ENO1 gene product binds to the c-*myc* promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1)

Salvatore Feo[a,b,*], Daniela Arcuri[a], Eugenia Piddini[a,1], Rosa Passantino[c], Agata Giallongo[c]

[a] *Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Viale delle Scienze, Parco D'Orleans, 90128 Palermo, Italy*
[b] *Centro di Oncobiologia Sperimentale, Viale delle Scienze, 90128 Palermo, Italy*
[c] *Istituto di Biologia dello Sviluppo del Consiglio Nazionale delle Ricerche, Via U. La Malfa 153, 90146 Palermo, Italy*

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Abstract The Myc promoter-binding protein-1 (MBP-1) is a 37–38 kDa protein that binds to the c-*myc* P2 promoter and negatively regulates transcription of the protooncogene. MBP-1 cDNA shares 97% similarity with the cDNA encoding the glycolytic enzyme α-enolase and both genes have been mapped to the same region of human chromosome 1, suggesting the hypothesis that the two proteins might be encoded by the same gene. We show here data indicating that a 37 kDa protein is alternatively translated from the full-length α-enolase mRNA. This shorter form of α-enolase is able to bind the MBP-1 consensus sequence and to downregulate expression of a luciferase reporter gene under the control of the c-*myc* P2 promoter. Furthermore, using α-enolase/green fluorescent protein chimeras in transfection experiments we show that, while the 48 kDa α-enolase mainly has a cytoplasmic localization, the 37 kDa α-enolase is preferentially localized in the cell nuclei. The finding that a transcriptional repressor of the c-*myc* oncogene is an alternatively translated product of the ENO1 gene, which maps to a region of human chromosome 1 frequently deleted in human cancers, makes ENO1 a potential candidate for tumor suppressor.

Key words: Enolase; DNA binding; Transcriptional repression; Alternative translation

1. Introduction

The c-*myc* protooncogene is a critical factor in the control of cell proliferation, differentiation and apoptosis [1–3]. Perturbation of c-*myc* gene expression occurs in different tumors in a variety of species and this is thought to be a crucial step in tumorigenesis [2,4]. The pivotal role of c-*myc* in regulating cell proliferation and cell death is consistent with the observations that its expression is tightly controlled in normal cells and downregulation at both mRNA and protein level is a prerequisite for differentiation in many cell types [2]. The expression of the c-*myc* gene is extremely complex and is controlled by several mechanisms, including changes in transcription initiation and transcript elongation, as well as stability, turnover and translational state of mRNA [2]. At the level of transcription initiation multiple promoters, termed P0, P1, P2, and P3, have been characterized, although in normal and transformed cells the majority of mRNAs initiate at the P2 promoter [5]. Several *cis*-acting sequences have been identified both 5′ and 3′ to the major P2 transcription start site [5]. These sequences, although a few of them are located quite distant from the P2 promoter, regulate transcription initiation from P2 and include binding sites for many known transcription factors (reviewed in [2]). Some of these factors are expressed in a cell- and tissue-specific manner and some of them, such as Fos/Jun, Blimp-1, CTCF [5] and ZF87/MAZ [6], function as repressors of c-*myc* transcription from the P2 promoter. The Myc promoter-binding protein-1 (MBP-1) has been identified as a 37–38 kDa protein which binds just 5′ to the P2 TATA motif and negatively regulates both human and mouse c-*myc* promoter activities [7,8]. It has been shown that MBP-1 and TBP (TATA-binding protein) both bind to the minor groove of the c-*myc* P2 promoter [9], suggesting that MBP-1 may negatively regulate c-*myc* transcription by preventing the formation of a transcription initiation complex. It has been reported that MBP-1 is ubiquitously expressed and a single transcript has been detected in HeLa as well as in other cell lines [7]. MBP-1 cDNA shares 97% similarity with the cDNA encoding the α-isoform of the glycolytic enzyme enolase both in the coding region and in the 3′-untranslated sequence [10]. The enzyme is responsible for the interconversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway and it is present in mammalian tissues as three isoforms, α- β- and γ-enolase, that are encoded by three distinct genes [11–13]. The presence of two single-base insertions in the MBP-1 sequence results in a shift in the reading frame as compared to the α-enolase coding region [7,14]. These insertions affect the amino-terminal portion of the MBP-1 protein whereas the amino acid sequence of the carboxy-terminal region is identical to the corresponding α-enolase sequence (GDB accessions: M14328 and M55914). These observations and the chromosomal location of MBP-1 on human chromosome 1p36 [14,15], in close proximity of ENO1, the gene encoding α-enolase, strongly suggest that MBP-1 and α-enolase could be encoded by the same gene. Furthermore, the observation that no alternatively spliced forms or variant transcripts of the ENO1 gene have been detected in several cell lines and tissues [11,16] suggests that α-enolase and MBP-1 could arise from the same transcript. We show here that a single human α-enolase transcript encodes two proteins of 48 and 37 kDa that are targeted to different cellular compartments, based on initiation of synthe-

*Corresponding author. Fax: (39)-91-420361.
E-mail: feo@unipa.it

[1] Present address: European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69012 Heidelberg, Germany.

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sis at alternative in-frame AUGs. The 37 kDa alternatively translated isoform, which preferentially localizes in the cell nucleus, shares with MBP-1 the capability to downregulate transcription from the c-*myc* P2 promoter indicating an additional functional role for α-enolase.

2. Materials and methods

2.1. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from HeLa cells as previously described [17]. The following double-stranded oligonucleotides were used as probe and competitor in EMSAs: MP2 (5′-AGGGATCGCGCTGAGTATAAAAGCCGGTTTTCGGGG-3′) containing the binding site for MBP-1 [7], and BEE-1 (5′-AGCTGTTCTGAGTGGGGGAGGGGGCTGCGCCTGC-3′) containing an unrelated consensus. EMSAs were performed by incubating end-labeled probes (0.1 ng, about 40 000 cpm) with 5 μg of nuclear extracts and 2 μg of poly(dI-dC) (Pharmacia) in 15 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 5 mM spermidine (Sigma) as previously described [17]. When the GST–ENO1 protein was used, the binding reaction mixtures contained 0.5 μg of affinity-purified GST–ENO1, 0.5 μg of poly(dI-dC) and 1 μg of bovine serum albumin. DNA–protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel in 25 mM TBE (25 mM Tris pH 8.3, 20 mM boric acid, 0.5 mM EDTA) and visualized by autoradiography. For antibody interference, EMSAs were performed under the conditions described above except for the addition to the reaction mixture of 1 μl rabbit anti-α-enolase antiserum [10] or 1 μl of rabbit pre-immune serum.

2.2. Construction of luciferase reporter expression vectors and plasmids expressing wild-type and mutant α-enolase

The pGL-cmp luciferase reporter expression vector was obtained by blunt-end ligation into the *Sma*I site of the pGL3-basic vector (Promega) of a 860 bp *Pvu*II/*Pvu*II fragment derived from plasmid pMC41, which contains the human c-*myc* gene [18]. This fragment corresponds to region −350/+510, relative to the P1 transcription start site of the c-*myc* gene, and it has been shown to drive efficient transcription of reporter genes in several cell lines [19]. The pRCENO1 expression vector was obtained by cloning into the cytomegalovirus promoter-directed expression vector pRC/CMV (Invitrogen) a 1740 bp *Hin*dIII/*Xba*I fragment of the human α-enolase cDNA, containing the entire coding region and most of the 3′-untranslated region of the human α-enolase cDNA [10]. The pRCENO2 expression vector was obtained by inserting into the *Kpn*I and *Eco*RV sites of the pRC/CMV vector a 1790 bp *Kpn*I/*Nde*I-filled fragment of the human γ-enolase cDNA, containing the entire coding region and part of the 3′-untranslated region [20]. To generate the pRCENO1-97 expression vector a fragment of 1278 bp was obtained by PCR from the α-enolase cDNA using the oligonucleotide primers PSA-97 (5′-CCCAAGCTTACCATGGATGGAACAGAAAAT-3′) and revB5 (5′-CAGGAAACAGCTATGACCATGA-3′), the restriction site in the primer is underlined. After amplification with Pfu polymerase (Stratagene), in order to minimize mutations [21], the amplified fragment was digested with *Hin*dIII and *Xba*I and ligated into the pRC/CMV vector. To generate green fluorescent protein (GFP)-fused protein two DNA fragments were obtained by PCR from the pRCENO1 and pRCENO1-97 plasmids using oligonucleotide primers T7-P (5′-TGTAATACGACTCACTATAG-3′) and End-1 (5′-CGGGATCCTTGGCCAAGGGGTT-3′) in the condition described above. The amplified fragments were digested with *Hin*dIII and *Bam*HI and ligated into the pEGFP-N1 vector (Clontech) to obtain the pENO1-GFP and the pENO1-97-GFP expression vectors, respectively. The nucleotide sequence of the α-enolase DNA fragments generated by PCR was confirmed by sequencing.

2.3. Protein expression in bacteria and by in vitro transcription and translation

A 1.35 kb *Bgl*II–*Xho*I fragment encoding amino acids 10–433 was isolated from the α-enolase cDNA [10] and subcloned in the bacterial expression vector pGEX-3X (Pharmacia). The glutathione *S*-transferase–α-enolase fusion protein was overexpressed in *Escherichia coli* and affinity-purified by binding to glutathione-linked Sepharose beads according to standard procedures.

In vitro transcription was carried out with 2 μg of linearized pRCENO1, pRCENO1-97 and pRCENO2 plasmids using a mRNA capping kit (Stratagene). In vitro translation was performed with a commercially available rabbit reticulocyte lysate system according to the instructions of the manufacturer (Promega), in the presence of [^{35}S]methionine (Amersham). Translation products were fractionated on a 12% SDS–polyacrylamide gel and visualized by fluorography.

2.4. Immunoblot analysis

For immunoblot analysis total cell lysates (50 μg) obtained by extraction in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin and 0.5 μg/ml pepstatin, were resolved by electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted to a nitrocellulose membrane (Hybond-C, Amersham). The membrane was incubated with anti-α-enolase antiserum (1:5000) then with a secondary antibody conjugated to horseradish peroxidase (Amersham) (1:5000). The antigen–antibody complexes were visualized by enhanced chemiluminescence (Supersignal kit, Pierce).

2.5. Cell culture, transfection and luciferase assay

Human cervical carcinoma (HeLa), simian embryo kidney (COS-7) and the human histiocytic lymphoma (U-937) cell lines were obtained from the American Type Culture Collection (USA). The SV40-transformed human fibroblast (PAF), the human lymphoblastoid (GM1500), the Burkitt's lymphoma (Daudi) and the human T-cell leukemia (Jurkat) cell lines were from laboratory stocks. Cells were maintained in the following media containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin: RPMI 1640 (Sigma) for U-937, PAF, GM1500, Daudi and Jurkat and Dulbecco's modified Eagle's medium (DMEM, Sigma) for HeLa and COS-7. Transient transfection experiments were performed using FuGENE-6 transfection reagent according to the instructions of the manufacturer (Roche). Briefly, 1.5×10^5 cells were plated per 35 mm dish and transfected with 1 μg of pGL-cmp reporter vector, 0.01–1 μg of one of the pRC/CMV effector vectors and 0.2 μg of pRL-CMV vector (Promega), to monitor transfection efficiency. Forty-eight hours after transfection cells were harvested and cell lysates prepared in Passive Luciferase Buffer (Promega). The firefly and *Renilla* luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and luciferase activity measured with a TD-20/20 luminometer (Turner Design) equipped with an automatic dual injector system.

For visualization of GFP by fluorescence, COS-7 cells were plated on 35 mm tissue culture dishes containing glass coverslips and transfected with 1 μg of the GFP expression plasmids as described above. At 48 h post transfection the cells were washed once in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 20 min, followed by an additional rinse in PBS containing DAPI and observed using a fluorescent microscope.

3. Results

3.1. The ENO1 gene product specifically binds to the c-myc P2 promoter

MBP-1 has been shown to bind to a DNA sequence that contains the TATA box of the c-*myc* P2 promoter [7]. The protein shares considerable homology at the amino acid level with the glycolytic enzyme α-enolase. To investigate a possible correlation between MBP-1 and the ENO1 gene product, EMSAs were performed using nuclear extracts from HeLa cells and a 36 bp end-labeled oligonucleotide (MP2) containing the binding site reported for MBP-1 [7]. Two major DNA–protein complexes were observed (Fig. 1, lane 1). The formation of the slower migrating complex was abolished by competition in EMSAs with a 100-fold molar excess of unlabeled MP2 oligonucleotide (Fig. 1, lanes 2 and 3), while it was not affected by the use of an unrelated oligonucleotide (Fig. 1, lanes 4 and

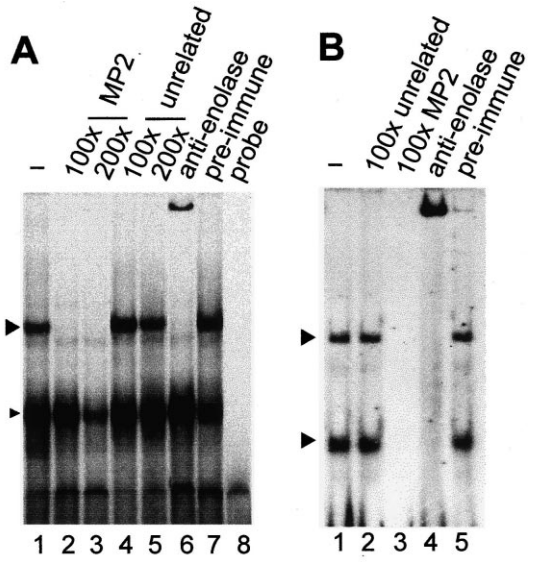


Fig. 1. EMSA analysis of endogenous nuclear proteins and recombinant GST–α-enolase. A: A labeled oligonucleotide (MP2) containing the MBP-1 binding site was incubated with nuclear extracts (5 μg) from HeLa cells (lanes 1–7). For competition in the EMSA a 100- and 200-fold molar excess of unlabeled MP2 oligonucleotide or an unrelated oligonucleotide was added to the binding reaction mixture. Addition of anti-α-enolase antibodies results in a supershift of the slowly migrating specific complex, while pre-immune serum has no effect (lanes 6, 7). The position of specific and non-specific complexes is indicated by large and small arrowheads, respectively. The faster migrating complex is probably due to an uneven ion front as detected in the absence of nuclear extract (lane 8). B: EMSA performed with affinity purified GST–α-enolase protein using a labeled MP2 oligonucleotide as probe. Competition in EMSA (lanes 2, 4) and antibody interference (lanes 4, 5) are as described above. The formation of two specific complexes, indicated by the large arrowheads, is due to partial degradation of the purified GST–α-enolase protein.

5), confirming the specificity of the binding. This DNA–protein complex was specifically supershifted when anti-α-enolase antibodies [10] were added to the binding reaction (Fig. 1, lane 6), while pre-immune serum had no detectable effects (Fig. 1, lane 7). Taken together, these results indicate that the DNA–protein complex observed contains a polypeptide immunologically related to α-enolase that is present in the nucleus. Similar results were obtained using nuclear extracts from different human cell lines indicating the ubiquity of this α-enolase-related protein (data not shown). To further investigate the identity of the enolase-related polypeptide present in the complex, a GST–α-enolase fusion protein purified from bacterial lysates was used in EMSAs. As shown in Fig. 1B the recombinant α-enolase specifically binds the MBP-1 consensus (Fig. 1B, lane 1) as assessed by competition with an excess of unlabeled MP2 oligonucleotide (Fig. 1B, lanes 2 and 3) and the formation of a supershifted DNA–protein complex following addition of anti-α-enolase antibodies (Fig. 1B, lanes 4 and 5). The presence of two differently retarded complexes is due to partial degradation of the purified GST–α-enolase protein, as established by SDS-polyacrylamide gel and Western blot with anti-α-enolase antibodies (data not shown). These data indicate the capability of α-enolase to bind to the c-*myc* P2 promoter.

3.2. α-Enolase mRNA encodes two alternatively translated polypeptides

Alignment of the amino acid sequence of α-enolase and MBP-1 indicated that the MBP-1 initiator methionine corresponds to a methionine at position 97 in α-enolase (Met-97). This methionine is encoded by an AUG codon that is enclosed in a good consensus for translation initiation [22] and is conserved in all the α-enolase isoforms isolated from different species, while it is absent in the β-enolase and γ-enolase isoforms, although the three isoforms share more than 90% similarity at the amino acid level in the same species [23]. In order to establish if this methionine could represent an alternative translation start site the entire coding region of α-enolase (ENO1) (aa 1–433), a partial cDNA lacking the nucleotides encoding the first 96 amino acids of α-enolase (ENO1–97) (aa 97–433) and the entire coding region of the γ-enolase (ENO2) (aa 1–433) were cloned in the mammalian expression vector pRC/CMV. The resulting plasmids were linearized and in vitro transcribed and translated. Two translated products were detected in the translation mixture programmed with ENO1 mRNA (Fig. 2A, lane 1), the major product was a polypeptide migrating with an apparent molecular mass of 48 kDa, corresponding to the entire α-enolase protein, the minor product was a polypeptide migrating with an apparent molecular mass of 37 kDa. A unique product migrating as a 37 kDa polypeptide was obtained in the translation mixture programmed with ENO1-97 (Fig. 2A, lane 2), whereas a single 48 kDa polypeptide was obtained with ENO2 (Fig. 2A, lane 3) that has a leucine UUG codon (leucine) instead of a AUG codon (methionine) at position 97. Incubation of the translation mixtures at room temperature for increasing

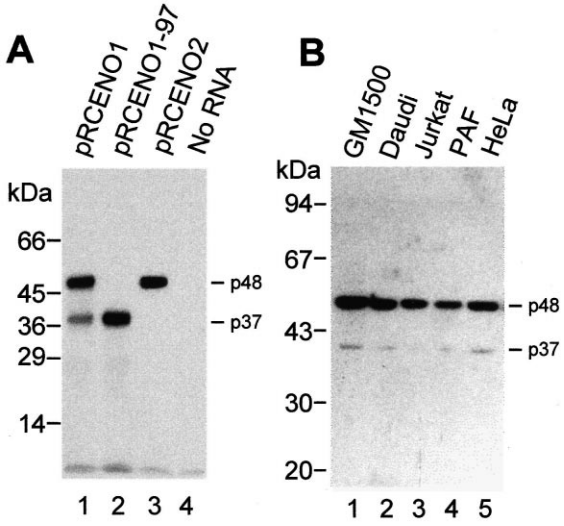
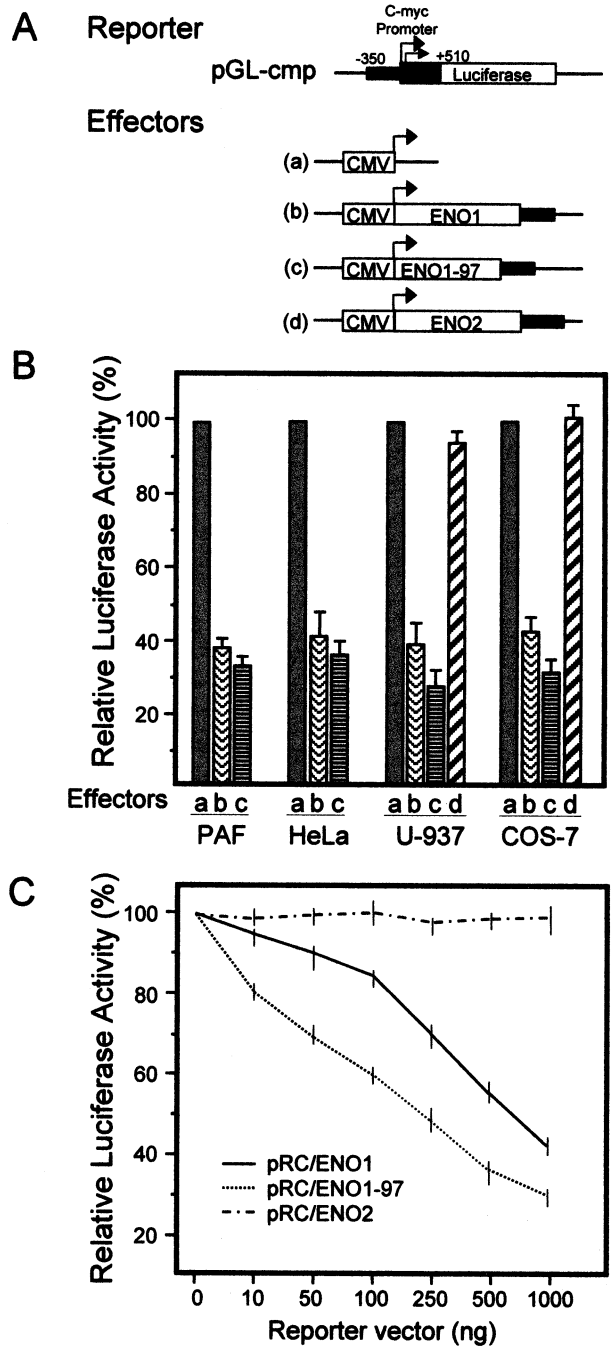


Fig. 2. A: Analysis of in vitro translated proteins. In vitro transcribed RNAs from the full-length α-enolase cDNA (ENO1), a partial α-enolase cDNA starting from the AUG triplet encoding methionine 97 (ENO1–97) and a full-length γ-enolase cDNA (ENO2) were translated in a rabbit reticulocyte system and products resolved on a SDS-polyacrylamide gel (12% acrylamide) as described in Section 2. As negative control a translation mixture with no addition of exogenous RNA (No RNA) was run on the same gel (lane 4). Molecular size markers are indicated on the left. B: Western blot analysis of total cell extracts. Lysates from different human cell lines (indicated at the top) were resolved on an SDS-polyacrylamide gel (10% acrylamide), transferred to nitrocellulose and the filter incubated with anti-α-enolase, as described in Section 2. Molecular size markers are indicated on the left.

lengths of time and subsequent Western blot excluded the possibility that the 37 kDa polypeptide could arise from proteolytic cleavage of the entire α-enolase protein (data not shown). These results suggest that a bona fide internal translation site is present in the full-length α-enolase mRNA and most likely corresponds to the AUG encoding Met-97 as suggested by the detection of a protein with the expected molecular mass of 37 kDa. Translation initiation from this internal AUG codon is about 20-fold less efficient than the translation initiation obtained from the first AUG codon of the α-enolase mRNA. We decided to investigate if an α-enolase related peptide of 37 kDa is present in vivo in human cells expressing α-enolase. Western blot of total protein extracts from different human cell lines performed with anti-α-enolase antibodies clearly indicates the existence of a 37 kDa protein immunologically related to α-enolase whose relative amount to the 48 kDa α-enolase is consistent with what was observed in the in vitro experiments (Fig. 2B).



3.3. α-Enolase acts as a repressor of the c-myc promoter transcriptional activity

To address the question if α-enolase and/or the related 37 kDa protein, encoded by the same mRNA but resulting by translation initiation from an internal AUG codon, may play a negative regulatory role in transcriptional regulation of the c-*myc* gene, as has been reported for MBP-1 [24,25], ENO1, ENO1–97 and ENO2 proteins were overexpressed in transfection assays with a luciferase reporter plasmid containing the promoter region (nt −35–+650) of the human c-*myc* gene. In different cell backgrounds, the expression of both ENO1 and ENO1–97 resulted in a consistent reduction (55–75%) of the luciferase activity relative to the activity detected in cells transfected with the parental, insertless expression vector (Fig. 3B). This reduction was not observed when ENO2 was overexpressed in the same cell context and with the same reporter plasmid (Fig. 3B). As a control, the ability of each effector plasmid to produce the corresponding peptide(s) was assessed by Western blot of lysates from transfected cells with anti-α- and anti-γ-enolase-specific antibodies (data not shown). The results obtained suggest that the repression of luciferase activity consequent to overexpression of the ENO1 gene product may be caused by the alternatively translated 37 kDa protein encoded by this gene rather than by α-enolase itself. This hypothesis is further supported by the fact that in COS-7 cells cotransfected with lower amounts of effector plasmids (10–100 ng), a higher level of transcriptional repression was observed in the cells overexpressing ENO1–97 than in cells overexpressing ENO1 (Fig. 3C) as if a threshold amount of ENO1 mRNA was needed to produce enough effector protein. In conclusion, these experiments indicate that a 37 kDa protein alternatively translated from the α-enolase mRNA has the ability to repress transcription from c-*myc* P2 promoter.

3.4. Subcellular localization of ENO-GFP chimeric proteins overexpressed in COS-7 cells

To determine the subcellular localization of the proteins

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Fig. 3. Effect of overexpression of enolase proteins on transcriptional activation by the c-*myc* promoter. A: Schematic representation of reporter and effector plasmids used in transient transfection experiments. pGL-cmp contains the c-*myc* promoter and part of the first untranslated exon (black box) upstream of the firefly luciferase gene cloned in the pGL3 vector. Effectors a, b, c, and d correspond to the expression vector pRC/CMV and recombinant constructs pRC-ENO1, pRC-ENO1–97 and pRC-ENO2, respectively. B: Luciferase assays of different cell lines (indicated at the bottom) cotransfected with 1 μg of the pGL-cmp reporter plasmid and 1 μg of the insertless effector plasmid (a), or plasmid expressing the full-length α-enolase (b), or the amino-terminal shorter α-enolase (c), or the full-length γ-enolase (d). C: Luciferase assays of COS-7 cells cotransfected with 1 μg of the pGL-cmp reporter plasmid and increasing amounts (indicated at the bottom) of effector plasmids. Luciferase activities, corrected for differences in transfection efficiencies, are compared with activity observed with the insertless effector plasmid that was arbitrarily set at 100%. The data are averages of at least three independent experiments, and the error bars represent standard deviations.

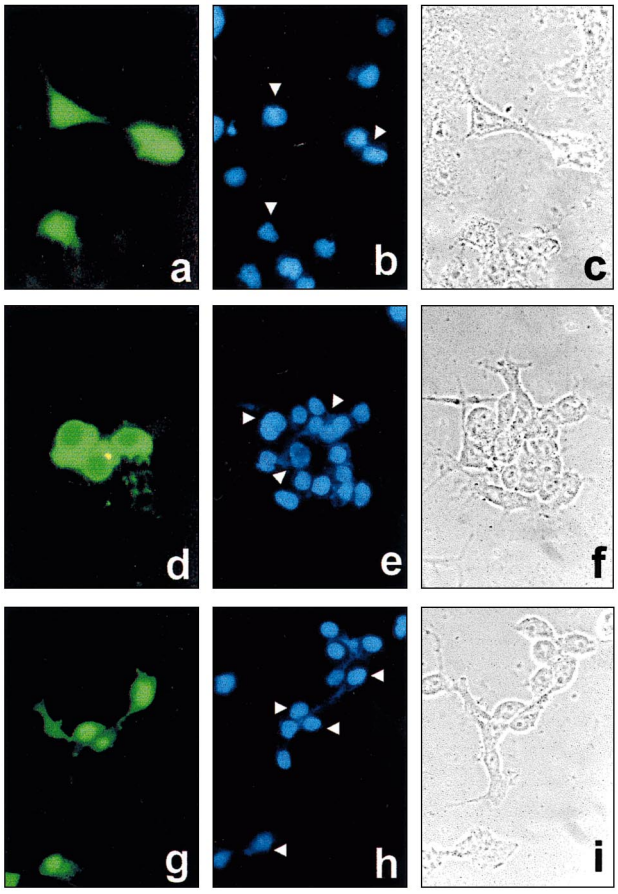

Fig. 4. Localization of ENO1–GFP chimeric proteins. COS-7 cells were transiently transfected with the pEGFP-N1 expression plasmid, encoding the wild-type GFP (a, b and c), the pENO1–GFP, encoding the entire α-enolase–GFP (d, e and f), and the pENO1–97–GFP, encoding the amino-terminal deleted α-enolase–GFP (g, h and i), as reported in Section 2. Forty eight hours post-transfection cells were fixed, counterstained with DAPI and visualized using a epifluorescence equipped microscope for GFP staining (a, d and g) or DAPI staining (b, e and h) or by phase contrast (c, f, and i). White arrowheads in b, e and h indicate nuclei of transfected cells.

overexpressed in the cells, the GFP gene was fused in frame to the 3′ end of both the full-length cDNA encoding α-enolase (pENO1–GFP) and the partial cDNA encoding the polypeptide spanning aa 97–433 (pENO1–97–GFP). The resulting fusion proteins have a predicted molecular mass of 75 kDa and 64 kDa, respectively, which is above the size exclusion limit (40–50 kDa) for passive diffusion of protein through the nuclear pores [26]. The expression plasmids were transiently transfected in COS-7 cells and after 48 h cells were treated to visualize the GFP. Cells transfected with the plasmid expressing the GFP are homogeneously stained (Fig. 4a), since GFP has a molecular mass of about 27 kDa and distributes approximately equally between the cytoplasm and nucleus. Different results were obtained when cells were transfected with the two plasmids expressing the GFP fused to the entire α-enolase protein (Fig. 4d) or to the polypeptide lacking the first 96 amino acids (Fig. 4g). The majority of the ENO1-GFP chimera is retained in the cytoplasm, whereas the ENO1–97–GFP chimeric protein is preferentially localized in the nuclei of the transfected cells (Fig. 4e). These experiments indicate that the deletion of the first 96 amino-terminal amino acids from α-enolase results in a preferential nuclear localization of the shorter polypeptide, compatible with its function of transcriptional repressor of the c-*myc* promoter.

4. Discussion

The aim of this work was to test if the gene encoding the glycolytic enzyme α-enolase, ENO1, could also encode MBP-1, which acts as a transcriptional repressor of c-*myc* P2 promoter activity. We have shown here that α-enolase and a short form that initiates from an internal methionine, presumably Met-97, have the ability to bind the TATA box region of the c-*myc* P2 promoter and to reduce the expression of a luciferase reporter gene under the control of the c-*myc* promoter in transient transfection experiments. All these features are shared by MBP-1 [7,9]. Consistent with the high similarity (about 97%) existing between MBP-1 and α-enolase cDNA nucleotide sequences and with the colocalization of MBP-1 and ENO1 genes in the same region of human chromosome 1p36 [14,15], we propose that MBP-1 is a polypeptide alternatively translated from the full-length α-enolase mRNA. This hypothesis is further supported by the fact that in vitro transcription and translation of a full-length α-enolase cDNA results in the production of two polypeptides with apparent molecular weights of 48 kDa and 37 kDa respectively, while in vitro transcription and translation of full-length γ-enolase cDNA, whose coding region is more than 90% similar to α-enolase but lacks of the AUG codon specifying Met-97, results in the production of a single 48 kDa peptide. These data indicate that the methionine at position 97 in the α-enolase mRNA is the initiator methionine of the 37 kDa polypeptide. Eukaryotic translation initiation usually occurs at the first in-frame AUG codon that is in the optimal context [22], however, leaky scanning may produce more than one protein from a single mRNA, and in many instances the differentially translated products have different subcellular fates. For example, alternative initiation of translation determines cytoplasmic or nuclear localization of bFGF [27], as well as of the FGF-related protein Int-2 [28] and the prostatic protein probasin [29]. The data presented here indicate that the two products encoded by the ENO1 gene can have a different function and subcellular localization. Most of the α-enolase protein translated from the first AUG is present in the cytoplasm, but a consistent proportion of the shorter products initiated at the internal AUG codon is diverted to the nucleus. Enolase is a glycolytic enzyme and consistent with its primary function it is predominantly localized in the cytoplasm where it can be free or associated with the cytoskeleton with other glycolytic enzymes [30]. Although nothing is known about the domains of the protein responsible for the interactions with the cytoskeleton, we can speculate that this signal is localized within the first 96 amino acids of the protein and that the lack of these amino acids in the 37 kDa peptide might determine its translocation into the nucleus. Whether this translocation is directly controlled by a non-canonical nuclear localization signal or whether it is an indirect response remains to be determined. Furthermore, it is possible that a preferential translation of the 37 kDa products is linked to the differentiation stage of some cell types and regulated by an internal ribosomal entry site (IRES), as has been reported for PDGF2/c-*sis* mRNA [31]. A preliminary computer-assisted analysis predicts structural features within the 5′ coding region of

the α-enolase mRNA that are common to known IRES elements [32].

It has been reported that other glycolytic enzymes may be involved in a variety of cellular events in addition to their primary catalytic role [33,34], such as GAPDH, which has been found in the nucleus of certain cell types [35] and has been shown to bind DNA [36]. Several other functions have been reported for α-enolase: it is a heat shock protein in yeast [37], it has been identified as the eye lens crystallin τ in reptiles and birds [38], it has been described as a component of the centrosome in HeLa cells [39], it functions as a plasminogen receptor in human peripheral blood cells [16], it is one of the hypoxia-inducible proteins in human cells [40], an immunodominant antigen in *Candida albicans* [41], and it has even been identified as toxin B in *Clostridium difficile* [42]. This report indicates an additional functional role for α-enolase carried out by an alternatively translated product whose structural and functional characteristics strongly support its identity with MBP-1. Recently it has been shown that the c-*myc* downregulatory activity of MBP-1 resides in two hydrophobic domains located in the amino- and in the carboxy-terminal portion of the protein [25], both domains are present in the 37 kDa alternatively translated product of the α-enolase gene.

The gene encoding α-enolase (ENO1) maps to a region of human chromosome 1 (1p35–p36) that is often deleted in several human malignancies, including neuroblastoma, melanoma, pheochromocytoma, and carcinomas of the breast, liver and colon [43]. Tumor-specific deletions of distal 1p predict the presence of one or more tumor suppressor loci in this region. The finding that a transcriptional repressor of the c-*myc* oncogene is an alternatively translated product of the ENO1 gene makes ENO1 a potential candidate for tumor suppressor. This hypothesis is supported by the observations that overexpression of MBP-1 induces loss of anchorage-independent growth and suppresses tumor formation in athymic nude mice of human breast carcinoma cells [44], as well as inducing cell death in murine fibroblasts [8].

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