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Polyamines regulate eukaryotic initiation factor 4E-binding protein 1 gene transcription

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Abstract

Difluoromethylornithine-induced polyamine depletion produced a significant fall in the rate of 4E-BP1 gene transcription in IEC-6 cells, without a change in stability of the 4E-BP1 message. The effect was reversed by the addition of exogenous putrescine. Decreased 4E-BP1 gene transcription produced a concomitant fall in steady-state concentration of the 4E-BP1 protein. Segments of the 4E-BP1 gene 5′ flanking sequence were inserted into a GFP reporter construct. While all the segments containing the first 500 nucleotides 5′ to exon 1 were capable of driving GFP expression, two regions (between −2465 and −1965, and between −896 and 511) did so in a polyamine-dependent manner. Steady-state concentration of ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, was increased in response to polyamine depletion. These data provide a mechanism by which polyamines affect transcription of the 4E-BP1 gene, which in turn affect translation of ODC and perhaps other cap-dependent proteins.

Polyamines are organic cations found in virtually all tissues and cell types, both eukaryotic and prokaryotic. While it is well established that polyamines are essential to cellular growth and survival, the exact mechanism(s) by which they act remains unclear. Depending on cell type, eukaryotic cells may transport polyamines from their surrounding extracellular environment, or they may utilize the enzyme ornithine decarboxylase (ODC) to catalyze the conversion of the amino acid ornithine into the parent polyamine putrescine. Depletion of cellular polyamines stops cell cycle progression, producing cell cycle arrest in the G_1 phase of the cell cycle and preventing transition to the S phase [1–4]. Conversely, elevated ODC activity has been observed in aberrant growth and oncogenesis [5–8], and changes in concentration of both polyamines and ODC occur throughout the cell cycle [9]. Over-expression of ODC in normal cells induces cellular transformation, a process that is reversed by use of specific ODC inhibitors such as difluoromethylornithine (DFMO) [10]. The regulation of polyamines is, therefore, crucial for normal cell growth and division, in addition to their potential role in cancer. Cells treated with exogenous putrescine demonstrate a marked decrease in ODC activity, a classic example of end-product mediated inhibition [2]. Although several potential mechanisms for polyamine-mediated inhibition of ODC have been identified, including regulation at the post-transcriptional level and induction of antizyme [11], data presented below suggest a mechanism by which changes in polyamine concentration affects translation of not only ODC, but potentially many cellular proteins.

The majority of eukaryotic mRNA species are translated in a cap-dependent manner, referring to the fact that they require the formation of the eukaryotic initiation factor 4F complex (eIF-4F) in order to be translated [12–20]. These mRNAs are co-transcriptionally modified by the addition of a 7-methylguanosine, or

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cap, moiety to their 5′ terminus. A component of eIF-4F, eIF-4E, is the cap-binding protein and is the concentration limiting component required for activation of the eIF-4F translation initiation complex. Binding of eIF-4E to the 5′ cap is the rate-limiting step in translation of these proteins [21–23]. Once eIF-4E is bound to the 5′ cap, other proteins, starting with the scaffolding protein eIF-4G, begin assembling on the 5′ end of the mRNA and eventually form the eIF-4F complex. In turn, eIF-4E is held in limiting concentrations by binding proteins, the best studied of which is the translational repressor molecule 4E-binding protein 1 (4E-BP1), originally called PHAS-1 [24,25]. Both eIF-4E and 4E-BP1 are phosphoproteins, and are regulated by post-translational phosphorylation. Phosphorylation of 4E-BP1 decreases its affinity for eIF-4E, while phosphorylation of eIF-4E at Ser-209 alters its affinity for the cap structure and not 4E-BP1. eIF-4E's binding site for eIF-4G is competed for by 4E-BP1, thus 4E-BP1 prevents eIF-4F complex formation by preventing binding of eIF-4E to eIF-4G [26]. These multiple levels of control of eIF-4E and 4E-BP1 allow for varied responses depending on a variety of inputs. Earlier data from this laboratory on the effects of rapamycin on ODC activity suggested that polyamines might regulate the eIF-4F pathway and thereby modulate cap-dependent translation [20]. We reasoned that one possible mechanism responsible for a change in rate of translation could be through altering the amount of eIF-4E available for initiation of translation. As the availability of eIF-4E is controlled primarily by association with its binding protein 4E-BP1, we tested the hypothesis that polyamines were exerting their influence on translation by having an effect on steady state 4E-BP1 mRNA and protein concentrations by affecting 4E-BP1 gene transcription.

Materials and methods

Cell culture. Either IEC-6 (ATCC CRL-1592, passages 16–21) or NIH-3T3 (ATCC CRL1658) cells, as indicated, were cultured in Dulbecco's modified Eagle's medium plus 5% fetal bovine serum (FBS, for IEC-6 cells) or 5% calf serum (CS, for 3T3 cells) and were passaged weekly with trypsin/EDTA. Incubation was performed in a 5% CO_2, humidified atmosphere at 37 °C. Where applicable, cells were putrescine and spermidine depleted (referred to simply as "polyamine depleted") by daily treatment with 5 mM DFMO. For rescue assays, cells were treated with 10 μM putrescine either during DFMO treatment or following DFMO treatment as indicated. All cell cultures were subconfluent at the time of collection.

In vitro transcription. Antisense transcripts were generated from a full-length rat 4E-BP1 cDNA (JC Lawrence, University of Virginia) using the MAXIscript in vitro transcription kit (Ambion, Austin, TX). A rat β-actin template was used as a control. In experiments requiring radioactively labeled riboprobes (ribonuclease protection assays and mRNA half-life), [α-^{32}P]ATP (Perkin–Elmer, Boston, MA) was used in place of cold ATP. Full-length riboprobes were purified by electrophoresis and the band corresponding to the full-length probe was excised from the gel, eluted with 200 μl gel elution buffer (0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS), and allowed to incubate overnight at 37 °C with shaking to extract the RNA.

mRNA half-life. IEC-6 cells were treated with DFMO for 48 h or putrescine for 12 h following 48 h DFMO treatment. Cells were removed from plates by scraping at 0, 2, 6, and 12 h following Actinomycin D (10 μg/ml) treatment. Total RNA was isolated using the Trizol (Invitrogen, Carlsbad, CA) protocol, quantified, and subjected to slot blot analysis. Blots were exposed to HyperFilm (Amersham Biosciences, Piscataway, NJ), films were digitized, and densitometry was performed using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). Density measurements were used to calculate half-life of mRNA in three replicant experiments. Data were analyzed by ANOVA and when significant, Tukey's test was used as the post test. Differences were considered significant at $p < 0.05$.

Ribonuclease protection assay. The ribonuclease protection assay (RPA) was performed with the RPAII kit (Ambion). Two micrograms of total RNA from DFMO or DFMO followed by putrescine-treated IEC-6 cells was hybridized to a specific probe for 15 h at 65 °C. Unprotected RNA species were digested with 100 μl RNase A (250 U/ml) and T1 (10,000 U/ml). Protected fragments were subjected to electrophoresis on a 5% denaturing polyacrylamide gel (8 M urea in TBE). Bands were visualized by exposure of gels to film. Images were digitized and analyzed as described above. Change in 4E-BP1 hybridization was corrected for change in actin hybridization. Representative images are shown for experiments conducted in triplicate, and data were analyzed by *t* test of control versus DFMO treatment. Changes in band signal density were considered significant at $p < 0.05$.

Immunoblots. Steady-state levels of 4E-BP1, eIF-4E, and actin proteins were determined in IEC-6 cells treated with DFMO and DFMO followed by putrescine. Cells were collected in western collection buffer [50 mM Tris, pH 7.4, 5 mM EDTA, 25 mM NaF, 1 mM PMSF, and the protease inhibitors leupeptin, pepstatin, and aprotinin (10 μg/ml each)], sonicated, and cleared by centrifugation for 30 mim at 16,000*g* at 4 °C. Total protein concentration was determined by the method of Bradford using γ-globulin as a standard [27]. Samples were subjected to standard SDS–PAGE electrophoresis on 10% gels and transblotted to nitrocellulose using the rapid transfer protocol. Primary antibody incubation was for 4 h at 25 °C or overnight at 4 °C. Primary antibodies used were 4E-BP1 (JC Lawrence, University of Virginia; 1:1000), eIF-4E (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), ODC (Sigma–Aldrich, St. Louis, MO; 1:100), and actin (Oncogene Science, Cambridge, MA; 1:5000). Blots were visualized using the enhanced chemiluminescent protocol followed by exposure to Hyperfilm (Amersham Biosciences). Films were digitized and analyzed as described above. Representative immunoblots are shown for experiments conducted in triplicate, and data were analyzed by *t* test of control versus DFMO treatment. Changes in band signal density were considered significant at $p < 0.05$.

Nuclear run-off assay. Nuclei were isolated from control, DFMO, or DFMO followed by putrescine-treated IEC6 cells by scraping in nuclear homogenization buffer (20 mM Tris, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$). NP-40 was added to a final concentration of 0.15%, cells were homogenized, and nuclei were collected by centrifugation at 4 °C at 100*g*. Nuclei were resuspended in 1 ml resuspension buffer (40% glycerol, 50 mM Tris, pH 8.3, 0.1 mM EDTA, and 5 mM DTT). Nuclear run-off assays were performed using 250 μg nuclei, ATP, CTP, GTP, and [α-^{32}P]UTP at 25 °C for 30 min. Riboprobes for actin and 4E-BP1 as well as genomic DNA were blotted to Hybond membrane, cross-linked, and baked for 1 h at 65 °C. After pre-hybridization for 2 h at 60 °C, blots were hybridized in Hybrisol 1 for 48 to 60 h with total RNA that had been labeled in the reaction described above. Blots were treated as described above for RPA.

Green fluorescent protein reporter constructs. A 2499 base pair (bp) fragment of the 5′ flanking sequence of the 4E-BP1 gene (including 34 bp of exon 1) was generated by PCR, inserted into the pGlow T/A expression vector, and transformed into *Escherichia coli* according to

the manufacturer's protocol (Invitrogen). Plasmids were isolated and purified using the Wizard Mini-prep kit (Promega, Madison, WI), followed by restriction analysis or PCR to select for correct orientation of the insert.

3T3 cells growing in 100 mm dishes at approximately 60–80% confluence were transiently transfected with the construct using the standard calcium phosphate precipitation protocol. In order to account for differences in transfection efficiency across plates, a single plate of transfected cells was split into multiple 35 mm dishes (1×10^5 cells/dish) sufficient for all treatments within a replication, and the procedure was carried out in triplicate. This ensured that all dishes in each replication had approximately the same number of transfected cells. After plating, serum was removed from the transfected cells for 24 h to diminish GFP expression prior to beginning treatment and to ensure that all cells would be in approximately the same cell-cycle status. Cells were removed from the plates by lifting with 0.25% trypsin/EDTA, centrifuged at 500*g* for 5 min, washed, and resuspended in PBS. The suspension was transferred to 96-well assay plates, and fluorescence was measured using a SpectraMax Gemini XS Microplate Spectrofluorometer and SoftMax Pro software (Molecular Devices, Sunny Vale, CA).

A time-course study was conducted to verify a linear increase in fluorescence within the time course of 48 h DFMO-induced polyamine depletion. The construct described above was transfected into 3T3 cells which were cultured in serum-free DMEM (control) or in DMEM + 5% CS. Cells were collected and fluorescence was measured at 0, 2, 4, 7, 24, and 50 h, and fluorescence generated by the serum-treated cells was calculated relative to control cells.

To confirm a measurable response to 48 h DFMO treatment, the same construct was transfected and fluorescence was measured on 3T3 cells collected at 48 h following growth in DMEM with all combinations of serum or serum-free, 5 mM DFMO, and DFMO + 10 μM putrescine. For this study, fluorescence is given in arbitrary units.

A series of seven additional constructs were generated using the above-described protocol in order to localize the region(s) of the 4E-BP1 5′ flanking sequence responsible for polyamine responsiveness. Serum response was determined by measuring the change in fluorescence for serum-treated cells as compared to serum-starved cells. In order to standardize the response of cells to polyamine depletion during analysis of the various PCR fragments, fluorescence measured from cells treated with 5 mM DFMO for 48 h was calculated as a percentage reduction in fluorescence as compared to cells grown in DMEM plus 5% CS for each transfectant. Thus, the greater the reduction in measured fluorescence, the greater the response to polyamine depletion. The reduction in GFP production as a result of 48 h DFMO treatment is referred to herein as "polyamine responsiveness."

Comparison of sequence to a known polyamine response element. The 5′ flanking sequence of the mouse 4E-BP1 gene comprising approximately 3000 bp was sequenced by the Automated Sequencing Facility at the University of Tennessee. ClustalW (http://www.ebi.ac.uk/clustalw/) was used to compare the sequence of the mouse 4E-BP1 gene 5′ flanking sequence to a known human polyamine response element (PRE) [28].

Results

To determine the effect of polyamines on 4E-BP1 message and protein, total RNA and cytosolic protein were isolated from IEC-6 cells treated with either DFMO for 48 or 48 h of DFMO followed by exogenous putrescine treatment for up to 24 h. Forty-eight hours DFMO treatment has been shown by numerous laboratories to deplete cells of putrescine and spermidine [29].

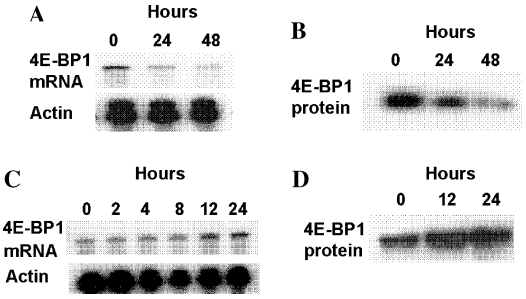


Fig. 1. Difluoromethylornithine (DFMO)-induced (5 mM) polyamine depletion decreases steady-state concentrations of (A) 4E-BP1 mRNA and (B) 4E-BP1 protein of IEC-6 cells. Addition of exogenous putrescine (10 μM) following DFMO-induced polyamine depletion returns steady-state concentrations of (C) 4E-BP1 mRNA and (D) 4E-BP1 protein of IEC-6 cells. Each experiment was performed three times with similar results and one representative experiment is illustrated.

ODC activity assays were also performed to verify the effectiveness of DFMO treatment as previously described [30]. DFMO decreased ODC activity in the presence of 5% FBS to that obtained in cells that had been deprived of serum for 48 h (data not presented). The steady-state level of 4E-BP1 mRNA in polyamine-depleted cells fell significantly compared to that of control cells (Fig. 1A), producing a parallel decrease in 4E-BP1 protein (Fig. 1B). Conversely, treatment of IEC-6 cells with exogenous putrescine following DFMO-induced polyamine depletion produced an increase in 4E-BP1 mRNA and an increase in 4E-BP1 protein (Figs. 1C and D, respectively). Thus, DFMO-induced polyamine depletion or addition of exogenous putrescine following polyamine depletion had a significant effect on both 4E-BP1 steady-state mRNA and protein levels. The same treatments had no effect on actin mRNA or protein.

The observed decrease in steady-state 4E-BP1 mRNA could be explained by one of two mechanisms: a decrease in 4E-BP1 mRNA stability or a decrease in 4E-BP1 gene transcription. The potential for a polyamine-induced change in stability of 4E-BP1 mRNA was examined in IEC-6 cells treated with DFMO or putrescine as described above followed by actD to prevent further transcription. In control cells treated with actD alone, the 4E-BP1 mRNA had a $t_{1/2}$ of 6.8 h. Neither DFMO (4E-BP1 $t_{1/2}$ = 5.9) nor DFMO followed by putrescine (4E-BP1 $t_{1/2}$ = 6.4) treatment produced a significant change in mRNA stability (Fig. 2). A representative slot blot is shown which clearly indicates no significant difference in 4E-BP1 $t_{1/2}$ between control cells and those cultured in 5 mM DFMO followed by 24 h 10 μM putrescine treatment. These data demonstrate that polyamines had no effect on 4E-BP1 mRNA transcript stability, suggesting a potential effect of polyamines at the level of 4E-BP1 gene transcription. Nuclear run-off assays were employed to measure alterations in the rate of de novo 4E-BP1 gene transcription.

4E-BP1 mRNA Half-life (min.)

	Mean	S.D.
Control (n=6)	6.9	0.3
Putrescine (n=3)	6.4*	0.5
DFMO (n=3)	5.9*	0.4

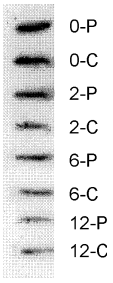


Fig. 2. Neither 48 h treatment of IEC-6 cells with 5 mM difluoromethylornithine (DFMO) nor addition of 10 μM putrescine after 48 h DFMO treatment significantly affected 4E-BP1 mRNA halflife. To the right is a representative slot-blot from which 4E-BP1 $t_{1/2}$ was calculated. Control cells (C) were grown for 48 h in complete medium, treated cells (P) were grown in complete medium plus 5 mM difluoromethylornithine for 48 h, followed by 10 μM putrescine for 24 h. Actinomycin D was applied, and cells were collected at the time points indicated. (*Not statistically different from control, $p > 0.05$.)

Nuclei isolated from cells incubated in DFMO for 48 h had marked decreases in the rate of 4E-BP1 gene transcription when compared to control (Fig. 3). Conversely, exogenous putrescine addition reversed the inhibition of gene transcription and produced a significant increase in the rate of de novo 4E-BP1 transcription. The rate of actin gene transcription was not altered from control. These data suggest the polyamine-dependent effect on 4E-BP1 was specific for 4E-BP1, and that polyamine-dependent changes in steady-state 4E-BP1 mRNA were due to modulation of the rate of 4E-BP1 gene transcription, and not due to changes in stability of existing 4E-BP1 mRNA.

To further characterize the ability of polyamines to modulate transcription of the 4E-BP1 gene, a GFP reporter construct was employed as described under Materials and methods. Approximately three kb of the 5′ flanking sequence immediately preceding exon 1 of the gene encoding mouse 4E-BP1 was sequenced and submitted to GenBank (Accession No. AY278989). The vector containing the complete sequence was used in preliminary studies to test the ability of the sequence to drive GFP expression. GFP is known to have a half-life

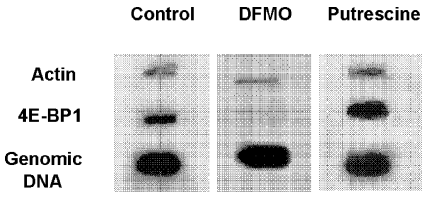


Fig. 3. Difluoromethylornithine (DFMO) (5 mM) decreases the transcription rate of the 4E-BP1 gene, while subsequent addition of putrescine (10 μM) returns transcription to control levels. Representative slot blots showing transcription rate of nuclei isolated from control, DFMO-treated (DFMO), and DFMO + putrescine (putrescine)-treated IEC6 cells. Cells were treated with DFMO for 48 h or with DFMO for 48 h plus treatment with putrescine for the subsequent 24 h. Experiment was performed three times with similar results.

in excess of 24 h [31], so all studies were carried out on cells that had been serum-deprived for 24 h prior to beginning treatment. To ensure our ability to adequately measure a GFP response in the 48 h time frame of these studies, preliminary experiments were conducted to determine the time-dependent response of transfected cells to stimulation with 5% CS (Fig. 4). Addition of serum to 24 h serum-deprived cells generated an approximate 4-fold increase in GFP expression after 50 h that was linear over time, indicating that the construct could be activated in this cell line and providing preliminary evidence for the presence of a serum response element in the 4E-BP1 5′ flanking sequence. We further used this construct to determine if GFP expression was responsive to DFMO-induced polyamine depletion, both with and without addition of serum (Fig. 5). Five millimolar of DFMO-induced polyamine depletion in the presence of serum (S + D) decreased fluorescence to approximately the same level as seen in serum-starved (SS) cells (Fig. 5A). There was no statistical difference in fluorescence between serum-stimulated (S) cells and those to which 10 μM putrescine was added in addition to the DFMO (S + D + P). Serum-starved cells were also treated with 5 mM DFMO either alone (SS + D) or with exogenous putrescine (SS + D + P) (Fig. 5B). Thus in this system, GFP expression was not polyamine responsive except in the presence of serum.

As a next step in our analysis, PCR fragments ranging from approximately 500 to 3000 bp (Fig. 6) were generated from the mouse 4E-BP1 gene 5′ flanking sequence and again inserted into the pGlow expression vector and transfected into 3T3 cells. There was a significant serum-induced increase in fluorescence for each fragment ($p < 0.05$), but there was no significant difference in serum responsiveness between fragments (data not shown). The proximal 511 bases upstream of the

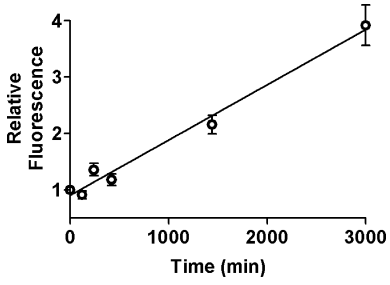


Fig. 4. A PCR fragment of the 5′ flanking sequence of the 4E-BP1 gene drives expression of a GFP reporter construct in a time-dependent, linear manner. A PCR fragment containing 2465 base pairs (bp) of the 5′ flanking sequence and 34 bp of exon 1 of the mouse 4E-BP1 gene was inserted into a pGlow expression vector and transfected into 3T3 cells. Cells were grown in Dulbecco's modified Eagle's medium without serum for 24 h, at which time 5% calf serum was added, and fluorescence measurements were conducted in a time course for 50 h. Bars represent mean of 3 replications (±SEM shown as error bars).

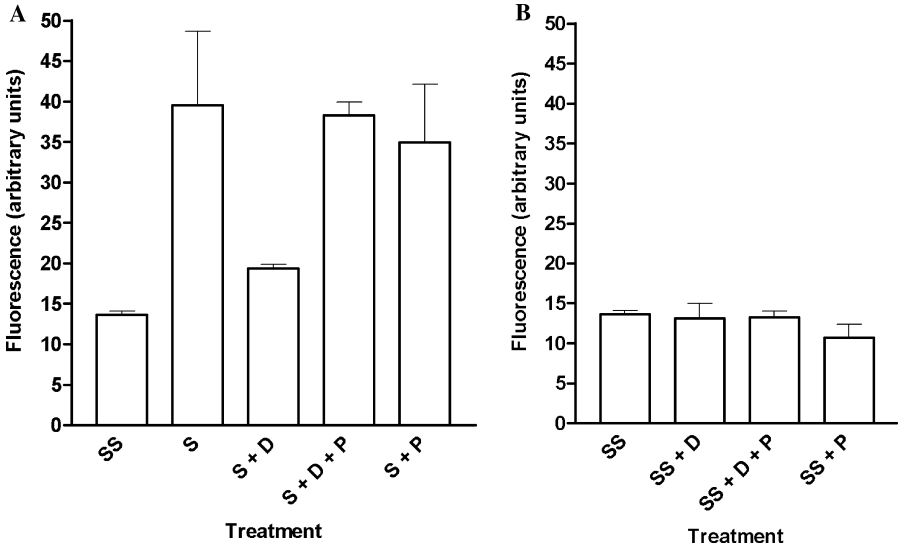
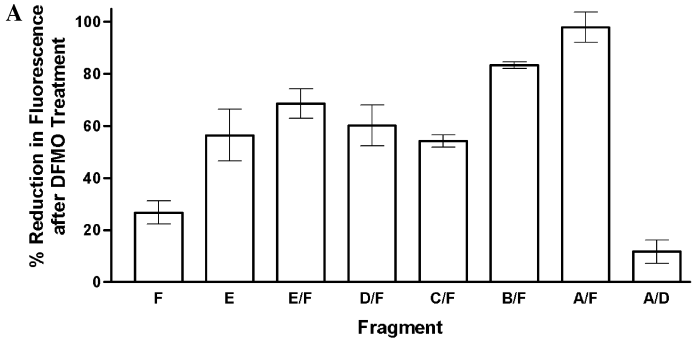


Fig. 5. A PCR fragment of the 5′ flanking sequence of the 4E-BP1 gene drives expression of a GFP reporter construct in a serum and polyamine-responsive manner. A PCR fragment containing 2465 base pairs (bp) of the 5′ flanking sequence and 34 bp of exon 1 of the 4E-BP1 gene was inserted into a pGlow expression vector and transfected into 3T3 cells. Cells were cultured for 48 h following 24 h serum depletion in Dulbecco's modified Eagle's medium plus the treatments described and fluorescence was measured. (A) Cells grown in 5% calf serum (S), serum + 5 mM DFMO + 10 μM putrescine (S + D + P), or serum + putrescine (S + P) showed significantly increased fluorescence as compared to serum-starved (SS) cells. Cells cultured in serum + DFMO (S + D) did not show a significant increase in fluorescence. (B) There was no significant change in fluorescence regardless of treatment when cells were cultured without serum. Bars represent mean of 3 replications (±SEM shown as error bars).



B

Fragment Name	Size (bp)	Left (5') Primer	5' Base	Right (3') Primer	3' Base
F	545	tgcccattcctcatcagatt	-511	atgtgcgaccttgtctgtga	+34
E	507	atcagcccacccttttcttct	-850	atcagcccacccttttcttct	-343
E/F	884	atcagcccacccttttcttct	-850	atgtgcgaccttgtctgtga	+34
D/F	1487	ctctggcagtcctggaact	-1453	atgtgcgaccttgtctgtga	+34
C/F	1989	attctcagggccatgtggt	-1955	atgtgcgaccttgtctgtga	+34
B/F	2499	atgtgcgaccttgtctgtga	-2465	atgtgcgaccttgtctgtga	+34
A/F	3020	ggaaggaaggaaggaaggaa	-2986	atgtgcgaccttgtctgtga	+34
A/D	2091	ggaaggaaggaaggaaggaa	-2986	aggaaatctggggcctctag	-896

C

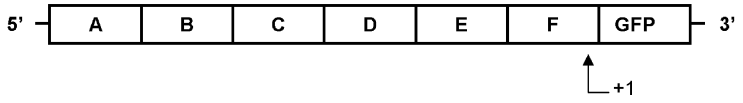


Fig. 6. (A) Two major polyamine responsive regions exist in the 5′ flanking sequence of the 4E-BP1 gene. PCR fragments were generated and inserted into the pGlow expression vector as described. Bars represent the percent reduction in fluorescence of cells grown in Dulbecco's modified Eagle's medium (DMEM) + 5% serum + 5 mM DFMO as compared to cells grown in DMEM + serum ($n = 3$, ±SEM shown as error bars). (B) Primers used to generate PCR fragments, size of fragment (bp), and start (5′) and ending (3′) base number relative to the transcription start site (+1). (C) Schematic illustration of fragments relative to GFP reporter sequence with transcription start site of 4E-BP1 gene indicated (+1) (not to scale).

transcription start site (−511 to +34, fragment F) was not polyamine responsive, in that DFMO treatment did not result in a change in measured fluorescence (Fig. 6). However, when approximately 900 bp were tested (−850 to +34, E/F), DFMO treatment decreased GFP expression as measured by fluorescence by approximately 60%. There was no change in the polyamine response when an additional 500 or 1000 bases (−1453 to +34, D/F or −1955 to +34, C/F) were added (as compared to fragment E/F). Interestingly, the sequence from −850 to −343 (E) showed approximately the same polyamine responsiveness (60% reduction in fluorescence) as fragment E/F when inserted immediately upstream of the GFP gene. We concluded from these data that a polyamine responsive sequence is likely located between −850 and −511 of the transcription start site, and the proximal bases (−343 to +34) are not necessary for transcription of the 4E-BP1 gene. When the sequence from −2465 to +34 (B/F) was tested, fluorescence was reduced in excess of 80%, which was significantly ($p < 0.05$) higher than for the shorter fragments. The fragment containing the sequence from 2986 to +34 (A/F) had virtually a 100% reduction in fluorescence as a result of DFMO treatment, but this value was not statistically different from the reduction seen for fragment B/F. These data indicate that a second, perhaps more important, PRE may exist between bases −2465 and −1955. The sequence from −2986 to −850 (A/D), which does not contain the proximal 850 bp, did not produce a significant change in fluorescence due to DFMO treatment. Using ClustalW, it was determined that four of the top matches to the human PRE were found at −2060 to −2052, −2111 to −2103, −2135 to −2127, and −655 to −647.

These data point to the possibility that a DFMO-induced decrease in 4E-BP1 gene transcription could result in an increase in cap-dependent translation, and in particular provide a mechanism by which polyamines regulate their own biosynthesis. To test this possibility, the effect of DFMO-induced polyamine depletion on steady-state concentration of ODC was also determined (Fig. 7). Depletion of polyamines from IEC-6 cells for 48 h resulted in a significant increase in ODC content.

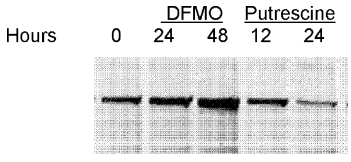


Fig. 7. Five millimolar of difluoromethylornithine (DFMO) treatment of IEC-6 cells for 48 h resulted in a significant increase in steady-state ODC content. Exogenous addition of putrescine (10 μM) following DFMO-induced polyamine depletion decreased steady-state concentration of ODC within 24 h. One representative immunoblot is shown from three replications showing similar results.

Addition of exogenous putrescine following polyamine depletion returned steady-state levels of ODC to control levels within 24 h.

Discussion

Polyamines are known to be essential for cellular growth and differentiation, and the data presented here provide compelling evidence for their role in regulating transcription of the 4E-BP1 gene. 4E-BP1 protein is a translational repressor that binds eIF-4E and inhibits the latter's ability to complete the eIF-4F translation initiation complex, thus inhibiting protein synthesis. We have demonstrated that DFMO-induced polyamine depletion results in a decrease in transcription of the 4E-BP1 gene, and therefore decreases the steady-state concentration of 4E-BP1 mRNA and protein. The decrease in steady-state 4E-BP1 protein has the potential to significantly increase the cellular content of free eIF-4E, the cap-binding protein. Although yet to be demonstrated experimentally, we hypothesize that the net result of decreased 4E-BP1 and the subsequent increase in free eIF-4E due to polyamine depletion could be a general increase in translation of proteins that are synthesized by the cap-dependent translation pathway. The addition of putrescine after 48 h DFMO-induced polyamine depletion reverses the effect and leads to increased 4E-BP1 gene transcription and increased steady-state 4E-BP1 mRNA and protein that should ultimately produce a fall in the initiation of translation of cap-dependent messages. Thus, polyamines may play a role in regulating translation of multiple proteins through their modulation of transcription of the gene encoding 4E-BP1. Indeed, it has been estimated that 80% of all eukaryotic mRNAs are translated by this pathway, suggesting polyamines may play an important role in regulating cellular translation of a large number of proteins.

The mechanism by which polyamines regulate gene transcription in general is an area of current, active research. A PRE has been identified in eukaryotic DNA, specifically in the promoter of the spermidine/spermine *N*1-acetyltransferase (SSAT) gene [28]. It is interesting that this enzyme is also involved in polyamine catabolism, downstream of the ODC enzyme. Cloning and sequence analysis of the 4E-BP1 5′ flanking sequence in *Mus musculus* by this laboratory has revealed the presence of several potential PRE elements in the 4E-BP1 gene upstream region. Our data correlate well with the in silico analysis of the 5′ flanking sequence for potential PREs. Three of the top four matches to the human PRE were found between −2135 and −2052, while the fourth was located between −655 and −647, the same segments that produced the increases in polyamine responsiveness. The fact that the bases proximal to the transcrip-

tion start site were necessary to see changes in GFP expression due to DFMO treatment in our system seems very reasonable. Sequence analysis indicates that multiple elements of control recognized by serum factors may be present in this segment.

In the human SSAT gene, three transcription factors/cofactors have been identified as binding partners which may be associated with the PRE [28,32,33]. These are nuclear factor E2-related factor 2 (Nrf-2), the mammalian homologue of the *Arabidopsis* COP 9 signalosome subunit 7a (CSN-7), and polyamine modulated factor 1 (PMF-1). These three *trans*-acting factors may interact in some manner to regulate SSAT gene transcription. The PMF-1 gene has been cloned in both human and mouse, and its expression has been shown to be regulated by polyamines [33,34]. The presence of polyamine responsive elements in the region 5′ to the start of the 4E-BP1 gene could be responsible for the polyamine-sensitive changes in transcription reported here. It is an intriguing possibility that polyamines could regulate the activity of these three *trans*-acting factors by having a direct effect on their assembly and subsequent interaction with the PRE. The polyamine-dependent self-assembly of nuclear factor NF-κB with its response element in transcription of proliferative genes as demonstrated in a breast cancer cell line is an example of such an affect [35].

Other investigators have proposed a role for polyamines in modulating histone acetylation, resulting in enhanced transcription factor accessibility to genomic DNA [36,37]. These data cannot eliminate the possibility that polyamines play a role in modulating histone acetylation. As has been shown, histones can associate with plasmid DNA [38]. It is not known if histones associate with the plasmid in this particular system. However, it seems unlikely that histone acetylation or de-acetylation could account for the changes in fluorescence demonstrated with this reporter system.

Polyamine-dependent regulation of transcription is not a new concept, but regulation of 4E-BP1 transcription may play an important, global role in cellular proliferation. Limited previous research has been conducted on factors related to regulation of 4E-BP1 gene transcription. In one other study, it was demonstrated that the transcription factor Egr1, induced by activation of either the ERK or p38 pathways, was capable of inhibiting expression of the 4E-BP1 gene in hematopoietic cell lines [39]. This raises the distinct possibility of the interaction of multiple factors in transcriptional control of 4E-BP1. However, the current work is the first to demonstrate a direct relationship between polyamine depletion and a decrease in 4E-BP1. This is also the first to determine the half-life of the 4E-BP1 message in IEC-6 cells, roughly 6 h. To date, the bulk of research on 4E-BP1 has focused on post-translational modification of the protein. In particular, the phosphorylation of both 4E-BP1 and eIF-4E obviously play important roles in determining the activity of these proteins [30,40–43]. Phosphorylation inactivates 4E-BP1, while it activates eIF-4E, and their phosphorylation results from activation of separate pathways. It is possible that under normal conditions, increased expression of 4E-BP1 may not lead to decreased cap-dependent translation, since it is possible that compensation in the form of increased phosphorylation of 4E-BP1 and/or eIF-4E may occur. This paper does not attempt to address the question of activity of 4E-BP1 or eIF4E. However, increasing the cellular content of 4E-BP1 with no change in the pathways leading to its phosphorylation should result in an increase in its association with eIF-4E, producing a corresponding fall in cap-dependent translation.

This paper further suggests a mechanism by which polyamines regulate their own biosynthesis. Polyamines exert product-mediated inhibition of ODC. When polyamines are elevated, ODC activity is decreased [2], and the decrease in 4E-BP1 following DFMO treatment could explain this phenomenon. If a decrease in 4E-BP1 results in increased availability of eIF-4E without the need to activate pathways leading to phosphorylation of 4E-BP1, translation of ODC and other mRNA transcripts that are translated by cap-dependent pathways could be increased. In this study, ODC steady-state concentration was significantly increased following polyamine depletion. This certainly appears plausible since a decrease in polyamines could trigger cells to increase their expression of the first enzyme in the polyamine biosynthetic pathway. On the other hand, addition of exogenous putrescine should decrease the necessity for biosynthesis, and indeed our data clearly demonstrate that putrescine addition to polyamine-depleted cells resulted in a decrease in steady-state ODC concentration.

These data provide clear evidence of a direct effect of polyamines on transcription of the 4E-BP1 gene. They demonstrate a mechanism by which polyamines could directly influence translation of a significant subset of cellular proteins. A mechanism is also proposed by which polyamines regulate their own biosynthesis, through regulating translation of the rate limiting enzyme of the polyamine biosynthetic pathway, ODC. The most widely accepted mechanism underlying regulation of ODC activity is mediated by the protein ornithine decarboxylase antizyme (AZ). AZ binds the cytosolic enzyme ODC and maintains it as an inactive monomer, thus inhibiting synthesis of new polyamines [44]. The AZ-ODC complex translocates to the 26S proteosome, where ubiquitin-independent proteolysis occurs [45]. Our data suggest a second mechanism whereby polyamines may regulate their own synthesis. By regulating the availability of 4E-BP1, polyamines modulate ODC translation. When intracellular polyamine concentrations are high, 4E-BP1 transcription is

increased. This would have the effect of allowing increased complex formation between 4E-BP1 and eIF-4E, limiting further ODC translation and leading to a reduction in cellular polyamines. Because of the potential significance of this mechanism, more extensive characterization of the 4E-BP1 gene 5′-flanking sequence and the effect of changes in 4E-BP1 gene transcription on global protein translation are logical next steps in the continuation of these studies.

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