Mxi1 Is a Repressor of the c-myc Promoter and Reverses Activation by USF*

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The basic region/helix-loop-helix/leucine zipper (B-HLH-LZ) oncoprotein c-Myc is abundant in proliferating cells and forms heterodimers with Max protein that bind to E-box sites in DNA and stimulate genes required for proliferation. A second B-HLH-LZ protein, Mxi1, is induced during terminal differentiation, and forms heterodimers with Max that also bind E-boxes but tether the mSin3 transcriptional repressor protein along with histone deacetylase thereby antagonizing Myc-dependent activation. We show that Mxi1 also antagonizes Myc by a second pathway, repression of transcription from the major c-myc promoter, P2. Repression was independent of Mxi1 binding to mSin3 but dependent on the Mxi1 LZ and COOH-terminal sequences, including putative casein kinase II phosphorylation sites. Repression targeted elements of the myc P2 promoter core (−35/−10), where it reversed transactivation by the constitutive transcription factor, USF. We show that Zn²⁺ induction of a stably transfected, metallothionein promoter-regulated mxi1 gene blocked the ability of serum to induce transcription of the endogenous c-myc gene and cell entry into S phase. Thus, induction of Mxi1 in terminally differentiating cells may block Myc function by repressing the c-myc gene P2 promoter, as well as by antagonizing Myc-dependent transactivation through E-boxes.

The c-myc gene is an immediate-early response gene, which is activated in quiescent cells by mitogenic stimuli. c-myc is required for the G₀ to G₁ transition (reviewed in Refs. 1–3), but in contrast to other immediate-early response genes, its expression continues beyond the G₀-G₁ transition at a lower constitutive level throughout the cell cycle, as cells proliferate (4–6). Myc also exerts G₂-S control by enhancing cyclin E/cdk2 activity (7, 8). Because inhibition of c-myc in proliferating cells arrests growth (9, 10), c-myc expression is required for continuous cell proliferation. Deregressed overexpression of c-Myc blocks terminal differentiation of a number of cell types. Likewise, cellular differentiation is normally accompanied by a decrease of c-Myc in a variety of cell lines (reviewed in Refs. 1–3). This suggests that the cellular choice between growth and differentiation depends upon a fine balance between c-myc stimulation and c-myc repression.

c-Myc is a basic region/helix-loop-helix/leucine zipper (B-HLH-LZ) transcription factor, which forms heterodimers with Max that bind to E-box Myc sites (11–15). Myc-Max heterodimers transactivate promoters with E-boxes (13, 14, 16), which in most cases are found in growth-promoting genes, including the ornithine decarboxylase gene (17–19), cdc25A gene (20), and the a-prothymosin gene (21, 22).

Myc-Max heterodimers form one arm of the Max interacting regulatory network. The other arm of this network is provided by Mad-Max heterodimers (reviewed in Ref. 2). Mad family proteins include Mad1, -3, and -4 and Mxi1 (Mad 2) (23–25). Mad-Max heterodimers also bind E-boxes, but repress transcription through their interaction with mSin3, a homolog of a yeast general transcriptional repressor (25–28). Histone deacetylases associated with mSin3 mediate Mad or Mxi1 transcription repression (29–31). Stimulation of transcription by Myc-Max at E-boxes is therefore balanced by transcription repression by Mad-Max.

Given the opposing actions of Myc-Max and Mad-Max, the cell may alter the activities of E-box-dependent genes by altering the relative levels of Myc and Mad, and this in turn may control proliferation. Indeed, in many instances of terminal differentiation, Mxi1 levels increase and c-Myc levels decrease as cells stop proliferating and differentiate (23–25, 32–37). Furthermore, Mxi1 antagonizes the Myc transformation function (27, 37–40). Mxi1-knockout mice demonstrate a cancer-prone phenotype, and an enhanced ability for several Mxi1-deficient cell types to proliferate (41). Thus, Mxi1/Mad proteins are essential in the regulation of normal and neoplastic growth.

The mechanism of inverse expression of the c-myc and mxi1 genes is not known. However, in vivo, c-myc expression ceases in many tissues during terminal differentiation, at the time that the mxi1 gene is activated. The c-myc gene is regulated at multiple levels including repression of transcription (42, 43). c-myc transcripts are initiated from two major start sites, P1 and P2, which are separated by 162 base pairs in the human c-myc gene. Because the great majority of c-myc transcripts arise from P2 (43), P2 is a likely target for transcriptional repression.

Myc is known to repress its own promoter, providing an autoregulatory loop that limits c-myc transcription (44–47). Myc also represses other promoters including the adenovirus-2 major late promoter and the promoters of the C/EBPα, gas1, and gadd45 genes (48–53). In fact, Myc may induce cell proliferation, at least in part, by repressing genes with growth arrest activity (54). This repression is opposed by USF, an ubiquitous transcription factor, which forms heterodimers with Max and stimulates transcription from E-boxes (55). USF is activated in quiescent cells by mitogenic stimuli (56, 57). The mechanism of USF repression of c-Myc involves the transcriptional repressor activity of Mxi1, which is independent of mSin3 and Mad interaction (58, 59). Several studies have implicated Mxi1 in the control of cell proliferation (58–60). Mxi1 may also function as a tumor-suppressor gene, as it is downregulated in some human tumors (61, 62). Mxi1 expression is regulated at multiple levels including transcriptional repression by Myc-Max heterodimers (29–31). The repression of c-myc by Mxi1 is mediated by the Mxi1 LZ and COOH-terminal sequences, including putative casein kinase II phosphorylation sites (63, 64). These results suggest that Mxi1 may play a critical role in the regulation of cell proliferation and tumorigenesis.

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The abbreviations used are: B-HLH-LZ, basic region/helix-loop-helix/leucine zipper; PCR, polymerase chain reaction; CMV, cytomegalovirus; kb, kilobase pair(s); aa, amino acid(s); Inr, initiator; CKII, casein kinase II.
B-HLH-LZ transcription factor (49, 50). The autoregulation of the c-myc promoter may be controlled by a similar Myc-dependent repression mechanism in continuously proliferating cells. However, an autoregulatory mechanism is inherently incapable of the complete shut down of c-myc promoter activity that takes place during terminal differentiation. Thus other mechanisms for c-myc regulation are likely to operate during terminal differentiation.

In this report, we show that the Mxi1 protein is a repressor of the c-myc promoter. Mxi1 represses the chromosomal c-myc gene 30-fold, even in the absence of other aspects of terminal differentiation. Repression of c-myc depends on a novel regulatory domain within the Mxi1 COOH terminus and targets the major c-myc promoter, P2. This repression is independent of the Mxi1 NH2 terminus and mSin3, and thus differs from the E-box repression function of Mxi1. Thus, Mxi1 represses genes required for proliferation through two distinct pathways: repression of E-box-dependent genes by a mechanism dependent on the Mxi1 amino terminus interaction with mSin3, and repression of the c-myc P2 promoter by a mechanism dependent on the Mxi1 carboxyl-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—To express Mxi1 in vivo, CMV-Mxi1 was constructed by subcloning a fragment from the full-length Mxi1 cDNA (a 2.4-kb EcoRI fragment) generated by PCR using a 5′-CCGGATTCCACCTCTAGGGCGGAAGATGAGCT (mxif) and a 3′-CCGGATTCCAGGGCGGAAGATGAGCT (mxir) primer as in the wild-type Mxi1 construction. CMV-Mxi1CT149 and CMV-Mxi1CT187 were constructed using the unchanged primer as the wild-type construction and a 3′ primer, (5′)-GGAGTCTTCAACTCCTTTTATGTTC (mx1r) and cloned into pCEP4-HA (Invitrogen). The following mutations were generated by PCR and expressed in pCDNA3. CMV-Mxi1NT36 was generated using a 5′-CCGGATTCCATGCGAGCCAGCCGCCGAGCTGCA and the same 3′ primer as in the wild-type Mxi1 construction. CMV-Mxi1CT149 and CMV-Mxi1CT187 were constructed using the unchanged primer as the wild-type construction and a 3′ primer, (5′)-GGAGTCTTCAACTCCTTTTATGTTC (mx1r) and cloned into pCEP4-HA (Invitrogen). The following mutations were generated by PCR and expressed in pCDNA3. CMV-Mxi1NT36 was generated using a 5′-CCGGATTCCATGCGAGCCAGCCGCCGAGCTGCA and the same 3′ primer as in the wild-type Mxi1 construction. CMV-Mxi1CT149 and CMV-Mxi1CT187 were constructed using the unchanged primer as the wild-type construction and a 3′ primer, (5′)-GGAGTCTTCAACTCCTTTTATGTTC (mx1r) and cloned into pCEP4-HA (Invitrogen). The following mutations were generated by PCR and expressed in pCDNA3. CMV-Mxi1NT36 was generated using a 5′-CCGGATTCCATGCGAGCCAGCCGCCGAGCTGCA and the same 3′ primer as in the wild-type Mxi1 construction. CMV-Mxi1CT149 and CMV-Mxi1CT187 were constructed using the unchanged primer as the wild-type construction and a 3′ primer, (5′)-GGAGTCTTCAACTCCTTTTATGTTC (mx1r) and cloned into pCEP4-HA (Invitrogen).

**FIG. 1. Terminal differentiation of 3T3L1 pre-adipocytes to adipocytes.** A, photomicrographs of cells on day 0 and day 7 of the terminal differentiation program. Note the accumulation of cytoplasmic fat droplets in day 7 terminally differentiated cells (55). Original magnification, ×100 or ×320 as indicated. B, Northern analysis of RNA obtained on different days of 3T3L1 differentiation. Twenty micrograms of total RNA per condition were analyzed with CDNA probes to human c-myc and then to c-myc after stripping. Ethidium bromide staining of the gel showed equivalent loading of lanes as indicated by the 28 S rRNA band. Lane 1, day 0; lane 2, day 1; lane 3, day 4; lane 4, day 7; lane 5, day 10; lane 6, day 14.

**Cell Culture—** NIH3T3 and 3T3L1 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.) supplemented with 100 units/ml penicillin and 100 g/ml streptomycin. Differentiation of 3T3L1 cells was performed as described in published procedures (55).

**Transfection Assays—** Transient transfections of NIH3T3 cells were performed in 10-cm dishes using the calcium phosphate co-precipitation method (56). A total of 25 μg of plasmid DNA was used in each transfection, consisting of 3–8 μg of reporter plasmid, 0.1 μg of CMV-βgal as an internal control plasmid to monitor transfection efficiency, a variable amount of expression plasmid adjusted with empty vector plasmid to equalize the amount of effector plasmid, and pB5 SK plasmid as carrier DNA. For stable transfections into NIH3T3 and 3T3L1 cells, 5 μg of MT-mxi1 or MT-neo were used. Stable transfected were selected in the presence of 800 or 1500 μg/ml G418 (Geneticin, Life Technologies, Inc.), respectively. Individual clones were expanded and used for experiments or cryopreserved. Selection enzyme digestion of P2(-2489)-luc into pGL2Basic. P2TATA-luc, P2SVL-luc, and SVL-luc were kindly provided by J. Lang.

Plasmid MT-mxi1 was constructed by inserting the human Mxi1 coding sequence (aa 1–228) tagged with a NH2-terminal Flag-epitope (DYKDDDDK) obtained by PCR amplification of a fragment from the human Mxi1 cDNA (24) into MT-neo (also known as pMTH globin.neo, kindly provided by S. Segal) in the sense orientation.

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LB9501 luminometer as described previously (49). Luciferase activity was normalized to β-galactosidase activity to account for variations in transfection efficiency. Normalization between experiments was done by setting the activity obtained with the empty expression vector equal to 1. -Fold change is calculated as the inverse of relative luciferase activity. A positive -fold change represents stimulation; a negative -fold change, repression. At least four determinations were made for each reporter/activator combination.

**RESULTS**

**mSin3-independent Repression of the c-myc Promoter by Mxi1**—The 3T3L1 cell line differentiates in culture from a proliferating preadipocyte cell to a non-proliferating postmitotic adipocyte (Fig. 1A) (57). Between days 1 and 7 of 3T3L1 differentiation, c-myc mRNA levels decrease and mxi1 messenger RNA expression is strongly induced (Fig. 1B) (57). These changes are reminiscent of the inverse relationship of mxi1 and mSin3 with c-myc expression.
FIG. 4. Effect of wild-type and mutant Mxi1 on P2(−2489)-luc or m4tk-luc. A, structure of Mxi1 wild-type and mutants. Mxi1 mutants were generated as described under “Experimental Procedures” and cloned into pcDNA3. ●, COOH-terminal putative casein kinase II phosphorylation sites. SID, mSin3 interaction domain. B-HLH-LZ, basic region/helix-loop-helix/leucine zipper DNA binding domain for interaction at the E-box. B, the amino-terminal 35-aa domain (containing the mSin3 interaction domain) is required for transcriptional repression of m4tk-luc, but not of P2(−2489)-luc. Ten micrograms of CMV-Mxi1, CMV-Mxi1NT36, or pcDNA3 were co-transfected with 5 μg of reporter plasmid P2(−2489)-luc or m4tk-luc. The experimental conditions that involved m4tk-luc described here and in C additionally contained CMV-Hm (2 μg) and CMV-Max (2 μg) which elevated the basal activity of m4tk-luc. The data indicate the average of at least three experiments with standard errors of the mean.
c-myc gene expression in other differentiation systems (24, 34, 36) and suggest a role for Mxi1 in the repression of c-myc. We therefore asked whether Mxi1 can repress the c-myc promoter. For these and subsequent experiments that employ transient transfection, we made use of NIH3T3 cells, which are the parent cells of 3T3L1. This was necessary because transfection of 3T3L1 preadipocytes was extremely inefficient, while NIH3T3 cells were readily transfected. We transiently transfected NIH3T3 cells with increasing amounts of the Mxi1 expression plasmid, CMV-Mxi1, and the repression target plasmid, P2(-2489)-luc (Fig. 2), which contains residues -2489 to +352 of the human c-myc promoter region (numbering relative to P2) linked to the firefly luciferase reporter gene. A cotransfected CMV-βgal expression plasmid provided an internal control for variations in transfection efficiency. In an initial experiment (Fig. 2), transient expression of Mxi1 reduced c-myc promoter activity 3-fold, but the tk promoter, a control, was relatively unaffected. Repression of c-myc by Mxi1 differed from repression of an E-box. While the Max protein is required for Mxi1 repression of the E-box (23, 24, 26, 27), Max expressed from the vector CMV-Max progressively reversed Mxi1 repression (Fig. 3A), rather than aiding the repression. Indeed, Max expression in the absence of Mxi1 greatly stimulated the c-myc promoter (Fig. 3A). As a control, Max synergized with Mxi1 to repress E-box-dependent transcription from plasmid m4tk-luc (Fig. 3B). Furthermore, the Mxi1 mutant, Mxi1NT36, which lacks the Mxi1 amino-terminal 35 amino acids required for mSin3 interaction (Fig. 4A) and for mSin3-dependent repression of E-boxes (26, 40), repressed c-myc promoter activity to the same extent as wild-type Mxi1 (Fig. 4B), although as expected, it could not repress the E-box-dependent activity of m4tk-luc (Fig. 4B). These observations indicate that Mxi1 represses the c-myc promoter and E-boxes by different mechanisms.

Repression Requires the Mxi1 LZ and COOH Terminus—The ability of Mxi1 to repress c-myc in the absence of the NH2-terminal mSin3 binding domain suggested that a different Mxi1 domain repressed c-myc. To locate this domain, we assayed repression of the c-myc promoter by mutants of Mxi1, which had an altered DNA binding domain or an altered carboxyl terminus. Western blotting confirmed that the mutants, which were epitope-tagged to distinguish them from endogenous Mxi1, expressed from the vector CMV-Max progressively reversed Mxi1 repression of c-myc (Fig. 3A), rather than aiding the repression. Indeed, Max expression in the absence of Mxi1 greatly stimulated the c-myc promoter (Fig. 3A). As a control, Max synergized with Mxi1 to repress E-box-dependent transcription from plasmid m4tk-luc (Fig. 3B). Furthermore, the Mxi1 mutant, Mxi1NT36, which lacks the Mxi1 amino-terminal 35 amino acids required for mSin3 interaction (Fig. 4A) and for mSin3-dependent repression of E-boxes (26, 40), repressed c-myc promoter activity to the same extent as wild-type Mxi1 (Fig. 4B), although as expected, it could not repress the E-box-dependent activity of m4tk-luc (Fig. 4B). These observations indicate that Mxi1 represses the c-myc promoter and E-boxes by different mechanisms.

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nous Mxi1, were expressed at comparable levels (Fig. 4E).

Mxi1ΔLZ, which lacks the leucine zipper (Δ aa 117–149), failed to repress either c-myc or the E-box-dependent plasmid, m4tk-luc (Fig. 4C). Mxi1ΔBR, which lacks the basic region (Δ aa 70–80), did not repress m4tk-luc (Fig. 4C), but retained approximately 50% of the wild-type activity for repression of P2(−2489)-luc (Fig. 4C). Thus, the Mxi1 leucine zipper, but not the basic region, was necessary for c-myc promoter repression.

The COOH-terminal domain of Mxi1 contains 80 amino acids distal to the LZ and is rich in serine and acidic residues. Within this region lie five potential casein kinase II (CKII) phosphorylation sites, grouped in three locations (Ser\textsuperscript{170}/Ser\textsuperscript{172}, Ser\textsuperscript{187},...
and Ser198/Ser200). Mxi1CT187, in which the COOH-terminal 41 aa (including two CKII sites) are lacking and a third CKII site is disrupted by severing Ser187 from its CKII recognition sequence, also failed to repress c-myc (Fig. 4C and D). However, the internal deletion mutant Mxi1Δ162–185 (Δ aa 162–185) repressed P2 (–2489) to 50% of the extent of the wild-type (Fig. 4D). Thus, the COOH-terminal 41 amino acids of Mxi1 (aa 187–228) contain a c-myc promoter repression domain that depends upon the adjacent serine-rich and acidic regions for full activity.

Mutant Mxi1Δ170/172(SS-AA), in which serines within CKII phosphorylation sequences at positions 170 and 172 were replaced by alanines, retained 42% of the repression activity while mutant Mxi1Δ198/200(SS-AA), in which the CKII consensus phosphorylation sites at Ser198 and Ser200 were changed to alanine, retained 36% (Fig. 4D). Thus, repression of the c-myc promoter was dependent upon CKII consensus phosphorylation sites within the COOH-terminal domain.

Mxi1 Repression of c-myc Requires Elements of the P2 Core Promoter—Although c-myc transcription is initiated from start sites P1 and P2, P2 accounts for over 90% of the activity (43). Plasmid P1myc-luc (residues –266 to –96; numbering relative to P2) contains the c-myc promoter while P2myc-luc (residues –96 to +352) contains P2. P1P2myc-luc, which contains both P1 and P2 (–266 to +352), and P2myc-luc both had much higher basal levels of activity than P1myc-luc and were repressed by Mxi1 to the same extent (Fig. 5A). In contrast, P1myc-luc was relatively resistant to Mxi1 repression. This indicates that the major target of Mxi1 is the P2 promoter.

We next defined components of P2 required for repression. P2TATA-luc (residues –150 to +10), which contains P2 but not P1, was also strongly repressed by Mxi1 (Fig. 5B), limiting the target of Mxi1 to a 160-residue region. When the core promoter, which encompasses the P2 TATA box, and the initiator (Inr) site (residues –35 to +10 of P2TATA-luc) (58) were replaced with the SV40 late Inr region (SV40 residues 199 to +399 relative to the SV40 origin) (59), repression by Mxi1 was reduced (Fig. 5B). The Inr region of the SV40 late promoter was used in this replacement because the SV40 late promoter is not subject to core promoter repression by Myc (59).2 The residual repression of P2SVL-luc was also seen with plasmid SVL-luc (Fig. 5B), which contains the SV40 late promoter initiator alone, and was therefore unrelated to control through c-myc promoter elements. Thus, Mxi1 represses the c-myc promoter through P2 core promoter elements located between residues –35 to +10.

The c-Myc protein also targets c-myc P2 core promoter elements to bring about its own negative autoregulation (47, 59). Myc repression of the adenovirus-2 major late promoter and the C/EBPα promoter is mediated by their respective Inr ele-

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2 L. Li and E. Ziff, unpublished observations.
ments (48, 49). Since the c-myc promoter has a candidate Inr for Myc-mediated transcriptional repression, we asked whether Mxi1 repression of the P2 promoter might involve the initiator element. To test this, two mutants of the P2 Inr region were assayed for repression by Mxi1. Mutant P2Inr.m1 contains a triple point mutation at residues +2, +3, and +4. Both sets of mutations reduced Mxi1 repression (Fig. 4C). The fact that repression was not entirely abolished suggests that other residues may be involved. Our observations suggest that Mxi1 repression of the P2 promoter involves, at least in part, an intact P2 Inr.

**Mxi1, but Not Its COOH-terminal Deletion Mutant CT149, Antagonizes USF-mediated Transactivation of P2—c-Myc core promoter repression (48–51, 59, 60) can be reversed by a second B-HLH-LZ protein, USF, which stimulates core promoter activity (49, 61, 62). USF stimulated the c-myc P1 and P2 promoters 2.7-fold in plasmid P2 (−514)-luc (residues −514 to

**FIG. 8. Induction of exogenous mxi1 expression blocked serum induction of the chromosomal c-myc in quiescent cells.** A, induction of mxi1 expression in cell line MT-mxi1.7, but not MT-neo, by zinc prevented serum induction of the c-myc gene. Cell lines MT-neo and MT-mxi1.7 were serum-deprived in Dulbecco's modified Eagle's medium supplemented with only 0.5% fetal calf serum for 48 h, then treated with ZnCl₂ (40 µM) 2 h prior to adding fetal bovine serum (final concentration of 20%) to each. Time 0' indicates the time of serum addition. Northern analysis was performed with cDNA probes to mxi1, c-myc, and gapdh. The filter was stripped after each hybridization. B and C, relative mRNA expression for c-myc and mxi1, respectively. Expression of mxi1 and c-myc relative to that of gapdh was determined by densitometric analysis. D, Western blots of cells treated as in A, demonstrating that zinc-induced Mxi1 expression blocks serum induction of the c-Myc protein expression. E–G, treatment with zinc in the MT-mxi1.7 cell line abrogated the serum induction of c-myc gene expression. The cell line MT-mxi1.7 was deprived of serum as above, then treated with or without ZnCl₂ (40 µM), and serum (20%) was added 2 h later. Time 0' indicates the time of serum addition. Northern analysis and densitometric analyses were performed as in panels A, B, and C.
+352) and P2 alone 8-fold in plasmid P2(-70)-luc (residues -70 to +10) (Fig. 6, A and B). Significantly, Mxi1 reversed the activation of the c-myc promoter by USF. Mxi1 repressed the USF activated P1 plus P2 promoters 6-fold, and the USF activated P2 promoter 14-fold (Fig. 6, A and B). Indeed, Mxi1 reduced the activities of both promoters to below basal levels. In contrast, mutant Mxi1CT149, which lacks the Mxi1 COOH-terminal tail (Fig. 4, A and D), was also ineffective in repressing USF-stimulated c-myc promoter activity (Fig. 6, A and D). These results indicate that USF strongly activates the c-myc P2 promoter, and that Mxi1 antagonizes this stimulation by a mechanism dependent on the Mxi1 COOH-terminal domain. This regulation can provide a 14-fold change in c-myc promoter activity.

Regulated Expression of Mxi1 Can Repress the Endogenous c-myc Gene—To measure repression of the chromosomal c-myc promoter by Mxi1 under more physiologically relevant conditions, we constructed cell lines derived from NIH3T3 and 3T3L1 cells with a stably transformed, Zn2+-inducible Mxi1 gene (MT-mxi1). In the clonal cell line MT-mxi1.7 (Fig. 7A), exogenous mxi1 mRNA (0.8 kb) is induced by Zn2+ 18-fold, but the endogenous Mxi1 transcript (2.4 kb) was not seen. No expression of either endogenous or exogenous Mxi1 mRNA was seen in control cells (Fig. 7B). Western analysis (Fig. 7C) indicated that Mxi1 protein was expressed by 3 h and peaked at 10 h following Zn2+ treatment.

To test whether Mxi1 represses the wild-type c-myc gene, the MT-mxi1.7 cell line, and a control cell line, MT-neo, which contains a Zn2+-regulated empty vector, were both deprived of serum for 48 h to induce the quiescent state. The growth medium was replenished with 20% fetal calf serum at time 0 h. To assure high levels of metallothionein promoter induction at the time of serum stimulation, ZnCl2 (40 μM) was added to both cell lines 2 h prior to serum addition (−2 h; Fig. 8, A and B). In the MT-neo control cell line, c-myc mRNA reached high levels by 1.5 h after serum addition (Fig. 8, A and B), consistent with serum induction of c-myc in quiescent cells (63, 64). In contrast,
induction of c-myc mRNA was blocked in the Mxi1-expressing cells. A low level c-myc activity at the time of serum addition resulted from the addition of Zn$^{2+}$ (compare −2 and 0 h lanes of MT-neo and MT-mxi1.7, Fig. 8A). Western analysis confirmed that c-Myc protein expression was also blocked (Fig. 8D). Serum induction of c-myc was also blocked by Mxi1 in MT-mxi1.7 cells, relative to an uninduced MT-mxi1.7 cell control (Fig. 8, E–G). In this experiment, the induction of c-myc mRNA was greatly attenuated by Mxi1, with densitometric quantitation revealing a 30-fold repression.

To determine whether Mxi1 can limit cell cycling, we employed four additional cell lines, L1-MT.neo, L1-MT-mxi1, L1-MT-nt36, and L1-MT-ct149, derived from 3T3L1 cells, the preadipocyte cell line in which Mxi1 expression is activated during terminal differentiation (Fig. 1). These lines express the wild-type or mutant forms of Mxi1 protein that were studied above by transfection. Fig. 9A shows that the S + G2/M phase population of cells had increased 2–4-fold 18 h following serum stimulation of zinc-treated MT-neo, MT-nt36, and MT-ct149 cells. In contrast, however, zinc-treated MT-mxi1 cells showed no increase in the percentage of cells in S + G2/M phases following serum stimulation. Western and Northern analysis shows that the mxi1 gene is zinc-inducible in each cell line, except the control line L1-MT.neo (Fig. 9, B and C). These observations demonstrate that Mxi1, in addition to repressing the c-myc promoter, blocks serum-induced cell entry into S phase following serum stimulation. Furthermore, expression of NT36 did not block cell cycle progression, although in transient assays it was capable of repressing the c-myc promoter. Likewise, mutant CT149 also did not block cell cycle progression, although it repressed E-box-dependent transcription in transient assays. These results indicate that inhibition of cell cycling by Mxi1 requires both the c-myc repression function provided by the Mxi1 COOH terminus and the E-box repression function provided by the Mxi1 NH$_2$ terminus.

**DISCUSSION**

**Effect of Mxi1 Expression on c-myc Gene Expression during Terminal Differentiation**—Expression of the c-myc gene maintains the ability of cells to proliferate and blocks or retards cellular differentiation. Because reduction of c-myc induces cell differentiation (2, 3) and constitutive c-myc expression blocks differentiation in a variety of cell lineages, including preadipocyte 3T3L1 cells (65), c-myc down-regulation may be a prerequisite for differentiation.

A second aspect of terminal differentiation is the induction of Mxi1, which was observed here during terminal differentiation of 3T3L1 cells, and which was coincident with a decrease of c-myc mRNA. Indeed, the mxi1 (or mad) and c-myc genes are inversely regulated in a large number of terminally differentiating systems, including leukemia cells (24, 32, 34), epidermal cells (25, 33, 36, 37), and neurons (25), as well as during mouse and zebrafish embryogenesis (35). A consequence of the decline in Myc and increase in Mxi1 and other Mad family proteins is a change in the relative levels of Myc-Max relative to Mad-Max heterodimers (32). This change leads to repression of E-box-dependent genes.

The current work shows that Mxi1 represses the c-myc promoter. This provides a second mechanism for repression of Myc-dependent functions. In differentiating HL60 and U937 cells, repression of the c-myb promoter has been attributed to a shut-off of c-myc transcription initiation (66). This mechanism is consistent with P2 repression shown here.

**Regulation at the P2 Core Promoter**—During the differentiation of monocytic cells (67, 68), two domains of the myc promoter, one between residues −606 and −101, upstream of P1, and a second between residues −2392 and −1396 (68) function in myc promoter repression. Our work defines a third domain, which overlaps the P2 transcription start site, a position where RNA polymerase II is retained in differentiating HL60 cells while c-myc mRNA decreases in vivo (42, 67). Myc also auto-
regulates its own expression through P2 (47, 59). Regulatory Functions of Mxi1, Myc, and USF—USF is a ubiquitous cellular transcription factor (69, 70), which we show stimulates the c-myc P2 core promoter. USF also stimulates the Ad-2 major late promoter and the C/EBPα promoter and reverses Myc-mediated repression (49, 61). The transcription factor TFII-I interacts with USF and with Myc to form complexes that bind promoter initiator elements which mediate activation or repression of transcription, respectively (48, 71). The opposing effects of USF and Mxi1 noted in this report suggest the possibility of the formation of USF and Mxi1 corepressor complexes.

USF may provide a relatively constant positive contribution to the basal activity of the c-myc promoter. Both Myc and Mxi1 appear to oppose this USF action. However, Myc and Mxi1 are likely to provide this action under different circumstances. During cell proliferation, when Myc is elevated and Mxi1 and other Mad family members are low, Myc would negatively regulate P2. During terminal differentiation, when Mxi1 and other Mad proteins are high and Myc is low, Mxi1 is likely to be the major repressor of P2. During the shift from proliferation to differentiation, repression would switch from the Myc-mediated mechanism to the Mxi1-mediated repression. We find in vivo a 10–20-fold difference between c-myc basal promoter activity in the unrepressed, USF-induced state and the Mxi1-repressed state. Myc autoregulation could control myc gene activity during continuous cell proliferation (9, 10). USF may provide a constitutive positive stimulus for c-myc expression under normal conditions, which can be regulated by Myc or Mxi1 depending on cell state. During terminal differentiation, reduction of Myc to the low levels (72) that are required for growth arrest would, by this model, be the consequence of Mxi1 repression of P2. Mxi1 would also repress E-box-dependent, growth-promoting genes via Mxi1-Max heterodimers.

Transcriptional Regulation by Mxi1: NH2- and COOH-termini Functions—The NH2-terminal domain of Mxi1 binds mSin3, the mammalian homolog of the yeast repressor, Sin3 (26, 27) and is required for E-box repression (25, 26, 40). Because a Mxi1 NH2-terminal truncation mutant repressed the c-myc promoter, c-myc promoter repression by Mxi1 must be independent of mSin3.

The carboxy-terminal sequences of Mxi1 distal to the leucine zipper are required for Mxi1 repression of the c-myc promoter. The COOH-terminal 41 amino acids of Mxi1 appear to be particularly important for myc repression, but are dispensable for E-box repression. The leucine zipper, however, is required for repression of both the c-myc promoter and E-boxes. c-Fos repression of the c-fos promoter requires phosphorylated, COOH-terminal 27 aa residues of c-Fos (73, 74). With Mxi1, CKII phosphorylation sites in the COOH terminus are also implicated in repression. These are grouped in three locations: I, Ser170/Ser172, II, Ser187, and III, Ser198/Ser200. Double mutations (Ser/Ser to Ala/Ala) of group I and III CKII sites in the Mxi1 COOH terminus resulted in 60% or greater loss of wild-type Mxi1 repression activity. Removal of peptide sequences containing the group II and III sites in mutant Mxi1CT187 eliminated repression activity altogether. The COOH terminus may bind a corepressor analogous to mSin3 that recruits a histone deacetylase. Alternatively, the Mxi1 COOH terminus might recruit a complex directly with a histone deacetylase as does Rb to repress transcription (75–77). It is also possible that the COOH terminus has the intrinsic ability to repress when in the phosphorylated state.

Biological Roles for Mxi1 in Cell Cycle Exit—Autoregulation of the c-myc gene may provide a fine control of Myc expression in proliferating cells. However, autoregulation would not be capable of establishing a definitive and complete shut down of Myc expression. During terminal differentiation, a more complete repression may be achieved by switching the mechanism of c-myc promoter repression from Myc autoregulation to repression by Mxi1. Because Mxi1 is induced during terminal differentiation and its expression is often accompanied by growth arrest, Mxi1 may provide a more complete and long lasting block of c-myc transcription than c-myc autoregulation, which restricts Myc protein to very low levels and potentiates the Mxi1 NH2-terminal transcriptional repressor function. The G1 phase cell cycle block by Mxi1 may result from effects of both repression of c-myc and effects of the Mxi1 NH2-terminal transcriptional repressor function. Finally, our results are consistent with the observations that ectopic expression of Mad1 inhibits cell proliferation at the G1 phase in human tumors (78), and in response to growth factor stimulation (79).

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