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Specific regulation by endogenous polyamines of translational initiation of *S*-adenosylmethionine decarboxylase mRNA in Swiss 3T3 fibroblasts

Michael W. WHITE,* Cathy DEGNIN,† John HILL and David R. MORRIS
Department of Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.

S-Adenosylmethionine decarboxylase (AdoMetDC) activity was elevated 18.8-fold in Swiss 3T3 fibroblasts which were depleted of cellular polyamines by using the inhibitor difluoromethylornithine (DFMO). Although the cellular level of AdoMetDC mRNA and the half-life of active AdoMetDC protein were also increased (4.3- and 1.5-fold respectively), together they could not account for the magnitude of the increase in AdoMetDC activity. These data suggested that the translation of AdoMetDC mRNA must be increased in the polyamine-depleted cells to account fully for the elevation in activity. The cellular distribution of AdoMetDC mRNA was examined in the polyamine-depleted cells, and it was found almost exclusively associated with large polysomes. In contrast, AdoMetDC mRNA in untreated controls was very heterogeneous, with the proportion associated with monosomes equal to that associated with large polysomes. The shift of the AdoMetDC message into large polysomes occurred within 18 h after addition of DFMO to the cultures and could be reversed by adding exogenous putrescine. The effect of polyamine depletion on AdoMetDC translation was specific, since there was no change in the distribution in polysomes of either actin mRNA or the translationally controlled mRNA encoding ribosomal protein S16 in the DFMO-inhibited cells. Thus the translational efficiency of AdoMetDC mRNA *in vivo* is regulated either directly or indirectly by the concentration of intracellular polyamines through a mechanism involving translational initiation, which results in a change in the number of ribosomes associated with this mRNA.

INTRODUCTION

It has been well established that the key enzyme of polyamine biosynthesis, AdoMetDC, is regulated in response to the levels of intracellular polyamines, principally putrescine and spermidine (reviewed in [1,2]). In general, an increase in intracellular putrescine or a decrease in the level of spermidine results in elevated AdoMetDC activity. The molecular details of this feedback mechanism have only recently begun to be elucidated with the development of cDNA and immunological probes. It has been demonstrated directly that depletion of polyamines in cell lines and in prostate leads to an elevation of the level of AdoMetDC mRNA [3,4] and to a lengthening of the half-life of AdoMetDC protein [5].

Recently, Kameji & Pegg [6] found that translation of AdoMetDC mRNA in reticulocyte lysates was inhibited by spermidine or spermine. However, the physiological significance of this observation with cell extracts was not established. In the present paper we provide evidence that changes in the intracellular levels of polyamines influence the translation of AdoMetDC mRNA in a fibroblast line and that the major effect is at the initiation step. This is similar to the translational regulation of AdoMetDC expression in response to mitogenic signals in T-lymphocytes that we described previously [7], in that regulation in both instances is at the level of translational initiation.

EXPERIMENTAL

Cell culture

Swiss 3T3 fibroblasts [8] were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum, non-essential amino acids, pyruvate, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Reagents

Difluoromethylornithine was a gift of Merrell Dow Research Institute, Cincinnati, OH, U.S.A. *S*-Adenosyl[*carboxy*-^{14}C]methionine (40 mCi/mmol) was purchased from ICN Biomedicals. Heparin sodium salt, phenylmethanesulphonyl fluoride, and cycloheximide were from Sigma.

Polysome fractionation and RNA analysis

Individual culture dishes of 3T3 cells (3×10^6 cells/150 mm-diameter culture dish) were harvested for polysome fractionation by first treating the cells for 5 min in growth medium (see above) containing 0.1 mg of cycloheximide/ml at 37 °C. This concentration of cycloheximide was included in all of the solutions used to harvest the cells before lysis. The cells were then washed with phosphate-buffered saline (PBS) (0.14 M-NaCl/5 mM-KCl/8 mM-Na_2PO_4/1.5 mM-KH_2PO_4, pH 7.2) and the cells were detached with 2 ml of a 0.25% (w/v) trypsin solution (Gibco) at 37 °C. The cells were then collected in PBS at 4 °C, containing 1 mM-phenylmethanesulphonyl fluoride to inhibit the trypsin and pelleted at 700 *g*. Cytoplasmic extracts were prepared as previously described [9] and applied to linear 0.5–1.5 M-sucrose gradients. The gradients were centrifuged at 4 °C in a Beckman SW40.1 rotor at 36000 rev./min for 110 min and then fractionated on an Isco Density Gradient Fractionator equipped with a 254 nm absorbance monitor. RNA was purified from the gradient fractions as previously described [9].

Total RNA was isolated from cell cultures by using the guanidinium isothiocyanate method of Chirgwin *et al.* [10]. Total

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50); DFMO, difluormethylornithine; PBS, phosphate-buffered saline.

* Present address and address for correspondence and reprint requests: Veterinary Molecular Biology Laboratory, Montana State University, Bozeman, MT 59717, U.S.A.

† Present address: Fred Hutchisen Cancer Center, 1124 Columbia, Seattle, WA 98104, U.S.A.

RNA and RNA purified from the sucrose-gradient fractions were analysed on Northern blots using ^{32}P-labelled probes as previously described [9]. AdoMetDC mRNA was detected by using a nick-translated bovine *Eco*R1/*Sau*-3A 180 bp cDNA fragment [7], S16 mRNA was detected by using a *Hha*-1 1100 bp mouse cDNA fragment [11], and actin mRNA was detected by using a bovine cDNA *Pst*1 1330 bp fragment [12]. Probe was removed from filters to be re-probed by washing the filters in water for 20 min at 100 °C [13] and then rehybridization as described above. The Northern blots were exposed to X-ray film at −80 °C, using a du Pont Cronex Lighting-plus intensifying screen, and autoradiographs exposed within the linear range of the film, were quantified by densitometric scanning.

RESULTS

AdoMetDC activity, mRNA levels and half-life

Cultures of Swiss 3T3 fibroblasts were treated with 5 mM-DFMO, and the levels of AdoMetDC activity were measured by using a standard $^{14}CO_2$-release assay using the substrate *S*-adenosyl[*carboxy*-^{14}C]methionine [14]. In parallel cultures, the cellular levels of AdoMetDC mRNA were determined by Northern analysis, loading equal amounts of total cellular RNA (see the Experimental section). A comparison of enzyme activity and mRNA level as a function of time after drug addition is given in Table 1. In agreement with previous reports, the depletion of intracellular polyamines with DFMO led to a substantial increase in AdoMetDC activity (18.8-fold by 72 h) and an elevation of cellular AdoMetDC mRNA (4.3-fold by 72 h). Our data are also consistent with those of Pajunen *et al.* [4], which indicate that the magnitude of the increase in AdoMetDC mRNA in response to DFMO inhibition does not account for the elevation in AdoMetDC activity. Thus the increase in mRNA level fails to account for the elevation of enzyme activity by 2.7-fold at 18 h and 4.3-fold at 72 h.

Since it has been demonstrated that the turnover of AdoMetDC can be affected by depleting intracellular polyamines, we estimated the half-life of active protein by monitoring the disappearance of AdoMetDC activity after inhibition of protein synthesis with 0.06 mg of cycloheximide/ml [15]. These experiments suggest a small increase in the half-life of AdoMetDC in cells treated with DFMO for 72 h (Table 1), but this can account for only a fraction of the discrepancy between enzyme activity and mRNA level.

Cytosolic distribution of AdoMetDC mRNA

Postnuclear supernatants were prepared from DFMO-treated and control cells. These extracts were fractionated by centrifugation in linear sucrose gradients, and AdoMetDC mRNA was analysed on Northern blots (see the Experimental section). The distribution of AdoMetDC mRNA in cells treated for 72 h with 5 mM-DFMO is shown in Fig. 1. Fraction 1 corresponds to the top of the sucrose gradient. In untreated 3T3 cells, the distribution of AdoMetDC mRNA is very heterogeneous, with nearly equivalent amounts of mRNA on monosomes (fraction 4) and on larger polysomes (fraction 9). By comparison, the distribution of AdoMetDC mRNA is dramatically altered in 72 h-DFMO-inhibited cells. AdoMetDC mRNA has nearly disappeared from fractions 3–5 and is found primarily on large polysomes contained in fractions 7–9. The Northern blots were washed and rehybridized for actin mRNA, and the results of the autoradiographic exposures are shown in Fig. 1. The peak hybridization signal in the control panel is in fraction 9, whereas the peak signal in the DFMO-treated panel is in fraction 8. These results indicate that actin mRNA is only slightly altered, if at all, by polyamine depletion and in a manner opposite to the shift in AdoMetDC mRNA.

The shift in the cellular distribution of AdoMetDC mRNA can be seen in cells treated for only 18 h with 5 mM-DFMO. The data in Fig. 2(*a*) demonstrate that, after only 18 h of exposure to DFMO, the distribution of AdoMetDC mRNA is essentially identical with that seen in the 72 h-treated cells (Fig. 1). Addition of putrescine to the cultures blocks the DFMO-induced shift in AdoMetDC mRNA distribution (Fig. 2*b*), demonstrating that the effect of the drug is mediated through polyamine depletion. It is noteworthy that, while the distribution of AdoMetDC

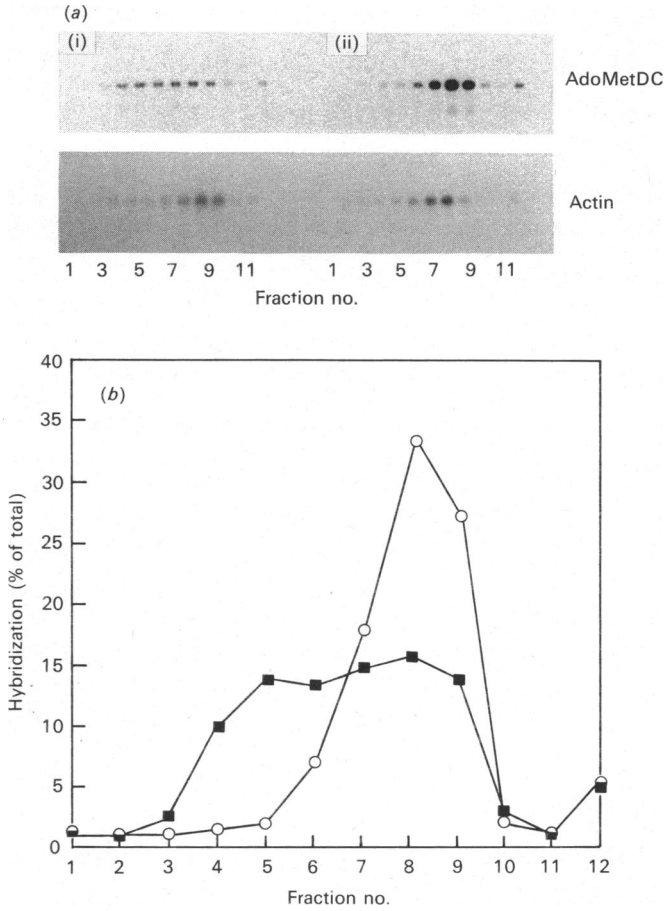

Fig. 1. Distributions of AdoMetDC and actin mRNA in polysomes from untreated and 72 h DFMO-treated 3T3 cells

Polysomes were fractionated in sucrose gradients, the RNA extracted, and Northern analysis was performed as described in the Experimental section. (*a*) Autoradiographs from Northern analysis: (i) untreated cells; (ii) 72 h-DFMO-treated cells. Quantitative results from the AdoMetDC autoradiograph are shown below (*b*); ○, untreated cells; ■, 72 h-DFMO-treated cells.

Table 1. Relative changes in AdoMetDC activity and mRNA levels in response to DFMO

Abbreviation: N/D, not determined.

Time (h)	Relative change: Enzyme activity	Relative change: mRNA levels	Enzyme half-life (min)
0	1.0	1.0	75
18	5.7	2.1	N/D
72	18.8	4.3	111

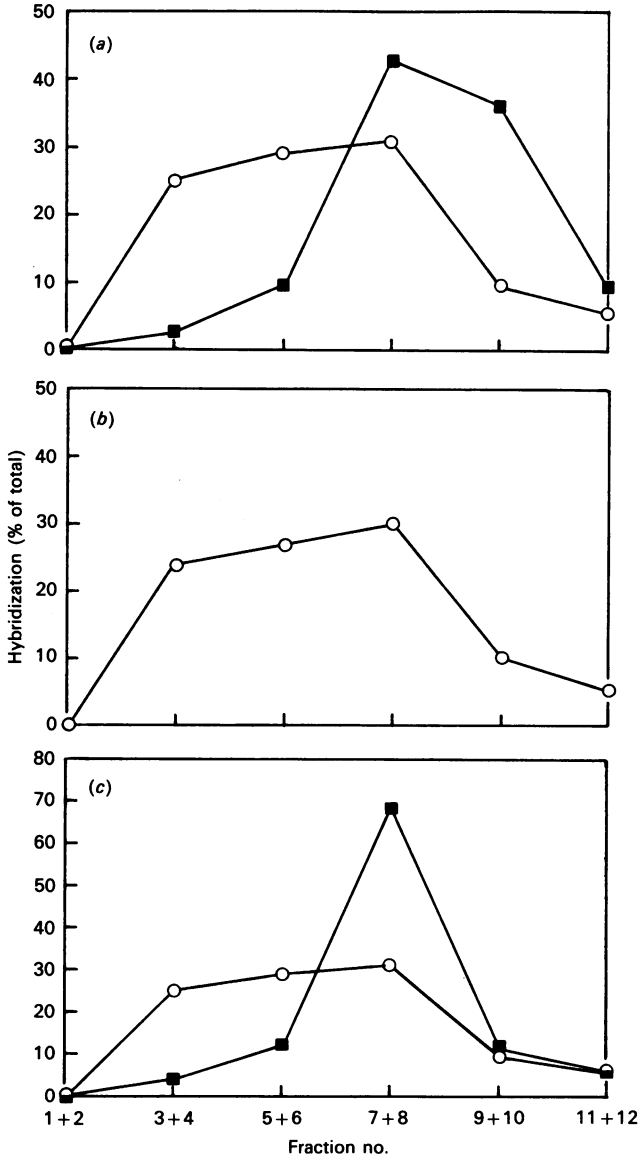

Fig. 2. Effect of various treatments on the polysome distribution of AdoMetDC mRNA in Swiss 3T3 fibroblasts

(*a*), (*b*) and (*c*) are the results from densitometric scans of the hybridization signals corresponding to AdoMetDC mRNA. (*a*) ○, Untreated cells; ■, cells exposed to 5 mM-DFMO for 18 h. (*b*) Cells exposed to a combination of 3 mM-putrescine and 5 mM-DFMO for 18 h. (*c*): ○, Untreated cells; ■, cells exposed to 0.75 μg of cycloheximide/ml for 15 min.

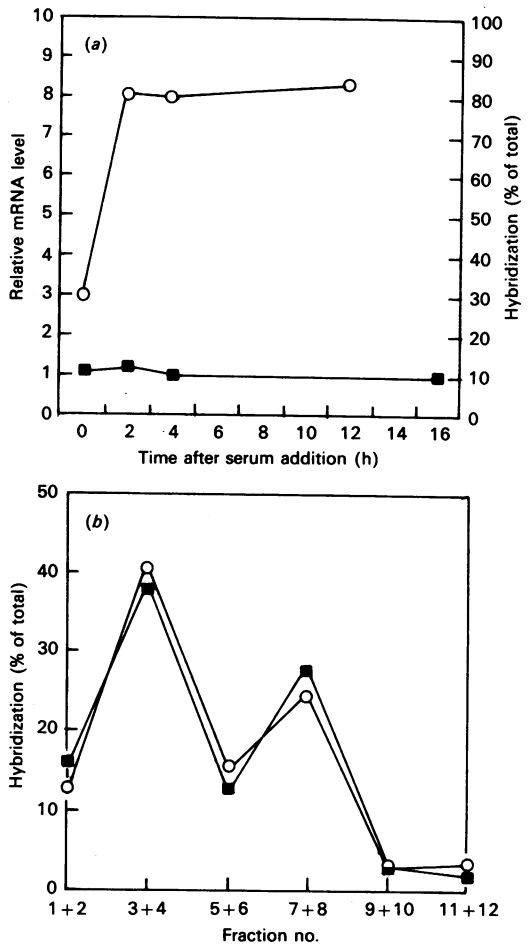

Fig. 3. Changes in the polysome distribution and total cellular levels of ribosomal protein S16 mRNA

(*a*) Polysome distribution of S16 mRNA (○) and the change in S16 mRNA levels (■) were analysed in 3-day 0.5%-serum-arrested and 10%-serum-activated 3T3 cells (see the Experimental section). The distribution of S16 mRNA in cytoplasmic extracts is expressed as the percentage of the total hybridization signal in polysomes. In the determination of S16 mRNA levels, 5 μg of total cellular RNA was loaded in each lane. (*b*) Northern blots from the experiment shown in Fig. 2(*a*) were reprobed with a ^{32}P-labelled mouse S16 cDNA fragment. ○, Untreated cells; ■, cells exposed to 5 mM-DFMO for 18 h.

mRNA has fully shifted within 18 h of DFMO treatment, the level of AdoMetDC mRNA has only achieved a fraction of its maximal increase (Table 1), suggesting that translational regulation of AdoMetDC synthesis is a more immediate response to polyamine depletion than is mRNA level.

The increase in the size of polysomes containing AdoMetDC mRNA in the DFMO-inhibited cells suggests that the rate of translational initiation of the AdoMetDC mRNA is elevated relative to elongation as a result of polyamine depletion. To duplicate this situation, we exposed 3T3 cells to a low concentration of cycloheximide, which selectively inhibits peptide elongation, resulting in an increase in the ratio of initiation to elongation [16]. Cells were treated with 0.75 μg/ml of cycloheximide for 15 min, and cytoplasmic extracts prepared and analysed on sucrose gradients as above. The quantification of the Northern blot from this experiment is shown in Fig. 2(*c*) and shows that the AdoMetDC mRNA has been completely shifted, by this short treatment with cycloheximide, to fractions 7 and 8, a result nearly identical with that seen with DFMO.

The lack of an increase in the translation initiation of actin mRNA after DFMO treatment suggested that the increased initiation of the AdoMetDC mRNA in polyamine-depleted cells was specific to this mRNA. We decided to explore the question of specificity with an mRNA known to be under translational control. Ribosomal-protein-S16 mRNA is translationally regulated in response to serum growth factors in Swiss 3T3 cells. As Fig. 3(*a*) shows, only 30% of the cellular S16 mRNA is found in polysomes in 3T3 cells, which were growth-arrested in 0.5% calf serum for 72 h. Within 2 h after activation with 10% serum, over 80% of the S16 mRNA is found in polysomes. This shift in S16 mRNA from mRNA–ribonucleoprotein particles into polysomes occurs in the absence of any change in the total cellular level of S16 mRNA (Fig. 3*a*). Using the S16 cDNA probe, we washed and rehybridized the Northern blots from Fig.

2(*a*) (18 h treatment of growing 3T3 cells with 5 mM-DFMO). The results of this experiment demonstrate that depletion of cellular polyamines with DFMO had no effect on the distribution of S16 mRNA (Fig. 3*b*).

DISCUSSION

Depletion of polyamines in Swiss 3T3 cells with the inhibitor of ornithine decarboxylase DFMO results in a major elevation of AdoMetDC activity, as has been found by others in other cell types and tissues [1,2]. The magnitude of this increase in AdoMetDC activity cannot be accounted for by either the elevation of mRNA level or an increase in enzyme stability, suggesting increased efficiency of translation of the AdoMetDC message. Kameji & Pegg [6] found that the polyamines spermidine and spermine inhibited the translation of AdoMetDC mRNA in reticulocyte lysates. In order to evaluate the possible regulation of AdoMetDC mRNA translation by polyamines in intact cells, we examined the distribution of this message in polysomes of normal and polyamine-depleted 3T3 cells. There was a profound movement of AdoMetDC mRNA into larger polysomes in DFMO-treated cells, which was reversed by restoring putrescine to the cultures. This effect was specific to AdoMetDC mRNA, since it was not seen with actin mRNA or the translationally controlled message coding for ribosomal protein S16. Thus these results demonstrate that endogenous polyamines regulate the number of ribosomes associated with AdoMetDC mRNA and hence the efficiency of translation of this message.

Feedback regulation of AdoMetDC expression by polyamines involves a mixture of at least three mechanisms: cellular level of AdoMetDC mRNA, efficiency of mRNA translation and rate of degradation of AdoMetDC protein. It is possible that each of these regulatory mechanisms comes into play at different endogenous polyamine levels. Thus some of these mechanisms may be important in controlling minor physiological fluctuations in polyamine levels, while others may only become involved under more rigorous conditions of polyamine depletion, such as those brought about by treatment of the cells with DFMO. Although no data are available concerning the dependence of the rate of AdoMetDC degradation on endogenous polyamine level, the results presented here suggest that translational control is at least as important as regulation of mRNA content at moderate degrees of polyamine limitation. By 18 h of DFMO treatment, the regulation of translational efficiency was fully invoked, based on the shift of AdoMetDC mRNA into large polysomes, whereas the increase in AdoMetDC mRNA was less than half that reached after 72 h of polyamine depletion. Thus it seems possible that regulation of translational initiation of AdoMetDC mRNA is quite sensitive to small fluctuations in polyamine level in cells. The extremely heterogeneous loading of ribosomes on to AdoMetDC mRNA seen in normal cultures may be a consequence of fluctuations in polyamine content from cell to cell, resulting in heterogeneity of translational initiation on AdoMetDC mRNA in the population as a whole.

The increase in average number of ribosomes associated with a molecule of AdoMetDC message must result from elevation of the ratio of translational initiation to elongation. This effect on polysome size was duplicated by treatment of the cells with low concentrations of cycloheximide, conditions which preferentially inhibit initiation relative to elongation. Although, in principle, the increase in polysome size could have been due to specific inhibition of elongation on AdoMetDC messages, it seems unlikely, since this situation would be unproductive in that it would not lead to an enhancement of the rate of AdoMetDC synthesis. It seems much more likely that intracellular polyamines specifically feedback-inhibit the initiation of translation on AdoMetDC mRNA and thereby inhibit synthesis of AdoMetDC protein. The specificity of this inhibition should be emphasized, since it is not seen with other messages, and it suggests that this regulation is governed by a sequence or sequences unique to the AdoMetDC mRNA. This inhibition of translational initiation could be mediated by a transacting factor, similar to the situation which has been proposed for the translational control of ferritin synthesis [17,18]. Alternatively, it could result from the specific interaction of polyamines with some aspect of AdoMetDC mRNA structure, as has been observed with other nucleic acids (reviewed in [19]).

The translation of AdoMetDC mRNA is also controlled by mitogenic signals in T-lymphocytes [7]. In this case, the AdoMetDC mRNA moves from very small polysomes in resting cells on to larger polysomes after mitogenic activation. Although mitogenic control and polyamine control of AdoMetDC mRNA translation both appear to be at the level of initiation, the mechanisms seem to be unrelated. In the present instance, DFMO lowers intracellular polyamines and inhibits growth, but AdoMetDC mRNA moves into larger polysomes. Conversely, mitogen stimulation of T-cells profoundly elevates intracellular polyamines [20], but AdoMetDC mRNA also moves into larger polysomes in this case as well. Whether these two distinct modes of control are mediated by the same sequences of the AdoMetDC message has yet to be determined.

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