

# Analysis of Myc Bound Loci Identified by CpG Island Arrays Shows that Max Is Essential for Myc-Dependent Repression

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## Summary

The *c-myc* proto-oncogene encodes a transcription factor, c-Myc, which is deregulated and/or overexpressed in many human cancers. Despite c-Myc's importance, the identity of Myc-regulated genes and the mechanism by which Myc regulates these genes remain unclear. By combining chromatin immunoprecipitation with CpG island arrays, we identified 177 human genomic loci that are bound by Myc in vivo. Analyzing a cohort of known and novel Myc target genes showed that Myc-associated protein X, Max, also bound to these regulatory regions. Indeed, Max is bound to these loci in the presence or absence of Myc. The Myc:Max interaction is essential for Myc-dependent transcriptional activation; however, we show that Max bound targets also include Myc-repressed genes. Moreover, we show that the interaction between Myc and Max is essential for gene repression to occur. Taken together, the identification and analysis of Myc bound target genes supports a model whereby Max plays an essential and universal role in the mechanism of Myc-dependent transcriptional regulation.

## Results and Discussion

### c-Myc Binds to the Regulatory Regions of Both Activated and Repressed Gene Targets

Delineating the pathway from Myc gene regulation to cellular functional changes has been hampered by the limited number of bona fide Myc-regulated genes identified. While expression arrays have shown many genes with altered expression when Myc is activated, few have been confirmed as direct Myc targets [1]. We recently used a cDNA microarray, followed by extensive analyses, to identify genes whose regulation is dependent

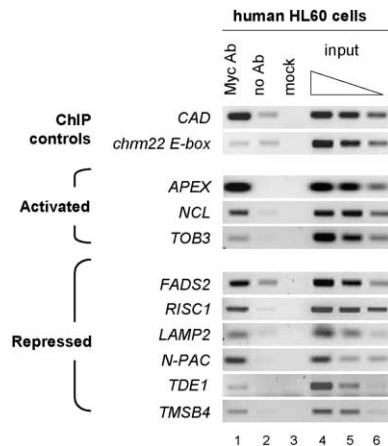
upon Myc [2, 3]. To evaluate if Myc bound to the regulatory regions of these genes, chromatin immunoprecipitation (ChIP) assays were conducted by using an anti-c-Myc antibody as described previously [4]. Myc binding to the 5' regulatory region of a known activated gene (*cad*) was detected in human HL60 cells, while no binding was evident at the negative control region, a chromosome 22 E box (*chr22 E box*) (Figure 1, compare lanes 1 and 2) [4, 5]. Binding was assessed by comparing the amount of promoter enrichment in the anti-Myc ChIP to the no antibody reaction. By this approach, Myc was shown to bind to each of the Myc-activated and -repressed genes analyzed. Taken together, the results show that these genes are directly bound and regulated by Myc. Identifying the repressed targets as bound and directly regulated by Myc was particularly insightful, as only two genes (*cdkn2b* and *cdkn1a*) had been previously shown to possess these characteristics [6, 7]. Interestingly, the Myc-dependent targets cloned in our expression array, originally identified in a rat fibroblast system, have been verified here as direct Myc targets in a human myelogenous leukemia cell system. These data suggests that at least a subset of bona fide Myc target genes will be regulated in a species- and cell type-independent manner and reinforce that ChIP analysis is a powerful approach to definitively identify genes bound by Myc.

### Identifying Genomic Loci Bound by c-Myc in HL60 Cells

Binding by Myc to the 5' regulatory region of a gene within native chromatin provides compelling evidence that the gene is a direct target of Myc regulation. To determine the regions of the human genome bound by Myc, we probed a microarray spotted with 7776 CpG island clones, CG-rich DNA associated with 5' gene regulatory regions, with Cy5-labeled chromatin isolated by ChIP from exponentially growing HL60 cells by using an anti-c-Myc antibody or no antibody as a control [8]. This approach yielded 235 clones that had a >3-fold normalized intensity in the anti-c-Myc immunoprecipitate as compared with the no antibody control in two independent experiments. After sequencing each clone, we determined the genomic location for 209 of 235 clones by BLAST searching the human genome (<http://www.ncbi.nlm.nih.gov/BLAST/>). This analysis indicated that the CpG island clones corresponded to 177 unique genomic loci, and the majority of these loci were located within promoter regions, as assessed by their proximity to the 5' end of the genes. A total of 107 of 177 loci are associated with genes of known or inferred function (see Figure S1 in the Supplemental Data available with this article online). Importantly, the loci include known Myc-activated (*ddx18*) and -repressed (*c-myc*) genes as well as several novel Myc target genes [9, 10].

To validate that the loci identified were bound by Myc, we performed conventional ChIP assays in HL60 cells. As ChIP controls, Myc is shown to bind the 5' regulatory

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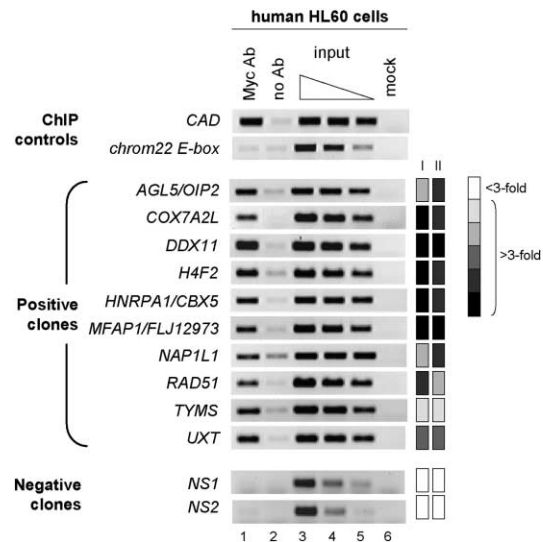


**Figure 1. Myc Binds to Activated and Repressed Genes in HL60 Cells**

ChIP assays with anti-c-Myc antibody (N-262, Santa Cruz) (lane 1) or no antibody (lane 2) from logarithmically growing human HL60 cells were performed as described. Additional control reactions include a no template mock IP (lane 3) and a 1×, 1/2×, and 1/4× titration of input chromatin (lanes 4–6). DNA was PCR amplified by using primers designed against the regulatory regions indicated. This includes an activated gene (*cad*) and an E box on chromosome 22 (*chrm22 E box*) that serve as a positive and negative control, respectively, for binding by Myc, as well as Myc-activated and -repressed genes previously identified as downstream of Myc by cDNA expression array analysis. Sequences for ChIP-PCR primers are available upon request.

region of *cad*, but not *chrm22 E box* (Figure 2). PCR primers were designed against the DNA sequence of 15 randomly selected positive clones, potentially representing Myc bound loci, as well as 2 negative clones, which did not specifically hybridize with a Myc-specific ChIP probe. All 15 positive clones were verified as being bound by Myc in growing, intact cells (Figures 2 and 3A). By contrast, the two negative controls were not bound by Myc (Figure 2). Thus, the combined ChIP and CpG island microarray approach generated a high signal to noise ratio and efficiently identified Myc bound loci.

To evaluate if the loci identified represented genes whose expression was regulated by Myc, reverse transcriptase (RT)-PCR was used to assess gene expression in logarithmically growing (LOG) HL60 cells as well as in cells triggered to differentiate by DMSO treatment (DMSO), conditions in which endogenous Myc protein levels are high and low, respectively (data not shown). RT-PCR analysis showed that the Myc bound loci correspond to targets that are Myc repressed (*dleu1*, *dleu2*, *d1s155e*, and *dkfzp586c1924*) and Myc activated (*ube2c* and *mthfd1*) (Figure 3B). Moreover, at least 34 additional clones bound by Myc have been identified as being Myc regulated by one or more of the recent expression array analyses (Figure S1, footnote 2) (<http://www.myc-cancer-gene.org/>; O'Connell and Sedivy, personal communication). While these analyses are not exhaustive, the results strongly suggest that these Myc bound targets warrant further study at an individual level of their regulation in response to Myc and their role in Myc biology. Taken together, these analyses suggest the Myc bound loci identified in this screen correspond to genes whose expression is indeed Myc regulated.



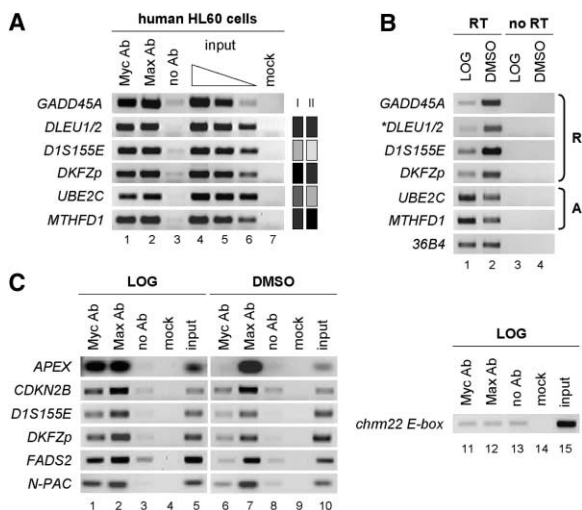
**Figure 2. Conventional ChIP Analysis Confirms that Myc Binds to the Novel Loci Identified by the Combined ChIP-CpG Island Microarray Screen**

ChIP assays with anti-c-Myc antibody (lane 1) or no antibody (lane 2) from logarithmically growing human HL60 cells. Additional control reactions include a 1×, 1/2×, and 1/4× titration of input chromatin (lanes 3–5) and a no template mock IP (lane 6). DNA was PCR amplified by using primers designed against a positive control (*cad*), negative control (*chrm22 E box*), and the sequences of ten randomly selected positive clones. Two negative clones that had hybridization ratios <3-fold were randomly selected and are also shown. The shaded bars to the right represent the fold binding observed on each of two independent microarray hybridizations (I and II) for each clone.

The genes identified fall into broad functional categories and reinforce the notion that Myc regulates a wide variety of cellular activities (Figure S1). Interestingly, groups of genes thought to participate in similar functions have also been identified. For example, in addition to the RNA helicase, dead-box protein *ddx18*, this screen reveals that *ddx11* is also Myc bound. In addition, a cohort of 20 genes whose regulatory region is bound by Myc as well as E2F, another potent regulator of cell cycle progression and apoptosis, has been identified (Figure S1, footnote \*) [8, 11]. An unexpected observation is the large number of bidirectional regulatory regions that are bound by Myc. This work reveals that the genes upstream and downstream of these regulatory regions can be coregulated by Myc. For example, both *hnrpa2b1* and *cbx3* are activated by Myc, whereas the putative tumor suppressors *dleu1* and *dleu2* are repressed by Myc. Indeed, a number of loci associated with leukemogenesis have also been identified in this screen by using myelogenous leukemia-derived HL60 cells (*dleu1*, *dleu2*, *meis1*, *meis2*, and *loc136319*). Identifying Myc bound loci has revealed many target genes that will be valuable reagents to explore the biological activities and mechanism of gene regulation by Myc.

**Max Is Present at Human Genomic Loci Bound by Myc**

To explore how Myc regulates transcription, we directly assessed the role of Max in the organization and regula-



**Figure 3. Max Binds to Myc Bound Genes before and after Myc Protein Recruitment**

(A) ChIP assays with anti-c-Myc (lane 1), anti-Max (C-124, Santa Cruz) (lane 2), or no antibody (lane 3) from logarithmically growing HL60 cells. Additional control reactions include a 1×, 1/2×, and 1/4× titration of input chromatin (lanes 4–6) and a no template mock IP (lane 7). *gadd45a* (shown at top) is a known Myc-repressed gene. Below are five randomly selected positive clones (*dleu1/2*, *d1s155e*, *dkfzp586c1924*, *ube2c*, and *mthfd1*), which were amplified by PCR by using primers designed against each locus. The shaded bars (to their right) represent the fold binding observed on each of two independent microarray hybridizations (I and II) for each clone.

(B) mRNA expression of genes whose regulatory regions are bound by Myc, as assessed by reverse transcriptase (RT)-PCR in logarithmically growing (lanes 1 and 3) and DMSO-treated (lanes 2 and 4) HL60 cells. A set of cDNA amplified under no RT conditions (lanes 3 and 4) are included as controls to evaluate genomic DNA contamination. Sequences for RT-PCR primers are available upon request. An asterisk indicates that the mRNA expression of *dleu1* and *dleu2*, referred to as *dleu1/2*, was similarly regulated; thus, a representative panel is shown. Genes that are repressed (R) or activated (A) by *c-myc* expression are indicated.

(C) ChIP assays with anti-c-Myc (lanes 1, 6, and 11), anti-Max (lanes 2, 7, and 12), or no antibody (lanes 3, 8, and 13) from LOG- and DMSO-treated HL60 cells. Additional control reactions include a no template mock IP (lanes 4, 9, and 14) and 1× input chromatin to control for the PCR reaction (lanes 5, 10, and 15). DNA was PCR amplified by using primers designed against Myc-activated (*apex*) and Myc-repressed (*cdkn2b*, *d1s155e*, *dkfp586c1924*, *fads2*, and *n-pac*) genes, as well as a negative control (*chrm22 E box*).

tion of Myc target genes. Myc and Max interact through their helix-loop-helix/leucine zippers, and this interaction is essential for Myc to activate transcription as well as to drive all its key biological activities tested to date [12, 13]. Interaction enables Myc:Max heterodimers to bind E box elements, CAC(A/G)TG, and activate transcription by at least two mechanisms: recruitment of histone acetyltransferase (HAT) or PTEFb complexes [5, 14–18]. By contrast, recent data suggest that Myc represses transcription by binding to and inhibiting activators at the core promoter region of target genes. For example, Myc can bind Miz-1 and block the transcriptional activation of *p15<sup>INK4b</sup>* (*cdkn2b*) and *p21<sup>CIP1/WAF1</sup>* (*cdkn1a*), whereas Myc can bind NF-Y and repress *pdgf-β receptor* [2, 6, 7, 19]. However, it remains unclear how target gene specificity is achieved for Myc repres-

sion. Moreover, the role of Max in the mechanism of Myc-dependent repression remains largely unexplored.

To assess if Max could bind to all or a subset of the novel Myc bound target genes, we performed anti-Max ChIPs in exponentially growing HL60 cells. Strikingly, Max bound to all genomic loci to which Myc was also bound. This included genes that were bound and activated in response to Myc (*mthfd1* and *ube2c*) and, importantly, also genes that were bound and repressed by Myc (*dleu1*, *dleu2*, *d1s155e*, *dkfzp586c1924*, *fads2*, and *n-pac*) (Figures 3A and 3C). Indeed, we have also performed ChIPs on the 21 genes described in Figures 1 and 2 and have not observed a single locus, including 14 known and novel Myc-repressed genes such as *gadd45a* and *cdkn2b*, at which Myc is bound in the absence of Max (Figures 3A and 3C; data not shown) [6, 20, 21]. Further analysis in HL60 cells under proliferating (LOG) and differentiated (DMSO) conditions shows that Myc binding to target genes is proportional to the level of Myc protein in the cells (Figure 3C; data not shown). Max can bind, either alone or in combination with other Max binding proteins, to Myc-activated genes prior to Myc binding and may specify which genes are activated by Myc. Consistent with this model, we show that Max binds to the 5' regulatory region of *apex*, a Myc-activated gene, in LOG- and DMSO-treated cells (Figure 3C). Interestingly, Max also binds to Myc repressed genes (*fads2*, *n-pac*, *dkfzp586c1924*, and *cdkn2b*) in a manner that is independent of Myc protein levels or the degree to which Myc is bound (Figure 3C). Neither Myc nor Max bound to the negative control region, *chrm22 E box*.

### Max Binds to Repressed Genes Independently of Myc

To investigate if Max could bind to regulatory regions of Myc-repressed genes in the absence of Myc protein, we performed ChIP assays in subconfluent, asynchronous Rat-1<sup>*c-myc*−/−</sup> cells [2]. We show Myc can bind to the regulatory regions of repressed genes (*c-myc*, *tde1*, and *gadd45a*) in Rat-1<sup>*c-myc*−/−</sup> cells that ectopically express Myc, but not in the vector only control (Figure 4A). By contrast, Max binds to each of these 5' regulatory regions in the *c-myc* null cells infected with either control retrovirus or retrovirus carrying and expressing human *c-myc* (Figure 4A). Neither Myc nor Max bound to the negative control region, glucokinase (*gck*) [17]. These data show that Max binding to repressed genes can occur in the absence of Myc protein. We also have observed this binding pattern at *cad*, a Myc-activated gene, and this pattern is reminiscent of previous observations with another Myc-activated target, *cyclin D2* [5, 18]. Determining how Max binds to loci, that do and do not contain E boxes, is an important question that we are investigating. Interestingly, none of the cofactors that we have analyzed thus far appears to be the common denominator at all Myc-repressed promoters (D.Y.L.M. and L.Z.P., in preparation). Taken together, we show that Max is bound to both Myc-activated and -repressed gene targets prior to Myc expression and recruitment.

### Max Is Essential for Gene Repression by Myc

To evaluate if heterodimerization by Myc and Max was essential for gene repression, we assessed gene regula-

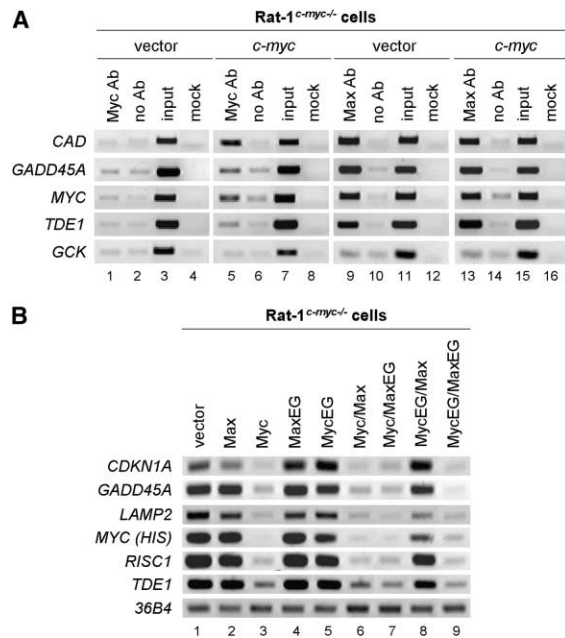


Figure 4. Max Can Bind to Genes Independently of Myc, and the Myc:Max Interaction Is Essential for Myc to Repress Gene Expression

(A) ChIP assays with anti-*c-Myc* (lanes 1 and 5), anti-*Max* (lanes 9 and 13), or no antibody (lanes 2, 6, 10, and 14) in *Rat1 c-myc*<sup>-/-</sup> cells infected with an ecotropic retrovirus carrying *c-myc* or empty vector. Additional control reactions include 1× input chromatin (lanes 3, 7, 11, and 15) and a no template mock IP (lane 4, 8, 12, and 16). DNA was PCR amplified by using primers against *Myc*-activated (*cad*) and *Myc*-repressed (*gadd45*, *c-myc*, and *tde1*) genes, as well as a negative control (*gck*).

(B) RT-PCR expression analysis of *Myc*-repressed genes in subconfluent, asynchronous *Rat-1 c-myc*<sup>-/-</sup> cells that ectopically express *Myc*, *Max*, *MycEG*, and/or *MaxEG*. *36B4* was assayed as a control for equivalent loading.

tion in *Rat-1 c-myc*<sup>-/-</sup> cells engineered to ectopically express *Myc*, *Max*, *MycEG*, and/or *MaxEG* by using ecotropic retroviruses [12]. *MycEG* and *MaxEG* proteins harbor mutations in their leucine zipper regions that enable them to bind efficiently to one another, but not to their wild-type counterparts. These tools have previously showed that the *Myc:Max* interaction is essential for *Myc* transformation, cell cycle progression, and apoptosis [12, 13]. Our previous work with these reagents showed that the *Myc:Max* interaction was required for *c-myc* autosuppression [22]. However, at the time, it was unclear if the *Myc:Max* interaction activated a gene whose product then repressed *c-myc* transcription, or if *Myc:Max* directly bound and repressed the *c-myc* promoter. Here, ChIP analysis shows that *Myc* autosuppression is a direct effect of *Myc:Max* binding to the 5' regulatory region of *c-myc* (Figure 4A) [22]. To investigate if the *Myc:Max* interaction was unique to *c-myc* autosuppression or if this union was a more global feature of *Myc* repression, RNA was collected from subconfluent, asynchronously proliferating cultures and was assayed by RT-PCR for endogenous levels of each *Myc*-repressed gene analyzed (*cdkn1a*, *gadd45a*, *risc1*, *tde1*, *lamp2*, and *c-myc(his)*). Ectopic expression of wild-type

*Myc* (*Myc*, *Myc* and *Max*, and *Myc* and *MaxEG*) repressed expression of the genes compared to cells infected with control retrovirus; this repression presumably occurred through interaction with endogenous *Max* protein (Figure 4B). By contrast, constitutive expression of *Max*, *MaxEG*, *MycEG*, or *MycEG* and *Max* was insufficient to trigger repression (Figure 4B). Importantly, cells that coexpressed *MycEG* and *MaxEG* showed repression of gene expression, which was comparable to wild-type *Myc* alone (Figure 4B). Thus, *Myc* repression of gene transcription requires a functional *Myc:Max* complex.

## Conclusions

The data shows that *Myc* bound target genes can be efficiently identified by combining ChIP and CpG island microarrays. Subsequent analyses of known and novel *Myc* targets showed that *Myc* and *Max* were cobound at all loci tested, which included 14 repressed genes. Further analysis of this association shows that *Max* was bound to repressed regulatory regions prior to *Myc*'s recruitment and that interaction between *Myc* and *Max* was essential for *Myc* to repress gene transcription. Taken together, we propose that the *Myc:Max* heterodimer is required for *Myc*-dependent transcriptional activation and repression, which leads to *Myc*-directed activities, including transformation.

## Supplemental Data

Supplemental Data including the list of human genomic loci bound by *Myc* are available at <http://images.cellpress.com/supmat/supmatin.htm>.

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