The Mad and Myc Basic Domains Are Functionally Equivalent*

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The Myc/Mad family of transcription factors plays a fundamental role in the regulation of cell proliferation, oncogenic transformation, and cell differentiation. However, it remains unclear whether different heterodimers, such as Myc/Max and Mad/Max, recognize the same or different target genes in vivo. We show by chromatin immunoprecipitation that Myc target genes are also recognized by Mad1 in differentiated HL60 cells. We also substituted the complete basic region of Myc for the corresponding region of Mad. Wild-type c-Myc was then compared with c-Myc(Mad-BR) in oncogenic transformation, regulation of cell proliferation, induction of apoptosis, activation of chromosomal condensation, and direct binding to chromosomal sites by chromatin immunoprecipitation. We find that the wild-type c-Myc and c-Myc/MadBR proteins have indistinguishable biological activity and target gene recognition in vivo. These data are consistent with a model in which Myc and Mad regulate a common set of target genes.

The c-myc gene encodes a transcription factor, c-Myc, that dimerizes with Max to serve as a master regulator of cell growth, cell division, and cell differentiation in all metazoans (reviewed in Ref. 1). Mutations that disrupt the regulated expression of c-myc (or the related N-myc and L-myc genes) are common, causative lesions in many human and animal cancers (2). In addition to its prominent link to cancer, c-myc is also required for timely progression through the cell cycle and is an essential downstream effector of mitogenic signals (3–5). The Myc family proteins function as transactivators when assayed on reporter constructs containing consensus binding sites or when the Myc N terminus is tethered to DNA through a heterologous DNA binding domain (6, 7). Recognition of specific chromosomal targets primarily involves the basic region, which forms an extended α-helix with helix 1 in the Max homodimer crystal structure (8, 9). Myc/Max heterodimers recognize a preferred consensus site ACCACGTGTT, although other related sites are also bound with varying affinity (10–12). The helix-loop-helix and leucine zipper domains dictate the highly specific dimerization of Myc with Max, which prevents dimerization with numerous other transcription factors that have related HLH and/or leucine zipper motifs (13). An increasing number of cellular targets for Myc/Max heterodimers have been defined (14) with compelling evidence that Myc protein can be cross-linked to target sites in vivo using chromatin immunoprecipitation (15–18).

More recently, the Max superfamily was extended to include the Mad and Mxi proteins that form alternate heterodimers with Max (reviewed in Ref. 19). Unlike Myc, Mad/Mxi proteins function as repressors of transcription and inhibitors of both cell proliferation and oncogenic transformation (20–22). Mad/Max heterodimers recognize consensus binding sites in vitro that are indistinguishable from those recognized by Myc/Max, suggesting that these alternate Max complexes may activate and repress common target genes. Although this is an appealing model, there remains only limited evidence to support it. Whereas Myc protein is expressed in all proliferating cells, Mad protein expression is more restricted and primarily found in terminally differentiated cells (23–26). Thus, in mitogen-deprived cells where Myc levels are low, there is no evidence that Mad supplants Myc to repress target genes. Some specific gene promoters, tert and cyclinD2, have been shown to bind to Mad1 protein in vitro, using HL60 cells, which differentiate and express high enough levels of Mad protein to cross-link (16, 18). In undifferentiated cells that express Myc rather than Mad, Myc was bound to the same promoters, suggesting that Myc and Mad can bind sequentially to common promoters. However, these are isolated examples so far, and no other promoters were examined. Further support for antagonistic functions of Myc and Mad/Mxi comes from mouse gene knockout experiments in which Mad and Mxi knockouts exhibit minor hyper-proliferative disorders or occasional tumors, suggesting that these genes can function as tumor suppressors (27–29). However, no homozygous mutations in mad or mxi genes have been found in human or animal cancers to establish any direct role for these proteins as tumor suppressors.

Previous studies compared Myc and Mxi/Mad binding with cellular promoters through either the introduction of Mad/Mxi-specific amino acid substitutions into the Myc basic region or the complete substitution of the Mad1 basic/HLH/LZ region into Myc (30, 31). Since the basic region is responsible (when dimerized with Max) for the recognition of DNA sequence elements at chromosomal sites, it was suggested that these mutants would offer insight into Myc versus Mad/Mxi target gene recognition and biological function. One study noted a substantial decrease in the transforming activity of the Myc(Mxi1-L) exchange mutants and a shift in the spectrum of genes regulated in human fibroblasts (30). From this, it was suggested that the biological activities of Myc and Mxi1 involve the regulation of both common and distinct sets of target genes.

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¶ The abbreviations used are: HLH, helix-loop-helix; LZ, leucine zipper; B, basic; PCNA, proliferating cell nuclear antigen; SHMT, serine hydroxymethyltransferase; mSHMT, mitochondrial SHMT; WT, wild type; CAD, carbamoyl phosphate synthase-aspartate transcarbamylase-dihydroorotase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; biHZ, basic helix-loop-helix-leucine-zipper.
governing diverse biological processes. A second study with a complete B/HLH/LZ exchange came to the different conclusion that DNA binding activity and target gene regulation in terms of cell proliferation were similar for the Myc and Mad1 proteins but that the induction of apoptosis was substantially different (31).

We were interested in a comparison of target gene regulation and biological activities of the Myc and Mad/Mxi basic regions. In particular, we noted that the Mad/Mxi1 proteins lacked conserved basic residues in the N-terminal half of the basic region that are common to the three Myc family proteins but that Mad/Mxi proteins have several highly conserved basic amino acids farther toward the N terminus that are not found in Myc proteins. To assess the basic region of Mad/Mxi1 proteins, we constructed a hybrid Myc protein in which the entire basic region of Myc was exchanged for the corresponding region of Mad1 while retaining the Myc HLH/LZ domain. We found that this Myc(Mad-BR) hybrid protein has the ability to oncogene-ically transform cells, induce apoptosis, and regulate cellular promoters that is indistinguishable from that of Myc family proteins. We also showed that Mad1 protein can bind to several additional Myc target genes in vivo. Therefore, we conclude that there is no inherent structural difference between the Myc and Mad/Mxi1 basic regions that would promote the regulation of distinct sets of cellular target genes.

MATERIALS AND METHODS

Vectors—Expression plasmids were created by standard methods in retroviral expression vector pLXSH and verified by sequence analysis. Details of individual constructs are available upon request.

Cell Culture, and Retroviral Infection—The human promyelocytic leukemia cell line HL60 was grown in RPMI 1640 medium containing 10% fetal calf serum and 2 mM L-glutamine. To induce cell differentiation, cells at a density of 0.4 × 10^6 cells/ml were treated with 1.25% v/v Me2SO for 48 h. Normal human fibroblasts (IMR90), rat c-myc-null fibroblasts (HO15.19 cells), and retroviral producer PhoeNX cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Retroviral infection of HO15.19 and IMR90 cells and fixed in 70% ethanol at 4°C overnight. The fixed cells were pelleted, and the pellet was resuspended in 1 ml of phosphate-buffered saline +0.5% fetal calf serum and fixed in 70% ethanol at −20°C overnight. The fixed cells were pelleted and resuspended in 500 μl of phosphate-buffered saline +0.5% fetal calf serum and an equal volume of the DNA extraction buffer (192 ml, 0.2 M NaHPO4; 8 ml, 0.1 M EDTA, final pH of 7.5) was added to each sample followed by incubation for 5 min at room temperature. The cells were pelleted and resuspended in 800 μl of phosphate-buffered saline +0.5% fetal calf serum supplemented with 8 μl of propidium iodide (1 mg/ml in 20 mM sodium citrate) and 4 μl of RNase A (DNase free, 10 mg/ml) in a 1 ml total volume. The cells were incubated for 30 min at 37°C and analyzed on a BD Biosciences FACScan fluorescence-activated cell sorter.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation assay on logaritically growing and differentiating HL60 cells was performed according to Xu et al. (18). Conditions for the chromatin immunoprecipitation assay on HO15.19 cells were described previously (32). The only crystal structure from this gene family is of Max/Max homodimers in which the proximal basic residues are in a helical structure and have been deduced from base contacts (8, 9). However, phosphorylation of serine 11 in Max inhibits DNA binding activity (35), indicating that acidic amino acids in the basic region can dramatically influence its function. (35). The Mad/Mxi basic region extends over only 13 amino acids. The Myc protein could be efficiently cross-linked to the Myc basic region extends over only 13 amino acids. The Myc basic region extends over only 13 amino acids. The Myc basic region extends over only 13 amino acids. The Myc basic region extends over only 13 amino acids. The Myc

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RESULTS

Mad binds to Myc Target Genes in vivo—To explore the binding of Mad1 protein in vivo, we took advantage of HL60 cells, which produce Mad1 protein after being induced to differentiate with Me2SO. We analyzed the binding of Mad1 protein to a number of chromosomal Myc targets in vivo by chromatin immunoprecipitation (Fig. 1). We found that Mad1 protein could be efficiently cross-linked to the cad, cdk4, hsp60, mShmt, and Id2 promoters in differentiated HL60 cells where Mad1 expression is induced, but not in the same cells before inducing differentiation where Mad1 is not expressed. All of these genes have been shown previously to bind to Myc in several different cell types. Nonspecific PCR amplification of DNA from the β-globin gene, which does not bind to Myc, served as a negative control for normalization. These data add to the previous demonstration that Myc and Mad1 bind to the promoters of tert and cyclinD2 in logarithmically growing and differentiated cells, respectively (16, 18).

Exchange of Myc and Mad Basic Regions—Different antibodies are used for Myc and Mad1 chromatin immunoprecipitation; therefore, we cannot determine whether binding to cellular targets is quantitatively equal between the two proteins. To dissect the contribution of the basic DNA recognition domain alone, we decided to test the DNA binding activity of the Myc and Mad basic regions through functional assays. A comparison of the basic regions of the Myc and Mad/Mxi families is shown in Fig. 2. Both protein families have a bipartite basic region that extends −20 to −25 amino acids N-terminal to helix 1. The only crystal structure from this gene family is of Max/Max homodimers in which the proximal basic residues are in a helical structure and have been deduced from base contacts (8, 9). However, phosphorylation of serine 11 in Max inhibits DNA binding activity (35), indicating that acidic amino acids in the basic region can dramatically influence its function. (35). The Mad/Mxi basic region extends over only 13 amino acids, whereas the Myc basic region extends over only 13 amino acids. The Myc

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SC-26 (5'-GCTCGGTTGGC-CAATCCTCGA-3'), human end-specific primers hCD4-CPs (5'-GAGGAGGCGC-ATACAGAGTG-3'), and hCD4-CPs (5'-GAGGAGGCGCA-CTACAAGTG-3'); human hs6p-specific primers hHS6P-CPs (5'-CTCCGCCTGACTCCTA-3') and hHS6P-CPs (5'-GAGGAGGAGAGGCCC-ATCCT-3'); human cdk4-specific primers hCDK4-CPs (5'-CTCCGTTGCTGTTGTAGTT-3') and hCDK4-CPs (5'-TAGAAGGGCCCCTCCTC-3'). To normalize samples by the amount of nonspecific DNA, we amplified a region in the promoter of the rat pten gene, PCNas (5'-CGAAG- CACCCAGGTAATGTT-3') and PCNbs (5'-ATCGTACGGTGTTTGA- GC-3'), or in the third intron of the human β-globin gene, SC-46 (5'-AT- CCTCC-TCACACGTCTCCT-3') and SC-47 (5'-TGGTCACCTACCC- TTCCTT-3'). One of the primers in each pair was end-labeled with 32p-ATP. Amplification was performed in a T3 Thermocycler (Biometra®) for 31 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s followed by a final extension step at 72°C for 5 min. The optimal number of cycles for exponential amplification was determined by kinetic analysis. PCR products were resolved on a 4% or 6% polyacrylamide gel. PCR products were quantitated and normalized using the Ame- sham Biosciences phosphorimaging system.
and Mad/Mxi basic regions are both bounded by proline or glycine, which are likely positions for a domain boundary. Given these structural considerations, we decided to test the Mad basic region function by exchanging 24 amino acids of mouse c-Myc with 21 amino acids of mouse Mad1, using an N-terminal proline as one boundary and an invariant leucine in helix 1 as the C-terminal boundary. Within the domain exchanged, 9 out of the 25 amino acids are identical or conservative substitutions.

Ectopic transient expression of WT c-Myc and Myc(Mad-BR) gave equivalent levels of protein and equivalent binding to E-box elements in electrophoretic mobility shift assay (data not shown). To assess the ability of the two proteins to promote cell proliferation, we reconstituted Myc-null rat fibroblasts with each Myc protein and established that they were expressed at similar levels (Fig. 3A). We then examined the cell morphology (Fig. 3B) and growth rate (Fig. 3C). Both WT c-Myc and Myc(Mad-BR) fully rescued the growth defect in Myc-null fibroblasts, giving doubling times of 18 and 19 h, respectively, which is equivalent to the doubling time (18 h) of the parental cells with WT c-myc genes. In contrast, the Myc-null fibroblasts have a doubling time of ~45 h. We also tested the relative fraction of cells in each phase of the cell cycle by propidium iodide staining (Fig. 4). When compared with the pronounced accumulation of cells in G1 in the Myc-null cells (69%), both c-Myc and Myc(Mad-BR) had an equivalent reduction in G1 fraction (52 and 50%, respectively). Cells reconstituted with c-Myc and Myc(Mad-BR) had similar increases in S phase cells (20 and 21% respectively) when compared with myc-null cells (11%). Thus, the Myc and Myc(Mad-BR) proteins rescue the cell cycle defect in Myc-null cells to an equivalent extent.

To compare the oncogenic activity of Myc(Mad-BR) with WT c-Myc, we tested each construct for focus formation in primary rat embryo cells in cooperation with an H-ras(G12V) oncogene. Both WT c-Myc and Myc(Mad-BR) induced equivalent numbers of foci, ~15/dish for each (Fig. 3D). Similar results were obtained for three independent assays. The morphology of the foci was indistinguishable between WT and the exchange mutant (data not shown). We conclude that the Mad basic region has an equivalent biological function to that of c-Myc. Since the proliferative and oncogenic activities of c-Myc are presumably a manifestation of the complex sets of target genes that it regulates, we infer that all of the critical functional targets recognized by the c-Myc basic domain are also recognized by the Mad basic domain.
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Myc and Myc(Mad-BR) Induce Equivalent Apoptosis—As a final test of biological activity, we examined the induction of apoptosis by the Myc and Myc(Mad-BR) proteins. The Myc-null cells were reconstituted with each protein using a long terminal repeat-driven reconstitution vector, which is sustained in expression when serum survival factors are withdrawn. This constitutive Myc expression induces apoptosis, unlike in the parental Rat1 (thioguanine-resistant (TGR) subclone) cells where Myc is down-regulated upon serum withdrawal. As reported previously, the Myc-null cells have no detectable apoptosis when starved in 0.1% serum for 24 h. Both floating and adherent cells were harvested and analyzed by flow cytometry to determine the percentage of apoptotic cells. M1, M2, M3, and M4 represent gating for sub-G1, G1, S, and G2/M populations, respectively. B, quantitation of the fraction within each gate in panel A.

Myc and Mad Basic Regions Recognize Equivalent Chromosomal Targets—The data above support a model in which the Myc and Mad basic region recognize an equivalent set of genes. Quantitative PCR was performed with cDNA synthesized on total RNA isolated from cells expressing the empty vector, Myc, or Myc(Mad-BR) proteins using radiolabeled oligonucleotides specific for the rat genes shown on the right. Quantification of the PCR products was performed using an Amersham Biosciences phosphorimagery system. Numbers below a panel indicate the fold induction determined by dividing an hsp60, nm23, nucleolin, mSHMT, cad, or gapdh-specific signal by a corresponding gapdh-specific signal and by the ratio of these signals in the vector lane. B, wild-type Myc and Myc(Mad-BR) bind equivalently to the same chromosomal sites in rat fibroblasts. HO15.19 cells expressing empty vector (vector), WT c-Myc (indicated by Myc), and Myc(Mad-BR) were cross-linked and lysed, and chromatin was immunoprecipitated with c-Myc-specific (M) or Gal4-specific (G) antibodies followed by the reversal of the cross-linking and DNA isolation. Isolated DNA was used in PCR with radiolabeled oligonucleotides flanking Myc binding sites in the promoter of the genes indicated on the right side. For negative controls, two types of PCR were performed. The first used oligonucleotides flanking CACGTG sites that do not interact with c-Myc in the promoter of rat glucokinase gene (GLU). Another PCR was performed with oligonucleotides flanking a DNA region containing no CACGTG sites in the rat pena gene. Quantification of the PCR products was performed by using the Amersham Biosciences phosphorimagery system. Numbers below a panel indicate the fold difference in the signal intensity determined by dividing a given PCR signal by a corresponding pena-specific signal and by the ratio of these signals in the Gal4 lane.

As a definitive test of the in vivo function of the basic region, we assayed the direct binding of the different proteins to chromosomal sites by chromatin immunoprecipitation. We analyzed the binding of Myc proteins to chromosomal promoters in reconstituted Myc-null cells, with cells that lack Myc protein as controls. Formaldehyde cross-linked chromatin was immunoprecipitated with anti-Myc antisera or a control anti-GAL4 antisera, and the immunoprecipitated DNA was analyzed for the presence of four target genes (hsp60, nm23, nucleolin, and mshmt). For comparison, we analyzed a CACGTG site in the glucokinase gene, which does not bind to Myc and also the pena gene, which lacks Myc binding sites (Fig. 5B). There was a reproducible enrichment (4–11-fold) of Myc target gene sequences in the anti-Myc immunoprecipitations in Myc-recon-
stituted cells when compared with the signal with the control anti-GAL4 antiserum. No similar enrichment was observed for the two genes that do not bind to Myc in vivo. Most significantly, the binding of the Myc(Mad-BR) protein to chromosomal promoters was indistinguishable from that of WT c-Myc. The small differences in signal between c-Myc and Myc(Mad-BR) for the specific experiment shown were not found reproducibly, and the signals for different genes were not consistently higher for one protein versus the other. No significant enrichment of any gene sequences was observed with the anti-Myc antiserum in the original Myc-null cells when compared with the anti-GAL4 control.

**DISCUSSION**

In this study, we have conducted a thorough analysis of the DNA recognition properties of the Myc and Mad1 basic regions. We conclude that there is no measurable difference in DNA site recognition in vivo between the Myc and Mad1 basic regions, and therefore, that these two domains can recognize equivalent sets of target genes. The precise fusion proteins that we analyzed and their resulting biological activities differ significantly from two previous reports (30, 31) that addressed similar questions. It is informative to compare and contrast each study to understand the precise contribution of the basic region to target gene selection by the Myc/Mad family of proteins.

Our conclusions differ most significantly from a previous study by O'Hagan et al. (30) that attempted to create a Mxi-like basic region within Myc through the mutation of 2–4 amino acids in the Myc basic region to those found at the corresponding positions in Mxi (and Mad) proteins. The most disruptive mutation in this study was R357S (numbered from the human c-Myc initiation methionine), which changes one of the basic amino acids in Myc into a non-basic residue. It seems quite possible that target gene recognition by Myc is dependent on an extended set of basic amino acids within this region, and thus, this mutation debilitates Myc function. The basic region exchange used in our study included two additional basic amino acids farther toward the N terminus. We speculate that these amino acids provide important nonspecific DNA interactions with the phosphate backbone to increase target gene recognition in vivo. It is also possible that other conformational aspects of the Mad1 basic region are preserved in the more extended Mad/Myc exchange. This interpretation is supported by the partial restoration of transforming activity in the Myc(R357S/V361E) double mutant, which exchanges two positions of the Myc basic region with the corresponding residues from Mxi1 and Mad1 (30). The indistinguishable transforming and proliferative activity of the Myc and Myc(Mad-BR) exchange mutant in our study argues that the basic regions of Myc and Mad1 can recognize the same critical target genes in vivo.

Our findings also contrast a more recent report that the Myc and Mad1 B/HLH/LZ domains differ in apoptotic potential but not in proliferative activity (31). We find that the c-Myc and Myc(Mad-BR) proteins induce indistinguishable levels of apoptosis, implying that these two proteins activate or repress the same target genes that promote apoptosis in the absence of survival factors. The simplest explanation for the differences between the two studies is the potential contribution of the HLH/LZ domain to biological activity since our study exchanged only the extended basic region and not the entire DNA binding/Max dimerization domain. Structural studies imply that the helix 1 and loop domains can affect DNA binding activity (37, 38), although no differences in DNA binding between WT c-Myc and the Mad bHZ exchange were found using in vitro assays (31). Of more interest are reported differences in protein interactions with the HLH/LZ domains, where Myc can bind to Miz1 and Mad1 cannot (39). We also note a significant difference in the ability of the previously described bHZ exchange protein to rescue the growth defect in Myc-null cells. We find indistinguishable cell doubling times for Myc and Myc(Mad-BR), whereas the growth rate for the Myc(MadBHZ) exchange was less than for WT c-Myc (31). Even greater differences were found in the rescue of the cell cycle defect. In the Myc(MadBHZ) study, 48% of cells were in G1 for the Myc(MadBHZ) exchange when compared with 58% for Myc-null cells and 28–29% for both Myc reconstituted and parental Rat1 cells (31). Similarly, the S phase fraction was 25% for Myc-null, 43% for WT c-Myc, and 37% for Myc(MadBHZ). In contrast, we find indistinguishable profiles for G1-S-G2/M between Myc and Myc(Mad-BR), similar to the results for cell doubling time. Thus, the Myc(MadBHZ) exchange protein only rescues part of the growth defect in Myc-null cells. These differences between our study and the bHZ exchange study may be attributable to specific protein-protein interactions with the HLH and/or LZ domains rather than to any differences in target gene recognition. It is also possible that the defect in apoptosis noted in the Myc(MadBHZ) mutant is coupled to the defect in cell cycle rescue.

The most important question that remains unresolved is whether or not Myc and Mad/Mxi regulate the same target genes under normal developmental conditions. Our study provides the most extensive evidence to date for the direct binding of Myc and Mad1 to the same target genes in vivo. Since the endogenous Myc and Mad/Mxi proteins differ in antibody reactivity, it is impossible to provide an accurate quantitative assessment of the relative binding efficiency of each protein at individual targets, and it is also difficult to assess the activity of each protein once binding has occurred. However, in the differentiating HL60 cells that we studied, Mad1 binds to all of the Myc target genes we tested under the differentiated/growth arrest conditions where Mad1 is induced.

Our data support a model in which Myc/Max and Mad/Max heterodimers recognize equivalent target genes in vivo. The primary biological difference between these complexes is their precise regulation and their recruitment of nuclear cofactors, i.e., activators versus repressors (reviewed in Ref. 19). Mad/Mxi family proteins are mainly expressed at distinct stages of development and differentiation, almost all of which are associated with growth arrest. An exception to this is the nearly ubiquitous expression of Mxi1 and the S phase-specific expression of Mad3. In addition, Mxi1-deficient primary cells have a higher colony-forming ability when plated at low density, suggesting an enhanced proliferative capacity (27). In contrast, Myc proteins are expressed in all dividing cells and repressed under virtually all growth arrest conditions. Thus, in many cells, target genes will be down-regulated in non-dividing cells by the absence of Myc activation but not through active repression by Mad/Mxi. Furthermore, we note that most Myc target genes are still expressed basally even in the complete absence of Myc, such as in Myc-null fibroblasts (33, 40). This Myc-independent target gene expression is likely due to promoter elements that bind other nuclear factors, and the Myc-dependent induction of its targets is usually quite modest (3-fold) in log phase cells (33, 40). One factor with a potential role in this system is Mnt, which is simultaneously expressed in dividing cells with Myc. Mnt can bind the Sin3A corepressor and block Myc function (41), and Mnt/Max complexes are significantly more abundant than Myc/Max complexes in nuclear extracts (26, 42). It is not known whether Mnt/Max heterodimers constitutively repress Myc target genes and act to down-regulate their expression when Myc is repressed. Moreover, induction of Mad/Mxi under conditions where Myc is already down-regulated may recruit additional repressive cofactors to shut down.
target gene promoters below their level of basal transcription. Yet another complication in this system is the upstream stimulatory factor (USF) transcription factor, which has been shown to bind to many Myc/Max binding sites under conditions when Myc is not expressed (43). Our data are consistent with a regulatory factor (USF) transcription factor, which has been

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