MAPPING THE FUNCTIONAL DOMAINS INVOLVED IN DOWN-REGULATION OF THE c-myc PROTOONCOGENE*

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Aruna Subramanian‡ and Donald M. Miller§

From the ‡Comprehensive Cancer Center and Department of Biochemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294-3300 and the §James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky 40206

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† To whom correspondence should be addressed: 529 S. Jackson St., Louisville, KY 40206; Tel.: 502-562-4790; Fax: 502-562-4368; E-mail: donaldm@uhl.org.

1 The abbreviations used are: MBP, Myc-binding protein; EMSA, electrophoretic mobility shift assay; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; CMV, cytomegalovirus; DOTAP/DOPE, 1,2 dioleyl glycero 3-phosphoethanolamine/3-trimethyl ammonium propane.

2 D. Chaudhary, A. Subramanian, R. Ray, and D. M. Miller, submitted for publication.

3 A. Subramanian, J. O. Trent, and D. M. Miller, unpublished data.

Structural Analysis of α-Enolase

Myc-binding protein-1 (MBP-1) is a 37-kDa protein with sequence homology to the 3’ portion of the α-enolase gene. α-Enolase is a 48-kDa protein, which plays a critical role in the glycolytic pathway. MBP-1 binds to the c-myc P2 promoter and down-regulates c-myc expression. We have investigated the role of α-enolase in regulation of the c-myc protooncogene. RNase protection assay shows that α-enolase is transcribed into a single RNA species in HeLa cells. A start codon, 400 base pairs downstream of the α-enolase ATG, corresponds to the MBP-1 ATG, suggesting that MBP-1 is an alternative translation initiation product of the α-enolase RNA. Domain mapping was performed using constructs containing truncations of the α-enolase gene. In vitro binding to the c-myc gene was abolished after deletion of the N-terminal portion of α-enolase. In order to determine the relationship between DNA binding activity and transcription inhibition, we performed co-transfection assays in HeLa cells. These studies confirmed that an N-terminal deletion of α-enolase is unable to down-regulate c-myc promoter activity. Our data suggest that α-enolase plays an important role in regulation of c-myc promoter activity in the form of an alternative translation product MBP-1, which is distinct from its role as a glycolytic enzyme.

The c-myc protooncogene is a DNA-binding phosphoprotein that plays an important role in the regulation of cell growth and differentiation (1, 2). Regulation of c-myc gene expression is quite complex and involves several mechanisms, including changes in transcription initiation and elongation, RNA stability and turnover, and translation (3, 4). Overexpression of the c-myc gene is a common characteristic of many malignant cell types (5). The human c-myc protooncogene contains two TATA box sequences separated by about 165 base pairs located near the 5’ end of the first exon (6). The transcription of c-myc from P1 and P2 is regulated by a composite of positive and negative elements located both upstream and downstream of the promoters (7–10).

A human cDNA clone encoding MBP-1 was detected by screening a HeLa cell cDNA library. The Myc-binding protein-1 (MBP-1)1 is a 37-kDa human c-myc promoter-binding protein that binds in a region +123 to +153 relative to the c-myc P2 promoter (11). MBP-1 is a negative regulator of c-myc expression and binds in the minor groove of the c-myc P2 promoter simultaneously with the TATA-binding protein (12). Consistent with its negative regulation of c-myc and as a potential tumor suppressor protein, transfection of human breast carcinoma cells with MBP-1 cDNA results in inhibition of tumor formation in nude mice (13). Exogenous expression of MBP-1 has been suggested to play an important role in the regulation of human immunodeficiency virus-1 replication in infected cells (14). Careful sequence reanalysis of MBP-1 has shown that it has extensive homology to the sequence of the 3’ portion of the α-enolase gene (11, 15).

Enolase is the glycolytic enzyme that catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high energy intermediates that generate ATP in glycolysis (16). The MBP-1 cDNA shares sequence homology with the α-enolase cDNA, which encodes a 1.8-kb mRNA and a polypeptide of about 48 kDa. The high degree of sequence homology is confined to the 1.4-kb 3’ region of α-enolase and the full-length 1.4-kb MBP-1 and suggests that α-enolase and MBP-1 are both products of the α-enolase gene.2 The presence of an ATG start codon followed by the Kozak sequence suggests that MBP-1 may be the product of alternate translation initiation from an in frame internal translation initiation site 400 bp downstream on the α-enolase cDNA (Fig. 1A).

Western blot analysis using an antibody specific to non-neuronal enolase from human brain (Biogenesis) has identified both 48- and 37-kDa proteins in HeLa nuclear extracts (Fig. 1B). The cellular localization of α-enolase is known to be predominantly cytosolic. The function of MBP-1 as a down-regulator of c-myc gene expression suggests that it would be localized in the nucleus. HeLa cell extract made using Promega reporter lysis buffer (prepared as described under “Experimental Procedures”) is primarily cytosolic and does not show the presence of MBP-1.

The presence of MBP-1 in nuclear extracts corroborates with its role in down-regulation of c-myc promoter activity. α-Enolase constructs are able to down-regulate c-myc promoter activity, albeit to a lower extent than MBP-1.3 On the other hand, preliminary experiments indicate that MBP-1 does not have enolase enzyme activity.4 Here we have studied the structure-function relationship of α-enolase as a negative regulator of c-myc activity using DNA binding studies and transfection
**EXPERIMENTAL PROCEDURES**

RNsase Protection Assay—The α-enolase fragment from 181 to 600, containing the MBP-1 start site, was PCR-amplified and subcloned into the pBluescript II SK vector (Stratagene). In order to make an antisense RNA probe, the vector containing the 420-base pair α-enolase fragment was linearized with XhoI and in vitro transcribed with T3 RNA polymerase and [α-32P]CTP, using a Maxiscript kit (Ambion). Labeled probe was purified on 5% acrylamide, 8 M urea denaturing gel and eluted overnight at 37 °C. RNase protection assay was carried out using the RPA II kit (Ambion). The antisense cRNA probe (1 × 10^6 cpm) was hybridized overnight at 37 °C with increasing concentrations of HeLa nuclear extract and then digested with RNase A (0.5 units) and RNase T1 (20 units) for 30 min at 37 °C. Hybridization was also performed with in vitro transcribed α-enolase and MBP-1 RNA as controls. Following ethanol precipitation, protected fragments were separated on a 6% acrylamide, 8 M urea denaturing gel. The sizes of the protected fragment were determined by running a labeled Century RNA marker (Ambion) alongside.

Plasmid Construction—The N-terminal truncations of α-enolase were generated by PCR using upstream primers that contained the start codon. Downstream primers containing the stop codon were used to PCR-amplify the C-terminal deletions. The PCR products were then cloned directly into the PCR 2.1 vector using the TA cloning kit (Invitrogen). From PCR 2.1, they were excised and cloned directionally into the pCITE (Novagen) and pBluescript (Stratagene) vectors under control of the T7 promoter. The full-length α-enolase cDNA was also cloned into these vectors. The pCITE clones were used for in vitro transcription and translation, while the pBluescript clones were expressed in BL21 (DE3) cells. For transfection assays, the α-enolase cDNA and its deletion mutants were cloned into the pBCKCMV vector (Stratagene) under control of the CMV promoter.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange kit from Stratagene. Full-length α-enolase cloned into the pCITE vector was used as the template for mutagenesis of the MBP-1 ATG. Two oligonucleotide primers, each complementary to the opposite strands of the vector, and containing a G→C mutation were designed and extended by PCR following the manufacturer’s instructions. After incubating the PCR products with DpnI to digest dam-methylated E. coli DNA, the plasmid was transformed into competent cells. To make two G→C mutations, α-enolase DNA containing the first mutation was used as the template with a new set of primers containing the second mutation. The plasmid DNA obtained from the cells was sequenced to determine the presence of the mutations. The point-mutated α-enolase in the pCITE vector under control of the T7 promoter were linearized with XhoI downstream of the insert and used for in vitro transcription and translation. The point-mutated α-enolase DNAs were also cloned into the pBCKCMV vector for transient transfection assays.

**In Vitro Transcription and Translation**—The pCITE vector containing the full-length and truncated α-enolase cDNA under control of the T7 promoter was linearized at the XhoI site downstream of the coding sequence. RNA was generated by in vitro transcription using the ME-GAscript T3 kit (Ambion). RNA transcripts were quantified by absorbance at 260 nm and ethidium bromide staining on an agarose gel after verification of their integrity. In vitro translation in rabbit reticulocyte lysate (Red Nova lysate from Novagen) was performed as per instructions. Translation reactions were performed with [35S]methionine, and the in vitro translated products were analyzed directly by electrophoresis on a 12.5% SDS-polyacrylamide gel. This procedure was also followed for the point mutants of α-enolase generated by site-directed mutagenesis.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA was performed as described previously with some modification (17). Full-length α-enolase and its deletion mutants cloned into the pBluescript vector were expressed in BL21 (DE3) cells and isopropyl-1-thio-β-D-galactopyranoside induced as described previously (11). The induced proteins were separated on a 12.5% SDS-polyacrylamide gel and analyzed by Coomasie staining. The 45-bp double-stranded oligonucleotide (GGAGGAGATCGCGCTGTTAAGTGATATAAAAGCCGGTTTTCGGGGCTTTATCG) was used as unlabeled competitor. 15 μg of the extracts prepared from the induced cultures were incubated with the probe (2 ng, 10^6 cpm) in the EMSA buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 1 mM dithiothreitol, 0.05 mM EDTA, 2.5 mM MgCl2 and 6% glycerol) in the presence of 2 μg of poly(dI-dC)·poly(dI-dC) on ice for 30 min. Unlabeled competitor oligonucleotide or 2 μl of antibody (1 μg/μl) were incubated with the protein for 30 min on ice before addition of the labeled oligonucleotide. The resulting complexes were then separated on a native 5% polyacrylamide gel at room temperature in 1× Tris borate-EDTA at 10 V/cm. After electrophoresis, gel retardation was visualized by autoradiography.

**Cell Line**—The HeLa human cervical carcinoma cell line was stably transfected with the luciferase reporter gene under control of the c-myc P2 promoter. These stably transfected cells were called MYC1 cells. All transient transfection assays were performed in MYC1 cells.

**Transfection**—MYC1 cells were plated at an initial density of 5 × 10^4 cells/well of a 24-well plate in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Immediately prior to trans-
fection, cells were washed three times with sterile phosphate-buffered saline. Transient transfection of the MYC1 cells with pBKCVM clones of full-length α-enolase and its mutants was carried out using the lipid DOTAP/DOPE method. To normalize the transfection efficiency, the SV40-based β-galactosidase expression plasmid (SV40-β-gal) was co-transfected. To transfect one well, 8 μg of DOTAP/DOPE (1 μg/ml) was mixed with 1 μg of each vector and incubated at room temperature for 15 min. The liposome/DNA complexes were mixed with 500 μl of serum free media and added to each well. Plates were incubated for 4 h at 37 °C, 500 μl of Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum was then added, and cells were incubated for 24 h at 37 °C.

Luciferase Assays—Cell extracts were made by lysing the cells in each well of a 24-well plate with 100 μl of freshly diluted 1× Reporter lysis buffer (Promega, selected for its low background in protein assays), which allows extracts to be used for Western blot analysis, lucerase, and β-galactosidase assays. Lysis was performed for 30 min at room temperature with rocking. Lysate was transferred to a 1.5-ml polypropylene tube and centrifuged at 16,000 × g for 4 min to pellet cell debris. The HeLa cell extracts prepared in this manner are primarily cytoplasmic with minimal or no nuclear material. Supernatant was transferred to a fresh tube and protein concentration determined with the Bio-Rad protein assay kit according to the manufacturer’s protocol (Bio-Rad). Cell extracts were assayed for both β-galactosidase and lucerase activity at 24 h after transfection. 50 μl of extract was added to 10 μl of lucerase assay substrate (Promega) in a clear 12 × 75-mm polystyrene tube. Samples were read immediately on a luminometer (Analytical Luminescence Laboratory) and light production (relative light units) measured for 10 s. Each value of lucerase activity is from at least three independent experiments, each performed in triplicate.

Western Blotting—Cell extracts were made as described above from the MYC1 cells transfected with the pBKCVM expression vectors encoding full-length α-enolase or its mutants under control of the CMV promoter and analyzed by Western blot analysis. The proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (Millipore) membrane by electroblotting overnight (15 V). The α-enolase proteins were detected using an antibody specific to non-neuronal enolase from human brain raised in sheep as a host (Biogenesis) and a chemiluminescence kit (ECL detection, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. HeLa whole cell and nuclear extracts containing 5 μg of protein were also analyzed by Western blot analysis.

Northern Blotting—RNA was isolated from the transiently transfected MYC1 cells and analyzed for levels of expression of message from the transfected constructs by Northern hybridization. The probe was the 1.8-kb full-length α-enolase cDNA labeled with [α-32P]dCTP using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech). Hybridizations were performed at 42 °C overnight in 5 ml of formamide and 100 μl of denatured salmon sperm DNA. Filters were washed at moderate stringency (0.1× SSC, 1% SDS, 42 °C) and exposed to x-ray film overnight.

RESULTS

Expression of the α-Enolase Transcript—Although we have hypothesized that MBP-1 and α-enolase are alternate translation products of a single α-enolase mRNA, it is important to document this fact. RNase protection assay was used to analyze expression of the α-enolase transcript. A cRNA antisense probe of 420 nucleotides corresponding to nucleotides 181–600 of α-enolase, containing the MBP-1 start site at position 386, was synthesized together with Century RNA markers (Ambion). The radiolabeled cRNA probe was hybridized to total RNA derived from HeLa cells and to in vitro transcribed α-enolase and MBP-1 RNA as control (Fig. 2). In total HeLa cell RNA, a 420-nucleotide fragment was protected corresponding to the fragment observed in the in vitro transcribed α-enolase mRNA control lane. The MBP-1 control RNA protected a 220-nucleotide fragment that was not seen in the HeLa cell RNA. These data confirm our hypothesis that expression of the α-enolase gene gives rise to a single transcript and that an MBP-1-specific mRNA is not transcribed. This would also indicate that MBP-1 is not a product of alternative RNA splicing.

Site-directed Mutagenesis of MBP-1 ATG on α-Enolase DNA—In order to confirm that MBP-1 is a product of translation initiation from an internal ATG on the α-enolase cDNA, site-directed mutagenesis was performed (Fig. 3A). The ATG codon for methionine at position 97 of α-enolase was transformed into the ATC codon for isoleucine (Enomut1). This mutation failed to abolish translation of the MBP-1 protein from the α-enolase cDNA. This may have been due to the presence of another in frame ATG at position 377 of α-enolase, six bases upstream of the first ATG, coding for methionine 94, which could have been used for translation. After mutating methionine 94 to isoleucine (Enomut2), a 37-kDa MBP-1 protein band was still visible. Site-directed mutagenesis at both positions was performed on the same template DNA and the resulting α-enolase cDNA (Enomut3) translated into a single protein of 48 kDa (Fig. 3B).

In order to determine if the α-enolase protein could down-regulate c-myc promoter activity in the absence of MBP-1 translation, Enomut3 cloned under control of the CMV promoter was used in transient transfection assays as described under “Experimental Procedures.” Extracts from the MYC1-transfected cells were analyzed by Western blot, and similar levels of protein were observed (Fig. 3C). Luciferase assay results show that, although full-length α-enolase down-regulates c-myc promoter activity by about 40%, Enomut3, which does not generate MBP-1 on translation, is able to repress it by less than 20% (Fig. 4). This indicates that the c-myc down-regulating activity of α-enolase lies in the alternative translation product MBP-1.

Construction and Expression of α-Enolase Deletion Mu-
A

![Diagram](image)

**Fig. 3.** A, the drawing shows the locations of the separate site-directed mutations introduced into the α-enolase cDNA. Mutations were verified by nucleotide sequencing of the mutated regions. B, in vitro translation of α-enolase RNA. In vitro transcribed RNAs from MBP-1 cDNA and from the wild type and point-mutated α-enolase cDNAs were translated in a rabbit reticulocyte lysate system, and the products were analyzed as described in "Experimental Procedures". Lane 1, negative control; lane 2, wild type α-enolase generating α-enolase and MBP-1 proteins; lane 3, MBP-1; lane 4, ATG (coding methionine 97) → ATC mutant; lane 5, ATG (coding methionine 94) → ATC mutant; lane 6, ATG (coding methionine 94 and 97) → ATC mutants. MBP-1 translation is abolished. C, Western blot analysis. The MBP-1 cDNA and wild type and point-mutated α-enolase cDNAs under control of the CMV promoter were transfection into HeLa cells and expression assayed by immunoblotting with α-enolase antibody. The α-enolase and MBP-1 bands are indicated. Lane 1, pure human α-enolase positive control; lane 2, pBKCMV vector control showing endogenous α-enolase expression; lane 3, CMV-α-enolase; lane 4, CMVMBP-1; lane 5, CMV promoter controlled ATG → ATC mutations of methionines at position 94 and 97 of α-enolase (CMVEnomut3).

**Diagram Descriptions:**

1. **A**: Diagram showing the locations of the separate site-directed mutations introduced into the α-enolase cDNA. Mutations were verified by nucleotide sequencing of the mutated regions.
2. **B**: Diagram illustrating in vitro translation of α-enolase RNA. In vitro transcribed RNAs from MBP-1 cDNA and from wild type and point-mutated α-enolase cDNAs were translated in a rabbit reticulocyte lysate system, and the products were analyzed as described in "Experimental Procedures".
3. **C**: Western blot analysis showing expression of α-enolase and MBP-1 bands.

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**Fig. 4.** Regulation of c-myc promoter activity in MYC1 cells. The effect of MBP-1 and of wild type and point-mutated α-enolase on c-myc promoter activity was measured as luciferase activity in transfected MYC1 cells (see "Experimental Procedures"). To eliminate the influence of transfection efficiency, all data from the luciferase assays were normalized against β-galactosidase activity and presented as the mean ± S.D. from at least three independent experiments, each performed in triplicate. The activity of the c-myc promoter when co-transfected with control (empty) pBKCMV expression vector was assigned a value of 100.

**P2 Promoter**—It has been shown previously that MBP-1 binds to the c-myc P2 promoter (11). EMSA was performed with a 50-base pair labeled c-myc probe (see "Experimental procedures") and lysates from induced BL21(DE3) cells expressing α-enolase and its deletion mutants. The *in vitro* translated full-length and truncated α-enolase proteins were initially used for gel shift analysis. However, addition of any of the *in vitro* translated proteins to the 50-base pair labeled c-myc oligonucleotide caused a shift to the same extent in every lane. We soon realized that endogenous enolase from Rabbit reticulocyte lysate (in which the *in vitro* translation is carried out) interfered with the EMSA. In order to get around the problem of interference from endogenous α-enolase, bacterially expressed proteins were made and used in EMSAs. The crude bacterial extracts, when analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie-stained, indicated the presence of the translation products from full-length α-enolase and its deletion mutants. However, the smaller peptides due to translation from the internal initiation site on the C-terminal deletions, EnoΔ242–434 and EnoΔ373–434, were not observed (data not shown). A specific DNA-protein complex was visualized by autoradiography in all the α-enolase deletion mutants except EnoΔ1–236. The visible DNA-protein complexes were not disrupted upon addition of 100-fold molar excess of a mutant cold competitor (Fig. 6A). The unlabeled oligonucleotide used as competitor has been described previously (11) and has a mutation that prevents binding of MBP-1 to the DNA. A polyclonal α-enolase antibody was able to bind to and supershift the DNA-protein complexes (Fig. 6B). An antibody to human c-myc was unable to supershift the full-length α-enolase-DNA complex, indicating that the supershifts obtained using the α-enolase antibody are specific. Deletion of amino acids 1–236 of α-enolase prevents the protein from binding to the c-myc P2 promoter. These results indicate that the DNA binding region of the α-enolase protein lies between amino acids 96 and 236.

**Down-regulation of c-myc Promoter Activity by α-Enolase and Its Deletion Mutants**—MBP-1 has been shown to downregulate c-myc promoter activity (11). HeLa cells stably transfected with the luciferase reporter gene under control of the c-myc promoter (MYC1 cells) were transiently transfected with α-enolase and its deletions under control of the CMV promoter.
Northern and Western blot analysis confirmed expression of the mutated forms of α-enolase in the transfected cells (Fig. 7, A and B). Similar levels of RNA were expressed from α-enolase and its truncations in the MYC1 cells. Levels of protein expression from α-enolase and its deletion mutants were quantified by densitometry and found to be comparable. The shorter peptide of 26 kDa translated from the internal initiation site of the C-terminal deletions EnoD373–434 is also generated upon transfection into MYC1 cells and can be seen in Fig. 7B. The 15-kDa peptide generated by translation initiation from the internal ATG on EnoD242–434, which ran along with the dye front on the SDS gel, is not shown in Fig. 7B. However, the amount of protein translated from the internal ATG is far less than that from the first ATG of these constructs. Hence, the effect observed on c-myc promoter activity in transient transfection assays is thought to be predominantly due to the larger proteins.

The effect of α-enolase and its deletion mutants on c-myc promoter activity were measured as luciferase activities in transfected MYC1 cells. The results indicate that EnoD1–236 does not down-regulate c-myc promoter activity as efficiently as full-length α-enolase (Fig. 8). The MBP-1 protein down-regulates c-myc promoter activity by 65%. These results correspond with the EMSA results and show that the DNA binding and c-myc down-regulating activity of α-enolase lies between amino acids 96 and 236.

**DISCUSSION**

The role of α-enolase as a glycolytic enzyme has been very well characterized. The α-enolase gene is transcribed into a single RNA species, as proven by the RNase protection assay. Here we show that at least two proteins arise from the alternative usage of translation initiation sites present on the α-enolase mRNA. MBP-1, which negatively regulates c-myc promoter activity, was initially identified from a human cervical carcinoma cell expression library. Previous work (13) has demonstrated that this alternate translation product of the α-enolase gene acts as a tumor suppressor when transfected into human breast carcinoma cells, largely preventing anchorage-independent growth and the growth of tumors in nude mice.

We examined the MBP-1 and α-enolase cDNA sequences and observed complete sequence homology between the MBP-1 sequence and the 1.4-kb 3′ region of α-enolase. A potential translation initiation site at codon 97 of α-enolase was observed, and the sequence surrounding this ATG triplet exhibits an overall sequence homology to the Kozak consensus cassette (18).

In a construct in which the ATG at codon 97 on the α-enolase cDNA was mutated to ATC (Enomut1), MBP-1 translation was not abolished. Another in-frame ATG at codon 94 was observed and, after mutation of this ATG to generate Enomut2, α-enolase and MBP-1 continued to be translated. However, when both the ATGs were mutated (Enomut3), α-enolase was the sole product of translation. Because Enomut3 gives rise to just the 48-kDa α-enolase, we can exclude the possibility that MBP-1 arises from proteolytic cleavage of the complete protein. Western blot analysis of pure human α-enolase shows the presence of a single band of ~48 kDa. Even after incubation of the pure human α-enolase protein in a transcription/translation system, no smaller fragment the size of MBP-1 could be observed by Western blotting (data not shown). Full-length α-enolase cDNA after in vitro transcription/translation gives rise to
both α-enolase and MBP-1 protein bands. The ratio of these two proteins remains constant when checked on a gel after storage for a considerable period of time. These results further confirm that MBP-1 is not a product of proteolytic cleavage of α-enolase.

The single α-enolase mRNA is alternatively translated from methionine 94 or 97 to yield MBP-1. Our data do not allow us to distinguish whether MBP-1 is translated from the codon for methionine at position 94 or 97 on the α-enolase mRNA.

Of the two isoforms of α-enolase, MBP-1 better down-regulates c-myc promoter activity. In transient transfection assays in HeLa cells, α-enolase is unable to down-regulate activity of the c-myc promoter efficiently after mutation of the internal translation initiation site to prevent translation of MBP-1. The ~20% down-regulation of c-myc promoter activity observed

**Fig. 6.** A, electrophoretic mobility shift assay of 32P-labeled, 50-base pair c-myc P2 promoter with full-length α-enolase and its deletion mutants expressed in BL21(DE3) cells was performed as described under “Experimental Procedures.” The position of the unbound probe is indicated. Lane 1, labeled probe; lane 2, vector-transformed bacterial extract is used as control; lanes 3–7, 15 μg of bacterially expressed protein as indicated; lane 8, 100-fold molar excess of unlabeled 50-bp c-myc probe is added as competitor; lanes 9–13, 100-fold molar excess of unlabeled mutant Myc oligonucleotide is added as competitor to show the specificity of the DNA-protein complexes formed. The protein used in each reaction is indicated on the top of each lane.

**Fig. 7.** A, Northern blotting of mRNAs from transfected HeLa cells. Total RNA was purified and analyzed by Northern blotting as described under “Experimental Procedures” using the 32P-labeled, 1.8-kb α-enolase cDNA as probe. The plasmid used for transfection is indicated on the top of each lane. Size standard RNAs are indicated. Blots were stripped and reprobed with a labeled β-actin cDNA probe to control for errors in gel loading. B, expression of full-length α-enolase and deletion mutants in transfected HeLa cells was analyzed by immunoblotting with antibody to α-enolase. The positions of the bands in kDa are indicated.

**Fig. 8.** The effect of the α-enolase deletion mutants on c-myc promoter activity was measured as luciferase activity in transfected HeLa cells as described under “Experimental Procedures.” The data shown have been normalized to β-galactosidase activity and are the ±S.D. from at least three independent experiments each performed in triplicate.
after transfection with Enomut3 is due to the binding of
the full-length -enolase to the c-myc P2 promoter. These results
suggest that the -enolase gene is bifunctional, encoding two
proteins, one of which has a role in glycolysis and the other in
regulation of c-myc expression. Evidence to suggest that -enolase may have functions other
than as a glycolytic enzyme has been generated earlier in yeast,
other vertebrates, and mammalian cells (19, 20). These include
either a direct function or indirect role in processes such as
thermal tolerance, growth control, and hypoxia tolerance (21).
A structural role in the lens of some species has been exhibited
by -enolase (22). It also functions as a cell surface receptor for
plasminogen, resulting in enhanced plasminogen activation
and localization of the proteolytic activity of plasmin on cell
surfaces (23). The presence of -enolase on the surface of patho-
genic streptococci has recently been demonstrated (24). The
streptococcal surface enolase is thought to play an important
role in the disease process and in post-streptococcal autoim-
nune diseases.
Our results demonstrate that MBP-1 is a product of internal
translation initiation from the -enolase gene. Internal initia-
tion has been described for other genes such as those for C/EBP
and . Myc proteins are amplified in many tumors, and their 
expression has been described for other genes such as those for C/EBP
and . The existence of two imperfect CACGTG motifs (5 out of 6 bases match) in the
c-myc enhancer sequence (31–34). The two GATA-1 isoforms share identi-
fication of energy metabolism and growth control.
For blocking gluconeogenesis. Although there is no direct evi-
dence for the presence of two imperfect CACGTG motifs (5 out of 6 bases match) in the
the MBP-1 isoform of -enolase, which has been shown to down-regulate c-myc expression by 65%.
This result is consistent with the previously published work of Ghosh et al. (40), which demonstrated transcriptional repres-
sion activity in the N-terminal portion of MBP-1. Our data show,
however, that DNA binding activity correlates nicely with ability to inhibit transcription of c-myc. As seen by mu-
tating methionine 94 and 97, the c-myc down-regulating activ-
ity of -enolase is lost, by abolishing translation of the MBP-1 isoform. Thus, it is possible that the bifunctional role of -enolase could be modulated by the varying ratio of the two isoforms.
The existence of two -enolase isoforms with distinct func-
tions presents a unique example of a gene encoding proteins
with roles in metabolism and cell proliferation. Our data sug-
gest that, while the -enolase isoform functions as the glyco-
lytic enzyme, the N-terminal region of the MBP-1 isoform is
important in binding to and down-regulating expression of the
c-myc gene. The MBP-1 isoform has been shown to inhibit
anchorage-independent cell growth and tumor growth in nude
mice (13). The manner in which the coding capacity of the
- enolase mRNA for the two protein isoforms is regulated has
not been ascertained. Internal ribosome entry constitutes a
novel mechanism of gene expression regulation. This has been
shown in the case of FGF-2, whose CUG-initiated isoforms are
translationally activated in response to stress (41). Whether
this kind of initiation from the presence of an internal ribosome
entry site occurs in the case of MBP-1 remains to be
determined.

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