# Gene-target recognition among members of the Myc superfamily and implications for oncogenesis

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**Myc and Mad family proteins regulate multiple biological processes through their capacity to influence gene expression directly. Here we show that the basic regions of Myc and Mad proteins are not functionally equivalent in oncogenesis, have separable E-box–binding activities and engage both common and distinct gene targets. Our data support the view that the opposing biological actions of Myc and Mxi1 extend beyond reciprocal regulation of common gene targets. Identification of differentially regulated gene targets provides a framework for understanding the mechanism through which the Myc superfamily governs the growth, proliferation and survival of normal and neoplastic cells.**



## Introduction

Members of the Myc superfamily of basic-region helix-loop-helix/leucine zipper (bHLH/LZ) proteins regulate diverse cellular processes integral to the growth, survival and development of normal and neoplastic cells[1]. Max is an obligate heterodimeric partner for the Myc and Mad families[2,3]. c-, N- and L-Myc each have established roles in promoting oncogenesis[4], whereas members of the Mad family (Mad1, Mxi1, Mad3 and Mad4) have been shown to promote differentiation, block cellular growth and Myc-induced transformation, and suppress the development of cancer *in vivo*[5]. These contrasting properties are consistent with the biochemical findings that Myc/Max and Mad/Max heterodimers act antagonistically in E-box–dependent reporter assays, wherein Myc activates and Mad represses gene transcription[6–8]. These findings support the view that members of the Mad family oppose the actions of Myc through their competitive occupation of E-box recognition elements embedded in commonly regulated genes.

Basic-region residues known to provide critical contacts with the DNA major groove[9] are conserved among Myc, Max, Mad and the related bHLH/LZ proteins, USF, TFE-3 and TFE-B, which are all capable of recognizing the E-box consensus sequence CACGTG in engineered reporters. *In vivo* and *in vitro* site selection suggests that sequences flanking the E box may confer differential recognition among these proteins[10–12]. These studies indicate that Myc/Max preferentially binds E boxes flanked by a 5′ C and a 3′ G and fails to bind those flanked with a 5′ T or a 3′ A; in contrast, Max/Max possesses a more flexible binding profile[11]. Analogous flanking sequence selectivity exists for the *Saccharomyces cerevisiae* bHLH proteins, Pho4p and Cpf1p, which share homology with the basic regions of the Myc superfamily proteins[13]. Substitution of a single amino acid in the basic region can confer a Cpf1p-like binding profile on Pho4p (ref. 13). The analogous position exhibits great variance in amino acid residues among Myc, Mad and Max proteins.

Here we used basic-region swap experiments to address whether Myc-induced transformation targets and Mad/Mxi1-induced suppression targets are the same. Comparisons were made on the levels of oncogenic potential, E-box–dependent reporter transcriptional activity, modelling of the structure of the basic region and genome-wide scans of gene expression. Our studies revealed that the biological actions of Myc and Mxi1 involve the regulation of both common and distinct sets of genes governing diverse biological processes.

## Results

Amino acid residues in the basic region that have been shown previously to contact the nucleotide bases and phosphate backbone of DNA (ref. 9), and to recognize CACGTG sequences[9], were conserved among known members of the Myc superfamily (Fig. 1). Comparison of the basic regions of the Myc (c-, N- and L-Myc) and Mad families (Mad1, Mxi1, Mad3 and Mad4) and Max showed conserved differences among these subfamilies at positions 2, 6, 9 and 10. These specific residues point away from the DNA major groove and may be involved in interactions outside the canonical E-box consensus sequence.

### Basic-region residues underlie functional differences

To better understand the role of the basic region as a determinant of biological function of Myc superfamily members, we used site-directed mutagenesis to replace the basic region of c-Myc with that of N-Myc, L-Myc, Max, Mxi1 or the unrelated b-HLH-LZ protein TFE-B in an otherwise wild-type c-Myc molecule. We then assayed the c-Myc protein and its mutant derivatives for their ability to transform early passage rat embryo fibroblasts (REFs) in cooperation with activated H-RAS (G12V).

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| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| c-Myc | R | R | T | H | N | V | L | E | R | Q | R | R |
| N-Myc | R | R | N | H | N | I | L | E | R | Q | R | R |
| L-Myc | R | K | N | H | N | F | L | E | R | K | R | R |
| Max | R | A | H | H | N | A | L | E | R | K | R | R |
| Mad1 | R | S | H | H | N | E | M | E | K | N | R | R |
| Mxi1 | R | S | T | H | N | E | L | E | K | N | R | R |
| Mad3 | R | S | V | H | N | E | L | E | K | R | R | R |
| Mad4 | R | S | S | H | N | E | L | E | K | H | R | R |
| Mnt | R | E | V | H | N | K | L | E | K | N | R | R |
| TFE-B | K | D | N | H | N | L | I | E | R | R | R | R |

**Fig. 1** Comparison of the basic regions of Myc superfamily members. Amino acid sequences of the various basic regions were obtained using the Genetics Computer Group Sequence Analysis Software Package and aligned by visual fit. Numbers refer to the position within the 12-aa basic region. Shading indicates residues conserved among all family members. In addition, position 9 is a conserved basic residue, consistent with its interaction with the phosphate backbone. At position 2 of Myc family members there is a large, positively charged residue. Max contains an alanine at this position, and a conserved serine is found in Mad family members. At position 6, c-, N- and L-Myc, and Max contain a neutral amino acid, whereas Mad family members contain a conserved negatively charged glutamic acid. Mnt differs from Mad family members by the presence of a glutamic acid at position 2. Residues 6 and 9 are conserved among Mad family members.

In multiple independent experiments, c-Myc and c-Myc chimaeras containing N-Myc, L-Myc or Max basic regions generated similar numbers of foci with comparable transformed morphology (data not shown). In contrast, substitution of either the Mxi1 or the TFE-B basic region into c-Myc resulted in an average reduction in foci of 87% for c-Myc(Mxi1-BR) and of 78% for c-Myc(TFEB-BR) (Fig. 2*a*, $P<0.001$). Moreover, foci generated by c-Myc(Mxi1-BR)/RAS or c-Myc(TFEB-BR)/RAS exhibited a less transformed morphology and poor subcloning efficiency (Fig. 2*b*, and data not shown). c-Myc, c-Myc(Mxi1-BR) and c-Myc(TFEB-BR) were expressed at equivalent levels (data not shown) and were equally effective in activating E-box–dependent transcription (Fig. 2*c*). Finally, Mxi1 possessing the c-Myc basic region was tested for its ability to repress Myc/RAS transformation. Wild-type Mxi1 inhibited Myc/RAS-induced focus formation by 98% compared with 90% for Mxi1(c-Myc-BR) (consistent over three trials, $P<0.05$; Fig. 2*d*). These findings indicate that although functional equivalence exists among the basic regions of the Myc family, biological differences are evident among the c-Myc, Mxi1 and TFE-B basic regions.

The functional differences between the yeast bHLH proteins Cpf1p and Pho4p are determined solely by the basic-region residue at position 2 (ref. 13), and the analogous position encodes arginine in Myc, alanine in Max and serine in Mad. To test whether the position 2 residue confers distinct DNA-binding specificities among the Myc superfamily proteins, we compared c-Myc containing an arginine-to-serine substitution at position 2 (designated c-Myc(R2S-BR)) with c-Myc and Myc(Mxi1-BR) in a REF co-transformation assay. c-Myc(Mxi1-BR) and c-Myc(R2S-BR) yielded fewer foci relative to c-Myc (82% and 91% fewer, respectively; $P<0.001$; Fig. 3*a*)—thus supporting a role for the arginine at position 2 of the Myc basic region in oncogenic potency. A second substitution at position 6 (R2S/V6E) resulted in a partial restoration of oncogenic potency relative to the c-Myc(R2S-BR) mutant. The ability of a single mutation at position 2 of the Pho4p basic region to alter the target-gene specificity of this yeast protein[13] suggests that a change in target gene specificity likely accounts for the difference in biological activity between c-Myc and c-Myc(R2S-BR), and by extension c-Myc(Mxi1-BR). The partial rescue of transforming activity by the second site mutation suggests a complex interplay among these non-conserved residues of the basic region. Finally, positions 9 and 10 were not assayed specifically because these changes are conserved between Mad/Mxi1 and the oncogenically competent basic region of the Mnt protein[14].

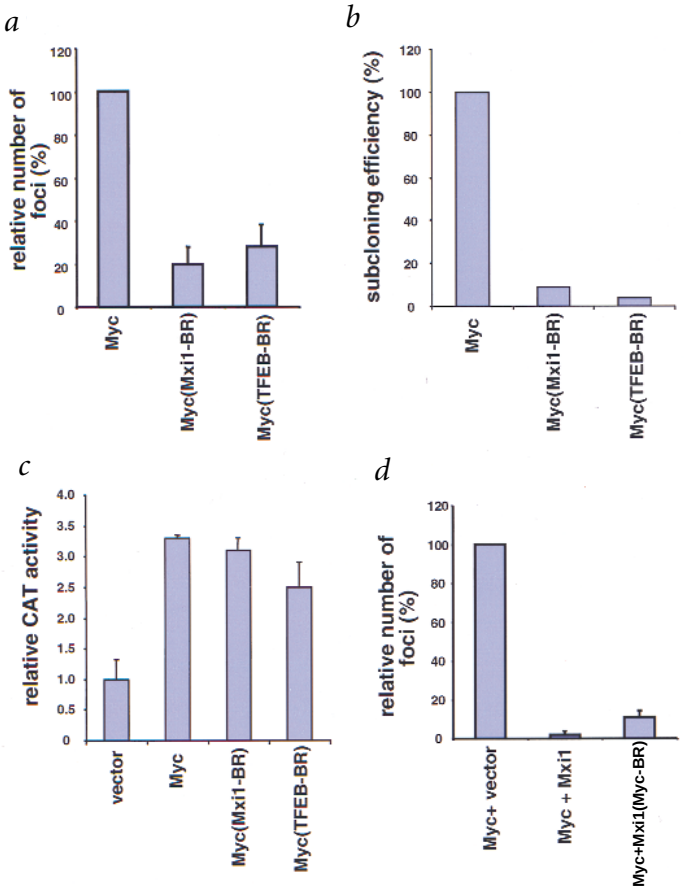


**Fig. 2** c-Myc and Mxi1 basic regions are not equivalent in transformation. ***a***, Primary rat embryo fibroblasts (REFs) were transfected with vectors encoding activated H-Ras and c-Myc or a chimaeric c-Myc containing the basic region of Mxi1 or TFE-B. Total number of foci are expressed as a percentage of the number obtained with activated H-Ras plus wild-type c-Myc. ***b***, Bars illustrate the relative capability of foci from the REF assay to establish permanent cell lines. The subcloning efficiency of foci derived from cells transfected with vectors encoding Myc(Mxi1-BR) or Myc(TFEB-BR) plus Ras was expressed relative to that of foci derived from cells transfected with vectors encoding Myc plus activated H-Ras. ***c***, Histogram representing the relative abilities of the indicated molecules to transactivate the min4CAT reporter bearing four E-box consensus sequences upstream of the thymidine kinase minimal promoter. Values were obtained by phosphorimager quantification of signal intensities and are expressed relative to the signal obtained with reporter plus empty vector. ***d***, REFs were triply transfected with Myc, activated Ras and empty vector, wild-type Mxi1-SR or chimaeric Mxi1-SR containing the basic region of c-Myc. The number of foci obtained was determined as described in (*a*).

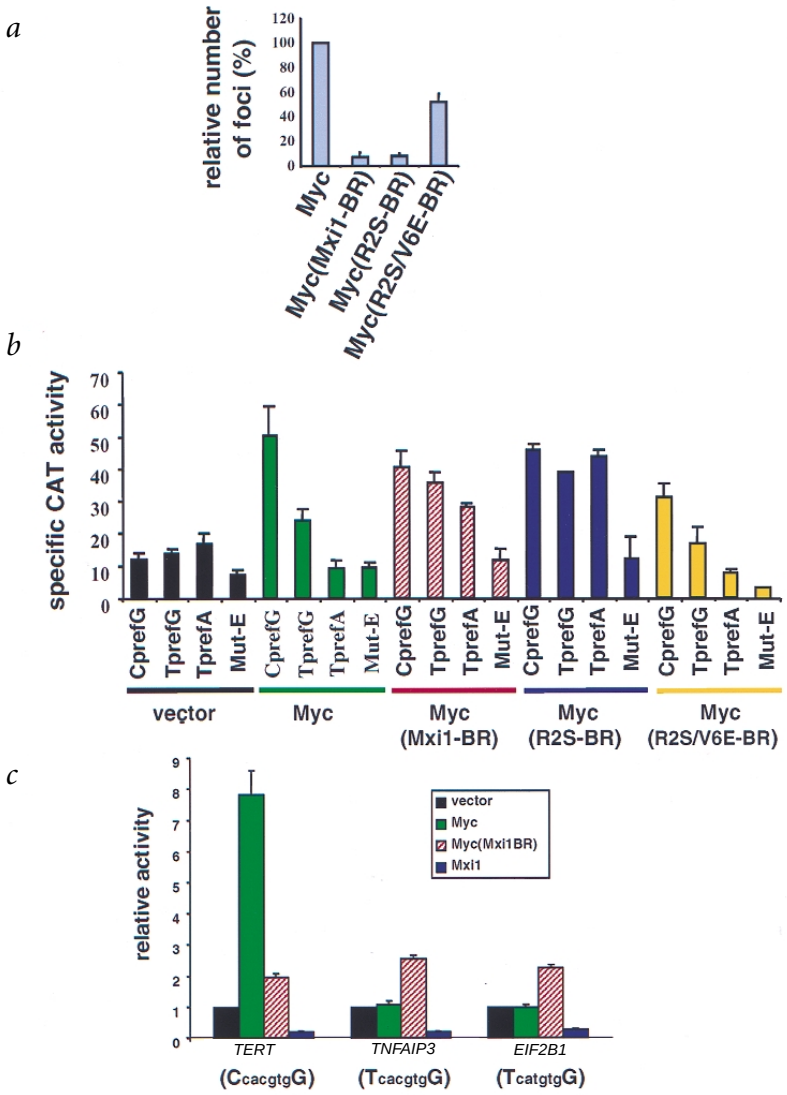


**Fig. 3** Specific residues in the Myc basic region determine biological activity. ***a***, Primary rat embryo fibroblasts (REFs) were transfected with vectors encoding activated H-Ras and c-Myc or a c-Myc chimaera containing the indicated mutations within the basic region. The number of foci obtained was determined as described in Fig. 2*a*. ***b***, Histogram representing the ability of wild-type c-Myc, or the basic region mutants described in the text, to transactivate CAT reporters bearing a single E box (CACGTG) flanked by C and G (CprefG), or T and G (TprefG), or T and A (TprefA), or a reporter bearing a mutant E box (CACGTA). Values were determined by phosphorimager quantification of signal intensities and corrected for transfection efficiency by β-galactosidase assay. ***c***, Histogram representing the ability of Myc, Myc(Mxi1-BR) and Mxi1-SR to regulate the *TERT*, *TNFAIP3* and *EIF2B1* promoters. Nucleotides flanking the E box are indicated below each promoter. Luciferase or SEAP values were determined by luminometer and corrected for transfection efficiency by β-galactosidase assay.

E-box flanking sequences confer differential DNA binding on Myc/Max heterodimers compared with Max/Max homodimers[11]. These differences prompted us to examine whether a similar distinction exists between c-Myc and Mxi1 basic regions. We transfected 293T cells with c-Myc, c-Myc(Mxi1-BR), c-Myc(R2S-BR) or c-Myc(R2S/V6E-BR) and one of four CAT reporter constructs linked to a single E box with different flanking nucleotides (CprefG, TprefG, TprefA) or to a mutant E box[11] (Mut-E).

Transfection of c-Myc enhanced CAT expression from the CprefG reporter fivefold (Fig. 3*b*) compared with a 1.5-fold increase from the TprefG reporter and no activity from the TprefA reporter (findings consistent with previous data[11]). c-Myc(Mxi1-BR) also increased expression from the CprefG reporter approximately fourfold, but differed from c-Myc by stimulating three- to fourfold activation of the TprefG and TprefA reporters. The reporter activation profiles of c-Myc(Mxi1-BR) and c-Myc(R2S-BR) were similar. Comparable results were also evident between c-Myc and c-Myc(R2S/V6E-BR), although c-Myc(R2S/V6E-BR) enhanced CprefG reporter activity threefold compared with fivefold for c-Myc. The shared inability of c-Myc and c-Myc(R2S/V6E-BR) to engage an E box flanked by T and/or A residues (Fig. 3*b*) correlates with their shared transforming activity (Fig. 3*a*). c-Myc(Mxi1-BR) and c-Myc(R2S-BR) both showed robust E-box–dependent reporter activity independent of the composition of flanking nucleotides (Fig. 3*b*) and both were weakly transforming (Fig. 3*a*).

To assess the physiological relevance of these reporter assays to the regulation of endogenous gene targets, we examined the ability of Myc, Myc(Mxi1-BR) and Mxi1 to regulate the activity of promoters of genes bearing E boxes in distinct contexts. The regulatory E box in the *TERT* promoter is flanked by a 5′ C and 3′ G (ref. 15), whereas the E boxes in the *TNFAIP3* (ref. 16) and *EIF2B1* (ref. 17) promoters are flanked by a 5′ T. Myc and Myc(Mxi1-BR) enhanced the activity of the *TERT* promoter, whereas Mxi1 repressed *TERT* promoter activity (Fig. 3*c*). Similarly, Myc(Mxi1-BR) enhanced the activity and Mxi1 repressed the activity of the *TNFAIP3* and *EIF2B1* promoters. In accord with the reporter assays using engineered E boxes, however, Myc did not regulate the *TNFAIP3* or *EIF2B1* promoters, confirming that flanking nucleotides affect Myc and Mxi1 E-box–binding specificity *in vivo*.

## Conformational changes induced by BR residues

To provide a structural basis for the capacity of position 2 to influence interactions with flanking E-box nucleotides, we modelled the structure of both the Myc and Mxi1 basic regions bound to DNA on the basis of the known crystal structure of the Max basic region bound to DNA (ref. 9). These modelling data predicted that the four amino acids that differ between the Myc and Mxi1 basic regions are positioned on the α-helical basic-region surface that faces away from the DNA (Fig. 4*a–d*). As such, these residues are not likely to be involved in direct interactions with the E-box DNA sequences. It has been proposed, however, that a T immediately 5′ of the E box may sterically hinder interactions between the basic region and the DNA (refs 11,13). The structure of the Max basic region bound to DNA suggests that a T immediately 5′ of the E box would protrude 0.7 Å farther into the major groove than C, G or A, and increase the surface area of the 5′ flanking nucleotide by 21.17 Å$^2$, thereby reducing the space between the basic region and the DNA (Fig. 4*e*,*f*). Effective interaction between Myc and an E box flanked with a 5′ T would necessitate that the basic-region helix of Myc occupies the same space as that of Max. If the Myc basic region is involved in either intra- or inter-molecular interactions with other protein mod-

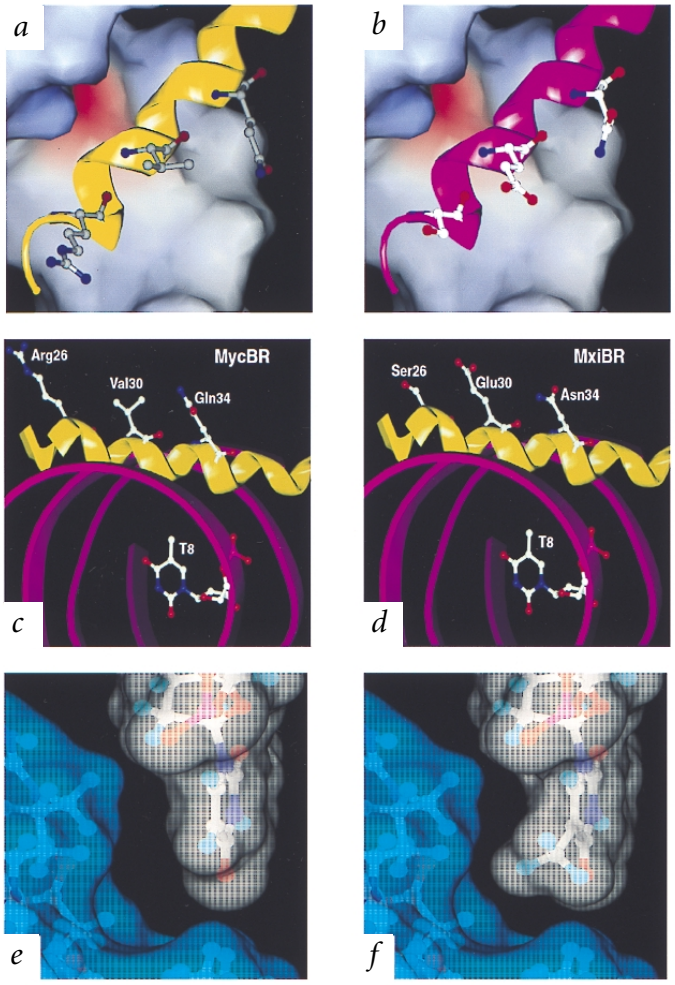


**Fig. 4** Interaction of Myc and Mxi1 basic regions with DNA. Van der Waals surface models illustrating the interaction of Myc (***a***, yellow helix) and Mxi1 (***b***, purple helix) basic regions with the E box are shown. ***c***,***d***, Ribbon models illustrating the interaction of Myc and Mxi1 basic regions (yellow helices) with the E box and a 5′-flanking thymidine, respectively. Positions 26, 30 and 34 defined for the Max basic region[9] are equivalent to positions 2, 6 and 10 described in the text. ***e***, The interaction of the basic region of Max (blue) with a uridine residue at the 5′ flank of the E box (white). Uridine was used to represent C, G or A because none of these contain methyl groups which extrude into the major groove. ***f***, The interaction of the Max basic region with a thymidine group at the 5′ flank of the E box. Models were determined computationally using the coordinates of the basic region from the Max/Max crystal structure[9].

ules, however, then a large arginine at position 2 would force the basic region deeper into the DNA major groove, resulting in a collision with the 5′ T. Substitution of the T with a C would remove the methyl group from the major groove of the DNA, allowing the basic region to interact more tightly with the DNA (Fig. 4*e*,*f*). In other words, the arginine in Myc would permit binding to E boxes flanked by a 5′ C, but not a 5′ T. The serine at position 2 of the Mxi1 basic region is 4.7 Å shorter than arginine, suggesting that the Mxi1 basic region would not be forced as tightly into the major groove, thereby enabling the Mxi1 basic region to bind to the E box independent of the identity of the 5′ flanking nucleotide. Thus, the size of the amino acid at position 2 of the basic region appears to dictate the suitability of an E-box context for members of the Myc superfamily.

### Common and distinct targets of Myc and Myc(MxiBR)

Our functional and structural studies predicted that the c-Myc and Mxi1 basic regions are likely to engage both common and distinct gene targets. To test this prediction, we used a cDNA microarray of 5,272 human cDNAs to compare gene expression profiles in IMR90 cells following induction of a c-Myc–oestrogen receptor (MycER) or c-Myc(Mxi1-BR)ER fusion protein. To identify genes that are directly regulated by MycER or Myc(Mxi1-BR)ER, we pretreated IMR90 cells with cycloheximide for 30 minutes before 4-hydroxy-tamoxifen (4-OHT) induction. Under these conditions, well-established c-Myc targets, including *ODC1* (encoding ornithine decarboxylase), *THBS1* (encoding thrombospondin), *DDX18* and *GAS1* (refs 19,20), were appropriately regulated by c-MycER (Table 1), thus providing an internal validation of the system. A comparative expression profile analysis in three independent experiments revealed only 8 shared targets versus 11 and 8 genes regulated specifically by Myc or Myc(Mxi1-BR), respectively (Table 1). Furthermore, in the few cases in which genomic sequences were available in the database, Myc-specific targets possessed a single E box flanked by 5′ C and 3′ G nucleotides. In contrast, Myc(Mxi1-BR)-specific targets contained an E box flanked by a 5′ T (data not shown). Moreover, representative gene targets regulated by the Myc(Mxi1-BR) chimaera were also repressed on induction of wild-type Mxi1-ER in IMR90 cells. These genes included *ODC1*, *Cul1*, *API2*, *Akt1*, *ZRP1* and *EIF2B1* (data not shown), thus attesting to the physiological relevance of the Myc(Mxi1-BR) gene target selection. Despite these correlations, further studies are required to establish whether all genes regulated by Myc(Mxi1-BR) are bone fide physiological targets of the Mxi1 basic region.

Myc and Myc(Mxi1-BR) regulated the expression of genes implicated in processes known to be affected by Myc, including proliferation, apoptosis, metabolism, and genomic stability[18–20]. When direct and indirect (non-cycloheximide) gene expression patterns were assessed, Myc enhanced expression of the anti-apoptotic genes *API2* (direct), and *Akt1* and *TNFAIP3* (indirect), and repressed the pro-apoptotic gene *CASH* (indirect; Table 1, and data not shown). In contrast, Myc(Mxi1-BR) enhanced expression of *TNFRSF6*/*TNFSF6* (indirect), and *API2* and *TNFAIP3* (direct), and repressed expression of survival genes such as *Akt1* and *IGF1* (direct), and *BTG2* and *BCL2A1* (indirect; Table 1, and data not shown). These differences in downstream gene expression predict that Myc(Mxi1-BR)-expressing cells would exhibit a higher level of apoptosis than c-Myc–expressing cells. To test this possibility, we treated IMR90 cells expressing c-MycER, Myc(Mxi1-BR)ER or the empty vector with 4-OHT for 24–48 hours in high serum (15%) and examined the extent of apoptosis by propidium iodide (PI) and annexin staining. Myc(Mxi1-BR)ER-expressing cells showed 76% apoptosis after 24 hours (Fig. 5). In contrast, c-MycER-expressing cells showed a minimal increase in apoptosis compared with the empty vector control (Fig. 5). Thus, differences between c-Myc and c-Myc(Mxi1-BR) regulation of apoptotic/survival genes correlates with differences in the observed rates of apoptosis, which are likely to contribute to the distinct transforming potential of these proteins.

## Discussion

The combined use of the ER system and cDNA microarray analysis verified that Myc and Mxi1 basic regions engage both common and distinct gene targets. A proportion of the genes

**Table 1 • Myc and Myc(Mxi1-BR) regulate common and unique downstream targets**

| Gene | Protein function | Myc | | | | Myc (Mxi1-BR) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | no CHX | | plus CHX | | no CHX | | plus CHX | |
| | | chip | northern | chip | northern | chip | northern | chip | northern |
| Common targets | | | | | | | | | |
| *ODC1* | metabolism | 2.1 | 2.5 | 1.9 | 1.9 | 2 | 2.7 | 1.9 | 1.8 |
| *API2* | inhibitor of apoptosis | 6.3 | 12.54 | 4.3 | 8.64 | 2.5 | 3.67 | 3.3 | 6.85 |
| *ZRP1* | cytoskeletal protein | 2 | 1.9 | 1.9 | 1.7 | 2.8 | 1.7 | 2.8 | 1.7 |
| *THBS1* | angiogenesis | 0.27 | 0.54 | 0.41 | 0.54 | 0.26 | 0.9 | 0.46 | 0.54 |
| *Gas1* | growth arrest | 0.5 | | 0.52 | | 0.4 | | 0.5 | |
| *Myc* | transcription factor | 0.44 | | 0.5 | | 0.32 | | 0.3 | |
| *Cul1* | component of E3 ligase complex | 2.1 | 2 | 1.9 | 1.9 | 1.9 | 2 | 2 | 1.8 |
| *CCNG2* | cell-cycle regulator | 0.43 | 0.13 | 0.5 | 0.51 | 0.56 | 0.38 | 0.62 | 0.6 |
| *Tert** | telomere maintenance | | | | 5.1 | | | | 2 |
| Myc-specific targets | | | | | | | | | |
| *DDX18* | RNA helicase | 2.25 | | 2 | | n/c | | n/c | |
| *CTIP* | transcription corepressor | 3.1 | | 4.1 | | n/c | | n/c | |
| *RPIA* | metabolism | 3.6 | 1.6 | 2.4 | 1.7 | n/c | 1 | n/c | 0.96 |
| *BLMH* | drug resistance | 3.1 | | 2.5 | | n/c | | n/c | |
| *UMPS* | metabolism | 1.8 | | 2.5 | | n/c | | n/c | |
| *TOP1* | DNA modification | 1.8 | 1.6 | 2 | 2.1 | n/c | 1.1 | n/c | 1.1 |
| *GRPE* | molecular chaperone | 2 | 4.6 | 2.4 | 2.1 | n/c | 1 | n/c | 1.1 |
| *SNRPD1* | small nuclear ribonucleoprotein | 1.7 | 2.3 | 1.8 | 1.7 | n/c | 1 | n/c | 1 |
| *Ches1* | cell cycle checkpoint regulator | 0.18 | 0.51 | 0.26 | 0.53 | n/c | 2.6 | n/c | 0.9 |
| *P2R4* | phosphatase | 0.6 | 0.65 | 0.41 | 0.51 | 0.37 | 0.38 | n/c | 0.94 |
| *IGF2R* | growth factor receptor | 0.33 | 0.6 | 0.55 | 0.4 | n/c | 1.1 | n/c | 1.2 |
| Myc (Mxi1BR)-specific targets | | | | | | | | | |
| *P40* | nucleolar protein | n/c | | n/c | | 2.8 | | 2.5 | |
| *EIF2B1* | translation | n/c | 1.3 | n/c | 0.9 | 2 | 1.8 | 3 | 2.7 |
| *P2P-R* | cell proliferation-related expression | 1.5 | 2.1 | n/c | 1 | 2.7 | 4.2 | 2.5 | 1.7 |
| *TNFAIP3* | anti-apoptotic | 2.8 | 1.9 | n/c | 0.97 | 6 | 1.5 | 7.5 | 3.1 |
| *SCL20A1* | retroviral receptor | 0.36 | 0.65 | n/c | 1 | 0.23 | 0.56 | 0.21 | 0.56 |
| *Akt1* | anti-apoptotic | 1.2 | 1.8 | n/c | 1 | 0.26 | 0.66 | 0.23 | 0.56 |
| *CG1I* | cell cycle | n/c | 1.1 | n/c | 0.9 | 0.33 | 0.6 | 0.35 | 0.6 |
| *RING3* | nuclear serine-threonine kinase | 0.4 | 1.3 | n/c | 1.2 | 0.24 | 0.5 | 0.35 | 0.6 |

The indirect and direct effects of Myc and Myc(Mxi1-BR) on gene expression as determined by microarray analysis (chip) or northern blot are shown. n/c, no change in expression was detected. Induction of c-Myc or c-Myc(Mxi1-BR) activity was performed with 2 μM 4-OHT in the absence (no CHX) or presence (plus CHX) of 100 μM cyclohexamide. Differentially expressed genes listed were consistently differentially expressed in three independent experiments or confirmed by northern blot if change in expression was detected in two of three microarray hybridizations. IMR90 cells containing the empty vector control did not exhibit any of these changes in gene expression on treatment with 2 μM 4-OHT. *Expression determined using RT–PCR.

differentially regulated by Myc or Myc(Mxi1-BR) are repressed by both, implying a role for the basic region in Myc-induced transcriptional repression. Our observations relate to the evidence that the oncogenic activity of Myc is linked to its capacity to repress expression of genes such as *Gas1* (ref. 21), *Ddit1* (ref. 22) and *FTH1* (ref. 23). Transcriptional repression by Myc occurs through interactions with the promotor initiator element (*Inr*) and is not mediated through the E-box consensus[23–25]. These observations, together with the differences in repression activity for Myc and Mxi1 basic regions, indicate a role for these residues in dictating physical interaction with distinct classes of *Inr* elements. This remains speculative, however, because the promoter sequences of the bulk of the repression targets identified here have not yet been defined.

The capacity of Myc, but not Myc(Mxi1-BR), to regulate directly the expression of genes encoding growth factor receptors (*IGF2R*), regulators of transcription (*CTIP*), RNA metabolism (*DDX18*), the cell cycle (*CHES1*), chromosomal integrity (*TOP1*) and metabolic pathways (*RPIA*, *UMPS*, *GRPE*), among others, is consistent with the view that the full transforming activity of Myc is dependent on the coordinate regulation of diverse genes or pathways. In addition it is also possible that the specific and 'inappropriate' regulation of Myc(Mxi1-BR) targets accounts for the impaired oncogenic potential of Myc(Mxi1-BR) and ultimately the anti-oncogenic activity of Mxi1. The ability of c-Myc(Mxi1-BR) and Mxi1 to directly repress *Akt1* expression is interesting because activation of *Akt1* suppresses apoptosis induced by c-Myc (ref. 26). It is also notable that c-Myc enhanced the expression of *Akt1*, albeit not directly, and thus may enhance survival and have a permissive role in the transformation process. In fact, c-Myc(Mxi1-BR)–expressing cells do exhibit higher levels of apoptosis than do c-Myc–expressing cells, a phenotype that accounts for reduced oncogenic activity.

Regulation of gene promoters by Myc or Mxi1 was dependent upon nucleotides flanking the regulatory E box in a manner consistent with the consensus for E-box flanking sequences determined for the Myc and Mxil basic regions in reporter assays (Fig. 3*b*). Furthermore, limited northern-blot analysis showed that Mxi1 repressed the expression of common and Myc(Mxi1-BR)-specific gene targets, but did not repress Myc-specific gene targets. Hence, these limited studies indicate that the Mxi1-BR in the context of the Myc(Mxi1-BR) chimaera engages E boxes in a manner relevant to the physiological function of the Mxi1 basic region in its normal structural setting.

The basic regions of Myc superfamily proteins are highly conserved and are critical in target gene selection and transforming activity. Our work indicates that the opposing actions of Myc and Mad family members extend beyond the prevailing view of Myc as transcriptional activator and Mad as transcriptional repressor of a common set of gene targets. Prominent aspects of Myc are its role as a repressor of gene expression and its poor overlap in target gene specificity with the Mxi1 basic region. Here, the correlation of gene targets unique to c-Myc or to c-Myc(Mxi1-BR) with their transforming activities indicate pathways which may be

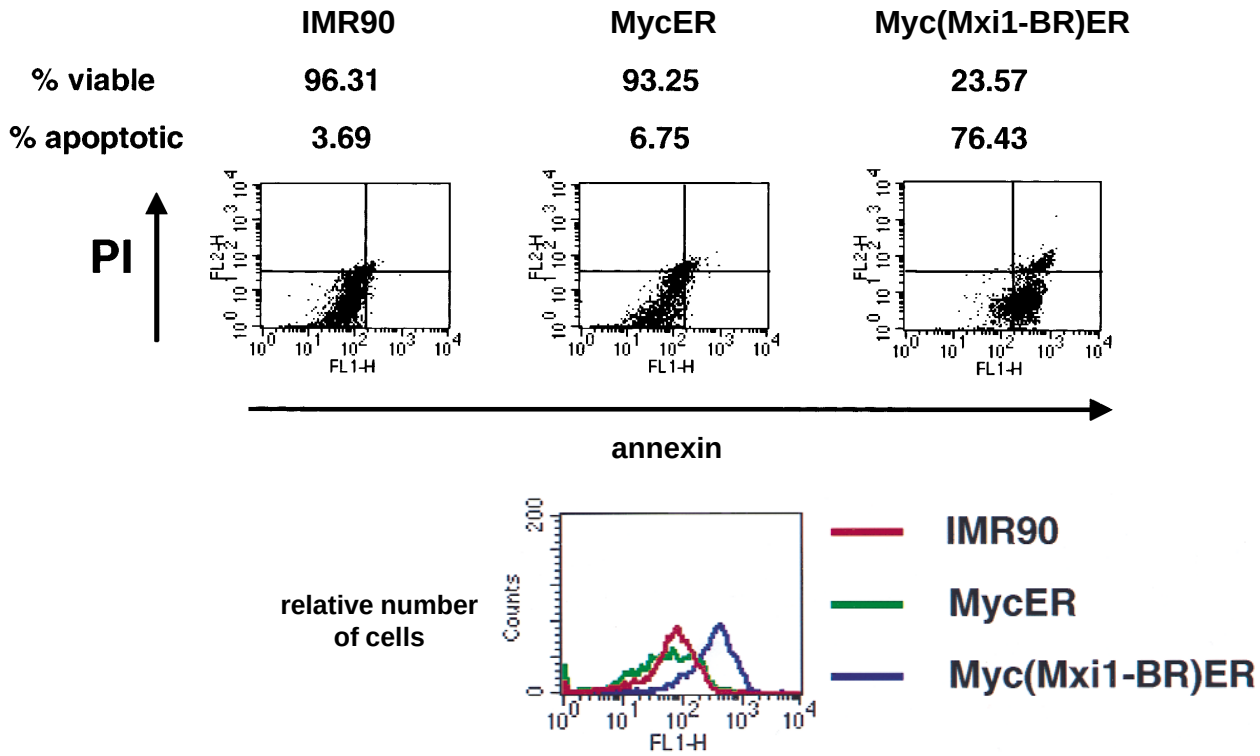


**Fig. 5** c-Myc and c-Myc(Mxi1-BR) differ in their capacity to induce apoptosis under high serum conditions. IMR90 cells encoding MycER, Myc(Mxi1-BR)ER or the empty vector were treated for 24 hours with 1 μM 4-OHT in the presence of 15% serum. The extent of apoptosis was determined by FACS.



engaged during transformation by the Myc oncoprotein. Conversely, c-Myc(Mxi1-BR)–specific gene targets suggest pathways which, if inappropriately regulated, might limit the transforming activity of Myc. As such, the combined genetic-genomic approach taken here provides a framework in which to begin a systematic functional analysis of those targets essential for Myc bioactivity, and to understand the functional inter-relationship among members of the Myc superfamily.

## Methods

**Construction of basic region chimaeras.** The sequence of the basic region of mouse *Myc* contained in the vector pKO-myc (ref. 27) was converted to that of N- or L-*myc*, *Mxi1* or *TFE-B* [**AUTHORS: PROVIDE APPROVED GENE SYMBOLS FOR ALL**] by overlap extension PCR (ref. 28). We used the flanking oligonucleotides LA4 (5′–CACTCACCAGCACAAC TACGC–3′) and LA7 (5′–GAACCGTTCTCCTTAGCTCTC–3′) to amplify PCR products from LA4 and LA5 (5′–CCTCCTGTTCTTTTCCAACTC GTTGTGTGTCGACCTCTT–3′) plus LA6 (5′–AAGAGGTCGACACAC AACGAGTTGGAAAAGAACAGG AGG–3′) and LA7 (*Mxi1* basic region); LA4 and KC9 (5′–CCTCCTCTTACGTTCCAAGAAGTTGTGGTTCTTC CTCTTG–3′) plus KC8 (5′–CAAGAGGAAGAACCACAACTTCTTGGAA CGTAAGAGGAGG–3′) and LA7 (L-*myc* basic region); LA4 and KC7 (5′–CTGACGTTCCAAGATGTTGTGATTCCGCCTC–3′) plus KC6 (5′–GAGGCGGAATCACAACATCTTGGAACGTCAGG–3′) and LA7 (N-*myc* basic region); and LA4 and KC4 (5′–CAAGAGGGCGCATCACAACGAG TTGGAACGTAAGAGGAGG–3′) plus KC5 (5′–CCTCCTCTTACGTTC-CAACTCGTTGTGATGCGCCCTCTTG–3′) and LA7 (*TFE-B* basic region). We cloned products of the flanking PCR reaction into the *Pvu*II-*Xho*I sites of pKO-myc. Similarly, NA7 (5′–GCGGAGGAATTCGAGCGGGTGC–3′) and LA3 (5′–GCCAAGAAGCTCGGGAAGGGTTC–3′) were used to amplify the products of NA7 and LA1 (5′–TCGTCGCTGCCTTTCCAACA CATTGTGTGTACGTCTGTT–3′) plus LA2 (5′–AACAGACGTACACA CAATGTGTTGGAAAGGCAGAGCAGC–3′) and LA3 to alter the sequence of the *Mxi1* basic region to that of *Myc*. The product of the flanking PCR was cloned into the *Sac*II-*Ssp*I sites of pV-Mxi1. We confirmed chimaeric molecules by sequencing.

**Cell culture.** IMR90, 293 and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.29 mg/ml), 0.03% penicillin and streptomycin and gentamycin sulphate (25 μg/ml). For the Myc induction studies in IMR90 cells, we exposed transduced cells to 4-OHT (2 μM) for 8 h in the absence or presence of cycloheximide (100 μM). Uninduced controls were treated with an equivalent volume of ethanol, the solvent for 4-OHT.

**REF cooperation assay.** We transfected REFs with expression constructs encoding H-RAS$^{G12V}$, pKO-myc or the indicated chimaeric molecule and pV-Mxi1-SR or pV-Mxi1(Myc-BR) by the calcium phosphate method[29]. Twenty hours after transfection plates were split 1 to 3 and media was changed every 3 d thereafter. Foci were stained with crystal violet and counted 8 d after transfection.

**Reporter assays.** We transfected NIH 3T3 cells using Lipofectamine reagent (Life Science Technologies) with a CAT reporter (100 ng) bearing 4 tandemly repeated CACGTG sequences upstream of the thymidine kinase minimal promoter; a luciferase reporter (100 ng) bearing nt –1,000 to +125 of *EIF2B1* (ref. 17), a SEAP reporter (100 ng) bearing nt –230 to +70 of the *TNFAIP3* promoter[16] or nt –2,482 to –1 of the *TERT* promoter[15]; pcDNA3 (300 ng; Invitrogen) encoding the indicated effector molecule or as empty vector control; and pCMX-β-galactosidase (200 ng), which served as an internal control for transfection efficiency. We transfected 293 cells using Lipofectamine reagent with the indicated reporter[11] (200 ng), pcDNA3 (600 ng) encoding the indicated Myc effector molecule and pCMX-β-galactosidase (200 ng). β-galactosidase activity was assayed by incubation of whole cell extracts with o-nitrophenyl β-D-galactopyranoside (ONPG) (400 μg/ml) in buffer containing $Na_2HPO_4$ (60 mM), $NaH_2PO_4$ (40 mM), KCl (10 mM) and $MgSO_4$ (1 mM) and relative transfection efficiencies determined by reading absorbance at 415 nm.

**Molecular modelling.** We obtained coordinates of the Max protein bound to the E box from the Protein Data Bank[9]. Mutations of the Max protein to match the sequences of Myc-BR, Myc-BR mutants and Mxi-BR were performed using the program O (ref. 30). The mutation of thymidine to uridine at position 8 of the bound DNA was made manually and protonated

using the Insight II program suite (Molecular Simulations). We subjected models containing the mutations to 200 cycles of conjugate gradient minimization with no experimental energy terms in CNS (A.T. Brunger *et al*, Crystallography and NMR System, Version 0.5). The resulting models were compared with the partial crystallographic structures of other bHLH/Z proteins, the bHLH/Z region of USF (ref. 31), the bHLH/Z region of Pho4p (ref. 32) from *S. cerevisiae* and the original Max structure, and assumed to be accurate. The minimized models allow one to assume that sequence changes within the basic region at the positions 2, 6 and 10 do not affect primary interaction with the DNA. Superimposition and inspection of the resulting models was performed using the program SPOCK (ref. 33).

**Retroviral infection.** The mouse ecotropic receptor (gift from S. Lowe[34]) was transduced into IMR90 fibroblasts and all subsequent transductions with ecotropic retrovirus were carried out as described[34]. We collected pBABE-MycER, pBABE-Myc(Mxi1-BR)ER, pBABE-Mxi1WR-ER and pBABE-Mxi1SR-ER viruses from transiently transfected ϕX cell lines.

**RNA preparation and microarray analysis.** We extracted RNA from IMR90 cells 8 h after induction by 4-OHT and prepared RNA using the RNeasy Midi kit (Qiagen) followed by extraction with Triazol (Life Science Technologies) and ethanol precipitation. Microarrays consisting of 5,272 sequence-verified cDNAs were printed on glass slides as described[35]. We converted RNA to cDNA labelled with either Cy3 or Cy5 dUTP, and hybridized as described[36] (protocols are available at http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/). After hybridization at 65 °C for 16 h, the slides were washed for 2 min each in 0.5×SSC, 0.01% SDS followed by 0.06×SSC at ambient temperature. Fluorescence intensities at each array element were measured using a custom designed laser confocal microscope with a scanning stage and a photomultiplier tube detector. Intensity data was integrated over 20-μm square pixels and recorded at 16 bits. We carried out image analysis with DeArray software using ratio normalization based on 88 pre-selected internal control genes. The 99% confidence interval for ratios was used to select significantly differentially expressed genes using a described algorithm[37].

**Northern-blot analysis.** We prepared RNA as described above. Total RNA (20 μg) was separated by electrophoresis in a 0.8% agarose, formaldehyde (2.2 M) gel and transferred onto nitrocellulose. We incubated membranes with $^{32}$P-labelled probes synthesized from the same fragments spotted on the cDNA microarrays.

**Apoptosis.** IMR90 cells encoding MycER, Myc(Mxi1-BR)ER or the empty vector were treated for 24 h with 1 μM 4-OHT. We determined the extent of apoptosis by FACS using the Annexin-V-Fluos kit (Boehringer) as per the manufacturer's protocol.

**Accession numbers.** Protein Data Bank: Max, 1AN2; USF, 1AN4; PHO4, 1A0A.

**Acknowledgements**

*We thank H. Land for the CAT reporter constructs; S. Lowe for the mouse ecotropic receptor; S. Hann for the pBabe-c-MycER plasmid; J. Chiorini for the eIF2β reporter; Y. Jiang and J.K. Lee for technical assistance; and members of the DePinho laboratory for helpful comments. R.C.O. is a recipient of a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. N.S.-A. is a recipient of a Special Fellowship from the Leukemia Society of America. R.A.D. is supported by grants (RO1HD28317, RO1EY09300) from the National Institutes of Health and is an American Cancer Society Research Professor. Support from the DFCI Cancer Core grant to R.A.D. is acknowledged.*

Received 11 November; accepted 13 December 1999.

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