Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs

Ildiko VERESS, Saeid HAGHIGHI, Anitta PULKKA and Antti PAJUNEN¹

Department of Biochemistry, University of Oulu, P. O. Box 400, FIN-90571 Oulu, Finland

We used differential display analysis to identify mRNAs responsive to changes in polyamine synthesis. As an overproducing model we used the kidneys of transgenic hybrid mice over-expressing ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, two key enzymes in polyamine biosynthesis. To identify mRNAs that respond to polyamine starvation, we treated Rat-2 cells with α -difluoromethylornithine, a specific inhibitor of polyamine biosynthesis. We isolated 41 partial cDNA clones, representing 37 differentially expressed mRNAs. Of these, 15 have similarity with known genes, five appear to be similar to reported expressed sequence tags and seventeen clones were novel sequences. Of the 35 mRNAs expressed differentially after α -difluoromethylornithine treatment, 26 were up-regulated. The expression of only three mRNAs was altered in the transgenic animals and all three were down-regulated. Determination of

mRNA half-life of three of the mRNAs up-regulated in response to polyamine depletion revealed that the accumulation results from stabilization of the messages. Because most of the transcripts identified from Rat-2 cells suffering polyamine starvation were accumulated, we conclude that polyamine depletion, while blocking cell growth, is stabilizing mRNAs. This may be due to the lack of spermidine for post-translational modification of the eukaryotic initiation factor 5A, which plays a major role in mRNA turnover. The coupling of mRNA stabilization with cell-growth arrest in response to polyamine starvation provides cells with an economical way to resume growth after recovery from polyamine deficiency.

Key words: differential display, α -difluoromethylornithine, Rat-2 cells.

INTRODUCTION

The polyamines spermidine and spermine and their precursor putrescine are organic polycations found in virtually all cells of higher eukaryotes. Studies with polyamine-deficient mutants and synthesis inhibitors have shown that polyamines are essential for normal cell growth and differentiation [1–3]. They have been implicated in many physiological functions including DNA replication, transcription and translation because they have profound and beneficial effects on these processes *in vitro*. It is difficult to distinguish which of these effects are polyamine-specific and which are due to the general cationic nature of these amines. However, intracellular polyamine concentrations usually rise before changes in DNA, RNA or protein synthesis can be detected, suggesting a role for polyamines in regulation of these processes [4]. Upon polyamine deprivation, cells cease to grow and proliferate, the protein and nucleic acid elongation rates are diminished, the fidelity of translation is impaired [5], and chromosomes may disintegrate in later stages of starvation [6]. Because the suppression of cell growth can be overcome by adding exogenous spermidine and spermine, it is apparent that a minimum amount of polyamines is needed to sustain growth processes. The effects of polyamines on cells are not exclusively beneficial, since an excessive polyamine accumulation has been shown to be toxic [7] and to induce apoptosis [8,9]. However, precise molecular mechanisms underlying these effects have not been resolved.

Intracellular polyamine homeostasis is highly regulated by both biosynthetic and catabolic enzymes [2,10–13]. The two key regulatory enzymes in their biosynthesis are ornithine decarboxylase (ODC; EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50). The catabolism of polyamines in eukaryotes is controlled by spermidine/spermine *N*¹-acetyltransferase. These enzymes are very strictly regulated *in vivo*, not only at the level of gene expression but also post-transcriptionally by the polyamines themselves. This reflects the key role of these enzymes in maintaining the polyamine levels between the physiological limits. However, polyamine homeostasis is not only maintained by synthesis and catabolism. There is also substantial evidence that the polyamine transport system plays a quantitatively important role in the homeostasis of polyamine pools *in vivo*. Depletion of intracellular polyamines by treatment with α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, enhances their uptake several fold [14,15]. In transgenic hybrid mice over-expressing both ODC and AdoMetDC the compensation mechanism that prevents intracellular accumulation of higher polyamines involves acetylation and increased export out of the cell [16]. Taken together, the mammalian cells have highly effective means to regulate intracellular polyamine concentrations according to their metabolic needs.

Many observations suggest strongly that a major portion of the intracellular polyamines are sequestered [13]. It has been estimated that over 90 % of intracellular spermidine and spermine

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, α -difluoromethylornithine; MGBG, methylglyoxal bis(guanyldrazon); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; eIF-5A, eukaryotic initiation factor 5A.

¹ To whom correspondence should be addressed (e-mail antti.pajunen@oulu.fi).

The nucleotide sequences reported have been deposited in the GenBank database (accession numbers AA856177–AA856211 and AI586376–AI586378).

Table 1 Intracellular polyamine content in DFMO-treated Rat-2 cells

Rat-2 cells were incubated for 90 min or 48 h in Dulbecco's modified Eagle's medium in the presence of 5 mM DFMO prior to determination of polyamine contents. Other details are provided in the Experimental section. Values are means $\pm$ S.D. for four independent experiments. Significance between DFMO-treated and controls: * $P < 0.001$.

	Incubation period	Polyamine concentration (pmol/ μ g of DNA)		
		Putrescine	Spermidine	Spermine
Control	90 min	99.93 $\pm$ 7.07	253.40 $\pm$ 28.97	125.05 $\pm$ 25.82
DFMO	90 min	57.77 $\pm$ 5.76*	246.10 $\pm$ 21.81	112.64 $\pm$ 26.92
Control	48 h	101.07 $\pm$ 19.76	198.02 $\pm$ 18.58	105.82 $\pm$ 22.23
DFMO	48 h	1.58 $\pm$ 0.58*	16.64 $\pm$ 6.52*	131.57 $\pm$ 15.93

Figure 1 mRNA differential displays

(A) mRNA differential-display autoradiograph of total RNA of DFMO-treated Rat-2 cells using exon 6- and 7-specific decamer primers that primed the amplification of a 257-bp cDNA fragment from the AdoMetDC mRNA (lane 2). PCR-amplification products of rat genomic DNA (lane 1) serve as size markers (*rAmd1* [26], *rAmdP2-3* [27] and *rAmdP1* [28]). The sequences of two pseudogenes, *rAmdP2* and *rAmdP3*, are co-linear with cDNA in the region that is amplified with these primers, giving amplification products of the same size. (B) Representative mRNA differential displays from DFMO-treated (lanes 1–4) and control Rat-2 cells (lanes 5–8). Four PCR reactions were performed for both groups and run in adjacent lines to minimize artifacts. Most of the cDNA bands produced were of equal intensity across all eight lines. Only bands altered with DFMO treatment that were consistent in each group were selected for analysis. Arrows mark examples of DFMO-treatment-induced and -suppressed bands.

are bound to cell constituents [17]. Thus even small changes in the rates of their synthesis, degradation or transport markedly affect the levels of free polyamines. The pool of free polyamines can fluctuate rapidly in response to intracellular signals and this pool might play a regulatory role in controlling tissue growth.

We have used mRNA differential display as an approach to investigate the physiological consequences of changes in the polyamine pool and to gain a better understanding of the mechanisms that underlie these responses. Rat-2 cells cultured in

the presence or absence of DFMO were compared for differences in expression of mRNAs under conditions of polyamine depletion. In addition, transgenic mice overexpressing two key enzymes in polyamine synthesis, ODC and AdoMetDC, and their non-transgenic litter-mates were compared to determine the changes in mRNA resulting from polyamine overproduction. Products amplified from mRNA species that appeared to be down- or up-regulated were selected for further analysis. In this report, we describe identification of 38 cDNAs expressed differentially in response to altered polyamine status.

EXPERIMENTAL

Cell line and animals

Rat-2 cells (A.T.C.C., Rockville, MD, U.S.A.) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin and 5% (v/v) fetal bovine serum in 5% CO₂/95% air at 37 °C. Subconfluent cultured Rat-2 cells were treated with 5 mM DFMO for 90 min or 48 h. For mRNA half-life assays cells were treated with 4 μ M actinomycin D for 0, 3, 6 and 9 h before harvesting total RNA. A transgenic mouse line overexpressing both ODC and AdoMetDC was obtained as described in [16]. RNA was isolated using the RNeasy kit (Qiagen) and treated with RQ1 RNase-free DNase (Promega Biotech) before mRNA differential display.

Analytical methods

Differential display was performed essentially as described in [18,19]. Briefly, 0.4 μ g of RNA was used in a reverse-transcription reaction using each of the four 1-bp-anchored 3' oligo(dT) primers [20] and 300 units of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (RT; Promega Biotech) in a 20- μ l vol. The reverse-transcribed cDNA (2 μ l) was used for each PCR reaction, performed using the same 1-bp-anchored 3' oligo(dT) primer and four 5' arbitrary oligonucleotides of 10 nucleotides in length [19]. The 20- μ l PCR reaction contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 μ M dNTP, 0.4 μ l of [α -³²S]dATP (1000 Ci/mmol), 2 μ M of 5' arbitrary oligonucleotides and 3' anchored dT oligonucleotides and 0.5 μ l of *Taq* polymerase. Parameters for PCR were 40 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 2 min, and extension at 72 °C for 30 s. Then 2.5 μ l of the PCR reaction mixture was loaded on to an 8% sequencing gel, and differentially amplified PCR fragments were visualized by exposing the dried sequencing gel to X-ray film. Candidate PCR products were excised from the gel and DNA eluted from the gel slices by boiling in TE buffer (10 mM Tris/HCl, pH 7.5, and 1 mM EDTA) for 10 min. The eluted DNA fragments were re-amplified by using the same primer pair and subsequently cloned into the TA cloning vector (Invitrogen). Inserts were sequenced by cycle sequencing on an ABI sequencer and DNA sequences analysed using the FASTA and BLAST computer programs. The isolated cDNA fragments were used as probes to determine differential expression by Northern-blot analyses.

For the Northern-blot analysis, total RNA was fractionated (15 μ g/lane) on a 1% agarose gel containing formaldehyde [21], blotted on to nitrocellulose and hybridized with cDNA fragments labelled with [α -³²P]dCTP using the random-priming method (Boehringer Mannheim).

The testis-expressed gene 261 (Tex261) cDNA [22] covering the coding sequence was produced by RT-PCR using sequence-

specific primers (5'-TCCTGTCCAATGCTGATTCC-3' and 5'-CCCAGCTACAGACTTTCTACCC-3') and inserted into a *NheI/BstXI* site of the pBK-CMV phagemid (Stratagene). Rat-2 cells were grown as described above. At 24 h after plating, cells were transfected with a total of 10 µg of pBK-CMV-*Tex261* by the calcium phosphate DNA precipitation using Hepes-buffered saline and standard protocol [23]. After 6 h cells were washed twice with PBS and the medium was replaced with 10 ml of fresh medium containing 5% fetal bovine serum. To monitor the effect of polyamine depletion on the stability of *Tex261* transcript in transfectant Rat-2 cells, medium was supplemented with 5 mM DFMO and/or 5 µM MGBG [methylglyoxal bis-(guanyldihydrazone)], an inhibitor of AdoMetDC. Total RNA was isolated 48 h after transfection.

Polyamine concentrations were determined essentially as described in [24] and DNA was quantified by the method described by Giles and Myers [25].

RESULTS

To identify mRNAs responsive to changes in polyamine biosynthesis, we performed RNA differential-display analysis using RNAs from Rat-2 cells subjected to short-term (90 min) or long-term (48 h) treatment with DFMO (5 mM). The purpose of choosing two treatment periods was that the long-term treatment may cause, in addition to specific effects, secondary effects due to the cessation of cell growth. Intracellular polyamine concentrations were determined to monitor the efficiency of DFMO treatment (Table 1). As expected there were no changes in spermidine or spermine concentrations after 90 min whereas putrescine concentration was decreased by about 30%. After 48 h putrescine was almost totally depleted and the spermidine concentration was reduced to about one-tenth that in control cells, whereas the spermine concentration remained unchanged.

To minimize errors in the differential-display procedure and to avoid selection of artifact bands as differentially expressed

Table 2 Summary of differential-display clones

Clone designations are coded from the anchor primer (e.g. CG = T¹²CG) and arbitrary primer (e.g. 16 = OPA16) followed by a band designation. Northern-blot analysis was used to confirm the regulated status of some of the clones (n.d., not determined). The differential-display clones that turned out to represent false positives and those that did not display any band in the Northern-blot analysis, apparently corresponding to very rare mRNAs, are denoted by 'not confirmed'. Three sequences which were the same both after the short-term and long-term treatments are denoted by *. Homology or identity for each clone, excluding the PCR primer sequence, was identified as the highest-scoring homologue from FASTA searches of the EMBL database (EMBL All library). Accession numbers for the homologues can be found under the EST/GenBank entry for each clone. EST, expressed sequence tag.

Clone	Accession no.	Size (bp)	Effect of DFMO treatment	Northern-analysis confirmation	Identity/homology
Short-term treatment (90 min)					
DFMO-CA-16-1	AA856203	146	Up	n.d.	Unknown
DFMO-CA-19-1	AA856207	326	Up	n.d.	Unknown
DFMO-CA-19-2	AA856208	148	Down	Confirmed	Mouse EST (MM97319)*
DFMO-CC-16-1	AA856178	207	Up	Confirmed	Rat mitochondrial adenine nucleotide translocator*
DFMO-CG-19-1	AA856183	220	Up	n.d.	Rat ribophorin II
DFMO-CT-16-1	AA856184	150	Down	n.d.	Rat mitochondrial 16 S rRNA
DFMO-CG-17-1	AA856189	162	Up	Not confirmed	Human EST (AA648550)*
DFMO-CG-17-2	AA856195	230	Up	n.d.	Unknown
Long-term treatment (48 h)					
DFMO-CA-16-2	AA856204	156	Up	Confirmed	Mouse CArG-box-binding factor
DFMO-CA-17-1	AA856205	127	Up	n.d.	Rat non-muscle myosin alkali light chain
DFMO-CA-18-1	AA856206	200	Up	n.d.	Unknown
DFMO-CA-19-3	AA856209	232	Up	n.d.	Unknown
DFMO-CA-19-4	AA856210	161	Up	n.d.	Unknown
DFMO-CA-19-5	AA856211	167	Up	Confirmed	Human EST (AA197287)
DFMO-CA-19-6	AA856177	131	Up	Confirmed	Mouse J1 protein (L3)
DFMO-CG-18-1	AA856179	122	Up	Not confirmed	Rat EST (AA799393)
DFMO-CG-18-2	AA856180	185	Up	n.d.	Mouse EST (AA386981)
DFMO-CG-18-3	AA856181	268	Down	n.d.	Rat collagen α-1 type I
DFMO-CG-18-4	AA856201	302	Down	n.d.	Rat lysosomal membrane glycoprotein
DFMO-CG-18-5	AA856182	348	Down	n.d.	Unknown
DFMO-CT-16-2	AA856185	132	Up	n.d.	Unknown
DFMO-CT-16-3	AA856202	200	Up	n.d.	Unknown
DFMO-CT-16-4	AA856186	154	Up	n.d.	Unknown
DFMO-CT-16-5	AA856187	150	Up	n.d.	Unknown
DFMO-CT-16-6	AA856188	151	Up	n.d.	Rat squalene synthetase
DFMO-CC-17-2	AA856190	175	Up	n.d.	Unknown
DFMO-CC-16-2	AA856191	188	Down	n.d.	Unknown
DFMO-CC-17-4	AA856192	200	Up	Confirmed	Rat mitochondrial acetoacetyl-CoA thiolase
DFMO-CC-17-3	AA856193	200	Up	n.d.	Unknown
DFMO-CC-17-5	AA856194	200	Up	n.d.	Unknown
DFMO-CG-17-3	AA856196	269	Down	Not confirmed	Cyr61
DFMO-CG-17-4	AA856197	272	Up	n.d.	Unknown
DFMO-CC-17-1	AA856198	286	Up	Not confirmed	Mouse circadian clock protein (Per2)
DFMO-CC-18-1	AA856199	351	Down	Not confirmed	Rat peptidylglycine α-amidating mono-oxygenase
DFMO-CT-18-1	AA856200	243	Down	n.d.	Unknown

Figure 2 Differential expression of RNAs in DFMO-treated and untreated Rat-2 cells assayed by Northern blotting

The eight cDNA clones shown to be displayed differentially are as follows: CarG-box-binding factor, acetoacetyl-CoA thiolase, ribosomal proteins L3 and 3Sa, ADP/ATP translocase, GAPDH, β -actin and the expressed sequence tag (DFMO-CA-19-2) showing a high similarity with *Drosophila melanogaster* acetyl-CoA synthetase. The amount and quality of total RNA loaded in the gels were examined by staining the transferred membrane with Methylene Blue [33].

transcripts, RNA samples prepared from four separate experiments were used for each display. The series of differential-display reactions of Rat-2 cells using T₁₂CM-anchored primers (where M is A, T, G or C) with four arbitrary decamers yielded 15 cDNA bands after 90-min treatment and 44 cDNA bands after 48-h treatment with a visible difference of intensity (Figure 1B). Of these bands, 43 showed increased intensity and 16 showed decreased intensity as a result of DFMO treatment. To confirm the reproducibility of the differential expression, we repeated the display using a new set of RNAs isolated from control and DFMO-treated cells. Of the differentially expressed cDNA bands, 35 were reproducible and these bands were subcloned and analysed subsequently by DNA sequencing. Three

of the sequences were present after both the short-term and long-term treatments.

It is known that DFMO treatment will cause the accumulation of AdoMetDC mRNA [26,27] and therefore, as a control to confirm the sensitivity and effectiveness of mRNA differential display under these conditions, we used a decamer pair that should prime amplification of a 257-bp PCR product from rat AdoMetDC (Figure 1A) [28–30]. A cDNA band of that size was observed, confirming the sensitivity of the method. Searches of the GenBank/EMBL databases with all 35 clones revealed that 13 of the sequences corresponded to known genes, five appeared to be similar to expressed sequence tags and seventeen clones had no significant similarity with any sequence in the databases (Table 2).

We subjected 11 of the 35 cDNA fragments to Northern-blot analysis. Six clones showed changes in gene expression that confirmed the differential-display pattern (Table 2 and Figure 2), five of them being increased and one of them decreased. As a positive control, the same filter was hybridized with β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probes. It has been shown that the steady-state level of β -actin mRNA increases in cultured mammalian cells upon DFMO treatment [31,32], and we have observed previously that GAPDH also gave repeatedly higher signals from DFMO-treated cells than control samples, although hybridization or ethidium bromide staining of 28 S and 18 S rRNA had shown comparable amounts of RNA in each lane. The Northern blot in the present study confirmed this accumulation of β -actin and GAPDH transcripts after DFMO treatment.

Although it is known that transgenic mice over-producing polyamines are able to maintain intracellular polyamine homeostasis by their acetylation and excretion [16], it is not clear whether there are other cellular consequences of the increased synthesis. Therefore we used as another experimental group transgenic mice overexpressing ODC and AdoMetDC versus their non-transgenic litter-mates. RNAs isolated from kidneys were used in differential display to map differences in gene-expression profiles. We detected only three differentially expressed cDNA bands when RNAs from the kidneys of transgenic and non-transgenic mice were used in differential display (Table 3) and all three transcripts were down-regulated. One of them, murine J1 protein mRNA (yeast ribosomal protein L3 homologue) [34,35], also showed altered expression in response to polyamine depletion in DFMO-treated Rat-2 cells where its amount was consistently increased (Table 2 and Figure 2). The second was B94 (mouse primary response gene mRNA [36]). The third down-regulated transcript coded for Tex261, which has been characterized from mouse testis where it is expressed preferentially in germ cells during and after meiotic differentiation [22].

Table 3 Summary of cDNA clones identified by differential display from the kidneys of transgenic versus non-transgenic mice

See Table 2 for explanations.

Clone	Accession no.	Size (bp)	Status in transgenic mice	Northern-analysis confirmation	Identity/homology
ODC-ADOMETDC-CA-17-1	AI586376	111	Down	Not confirmed	Mouse B94
ODC-ADOMETDC-CA-18-1	AI586377	257	Down	Confirmed	Mouse Tex261
ODC-ADOMETDC-CA-19-1	AI586378	156	Down	Confirmed	Mouse J1 protein (L3)

Figure 3 Effect of DFMO on the turnover of AdoMetDC mRNA

Rat-2 cells were untreated or treated with 5 mM DFMO 48 h before exposure to 4 μ M actinomycin D for the times indicated. Total RNA (0.4 μ g) was reverse-transcribed using an oligo(dT) primer. Aliquots of the RT reactions were used for PCR in the presence of 0.2 μ Ci [α - 32 P]dCTP as tracer. PCR reactions were run for 15 cycles using exons 6- and 7-specific primers (5'-CTGCAAAGGATGTCACG-3' and 5'-CAAGGTGGTCACAAATTTCC-3') at an annealing temperature of 57 °C. Reaction products were resolved on 6% polyacrylamide gel and exposed for autoradiography. Three experiments were performed that showed similar results.

Figure 4 Effect of DFMO on the turnover of GAPDH mRNA

Rat-2 cells were untreated or treated with 5 mM DFMO 48 h before exposure to 4 μ M actinomycin D for the indicated times. Total RNA was analysed by Northern blotting using a GAPDH-specific probe. Loading of RNA was monitored by staining the transferred membrane with Methylene Blue [33].

Because most of the differentially displayed transcripts were up-regulated in DFMO-treated cells we tested whether mRNA accumulation results from mRNA stabilization. Three of the up-regulated mRNAs were chosen for half-life analysis after actinomycin D treatment. Northern-blot and RT-PCR analyses showed that all of these three transcripts were stabilized in DFMO-treated cells. To measure the half-life of AdoMetDC mRNA we performed RT-PCR in the presence of radiolabel with the primers that amplified exons 6 and 7 (Figure 3). Scanning with a densitometer showed that AdoMetDC mRNA half-life in the control cells was about 3 h whereas in the polyamine-depleted cells it remained constant up to 6 h. Similarly, both GAPDH (Figure 4) and Tex261 (Figures 5A and 5B) transcripts were found to be clearly stabilized in DFMO-treated cells.

To confirm further the effect of polyamine depletion on stabilization of mRNAs we transfected Rat-2 cells with a chimaeric construct where the coding sequence of Tex261 was placed under the human cytomegalovirus promoter (Figure 5C). Polyamine depletion by treatment with DFMO decreased the degradation rate of the chimaeric transcript. The degradation rate was decreased further by treatment with both DFMO and MGBG.

Figure 5 Effect of DFMO on expression and turnover of Tex261 mRNA in Rat-2 cells

Total RNA was analysed by Northern blotting using a Tex261 probe. (A) Lane 1, untreated; lane 2, DFMO-treated (5 mM, 48 h). Loading of RNA was monitored by staining the transferred membrane with Methylene Blue [33]. (B) Effect of DFMO on turnover of Tex261 mRNA. Cells were untreated or treated with DFMO (5 mM) 48 h before exposure to actinomycin D (4 μ M) for the times indicated. (C) Effect of DFMO (5 mM) and/or MGBG (5 μ M) on the stability of the chimaeric Tex261 transcript. Cells were transfected with pBK-CMV-Tex261 and after 6 h cells were washed and the medium replaced with fresh medium with or without drugs. Total RNA was isolated 48 h after transfection. Lane 1, mock-transfected; lane 2, pBK-CMV-Tex261-transfected; lane 3, mock-transfected + DFMO; lane 4, pBK-CMV-Tex261-transfected + DFMO; lane 5, mock-transfected + MGBG; lane 6, pBK-CMV-Tex261-transfected + MGBG; lane 7, mock-transfected + MGBG + DFMO; lane 8, pBK-CMV-Tex261-transfected + MGBG + DFMO.

Figure 6 Effect of putrescine or spermidine on S3a mRNA accumulation in DFMO-treated Rat-2 cells

Cells were treated with DFMO (5 mM) 48 h before exposure to putrescine (50 μ M) or spermidine (50 μ M) for 1 h. Total RNA was analysed by Northern blotting using S3a-specific probe. Loading of RNA was monitored by staining the transferred membrane with Methylene Blue [33]. Lane 1, untreated; lane 2, DFMO-treated; lane 3, DFMO + putrescine; lane 4, DFMO + spermidine.

To obtain additional evidence that polyamine depletion itself is responsible for the stabilization of these mRNAs, we added back putrescine or spermidine to the DFMO-treated cells and analysed the content of the S3a (ribosomal protein) transcript by Northern blotting. After polyamine addition (1 h) the mRNA level was reduced to the control level (Figure 6), confirming that the mRNA stabilization is associated with polyamine depletion.

DISCUSSION

Numerous studies with polyamine-deficient mutants and synthesis inhibitors have confirmed that polyamines are essential for optimal cell growth [1,2,10,37,38]. As a result of polyamine deprivation, cells cease to grow and proliferate, but usually they

do not die [39,40]. The suppression of cell growth can be overcome by adding exogenous spermidine or spermine. Because DFMO treatment resulted in both up-regulated and down-regulated transcripts, these changes cannot be due solely to a general decrease in the transcription rate and thus just secondary effects due to the arrest of cell growth by DFMO. However, since most of the differentially displayed transcripts were up-regulated under polyamine depletion, this suggests the generality of the phenomenon. The fact that not all transcripts were stabilized by DFMO indicates a selective mechanism.

Interestingly, GAPDH mRNA, which is commonly used as an RNA loading control in Northern-blot analyses, was also accumulated following DFMO treatment. The appropriateness of the GAPDH gene as a loading control has also been questioned by others because of its susceptibility to the influence of growth factors such as transforming growth factor- β [41,42]. In addition to the transcripts stabilized by polyamine depletion reported here, there are several other examples where the treatment of mammalian cells with polyamine synthesis inhibitors leads to the stabilization of mRNAs such as transglutaminase [43], transforming growth factor- β [44], tubulin [32,45] and β -actin [32] mRNAs among others. Similar superinduction of most of these mRNAs has also been demonstrated by using protein-synthesis inhibitors [31,32,46,47]. Polyamine depletion also decreases general protein synthesis [5,38]. One possible reason for protein-synthesis inhibition by polyamine depletion could be that spermidine acts as a substrate for eukaryotic initiation factor 5A (eIF-5A) modification [48,49]. Because eIF-5A is necessary for cell proliferation, depletion of cellular polyamines suppresses growth by depleting the cellular content of modified eIF-5A [50]. Originally eIF-5A was thought to be a translation-initiation factor involved in the formation of the first peptide bond [51,52].

However, work by Kang and Hershey [53] has shown that marked depletion of this protein in yeast cells, while resulting in cell-cycle arrest, led only to a 30% drop in the first round of protein synthesis. This finding was confirmed by Zuk and Jacobson [54] using yeast deletants in which the *TIF51A* gene coding for eIF-5A was inactivated. Furthermore, they showed that this yeast deletant strain exhibited decreased mRNA decay rates with 30% inhibition of protein synthesis. A similar increase in the abundance of mRNAs was only observed with cycloheximide when inhibition of protein synthesis reached 80%. Taken together, these data suggest that eIF-5A plays a role in mRNA turnover in addition to that in protein synthesis. This can explain why the major changes among differentially displayed transcripts by DFMO treatment were transcript up-regulations. Polyamine-synthesis inhibitors arrest cell growth in G_1 [55–57]. Because eIF-5A depletion also blocks cell growth in the G_1 phase [58,59], this suggests that cessation of cell growth by polyamine-synthesis inhibitors results from the depletion of spermidine required for eIF-5A modification.

Recently Ross [60] presented a rationale for the stabilization of mRNAs in mammalian cells by translation inhibitors. He proposed that mRNA stabilization reflects a physiological process that occurs during each mitosis and is necessary for cell survival. Transcription [61,62] and translation [63–65] decline dramatically during a 1–2 h interval of mitosis and this translational repression somehow inactivates a critical component of the mRNA-degradation machinery. As a result, mRNA half-lives are prolonged during the interval when transcription is repressed. If labile mRNAs were not stabilized during mitosis they would be depleted as the cell entered G_1 . Stabilization during mitosis, or in response to translation inhibitors, thus preserves the capacity of the cell to synthesize essential proteins as it enters G_1 or recovers from inhibitor treatment. The coupling of mRNA stabilization with

translation arrest would also provide a response mechanism to stresses such as polyamine starvation.

REFERENCES

- Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- Marton, L. J. and Pegg, A. E. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35**, 55–91
- Morgan, D. M. L. (1990) *Biochem. Soc. Trans.* **18**, 1080–1084
- Marton, L. J. and Morris, D. R. (1987) in *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E. and Sjoerdma, A., eds.), pp. 79–105, Academic Press, Orlando
- Pohjanpelto, P. and Knuutila, S. (1982) *Exp. Cell Res.* **141**, 333–339
- Pierce, G. B., Parchment, R. E. and Lewellyn, A. L. (1991) *Differentiation* **46**, 181–186
- Packham, G. and Cleveland, J. L. (1994) *Mol. Cell. Biol.* **14**, 5741–5747
- Poulin, R., Pelletier, G. and Pegg, A. E. (1995) *Biochem. J.* **311**, 723–727
- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- Jänne, J., Alhonen, L. and Leinonen, P. (1991) *Ann. Med.* **23**, 241–259
- Casero, Jr., R. A. and Pegg, A. E. (1993) *FASEB J.* **7**, 653–661
- Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290
- Seiler, N. and Dezeure, F. (1990) *Int. J. Biochem.* **22**, 211–218
- Byers, T. L. and Pegg, A. E. (1990) *J. Cell. Physiol.* **143**, 460–467
- Heljasvaara, R., Veress, I., Halmekytö, M., Alhonen, L., Jänne, J., Laajala, P. and Pajunen, A. (1997) *Biochem. J.* **323**, 457–462
- Watanabe, S.-I., Kusama-Eguchi, K., Kobayashi, H. and Igarashi, K. (1991) *J. Biol. Chem.* **266**, 20803–20809
- Liang, P. and Pardee, A. B. (1992) *Science* **257**, 967–971
- Liang, P., Averboukh, L. and Pardee, A. B. (1993) *Nucleic Acids Res.* **21**, 3269–3275
- Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connell, R. P., Averboukh, L., Wang, F. and Pardee, A. B. (1994) *Nucleic Acids Res.* **22**, 5763–5764
- Meinkoth, J. and Wahl, G. (1984) *Anal. Biochem.* **138**, 267–284
- Lopez-Fernandez, L. A., Parraga, M. and del Mazo, J. (1998) *Biochem. Biophys. Res. Commun.* **242**, 565–569
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor
- Gilbert, R. S., Gonzalez, G. G., Hawel, III, L. and Buys, C. V. (1991) *Anal. Biochem.* **199**, 86–92
- Giles, K. W. and Myers, A. (1965) *Nature (London)* **206**, 93
- White, M. W., Degnin, C., Hill, J. and Morris, D. R. (1990) *Biochem. J.* **268**, 657–660
- Pajunen, A., Crozat, A., Jänne, O. A., Ihalainen, R., Laitinen, P., Stanley, B., Madhubala, R. and Pegg, A. E. (1988) *J. Biol. Chem.* **263**, 17040–17049
- Pulkka, A., Ihalainen, R., Suorsa, A., Riviere, M., Szpirer, J. and Pajunen, A. (1993) *Genomics* **16**, 342–349
- Heljasvaara, R., Laajala, P., Soininen, T. and Pajunen, A. E. (1996) *Mamm. Genome* **7**, 776–779
- Pulkka, A., Keränen, M.-R., Salmela, A., Salmikangas, P., Ihalainen, R. and Pajunen, A. (1990) *Gene* **86**, 193–199
- Celano, P., Bayli, S. B., Giardiello, F. M., Nelkin, B. D. and Casero, R. A. (1988) *J. Biol. Chem.* **263**, 5491–5494
- Kaminska, B., Kaczmarek, L. and Grzelakowska-Sztart, B. (1992) *FEBS Lett.* **304**, 198–200
- Herrin, D. and Schmidt, G. (1988) *Biotechniques* **6**, 196–197
- Peckham, I., Sobel, S., Comer, J., Jaenisch, R. and Barklis, E. (1989) *Genes Dev.* **3**, 2062–2071
- Petersen, R., Sobel, S., Wang, C. T., Jaenisch, R. and Barklis, E. (1991) *Gene* **101**, 177–183
- Wolf, F. W., Sarma, V., Seldin, M., Drake, S., Suchard, S. J., Shao, H., O'Shea, K. S. and Dixit, V. M. (1994) *J. Biol. Chem.* **269**, 3633–3640
- Kostura, M. and Craig, N. (1986) *Biochemistry* **25**, 6384–6391
- Heby, O. (1981) *Differentiation* **19**, 1–20
- Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290
- Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158
- Bhatia, P., Taylor, W. R., Greenberg, A. H. and Wright, J. A. (1994) *Anal. Biochem.* **216**, 223–226
- Virdi, A. S., Cook, L. J., Oreffo, R. O. and Triffitt, J. T. (1998) *Cell. Mol. Biol.* **44**, 1237–1246
- Balasundaram, D., Tabor, C. W. and Tabor, H. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4693–4697
- Wang, J. Y., Viar, M. J., Li, J., Shi, H. J., Patel, A. R. and Johnson, L. R. (1998) *Am. J. Physiol.* **274**, C522–C530
- Bellofatto, V., Fairlamb, A. H., Henderson, G. B. and Gross, G. A. (1987) *Mol. Biochem. Parasitol.* **25**, 227–238

-
- 46 Varedi, M., Ghahary, A., Scott, P. G. and Tredget, E. E. (1997) *J. Cell Physiol.* **172**, 192–199
- 47 Gong, Z. Y. and Brandhorst, B. P. (1988) *Mol. Cell Biol.* **8**, 3518–3525
- 48 Byers, T. L., Ganem, B. and Pegg, A. E. (1992) *Biochem. J.* **287**, 717–724
- 49 Byers, T. L., Wiest, L., Wechter, R. S. and Pegg, A. E. (1993) *Biochem. J.* **290**, 115–121
- 50 Tome, M. E. and Gerner, E. W. (1996) *Biochem. J.* **320**, 55–60
- 51 Kemper, W. M., Berry, K. W. and Merrick, W. C. (1976) *J. Biol. Chem.* **251**, 5551–5557
- 52 Benne, R., Brown-Luedi, M. L. and Hershey, J. W. (1978) *J. Biol. Chem.* **253**, 3070–3077
- 53 Kang, H. A. and Hershey, J. W. (1994) *J. Biol. Chem.* **269**, 3934–3940
- 54 Zuk, D. and Jacobson, A. (1998) *EMBO J.* **17**, 2914–2925
- 55 McConlogue, L. C., Marton, L. J. and Coffino, P. (1983) *J. Cell Biol.* **96**, 726–767
- 56 Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B. and Johnson, L. R. (1999) *Am. J. Physiol.* **276**, C684–C691
- 57 Kramer, D. L., Vujcic, S., Diegelman, P., Alderfer, J., Black, J. D., Bergeron, R. J. and Porter, C. W. (1999) *Cancer Res.* **59**, 1278–1286
- 58 Watson, P., Hanauske-Abel, H. M., Flint, A. and Lalande, M. (1991) *Cytometry* **12**, 242–246
- 59 Hanauske-Abel, H. M., Park, M. H., Hanauske, A. R., Popowicz, A. M., Lalande, M. and Folk, J. E. (1994) *Biochim. Biophys. Acta* **1221**, 115–124
- 60 Ross, J. (1997) *BioEssays* **19**, 527–529
- 61 Shermoen, A. W. and O'Farrell, P. H. (1991) *Cell* **67**, 303–310
- 62 Weisenberger, D. and Scheer, U. (1995) *J. Cell Biol.* **129**, 561–575
- 63 Fan, H. and Penman, S. (1970) *J. Mol. Biol.* **50**, 655–670
- 64 Tarnowka, M. A. and Baglioni, C. (1979) *J. Cell. Physiol.* **99**, 359–368
- 65 Bonneau, A.-M. and Sonenberg, N. (1987) *J. Biol. Chem.* **262**, 11134–11139
-

Received 14 July 1999/5 November 1999; accepted 6 December 1999