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Polyamine Depletion Up-Regulates c-Myc Expression, Yet Induces G_1 Arrest and Terminal Differentiation of F9 Teratocarcinoma Stem Cells

Lennart Frostesjö and Olle Heby*

Department of Cellular and Developmental Biology, Umeå University, S-901 87 Umeå, Sweden

Abstract The ornithine decarboxylase (ODC) gene is a transcriptional target of c-Myc. Exponentially growing cells usually exhibit high c-Myc levels and high ODC levels, whereas stationary phase cells and terminally differentiated cells have low levels of both proteins. Therefore, we were surprised to find that when F9 teratocarcinoma stem cells were blocked in the G_1 phase of their cell cycle and induced to differentiate by irreversible inhibition of the ODC activity, the expression of c-Myc was up-regulated instead of being down-regulated. During the course of differentiation, the c-*myc* gene was constitutively expressed, and c-Myc protein accumulated. In transfection experiments, using ODC promoter-reporter gene fusion constructs, the accumulation of c-Myc protein, resulting from polyamine depletion, led to increased reporter gene expression. This finding is consistent with the view that depletion of polyamines relieves the suppression that they exert on c-*myc* mRNA translation, causing an accumulation of c-Myc protein, which in turn transactivates its target gene, the bona fide ODC gene. Thus, the accumulation of an active c-Myc protein does not preclude differentiative events, nor does it override the growth arrest caused by polyamine depletion. These results suggest a new role for polyamines—as negative regulators of c-Myc expression. J. Cell. Biochem. 76:143–152, 1999.

Key words: differentiation; α-difluoromethylornithine; c-Myc; Max; ornithine decarboxylase; parietal endoderm; polyamines; retinoic acid

The polyamines spermidine and spermine, and their diamine precursor putrescine, play important roles in cell growth and differentiation [Cohen, 1998]. Inactivation of the polyamine biosynthetic enzymes, i.e., ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, and spermine synthase, by mutation or treatment with specific inhibitors, reduces the growth rate of all cell types studied to date [McCann and Pegg, 1992; Pegg and McCann, 1992; Marton and Pegg, 1995]. Conversely, overproduction of ODC, by transfection [Auvinen et al., 1992] or relief of translational repression [Shantz and Pegg, 1994; Shantz et al., 1996b], may induce neoplastic transformation. The relevance of these findings is emphasized by the fact that the malignant process is counteracted by specific inhibition of the ODC activity [Auvinen et al., 1992] and by expression of an ODC dominant-negative mutant [Shantz et al., 1996a]. ODC-overproducing NIH 3T3 cells injected into nude mice proved to be both invasive and angiogenic [Auvinen et al., 1997].

In view of the fact that ODC overproduction can induce neoplastic transformation, it is not unreasonable to assume that ODC inhibition might induce transition from a malignant to a normal phenotype. Indeed, when the ODC activity is irreversibly inhibited by treatment with α-difluoromethylornithine (DFMO) [Metcalf et al., 1978], mouse F9 teratocarcinoma cells differentiate from malignant stem cells to extraembryonic parietal endoderm-like cells, which have no further proliferative potential [Heby et al., 1983; Oredsson et al., 1985; Jetten and Shirley, 1985]. Physiological doses of all-*trans*-retinoic

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*Correspondence to: Olle Heby, Department of Cellular and Developmental Biology, Umeå University, S-901 87 Umeå, Sweden. E-mail: olle.heby@cdbiol.umu.se

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acid (RA) [Strickland and Mahdavi, 1978] induce a similar progeny, corresponding to the extraembryonic endoderm cells of the parietal yolk sac surrounding the early mouse embryo [Hogan et al., 1983]. The differentiation process is accompanied by dramatic changes in gene expression [Sleigh, 1992], which appear to be unique for each inducer [Bjersing et al., 1997], indicating that the teratocarcinoma stem cells possess the potential to use alternate pathways to reach the same goal—the terminally differentiated parietal endoderm cell. In each pathway, the F9 cells lose their tumorigenicity and their transformed phenotype.

The apparently unique mechanism by which DFMO treatment, and the ensuing polyamine depletion, causes the transition from stem cell to differentiating cell is not fully understood [Heby et al., 1983; Schindler et al., 1983; Oredsson et al., 1985; Jetten and Shirley, 1985; Schindler et al., 1985; Frostesjö et al., 1997; Bjersing et al., 1997]. DFMO is a highly specific inhibitor of ODC. It has been called a suicide inhibitor because it is converted from a reversible to an irreversible inhibitor by the target enzyme, ODC [Metcalf et al., 1978]. The fact that only ODC is capable of activating DFMO, by catalyzing its decarboxylation, explains the high specificity of the inhibitor. In the process, a highly reactive residue is generated, causing irreversible inactivation of ODC through interaction with either of two separate residues (Cys^{360} and Lys^{69} in the mouse enzyme), which form essential parts of the active site [Coleman et al., 1993]. As a consequence, the formation of the product, putrescine, stops. The putrescine pool is gradually depleted by conversion to spermidine, and the spermidine pool is subsequently depleted by conversion to spermine.

One possible explanation for DFMO-mediated induction of teratocarcinoma stem cell differentiation is that putrescine and spermidine possess growth-promoting effects, and that their depletion acts as a differentiation signal [Heby et al., 1983; Schindler et al., 1983, 1985; Oredsson et al., 1985; Jetten and Shirley, 1985]. Another possibility is that the dramatic accumulation of decarboxylated AdoMet, a consequence of the depletion of putrescine and spermidine, induces differentiation by preventing postreplicative methylation of promoters [Heby, 1995; Frostesjö et al., 1997]. Decarboxylated AdoMet is a substrate analogue that inhibits the transfer of methyl groups from AdoMet to CpG sites in DNA. Demethylation of CpG sites can preclude the binding of transcriptional repressors, causing activation of promoters that are repressed by methylation [Nan et al., 1998]. As a consequence, genes that are of importance for the transition from stem cell to differentiated cell may become active.

When analyzing the regulation of polyamine biosynthesis in F9 cells, we noted that the ODC mRNA level failed to decrease during growth deceleration, as had been expected on the basis of other studies [Lau and Nathans, 1985; Wallon et al., 1995]; instead, the ODC mRNA level increased during the course of DFMO-mediated growth arrest and differentiation. Because the ODC gene is a direct transcriptional target of c-Myc [Bello-Fernandez et al., 1993], it was of interest to determine whether c-Myc might play a role in the observed accumulation of ODC mRNA in the G_1-arrested and differentiating F9 cells. The responsiveness to c-Myc is mediated by two c-Myc:Max E-box sequences, each having a central CACGTG core, in the first intron of the mouse ODC gene [Bello-Fernandez et al., 1993].

c-Myc belongs to the basic helix-loop-helix leucine zipper family of DNA-binding proteins [Marcu et al., 1992; Henriksson and Lüscher, 1996; Lemaitre et al., 1996; Facchini and Penn, 1998] and regulates transcription through interaction with its obligate partner Max (Myc-associated factor x) [Blackwood and Eisenman, 1991]. c-Myc:Max heterodimers bind to E-box elements and may thus activate ODC expression [Bello-Fernandez et al., 1993]. Max can also associate with members of the Mad family (Mad 1–4) [Ayer et al., 1993, 1995]. By competitively binding to the same E-box elements, Mad:Max heterodimers can antagonize the activity of c-Myc:Max.

During the course of F9 cell growth deceleration and differentiation, induced by polyamine depletion, c-*myc* proved to be constitutively expressed, resulting in accumulation of c-Myc protein. In transfection experiments, using an ODC promoter-reporter gene fusion construct [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997], the accumulation of c-Myc was shown to stimulate the expression of the reporter gene. Therefore, and in view of the fact that *max* gene expression was up-regulated, whereas no *mad* mRNA was detectable, it is likely that c-Myc:Max complex formation is responsible for the observed increase in ODC

gene expression. The mechanism by which polyamine depletion causes increased c-Myc expression remains to be established, but other studies [reviewed by Heby and Persson, 1990] indicate that polyamines, either directly or indirectly, may repress transcriptional as well as translational events.

MATERIALS AND METHODS

Chemicals

D,L-α-difluoromethylornithine monohydrochloride monohydrate (DFMO) (MDL 72527) [Metcalf et al., 1978], was a kind gift from Marion Merrell Dow Research Institute (Cincinnati, OH, and Strasbourg, France). Methylglyoxal-bis(guanylhydrazone) (MGBG), a potent inhibitor of AdoMetDC, was purchased from Aldrich-Chemie.

Antibodies

The antibodies used were affinity purified. Anti-human c-Myc (a rabbit polyclonal IgG, 06-340), which cross-reacts with mouse, was from Upstate Biotechnology (Lake Placid, NY) and cyclin E (M-20, a rabbit polyclonal IgG reactive with cyclin E of murine origin was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Culture Conditions

F9 teratocarcinoma stem cells, which resemble the cells of the inner cell mass of the mouse preimplantation embryo [Hogan et al., 1983], and NIH 3T3 fibroblasts were used in the experiments. F9 cells, which exhibit a very low rate of spontaneous differentiation, were induced to differentiate by treatment with 5 mM DFMO or 1 μM RA [Heby et al., 1983; Oredsson et al., 1985]. During routine passages, monodisperse F9 cells were seeded into gelatinized tissue culture flasks at a density of 2×10^4 cells/ml of growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS). The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured every other day. For the experiments, monodisperse F9 cells were seeded into growth medium supplemented with either (1) 5 mM DFMO, (2) 5 mM DFMO + 5 μM MGBG, or (3) 1 μM RA.

Like F9 cells, NIH 3T3 cells were grown as monolayer cultures in DMEM, but with a lower FCS concentration (10%). Both cell lines were grown in the absence of antibiotics and were tested regularly to verify the absence of mycoplasma contamination. Growth media and FCS were purchased from Life Technologies.

Polyamine Analysis

The cellular content of putrescine, spermidine and spermine was determined by reversed-phase high-performance liquid chromatographic (HPLC) analysis as previously described [Seiler and Knödgen, 1985; Frostesjö et al., 1997].

Northern Blot Analysis

Total RNA, isolated from the cells according to a published method [Chomczynski and Sacchi, 1987], was fractionated by electrophoresis in 1% agarose gels containing 0.66 M formaldehyde in 1× MOPS buffer (10× MOPS = 0.2 M 3-[N-morpholino]propanesulfonic acid, pH 7.0, 50 mM sodium acetate, 10 mM EDTA), and transferred by vacuum blotting to a positively charged nylon membrane (Hybond-N^+, Amersham) using 20× SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0). The membrane was prehybridized at 65°C for ≥3 h in 5× SSPE (20× SSPE = 3.6 M NaCl, 0.2 M sodium phosphate, and 0.02 M EDTA), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml of salmon sperm DNA. Hybridization was performed at the same temperature for 18 h, using a solution of $1–10 \times 10^6$ cpm/ml of randomly labeled DNA probe, 0.1 mg/ml of salmon sperm DNA, and 0.5% SDS in 5× SSPE. Finally, the membrane was washed at 65°C for 3×20 min in a solution of 0.1% SDS in 0.2× SSPE.

DNA Probes

The following DNA probes were used in Northern blot analyses to analyze the expression of c-*myc*, *max*, and *mad* mRNAs: (1) a 600-bp *Eco*RV-*Cla*I fragment from a human c-*myc* cDNA [Bentley and Groudine, 1986], (2) a 550-bp *Eco*RI fragment from a human *max* cDNA (pVZ1) [Blackwood and Eisenman, 1991], and (3) a 913-bp *Xho*I fragment from a mouse *mad* cDNA [Ayer et al., 1993, 1995]. For the detection of ODC mRNA, a 1.1-kbp *Hin*dIII-*Cla*I fragment from a human cDNA clone (pODC10/2H) [Hickok et al., 1987] was used. In F9 cells, collagen type IV α-1 mRNA and tissue plasminogen activator (tPA) mRNA were used

as markers of parietal endoderm differentiation [Frostesjö et al., 1997]. To ascertain that equal amounts of RNA were loaded in the slots of the gel, a 0.55-kb human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *Hin*dIII-*Xba* I fragment (American Type Culture Collection) was initially used as a probe. However, in agreement with another study [Bjersing et al., 1997], the GAPDH mRNA content was found to decrease during F9 cell differentiation, particularly in RA-treated cells. Ethidium bromide staining of the 18S and 28S rRNA bands proved to be a more suitable monitor of equal loading in this experimental cell system.

Western Blot Analysis

F9 cell cultures were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 0°C by adding 5 vol of lysis buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, and 1% Nonidet P-40 (NP-40). After centrifugation at 12,000 × g (2 min, 4°C), the supernatant was collected and stored at −70°C. The soluble proteins were denatured in 2% SDS and 5% (vol/vol) 2-mercaptoethanol (100°C, 3 min) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a gel containing 0.1% SDS. The proteins were transferred to a nitrocellulose membrane (Hybond Enhanced Chemiluminescence [ECL] Western, Amersham) by electroblotting (100 mA, 4°C, overnight) in a Bio-Rad Transfer Blot-Cell. The electrode buffer contained 50 mM Tris base, 384 mM glycine, 0.01% SDS, and 20% (vol/vol) methanol [Towbin et al., 1979]. Subsequently, the membrane was suspended overnight at 4°C in TBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing 5% nonfat dry milk and 3% FCS in order to block unspecific binding of antibodies. The membrane was then incubated for 8 h at 4°C with the primary antibody (diluted in TBST [0.1% Tween-20 in TBS] containing 15% FCS, 5 mM EDTA and 0.1% sodium azide) and finally for 1 h at room temperature with affinity-purified and species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) diluted in TBST containing 3% nonfat dry milk. After adding detection reagents (ECL+, Amersham), chemiluminescence was recorded on Hyperfilm-ECL (Amersham).

Analysis of c-Myc Transactivation Efficiency by Transient Transfection

F9 cells were seeded into 6-well tissue culture dishes (Falcon), each well receiving 1.25×10^5 cells in 2 ml of growth medium. After 18 h of growth, the subconfluent cells were washed in Opti-MEM® 1 reduced serum medium (Life Technologies) and overlaid with a suspension of 2 µg of plasmid DNA (the constructs are described below) and LIPOFECTAMINE transfection reagent (Life Technologies) in Opti-MEM® 1. To monitor the transfection efficiency, the cells were co-transfected with 0.1 µg of pSV-β-galactosidase control vector (Promega, Madison, WI). When the cells had been incubated for 6 h at 37°C, an equal volume of growth medium containing 40% FCS, was added; 18 h later, the medium was changed to regular growth medium (with or without 5 mM DFMO). After 48 h in the absence or presence of DFMO, the transfected cells were harvested, washed in PBS, and lysed in 250 µl of reporter lysis buffer (Promega). Luciferase assays were performed using 20 µl of cell extract and 100 µl of luciferase assay reagent (Promega). The luciferase activity was measured using Luminometer model TD-20/20 (Turner Designs). The β-galactosidase activity was measured spectrophotometrically.

For transfection, the following ODC promoter-reporter gene fusion constructs were used: ODCΔLucS$^-$ (wild-type) and ODCΔLucS$^-$5A (double mutant) [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997]. Both constructs contained the proximal promoter (264 nucleotides upstream of the transcription start site), exon 1, intron 1 (with the two c-Myc:Max E-boxes), and a portion of exon 2 of the mouse ODC gene (from the *Stu*I site at −264 to the *Sal*I site at +2170 in exon 2) in front of a luciferase reporter gene. The ODCΔLucS$^-$ construct was generated by cloning the mouse ODC sequence into pGEM-*luc* (Promega), which contains the luciferase gene from *P. pyralis*. The ODCΔLucS$^-$5A construct contains the same sequence, except that the two intronic c-Myc:Max E-boxes were altered from CACGTG to CAC<u>C</u>TG). Mutation of both binding sites is known to abolish ODC transactivation by Myc [Bello-Fernandez et al., 1993].

RESULTS

When F9 cells are treated with 5 mM DFMO, they become depleted of their polyamines (Fig. 1) and are consequently stopped in the G_1 phase

and induced to differentiate into parietal endoderm cells [Heby et al., 1983; Oredsson et al., 1985; Jetten and Shirley, 1985; Frostesjö et al., 1997]. The cellular putrescine and spermidine contents are maximally reduced by day 1, and that of spermine by day 2 (Fig. 1). Figure 2 shows that the c-*myc* mRNA content remained largely unchanged in the growth-arrested and terminally differentiating F9 cells. When differentiation was instead induced by treatment with 1 μM RA, the c-*myc* mRNA content decreased significantly and was below the level of detection by day 6 (Fig. 2). In fact, one of the earliest identified changes during RA-induced F9 cell differentiation is the decrease in c-*myc* mRNA content, reportedly occurring within 3 h of addition of the inducer [Griep and DeLuca, 1986].

The DFMO-mediated depletion of putrescine and spermidine causes a dramatic accumulation of decarboxylated AdoMet, a metabolite that has been shown to interfere with DNA methylation [Frostesjö et al., 1997]. When the accumulation of decarboxylated AdoMet was prevented by treatment with 5 μM MGBG, a potent inhibitor of AdoMetDC, the c-*myc* mRNA level was unaffected (Fig. 2). Thus, our finding that c-*myc* expression is not down-regulated, as in the case of RA treatment, cannot be explained by decarboxylated AdoMet-mediated interference with methylation of, for example, CpG sites in the c-*myc* promoter. If down-regulation of c-*myc* mRNA expression were contingent on methylation of CpG sites in its promoter, prevention of such methylation events by decarboxylated AdoMet could have served to maintain the activity of the c-*myc* gene. Instead, the maintenance of c-*myc* gene expression seems to be a consequence of the decrease in cellular polyamine content. Whether this effect is direct or indirect remains to be determined.

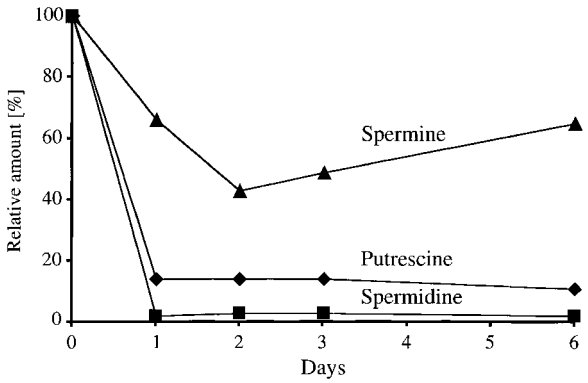

Fig. 1. F9 cells become polyamine-depleted when their ornithine decarboxylase (ODC) activity is irreversibly inhibited by α-difluoromethylornithine (DFMO) treatment. The F9 cells were cultured in the presence of 5 mM DFMO and their polyamine content was determined by reversed-phase high-performance liquid chromatography (HPLC) analysis; 100% is equivalent to a cellular content of 0.53, 3.75, and 2.21 fmol of putrescine, spermidine, and spermine, respectively.

Although it has been concluded from many studies that down-regulation of c-*myc* expression is sufficient and necessary for differentiation to occur [for reviews, see Marcu et al., 1992; Henriksson and Lüscher, 1996; Lemaitre et al., 1996; Facchini and Penn, 1998], our observation is clearly an exception to this general rule. Therefore, it was important to determine whether the observed constitutive expression of c-*myc* mRNA resulted in a significant amount of c-Myc protein, capable of transactivating its target genes (e.g., the ODC gene).

The amount of mRNA encoding the c-Myc partner, Max [Blackwood and Eisenman, 1991], increased significantly during treatment with 5 mM DFMO (Fig. 2), while the mRNA encoding Mad1 [Ayer et al., 1993, 1995], another partner of Max, remained undetectable, as did the Mad1 protein (not shown). Consequently, the differentiating F9 cells possess the transcripts required for the production of c-Myc:Max heterodimers, which are responsible for transactivation of E-box-containing genes such as the ODC gene [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997]. Mad:Max complexes, which would be capable of suppressing the same genes, including the ODC gene, by competing for the E-box elements, are likely to be sparse in polyamine-depleted F9 cells, because *mad* transcripts were not detectable by Northern blot analysis, despite prolonged exposure, nor was Mad1 protein detectable by Western blot analysis.

When F9 cells were instead induced to differentiate by treatment with RA + dibutyryl cyclic adenosine monophosphate (cAMP), c-*myc* expression decreased, *max* expression was unaltered, and the expression of *mad*1 mRNA and Mad1 protein was below the level of detection [Larsson et al., 1997]. In view of these differences between DFMO-mediated and (RA + dibutyryl cAMP)-mediated F9 cell differentiation, it appears that the stem cells possess at least two alternate pathways for parietal endoderm formation.

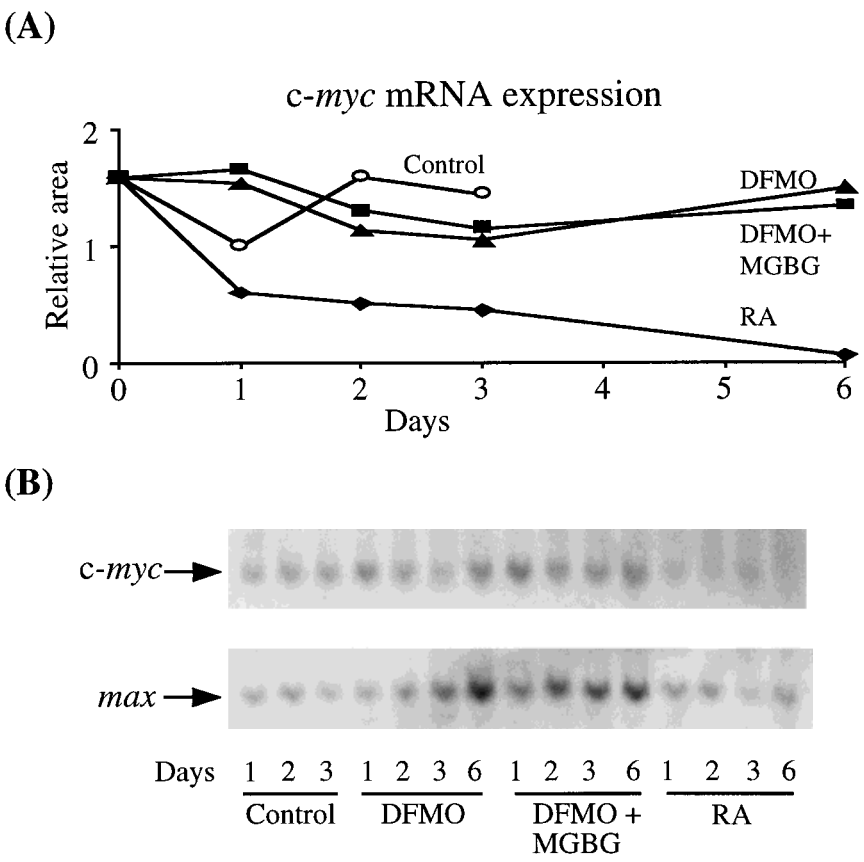

Fig. 2. The expression of the c-*myc* proto-oncogene is constitutive, and that of *max* is up-regulated during F9 cell differentiation induced by polyamine depletion. When differentiation was instead induced by retinoic acid (RA) treatment the expression of both c-*myc* and *max* mRNA decreased. The relative amounts of c-*myc* mRNA were obtained by densitometric scanning **(A)** of an autoradiogram resulting from Northern blot analysis of c-*myc* expression **(B).** Northern blot analysis was also used to monitor *max* expression **(B).** The F9 cells were cultured in the absence (control) or presence of 5 mM α-difluoromethylornithine (DFMO), in a combination of 5 mM DFMO and 5 μM methylglyoxal-bis(guanylhydrazone) (MGBG) or in 1 μM RA. Equal loading of the gel was ascertained by ethidium bromide staining of the 18S and 28S rRNA bands (data not shown).

During DFMO-mediated G_1 arrest and parietal endoderm differentiation, the constitutively expressed c-*myc* mRNA was translated into gradually increasing amounts of c-Myc protein, mainly the 67-kDa isoform (Fig. 3). To determine whether this accumulation of c-Myc protein was capable of transactivating one of its transcriptional targets, the ODC gene, F9 cells were transiently transfected with ODC promoter-reporter gene fusion constructs, either ODCΔLucS$^-$ (wild-type) or ODCΔLucS$^-$5A (double mutant) [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997]. Both reporter constructs contained the proximal promoter (264 bp of sequence upstream of the transcription start site), exon 1, intron 1 (with the two c-Myc:Max E-boxes), and a portion of exon 2 of the mouse ODC gene (−264 to +2170) in front of a luciferase reporter gene (Fig. 4A). As expected, on the basis of the accumulation of c-Myc in polyamine-depleted F9 cells, the wild-type reporter gene construct (ODCΔLucS$^-$) was induced by treatment with DFMO (Fig. 4B). However, in F9 cells transiently expressing the mutated counterpart (ODCΔLucS$^-$5A), in which the two E-boxes were altered from CACGTG to

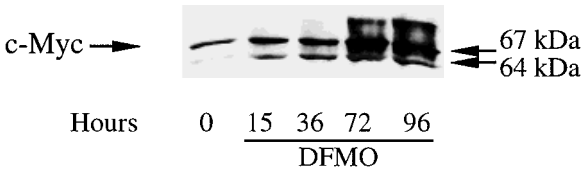

Fig. 3. The amount of c-Myc protein increases during F9 cell differentiation induced by polyamine depletion. The 67-kDa species was the major isoform and exhibited a greater increase than did the 64-kDa species. Total cell lysates, obtained from F9 cells cultured in the absence (0 h) or in the presence of 5 mM α-difluoromethylornithine (DFMO) for 15, 36, 72, or 96 h, were analysed by Western blotting. Cyclin E, a protein exhibiting constitutive expression during DFMO treatment (Frostesjö et al., submitted for publication), served as a control (data not shown).

CACCTG and thus prevented from binding c-Myc:Max heterodimers, polyamine depletion had no stimulatory effect on reporter gene activity (Fig. 4B).

Figure 5 shows that the accumulation of c-Myc, seen in F9 cells that were arrested in G_1 and induced to differentiate by polyamine depletion, was accompanied not only by up-regulation of ODC promoter-reporter gene fusion con-

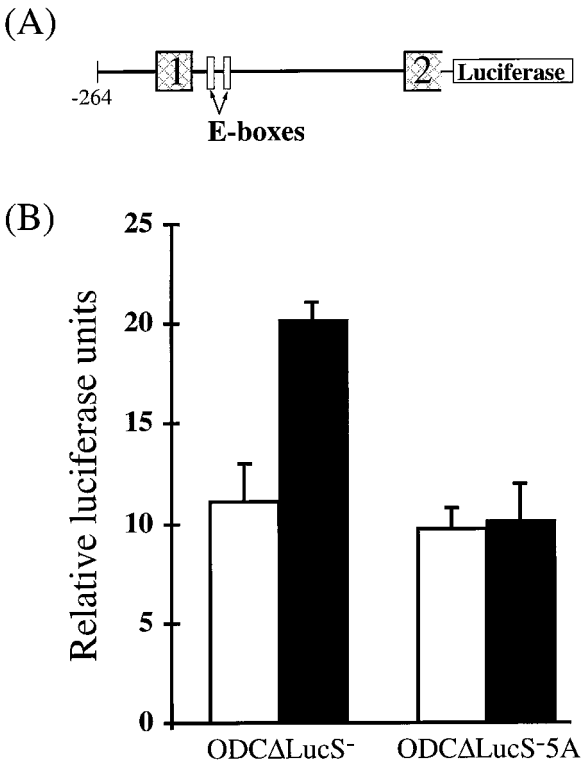

Fig. 4. The ornithine decarboxylase (ODC)-transactivating efficiency exerted by c-Myc is increased during F9 cell differentiation induced by polyamine depletion. F9 cells were transiently transfected with an ODC promoter-reporter gene fusion construct, ODCΔLucS⁻ (wild-type) or ODCΔLucS⁻5A (double mutant) [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997]. **A:** Both reporter constructs contained the proximal promoter (264 bp of sequence upstream of the cap site), exon 1, intron 1 (with the two c-Myc:Max E-boxes), and a portion of exon 2 of the mouse ODC gene (from the *StuI* site at −264 to the *SalI* site at +2170 in exon 2) in front of a luciferase reporter gene. In the mutant construct, ODCΔLucS⁻5A, both E-boxes were altered from CACGTG to CACCTG, and thus prevented from binding c-Myc:Max heterodimers. **B:** After transfection, the F9 cells were grown in the absence (open bars) or in the presence (closed bars) of 5 mM α-difluoromethylornithine (DFMO) for 2 days. To normalize transfection efficiency, the F9 cells were co-transfected with a control reporter gene (pSV-β-galactosidase).

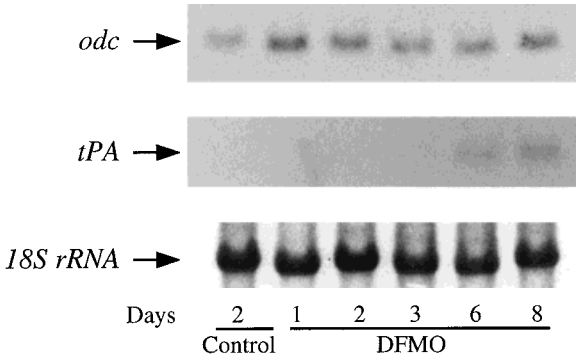

Fig. 5. Expression of the ornithine decarboxylase (*odc*) gene, a gene that is subject to direct transactivation by c-Myc, is upregulated during F9 cell differentiation induced by polyamine depletion. Total RNA was prepared from F9 cells grown for 2 days in the absence (control) or for 1, 2, 3, 6, or 8 days in the presence of 5 mM α-difluoromethylornithine (DFMO). The cellular content of ODC mRNA was studied by Northern blot analysis. The expression of tissue plasminogen activator (*tPA*) mRNA served as a differentiation marker, and ethidium bromide staining of 18S rRNA was used to ascertain equal loading.

structs, but also of the bona fide ODC gene. The ODC mRNA level was thus significantly elevated in DFMO-treated, as compared with untreated, F9 cells. The fact that the accumulation of c-Myc protein and ODC mRNA was not proportional indicates that other factors that regulate ODC gene expression (see below) may be affected by polyamine depletion as well.

Thus our present data suggest that polyamines are negative regulators of c-*myc* expression. Moreover, c-*myc* expression is autosuppressed by its own product, c-Myc [Facchini et al., 1997; Facchini and Penn, 1998]. This negative autoregulation is a homeostatic control mechanism whereby c-Myc protein suppresses transcription initiation from the c-*myc* promoter in a concentration-dependent manner [Facchini et al., 1997; Facchini and Penn, 1998]. Using a cell line that exhibits no autosuppression by c-Myc, we addressed the question whether polyamine depletion would lead to a more significant induction of c-*myc* in such cells than in F9 cells. NIH 3T3 cells, which do not demonstrate autosuppression by c-Myc [Facchini and Penn, 1998], were treated with 5 mM DFMO and subjected to Northern blot analysis, using a c-*myc* probe. A remarkable increase in the amount of c-*myc* mRNA was observed in the polyamine-depleted cells (Fig. 6), emphasizing the importance of the suppression exerted by the polyamines not only on c-*myc* translation, but on c-*myc* transcription as well.

DISCUSSION

c-Myc and ODC are both required for cell growth. Therefore, c-Myc and ODC levels usually show a positive correlation with the cellular growth rate; when cells become quiescent, both proteins virtually disappear [Henriksson and Lüscher, 1996; Heby, 1981]. When G_0/G_1 cells are stimulated with mitogens, the induction of c-Myc in early G_1 precedes that of ODC. This finding is consistent with the fact that c-Myc is a transactivator of the ODC gene [Bello-

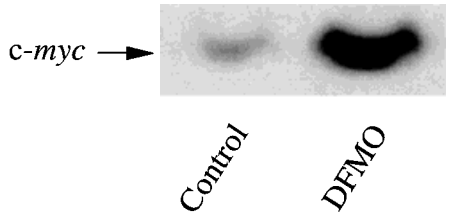

Fig. 6. NIH 3T3 cells, which reportedly lack c-Myc autosuppression [Facchini et al., 1997; Facchini and Penn, 1998], exhibit a dramatic accumulation of c-*myc* mRNA when depleted of their polyamines. Total RNA, isolated from exponentially growing NIH 3T3 cells (control), and from NIH 3T3 cells cultured in the presence of 5 mM α-difluoromethylornithine (DFMO) for 3 days (DFMO), were subjected to Northern blot analysis. Ethidium bromide staining of 18S rRNA was used to ascertain equal loading (data not shown).

Fernandez et al., 1993]. It should be emphasized that the control of ODC gene transcription is complex and that other transcription factors are also involved [Moshier et al., 1992], including c-Fos [Wrighton and Busslinger, 1993], cAMP-dependent factors [Palvimo et al., 1991; Abrahamsen et al., 1992; Palvimo et al., 1996], interferon regulatory factor 1 [Manzella et al., 1994], and Wilms' tumor suppressor WT1 [Moshier et al., 1996]. The two c-Myc:Max E-boxes in the first intron of the ODC gene are responsible for c-Myc binding and activation of the ODC promoter [Bello-Fernandez et al., 1993]. These elements are conserved among mammals.

The increased expression of ODC mRNA, observed during DFMO-mediated cell cycle arrest and differentiation of F9 cells, is at variance with the results obtained in other experimental systems [Wallon et al., 1995]. Thus, even though ODC mRNA has a long half-life [Wallon et al., 1995], its cellular level has been shown to decrease after growth arrest and differentiation [Lau and Nathans, 1985; Wallon et al., 1995]. In view of the fact that c-Myc functions as a direct and required regulator of the ODC gene [Bello-Fernandez et al., 1993], we considered it important to determine whether c-Myc might be involved in the observed up-regulation of ODC mRNA expression in DFMO-treated F9 cells. Despite the reportedly short half-lives of c-*myc* mRNA and c-Myc protein [Celano et al., 1988; Henriksson and Lüscher, 1996], which ensure that c-Myc expression is rapidly down-regulated, we found the cellular c-*myc* mRNA to be constitutively expressed and the c-Myc protein to increase during the DFMO-mediated G_1 arrest and the ensuing terminal differentiation of F9 cells. Interestingly, the half-life of c-Myc protein (estimated after cycloheximide addition) exceeded 2 h in F9 cells (not shown), which is considerably longer than that of other cell types [Henriksson and Lüscher, 1996]. However, the accumulation of c-Myc protein that occurs during DFMO-mediated growth arrest and differentiation of the F9 cells is not attributable to a change in half-life (not shown).

Up-regulation of c-Myc expression is consistent with the observed stimulation of the ODC gene, one of its targets, but clearly in contrast to most other studies. Thus, c-Myc, which is believed to promote continuous entry of cells into the S phase, is usually down-regulated upon growth deceleration and terminal cell differentiation [Marcu et al., 1992; Henriksson and Lüscher, 1996]. Down-regulation of c-*myc* expression is also seen when human colon carcinoma cells are depleted of their polyamines [Celano et al., 1988], and when F9 cell differentiation is induced by treatment with RA + dibutyryl cAMP [Campisi et al., 1984]. Moreover, when the amount of c-Myc protein is reduced by transfection with plasmids expressing antisense c-*myc* sequences, F9 cells are induced to differentiate, suggesting that down-regulation of c-Myc is sufficient for F9 cell differentiation [Griep and Westphal, 1988].

The present study suggests that there are means by which a cell can uncouple constitutive, even increased, expression of c-Myc from cell cycle progression (i.e., that cells can achieve a stop in their cell cycle and enter a differentiative pathway despite the presence of an active c-Myc proto-oncogene). This finding is in agreement with the results of another study, demonstrating that enforced expression of c-*myc* mRNA does not inhibit terminal differentiation of F9 cells [Nishikura et al., 1990]. However, quite the opposite result—that transfection of F9 cells with a plasmid expressing the c-*myc* gene under control of a surrogate promoter results in cell clones that are resistant to RA-mediated differentiation—has also been obtained [Griep and Westphal, 1988].

In addition to the demonstration of an uncoupling of c-Myc expression from cell cycle progression, our study may have demonstrated a novel biological role for c-Myc, as an inducer of one or several events essential for the transition from a stem cell to a terminally differentiated extraembryonic parietal endoderm cell. In fact, evidence for such a role was recently reported for

another cell type [Gandarillas and Watt, 1997]. When c-Myc was constitutively expressed in primary human keratinocytes, there was a progressive reduction in growth rate and a marked stimulation of terminal differentiation, suggesting that c-Myc may be a factor that regulates exit from the stem cell compartment into the differentiation program [Gandarillas and Watt, 1997].

Taken together, our present results, and those of a few other recent studies, indicate that c-Myc may play a role not only in promoting cell cycle traverse, and in preventing differentiation, but also in opposing these events, at least in certain types of cells and under certain conditions. The explanation for these apparently contradictory findings may be concealed among new members in the family of c-Myc partners [Sakamuro et al., 1996; Wechsler-Reya et al., 1998; Facchini and Penn, 1998].

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