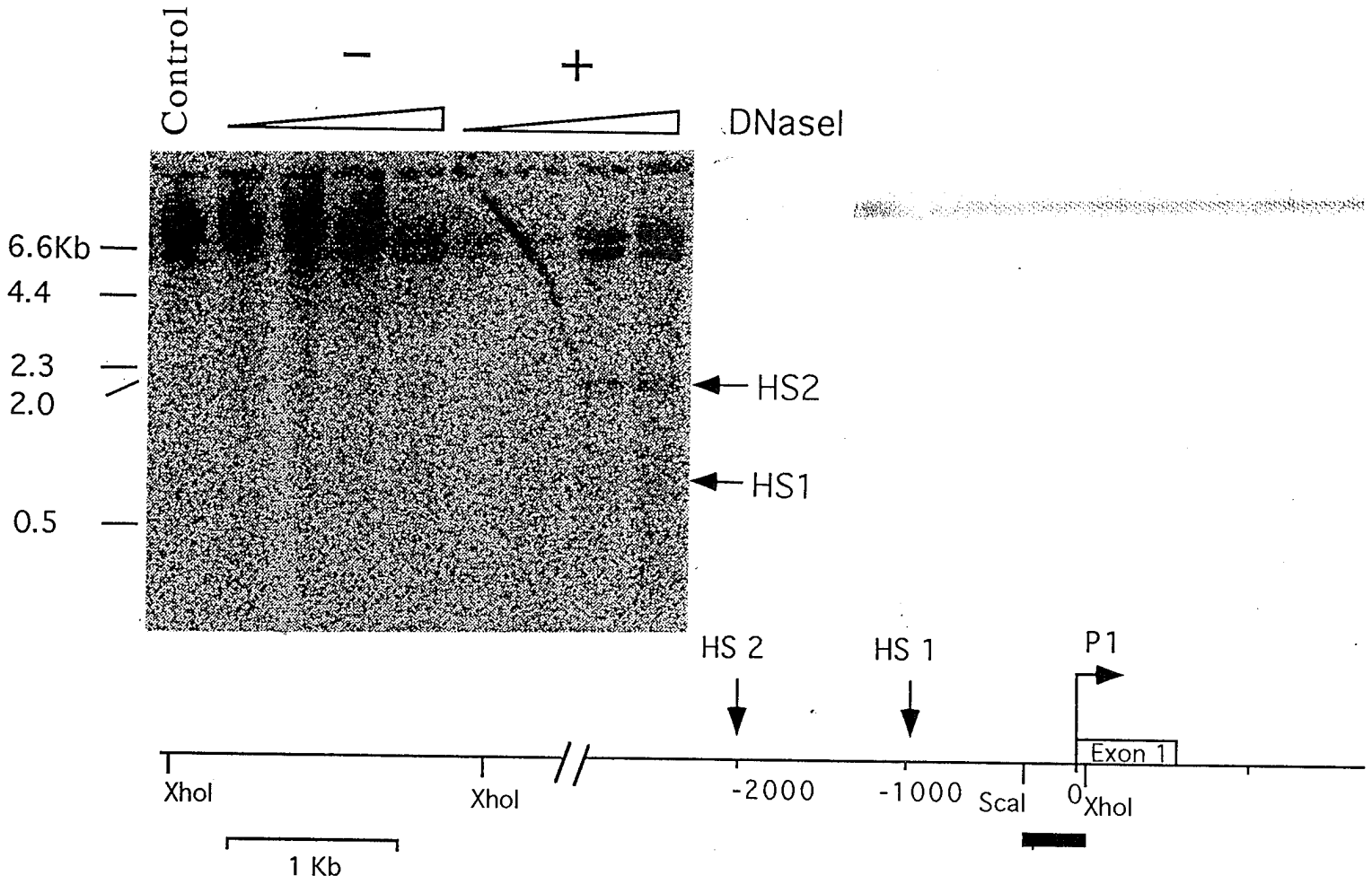


Figure 7A

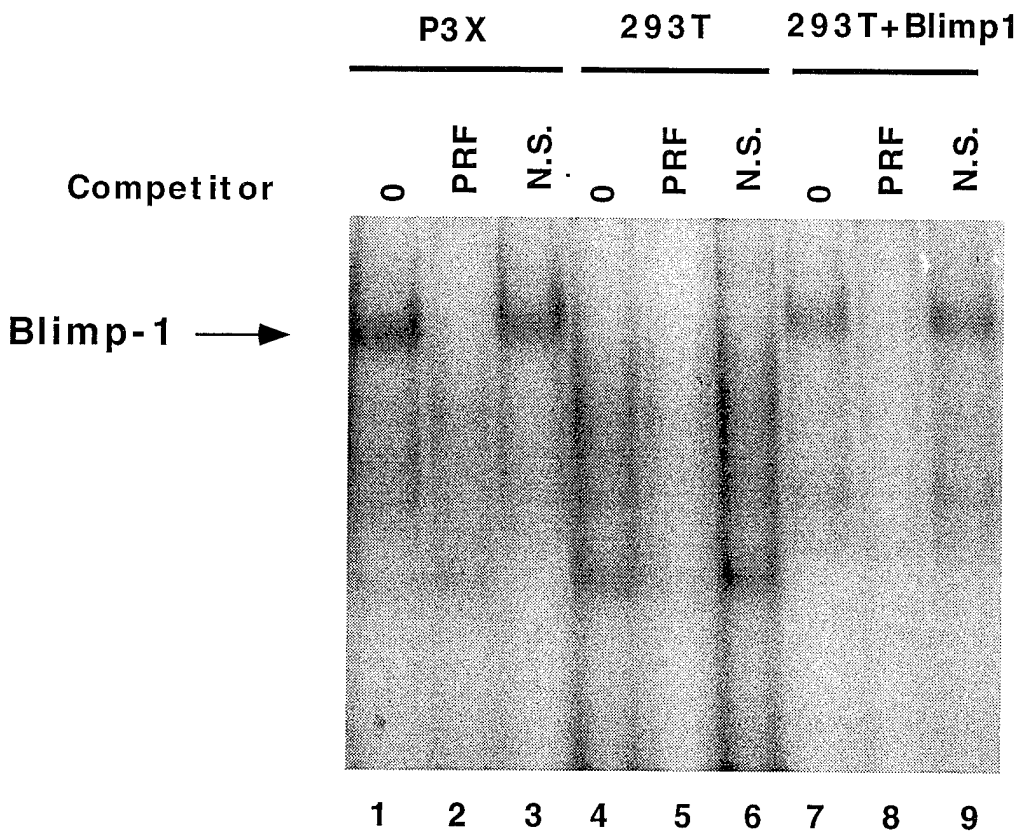
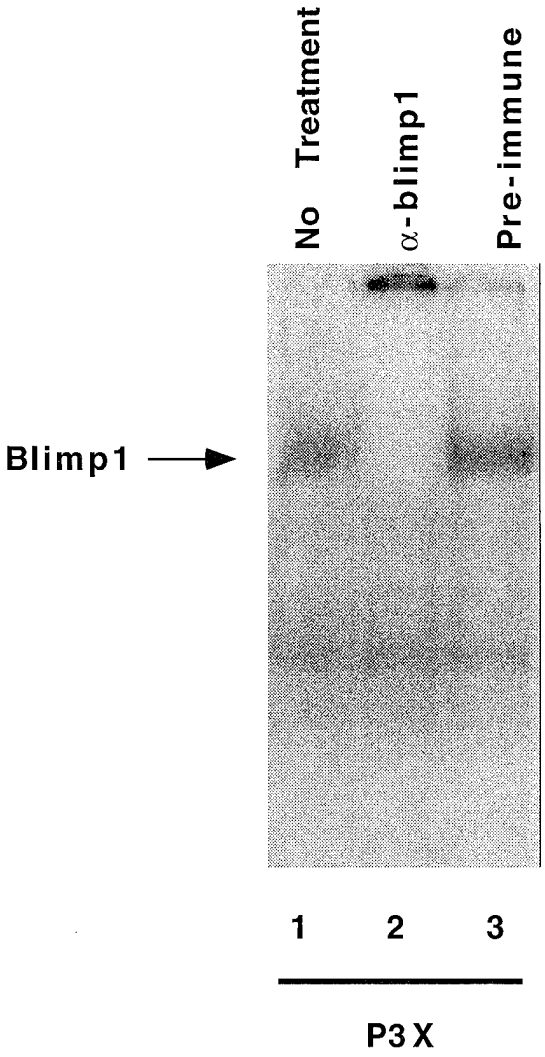

P3X
293T
293T+Blimp1
Competitor
0
PRF
N.S.
0
PRF
N.S.
0
PRF
N.S.
Blimp-1
1
2
3
4
5
6
7
8
9

Figure 7B



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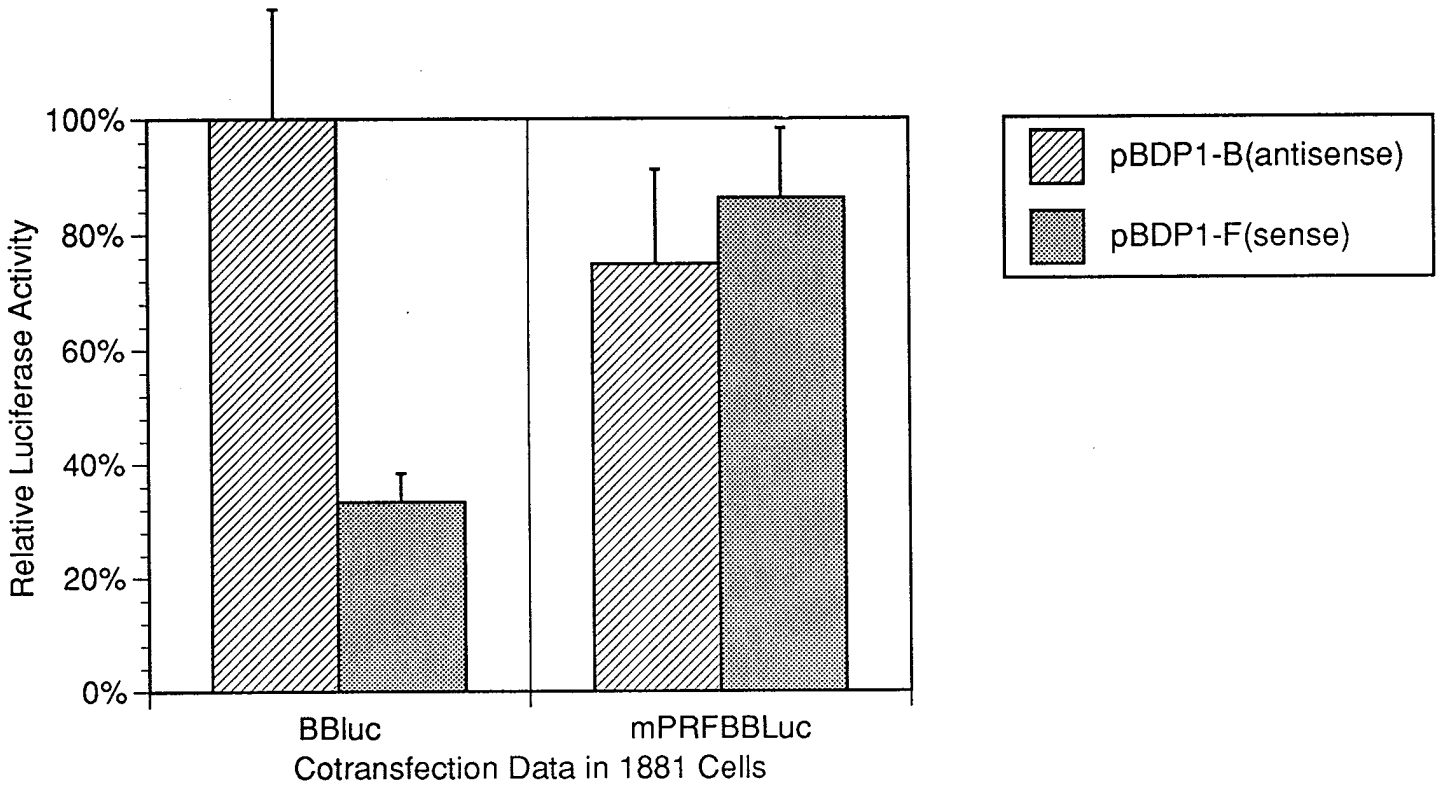
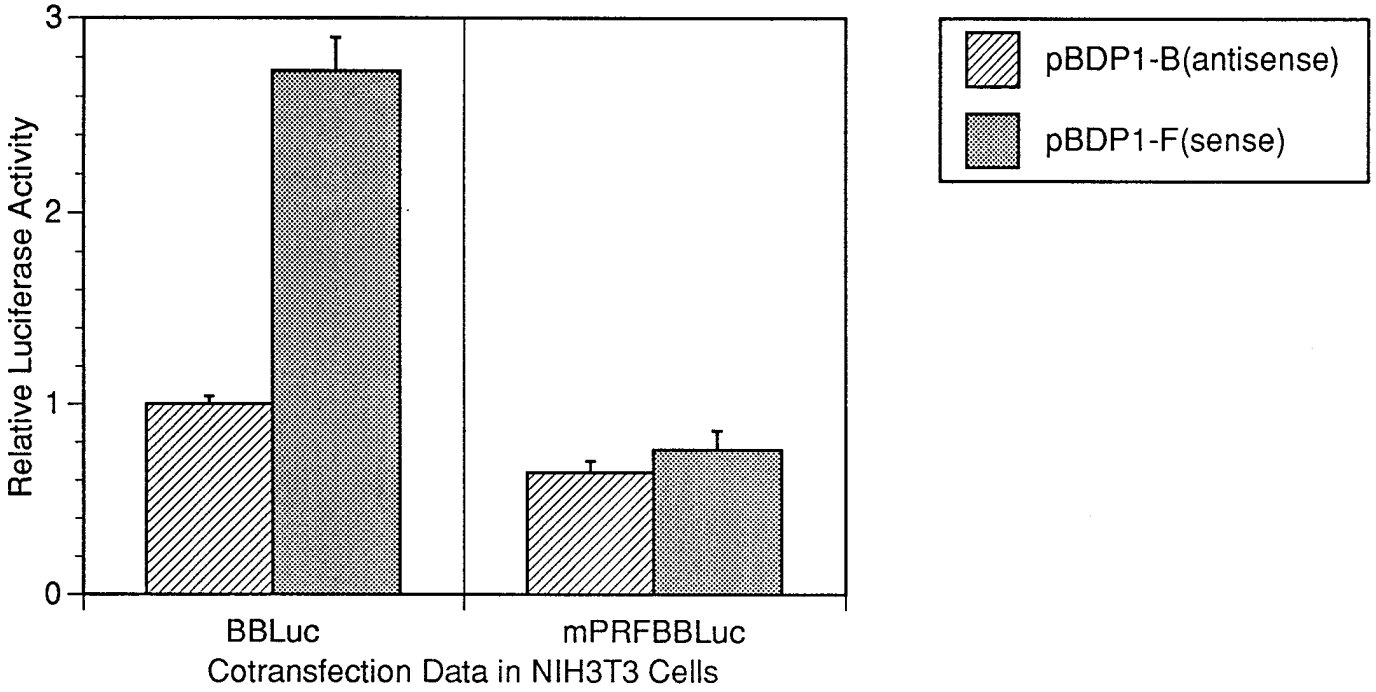

100%
80%
60%
40%
20%
0%
Relative Luciferase Activity
BBluc
mPRFBBLuc
Cotransfection Data in 1881 Cells
pBDP1-B(antisense)
pBDP1-F(sense)

Figure 8B


3
2
1
0
Relative Luciferase Activity
BBLuc
mPRFBBLuc
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pBDP1-F(sense)