THE ROLE OF THE TRANSACTIVATION DOMAIN IN C-MYC MEDIATED CELL CYCLE PROGRESSION AND TRANSFORMATION

By

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Dedicated to Paul West-Osterfield,
Linda West and Milton West.
Without their love and unending support,
I would have not gotten this far.

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LIST OF ABBREVIATIONS

a.a. – amino acid

Ab - antibody

ARF – alternate reading frame

B – basic region

BAF53 – Brg1-associated factor

BR – basic region

c-MycS – c-Myc short form

C-terminal – carboxyl terminal

CDK – cyclin dependent kinase

ChIP – chromatin immunoprecipitation

CS – calf serum

CTD – carboxyl terminal domain

DKO – double knockout (p53-/-, p19 ARF -/-)

DMEM – Dulbecco's minimal essential media

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

EMS – E-box Myc sequence

ER – estrogen receptor

EtOH - ethanol

FBS – fetal bovine serum

GSK-3 – glycogen synthase kinase-3

HAT – histone acetyltransferase

HLH-LZ – helix-loop-helix leucine zipper

hsp – heat shock protein

K – lysine

KCl – potassium chloride

MB0 - Myc box 0

MBI – Myc box 1

MBII – Myc box II

MBIII – Myc box III

MBIV – Myc box IV

MEF – mouse embryonic fibroblast

mRNA – messenger RNA

MTT – 3(3-(4,5-dimethylthiazol-2-yl))-2,5-dipheny tetrazolium bromide

N-terminal – amino terminal

NaCl – sodium chloride

NaF – sodium fluoride

Na_3VO_4 – sodium orthovanadate

OHT – hydroxytamoxifen

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PMSF – phenylmethylsulfonyl fluoride

PR – phenol red

pTEF – positive transcription elongation factors

Puro - puromycin

Q – glutamine

qPCR – quantitative polymerase chain reaction

REF – rat embryonic fibroblast

RNA PolII – RNA Polymerase II

S – serine

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

T – threonine

TBS – Tris buffered saline

TE buffer– Tris/EDTA buffer

TRRAP – transformation/transcription domain-associated protein

TRD – transregulatory domain

Ub – ubiquitin

WT – wild type

CHAPTER I

INTRODUCTION

Biology and molecular functions of c-Myc

c-Myc is an oncogene found overexpressed or misregulated in many different tumor types. In addition to c-Myc, several other Myc proteins exist in vertebrates as a result of two gene duplication events about 400 million years ago, very early in vertebrate evolution (Atchley and Fitch, 1995). Induction of c-Myc expression generates highly invasive tumors in mice that regress in a tissue specific manner (Felsher and Bishop, 1999a; Jain et al., 2002; Pelengaris et al., 2002). Overexpression of Myc induces apoptosis in vitro and in vivo in the absence of survival factors (Askew et al., 1991; Bissonnette et al., 1994; Evan et al., 1992), which can be inhibited by Bcl2 (Fanidi et al., 1992). Myc also inhibits cellular differentiation (Coppola and Cole, 1986), shortens the cell cycle phases G1 and G2 (Karn et al., 1989), increases cell size in specific cell types (Johnston et al., 1999; Piedra et al., 2002; Trumpp et al., 2001) and induces genomic instability and reactive oxidative species (Felsher and Bishop, 1999b; Li and Dang, 1999; Tanaka et al., 2002; Vafa et al., 2002). c-Myc is also an essential gene necessary for specific facets of normal cellular function. Absence of the *c-myc* gene in knockout mice results in early embryonic lethality at 10.5 days with diminished size as compared to their wild-type littermates, failure of neural tube closure and several heart defects (Davis et al., 1993). In immortalized fibroblasts where the *c-myc* gene has been removed by homologous recombination, absence of c-Myc results in a two to three fold increase in cellular doubling time, although

proliferation is not completely halted (Mateyak et al., 1997; Schorl and Sedivy, 2003).

One of the molecular functions mediated by c-Myc is the upregulation of specific target genes. It is a 439 amino acid protein of the basic helix-loop-helix leucine-zipper class of transcription factors. Interaction with the major groove of DNA is mediated by the basic region (BR) of c-Myc, while the helix-loop-helix leucine zipper (HLH-LZ) domain mediates heterodimerization with another bHLHLZ protein Max, both located within amino acids 355-439 as shown in Figure 1 (Ferre-D'Amare et al., 1993; Nair and Burley, 2003). Transcriptional upregulation of target genes by Myc requires heterodimerization with Max (Amati et al., 1993; Blackwell et al., 1990; Blackwood et al., 1992; Gartel and Shchors, 2003; Prendergast et al., 1991). Myc and Max bind to E-box Myc Sequences (EMS) (CA(C/T)GTG) embedded in the non-methylated CpG rich regions of c-Myc upregulated target gene promoters (Fernandez et al., 2003; Mao et al., 2003; Prendergast and Ziff, 1991). However, not all target genes regulated by c-Myc require EMS binding. In addition to Myc's role in upregulation, it also actively represses other subsets of target genes through at least two different mechanisms (Claassen and Hann, 1999; Oster et al., 2002).

As seen in Figure 1, the c-Myc molecule contains several highly conserved regions of the N-terminal domains in addition to the HLH-LZ domains. In addition to the DNA binding and heterodimerization domain, c-Myc also contains an amino terminal transregulatory domain (TRD) from 1-143 that plays a critical role in c-Myc biology. Within the TRD are two highly conserved domains, identified as Myc Box I (MBI) from 45-63, and Myc Box II (MBII) from 129-143 (Oster et al., 2002). Further studies have found other conserved regions,

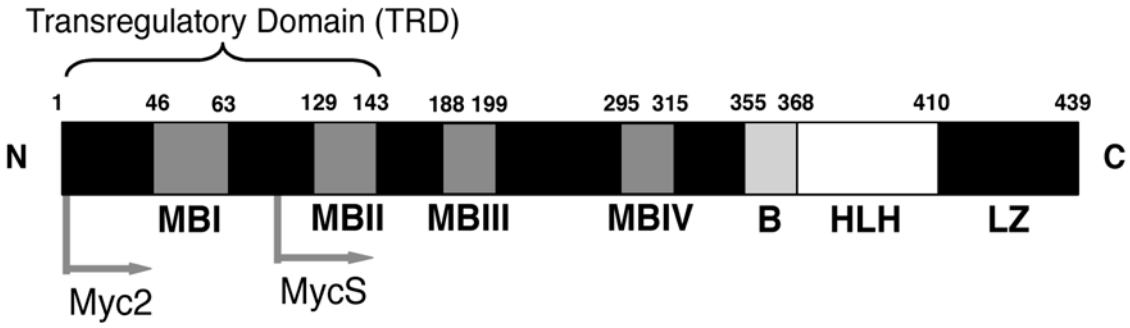


FIG. 1. Schematic of the significant domains within the c-Myc molecule. The numbers above represent the amino acid that borders each significant. Also noted above the Myc diagram is the region defined as the transregulatory domain. The arrows below signify the location where translation of the full-length c-Myc2 or the natural alternative translational start site c-MycS start.

identified as Myc Box III (MBIII) from 188-199 and Myc Box IV (MBIV) from 295-315 (N-Myc 317-337)(Cowling et al., 2006; Herbst et al., 2005). These Myc boxes are virtually identical and remain highly conserved in most other Myc molecules from other genes (Atchley and Fitch, 1995). Myc boxes appear to mediate different molecular and biological functions. Thus we will take a detailed look at each domain, identifying interactions with specific binding cofactors and how this correlates with specific and separable biological functions of c-Myc.

Myc Box I

The most N-terminal domain identified thus far is Myc Box I (MBI), which resides within amino acids 45-63. This region appears to be important in Myc-mediated enhancement of transcriptional elongation. In studies scrutinizing the factors that bound to the *cad* target gene promoter, substantial levels of RNA Polymerase II (RNA PolII) were found at the *cad* promoter even though no

upregulation of CAD was occurring at this stage, regardless of the presence or absence of Myc. (Eberhardy and Farnham, 2001). Following Myc induction, the RNA PolII C-terminal tail showed increased hyperphosphorylation in addition to an increase in the accumulation of RNA PoII found at the 3' end of the *cad* target gene. Together, these indicate a role for Myc in the processivity of RNA PolII elongation, rather than just initiation of transcription (Eberhardy and Farnham, 2001). One of the complexes known to be involved in transcriptional elongation is the pTEFb complex (Figure 2). pTEFb is a multi-cofactor complex comprised of the active subunit pair of CDK9 and Cyclin T1. It promotes the elongation of RNA PolII transciption post-initiation. Cyclin T1 was found to directly bind to a small region of Myc encompassing MBI (Eberhardy and Farnham, 2002). Further evidence suggests that the recruitment and activity of pTEFb is a major mechanism mediating some Myc activity, since Myc-mediated proliferation and apoptosis were inhibited upon Myc induction in the presence of a pTEFb specific inhibitor (Kanazawa et al., 2003). One study also implicated this complex in Myc-induced upregulation of Cyclin D2 and its involvement in cell size increase. In tamoxifen-induced myocardium targeted conditional *myc* knockout mice with hemodynamic stress, the myocardium hypertrophic response was attenuated upon tamoxifen induction. This response was independent of Cyclin D2, although Cyclin D2 was a requisite for cell cycle re-entry (Xiao et al., 2001; Zhong et al., 2006).

F-Box proteins also appear to play a critical MBI-dependent role. Fbw7 is an F-Box protein involved in ubiquitination of Myc and its subsequent degradation. Specifically Fbw7 could only interact with an MBI peptide containing phosphorylated T58/S62 (Figure 2) (Yada et al., 2004). Depletion of

Fbw7 also increased the transactivation of an EMS reporter gene, although it is unclear whether Fbw7 plays a direct molecular role in the inhibition of transactivation or simply stabilizes Myc levels (Yada et al., 2004). Further studies should enlighten Fbw7's role in transcriptional regulation and elucidate MBI's role in transcriptional regulation.

Myc Box II

The next highly conserved domain after MBI is Myc Box II (MBII) at amino acid 129 to 143. MBII has also been shown to be critical for c-Myc-induced repression, although the specific cofactors mediating repression that interact with this region are not agreed upon (Kleine-Kohlbrecher et al., 2006). A complicating factor in the study of repressed target genes, similar to that of upregulated target genes, is that repression is also governed by several potential mechanisms. Miz1-mediated repression is one of the most thoroughly studied of these mechanisms. Miz1 is a transcription factor that activates specific target genes such as p21; however, Myc binding causes repression of Miz-1 activation (Wanzel et al., 2003). However, the identity of the inhibitory complexes that Myc brings to Miz1 at Miz1 binding sequences is still disputed, as is whether Miz interaction is even necessary for Myc target gene repression. Binding occurs within the c-Myc C-terminus (Figure 2), and it is unclear why the MBII region would be necessary for repression. Recent findings have also implicated Dnmt3a, a histone methyltransferase, as a c-Myc cofactor. In cells where Dnmt3a was depleted, repressed target genes such as p21 were derepressed, while EMS-containing Myc-upregulated target genes remained unchanged (Brenner et al., 2005). In addition, p21 repression was sensitive to DNA methyltransferase

inhibitor and Dnmt3a was found to directly interact with the MBI and MBII regions in addition to Miz1 (Brenner et al., 2005). Dnmt3a also interacts with HDAC1, a histone deacetylase (Fuks et al., 2001). Just as the ATP-dependent DNA helicase-like cofactors of TIP48/49 have been found on Myc-upregulated target gene promoters, it is possible that the TIP48/49 molecules could be involved in target gene repression, specifically repression of Miz1 mediated CKI target genes that govern cell cycle progression (Bellosta et al., 2005; Etard et al., 2005). However, the exact function of TIP48/49 at the target gene promoter is

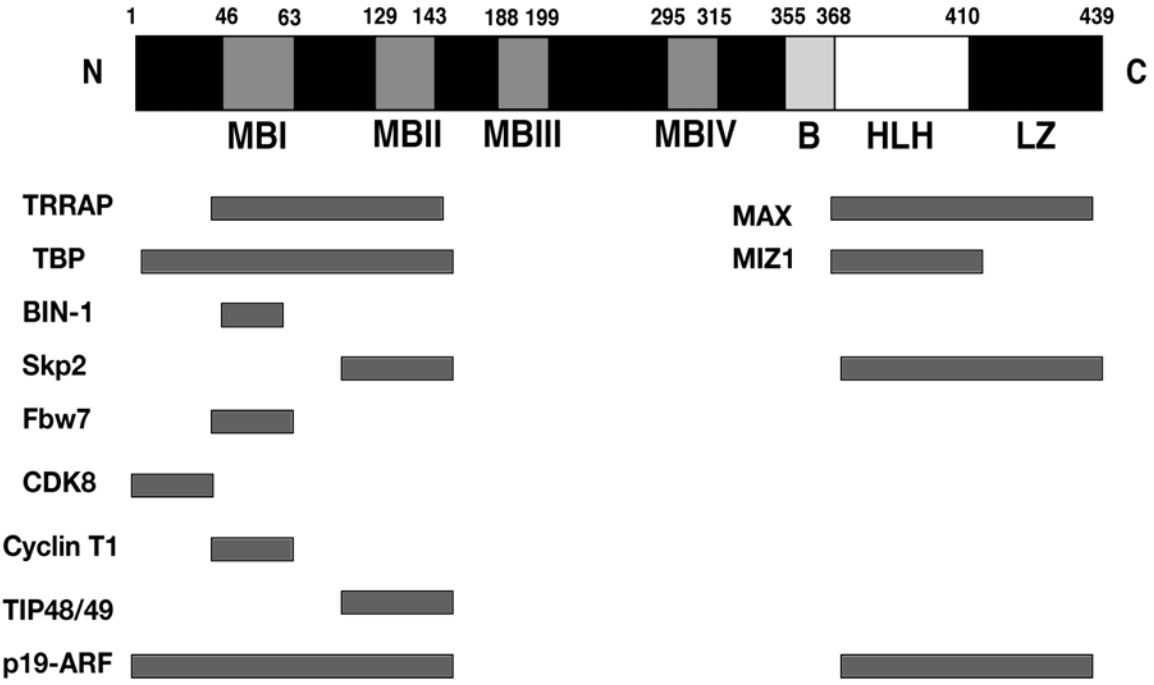


FIG. 2. Locations of c-Myc binding cofactors. This diagram shows the numerous known cofactors that are able to bind to c-Myc and their location of binding within the Myc molecule. Note that most cofactors interact either within the TRD or the bHLHLZ domain. Some cofactors, such as Skp2 or p19-ARF, bind at both the TRD and bHLHLZ domain.

unclear, and it is also unclear whether promoter specific context can determine the transcriptional outcome.

In addition to a strong role in target gene repression, MBII is also involved in target gene upregulation. A major molecular function of c-Myc linked to the MBII region is histone acetylation. Initial findings found the co-binding of a scaffolding protein called TRRAP to the c-Myc N-terminus. A TRRAP fragment from 1899-2401 amino acids, which bound to Myc, was able to slow down cell cycle progression by nearly three fold in one neuroblastoma cell line. However, in another cell line, the effects on cell cycle progression were not evident (Dugan et al., 2002; Park et al., 2001). Another fragment of TRRAP, a.a. 1261-1579, a fragment able to inhibit Myc/Ras cotransformation, did not interfere with progression of the cell cycle (McMahon et al., 1998; Park et al., 2001). Several studies have shown a critical role for TRRAP and the resulting histone acetylation in the upregulation of some Myc-mediated target genes. Myc, TRRAP, and acetylated Histone 3 and Histone 4 were all found at the *cyclin D2* promoter and recruitment was dependent on the integrity of MBII (Bouchard et al., 2001). However, other studies have shown that recruitment of TRRAP, and its mediation of histone acetylation, was not necessary to facilitate upregulation of some other target genes (Nikiforov et al., 2002; Zhang et al., 2006)

Other molecules associated with TRRAP have been targeted for further scrutiny in c-Myc molecular mechanisms. One of them is hGCN5 which is partially involved in Myc-Ras cotransformation (McMahon et al., 2000; Park et al., 2001). Two other cofactors were also found to associate with MBII, TIP48 and TIP49. Studies found that TIP48 was able to bind the MBII region in addition to a region within amino acids 1-96 (Wood et al., 2000). Functional TIP49 was a

critical component in Myc induced apoptosis in some systems and cell cycle progression. This is in contrast to the inhibitory TRRAP fragments that were not involved in Myc-induced apoptosis (Bellosta et al., 2005; Dugan et al., 2002). Further studies have shed light on the molecular function of TIP48 and TIP48 interaction. One study found that TIP48 and TIP49, in conjunction with Myc, were co-recruited to the nucleolin promoter, a well known upregulated Myc target gene, although the role of TIP48/49 role on the promoter is not known (Frank et al., 2003). The actin related, chromatin-modifying protein BAF53 also bound to MBII and a region preceding MBII. It formed distinct complexes with TIP48/49, TRRAP, another HAT and the human SWI/SNF complex (Park et al., 2002). Deletion of one segment of BAF53 impaired cotransformation by inhibition of histone acetyltransferase recruitment and reduced TIP49 binding, even as TRRAP binding was unaffected (Park et al., 2002). Indeed, the potential role of histone acetylation in target gene upregulation, the TIP's and BAF53's role in other c-Myc function and target gene regulation remains to be clarified.

Aside from the thoroughly studied role of histone acetylation and possible chromatin modification at target gene promoters, some studies have shown a connection between MBII and upregulation of target genes through other mechanisms. In addition to the F-box protein Fbw7 found to interact with a phosphorylated MBI, another F-box cofactor called Skp2 was found to bind to MBII as well as to the HLH-LZ of c-Myc (von der Lehr et al., 2003b). The presence of Skp2 appeared to be a component in the activation of several upregulated Myc target genes in addition to being present with other proteasomal subunits on endogenous promoters of upregulated target genes (Kim et al., 2003; von der Lehr et al., 2003b). However, Skp2 was also able to

interact with Cyclin T1 to induce the ubiquitination and destruction of CDK9 in the pTEFb complex (Kiernan et al., 2001). In addition, Skp2 actually enhanced Myc-induced cell cycle progression through S-phase, although whether this was directly through target gene regulation or assistance in degradation of cell cycle proteins is unclear. Given the data, it is theorized that ubiquitination may actually be necessary to activate transcription while simultaneously ensuring their quick destruction. This hypothesis, that ubiquitination induced by Skp2 increases transcription of target genes, is referred to as "licensing". (Salghetti et al., 2000; von der Lehr et al., 2003a)

Myc Box III and Myc Box IV

In addition to the well studied MBI and MBII, other conserved regions have been found that also contribute to Myc-mediated activity. One region encompasses amino acids 188-199, and has been termed MBIII. MBIII plays a critical role in lymphomagenesis, as well as anchorage-independent growth of Rat1a cells. However, it had little if any involvement in cell cycle progression or rescue of the slow-growing *myc* null phenotype in HO16 immortalized fibroblasts (Herbst et al., 2005). Unlike MBI and MBII that are required for apoptotic induction, MBIII appears to mediate quite the opposite. In the presence of apoptosis-inducing agents, deletion of MBIII increased apoptosis in both HO16 fibroblasts and primary cells. Survival increased with ectopic expression of Bcl2 (Herbst et al., 2005). This is the only Myc box identified thus far that appears to play a role in Myc-induced cell survival rather than apoptosis.

Although MBIII mediates specific biological functions, less is known about the molecular mechanisms correlated with this region or even the cofactors

that bind to this region. MBIII is a critical component in target gene repression and slightly contributes to target gene upregulation of some target genes (Herbst et al., 2005). This region also contributes to protein turnover under specific cellular conditions. Deletion of a region referred to as the D-Box, which overlaps MBIII, results in a more stable Myc protein without significant increases in ubiquitination. Even though the D-Box/MBIII is a potent degradation domain in the primary cell REFs, in immortalized fibroblasts degradation is regulated through a different region altogether (Herbst et al., 2004). Whether this difference in cellular ubiquitination and turnover mediated by MBIII is important to the molecular function of c-Myc remains to be determined.

Further downstream of MBIII lies another conserved region termed MBIV, which is located within amino acids 317-337, just before the basic region responsible for DNA binding. Although not wholly involved in transformation, MBIV does appear to have some control over specific facets of transformation. Deletion of MBIV did not affect Myc/Ras cotransformation in primary rat fibroblasts, nor did it affect anchorage independent growth in immortalized Rat1a cells, but the Rat1a cells retained their contact inhibition (Cowling et al., 2006). Removal of MBIV did not affect the hyperproliferation of already cycling cells, but it did affect cell cycle progression of quiescent cells as evidenced by an accumulation of cells in G2, and inhibited apoptosis (Cowling et al., 2006). This shows a critical difference between progression of the cell cycle and permission to progress through the cell cycle. MBIV deletion also diminished transactivation and repression, but this did not necessarily apply to all target genes. This could be explained simply by reduced DNA binding by deleting the MBIV (Cowling et al., 2006). What subsets of target genes are governed through

the mediation of MBIV-dependent mechanisms remains to be determined. Which cofactors bind to this region or if any cofactors interact with this region is also unknown. However, study of this region could elucidate the specific target genes responsible for the requirements of cotransformation and re-entry into the cell cycle as compared to progression through a pre-established cell cycle.

Function of the N-terminal 41 amino acids

Although the aforementioned Myc regions have been thoroughly studied, the N-terminal 41 amino acids within the transregulatory domain may also be important for Myc biology. Suggestion of the potential significance of this region is found in a screen of familial breast cancer. The genetic screening of Polish and German populations (excluding those testing positive for BRCA1/2 mutations) found a high prevalence of Asn11Ser point mutations present in *c-myc*, which is a highly conserved amino acid across species, although its role in carcinogenesis is unknown (Wirtenberger et al., 2005). One potential cofactor whose binding may be significant to this region is CDK8, a component of the Mediator holoenzyme complex. CDK8 has been shown to bind to amino acids 1-41 of c-Myc (Eberhardy and Farnham, 2002). CDK8 with Cyclin C are two of the components of the Mediator complex (Biddick and Young, 2005). The Mediator complex interacts with numerous cofactors that mediate phosphorylation of the RNA PolII carboxyl tail at distinct sites from those phosphorylated by the pTEFb complex (Kobor and Greenblatt, 2002). This phosphorylation can regulate recruitment of RNA PolII to gene promoters. However, several studies on Mediator have not conclusively determined whether Mediator acts as a positive or negative transcriptional initiator. Specifically, CDK8 and Cyclin C were found

to be negative regulators of transcription by phosphorylating the RNA PolII carboxyl tail before its arrival on the promoter, although contrasting evidence indicates that Mediator complexes containing CDK8 participated in activation of reporter genes (Furumoto et al., 2007; Sano and Schneider, 2003).

Other cofactors: post-translational modification and p19ARF binding

One important means of regulating c-Myc activity is through post-translational modification. In particular, phosphorylation has been shown to play a significant role in c-Myc stability and function. Several phosphorylation sites have been found in the N-terminal region of c-Myc (Henriksson et al., 1993; Lutterbach and Hann, 1994; Lutterbach and Hann, 1997; Pulverer et al., 1994) One site with noteworthy effects on biological function is the phosphorylation site at T58. GSK3α/ß was found to phosphorylate T58 which was highly dependent on prior phosphorylation of S62 (Gregory et al., 2003; Lutterbach and Hann, 1994; Sears et al., 2000). Mutations of this threonine to an alanine enhanced c-Myc-mediated primary cell cotransformation with Ras (Chang et al., 2000; Pulverer et al., 1994), although rescue of the slow growth phenotype of *myc*-null immortalized fibroblasts was restored regardless of the mutation at T58 (Chang et al., 2000). Some evidence has also shown that although transactivation at the EMS was not enhanced by mutation of T58, repression of gadd45, a repressed target gene, was diminished (Conzen et al., 2000; Lutterbach and Hann, 1994).

An additional post-translational modification closely associated with c-Myc phosphorylation is ubiquitination. c-Myc has been shown to be highly ubiquitinated which mediated c-Myc proteolysis by the proteasome (Gregory

and Hann, 2000). Specifically, the N-terminal domain was found to be the critical domain for ubiquitination (Gregory et al., 2003) Two cofactors involved in ubiquitination and protein destruction, Skp2 and Fbw7, have been shown to interact at the N-terminal domain, with Fbw7 interacting in a phosphorylation-specific fashion (Kim et al., 2003; Yada et al., 2004) However, the effect of these two cofactors had different effects on Myc-induced transactivation. The presence of Skp2 appeared to be an activating component in transactivation, while depletion of Fbw7 appeared to increase transactivation. The exact role of ubiquitination in this process is still unclear (Kim et al., 2003; von der Lehr et al., 2003b; Yada et al., 2004).

p19ARF is a tumor suppressor encoded by the INK4a locus that also codes for $p16^{Ink4a}$, although ARF is translated in an alternate reading frame. ARF was found tightly bound to c-Myc within the first 150 amino acids and the C-terminus of Myc (Qi et al., 2004). This association of ARF with c-Myc had a significant effect on c-Myc biology. The presence of ARF severely inhibited hyperproliferation in Rat1a cells, and soft agar growth in p53 -/-, p19ARF -/- (DKO) fibroblasts while enhancing Myc-induced apoptosis (Qi et al., 2004). ARF also inhibited the entry of quiescent HaCat cells into S phase normally induced by c-Myc (Datta et al., 2004). Although the presence of ARF did not affect repression of c-Myc target genes, ARF severely inhibited the transcriptional activation of several target gene promoters and the upregulation of several target genes. Further evidence of a role in transcription was shown by the presence of ARF at the promoter of several upregulated target genes (Qi et al., 2004). The exact mechanism of how ARF influences Myc in target gene regulation is unknown.

Studies in separation of function: MycS

One approach to studying the function of MBI is through examination of a naturally occurring c-Myc variant, MycS. MycS is generated through an alternate ATG start site 300bp from the full length Myc start site; therefore it is missing the N-terminal 100 amino acids, including MBI (Spotts et al., 1997). When expressed in immortalized Rat1a fibroblasts, MycS-induced hyperproliferation, apoptosis, soft agar growth in Rat1a cells, and rescued the slow growth phenotype of immortalized *myc* null rat fibroblasts, similar to full length wild type Myc2 (Oster et al., 2003; Xiao et al., 1998). However, unlike Myc2, MycS was severely impaired in inducing cell cycle progression of quiescent, primary fibroblasts in the absence of serum, as well as being impaired in apoptotic activity and the cotransformation of primary cell in conjunction with activated Ras (Hirst and Grandori, 2000; McMahon et al., 1998). In Drosophila imaginal disc cells, MycS could not induce any apoptosis whatsoever, even with two copies of the *mycS* transgene present (Benassayag et al., 2005). In contrast, when MycSER was expressed in primary human WI38 cells under higher serum conditions, MycSER initially outperformed Myc2ER in proliferation, then prominent apoptosis slowed down MycS proliferation, although hyperproliferation still occurred (Hirst and Grandori, 2000). In Drosophila, while MycS induced cell cycle progression similar to dMyc and Myc2, increases in cell size were never seen with MycS, which lead to decreased progeny size (Benassayag et al., 2005). Therefore it appears that the N-terminal 100 amino acids play a significant role in the progression of the cell cycle from a quiescent

state. However, in immortalized cells, which do not enter G0, this specific function may have less importance.

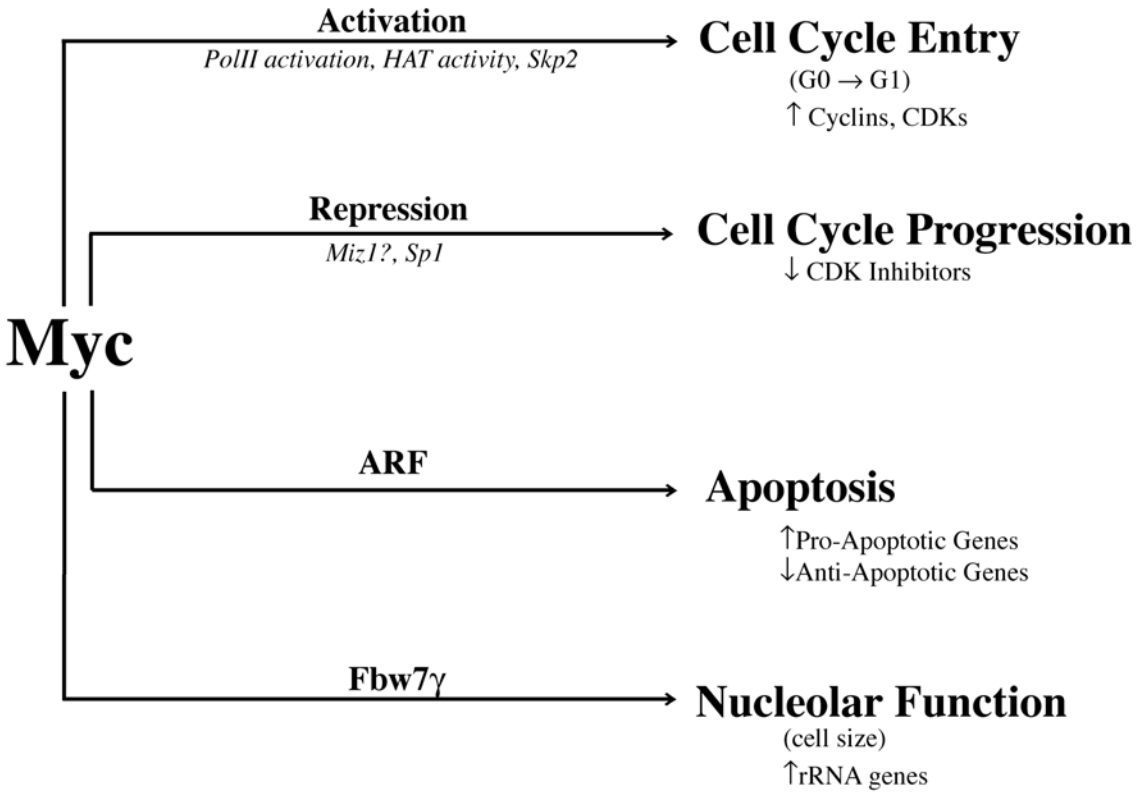


FIG. 3. Biological pathways mediated by c-Myc. The flow chart shows some of the predominant biological functions of c-Myc and how separate molecular functions may mediate each biological effect. Within each pathway are listed either the cofactors (ARF or Fbw7γ) or molecular functions (activation, repression) that are believed to mediate each function

In addition to the biological differences found between Myc2 and MycS, the molecular mechanisms vary as well. Most noteworthy, MycS did not transactivate using transient reporter assays. However, within the same assay MycS repressed downregulated target gene promoters similar to Myc2 (Xiao et al., 1998). When the results were compared to endogenous target genes, in contrast to a transient reporter gene, the results were a bit more ambiguous.

When upregulated target gene levels were assessed in immortalized mammalian cells and Drosophila, MycS showed less pronounced upregulation and downregulation in some target genes. The effect of MycS on target genes in primary cells is still not firmly established (Benassayag et al., 2005; Hirst and Grandori, 2000). This indicates that the mechanisms that mediate activation of a transiently expressed target gene are not always the same as those that mediate target gene upregulation. Although these studies do uncover a bit of the mechanisms mediating the functions of Myc, we cannot exclude other regions within those first 100 amino acids that may have a significant value.

Purpose and hypothesis

Previous research has shown that MycS, which is missing the first 100 amino acids including MBI, can induce some biological functions of c-Myc in immortalized cells but is unable to induce biological effects in primary cells. This indicates that specific regions can mediate distinct functions of c-Myc. I hypothesized that the first 100 amino acids have a role in primary cell transformation and cell cycle progression. My project specifically assessed the significance of specific domains on c-Myc-induced transactivation and on c-Myc induced target gene upregulation and c-Myc-induced transformation and cell cycle progression. In addition, I assessed whether these regions were necessary or sufficient for specific c-Myc functions and also how modifications of these regions could potentially affect c-Myc activity.

CHAPTER II

METHODS

Constructs

Stable cell lines expressing inducible c-Myc, c-Myc deletion mutants, and minimal Myc constructs utilized the retroviral vector pBabe-puromycin (puro). Hydroxytamoxifen (OHT)-inducible Myc2ER in pBabe-puro was used as a template for all of the previously described c-Myc constructs (Xiao et al., 1998). The Ψ2 retroviral packaging cell line was maintained in Dulbecco's minimal essential media (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For cotransformation experiments, CßS-wtMyc, empty vector CßS and a genomic fragment containing HRasG12V cloned into pSP6 were used, which were a generous gift from Michael Cole (Dartmouth). p19ARF in pcDNA3 was derived from p19-ARF in pRC-CMV (Qi et al., 2004). The reporter gene pFR-Luc was supplied by Stratagene, which was used for all Gal4 assay experiments. pGL4.14 (luc2/hygro) used for stably integrated 2X EMS was supplied by Promega.

Tissue culture

Rat1a fibroblasts were maintained in DMEM containing 10% calf serum, penicillin/streptomycin and 2.5 μg/ml puromycin. Mouse DKO (p53 -/-, p19ARF -/-) fibroblasts were maintained in phenol red – (PR-) DMEM containing 10% charcoal stripped calf serum (CS), penicillin/streptomycin and 2.5 μg/ml puromycin or 50 μg/ml hygromycin for Myc2ER. Primary Fisher 344

rat embryonic fibroblasts (REF), which were harvested at embryonic day 12.5 and passaged twice before shipment were supplied by Artis Optimus. They were passaged in DMEM containing 10% FBS, 10 mM glutamine, penicillin/streptomycin and nonessential amino acids. Cos7 cells were maintained in DMEM containing 10% CS and penicillin/streptomycin. Monoclonal *myc* -/- HO16 cell lines were maintained in DMEM containing 10% FBS, penicillin/streptomycin, sodium pyruvate and 3 μg/ml puromycin.

Western blotting reagents and analysis

For analysis of c-Myc expression in whole cell lysates, one 10 cm plate of confluent cells was lysed with 750 μl of ice-cold Ab Buffer (Hann et al., 1983), sonicated for 10 seconds on ice and clarified by centrifugation at 10000 rpm for 5 minutes. Protein concentrations were determined using the DC Protein Assay kit as directed (BioRad.) Equal quantities of each lysate were loaded on 12.5-15% polyacrylamide gels and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Rainbow Broad-Range Molecular Weight Ladder (Amersham) used as a molecular weight standard. After complete electrophoresis, the gel was then transferred to a nitrocellulose membrane and pre-blocked with 5% powdered milk in Tris buffered saline (TBS) with 0.1% Tween-20 for Western analysis.

Antibodies used for Western analysis include a sheep antibody against full length c-Myc, a rabbit antibody against full length c-Myc, a monoclonal Gal4 antibody targeted to the first 146 amino acids (Upstate) and rabbit anti-Cyclin T1 (Santa Cruz.) Blots were visualized using the Odyssey IR scanning system (Licor). In order to resolve immunoreactive proteins with the Odyssey scanner,

secondary antibodies conjugated to infrared spectra fluorophores were utilized. These secondary antibodies included donkey anti-goat AlexaFluor 680, goat anti-mouse AlexaFluor 680, donkey anti-sheep AlexaFluor 680 (Invitrogen) and donkey anti-rabbit IRDye 800 (Rockland).

Gal4 cloning

The plasmid used to generate Gal4-Myc fusion proteins was a pcDNA3 containing the coding sequence for the DNA binding domain of the yeast Gal4 transcription factor (amino acids 1-146) and a multiple cloning site 3′ of the Gal4 coding sequence originally derived from Ptashne et. al. (Farrell et al., 1996; Nevado et al., 1999). c-Myc in pcDNA3 was used as a template to generate PCR fragments containing a 5′ BamH1 and 3′ Xba1 as restriction sites to insert into the 3′ end of the Gal4 coding sequence. The primers listed below, synthesized by IDT-DNA, were used to generate each fragment of c-Myc in different combinations,

Fwd Myc2-Gal4	5′ GGATCCCGATGCCCCTCAAGG 3′
Fwd MycS-Gal4	5′ GGATCCAGATGATGACCGAG 3′
Fwd Mycdel150-Gal4	5′ GGATCCTGGCCTCCTACCAGG 3′
Rev MycTRD 262 Gal4	5′ GGTTTGCCTCTTCTCCACAGATCTAGATT-AAATTTC 3′
Rev MycGal4 1-100	5′ GGTTCTAGATTACAGCTGATCGGCG 3′
Rev MycGal4 1-62	5′ AGCGTCTAGATTAGGACAGGGGC 3′
Rev MycGal4 47-100	5′ CCGGGATCCGTGAGGATATCTG 3′

Rev MycGal4 1-46	5′ TCCATCTAGATTAACTGGGCGCGG 3′
Fwd MycGal4 63-100	5′ GCGGATCCCCCCGAGCCGCC 3′

The PCR amplified Myc fragments were gel purified, digested with Xba1 and BamH1 and repurified using the Qiagen Gel Extraction Kit as directed by the manufacturer. Xba1/BamH1 digested and calf intestinal phosphatase-treated Gal4 pCDNA2 vector and each fragment were ligated together using Quick Ligation Kit as directed (New England Biolabs). Two microliters of each transformation mixture were transformed into DH5α cells and selected with ampicillin. Ampicillin resistant clones were isolated and the desired constructs were verified with restriction enzyme digestion and nucleotide sequencing.

Minimal Myc cloning

For each of these constructs, Myc2ER in pBabe-puro was used as the parental template and vector. PCR amplification using the primers listed below (IDT-DNA) was used to amplify specific segments containing each fragment of the N-terminus, with an Sml1 restriction site used to ligate the N-terminal and C-terminal sequences together.

Rev Myc100Sml1	5′ GGTCTTGAGCTCCAGCTGATCGGC 3′
Rev Myc156Sml1	5′ GTCTTTCTTGAGAGCCTGGTAGGA 3′
Rev Myc46Sml1	5′ TATCCTTGAGGGGCGCGG 3′
Fwd kozMyc Sml1	5′ AGCAAACCTGGATCCGCCGCCACC ATGCTCAAGAGG3′

Fwd kozdl156BamH1 5′ GAGAAGGGATCCGCCGCCACCATG GCGCGCAAAGA 3′

Fwd koz Myc2 BamH1 5′ CCCGGATCCAGCCGCCACCATGCC CCTCAACGT 3′

3' Jcn MycER BamH1 5′TAGGTCAGGATCCGCACCAGAG

Myc2ER was digested with BamH1 and calf intestinal phosphatase in order to remove the Myc2 sequence and open up pBabe-puro vector. One N-terminal fragment, one C-terminal fragment and the vector were ligated in an overnight ligation, transformed into DH5α cells and plated on ampicillin plates to select for resistant clones. Ampicillin resistant clones were selected and verified for construct placement and appropriate direction with restriction digest and nucleotide sequencing.

Deletions and point mutations

Coding sequences for *myc* gene transcripts and *c-myc* transcripts within other species were derived from GenBank and translated using MacVector nucleotide analysis program. The protein sequences 5′ of the MBI were aligned with each other using MacVector software and the ClustalW algorithm (Thompson et al., 1994). Results from these alignments determined the deletions studied, which included deletion of MB0, MBI, the PolyQ domain and a combination of the MB0 and PolyQ domain. Stratagene's Site Direction Mutagenesis kit was used as directed by the manufacturer to delete each region as noted in the forward and reverse primers noted below.

MBI Deletion

Forward: 5′ CTGCAGCCGCCCGCGCCCCCGAGCCGCCGCTC 3′

Reverse: 5′ GAGCGGCGGCTCGGGGGCGCGGGCGGCTGCAG 3′

MB0 Deletion

Fwd: 5′ CAACGTGAACTTCACCAACAATTTCTATCACCAGCAACAG 3′

Rev: 5′ CTGTTGCTGGTGATAGAAATTGTTGGTGAAGTTCACGTTG 3′

PolyQ Deletion

Fwd: 5′ CTGCGACGAGGAAGAGCTGCAGCCGCCCGCG 3′

Rev: 5′ CGCGGGCGGCTGCAGCTCTTCCTCGTCGCAG 3′

MB0PolyQ Deletion

Fwd: 5′ CAACGTGAACTTCACCAACCTGCAGCCGCCCGCG 3′

Rev: 5′ CGCGGGCGGCTGCAGGTTGGTGAAGTTCACGTT G 3′

For all Gal4-Myc deletions, Gal4 Myc1-100 in pcDNA3 was used as a template while full-length c-Myc deletions utilized c-Myc in the CßS and CTF expression vector as a template. After complete amplification of the entire length of the plasmid template, Dpn1 digestion was performed to digest the bacterially derived methylated DNA. The resulting reaction was transformed into DH5α cells with ampicillin selection. Positive clones were isolated and confirmed with restriction enzyme digestion and nucleotide sequencing. Deletion mutants in CßS were used as a template to amplify the *myc* coding sequence with the restriction enzyme sites necessary to insert the c-Myc fragment into the retroviral

pBabe-puro expression vector (5′ BamH1, 3′ EcoR1) and the inducible construct pBabe-puro MycER (5′ and 3′ BamH1). Appropriate digestion was performed on both the inserts and vectors, and the insert and vector were ligated together in an overnight reaction at 12˚C. After transformation and ampicillin selection, ampicillin resistant colonies from the pBabe-puro ligation were confirmed with restriction enzyme digestion and subsequent nucleotide sequencing. Point mutants of Gal4-Myc100 containing T58A, or K51R and K52R were generated by Mark Gregory (Vanderbilt) using the same mutagenesis methods as described above for the Gal4-Myc deletions.

Establishment of stable cell lines

Retroviral infection using constructs in the pBabe-puro vector were used for stable Myc expression in Rat1a, DKO, or HO16 cells. Ψ2 cells at 50% confluence were transiently transfected with pBabe-puro constructs using Fugene6 (Roche) as directed. The next day, the old media was removed and replaced with 6 ml DMEM with 10% FBS. 24 hours later, the viral media was collected, filtered and added to two 10 cm plates containing Rat1a or DKO fibroblasts at 50% confluence. Two days post-infection, each cell line was selected using 10 μg/ml puromycin for two weeks. Once protein levels were assessed through Western blot analysis, the puromycin concentration was reduced to maintenance concentrations.

Gal4 and stably integrated EMS reporter assays

18 hours before transfection, a confluent plate of Cos7 cells was split to 80% density in 6-well plates. The next day calcium phosphate precipitation was

used for transfection (Graham and van der Eb, 1973; Wigler et al., 1978) of Cos7 cells with Gal4-Myc in pcDNA3 for approximately equivalent expression (based on previous optimizations, approximately 1μg to 1.5μg) and 1 μg of the Gal4 recognition sequence in pFR-Luciferase (Stratagene). Six hours post transfection the media was changed to fresh 10% CS DMEM. Cells were lysed 2 days after transfection with 160μl of Passive Lysis Buffer (Promega) for 30 minutes at 4˚C. For experiments assessing levels of stably integrated EMS reporter activity, 2X EMS in pGL4.14 (luc2/hygro) was transfected into stably expressing Rat1a fibroblasts expressing minimal Myc molecules using Fugene6 as directed (Roche). Two days post transfection the cells were selected with a combination of 3 μg/ml puromycin and 100 μg/ml hygromycin and regrown until they reached high density. 24 hours before harvest, all Rat1a samples were induced with 1 μM OHT. The resulting lysate of all samples was collected, with 60 μl retained for Western blot analysis to assess Gal4-Myc or Myc expression levels. The remainder was assayed for total luciferase activity using the BrightGlo Luciferase Assay kit (Promega) as directed and Pharmigen's LumiLight single tube luminometer. Samples were read in triplicate. Results are expressed in fold difference over Gal4 activity. Although expression levels of Gal4-Myc were relatively similar, luciferase values were also normalized against the relative expression of each construct within the assay sample using the protein densitometry function in the Licor Odyssey software.

Myc/Ras cotransformation

Low passage primary rat embryonic fibroblasts (Artis Optimus) were thawed and passaged twice to expand the cells before the start of the experiment.

Cotransformation experiments utilizing these cells were based on protocols performed by Michael Cole (McMahon et al., 1998). $8*10^5$ cells were plated onto 10 cm plates from confluent 10 cm plates. 24 hours later, 12.5 μg CßS-Myc2, ΔMB0, ΔMBI or empty CßS were transfected by calcium phosphate precipitation with an equal amount of a genomic fragment containing H-Ras G12V in pSP6. The next day, the media was removed and replaced with 8 ml of DMEM containing 4% FBS. The media was changed every 3 days until the end of the experiment. Foci appeared after 1 week of incubation. Fourteen days after transfection, each plate was rinsed once with PBS, fixed with ice-cold methanol for 10 minutes, rinsed additionally with distilled water, and stained using methylene blue for 2 minutes. After two additional water rinses the plates were air-dried overnight. Visible colonies were counted using BioRad's ChemiDoc and Quantity One software with the same reading parameters for every plate. The numbers of colonies and the surface areas of visible colonies were tallied and averaged together. These experiments were repeated at least twice.

Rat1a proliferation and apoptosis assays

For proliferation experiments, $2*10^4$ Rat1a cells expressing wild type Myc or a deletion mutant were seeded in triplicate 12-well plates with 1.5 ml DMEM containing 10% CS and 2.5 μg/ml puromycin. Cells were fed every other day with fresh 10% CS DMEM. To differentiate between living and dead cells, adherent cells were trypsinized from the plate, while floating cells were collected and the volume reduced by centrifugation. Triplicate counts were performed using a hemocytometer. Counts from the adherent and floating cells were

combined into the total cell count and averaged together with the standard deviation shown for each time point.

The MTT assay was an additional assay used to quickly assess net increases in cellular numbers over time. 1500 Rat1a cells expressing each minimal Myc clone were plated in each well of a 96 well plate well with 10% FBS DMEM. The next day, media containing 0.25 μM OHT or an equal volume of vehicle, ethanol (EtOH) was added to the wells at Day 0. Samples were tested every 24 hours for a period of 4 days. On the day of harvest, MTT was added to each well to a final concentration 0.1 mg/ml MTT in 10% FBS DMEM and incubated at 37˚C for 6 hours. After incubation, the MTT media was removed and 100 μl DMSO was added to develop the color for 30 minutes at room temperature. The microplates were read using the BioTek Plate reader at an absorbance of 595 nm.

Myc-induced apoptosis experiments with immortalized Rat1a fibroblasts were performed similarly to the proliferation experiments. In contrast 10^5 cells were plated into each well of a 6-well plate with 10% CS DMEM. Twenty-four hours later, the cells were shifted to a media containing 1% CS and 1 μM OHT. Samples were counted every day for a period of 2 days post serum withdrawal. Total living and dead cell populations were collected and counted by hemocytometer. The results are expressed as a percentage of dead cells against total number of cells within that sample with the standard deviation for each sample noted.

Immunoprecipitation

For experiments involving immunoprecipitation of MycER to resolve Cyclin T1 binding, two plates of Myc2ER, MycSER or empty vector Rat1a

fibroblasts were lysed with Low Stringency Buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.45% NP-40, 2mM EDTA, 10 mM NaF, 0.1 mM $NaVO_4$, 10 μg/ml aprotinin, 9 mM iodoacetamide, 0.1 mM PMSF), sonicated for 10 seconds on ice and clarified by centrifugation at 10000 rpm for 5 minutes. Protein concentrations were determined using the DC Protein Assay kit as directed (BioRad.) 200 μg total protein was saved for straight Western analysis while 2 mg of total protein were brought up to a final volume of 1.8 ml with Low Stringency buffer and protease inhibitors. Each sample was pre-cleared with 60 μl of Staph A blocked with 100ng/ml BSA (Upstate) before primary antibody incubation. 4-5 μg of sheep anti-Myc antibody was used with rotation at 4˚C overnight. The next day, antibody complexes were retrieved with 40 μl Staph A membranes for 30 minutes on ice. After incubation each sample was washed a total of 5 times with ice-cold Low Stringency buffer. Immunocomplexes were eluted with 80 μl of 2X Laemmli buffer and boiled for 5 minutes. Proteins were resolved on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, blocked with 5% milk in TBS and probed either for c-Myc, or CyclinT1. Blots were exposed using the Odyssey scanner (Licor).

RNA collection and quantitative PCR (qPCR) analysis

10^6 DKO's expressing Myc2ER, ΔMB0ER, ΔMBIER or empty vector were plated into a 10 cm plate. Forty hours post plating, each plate was shifted to PR-DMEM containing 0.1% stripped CS for 72 hours. After the 72 hours, 2 μM OHT was added for 0, 4, 8 and 12 hours. In the experiments comparing Myc2ER and MycSER upregulation levels, 10 cm plates were seeded to high density and dosed with 5 μM OHT for 0, 2, and 4 hours. RNA was isolated using the

Absolutely RNA Kit with DNase digestion as directed (Stratagene). DKO samples were harvested by trypsinization and centrifugation of the cells and frozen at –80˚C. Total RNA was purified from the frozen cell pellet using the miRNeasy Mini kit as directed (Qiagen). After purification and quantitation, 4μg total RNA was treated with DNase as directed to remove any genomic DNA within the sample (Ambion). Subsequently, the sample was converted to cDNA using iScript reverse transcriptase kit as directed (BioRad). Each cDNA sample was diluted 1:5, with 5 μl used to quantitate with the iCycler and iQ SYBR Green Supermix (Bio-Rad) and 10 pmol of a forward and reverse primer as listed below. Levels were calculated based on a standard curve using serial dilutions of DKO cDNA from previous experiments. Relative values were compared to the actin levels within each sample and to the 0 hr time point level of each cell type. The mean of triplicate samples +/- the standard deviation was graphed.

Actin	Fwd: 5′ GCTGTGCTATGTTGCTCTAG 3′
	Rev: 5′ CGCTCGTTGCCAATAGTG 3′
HSP60	Fwd: 5′ GGTGGCCTCCTTGCTAACTAC 3′
	Rev: 5′ CCCATTCCAGGGTCCTTCTCTT 3′
CDK4	Fwd: 5′ TCACGCCTGTGGTGGTTAC 3′
	Rev: 5′ CGGGTGTTGCGTATGTAGAC 3′
CAD	Fwd: 5′ TGGACATCTTCACCATTCG 3′
	Rev: 5′ CAGCTTCGGTGCAAGTAGG 3′
eIF4E	Fwd: 5′ CAGCAGAGACGGAGTGACC 3′
	Rev: 5′ CCATCAGCAAGAGGACAGC 3′

Nucleolin	Fwd: 5′ATCAGATTAGTCAGCCAGGATC 3′
	Rev: 5′ TCTCCAGTGTAGTAGAGTGAAAC 3′
αProthymosin	Fwd: 5′ GAGACGCACCTGCCAATG 3′
	Rev: 5′ CTCCATCTTCTTCCTCACCATC 3′

CHAPTER III

DISCOVERY OF A NOVEL REGION WITHIN THE FIRST 46 AMINO ACIDS OF C-MYC AND ITS SIGNIFICANCE IN BIOLOGY AND MOLECULAR FUNCTION OF C-MYC

Introduction and Rationale

Early studies with MycS showed that it was unable to transactivate an EMS-containing reporter gene or cotransform primary fibroblasts with activated Ras, implicating the importance of the first 100 amino acids in transactivation and specific c-Myc biological functions. Although previous experiments examined this region, the results examining the transactivational activity of domains within this region were inconsistent and controversial. Some of the complicating factors in the analysis of Myc target gene promoters included weak signals and the influence of other repressive cofactors also able to bind to EMS sequences, such as Mad or Mnt.

As a means to control for cofactor competition for EMS sites and weak binding to the EMS, the C-terminal DNA binding domain of c-Myc was substituted for the DNA binding domain of the yeast Gal4 transcription factor. Rather than using Myc target gene promoters, a reporter gene containing the recognition sequence for the Gal4 DNA binding domain was used to assess transactivation. This experiment, referred to as the Gal4 assay, is a powerful and robust tool for assessing transactivation. It allows the dissection of specific components of a transcriptional transregulatory domain while utilizing a well-studied and strong DNA binding domain with a known recognition sequence to generate strong, definitive signals.

Results

Transactivation by fragments of the c-Myc N-termini

In order to assess the regions of the TRD with high transactivational activity, a Gal4 screen of the Myc TRD was initiated. Among the N-terminal regions tested were the full c-Myc N-terminal domain of 1-262, a mutant containing a truncation of the first 100 amino acids similar to MycS, and another mutant containing a truncation of the first 150 amino acids (Figure 4). Using a cotransfected Gal4 reporter gene, the results showed that Gal4-Myc101-262 had

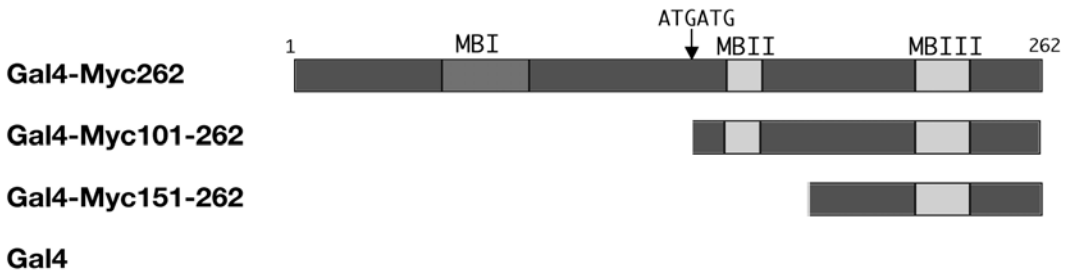


FIG. 4. Fragments of the c-Myc N-terminal domain to be tested for transactivation. Shown are fragments of the c-Myc N-terminal domain fused to the carboxyl terminal of the Gal4 DNA binding domain for initial experiments assessing the activity of regions after the MycS initiation site.

minimal activity in comparison with the full-length transregulatory domain of amino acids 1-262 of c-Myc (Figure 5). Gal4-Myc151-262 showed even less activity than Gal4-Myc101-262. Expressed protein levels were roughly equal to each other, indicating that excess or lack of activity was not due to extreme variations in Gal4 fusion protein levels (Figure 5). Based on these results, we conclude that the transactivation potential in the full transregulatory domain

does not involve Myc 101-262 or MBII. This result agrees with our previous assessment of MycS transcriptional activity.

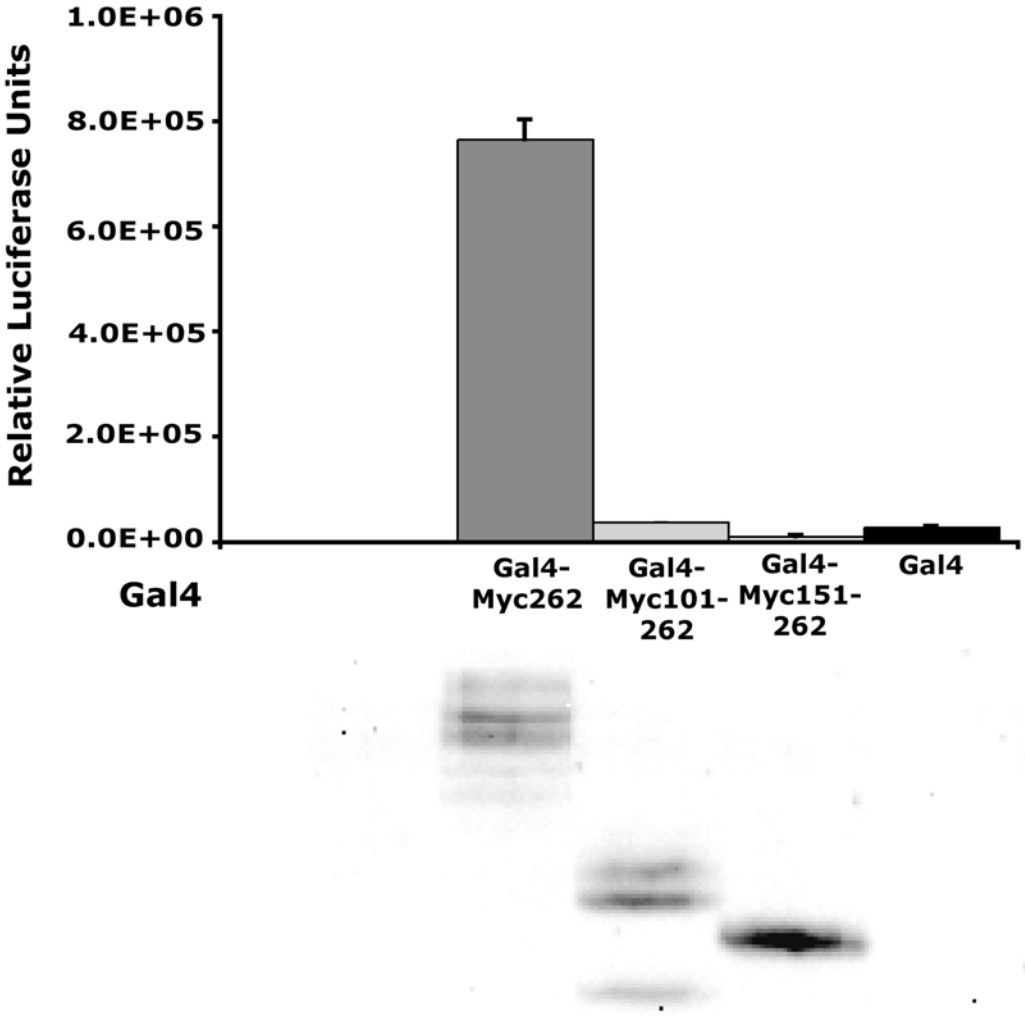


FIG. 5. Transactivation is dependent on the first 100 amino acids. A Gal4 assay was performed with Cos cells transfected with Gal4-Myc262, Gal4-Myc101-262, Gal4-Myc151-262 or empty Gal4. Samples were processed and analyzed as described in Methods. The bar graph shows the transactivation produced by each construct, expressed in relative luciferase units. Below, a Western blot probed with sheep anti-c-Myc shows expression of Gal4-Myc proteins in each sample.

Next, the contribution of other regions in the N-terminal 100 amino acids was assessed for activity. Using the Gal4 assay system, we subdivided the first 100 amino acids into smaller regions inclusive and exclusive of MBI (Figure 6). Each of these domains was fused to the Gal4 DNA binding domain and assessed for its specific activity.

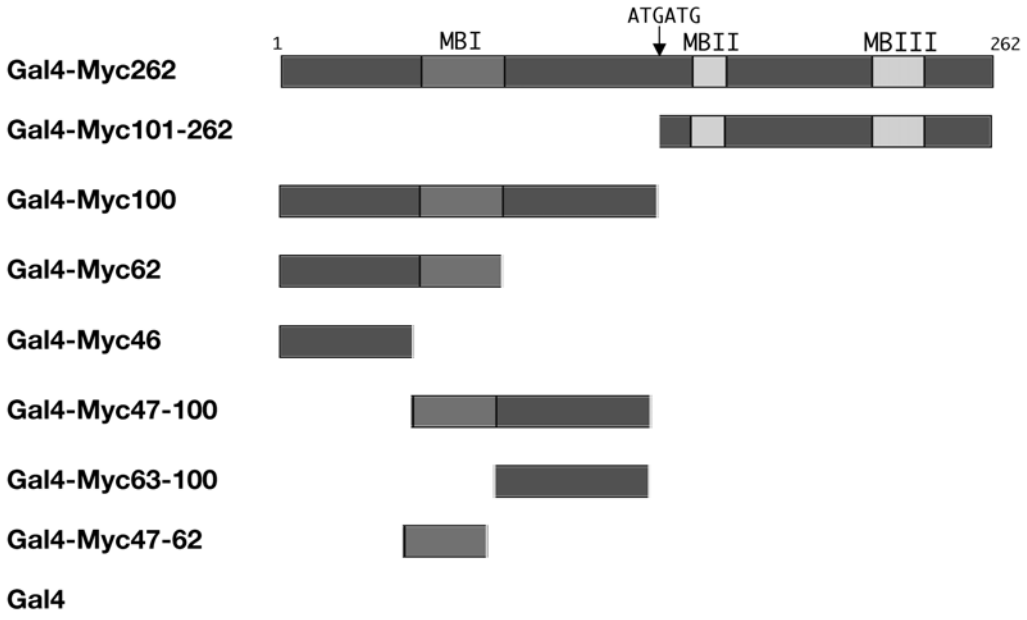


FIG. 6. Fragments within the c-Myc a.a. 1-100 transactivation domain to be tested for transactivation. These segments were tested to further isolate the regions with the most activity within the Gal4 assay. The regions tested resided before and after MBI, and were inclusive and exclusive of MBI.

Our results showed that the first 100 amino acids and the first 62 a.a. transactivated the most, significantly more than Gal4-Myc262 (Figure 7), suggesting that the first 62 a.a., which retains MBI, contains the transactivation domain. Negligible activity was found in the region from a.a. 63-100. Interestingly, a remarkable level of transactivation was found in Gal4-Myc46, a

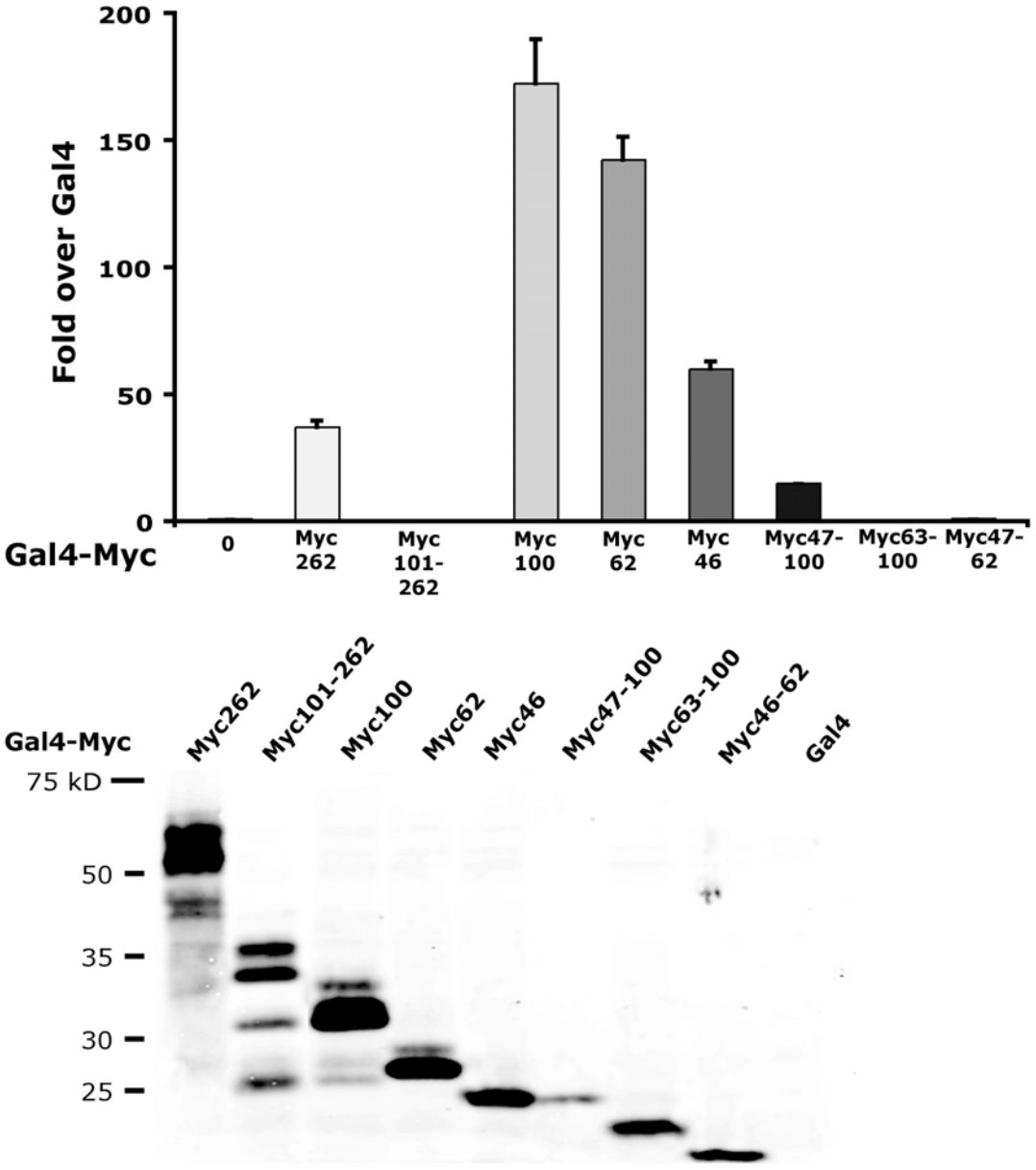


FIG. 7. Transactivation by fragments of the c-Myc N-terminal 100 amino acids. Cos cells were transfected with the Gal4-Myc constructs noted in Figure 6 and assayed as previously described. The bar graph shows the transactivation of each construct, expressed as the fold increase over the activity of Gal4 by itself and normalized to the Gal4-Myc levels detected in the protein blot. Below, Western analysis shows the levels of Gal4-Myc present in each sample tested.

region devoid of any known Myc boxes. Although some of the protein levels were variable, normalization to fusion protein levels revealed that the overall trends remained the same. Since Gal4-Myc262 has less activity, we hypothesize that there is a repression domain within a.a. 101-262.

Isolation of a novel c-Myc high homology domain

To determine if there were any conserved domains within Myc a.a. 1-45, amino acid sequence alignments were performed comparing c-Myc proteins from other species as well as mouse proteins from different *myc* genes, such as

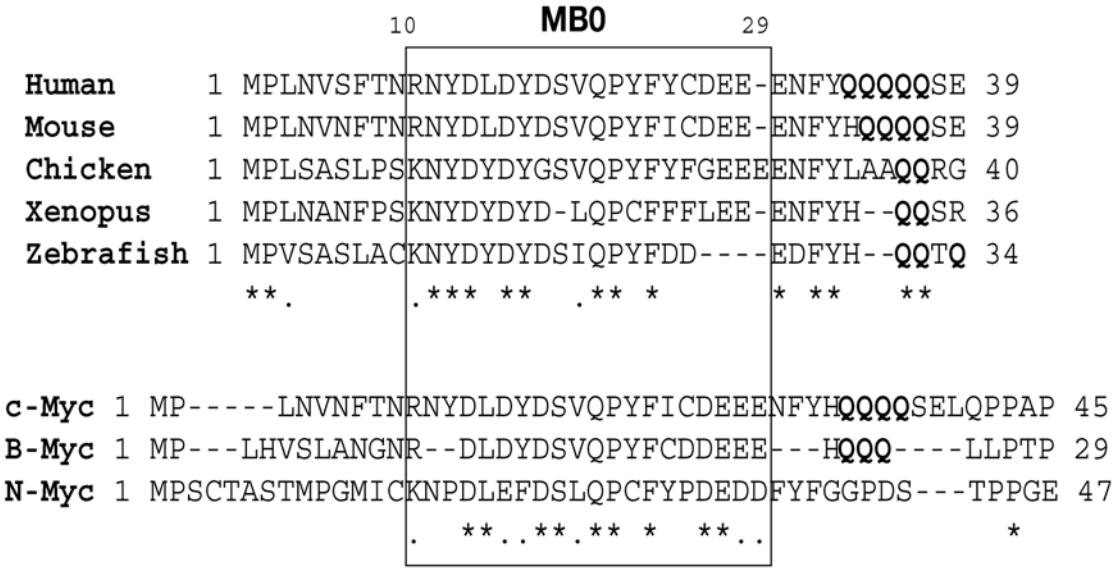


FIG. 8. Sequence Alignment Reveals a Novel Region of High Homology. NCBI protein sequences from the first 45 amino acids of c-Myc from several species and other *myc* genes were collected and compared against each other. Stars identify exact amino acid matches, periods denote chemically similar amino acids. Myc Box 0 (MB0) is defined by the box containing amino acids 10-29 of mouse c-Myc. Another region of high homology, the polyglutamine domain (Poly Q) is in boldface.

N-Myc and B-Myc. For these alignments, MBI was specifically excluded to ensure that the alignment software did not exclude regions of homology because of the strong homology found in MBI. The alignment uncovered a previously unreported region of high similarity within amino acids 10-29 of mouse c-Myc, which will be referred to as MB0 (Figure 8).

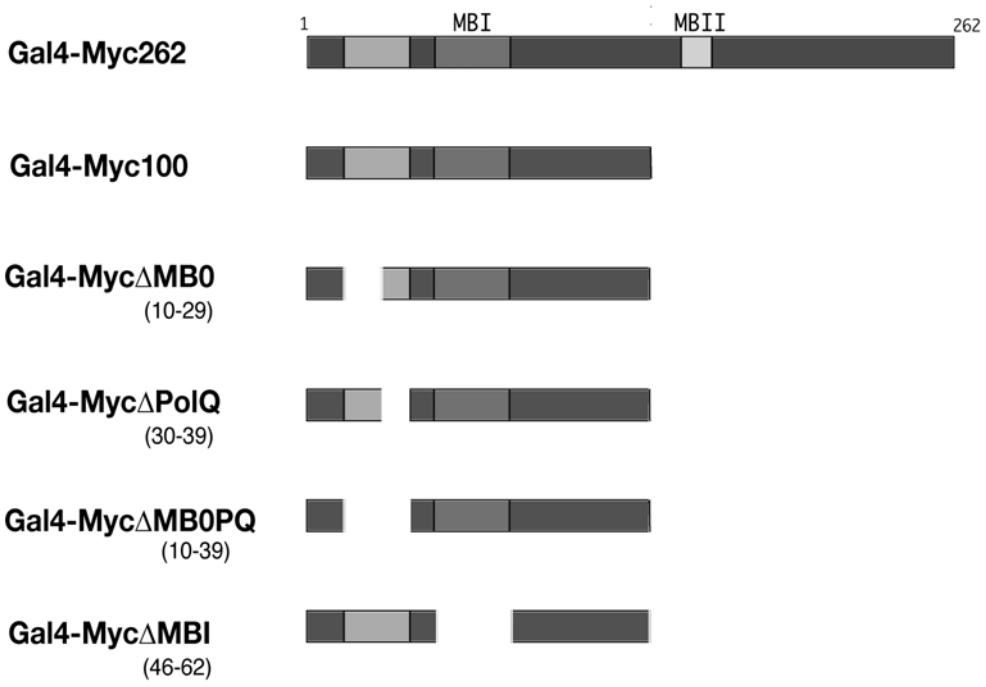


FIG. 9. Deletions within c-Myc a.a. 1-100 used to assay MB0 activity. The MB0 domain as defined in Figure 8 was deleted from the wild type Gal4-Myc100 in addition to MBI from a.a. 46-62, the polyglutamine domain from a.a. 30-39, and the combined region of MB0 and the polyglutamine domain from a.a. 10-39.

Further BLAST searches using the MB0 protein fragment found no other proteins that contained this domain aside from other Myc molecules. Next to MB0, a distinctive poly-glutamine tract was also found (PolyQ). Given the location of these homologous regions in the first 46 amino acids, we wanted to

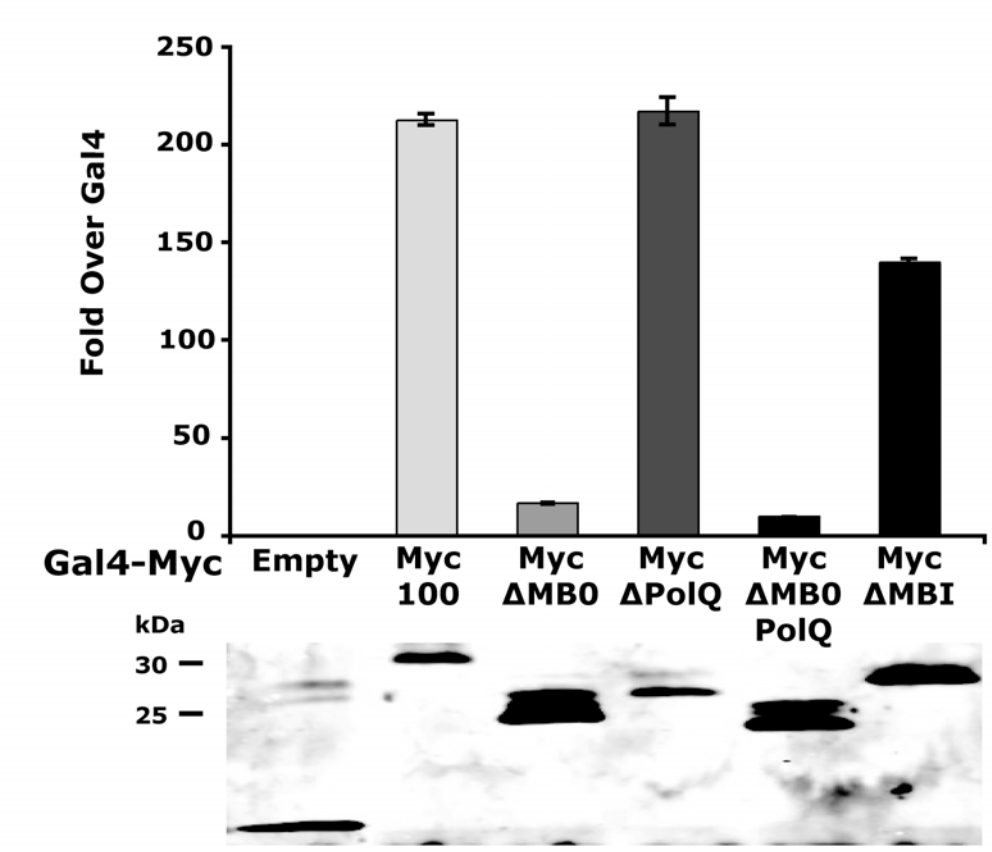


FIG. 10. MB0 and MBI are critical for transactivation by Gal4-Myc100. The Gal4 assay was performed as described previously to assay the activity of Myc100 with targeted deletions. The bar graph shows the fold activity of Gal4-Myc over Gal4 and normalized to levels of Gal4-Myc protein present within the sample. Below, Western analysis was performed using the monoclonal Gal4 antibody to show levels of Gal4-Myc in each sample.

determine whether they had a role in transactivation. Starting with the Gal4-Myc100 as a template, deletions of MB0, MBI, the PolyQ domain, and a combination of the MB0 and PolyQ domain were generated (Figure 9). The Gal4 assay results showed that deletion of MB0 substantially decreased transactivation compared to Gal4-Myc100 (Figure 10). In contrast, deletion of the PolyQ domain had a relatively small effect by itself or in combination with deletion of MB0. Deletion of MBI also caused a decrease of transactivation,

although not as significant as the reduction of transactivation by MB0 deletion. We conclude that MB0 and MBI were both necessary for transactivation. In contrast, the Poly Q tract was not necessary for transactivation.

The significance of MB0 in c-Myc biological activities

Once a correlation between transactivation and MB0 was established through the Gal4 assay, we wanted to correlate the region to specific c-Myc biological functions. To start, full-length versions of each deletion were constructed and cloned in an expression vector for cotransformation in addition to constitutive and inducible deletion constructs cloned into retroviral vectors for stable transfection of immortalized Rat1a cells and p53 -/-, p19ARF -/- DKO MEFs (Figure 11). These stable clones were used to assess activity of these deletions in variable biological and molecular functions of c-Myc.

FIG. 11. Deletions made within full-length c-Myc. Deletion of both MB0 and MBI were made in full-length c-Myc in the appropriate expression vector for utilization in experiments assessing c-Myc biology and molecular function.

We first assessed the effects of MB0 deletion on Myc biology using one of the most stringent assays for Myc function, the Myc/Ras cotransformation assay. This assay assesses the ability of Myc to induce foci in a monolayer of low passage primary rat fibroblasts with a constitutively activated Ras molecule. When Myc2, ΔMB0 and ΔMBI were highly overexpressed in low-passage REFs,

the results showed that Myc2 induced numerous foci of variable sizes (Figure 12). In contrast, both ΔMB0 and ΔMBI failed to produce the number of foci found in the Myc2 plates. The few colonies that did emerge in ΔMB0 and ΔMBI had a relatively consistent size and were substantially smaller than foci induced by Myc2 expression (Figure 12 charts). From these experiments we can conclude that that MB0 was a necessary domain in the cotransformation of primary fibroblasts.

We also wanted to determine whether deletion of the MB0 or MBI simply formed inactive proteins. In previous experiments, MycS induced proliferation similarly to Myc2 in Rat1a immortalized cells (Xiao et al., 1998). These experiments showed that neither MBI, nor MB0 were required for Rat1a hyperproliferation. To test this with targeted deletions, Rat1a cells were infected with Myc2, ΔMB0, or ΔMBI deletions and compared to the empty vector control. The results indicated that both ΔMB0 and ΔMBI induced hyperproliferation as well as wild type Myc2 (Figure 13). Western analysis of the Rat1a cell lines showed that even with higher expression of ΔMB0 and ΔMBI, they still had substantial proliferative activity in Rat1a cells (Figure 13). We conclude that MB0 and MBI were unnecessary for hyperproliferation in this cell line and that deletion of MB0 or MBI did not result in a completely non-functional protein.

The role of MB0 in c-Myc target gene upregulation

We also wanted to assess the significance of MBO and MBI in other molecular and biological functions of c-Myc. To accomplish this, Myc2, ΔMB0 and ΔMBI were fused to a partial domain of the estrogen receptor (ER). This fusion allows activation of c-Myc in the presence of hydroxytamoxifen (OHT)

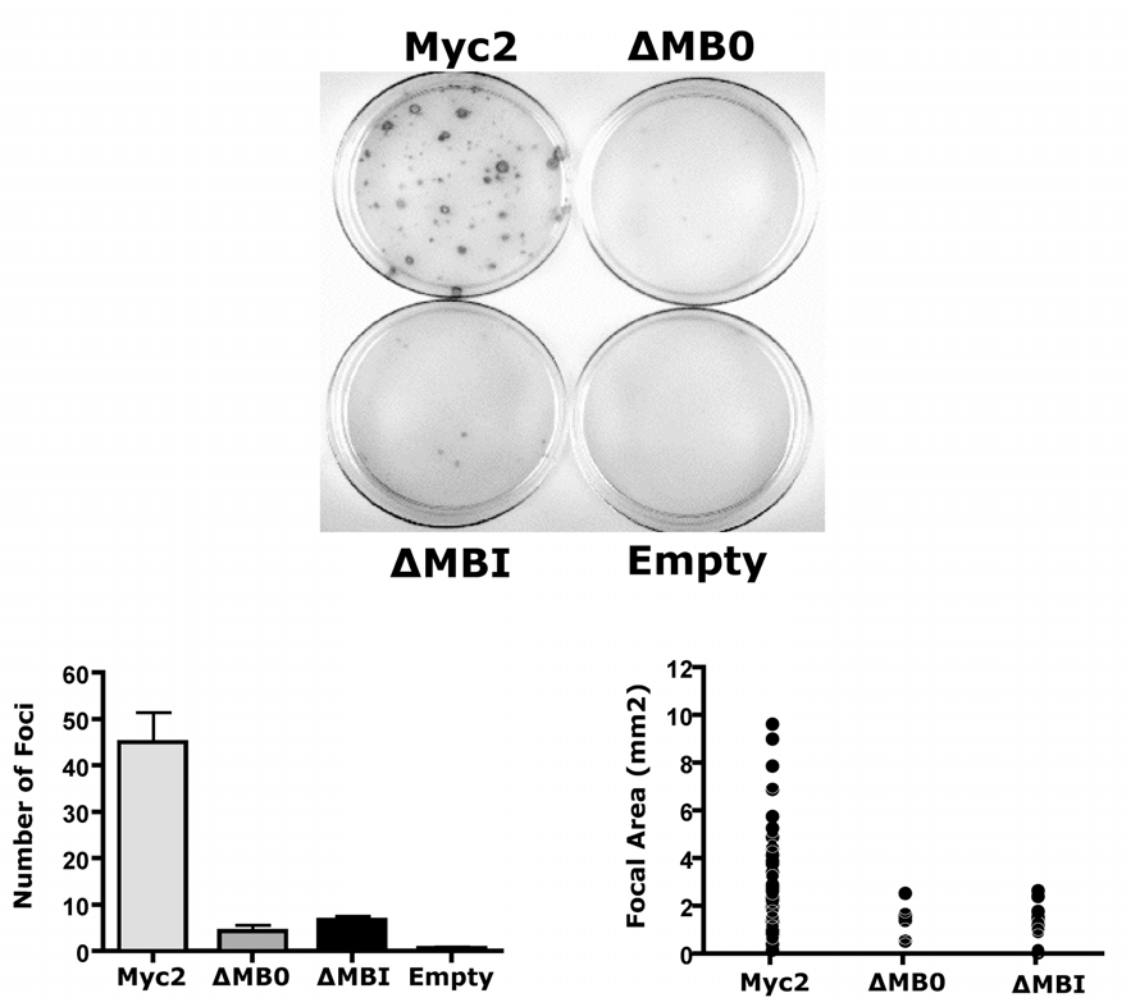


FIG. 12. Deletion of MB0 and MBI impairs Myc/Ras cotransformation in primary rat fibroblasts. Low passage primary rat fibroblasts were plated and transfected as described in Methods with CßS-Myc, ΔMB0, ΔMBI or empty vector in addition to H-Ras G12V genomic fragment. Shown is a representative plate from each Myc cotransformation assayed. Below are charts representing of the number of visible foci scored and the area of individual foci.

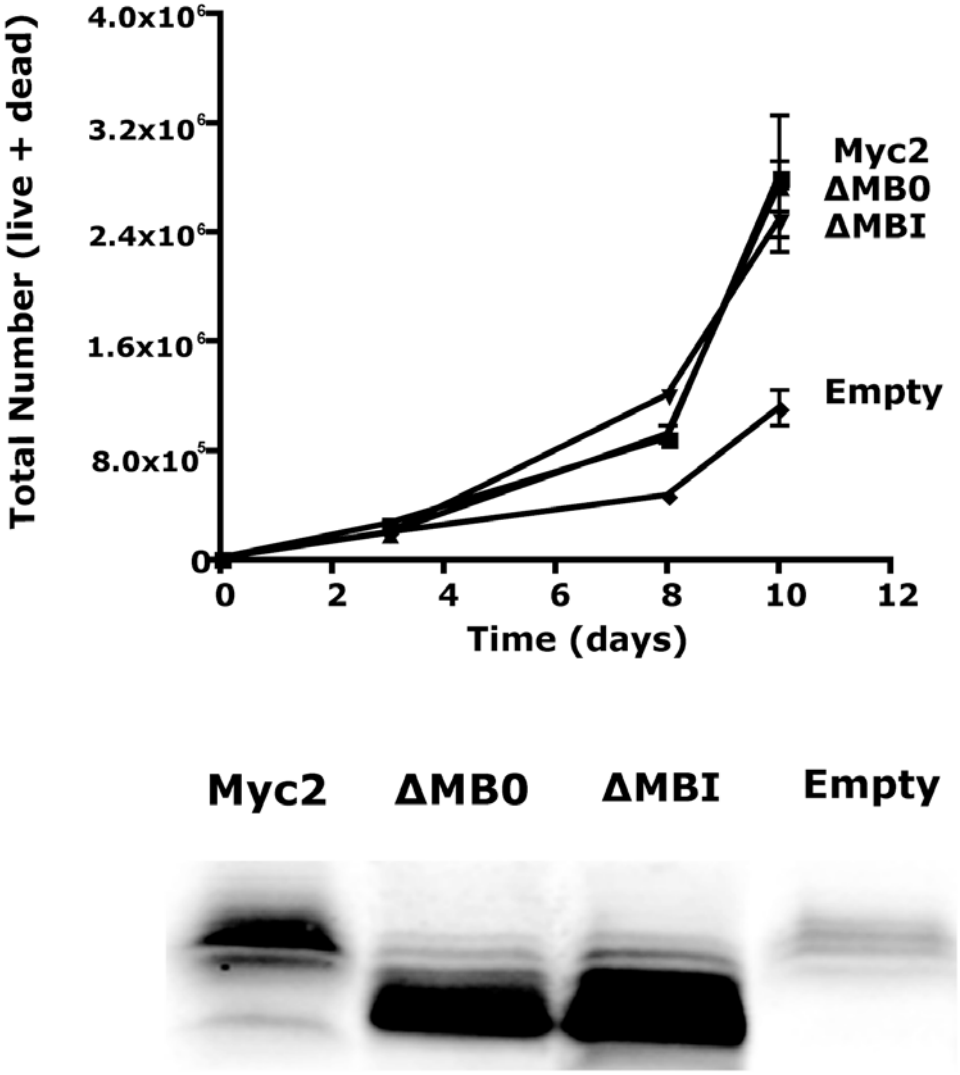


FIG. 13. MB0 and MBI are not necessary for Myc-induced hyperproliferation in Rat1a fibroblasts. Immortalized polyclonal Rat1a cells stably expressing wild type Myc2, ΔMB0 or ΔMBI were assayed for proliferative rate against the empty vector Rat1a control. The bar graph shows the total number of cells over time. Below, Western analysis shows Myc expression in each Rat1a cell line used.

(Facchini et al., 1997; Littlewood et al., 1995) Stably expressing DKO MEF cell lines were generated as previously described in Methods. As seen in Figure 14, polyclonal stable cell lines were generated that expressed approximately equal levels of inducible Myc.

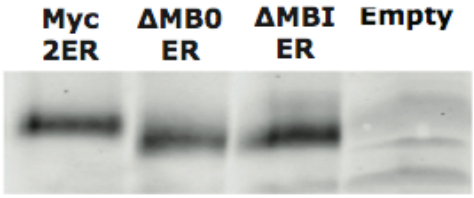


FIG. 14. Stable expression of OHT-inducible c-Myc. Western blotting using rabbit anti-Myc shows equivalent expression of inducible Myc2ER, ΔMB0ER, ΔMBIER in comparison to empty vector control in DKO fibroblasts. Polyclonal cell lines were matched based on comparable levels of MycER expression.

Within the Gal4 promoter assay, deletion of MB0 substantially decreased the transactivation of the reporter gene. We wanted to correlate this activity with the upregulation of endogenous c-Myc target genes. To address whether MB0 affects target gene upregulation DKO fibroblasts expressing Myc2ER, ΔMB0ER and ΔMBIER were serum-starved for 3 days and treated with OHT at several time points, harvested for total RNA, and subjected to qPCR. The results show that while both Myc2ER and ΔMBIER both upregulated c-Myc target genes *hsp60* and *CDK4*, ΔMB0ER did not induce upregulation of either target gene over a 12 hour time period (Figure 15). Additionally, ΔMBIER induced the upregulation of these two target genes more robustly than Myc2ER. From these results we can conclude that MB0 is critical in the upregulation of at least some c-Myc target genes.

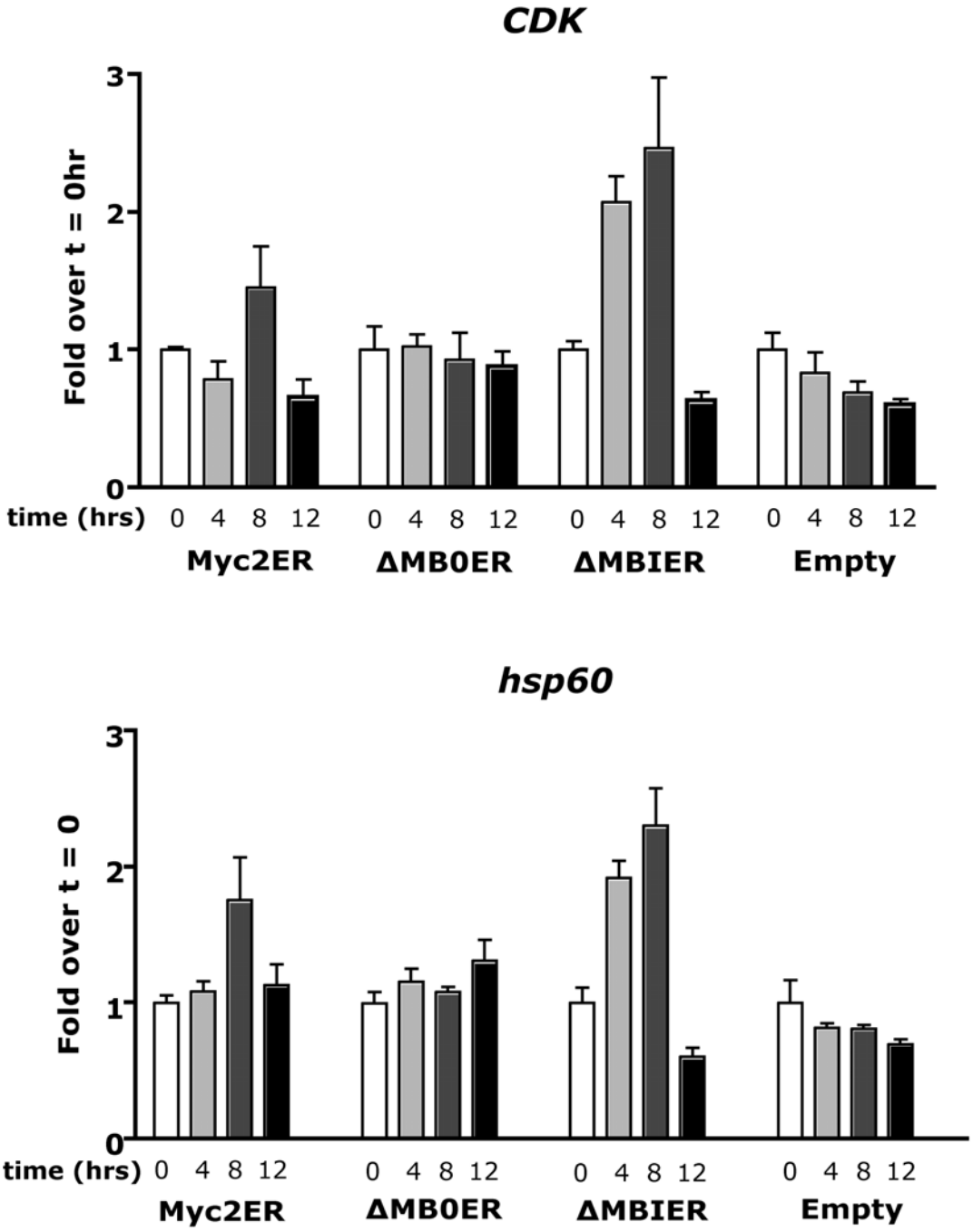


FIG. 15. ΔMB0ER is unable to upregulate *hsp60* or *CDK4*. Stable DKO cell lines as shown in Figure 14 were induced with 2 μM OHT for 0, 4, 8 and 12 hours. Quantitative PCR was used to assess the levels of target gene RNA/cDNA in each sample. The chart shows the upregulation of *CDK4* and *hsp60* normalized to *ß-actin* levels and shown as a fold difference over target gene levels at 0 hours.

Conclusions

Our results show that the region missing in MycS, Myc 1-100 has the highest transactivation capability. In addition, the region preceding MBI, Myc 1-46, showed approximately half the activity observed in Myc 1-100 or Myc 1-62. Protein sequence analysis of Myc 1-45 revealed a previously unreported region of high sequence similarity in a region rarely examined in the Myc field. Deletion of MB0 from the highly active Gal4-Myc100 resulted in a drastic decrease in transactivation of the Gal4 reporter gene, more than was observed with deletion of MBI in the same Gal4-Myc100 fusion protein. We conclude that this new Myc box, MBO, was a necessary component for Gal4 reporter transactivation.

Subsequent biological experiments showed that MB0 was necessary for primary cell cotransformation in conjunction with H-Ras G12V. Deletion of either MB0 or MBI reduced both the numbers of foci and the surface area of foci that did emerge. We can also conclude that deletion of MB0 did not result in the misfolding of Myc such that it generated a functionally inactive protein. This was evident in ΔMB0-induced hyperproliferation observed in Rat1a fibroblasts that was comparable to Myc2.

We can also conclude that MB0 was a necessary domain for the upregulation of at least two well-known Myc target genes, *CDK4* and *hsp60*, as evidenced by the lack of upregulation of *CDK4* and *hsp60*. In addition, ΔMBIER upregulated those same target genes more than Myc2ER. This contrasts significantly from the results shown in the Gal4 assay comparing Gal4-MycΔMB0 and Gal4-MycΔMBI. It is possible that the mechanism mediating the upregulation of the two target genes tested is through a transactivation-

independent pathway. Assessment of additional target genes with well-defined upregulation mechanisms will clarify the molecular roles of MBI and MB0.

CHAPTER IV

REGIONS OF THE C-MYC TRANSREGULATORY DOMAIN SUFFICIENT TO INDUCE C-MYC MOLECULAR AND BIOLOGICAL EFFECTS

Introduction and Rationale

It has become clear that some components of c-Myc molecular and biological function can be isolated to discrete regions of the transregulatory domain. In the previous chapter, we determined that specific regions of the TRD were necessary for some biological and molecular activities of c-Myc. Indeed we found a new Myc box that is critical for Myc transactivation and cotransformation with Ras. However these studies can only define the necessity of these regions. Several regions of c-Myc may be required to work together to mediate biological effects. None of these studies determine the sufficiency of the N-terminal domains to induce specific c-Myc functions.

The best approach to addressing sufficiency is to dissect c-Myc to minimal components while retaining other regions that are critical for all c-Myc functions. These destructuring methods have been used to great success with other transcription factors. Wild type Gal4 is a yeast transcription factor with a well-defined consensus sequence. Studies utilizing the Gal4 assay took advantage of the successful separation of the DNA binding domain from the transregulatory domain of Gal4. We pursued this strategy and applied it to c-Myc, removing specific regions, while retaining other regions of c-Myc. We assayed their function within the context of Myc-mediated molecular and biological function.

Results

Target gene regulation by c-MycS

We studied the sufficiency of MBII in the upregulation of endogenous target genes by assessing the extent of MycS-induced target gene regulation in an immortalized cell line lacking the *c-myc* gene (HO16). Myc2ER and MycSER were induced for 4 hours with 1 μM OHT. The results show that MycSER induced *α-prothymosin*, *eIF4e* and *nucleolin* target gene expression, albeit to a lesser extent than Myc2ER of (Figure 16). However, MycS still maintained a degree of activity that is significant over empty vector, specifically in *CDK4* and *cad* to levels comparable to Myc2ER. Additionally, MycSER and Myc2ER were expressed at comparable levels in each sample. This data shows that MycS can upregulate some target genes comparable to Myc2, while others were upregulated more weakly by a mechanism that is independent of transactivation.

CyclinT1 binding to c-MycS

Considering that c-MycS lacks MBI, it should not interact with pTEFb. To verify that MycS does not interact with pTEFb, we examined CyclinT1 binding, a component of the pTEFb elongation complex described in the Background chapter that bound MBI. Rat1a cells expressing inducible Myc2ER and MycSER were utilized for coimmunoprecipitation of Myc (both S and 2) and subsequent blotting for Cyclin T1. Results confirmed that Cyclin T1 bound to Myc2ER, while it was unable to bind to MycSER (Figure 17). The GABAα1, an unrelated antibody control, showed that Cyclin T1 interaction with Myc2 was not

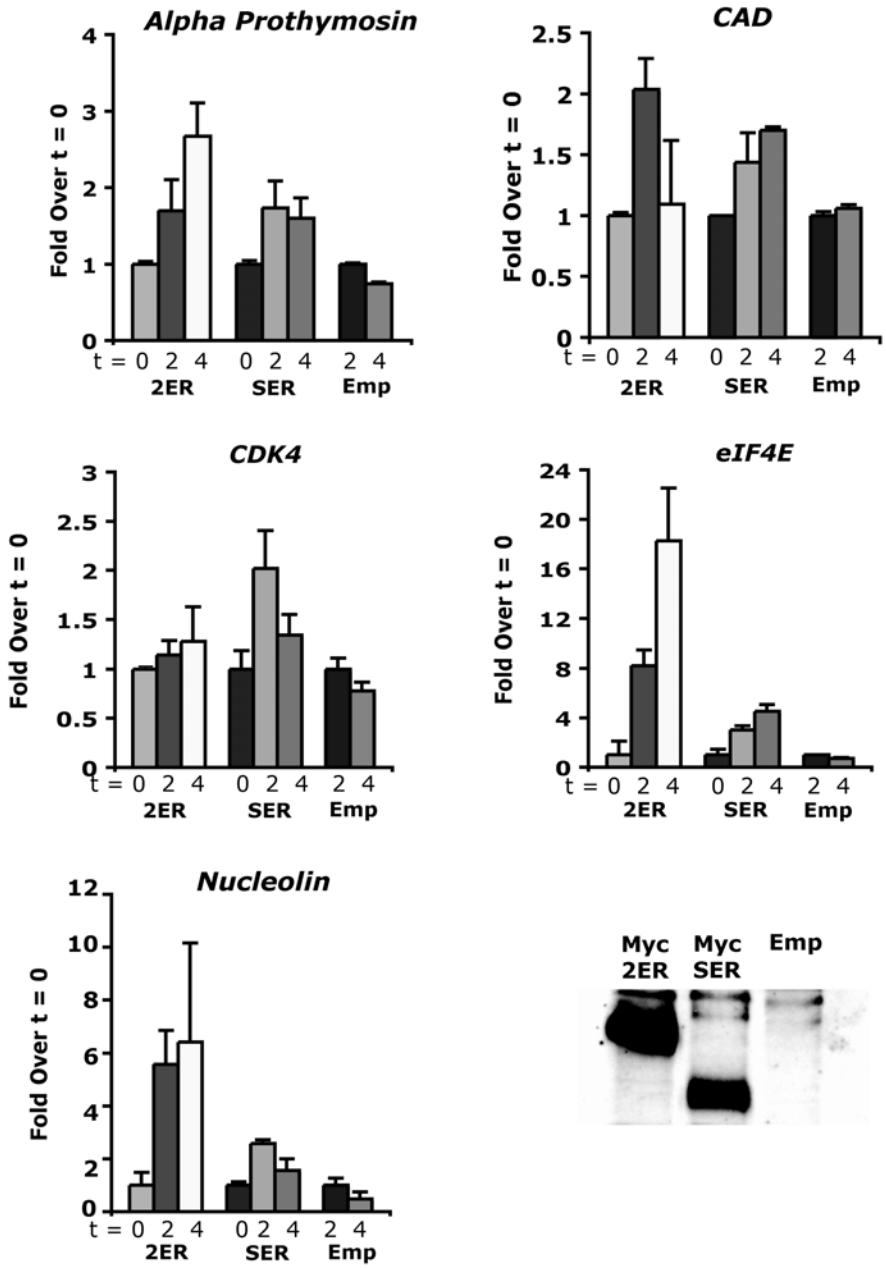


FIG. 16. Upregulation of Target Genes by MycSER. HO16 fibroblasts stably expressing Myc2ER and MycSER were induced with 5 μM OHT for 0, 2 and 4 hours. Each sample was prepared for total RNA and quantitated as indicated in Methods. Bar graphs of several target genes show levels of upregulation normalized to ß-actin levels within the samples and charted as a fold difference over the 0 time point. Below, a Western blot shows the expression of Myc2ER and MycSER in each sample.

because of non-specific binding. Hence we can conclude that MycS was not to bind Cyclin T1.

Construction and transactivation by minimal Myc

Two domains of interest are absent from MycS, MBI and MB0. Although these regions may be unnecessary in the functions mediated by MycS, it is unclear whether these domains are sufficient for c-Myc functions. To address this we fused discrete regions of the c-Myc TRD to the C-terminal domain containing the basic region and helix-loop-helix-leucine zipper, referred to as the CTD (Figure 18). As a negative control, the CTD with no Myc N-terminus

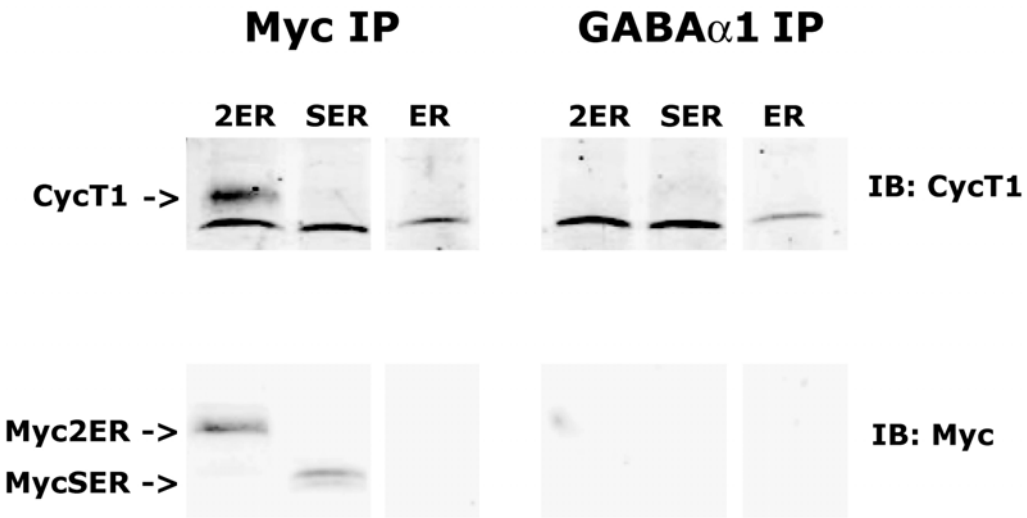


FIG. 17. Cyclin T1 is unable to bind to MycSER. Samples from Rat1a lysates stably expressing Myc2ER, MycSER and empty vector were treated with 1 μM OHT for 24 hours prior to harvesting. Immunoprecipitation was performed as described in Methods. Western blots were screened with polyclonal anti-Cyclin T1 to detect Cyclin T1 in Myc complexes or sheep anti-Myc to confirm immunoprecipitation of MycER. Also shown is a negative control immunoprecipitation of GABAα1, which is not expressed in Rat1a fibroblasts.

was generated. These minimal Myc molecules excluded MBIII and MBIV and selectively included specific Myc boxes to be tested. We then wanted to determine what biological functions they retained.

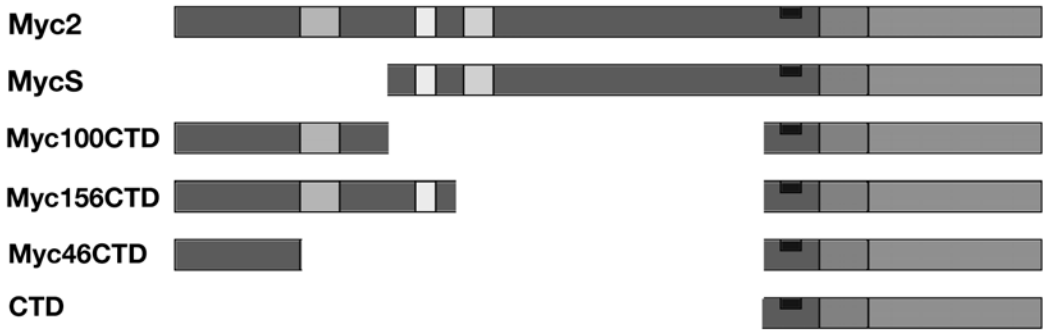


FIG. 18. Minimal Myc molecules. Shown are diagrams of wild type Myc2, the truncated c-MycS, and specific regions of the N-terminal domains of c-Myc fused to the C-terminal bHLHLZ domains.

Once introduced into pBabe-puro, each minimal Myc construct was retrovirally expressed in Rat1a fibroblasts to generate stably expressing polyclonal cell lines. Results from Western analysis of whole cell lysates derived from these stable clones indicated that Myc2ER and MycSER expressed at roughly equivalent levels. Myc100CTDER and Myc46CTDER were expressed at slightly lesser levels than Myc2ER (Figure 19). Unfortunately, several attempts to express CTDER on its own failed to generate a stable cell line. It is possible that the CTDER provides enough of a growth disadvantage to prevent establishment of a stably expressing cell line. Empty vector was used in the place of CTDER as a negative control.

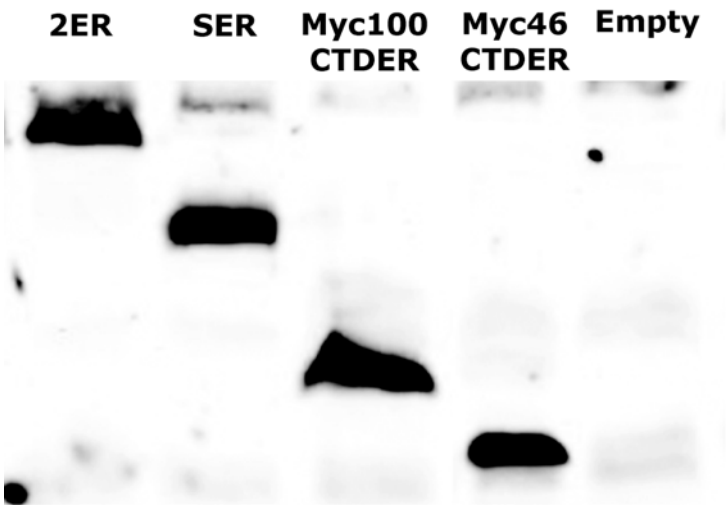
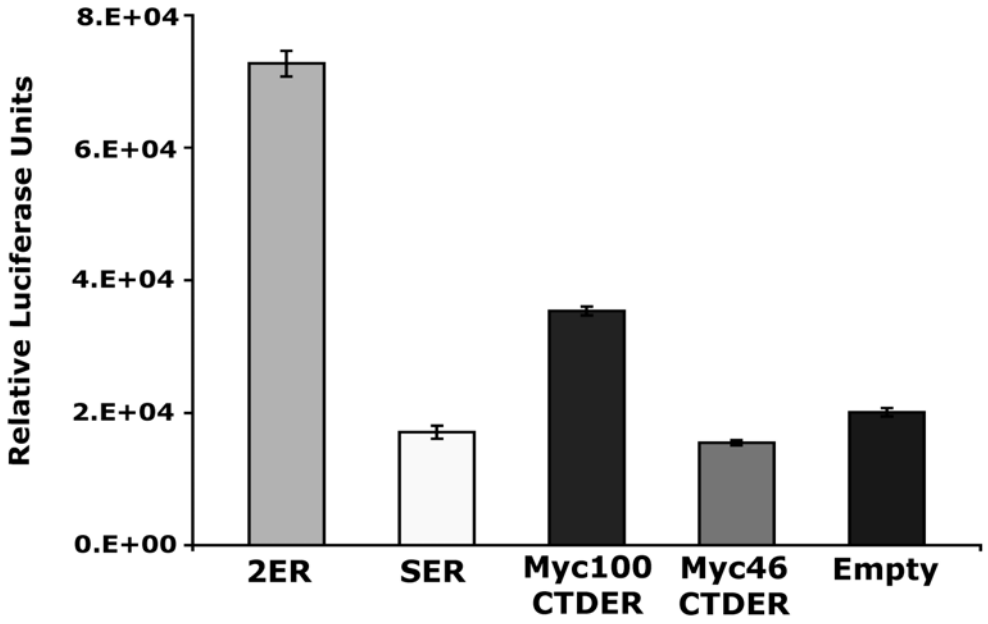


FIG. 19. Stable expression of minimal Myc proteins. OHT inducible minimal Myc molecules cloned into a retroviral expression vector and infected into immortalized Rat1a fibroblasts to generate stably expressing polyclonal cell lines. The Western blot shows levels of minimal Myc expression.

We wanted to determine whether minimal Mycs would induce transactivation in a stably integrated 2X EMS reporter gene as opposed to a transient reporter gene. A chromatin-integrated promoter would show the levels of transactivation within the context of chromatin modifying cofactors. Our results indicate some differences in the activity of the EMS reporter. Wild type Myc2ER had the highest activity, while MycS activity on the promoter was almost the same as the basal activity found in the empty vector control (Figure 20). However, the minimal Myc Myc100CTDER retained about half of the transactivation activity of Myc2ER. In contrast to previous Gal4 assays that showed the activity of Gal4-Myc46 to be roughly half that of Gal4-Myc100, Myc46CTDER was as inactive in transactivation as MycSER. We can conclude



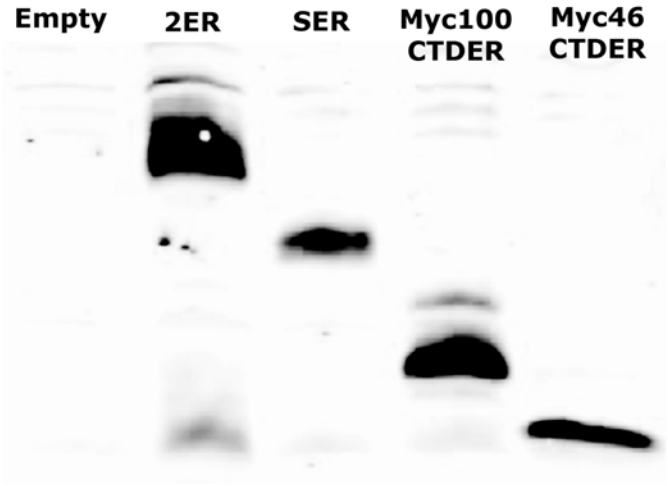


FIG. 20. Some minimal Myc molecules transactivate a stably integrated EMS. Rat1a cells expressing inducible minimal Myc were transfected with a 2X EMS reporter gene with a hygromycin resistance marker to allow for selection of integrated 2XEMS. 24 hours before harvest, all samples were induced with 1 μM OHT, lysed and quantitated as noted in Methods. The bar graph shows the levels of transactivation induced by each minimal Myc. Below, a Western blot shows the levels of minimal Myc expressed in each sample.

that at least some transactivation ability remains in Myc100CTDER within the context of a chromosomally integrated reporter gene, while Myc46CTDER lost its transactivation potential. Perhaps MB0 and MBI are both required for Myc's transcriptional activity. The activity of MBI alone has not yet been examined.

Biological activity of minimal Myc proteins

Since some transcriptional activity was shown in at least one of the minimal Myc molecules, we wanted to assess the sufficiency of each of these

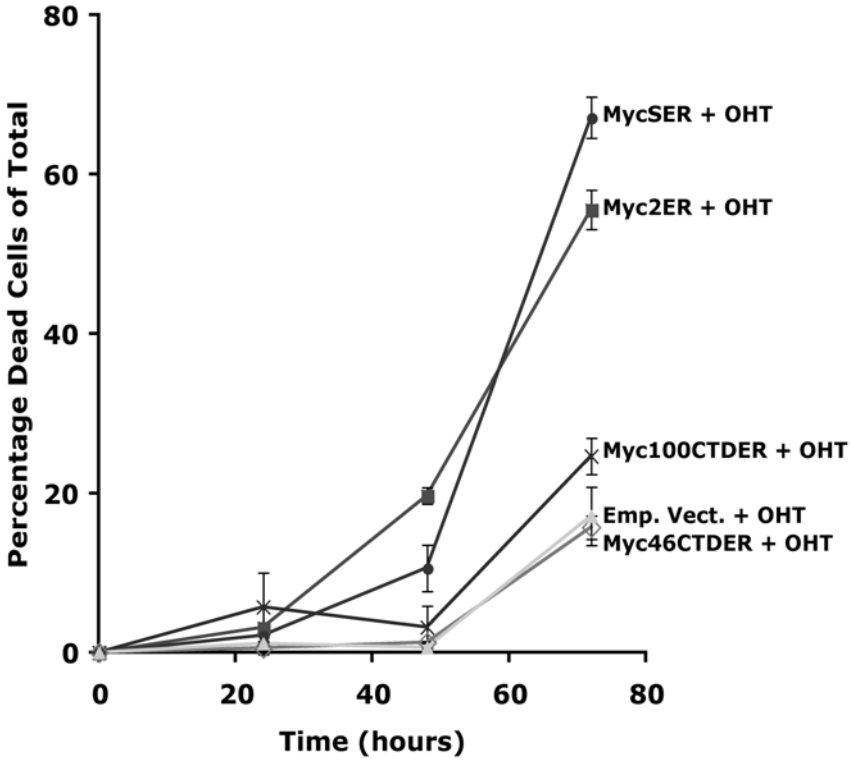


FIG. 21. Only Myc2ER and MycSER induce apoptosis. 24 hours after stably expressing Rat1a cells were plated, DMEM containing 1% serum and 1 μM OHT was added to all cells and counted daily. The chart shows the percentage of dead cells within the sample.

minimal N-terminal domains to induce c-Myc biological activities. One of the well-known effects of c-Myc induction is apoptosis in the absence of serum. Previously, apoptosis has been correlated with a functional MBII, as MycS was able to induce apoptosis in immortalized fibroblasts. The data showed that while both Myc2ER and MycSER induced apoptosis efficiently upon incubation with OHT, neither Myc100CTDER nor Myc46CTDER induced apoptosis under the same conditions (Figure 21). From these results we can conclude that the c-Myc 1-100 and 1-46 are not sufficient for the induction of apoptosis.

Myc-induced hyperproliferation is another biological effect of MycS expression in immortalized fibroblasts. We next wanted to determine whether the c-Myc 1-100 or 1-46 were also sufficient for hyperproliferation. For these experiments, the MTT assay was used to assess net proliferation. The results showed a very slight increase in proliferation with Myc100CTD and Myc46CTD over the empty vector control (Figure 22). Additional experiments confirmed that Myc46CTD was sufficient to induce hyperproliferation when activated with OHT (Figure 23). Although these assays can assess a net level of proliferation, it does not account for cells that apoptose during the experimental time course. At minimum, we can conclude that the first 100 or 46 amino acids may be sufficient to induce at least some hyperproliferation in Rat1a cells.

Conclusions

From our assay results of MycS-induced target gene regulation, we can conclude that MycSER was able to upregulate several target genes, although the upregulation of several of them was weaker compared to Myc2. This effect was not likely due to the recruitment of Cyclin T1 or potentially pTEFb to the site of

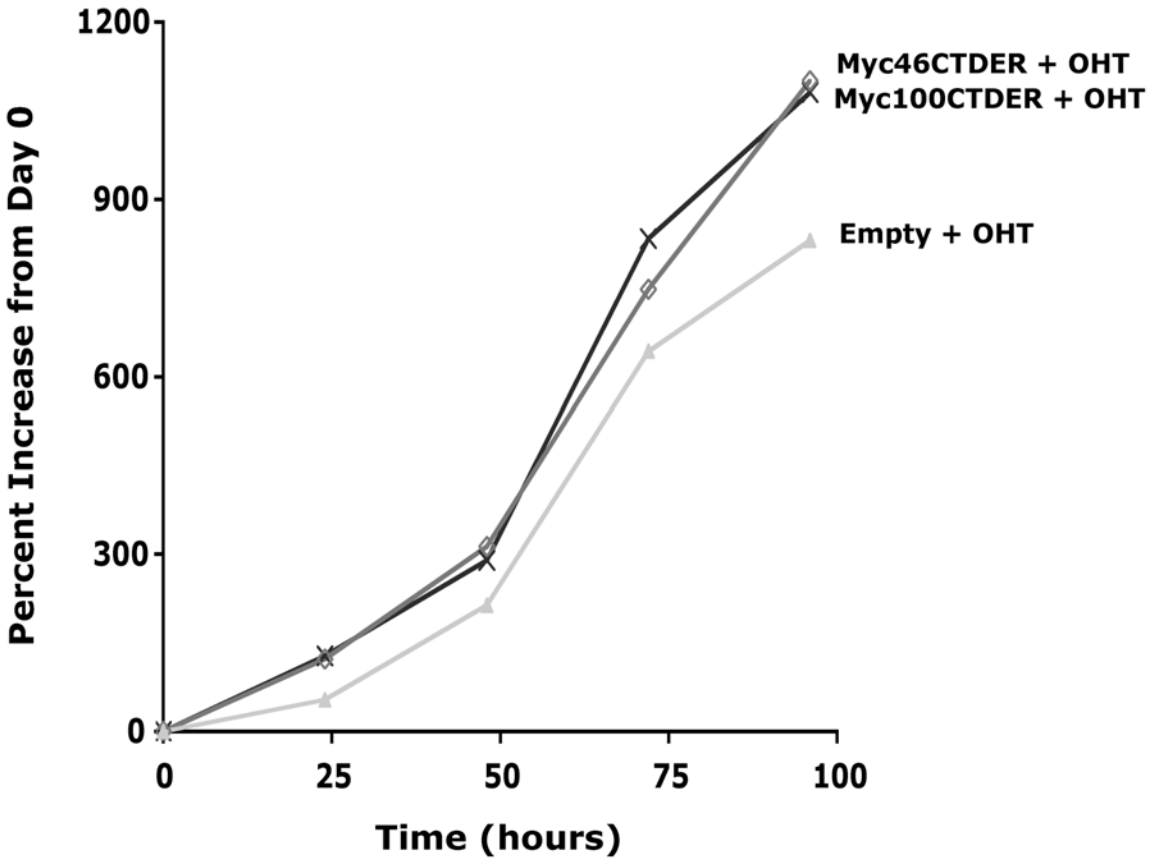


FIG. 22. Myc100CTDER and Myc46CTDER show increased proliferation. Equal numbers of clonal Rat1a fibroblasts were plated into a microplate in 20% FBS DMEM and the next day the plate was treated daily with 1 μM OHT. Each plate was assayed for live cell metabolism of MTT and charted as a percentage increase over the absorbance observed at Day 0.

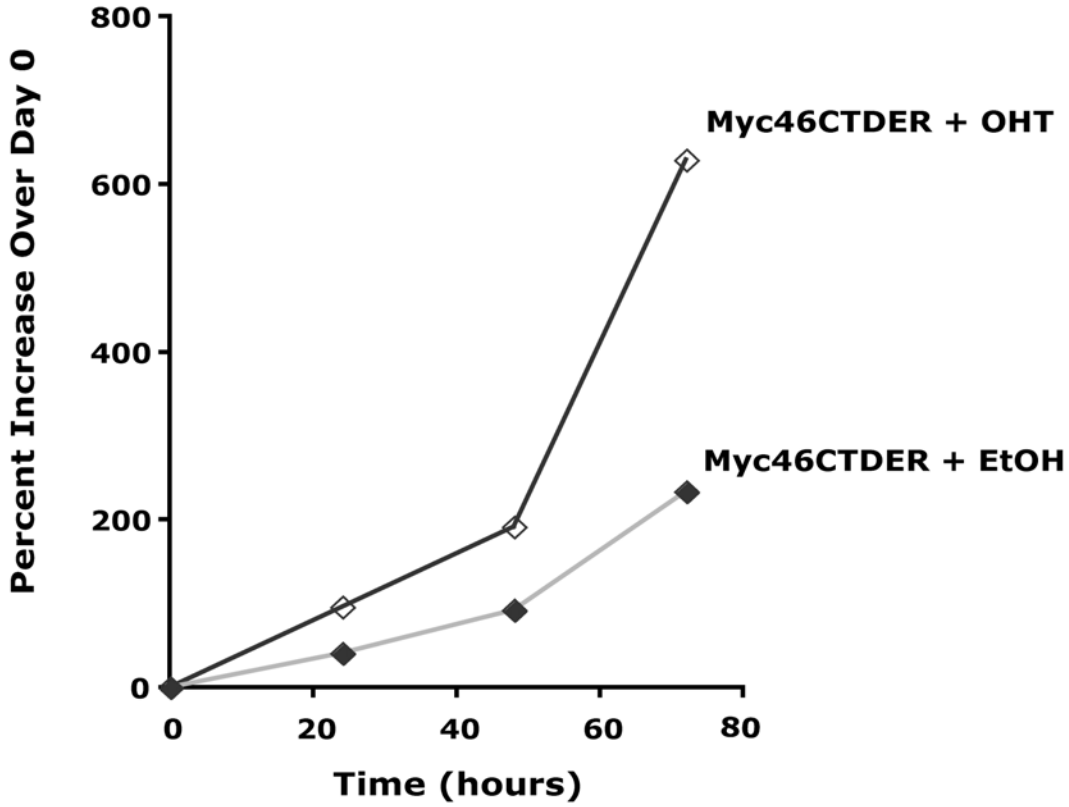


FIG. 23. OHT-induced Myc46CTDER shows increased proliferation over non-induced control. 24 hours after plating Rat1a cells expressing Myc46CTDER in 10% FBS DMEM were treated with 0.25 μM OHT or ethanol (EtOH) starting at Day 0. Each plate was assayed similarly to those in Figure 24.

MycSER-induced target gene upregulation, as found in immunoprecipitation of MycSER. The results of MycS induced endogenous target gene upregulation also did not correlate with the activity found in the chromatin-embedded 2XEMS reporter gene. While Myc2ER had the highest 2XEMS transactivation of all proteins tested, MycSER transactivation was roughly that found in the empty vector negative control. In contrast to the activity shown in MycSER, the activity of a minimal Myc containing only the first 100 amino acids of c-Myc was roughly half that of wild-type Myc2. This was a significantly different result from that seen in the Gal4 assay in Chapter III. A region with high activity in the same Gal4 assay, Myc 1-46 had virtually no activity within the chromatin bound EMS reporter. The lack of activity within the first 46 amino acids indicated that this region was insufficient to induce transactivation of a chromatin-embedded promoter without MBI.

Experiments utilizing minimal Myc proteins resulted in some interesting insights into Myc-induced function. The results from the apoptosis assays reinforce the importance of MBII in Myc-induced apoptosis. It is clear that neither the first 100 or first 46 amino acids were sufficient to induce apoptosis. In contrast, both minimal Myc proteins tested induced a statistically significant increase in proliferative activity over the empty vector negative controls, but the induced hyperproliferation was much less than with Myc2. The proliferation results of Myc100CTDER and Myc46CTDER were in significant contrast to previously published data indicating that in the Rat1a system, none of the first 100 amino acids were sufficient for proliferation.

CHAPTER V

THE INFLUENCE OF P19ARF AND POST-TRANSLATIONAL MODIFICATION ON C-MYC-INDUCED TRANSACTIVATION

Introduction and Rationale

As discussed in the background section, the post-translational modification of c-Myc has been suggested to play a role in induction of c-Myc activity. Specifically, ubiquitination by Skp2 was shown to contribute to target gene upregulation (von der Lehr et al., 2003b). In addition to ubiquitination, phosphorylation of c-Myc is also a tightly regulated modification of c-Myc as demonstrated in Figure 24. However modifications such as phosphorylation and ubiquitination also alter the stability of the protein as well. Using the Gal4 assay system to specifically assay transactivation we used specific regions containing post-translational modifications and mutated them to prevent the modification.

In addition to post-translational modifications, other binding proteins, specifically ARF, may play a role in transactivation. Previous studies have found that ARF binds to Myc and is recruited to c-Myc target gene promoters and can cause a strong inhibition of target gene upregulation (Qi et al.). ARF has also been shown to bind to not only the C-terminal of c-Myc but also the N-terminal 145 amino acids; however, given the two binding sites of ARF, it is unclear as to whether the c-Myc N-terminal binding region or the C-terminal binding region is the region responsible for the observed changes in target gene regulation. Utilizing the Gal4 assay to specifically assay transactivation allowed us to determine whether ARF inhibited the upregulation of c-Myc target genes through inhibition of transactivation or through some other mechanism.

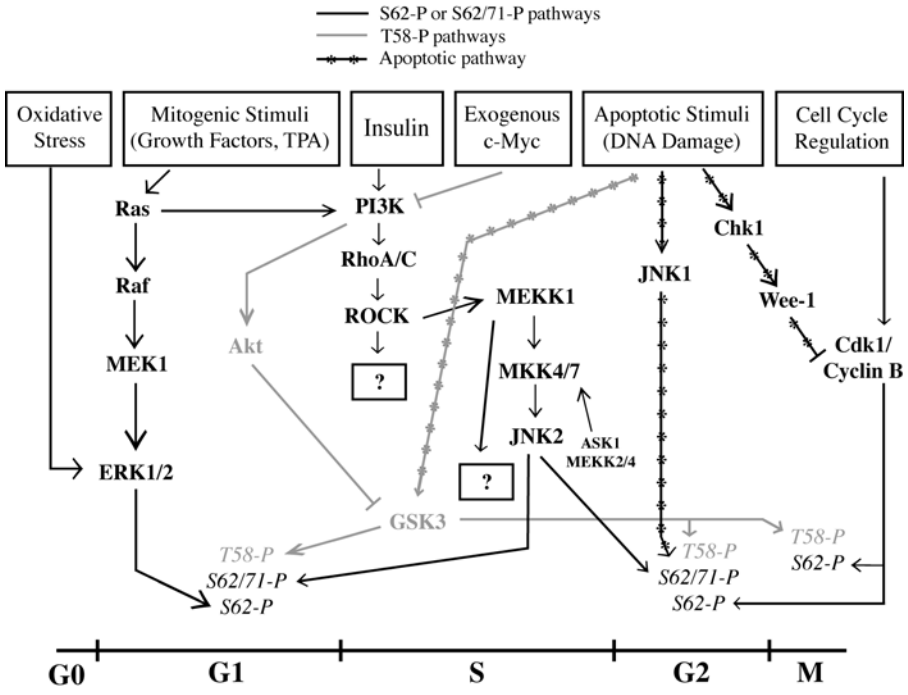


FIG. 24. Regulation of c-Myc phosphorylation. The schematic shows the pathways involved in the regulation of c-Myc phosphorylation within the cell and the time within the cell cycle that these phosphorylation events occur.

Results

The effects of ARF on c-Myc N-terminal transactivation

We first wanted to determine whether ARF co-expression affected transactivation of a reporter gene using specific regions, but it is possible that ARF binds at one or multiple sites within the N-terminal 156 a.a. of c-Myc. To test this, we performed Gal4 assays using the first 100, 63 and 46 amino acids of

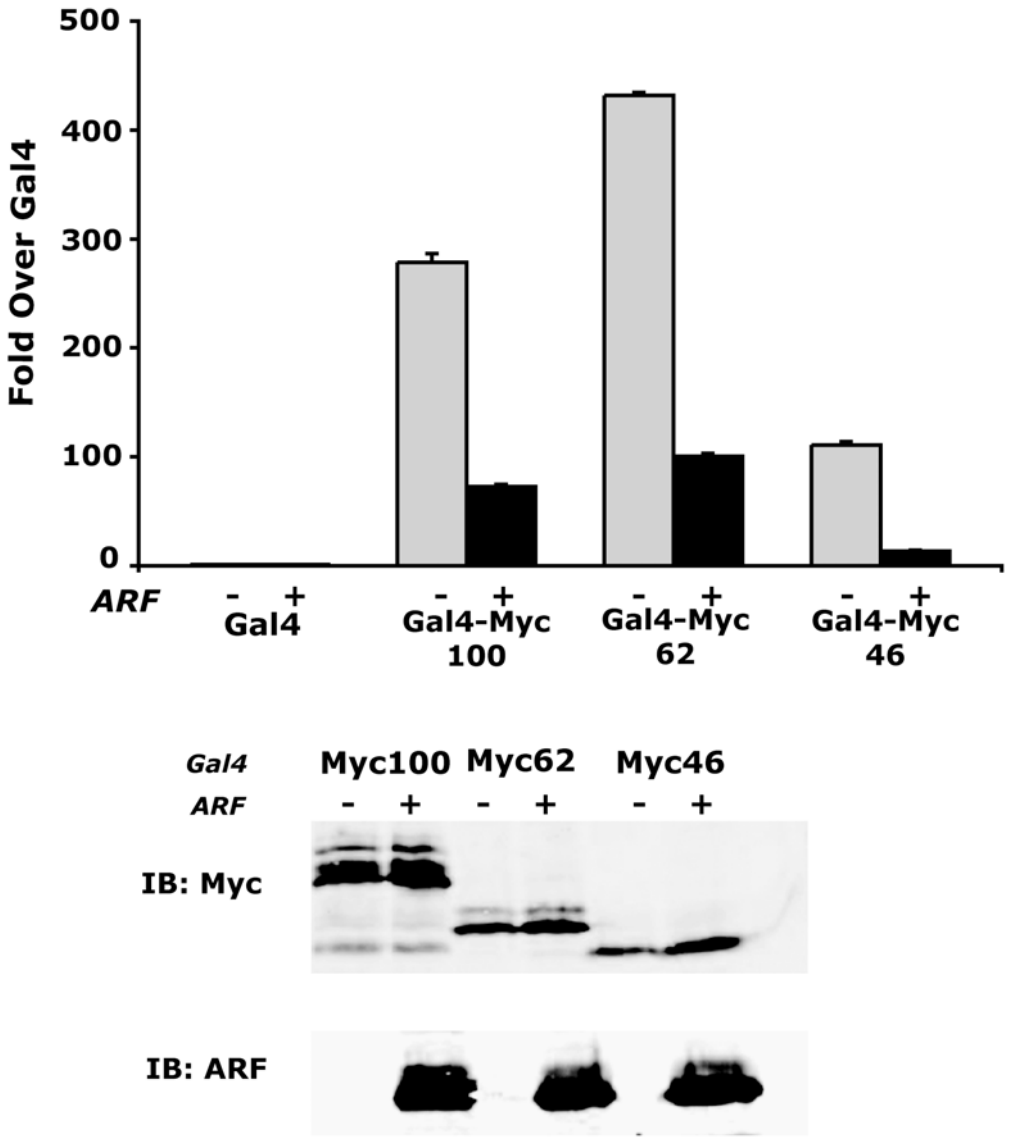


FIG. 25. Transactivation by Gal4-Myc constructs in the presence of ARF. Gal4-Myc, ARF and the Gal4 reporter gene were transfected and prepared as described in Methods and assayed for luciferase activity. The bar graph shows the levels of transactivation in each sample with and without ARF. Western blots with equal volumes of lysate and probed with sheep anti-Myc show equivalent levels of Gal4-Myc construct expressed.

Myc. Gal4-Myc100, Gal4-Myc63, or Gal4-Myc46, the Gal4 reporter gene, and exogenous p19ARF, or its empty vector, pcDNA3, were cotransfected into Cos7 cells, then assessed for luciferase activity. Results showed that when ARF was overexpressed, the transactivation of the reporter genes was diminished as compared to the Gal4 fusion protein without ARF expression (Figure 25). As

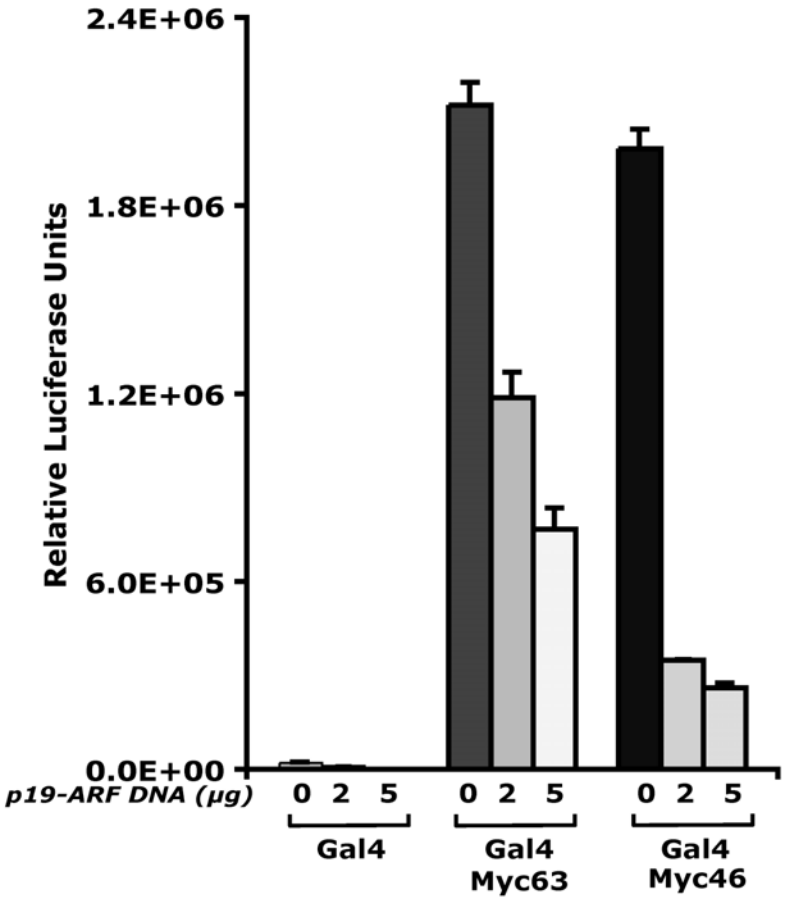


FIG. 26. Transactivation by Gal4-Myc63 and Gal4-Myc46 in the presence of increasing ARF. Gal4-Myc constructs, the Gal4 reporter gene and increasing levels of ARF were transfected and assayed for luciferase activity as described in Methods. The bar graph shows the level of transactivation in the presence of varying levels of ARF.

seen in the corresponding Western blot, this diminished transactivation was not the result of less Gal4 fusion protein being expressed in the system. In fact slightly greater levels in the presence of ARF still induced less activity.

To confirm this finding, the dose dependent effect of ARF was assayed. Increasing levels of ARF were transfected in conjunction with each Gal4 fusion protein and reporter gene. Results indicate that increased levels of ARF caused a greater inhibition of transactivation. This inhibitory effect was seen in all samples, including the samples expressing only Gal4, even though the basal activity of Gal4 is independent of the transregulatory domain of Myc. However it appears the effect of ARF inhibition was more pronounced in the Gal4-Myc46 than the Gal4-Myc63, the region containing MBI (Figure 26). Taken together, these results suggest that ARF is able to inhibit target gene transactivation at least through MB0. The effects on MBI alone need to be examined.

Post-translational modification and transactivation

To determine the role of post-translational modifications on transactivation, specific amino acids where these modifications occur were mutated. For these studies, we chose to utilize the Gal4-Myc with the highest transactivation activity, Gal4-Myc100. This region includes MBI, which has phosphorylation sites known to influence c-Myc biological function, including Thr58 and Ser62 (Figure 27)(Hann, 2006). In addition, there are potential ubiquitination sites as well as seen in Figure 29 at Lysine 51 and 52.

For these studies, site directed mutagenesis was used to not only change the critical T58 phosphorylation site from threonine to alanine, a double mutation was made as well that changed both lysines within the first 100 amino

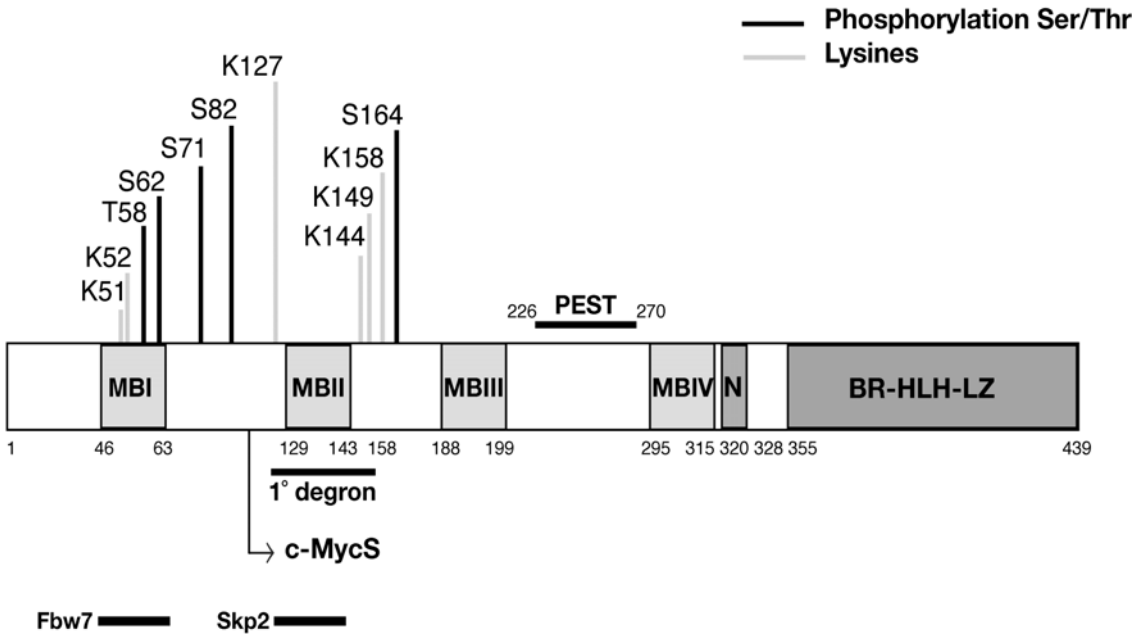


FIG. 27. Potential sites of post-translational modification within c-Myc. The schematic of the full-length c-Myc protein shows the location of potential modification sites of lysines, serines and threonines. The binding region of ubiquitination cofactors Fbw7 and Skp2 interact at MBI and MBII respectively.

acids (K51 and K52) to arginines. Elimination of lysines at these sites will prevent the modifications that may occur there, such as ubiquitination, acetylation or even methylation. These Gal4-Myc100 fusion proteins, referred to as Gal4-Myc100T58A and Gal4-Myc100KKRR, were compared to the unmutated Gal4Myc100 in a Gal4 assay to determine their effects on transactivation. Results indicate that both Gal4-Myc100T58A and Gal4-MycKKRR expressed at higher levels than the wild type Gal4-Myc100. When the results of the Gal4 assay were adjusted to total Gal4 protein levels, the mutation caused a decrease in the activity by roughly half (Figure 28). Therefore the results suggest that modifications at T58 and K51K52 do have some involvement in transactivation.

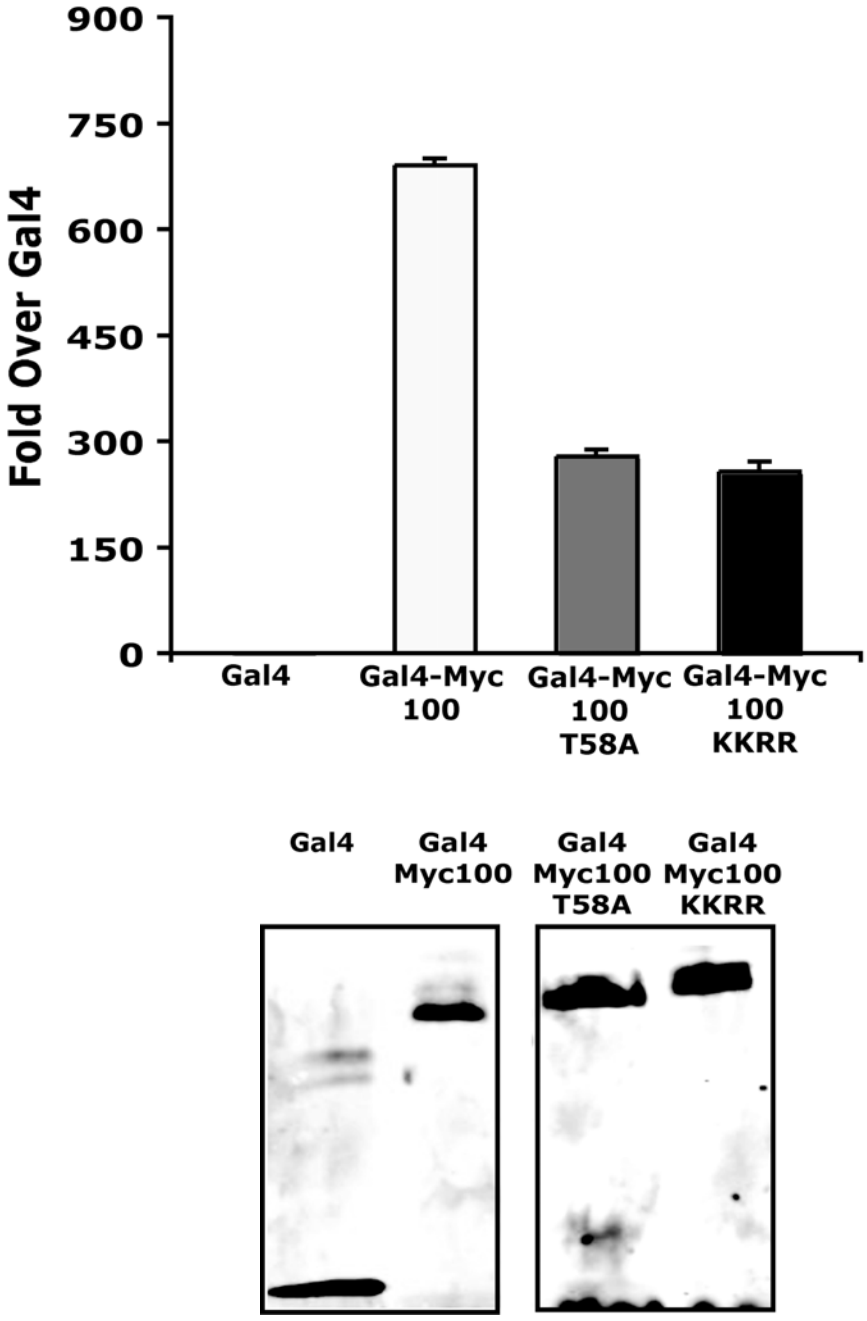


FIG. 28. Transactivation by Gal4-Myc100 upon alteration of modification sites. Gal4-Myc100 and the same construct with point mutations at T58 or K51 and K52 were transfected into Cos cells for a Gal4 assay as previously described. The bar graph shows the change in fold activity over Gal4. Western blots show the levels of Gal4-Myc protein in each sample.

Previous studies had found both ubiquitin ligases and proteasomal components localized to c-Myc target gene promoters upon Myc induction (Kim et al., 2003; von der Lehr et al., 2003b) These components are believed to enhance transactivation, through the "licensing" of transcriptional initiation. We wanted to test this licensing hypothesis by using the Gal4-Myc100. To block proteasomal activity MG132, a proteasomal inhibitor, was used. Gal4-Myc46 and Gal4-Myc100, lacking K51 K52 or T58 were also used. To normalize for the stabilization of proteins, an independently expressed ß-Gal was cotransfected with the Gal4 construct and reporter construct. When each sample was treated with either MG132 or its vehicle, DMSO, transactivation was assayed in the presence of MG132. When these levels were adjusted for increases in protein, the results reflected a consistent decrease in transactivation (Figure 29). Gal4-Myc100 showed the greatest decrease in activity upon MG132 addition. The mutants T58A and KKRR showed some inhibition of activity, but it was less than the inhibition of Gal4-Myc100. Gal4-Myc46 was also significantly inhibited in the presence of MG132. Overall these results show a decrease in transactivation in the presence of MG132 that was significantly lessened when T58 was mutated.

Conclusions

The data indicate that ARF interfered with target gene transactivation by the Myc1-100 fragment and even the Myc1-46 fragment. Gal4-Myc46 was the construct that showed the greatest inhibition of transactivation. The effect of ARF, and specifically the effect on the first 46 amino acids, may clarify the role of transactivation in c-Myc target gene upregulation. It has already been shown that ARF is recruited to c-Myc target gene and that ARF blocks the upregulation of c-

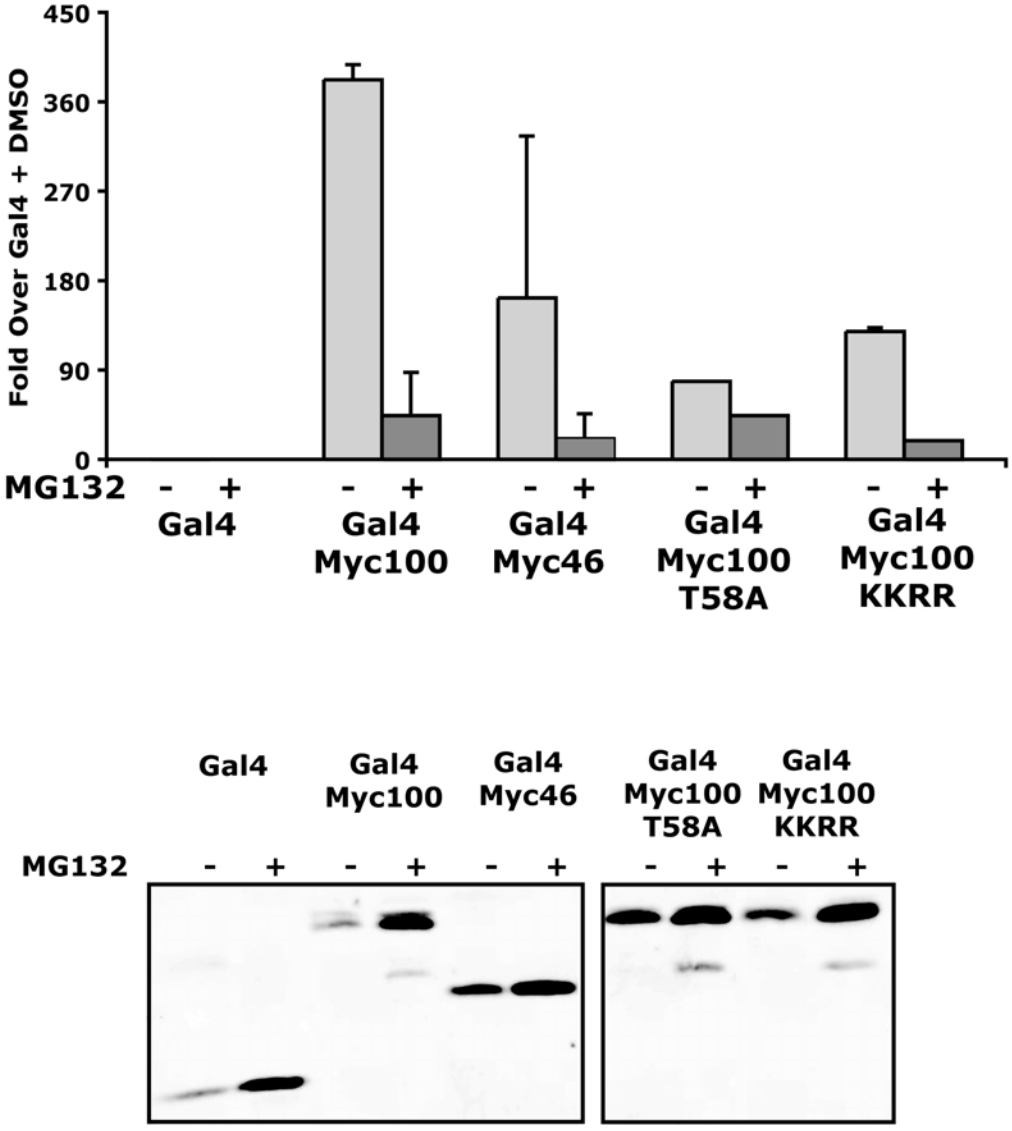


FIG. 29. Transactivation by Gal4-Myc100 constructs in the presence or absence of proteasomal inhibition. Gal4 assays testing Gal4-Myc100 and the point mutants T58A and KKRR were compared to Gal4-Myc46 in the presence and absence of MG132. Eight hours before harvest each sample was incubated with either MG132 or the vehicle, DMSO. The bar graph shows the fold change in activity over Gal4 after normalization with ßGal. Western blots show the levels of Gal4-Myc within the sample at each condition.

Myc target genes. Whether this effect blocks recruitment of specific activating cofactors or recruits inhibitory cofactors to the promoter is unknown and not clarified in our results. These studies also do not address the potential role of the C-terminal ARF binding on target gene upregulation. The C-terminus of c-Myc is critical for heterodimerization with Max and binding to the DNA at the EMS. The interruption of this DNA interaction could easily be affected by ARF binding to the C-terminal of Myc.

Our data also suggest that post-translational modifications can alter transactivation. Prevention of modification through mutation of T58 or K51 and K52 diminished transactivation to some degree but did not completely eliminate it. Lack of either modification appeared to have similar effects. Even though the extent of activity of each mutation is similar doesn't exclude the possibility of transactivation being mediated by separate mechanisms that may result in similar transcriptional activity. Additionally, mutations of these sites do not exclude the possibility of more promiscuous phosphorylation or ubiquitination at other sites within the Gal4 domain that may affect transactivation at the Gal4 promoter.

In the presence of MG132, transactivation is diminished in every Gal4-Myc tested, but the greatest extent of diminished activity was found with Gal4-Myc100. Less inhibition was found in Gal4-Myc46 and Gal4-MycKKRR. However, the mutation with the greatest effect on MG132-mediated inhibition of transactivation was found with Gal4-MycT58A, where phosphorylation is blocked by the mutation to alanine. Under the assay conditions, it also appears that the protein levels of Gal4-Myc46, Gal4-MycT58A and Gal4-MycKKRR are also slightly more abundant than Gal4-Myc100. One complicating factor to

interpretation of the MG132 data is the global effect of MG132 on all proteasomes, not just the ones potentially interacting on the promoters of target genes. The inhibition of proteasomes allows for the accumulation of unstable proteins, including common transcriptional cofactors. These cofactors will accumulate and could increase the overall transactivation potential by increasing limiting components or it could be inhibitory by overloading the system with cofactors that could behave in an inhibitory fashion.

Given all these caveats, the common thread that links all these results is that disruption of the ubiquitin-mediated proteasomal mechanism inhibited Myc-induced transactivation, either through mutation of lysines, inhibition of the proteasome or eliminating T58 phosphorylation which controls Fbw7-mediated ubiquitination. Thus, the proposed licensing hypothesis may have merit.

CHAPTER VI

DISCUSSION

Transactivation by c-Myc N-terminal regions

Our data has shown that of all the regions tested within the c-Myc TRD, the region with the highest level of transactivation activity was found within the first 62 amino acids. These observations are relatively consistent with several studies that showed a high level of activity with the first 100 amino acids (Cziepluch et al., 1993; Kato et al., 1990). Relatively minor discrepancies do exist. These discrepancies could simply be due to the inadvertent retention of inhibitory domains and variability in the exact regions deleted or tested (Kato et al., 1990). Our data also revealed a significant level of transactivation found in the first 46 amino acids of c-Myc, approximately 40% of the activity of the Myc 1-100. Additional studies have confirmed that this region has activity, although no specific domain within this region has ever been identified. For instance, a promoter assay utilizing the CCL6 promoter showed that both c-MycΔ2-42 and ΔMBI were deficient in CCL6 transactivation (Eberhardy and Farnham, 2002; Yi et al., 2003). However, using a 2XEMS reporter gene integrated into the chromatin, we found that Myc46CTD was unable to induce the 2XEMS reporter, in contrast to the results seen with Gal4-Myc46. Additional reports also support the importance of promoter context in the transactivation by Myc 1-46 (Eberhardy and Farnham, 2002; Yi et al., 2003).

Analysis of the region of c-Myc N-terminal to MBI uncovered a conserved region of high sequence similarity between other Myc proteins of other species

and other Myc family member proteins. This region was identified as MB0. Subsequent removal of this region resulted in dramatically decreased transactivation of a Gal4 reporter gene. These results were similar to what was found in a previous study that dissected the N-Myc N-terminal TRD. The addition of sequences corresponding to the MB0 or MBI region in N-Myc with previously deleted MB0 and MBI caused an increase in transactivation by almost approximately two-fold for each (Cziepluch et al., 1993). However, our studies were the first to identify a conserved domain critical for c-Myc activity within the region of c-Myc spanning a.a. 1-46.

Target gene regulation by c-Myc N-termini

Although our studies with MB0 have shown a significant role in Myc mediated transactivation, additional studies were pursued to correlate this region to target gene regulation and the mechanisms that mediate this regulation. Indeed we have shown that two well known target genes, *CDK4* and *hsp60*, rely on the presence of MB0 for their upregulation in DKO fibroblasts. In contrast, deletion of MBI actually increased the level of upregulation of both genes, even above wild type Myc levels. Discrepancies in transactivation versus upregulation are commonplace. MycS, which is defective in transactivation, can upregulate some target genes in primary and immortalized cells (Cowling and Cole, 2008; Hirst and Grandori, 2000; Xiao et al., 1998). These results were confirmed in our studies comparing MycSER induction of some target genes with wild type Myc2ER induction. However, some target genes were not as upregulated by MycS as compared with Myc2 induction.

Although, several binding proteins have been shown to interact at the TRD of c-Myc, none have been confirmed to interact with MB0 at this point. The cofactor with the highest probability of MB0 interaction is CDK8, which was found to interact with c-Myc 1-41 (Eberhardy and Farnham, 2002). Although TRRAP interaction was shown to be MBII dependent, numerous secondary binding regions have been shown within the first 100 amino acids, including regions encompassing MB0. Deletion of any of these regions prevented TRRAP interaction with c-Myc (McMahon et al., 1998; Park et al., 2001). In addition MBII was a necessary component in target gene upregulation, clearly contrasting the transactivation mechanisms shown by promoter assays (Nikiforov et al., 2002; Zhang et al., 2006). Our data also showed that Cyclin T1 was unable to bind to MycS, which is consistent with previous studies showing interaction of Cyclin T1 at MBI (Eberhardy and Farnham, 2002). Therefore MycS-induced upregulation of target genes must be mediated by mechanisms independent of Cyclin T1 or pTEFb. It is possible that Cyclin T1 could interact with MB0, however further experimentation needs to be performed to conclude this. There is substantial evidence that c-Myc target genes are not all regulated by the same mechanism (Nikiforov et al., 2002).

Regulation of c-Myc biology by regions of the c-Myc TRD

Assessment of the necessity of MB0 in c-Myc biology started with the most stringent biological effect of Myc activity, the Myc-Ras cotransformation assay. In addition, we examined the least stringent assays of Myc biological activity in Rat1a fibroblasts. Previously shown, both MycS and ΔMBI were less efficient at focal formation in primary rat fibroblasts (MacGregor et al., 1996;

McMahon et al., 1998). We have now shown that MB0 is essential for Myc-Ras cotransformation. In contrast, previous studies with MycS showed enhanced proliferation, apoptosis, and anchorage independent growth in immortalized Rat1as and rescue of the slow growth phenotype in *myc*-null HO16 cells (Cowling and Cole, 2008; Xiao et al., 1998). We found that deletion of MB0 also affected c-Myc biology similarly to MycS in the aforementioned biological effects, as expected.

In Rat1a immortalized fibroblasts, we found that neither MB0 nor MBI were necessary to induce hyperproliferation. Biological assays using minimal Myc constructs also show that, although deficient in apoptotic induction, both Myc100CTD and Myc46CTD induced some degree of Rat1a hyperproliferation. Although these studies suggest the importance of the N-terminal domain in Myc-induced Rat1a hyperproliferation, one alternate possibility that may explain this effect is that the activity induced in Rat1a is dependent on the C-terminal region involved in DNA binding and heterodimerization. These minimal Myc proteins could also behave as a dominant negative Myc, but negative effects on proliferation were not seen. Induction of the Myc CTD with no c-Myc N-terminus attached should clarify the significance of the N-terminal domain in hyperproliferation.

c-Myc has been shown to induce re-entry into S phase from a quiescent state (Eilers et al., 1991). Previous findings of the effect of ΔMBI and ΔMB0 on cell cycle re-entry from serum-starved DKO fibroblasts were inconclusive (data not shown). Other findings indicate a critical role for Myc boxes in cell cycle re-entry, although the exact Myc box responsible is not firmly agreed upon. Deletion of MBII has been shown to prevent cell cycle re-entry from quiescence

(Bush et al., 1998; Conzen et al., 2000; Herbst et al., 2005; Nikiforov et al., 2002) However, other models do show a role for the N-terminal 100 a.a. of c-Myc in cell cycle regulation using MycS. These differences were observed under two cellular conditions; cycling immortalized cells, and fully quiescent primary cells. MycS induced the continuation of the cell cycle in cycling WI38 primary cells, MycS was unable to induce re-entry into the cell cycle from a quiescent state (Hirst and Grandori, 2000) Although specific deletion of only MBI was never studied in this primary cell system, these studies don't exclude MB0 from playing a role in cell cycle re-entry.

c-Myc post-translational modification and ARF binding

ARF was previously shown to tightly bind the first 150 amino acids of c-Myc in addition to sites at the C-terminal domain (Qi et al., 2004) This interaction not only shut down the upregulation of some Myc target genes, it also prevented transactivation of target gene promoter assays. However, whether ARF mediated inhibition of transactivation is mediated through the N-terminal or C-terminal sites of c-Myc remains unclear. Gal4 assays utilizing Gal4-Myc were performed to assess the significance of c-Myc N-terminal ARF binding. Indeed, we showed that although coexpression of ARF reduced transactivation in all Gal4-Myc proteins tested, Gal4-Myc46 showed the most profound inhibition of transactivation in the presence of increasing levels of ARF. However, whether ARF conclusively binds to the N-terminal 46 amino acids or binds at several locations within the N-terminal 150 amino acids remains to be clarified.

Mutation of either Lys51 and 52 or Thr58 within Gal4-Myc100 resulted in diminished transactivation. This is in contrast to a study that found no difference in transactivation by c-Myc containing a T58A mutation (Lutterbach and Hann, 1994). The difference in results may be a result of the robustness of the Gal4 assay. The Gal4 assay may have revealed differences in transactivation that were masked by the weak signal normally observed in EMS promoter assays. However, mutation of Thr58 did appear to be significant when proteasomal activity was inhibited by MG132. Although MG132 had a global effect on transactivation of the Gal4 reporter gene, the greatest reduction of transactivation was found with Gal4-Myc100. Less inhibition was found with Gal4-Myc46 and Gal4-MycKKRR while the least inhibition of transactivation was found with Gal4-MycT58A. This specific mutation would prevent the binding of Fbw7 to c-Myc, as the phosphorylation required for Fbw7 binding would never occur. However, no direct role in transactivation has been linked to Fbw7 recruitment to c-Myc. The specific molecular mechanism mediating transactivation by Myc 1-46 may be independent of any mechanism that requires modification of either lysine or T58 or S62.

Although mutation of K51 and K52 prevents ubiquitination at these sites, it is unclear how ubiquitination is involved in transactivation and whether it is a critical component of transactivation or simply the result of proteasomal mediated licensing. Proteasomal subunits, Myc-dependent Skp2 recruitment, and ubiquitinated proteins were simultaneously found localized to upregulated target gene promoters (von der Lehr et al., 2003b). How these components rely on each other to induce transactivation or target gene activation is also unclear.

Future Directions

Although several cofactors bind MBI and MBII, no other cofactors have been isolated to the first 46 amino acids other than CDK8. Knowledge of cofactor interaction can further clarify the molecular mechanisms that mediate c-Myc's function. In conjunction with assessing the interaction of CDK8 with MB0, large-scale coimmunoprecipitations could be used to isolate Myc and ΔMB0 along with their binding cofactors. Mass-spectrometry will be used to initially identify the proteins bound to each Myc protein tested. The resulting data could be compared to wild-type Myc immunocomplexes to generate a list of potential binding proteins at each Myc box. In addition to cofactor binding, a large-scale survey of target gene regulation would provide a much greater understanding of the role of MB0 in c-Myc target gene regulation. Microarray experiments would compare the transcriptional activity of inducible wild-type Myc with ΔMB0 or ΔMBI. Subsequent studies would focus on target genes and cofactors in Myc2ER that differ in ΔMB0.

Although MB0 has been found in several Myc molecules not all of them contain this region, as shown by protein sequence alignment with L-Myc (Figure 30). L-Myc is a molecule whose functional divergence from c-Myc is not well understood. Some of the differences in L-Myc function include reduced cotransformation, reduced transactivation of a Gal4 reporter gene, alternate mechanisms of target gene upregulation and diminished tetraploidy in p53 -/- MEFs (Barrett et al., 1992; Nikiforov et al., 2002; Yi et al., 2003; Yin et al., 1999). Further experimentation should be able to determine whether the lack of a conserved MB0 results in the differences in L-Myc function and would define the biological functions mediated by MB0. To test this c-Myc MB0 would be fused to

the N-terminus of L-Myc. Myc Ras cotransformation would be assessed in addition to molecular interactions where c-Myc and L-Myc differ, such as binding to TRRAP, histone acetylation, or activation of TRRAP dependent genes.

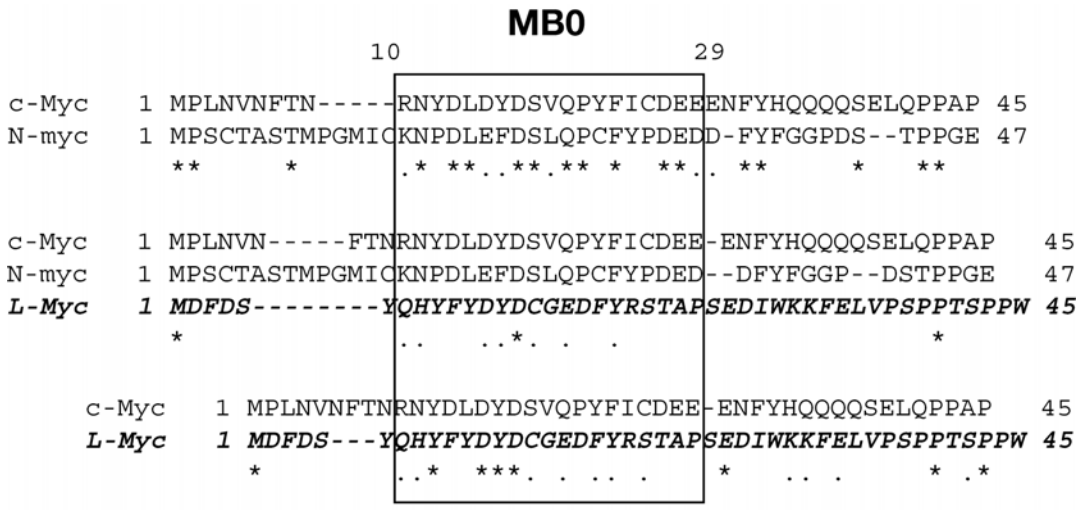


FIG. 30. Conserved MB0 not found in L-Myc. Sequence alignment of L-Myc in comparison with the homology found in c-Myc and N-Myc pre-MBI N-termini. The region defining MB0 is boxed in as shown.

Although some of the biological effects of MB0 have been assessed, further biological studies would complete the picture of the role of MB0 in c-Myc biology. Myc-induced apoptosis would be assessed in both immortalized and primary fibroblasts. Although the role of MB0 in primary cell cotransformation has been defined, the effect of deleting MB0 on tumorigenesis is unknown. To test tumorigenicity, Rat1a cells expressing wild type Myc or ΔMB0 would be injected into nude mice to determine tumor size, metastatic potential or length of survival. Another method of assessing tumorigenesis is through Myc-induced lymphomagenesis. A previous study noted that expression of c-Myc-ΔMBI in

murine hematopoetic stem cells (HSC) generated a different tumor type than HSCs expressing elevated levels of wild type Myc (Herbst et al., 2005). Myc2, ΔMB0 or ΔMBI overexpression in HSC's would be induced by an IgG heavy chain enhancer transgene (Eμ). Aside from survivability and manifestation of disease, determination of cellular fate may indicate additional functions mediated by MB0.

The isolation of minimal regions of c-Myc has initiated a different method to test the sufficiency of specific domains to c-Myc function. Some of the biological functions remaining to be defined are the rescue of the slow-growth phenotype of *myc*-null HO16 cells and soft agar growth in Rat1a fibroblasts. For these experiments establishing a stable cell line expressing a negative control CTD will be highly desirable for defining regions sufficient for function. Having this control would solidly determine whether the effects mediated by the minimal Myc are the result of its transregulatory domain fragment, or simply a function of the C-terminus.

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