Using cDNA microarrays, we recently identified a large number of transcripts that are regulated differentially by the c-Myc oncoprotein in myeloid cells. Here, we characterize one of these, termed MT-MC1 (Myc Target in Myeloid Cells-1). MT-MC1 is a widely expressed nuclear protein whose overexpression, unlike that of c-Myc targets reported previously, recapitulates multiple c-Myc phenotypes. These include promotion of apoptosis, alteration of morphology, enhancement of anchorage-independent growth, tumorigenic conversion, promotion of genomic instability, and inhibition of hematopoietic differentiation. The MT-MC1 promoter is a direct c-Myc target; it contains two consensus E-box elements, both of which bind c-Myc-Max heterodimers. Mutation of either site abrogates DNA binding by c-Myc-Max and renders the promoter c-Myc unresponsive. Finally, MT-MC1 regulates the expression of several other c-Myc target genes. MT-MC1 represents a proximal and direct c-Myc target that recapitulates many of the properties typically associated with Myc oncoprotein overexpression.

The c-Myc oncoprotein exerts considerable control over transformation, differentiation, apoptosis, and cell cycle progression (1–4). More recently, c-Myc has been shown to promote growth and angiogenesis (5, 6) and to induce genomic instability (7–9). These diverse effects have suggested that the c-Myc oncoprotein in particular, and other members of the Myc oncogene network in general, influence the expression of a large and diverse subset of cellular genes. This is consistent with the concept that c-Myc is a transcription factor capable of activating or repressing specific genetic targets (10, 11). Many putative target genes have been identified over the past several years by a variety of methods. However, the recent dramatic expansion of these genes largely reflects the use of DNA microarray technology, which allows the simultaneous assessment of several thousand expressed sequences (12–15). Many of the target genes encode proteins that participate in processes known to be regulated by c-Myc. However, only a small number of these have actually been demonstrated to recapitulate any of the known c-Myc phenotypes. Furthermore, in all cases so far reported, overexpression of the target cDNA is able to impart only a limited number of c-Myc-like features to cells. For example, overexpression of ornithine decarboxylase is both transforming and pro-apoptotic (16, 17), whereas overexpression of CDK4 partially restores cell cycle progression in c-Myc null fibroblasts (18) Several other targets have been reported to be transforming and/or tumorigenic or to affect cellular growth properties (19–22). Such studies, coupled with the fact that many targets appear to encode proteins with common functions, have suggested that reconstruction of the complete c-Myc phenotype requires the concurrent deregulation of multiple target genes, many of which possess overlapping functions. An example of such cooperativity is that between the c-Myc targets rcl and lactate dehydrogenase-A, neither of which is tumorigenic when overexpressed individually in Rat1a fibroblasts (19, 20) but which are tumorigenic when coexpressed (23).

We have also used cDNA microarrays to identify a large number of both positive and negative c-Myc target transcripts in murine myeloid cells (14). We have shown that the regulation of these genes depends upon a functional c-Myc transactivation domain, comprising the N-terminal approximately 150 amino acids of the protein. Small deletions within the transactivation domain lead to the loss of the ability of c-Myc to regulate defined subsets of these target genes properly and to execute some biological functions.

In the present work, we have studied one such c-Myc target gene in greater detail. Transcripts for this gene (hereafter referred to as c-Myc Target in Myeloid Cells-1 (MT-MC1)) are highly up-regulated by c-Myc overexpression in the 32D murine myeloid cell line (14). Except for a putative nuclear localization signal, the highly conserved MT-MC1 protein contains no obvious structural or functional motifs, although it shares homology with a small putative DNA helicase encoded by the nuclear polyhedrosis virus of Bombyx mori (24, 25). We report here that overexpression of MT-MC1 affects morphology, apoptosis, genomic stability, and differentiation. The MT-MC1 promoter is also a direct c-Myc target. Together, these studies implicate MT-MC1 as an important and proximal c-Myc-responsive gene, which mediates many of the known phenotypic features associated with c-Myc overexpression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Rat1a fibroblasts and Friend erythroleukemia (F-MEL) cells were routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected with FuGENE 6 transfection reagent according to the manufacturer's instructions. Transfected cells were selected using 5 μg/ml G418 or 0.5 μg/ml HAT media.

**Northern and Western Blot Analysis—**Total RNA was isolated using the RNAqueous kit (Ambion). Northern blots were hybridized with 1 μg of MT-MC1-specific cDNA and exposed to an x-ray film for 24 h. Western blots were probed overnight with 1 μg of MT-MC1-specific cDNA and then probed with 2 μg of c-Myc-specific cDNA and exposed to an x-ray film for 24 h.

**Immunofluorescence—**For double labeling, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100, and then stained with fluorescein isothiocyanate (FITC)–conjugated Anti-Myc antibodies (Santa Cruz) and Texas red–conjugated secondary antibodies. For single labeling, cells were stained with the antibodies as above, followed by FITC-conjugated secondary antibodies. Cells were analyzed on a Zeiss confocal microscope.

**Immunocytochemistry—**Cells on glass coverslips were stained with 1 μg/ml Hoechst 33258 and 2 μg/ml anti-c-Myc antibodies (Santa Cruz) as described (26) and analyzed on a Zeiss confocal microscope.

**DNA Affinity Chromatography—**DNA affinity chromatography was performed as described (27).

**Cell Cycle Analysis—**The cell cycle was analyzed by flow cytometry as described (28).

**Assays for Apoptosis—**Apoptosis was measured by a fluorometric assay (29) or TUNEL assay (30).

**Cell Migration Assay—**A scratch assay was performed as described (31).

**Assays for Transformability—**Cell transformation was measured in soft agar as described (16).

**Short Hairpin RNAs—**Short hairpin RNAs were synthesized by Thermo Fisher Scientific using the provided primer oligos. The RNAi cocktail mix was prepared as described (32).

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**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY09114.

1 The abbreviations used are: MT-MC1, c-Myc target in myeloid cells-1; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; EST, expressed sequence tag; FBS, fetal bovine serum; F-MEL, Friend erythroleukemia; GFP, green fluorescent protein; IL-3, interleukin-3; ORF, open reading frame; TUNEL, terminal nucleotidyl transferase-mediated UTP nick end labeling.
fied Eagle's medium supplemented with 2 mM glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (all from Invitrogen). COS-7 cells were cultured similarly except that 10% supplemented calf serum (Invitrogen) was used instead of FBS. 32D murine myeloid cells were cultured in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% Penicillin-Streptomycin-Glutamine from the interleukin-3 (IL-3)-producing WEHI-3B cell line. 32D-c-Myc and 32D-neo cells have been described previously (26, 27). The former were obtained after stable transfection with a c-Myc expression vector, whereas the latter were obtained after transfection with the empty parental vector. For some experiments we used previously described pooled clones of 32D-c-Myc and 32D-neo, and the all lines stably transfected with a puromycin-selectable expression vector for cyclin B1, or with the empty parental vector (32D-c-Myc/cyclin B, 32D-c-Myc/puro cells, 32D-neo/cyclin B, or 32D-neo/puro cells) (Ref. 9). We also established a pooled population of 32D-MT-MC1 cells transfected with the same cyclin B1 or parental vectors. All cultures were split at least twice weekly to maintain continuous logarithmic growth. Transfections of 32D and F-MEL cells were performed by electroporation as described previously using plasmid DNAs linearized in the vector backbone (26–28). Transfections into COS-7 and Rat1a cells were performed with LipofectAMINE (Invitrogen) using supercoiled DNA. Selection for stably transfected cells was accomplished by cotransfecting either 32D-neo or 32D-c-Myc cells with 5 μg of each of the above linearized wild-type or mutant promoter-luciferase vectors together with a puromycin-selectable expression vector for cyclin B1 (Stratagene). Each transfection was verified by DNA sequencing. Luciferase assays were performed as described previously (7) after first adjusting the amounts of cellular lysates for differences in β-galactosidase activity.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Two E-boxes were identified in the MT-MC1 promoter region, the first (“A”; CAGCGTG) located at position −1351 relative to the start of transcription, and the second (“B”; CATGTT) located at position −127. The following oligonucleotides and their reverse complements (not shown), containing either the wild-type (WT) or mutant (mut) E-box were synthesized: A (WT), 5′-GTC TCG ATA CATG TCT CTA GAC-3′; A (mut), 5′-GTC TCG ATA CATG TCT CTA GAC-3′; B (WT), 5′-TCT CGA GGC AGT GTG GAA TTC CAA-3′; and B (mut), 5′-TCT CGA GGC AGT GTG GAA TTC CAA-3′ where italicized bases denote the wild-type or mutant E-box. 100 ng of each oligonucleotide was end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 50 μCi of [γ-32P]ATP (specific activity >3,000 Ci/mmol, PerkinElmer Life Sciences) to specific activities of at least 2 × 106 dpm/μg. Each oligonucleotide was then annealed with a 10-fold molar excess of its unlabeled complementary strand in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA by heating the mixture to 80 °C and allowing it to cool to ambient temperature over 2–3 h. For competition experiments with unlabeled double-stranded oligonucleotides, equimolar amounts of each unlabeled competitor strand were added. Recombinant Myc and Max proteins were purified to >90% purity using heparin-Sepharose affinity chromatography (30). Max(53) is the 151-amino acid isoform of Max which binds DNA as a heterodimer, but not as a homodimer, in association with c-Myc (30, 31). EMSAs were performed with ~20 pg of [32P]-labeled double-stranded oligonucleotide. Recombinant proteins (~20 ng of each) were added to a final volume of 20 μl in binding buffer and incubated at 40 °C for 20 min before electrophoresis.

**Erythroid Differentiation of F-MEL Cells**—Erythroid differentiation of F-MEL cells was induced by plating the cells into fresh medium at a density of 1×105 cells/ml followed 1 day later by the addition of dimethyl sulfoxide to a final concentration of 1.5%. The extent of differentiation was assessed by benzidine staining as described previously (28).

African green fluorescent protein (GFP)-expressing cells was performed as described previously (29).

**Plasmids and Reporter Gene Assays**—All plasmid DNAs were purified using Qiagen kits (Invitrogen). Selection for stably transfected cells was accomplished by cotransfecting either 32D-neo or 32D-c-Myc cells with 5 μg of each of the above linearized wild-type or mutant promoter-luciferase vectors together with a puromycin-selectable expression vector for cyclin B1 (Stratagene). Each transfection was verified by DNA sequencing. Luciferase assays were performed as described previously (7) after first adjusting the amounts of cellular lysates for differences in β-galactosidase activity.
RESULTS

Characterization and Expression Pattern of the MT-MC1—We reported previously the results of a cDNA microarray analysis that compared the gene expression profile of control 32D murine myeloid cells (32D-neo cells) with that of 32D cells stably transfected with a c-Myc expression vector (32D-c-Myc cells) (14). A number of differentially expressed transcripts were identified and confirmed by Northern blotting. MT-MC1 was characterized previously only as an expressed sequence tag (EST) (Incyte Pharmaceuticals EST 444412). We again verified that MT-MC1 is highly up-regulated in 32D-c-Myc cells (Fig. 1A, top panel, and Ref. 14). The cDNA sequence showed that its 3′-end terminated with a poly(A) sequence 22 residues in length and was preceded by two identical consensus polyadenylation signals (ATTAAA). A second, approximately 1.5-kb cDNA, obtained by screened a murine lung cDNA library, contained a 3′-end that extended beyond the sequence of the longest MT-MC1 cDNA. The original cDNA (Incyte EST 444412) was PCR-amplified from the region between nucleotides 1002 and 1530 of the sequence shown in Fig. 1A. The highest levels of expression were seen in lung (Fig. 1B, top panel), skeletal muscle (Fig. 1C, row e), heart (Fig. 1C, row d), and prostate (Fig. 1D, row d). Additional tissues consisted of brain (Fig. 1D, row a), eye (Fig. 1D, row b), liver (Fig. 1D, row c), kidney (Fig. 1D, row f), smooth muscle (Fig. 1D, row g), eye (Fig. 1D, row d), thymus (Fig. 1D, row f), submaxillary gland (Fig. 1D, row e), testis (Fig. 1D, row g), thymus (Fig. 1D, row f), submaxillary gland (Fig. 1D, row e), testis (Fig. 1D, row g), and uterus (Fig. 1D, row d).

To confirm that the larger transcript shown in Fig. 1A contained the additional 3′-sequence encoded by the longer of the two cDNAs, an identical Northern blot was hybridized with a 525-bp cDNA fragment derived from the region downstream of the poly(A) signal at position 902. As expected, only the larger transcript hybridized this fragment (Fig. 1A, middle panel). These results indicate that the two transcripts shown in the top panel of Fig. 1A are the result of alternative polyadenylation. A comparison of our longest cDNA with that of the longer transcript shown in Fig. 1A suggests that an additional 500–600 bp are absent from the cloned sequence.

The reading frame of the cDNA extended to the 5′-end of the molecule depicted in Fig. 1B. However, additional sequencing of genomic DNA, combined with nuclease protection and primer extension analyses, indicated that transcription initiated only about 50 nucleotides upstream of this sequence (not shown). Therefore, we tentatively assigned the first ATG at position 77 of Fig. 1B as the translational start site.
This codon resided within the context of an excellent Kozak consensus element (ACXCATG) (35). Conceptual translation of the cDNA sequence beginning at this site indicated the presence of a 188-amino acid protein. The predicted size of the protein (21.2 kDa) corresponded well with the observed apparent molecular mass as determined by SDS-PAGE of in vitro translated product (not shown).

A computer-assisted data base search for structural motifs within the MT-MC1 protein showed two putative cAMP phosphorolytic sites (amino acids 49–53 and 110–114), two casein kinase II phosphorolytic sites (amino acids 81–85 and 155–159), and four putative protein kinase C phosphorylation sites (amino acids 46–49, 61–64, 113–116, and 165–168). A potential bipartite nuclear localization signals (RRRR, amino acids 47–50) and RRPR, amino acids 62–65) was also noted (36). Sequence homology to other proteins in the GenBank, SwissProt, and EMBL data bases revealed MT-MC1 to be highly homologous (79% identity, 84% similar) to a putative human protein encoded by a cDNA clone of unknown function in the human genome (GenBank accession no. XP015265) that maps to chromosomal region 6q25, a region deleted in some lymphoid malignancies.

Both the human and mouse sequences initiated from the identical methionine codon of their conceptual reading frames, thus lending additional credence to our selection of this amino acid as the true initiation site. Additional homology (27% identity, 48% similarity) was noted between amino acids 15–109 of MT-MC1 and amino acids 133–222 of a putative Saccharomyces cerevisiae open reading frame of 276 amino acids (GenBank accession no. YGL096w) located on chromosome VII, which bears similarity to the Cup9p protein involved in copper homeostasis. However, the most significant degree of homology (50% identity, 53% similarity) was between amino acids 45–93 of MT-MC1 and amino acids 17–56 of the 65-amino acid-long nucleocapsid DNA-binding protein, p6.9/ORF100, of the B. mori nuclear polyhedrosis virus (GenBank accession no. NP047501.1).

Hybridization of the MT-MC1 coding sequence to a mouse RNA master blot (Fig. 1C) (CLONTECH) indicated that MT-MC1 was widely expressed, albeit at generally low levels.

Expression and Nuclear Localization of the MT-MC1 Protein Product—To obtain greater insight into the function of MT-MC1, we expressed it transiently as a full-length GFP fusion protein in COS-7 cells. Western blotting of total cell lysates showed the presence of the appropriately sized fusion protein (Fig. 2A, right lane). GFP-MT-MC1 was largely localized to the nucleus of the transfected cells, whereas control GFP was expressed much more diffusely (Fig. 2B).

In addition to the above transient transfection studies, we also created Rat1a fibroblast and 32D myeloid cell lines that stably expressed full-length, Myc epitope-tagged MT-MC1 protein. Immunofluorescence staining of the former cells confirmed the nuclear localization of MT-MC1 (Fig. 2B). Western analysis of individual Rat1a clones and of pooled 32D transfectants revealed the presence of the appropriately sized protein in all cases (Fig. 2D).

MT-MC1 Expression Does Not Affect Growth but Promotes Apoptosis—The c-Myc oncoprotein can exert profound effects upon growth, apoptosis, morphology, differentiation, and transformation (1–4). Therefore, we first determined whether overexpression of MT-MC1 in the Rat1a or 32D cells depicted in Fig. 2D affected their growth properties. As seen in Fig. 3A, all of the MT-MC1-expressing cells showed growth rates indistinguishable from those of control cell lines transfected with the empty parental vector as well as from those expressing c-Myc. We conclude that MT-MC1 does not significantly affect the proliferative capacity of these cells.

c-Myc overexpression may promote apoptotic cell death, both in Rat1a fibroblasts deprived of serum and in 32D cells deprived of IL-3 (37, 38). We therefore next studied the responses of the various MT-MC1-overexpressing cell lines after removal of their respective growth factors. As seen in Fig. 3, B–D, MT-MC1 overexpression caused a marked acceleration of apoptosis in both cell types. From these experiments, we conclude that the deregulated expression of MT-MC1 in two different cell types promotes apoptosis in response to growth factor deprivation.

MT-MC1 Promotes Genomic Instability—A recently described property of ectopic c-Myc overexpression is its ability to promote genomic instability (7, 9, 41, 42). In 32D myeloid cells, and in certain epithelial cell types, this is manifested by the acquisition of tetraploidy after exposure to mitotically poisons such as nocodazole or Colecemid (7, 9, 42). However, c-Myc overexpression by itself is generally insufficient to promote tetraploidy in the absence of such poisons unless accompanied by the concurrent inactivation of the p53 tumor suppressor or by the coexpression of ectopic cyclin B1 (7, 9). We compared the DNA profiles of various 32D cell lines (7, 9) either during
Fig. 3. Growth and apoptosis of cells expressing MT-MC1. A, growth curves of Rat1a and 32D cells. Left panel, Rat1a-neo cells, Rat1a-MT-MC1 clones 3, 4, and 16, or a clone of Rat1a cells expressing c-Myc (34) were plated in six-well plates at a concentration of $5 \times 10^4$
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MT-MC1 Inhibits Erythroid Differentiation—One of the hallmark features of c-Myc is its ability to inhibit differentiation in various \textit{in vitro} systems (1). This property was first identified in F-MEL cells, which have the capacity to differentiate along the erythroid pathway after exposure to chemical agents such as dimethyl sulfoxide (28, 43). To determine whether MT-MC1 could also inhibit F-MEL differentiation, we generated stable cell lines after transfection with either the MT-MC1 expression vector or the empty vector alone. We confirmed that the pooled stable transfectants expressed epitope-tagged MT-MC1 and were impaired in their ability to differentiate (Fig. 7).

The MT-MC1 Promoter Is a Direct c-Myc Target—We cloned and sequenced more than 2.4 kb of the MT-MC1 promoter. Multiple binding sites for the hematopoietic-specific transcription factors GATA1–3, TCF-2/NrF-1, MZF-1, and IK-2/LyF-1 (44–46) were identified within this region (Fig. 8A). Two E-box elements that conform to the consensus c-Myc binding site CACGTG were identified at positions 1278 and 74 relative to the putative start of mRNA transcription. These were designated sites A (CACCGR) and B (CATGTG), respectively. Two putative TATA elements were also localized near the 5'-end of the longest cDNA. Primer extension analysis and S1 nuclease mapping indicated that the more 5' of the two served as the primary site for directing mRNA transcription (not shown).

To assess the importance of the two E-box elements for c-Myc-Max binding \textit{in vitro}, we performed EMSAs using 32P-labeled, synthetic double-stranded oligonucleotides corresponding to the A and B sites. These were incubated with purified c-Myc-Max heterodimers and subjected to non-denaturing gel electrophoresis (30). As seen in Fig. 8, B and C (lanes 3 and 4), and as reported previously (30, 31), neither c-Myc nor the 151-amino acid isoform of Max (Max(S)) alone bound to either oligonucleotide, whereas both oligonucleotides were retarded significantly by c-Myc-Max complexes (lane 5 in each panel). Competitive titration for binding was seen at the lowest concentrations of cold input double-stranded oligonucleotides containing the wild-type E-box elements, whereas no competition was observed with a nearly 100-fold excess of cold mutant oligonucleotides (compare lanes 8 and 9). As expected, 32P-labeled double-stranded oligonucleotides encoding mutant A or B elements did not bind c-Myc-Max(S) heterodimers (lane 10).

To examine the functional relevance of the E-box elements, each of the individual mutations described above, or the double (A + B) mutant, was introduced into the full-length promoter sequence depicted in Fig. 8A. Luciferase reporter vectors containing the wild-type or mutant promoter elements were then generated and stably expressed in 32D-neo or 32D-c-Myc cells along with a \textit{β}-galactosidase expression vector as a way of normalizing for differences in transfection efficiencies among the various cell lines (2.2-fold). Based on these adjusted luciferase activities, the wild-type promoter was more than 8-fold more active in 32D-c-Myc cells than in 32D-neo cells (Fig. 8D). In contrast, all three of the mutant MT-MC1 promoter-luciferase reporters showed a reduced basal activity in 32D-neo cells and failed to be up-regulated in 32D-c-Myc cells.

The lower basal activities of each of the mutant promoters in

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cells/well in 10% serum. Individual wells were treated with trypsin daily, and the total number of viable cells/well was determined by manual counting. \textit{Right panel}, 32D-neo, 32D-MT-MC1, or 32D-c-Myc cells (14) were plated at a concentration of 5 x 10^4 cells/ml in a total volume of 2 ml in six-well plates. Viable cell counts were performed daily. B, apoptosis of cells expressing MT-MC1. \textit{Left panel}, Rat1a-neo cells, Rat1a-MT-MC1 clone 3 cells, and Rat1a-c-Myc cells were grown to ~50% confluence. The cells were then maintained for the remainder of the experiment in serum-free medium. Individual wells were treated with trypsin daily, and the total number of viable cells remaining was determined by trypsin blue exclusion. \textit{Right panel}, logarithmically growing 32D-neo and 32D-MT-MC1 cells were replated at a density of ~5 x 10^4 cells/ml into fresh medium containing 10% FBS but lacking IL-3. Viable cell counts were then performed at the indicated times. All points represent the average of triplicate determinations ± 1 S.E. C, phase-contrast micrographs and TUNEL assay results of Rat1a-neo and Rat1a-MT-MC1 clone 3 cells 4 days after removal of serum. D, phase-contrast and TUNEL assays of 32D-neo and 32D-MT-MC1 cells 16 h after plating in medium lacking IL-3. Although only the results with Rat1a-MT-MC1cl.3 cells are shown here, very similar results were observed with clones 4 and 16 (not shown).
FIG. 4. MT-MC1 promotes genomic instability. Propidium iodide-stained nuclei from the indicated logarithmically growing or Colcemid-treated 32D cell lines (Refs. 7 and 9) were analyzed by a fluorescence-activated cell sorter. Colcemid treatment (60 ng/ml) was for 16 h. Each experiment was performed at least three times with similar results. Typical histograms are presented. 2n, 4n, and 8n (tetraploid) populations are indicated.
32D-neo cells suggested that in addition to the wild-type promoter being regulated by c-Myc overexpression, it might also be regulated by endogenous levels of the oncoprotein. To test this, 32D-neo or 32D-MT-MC1 cells, each stably transfected with the wild-type or mutant promoter-luciferase vectors, were assayed for luciferase activity either during log phase growth or after 12 h of IL-3 deprivation. This length of time was chosen because it provides sufficient opportunity for the extremely
labile endogenous c-Myc protein ($t_{1/2}$ approximately 30 min) to be depleted, yet is well before any detectable apoptosis occurs (26, 27). We reasoned that if the wild-type MT-MC1 promoter were under the control of endogenous c-Myc, its level of expression should be reduced in 32D-neo cells deprived of IL-3, whereas in 32D-c-Myc cells, it would continue to be expressed. We monitored twice weekly for evidence of tumor growth. Each group contained five animals. Curves represent the average tumor size of each group of mice ± 1 S.E. B, gross appearance of tumors formed by Rat1a-c-Myc and Rat1a-MT-MC1 cells 70 days after inoculation. C, histologic appearance of tumors formed by Rat1a-c-Myc cells and Rat1a-MT-MC1 cells. The upper panels demonstrate the well encapsulated, dense, and relatively avascular nature of each tumor. Each of the lower panels demonstrates the relatively homogeneous and fibroblastoid appearance of typical cross-sections.
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Fig. 7. MT-MC1 inhibits erythroid differentiation. F-MEL cells were transfected by electroporation with linearized vectors encoding Myc epitope-tagged MT-MC1 or with the empty neo parental vector. Stable transfectants were selected in G418, pooled, and then exposed to 1.5% dimethyl sulfoxide. The percentage of benzidine-positive cells was determined daily (28). Each point represents the average of triplicate determinations ± 1 S.E. Inset, Western blots from each of the F-MEL cell lines showing expression of MT-MC1 protein or actin as a control for protein loading.

at elevated levels. Indeed, as seen in Fig. 8E, relative luciferase levels in 32D-neo cells were reduced —3-fold in response to IL-3 deprivation, whereas they remained unchanged in 32D-c-Myc cells. These results, together with those depicted in Fig. 8D, suggested that the MT-MC1 promoter is responsive not only to overexpressed c-Myc but to endogenous levels of the oncprotein as well. We were unable to determine the effect of endogenous c-Myc levels on any of the mutant MT-MC1 promoters because their basal level of expression was too near base line to obtain meaningful results (not shown). Nonetheless, these results indicate that the MT-MC1 promoter contains two E-box elements, each capable of binding c-Myc-Max complexes and controlling the level of promoter activity in response to overexpressed c-Myc and probably also in response to endogenous c-Myc.

Finally, as an independent way of confirming that the MT-MC1 promoter was a direct transcriptional target for c-Myc, we established a 32D cell line stably transfected with the Myc ER™ expression vector that encodes an inactive human c-Myc protein fused to a mutant estrogen receptor (47). The activation of c-Myc by the addition of 4-hydroxytamoxifen, resulted in the high-level expression of MT-MC1 transcripts, both in the absence and presence of concurrently added cycloheximide (Fig. 8F). Therefore, the up-regulation of MT-MC1 by c-Myc can occur in the absence of intervening protein synthesis.

MT-MC1 Regulates the Expression of Some c-Myc Target Genes —The foregoing results indicate that MT-MC1 and c-Myc regulate several common biological properties. It was therefore of interest to determine whether these proteins also regulated common c-Myc target genes. We have shown previously that ornithine decarboxylase enzyme activity directly mirrors the activity of c-Myc (14, 31). Therefore, cell extracts were prepared from Rat1a-neo, Rat1a-c-Myc, and Rat1a-MT-MC1 cells and assayed for ornithine decarboxylase enzyme levels. As shown in Fig. 9A, ornithine decarboxylase activity was nearly 9-fold higher in Rat1a-MT-MC1 cells and 5-fold higher in Rat1a-MT-MC1 cells, respectively, than in Rat1a-neo cells. In other experiments, total RNAs were isolated from Rat1a-c-Myc and Rat1a-MT-MC1 tumor cells or from Rat1a-neo cells and used to prepare Northern blots. These were then hybridized with several radiolabeled cDNAs encoded by c-Myc target genes. These included the chemokine platelet factor 4 (48); CD9, a member of the tetraspanin family (49); SM-20, an immediate early response transcript in arterial smooth muscle cells (50); lactate dehydrogenase-A (20); and EST 425279 (14). With the exception of the last, which is dramatically down-regulated by c-Myc in myeloid cells (14), all of these cDNAs correspond to transcripts previously shown to be positively regulated by c-Myc (14, 51). As seen in Fig. 9B, transcripts for platelet factor 4 and CD9 were up-regulated equally in both Rat1a-MT-MC1 and Rat1a-c-Myc cells but remained at low or undetectable levels in Rat1a-neo cells. SM-20 transcripts were up-regulated in Rat1a-MT-MC1 cells but not in Rat1a-c-Myc cells or Rat1a-neo cells, whereas lactate dehydrogenase-A transcripts were not up-regulated by either c-Myc or MT-MC1. Finally, EST 425279 transcripts were reduced dramatically in both Rat1a-MT-MC1 and Rat1a-c-Myc cells. Thus, the gene deregulation that occurs as a result of c-Myc overexpression is, at least to some degree, mimicked by the overexpression of MT-MC1.

In other experiments, we investigated the possibility that the observed biological effects of MT-MC1 might be the result of a positive feedback deregulation of c-Myc or other members of the Myc network (1). Therefore, some of the above blots were also rehybridized with cDNA probes corresponding to these genes. Rat1a-c-Myc cells demonstrated increased expression of c-Myc as expected; however, only basal levels of the transcript were seen in Rat1a-MT-MC1 cells and in Rat1a-neo. In no case were transcripts for either N-myc or L-myc detected. We also hybridized blots with cDNAs for Max (31, 33) and for each of the four known members of the Mad family, Mad1, Mxi1, Mad3, and Mad4 (52–54). Transcripts for Max and Mxi1 were expressed at equivalent levels in each of the three cell lines, whereas transcripts for Mad1 and Mad3 were not detected. A slight increase (approximately 2-fold) of Mad4 transcripts was seen in Rat1a-MT-MC1 and Rat1a-c-Myc cells in two of three experiments. From these experiments, we conclude that the common biological properties imparted by c-Myc and MT-MC1 do not involve significant deregulation of other major members of the Myc network.

DISCUSSION

The search for c-Myc-responsive genes has benefited enormously from the recent widespread application of DNA microarray technology (12–15, 55). The identification of a large number of both positive and negative c-Myc molecular targets has permitted the classification of many of their encoded proteins into generic functional classes pertaining to growth and metabolism, cell cycle control, intracellular signaling, differentiation, and adhesion (12, 51). Nonetheless, many c-Myc targets have resisted such simple categorization, and the vast majority have yet to be shown to recapitulate any of the known c-Myc phenotypes.

Given the large number of important cellular processes affected by members of the Myc network, together with the apparent complexity of the target gene population, it is remarkable that any individual target gene-encoded protein could recapitulate even a single c-Myc-like activity. Yet, several positive targets have been reported to possess such functions, although they are generally somewhat less potent than c-Myc itself. For example, the overexpression of ornithine decarboxylase, lactate dehydrogenase-A, Tmp, rcl, and HMG/AY have been reported to be transforming and/or tumorigenic (16, 19–22, 37), whereas ornithine decarboxylase and lactate dehydrogenase-A are also pro-apoptotic (17, 20). The overexpression of negatively regulated c-Myc targets such as p21Cip1 and gadd45 may block cell cycle entry (56, 57), whereas overexpression of the cyclin-dependent kinase inhibitor CDK4 can partially rescue the cell cycle progression defect of c-Myc null fibroblasts.
FIG. 8.  A, structure of the MT-MC1 promoter. White rectangle, 5'-flanking region; black rectangle, transcribed region. Two putative TATA elements are indicated. Based upon primer extension and nuclease protection assays, the upstream TATA element appears to be utilized
FIG. 9. A, regulation of ornithine decarboxylase levels in Rat1a-neo, Rat1a-c-Myc, and Rat1a-MT-MC1 cells. The indicated cells were harvested in log phase growth and assayed for the expression of ornithine decarboxylase (ODC) as described previously (51). The results show the average of triplicate determinations ± 1 S.E. B, regulation of c-Myc target gene transcripts by MT-MC1. Rat1a-neo and Rat1a-MT-MC and Rat1a-c-Myc tumor cell RNAs were used in Northern blotting experiments. Identical blots were probed with cDNAs corresponding to the coding regions of the indicated genes, several of which have been previously shown to be deregulated by c-Myc overexpression (14, 20). At the end of the experiment the blot hybridized with EST 425279 was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe as a control for RNA loading. WT, wild-type; PF4, platelet factor 4; LDH-A, lactate dehydrogenase-A.

 preferentially (not shown). The locations of two putative E-box elements, CACGTG (element A) and CATGTG (element B) are denoted, as are additional putative transcription factor binding sites. B, EMSAs of the E-box A site. Approximately 20 pg of a 32P-labeled double-stranded 18-bp oligonucleotide containing the element A E-box described above was used for each EMSA. Additions included nothing (e.g. oligonucleotide alone, lane 1), 20 ng of recombinant c-Myc protein (lane 3), 20 ng of recombinant Max(S) (lane 4), or 20 ng each of c-Myc + Max(S) (lane 5) (50). Lanes 6–8 are the same as lane 5 except that increasing amounts of unlabeled mutant A double-stranded oligonucleotide competitor were added. Lane 9 is the same as lane 5 except that 25 ng of cold A competitor was added. Lanes 3 and 10 show 32P-labeled double-stranded mutant A oligonucleotide in the absence or presence of c-Myc and Max(S), respectively. C, EMSAs of the E-box B site. All reactions were performed as described in B except that 32P-labeled wild-type or mutant B site E-box 18-mers were used. D, luciferase assays. 32D-neo or 32D-MT-MC1 cells were cotransfected with 1 μg of linearized pCMV-β-galPuro and 5 μg of linearized luciferase expression vectors containing the promoter region shown in A. The promoter contained the wild-type E-box elements or the A, B, or A+B mutations. Stable transfectants were pooled and assayed for luciferase after first correcting for differences in β-galactosidase activity (1 <2-fold differences in all cases). The results show the average of three independent experiments ± 1 S.E. E, the MT-MC1 promoter is sensitive to endogenous levels of c-Myc. 32D-neo and 32D-c-Myc cells, each stably transfected with the wild-type MT-MC1 promoter-luciferase reporter vector, were assayed for luciferase activity either in the presence of IL-3 or 12 h after its removal. Note that in 32D-neo cells the activity of the promoter was reduced significantly in the absence of IL-3 (second versus first bar), whereas in 32D-c-Myc cells, no such regulation was observed (third and fourth bars). Although the results in the third and fourth bars are expressed relative to those in the first bar, absolute luciferase activities were ~6-7-fold greater, thus confirming the results shown in D. The results shown are the average of three independent determinations ± 1 S.E. F, MT-MC1 expression is up-regulated by c-Myc in the presence of cycloheximide. 32D cells were stably transfectected with the pBABE-puroMycER™ expression vector. Pooled puromycin-resistant clones were then induced with 250 ng 4-hydroxytamoxifen in the presence or absence of 10 μg/ml cycloheximide for 6 h. Total RNA was then extracted and subjected to Northern blotting and hybridization with a coding region cDNA probe for MT-MC1 (upper panel) or stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (lower panel).
on either growth factor requirement (Fig. 3A), or cell size (5).

This less than complete c-Myc phenotype of MT-MC1 is in keeping with the prevailing notion that the participation of many other genes is necessary for achieving the full range of c-Myc properties (51). It also suggests that cells expressing MT-MC1 might be used in complementation assays as a way of determining whether other c-Myc targets can fully recapitulate the c-Myc phenotype.

We have found that MT-MC1 overexpression neither induces c-Myc nor deregulates other members of the Myc oncoprotein network. However, MT-MC1 does regulate certain c-Myc target genes (Fig. 9). These observations, as well as others in this report, are consistent with a model in which c-Myc is a direct upstream transcriptional activator of MT-MC1. However, the well known ability of c-Myc to activate some target genes in the absence of intervening protein synthesis suggests that de novo synthesized MT-MC1 is not obligatory for c-Myc transcriptional activity. Thus, c-Myc and MT-MC1 may act independently of one another, perhaps amplifying or complementing one another’s activities. Alternatively, the full function of c-Myc might require only preexisting, endogenous levels of MT-MC1. Such cooperativity between c-Myc and MT-MC1 might provide another explanation as to how the expression of some targets can, in the face of protein synthesis inhibition, continue well beyond the point of detection of the short lived c-Myc protein (12, 19, 22).

Despite the ability of MT-MC1 to regulate at least some c-Myc targets, we do not yet understand how this occurs. We also do not know whether MT-MC1 binds directly to regulatory elements within these genes or whether the regulation is more indirect, perhaps involving an interaction between MT-MC1 and other transcription factors or coactivators. It will clearly be important in future work to delineate the precise mechanistic differences between c-Myc and MT-MC1 with regard to their effects on c-Myc target gene expression.

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