

Cell Density and Paradoxical Transcriptional Properties of c-Myc and Max in Cultured Mouse Fibroblasts

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Abstract

Deregulated expression of the c-Myc oncoprotein occurs in several human malignancies. The c-Myc protein behaves as a transcription factor, and undoubtedly its role in carcinogenesis involves its ability to affect the expression of genes involved in cell growth. c-Myc has been reported to both activate and repress transcription in transient transfection experiments using reporter constructs bearing multiple copies of the c-Myc binding site, CAC(G/A)TG. We investigated these apparently paradoxical effects of c-Myc by determining if they arose from differences in the cell proliferation states of transfected cells. We found that endogenous c-Myc protein levels vary inversely with the degree of cell confluency, such that at low cell confluency, where endogenous levels of c-Myc are high and presumably endogenous levels of Max are limiting, exogenous c-Myc fails to affect basal transcription. In cells at high cell confluency, in which endogenous c-Myc levels are low, exogenous c-Myc augments transactivation by titrating the relative excess endogenous Max. These observations suggest that the apparently paradoxical behavior of c-Myc in transfection experiments is partially dependent on ambient cellular levels of c-Myc. (*J. Clin. Invest.* 1995. 95:900–904.) **Key Words:** helix-loop-helix leucine zipper protein • transient transfection • transactivation • cell proliferation

Introduction

The *myc* family of protooncogenes includes *c-myc*, *N-myc*, and *L-myc*. Expression of *c-myc* occurs in widespread proliferating tissues, but *L-myc* and *N-myc* expression seems to occur principally in selective developing fetal tissues (1). The *c-myc* gene product is a transcriptional activator that plays critical roles in the control of both cell proliferation (2–4) and apoptosis (5–7). Deregulated expression of *c-myc* occurs in several human

malignancies including colorectal cancer, small cell cancer of the lung, and B and T cell leukemias (9). Altered *c-myc* expression can arise from chromosomal translocation, gene amplification or mutation. The link between *c-myc* and neoplasia is strengthened by the observation that coexpression of *c-myc* with the activated *ras* oncogene causes the transformation of rat embryo fibroblasts (10, 11). The overlap of structural regions of the c-Myc oncoprotein necessary for c-Myc mediated transformation with those required for its transcriptional properties (12–15), suggests that c-Myc transforms cells by altering expression of genes involved in cell proliferation. Identification of the ornithine decarboxylase (ODC) gene as a c-Myc putative target (16) further supports this hypothesis, as overexpression of ODC in fibroblasts results in their transformation (17). Thus, in the quest to understand how c-Myc potentiates neoplastic transformation, much effort has been dedicated to the study of c-Myc as a transactivator.

The structure of the c-Myc oncoprotein typifies those proteins that comprise a family of transcription factors, known as the basic helix-loop-helix leucine zipper (bHLH-LZ)¹ proteins. The basic domain at the carboxy terminus allows the protein to recognize and bind a specific hexameric sequence of DNA, CAC(G/A)TG known as an enhancer box or E-box. Myc dimerizes with another protein called Max through the HLH-LZ structure (18–20). Myc:Max heterodimers bind to the E-box resulting in transactivation of genes that contain E-boxes within their promoter or intronic sequences (21). Heterodimerization with Max is not only necessary for c-Myc transactivation but also is required for c-Myc oncogenic activity (15). In addition to forming heterodimers with Myc, Max dimerizes with itself (19, 22) and with two newly identified bHLH-LZ proteins, Mad (23, 24) and Mxi1 (25). Max:Max and Mad:Max dimers antagonize c-Myc mediated transcriptional activation by binding to the E-box (26, 23). *max* is expressed constitutively (27), but expression of *mad* and *mxi-1* increase in differentiating cells as *myc* expression falls (24, 28). Alterations in c-Myc, Mad, and Mxi-1 levels that occur with changes in cell proliferation may subsequently affect ratios of Max inhibitory dimers (Max:Max, Mad:Max, and Mxi-1:Max) to Myc:Max heterodimers. Changes in the ratio of activating to inhibitory dimers relative to the rate of cell proliferation could represent a form of transcriptional regulation (9, 29).

Although most studies suggest that c-Myc behaves as a transactivator, variable degrees of transactivation and even tran-

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1. *Abbreviations used in this paper:* bHLH, basic helix-loop-helix leucine zipper; CRM, cross-reactive material; ODC, ornithine decarboxylase.

scriptional repression by c-Myc have been reported (26, 30, 31). To investigate this variability, we studied the effect of changes in cell confluency at the time of transfection on c-Myc mediated transactivation. We found that c-Myc levels vary proportionally with the rate of cell proliferation and inversely to the degree of cell confluency. Further, variations in endogenous levels of c-Myc affect c-Myc mediated transcriptional activity in transient transfection experiments.

Methods

Construction of plasmids. pE₁₀CAT is a chloramphenicol acetyltransferase gene reporter plasmid driven by the E₁b minimal promoter that bears a decameric repeat of the core CACGTG sequence. A 43-bp oligonucleotide (5'-TCGACCTGCAGGCATGCAAGCTTACCCGGT-CACGTGGCCTACC-3') duplex bearing XhoI and SalI ends was concatenated using DNA ligase. A concatemer was subcloned into pE1b-CAT (gift of J. Lillie, Worcester, MA) and sequenced to verify that a decameric repeat had been generated. The 5' end of the decamer is located 454 bp upstream from the minimal promoter.

pMLVMyc, pΔ106-143, pΔHLH (D371-412) and pΔLZ (D413-433) are comprised of the Moloney murine leukemia virus LTR driving a 2.7 kb genomic *myc* fragment or *myc* mutants as described (13). pRSVMax is composed of the Rous sarcoma virus LTR driving p21 Max cDNA, gift of E. Blackwood and R. Eisenman, described in (32). pGM41-143 and pG₅E1bCAT (composed of a pentameric repeat of the GAL4 DNA binding site upstream of the minimal promoter driving the CAT gene) plasmids have been previously described (12).

Transfection experiments. Mouse LTK⁻ fibroblasts were grown in DMEM/high glucose with 10% fetal calf serum. Cells were seeded at different densities on 100-mm plates to achieve confluencies of 20, 50, and 90% by the following day. Cells grown to 20% (2.9×10^6 cells), 50% (7.3×10^6 cells) and 90% (14.5×10^6 cells) were transfected on day 1 using the calcium-phosphate precipitation method and subjected to 10% DMSO shock on day 2 (33). The medium was changed on day 3, and CAT activity was determined using the phase extraction method on day 4 (34).

DNA dot blot hybridization was performed to determine transfection efficiency. Fibroblasts were plated as described to achieve densities of 20, 50, and 90% and were transfected with 1.5 μg of a CAT reporter plasmid. After standardizing samples by cell number, DNA was extracted using the Hirt's lysis method (35) and was subjected to dot blot hybridization to a randomly primed ³²P-labeled CAT gene probe as described (36).

Radioimmunoprecipitation of c-Myc and Max. Radioimmunoprecipitation was performed using cells that were metabolically labeled with [³⁵S]methionine for 4 h. Cells were pelleted by centrifugation and solubilized in RIPA (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris) containing 0.1 mM phenylmethylsulfonylfluoride. Lysates were cleared by centrifugation and immunoprecipitated with rabbit polyclonal Max and c-Myc antibodies, raised against full-length bacterially expressed Max and c-Myc proteins. To normalize for the difference in the number of cells, samples were standardized by the amount of total protein labeled (12). Proteins were resolved on a 10% SDS-polyacrylamide gel.

Results

We sought to determine whether cell confluency could affect basal E-box driven transcription since cell density at the time of transfection is not always rigorously controlled in practice. Mouse LTK⁻ fibroblasts were grown to 20, 50, or 90% confluencies and transfected with the reporter, pE₁₀CAT. pE₁₀CAT contains a decameric repeat of the E-box, CACGTG, located upstream of the adenoviral E1b minimal promoter driving the

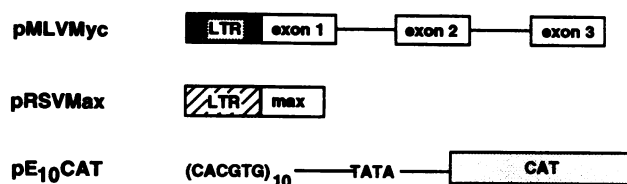


Figure 1. Schematic representation of effector and reporter constructs. The pE₁₀CAT reporter contains ten tandem repeats of the CACGTG site.

chloramphenicol acetyltransferase gene (Fig. 1). pBluescript II KS⁻ (pBSIISKS) was used to control for total amounts of input DNA. Cotransfection of pE₁₀CAT with pBSIISKS⁻ resulted in basal transcriptional (CAT) activity that decreased when cultures at higher cell confluencies were transfected (Fig. 2 A, *black bars*). Similar results were obtained using another fibroblast cell line, Rat 1a, at 20 and 90% confluencies (data not shown). DNA dot blot hybridization was performed on transfected LTK⁻ cells at both confluencies using a radiolabeled CAT probe to quantitate the total amount of reporter plasmid DNA transfected per plate. The amount of transfected reporter DNA per cell recovered from each plate was equivalent suggesting that alterations in the endogenous levels of transcription factors such as c-Myc and Max, rather than transfection efficiencies, contribute significantly to the differences in basal reporter gene activity (data not shown).

To determine if cell confluency specifically affected c-Myc mediated transactivation, we cotransfected pE₁₀CAT with pMLVMyc, a genomic Myc expression vector. When c-Myc was overexpressed, transcriptional activity failed to change ($P < 0.05$) in cells grown to 20% confluency, yet activity rose significantly from basal levels in cells grown to 90% confluency (Fig. 2 A, *shaded bars*). In 90% confluent cells, c-Myc did not increase the activity of SV-βgal, a β-galactosidase expression vector containing the SV40 promoter (data not shown). The failure of exogenous Myc to transactivate at low confluency is not due to limited levels of TATA-binding-protein (TBP) associated factors (TAFs), since a GAL4-Myc chimeric protein is fully active at that cell density. Cotransfection of 20% confluent cells with a GAL4 responsive reporter, pG₅E1bCAT, and a GAL4-Myc activator, GM(41-143) [composed of GAL4 DNA binding domain and c-Myc amino acids 41-143 (2)], resulted in a greater than 100 fold rise in transcriptional activity (Fig. 2 B). GM(41-143) was also active in cells grown to 90% confluency (data not shown).

In contrast to pMLVMyc, transfection of plasmids encoding c-Myc mutants lacking the helix-loop-helix region (pΔHLH) or the leucine zipper region (pΔLZ) had no effect on transactivation at 90% confluency (data not shown). A reporter lacking the E-box sequence yielded undetectable CAT activity with either pMLVMyc or pΔHLH under the same conditions (data not shown). We surmised that the introduction of high levels of c-Myc into cells at 20% confluency did not increase transactivation of the reporter because endogenous levels of c-Myc may be high relative to Max in rapidly proliferating cells. In such a milieu, excessive exogenous c-Myc fails to activate the reporter because the amount of endogenous Max may be limiting. Conversely, the rise in transcriptional activity with exogenous c-Myc seen in the cells transfected at 90% confluency could result

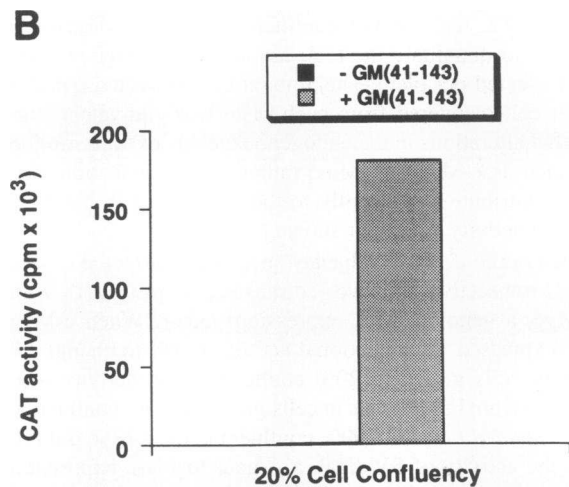
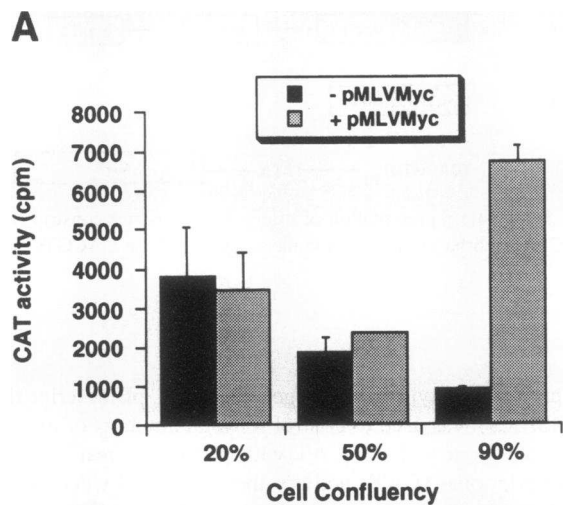


Figure 2. (A) Effect of cell confluency on c-Myc transcriptional activity. Cells were cotransfected with 1.5 μ g of the reporter construct and either 15.0 μ g pMLVMyc and 15.0 μ g pBSIIKS⁻ (shaded bars) or 30.0 μ g pBSIIKS⁻ alone (solid bars). Results reported represent the average CAT activity obtained from duplicate experiments. (B) TBP-associated factors at 20% confluency are not limiting. Mouse L cells at 20% confluency were transfected with 10 μ g pG₅E1bCAT and 5.0 μ g pGM(41-143).

from excess endogenous Max relative to the limiting amount of endogenous c-Myc.

We performed immunoprecipitation experiments with untransfected cells to determine if the difference in transactivation between cells grown to 20 and 90% confluency is related to alterations in endogenous levels of c-Myc and Max. We controlled for the difference in the number of cells by normalizing for total [³⁵S]methionine incorporation. Max levels remained constant despite changes in cell confluency (Fig. 3, 21-kD band, lanes 1–3). c-Myc levels (Fig. 3, 64–67-kD bands, lanes 7–9), decreased appreciably with increasing cell confluency. Identical patterns of protein expression were observed when we controlled for differences in sample loading by immunoprecipitating with both antibodies simultaneously (Fig. 3, lanes 4–6).

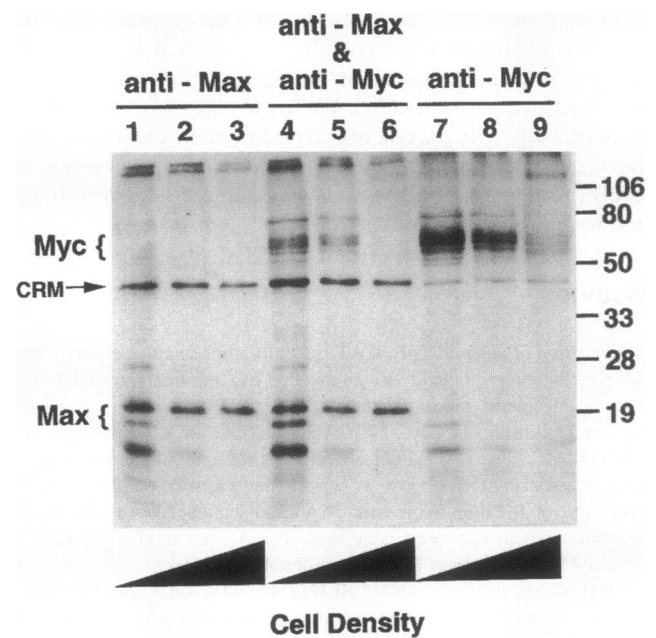


Figure 3. Immunoprecipitation of Myc and Max at various cell densities. [³⁵S]methionine-labeled cells were grown to 20, 50, or 90% cell densities, and labeled derivatives were immunoprecipitated with either polyclonal Max antibody (lanes 1–3), Myc antibody (lanes 7–9) or both (lanes 4–6). Molecular size (kD) markers are indicated on the right. Cell confluency is indicated at the bottom of each lane. CRM, cross reactive material.

Myc levels appear lower in lanes 4–6 than in lanes 7–9 because the amount of staphylococcal protein A was limiting relative to the amounts of anti-Myc and anti-Max antibodies. An as yet unidentified 43-kD polypeptide (labeled CRM) was also seen in all lanes and is present in the immunoprecipitations done by other investigators (29). The decrease in Myc levels that occurs with increasing cell confluency was also detected by Western immunoblotting in this cell line as well as in Rat 1a cells grown to 20 and 90% confluencies (data not shown).

To determine whether endogenous c-Myc levels are functionally in excess of endogenous Max at low cell confluency, we performed transfection experiments in which Max was overexpressed. Cells grown to 20% confluency were transfected with the reporter construct and increasing amounts of a Max expression vector, pRSVMax (Fig. 4 A). With increasing amounts of exogenous Max, transcription of pE₁₀CAT first increased and then declined below basal levels in 20% confluent cells. These results suggest that the Max expression vector alone can activate transcription from the CAT reporter, presumably by titrating excess endogenous c-Myc. At high input Max plasmid DNA, however, transcription was inhibited to below basal levels suggesting that excess Max inhibits transcription. The biphasic behavior of exogenous Max at low cell confluency cannot be explained simply by the presence of Mad, suggesting that overexpressed Max may inhibit transcription through Max homodimer formation. We then performed the same experiment using cells at 90% confluency. There was no significant change in reporter activity from basal levels with any amount of input pRSVMax alone (Fig. 4 B). However, if 90% confluent cells were cotransfected with a constant amount of pMLVMyc (15.0 μ g) and increasing concentrations of pRSVMax (0 to 5.0 μ g),

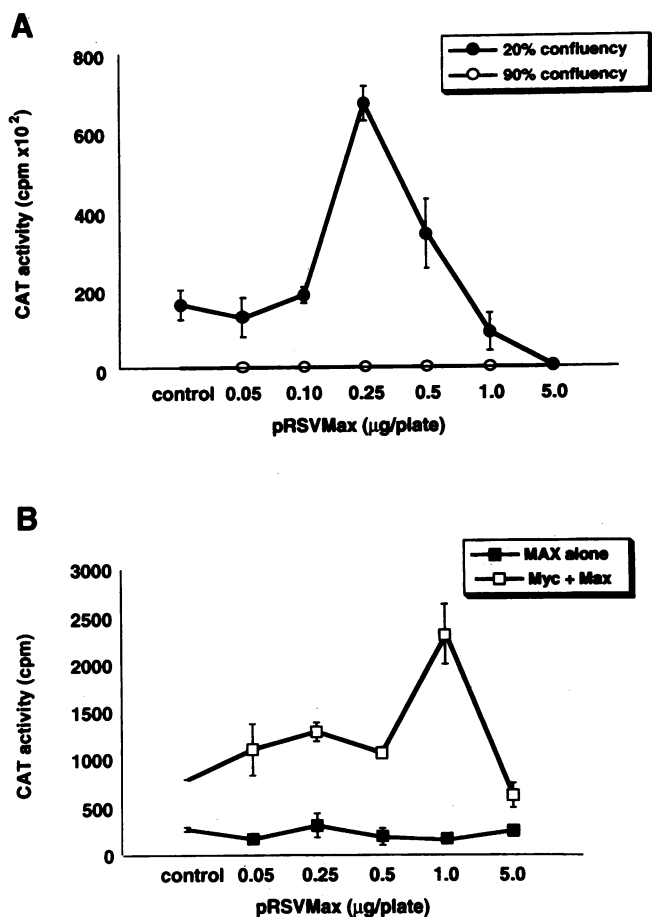


Figure 4. (A) Exogenous Max transactivates in 20% confluent mouse fibroblasts. All cells were transfected with 1.5 µg pE₁₀CAT and between 0.05 and 5.0 µg pRSVMax. Transfections were performed in quadruplicate. Closed circles represent CAT activity in cells grown to 20% confluency. Open circles represent cells grown to 90% confluency. Bars indicate standard errors. "Control" designates addition of pBSIISK⁻ alone. (B) Exogenous Myc titrates exogenous Max in 90% confluent cells. Cells at 90% confluency were transfected with 1.5 µg pE₁₀CAT and between 0.05 and 5.0 µg pRSVMax with or without 2.0 µg pMLVMyc. Transfections were performed in quadruplicate. Closed squares represent CAT activity in the absence of pMLVMyc. Open squares represent CAT activity in the presence of pMLVMyc. Bars indicate standard errors.

transcriptional activity first increased then decreased in a pattern that mimicked that seen with cells at 20% confluency transfected with pRSVMax alone. We conclude that although exogenously expressed c-Myc activates transcription at high cell densities, an appropriate amount of exogenous Max further augments transcription. If the level of exogenous Max exceeds that of c-Myc, transcription is then repressed.

Discussion

The study of the function of c-Myc as a transcription factor is essential to understanding its role in human carcinogenesis. Variable degrees of c-Myc transcriptional activity in transient transfection experiments have been observed, adding to the confusion about how c-Myc affects expression of target genes. We

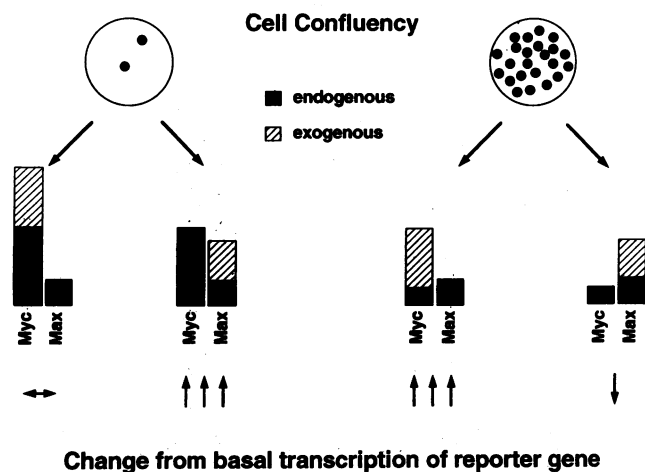


Figure 5. Schematic diagram of the paradoxical effect on c-Myc transcriptional activity caused by exogenous Myc or Max. Cells at low confluency but with high rates of cell proliferation express high levels of endogenous Myc such that when exogenous Myc is introduced, transcriptional activity of the reporter gene is unchanged. When ectopic Max is present, CAT activity increases above basal levels. These findings are in contrast to those found with cells at 90% confluency. The introduction of exogenous Myc leads to a rise in transcriptional activity above basal levels, but exogenous Max causes no change. The difference in transcriptional activity seen for cells grown to various confluencies reflects the difference in endogenous levels of Myc.

have now determined that cellular context is a significant factor influencing the activity of transcription factors in transient transfections. We observed that cell density contributes dramatically to c-Myc transcriptional activity because endogenous levels of c-Myc fall as cell density rises (Fig. 5). In rapidly proliferating cells grown at low confluency, exogenous c-Myc does not add to the high level of basal transcription of reporter constructs presumably because endogenous Max is limiting; in fact, exogenous Max can augment transcription by titrating excess endogenous c-Myc. In contrast, when the rate of proliferation is low, as in cells grown to high confluency, c-Myc, but not Max, can augment transcriptional activation from basal levels presumably because endogenous c-Myc is limiting. Our results support the proposed model that control of transcriptional regulation by c-Myc is dependent on changes in ratios between c-Myc activating dimers and Max inhibitory dimers (12, 29). Though we have observed that endogenous levels of c-Myc contribute to variability in c-Myc mediated transactivation, c-Myc may also repress transcription through its putative interaction with the initiator element (37–39). Our findings suggest that the study of other growth-related transcription factors may be affected by variations in endogenous levels dependent on the cell density.

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