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# Regulation of *S*-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content

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The effects of addition of exogenous spermidine and spermine and of two inhibitors of polyamine biosynthesis, α-difluoromethylornithine (DFMO), which decreases spermidine concentrations, and n-butyl-1,3-diaminopropane, which depletes spermine, on the expression of *S*-adenosylmethionine decarboxylase (AdoMetDC) activity were studied in mammalian cell lines (HT29, CHO and COS-7). AdoMetDC levels were inversely related to the polyamine content, and spermine was the more potent repressor of AdoMetDC activity, but only spermidine affected the amount of AdoMetDC mRNA. Transfection of COS-7 cells or CHO cells with plasmid constructs containing a chloramphenicol acetyltransferase (CAT) reporter gene driven by portions of the AdoMetDC promoter region indicated that CAT expression was altered by spermidine, but not by spermine, suggesting that there is a spermidine-responsive element in this promoter. Transient transfection of COS-7 cells with pSAMh1, a plasmid containing the AdoMetDC cDNA in a vector with the SV40 promoter and origin of replication, led to a large increase in AdoMetDC expression. Although treatment of COS-7 cells with n-butyl-1,3-diaminopropane greatly increased endogenous AdoMetDC activity, the spermine depletion brought about by this inhibitor did not stimulate AdoMetDC expression from pSAMh1. The pSAMh1 cDNA is missing 72 nucleotides from the 5′ end of the AdoMetDC mRNA, and it is possible that translational regulation by spermine involves this region. The expression of AdoMetDC from pSAMh1 in COS-7 cells was greatly inhibited by DFMO treatment, although endogenous AdoMetDC activity was increased. The expression of other plasmids containing the SV40 origin of replication was also inhibited by DFMO in COS-7 cells, but not in CHO cells. DFMO treatment did not interfere with the expression of plasmids driven by the RSV promoter. These results suggest that low spermidine levels interfere with the replication of plasmids containing the SV40 origin of replication.

## INTRODUCTION

The polyamine-biosynthesis pathway is a useful target for the design of chemotherapeutic agents. Depletion in polyamine levels has been shown to lead to decreases in cellular growth and alterations in cellular differentiation (Pegg & McCann, 1982; Pegg, 1986, 1988; Jänne *et al.*, 1991). Although most of the work has been carried out with compounds blocking the pathway at the ornithine decarboxylase (ODC) step, inhibitors of *S*-adenosylmethionine decarboxylase (AdoMetDC), which are now becoming available (Pegg *et al.*, 1988*a*; Kramer *et al.*, 1989; Casara *et al.*, 1989), may have some advantages, since this enzyme catalyses the rate-limiting step in the production of spermidine and spermine and forms an important branch point that commits *S*-adenosylmethionine to a role in polyamine synthesis (Pegg, 1984, 1986). However, both ODC and AdoMetDC levels are tightly regulated in mammalian cells. Compensatory increases in the levels of these enzymes may be of major importance in limiting the response to inhibitors of polyamine biosynthesis. Conversely, the use of polyamine analogues that lower normal polyamine levels by acting as repressors of ODC and AdoMetDC provides another approach to chemotherapy (Bergeron *et al.*, 1988; Porter & Bergeron, 1988). It is apparent that more information on the mechanism by which polyamine levels regulate AdoMetDC is needed to determine the best way to design and implement such therapeutic strategies.

Increases in AdoMetDC activity in response to inhibitors of ODC have been known for some time (reviewed by Pegg, 1984; Pegg *et al.*, 1988*b*; White & Morris, 1989). An even greater increase in the AdoMetDC protein is seen when irreversible inactivators of AdoMetDC are used to deplete cellular polyamines (Madhubala *et al.*, 1988; Persson *et al.*, 1989*a*; Autelli *et al.*, 1991). However, the mechanism by which these changes are brought about and the relative roles of spermidine and spermine in the regulation of AdoMetDC are not well understood. Although it has been suggested that polyamines influence the translation of AdoMetDC mRNA (Kameji & Pegg, 1987; Persson *et al.*, 1989*b*; Porter *et al.*, 1990; White *et al.*, 1990), there is also evidence for a change in mRNA levels in polyamine-depleted cells that suggests an effect on transcription (Shirahata & Pegg, 1986; Pegg *et al.*, 1988*b*; White & Morris, 1989).

In order to provide more information on these questions, we have examined the effects of inhibitors of putrescine synthesis and of spermine synthesis both on endogenous AdoMetDC expression and on AdoMetDC expression in cells that had been transfected with a cDNA plasmid for human AdoMetDC, pSAMh1 (Pajunen *et al.*, 1988). We used α-difluoromethylornithine (DFMO), which inhibits ODC activity, to prevent putrescine synthesis. Exposure to DFMO depletes putrescine and spermidine in cells, but has little effect on spermine (Pegg, 1986). In order to deplete spermine selectively, we used n-butyl-1,3-diaminopropane (BDAP), a potent inhibitor of spermine

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50); ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, α-difluoromethylornithine; BDAP, n-butyl-1,3-diaminopropane; CAT, chloramphenicol acetyltransferase.

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synthase (Baillon *et al.*, 1989). Polyamines were restored in cells treated with these inhibitors by addition of exogenous polyamines to the culture medium. Aminoguanidine, which is an inhibitor of amine oxidases (Pegg, 1986; Morgan, 1987), was added to these cultures to prevent the generation of toxic aldehydes from the added polyamines by oxidases present in the culture medium. We also studied the effects of changing the polyamine levels on the expression of a chloramphenicol acetyltransferase (CAT) reporter gene driven by portions of the AdoMetDC promoter. These results provide additional information on the mechanism of regulation of AdoMetDC and show clearly that spermidine and spermine have different sites of action to lower enzyme levels. Another finding arising from these experiments was that the normally high level of expression of proteins from transfection of plasmid constructs, which replicate in COS-7 cells because they contain the SV40 origin of replication, did not occur in cells depleted of spermidine.

## MATERIALS AND METHODS

### Materials

The antisera to human AdoMetDC and mouse ODC were raised in rabbits as described previously (Shirahata & Pegg, 1985). [$^{32}$P]CTP (800 Ci/mmol) and [$^{32}$P]UTP (800 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. *S*-Adenosyl[*carboxy*-$^{14}$C]methionine (50 mCi/mmol) was from Dupont–New England Nuclear, Boston, MA, U.S.A. DFMO was provided by the Merrell Dow Research Institute, Cincinnati, OH, U.S.A. BDAP was generously given by Dr. J. K. Coward, Department of Chemistry, University of Michigan, Ann Arbor, MI, U.S.A. pSV-CAT was a gift from Dr. T. Jorgenson, Lombardi Cancer Center, Washington, DC, U.S.A. SV40-CAT was purchased from Promega Corp., Madison, WI, U.S.A. Restriction enzymes were from Promega and Bethesda Research Laboratories, Bethesda, MD, U.S.A. Other biochemical reagents used were purchased from: Sigma Chemical Co., St. Louis, MO, U.S.A., Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A., Pharmacia LKB, Piscataway, NJ, U.S.A., and Bio-Rad, Richmond, CA, U.S.A.

### Preparation of AdoMetDC constructs

Two AdoMetDC promoter fragments were inserted in front of the bacterial CAT gene as described elsewhere (Palvimo *et al.*, 1991; Maric *et al.*, 1992). The promoter constructs started from either nucleotide −1505 or −173 and included the first 103 or 112 nucleotides of the 5′-untranslated region of AdoMetDC mRNA respectively. (The sequence of the human AdoMetDC gene is deposited in GenBank under accession numbers M88003–M88006). The DNA fragments were inserted into a promoterless CAT reporter vector (pPLCAT) containing a polylinker sequence in front of the CAT gene (Palvimo *et al.*, 1991). The shorter DNA fragment (−173/+112) was cloned into the *Sma*I site of pPLCAT vector, resulting in a construct termed p7662-CAT, whereas the longer fragment (−1505/+103) was inserted between the *Kpn*I and *Hin*dIII sites of pPLCAT and was designated p5604-CAT. The preparation and complete sequence of pSAMh1 have been described elsewhere (Pajunen *et al.*, 1988).

### Cell culture and transfection

SV-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (MEM) containing 3% horse serum and 2% fetal-calf serum. For some experiments, these cells were grown for 48 h in the above medium and then incubated in the same medium without serum for 24 h. Serum-containing medium was then added, and cell samples were collected at various time points for determination of AdoMetDC activity or mRNA. Where applicable, DFMO, spermidine or spermine was added at the same time as the serum. COS-7 and HT29 cells were grown in Dulbecco's MEM supplemented with 10% fetal-bovine serum, 20 mM-$NaHCO_3$ and 12 $\mu$g of penicillin/ml + 12 $\mu$g of streptomycin/ml. CHO cells were grown in $\alpha$MEM with the same additions. All cultures were grown on monolayers in 6 cm-diameter plates in an atmosphere of air/$CO_2$ (19:1) at 37 °C. In experiments in which polyamines or inhibitors of polyamine biosynthesis were added to the cultures, 1 mM-aminoguanidine was included in the medium to inhibit the action of oxidases present in the serum.

The plasmids were transfected into COS-7 and CHO cells by the calcium phosphate method (Gorman, 1985). Cells were plated at a density of 200000 cells/6 cm plate and allowed to grow for 24 h before transfection. Cells were transfected with 10–15 $\mu$g of DNA for 4 h, followed by a 3 min glycerol shock at 37 °C. After the medium was changed, the cells were allowed to grow for 48 h, and then harvested. BDAP (50 $\mu$M), spermine (10 $\mu$M) and spermidine (10 $\mu$M) were added at the time the cells were plated. DFMO (5 mM) was added 3 h before transfection. Mock-transfected cells were treated in the same way, except that DNA was not added to the transfection mixture. Non-transfected cells were plated at a density of 200000 cells/6 cm plate and allowed to grow for 24 h, when the medium was changed. Cells were harvested after growing for a further 48 h, and the level of AdoMetDC activity or protein or CAT protein was measured as described below. The results were not corrected by using a control plasmid to measure transfection efficiency, since the purpose of the studies was to test the effects of alterations in polyamine content on expression from these plasmids. All results are given as means ± S.D., and part of the variation may be due to differences in transfection efficiency, but this was not sufficiently great to compromise the experimental design.

### Preparation of cell extracts

Cells harvested for enzyme assays or Western blots were washed three times with cold phosphate-buffered saline. Then 200 $\mu$l of harvest buffer (50 mM-Tris/HCl, pH 7.5, 2.5 mM-dithiothreitol, 0.1 mM-EDTA) was added to each plate and the monolayers were scraped into 1.5 ml tubes by using a rubber policeman. The cells were lysed by placing the tubes into liquid nitrogen and then into cool water three times. The samples were centrifuged at 17000 ***g*** for 20 min at 4 °C and the supernatants stored at −70 °C.

Samples for RNA were harvested by washing three times with ice-cold phosphate-buffered saline, then scraping the cell suspension into a sterile 30 ml centrifuge tube (5–10 plates were combined for each determination). The tubes were centrifuged at 4000 ***g*** for 10 min at 4 °C. RNA was isolated from the pellets by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987).

Cells harvested for polyamine analysis were washed three times with ice-cold phosphate-buffered saline, then extracted with 10% (w/v) trichloroacetic acid. The cell suspension was scraped into 1.5 ml tubes and spun for 10 min in a Microfuge. Samples were used for the determination of polyamine content as described previously (Pegg *et al.*, 1989).

### Immunoblotting

SDS/PAGE was performed by the method of Laemmli (1970). For the detection of proteins reactive with anti-AdoMetDC or anti-ODC polyclonal antibodies, proteins were electrotransferred to a nitrocellulose sheet (Schleicher and Schuell) in a blotting apparatus (Bio-Rad) overnight at 30 V and 4 °C. Western blot

analysis was performed by the ECL method as described by the manufacturer (Amersham).

### Determination of mRNA content

For Northern blots, the RNA was separated on 1% agarose gels containing 0.66 M-formaldehyde and electroblotted on to Duralon-UV (Stratagene, La Jolla, CA, U.S.A.). RNA was cross-linked to the membranes by using u.v. light with a Stratalinker (Stratagene). Slot-blots on to Duralon-UV or nitrocellulose were performed with a Slot-Blot manifold (Schleicher and Schuell) according to the manufacturer's instructions. RNA was cross-linked to the membranes as above. SP6 RNA polymerase and [$^{32}$P]CTP were used to prepare a cRNA probe complementary to the mRNA for human AdoMetDC by using pCM9 (Stanley *et al.*, 1989) that had been linearized with restriction enzyme *Csp* 45 I as a template.

For AdoMetDC mRNA analysis using Northern blots, prehybridization (1–4 h) and hybridization (overnight) were performed at 60 °C in 50% formamide/6 × SSC (1 × SSC = 150 mM-NaCl/15 mM-sodium citrate, pH 7.0)/0.1% SDS/0.1% Tween 20 plus 100 μg of tRNA/ml. Probe was added to the hybridization buffer at a concentration of $10^6$ c.p.m./ml. When AdoMetDC RNA content was analysed by slot-blots, the samples (5–10 μg) were prehybridized for 1 h at 65 °C in a buffer consisting of 50% formamide, 20 mM Pipes, pH 6.5, 0.8 M-NaCl, 1% SDS and 100 μg of salmon sperm DNA/ml, and then hybridized overnight in the same buffer containing the $^{32}$P-labelled probe prepared as above except in the presence of [$^{32}$P]UTP. Equality of loading of the samples on Northern and dot blots was determined by measurement of the rRNA (Pajunen *et al.*, 1988). For slot blots detecting CAT RNA, the membranes were hybridized with a [$^{32}$P]cDNA probe prepared from the 7662-CAT plasmid, which had been linearized with *Kpn*I, by using a multi-prime kit (Amersham) according to the manufacturer's instructions. Although the 7662-CAT plasmid contains 112 nucleotides of the 5′ untranslated region of AdoMetDC mRNA, the intracellular levels of AdoMetDC were sufficiently low to prevent any significant background signal from co-hybridization to the CAT probe. All blots were washed 5 × 20 min in 0.1 × SSC/0.1% SDS at 65 °C, and all autoradiograms were scanned with a laser densitometer.

### Enzyme and protein assays

AdoMetDC activity was assayed by measuring the release of $^{14}CO_2$ from S-adenosyl[*carboxy*-$^{14}$C]methionine (Shirahata & Pegg, 1985). One unit of enzyme activity represents the formation of 1 nmol of $^{14}CO_2$/30 min per mg of protein. Protein was measured by the method of Bradford (1976). CAT protein was measured in soluble cell extracts with an e.l.i.s.a. kit (5prime-3prime, Inc., West Chester, PA, U.S.A.) according to the manufacturer's instructions.

## RESULTS

### Effect of polyamines on stimulation of AdoMetDC by serum

When serum-starved SV-3T3 cells were induced to grow by the addition of serum-containing medium, the AdoMetDC activity increased 7–8-fold with a peak 6 h after induction (Fig. 1). At 24 h after induction, the AdoMetDC activity had almost returned to that of uninduced cells. Variations in the amount of AdoMetDC mRNA followed a similar pattern, although the maximal increase was only about 5-fold (Fig. 1). Two species of mRNA were identified on Northern blots, with approximate sizes of 3.4 and 2.1 kb. These forms probably arise from the use of alternative polyadenylation signals and, as previously reported (Pajunen *et al.*, 1988) the larger form accounted for most (approx. 90%) of the total. There was no significant change in the relative proportions of these two species after serum stimulation or application of polyamines (results not shown), and the results shown in Fig. 1 and Table 1 are the sum of the amounts of each of the two species.

The addition of spermidine or spermine decreased the serum-induced increase in AdoMetDC, but the effects of the two polyamines were quite distinct (Table 1). Spermidine was less effective, decreasing the induction of activity by only 60% even when added at concentrations of 50 μM. Spermidine also decreased the level of AdoMetDC mRNA by about the same amount. In contrast, addition of 5 μM-spermine completely prevented the rise in AdoMetDC activity, but had no effect on the mRNA level (Table 1).

### Effect of spermidine depletion on intracellular AdoMetDC activity and polyamine levels

The results given above support the concept that spermidine levels regulate AdoMetDC activity and that at least part of this effect is due to changes in the content of AdoMetDC mRNA. In order to use the plasmid constructs to investigate the mechanism of this phenomenon in more detail, it was necessary to use COS-7 and CHO cells as recipients of transfected plasmid DNA. We therefore tested the extent to which spermidine depletion (induced by DFMO) influenced endogenous AdoMetDC expression in

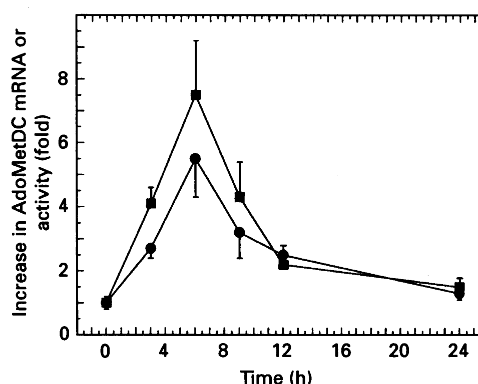


**Fig. 1. Effect of serum stimulation on AdoMetDC**

SV-3T3 cells were serum-starved for 24 h and then stimulated with 2% fetal-bovine serum/3% horse serum as described in the Materials and methods section. The AdoMetDC activity (■) and mRNA content (●) were then measured. Results are expressed as a multiple of the value at zero time and are shown as means ± S.D. for at least four measurements. There was little change (less than 20%) in these values over this time period if serum was not added.

**Table 1. Effect of polyamines on serum stimulation of AdoMetDC**

Results are the means of three experiments which agreed within ± 20%.

| Treatment | AdoMetDC activity (% of control) | AdoMetDC mRNA (% of control) |
|---|---|---|
| None, 0 h | 100 | 100 |
| No serum, 6 h | 110 | 94 |
| Serum, 6 h | 763 | 509 |
| Serum + 5 μM-spermidine | 421 | 282 |
| Serum + 50 μM-spermidine | 355 | 260 |
| Serum + 5 μM-spermine | 129 | 525 |
| Serum + 50 μM-spermine | 95 | 490 |

**Table 2. Effect of DFMO on AdoMetDC and polyamines in HT29, CHO and COS-7 cells**

Cells were grown for 24 h as described in the Materials and methods section. The medium was then changed and the cells were allowed to grow for 48 h, when they were harvested and assayed for AdoMetDC activity and polyamine content. Spermidine was added at the time the cells were plated, and DFMO was added when the medium was changed. Results shown are the means ± S.D. of three or four separate experiments or the mean of two experiments.

| Cell line | Treatment | AdoMetDC activity (units/mg) | Putrescine (nmol/mg) | Spermidine (nmol/mg) | Spermine (nmol/mg) |
|---|---|---|---|---|---|
| HT29 | None | 2.4 ± 0.3 | 9.0 ± 2.2 | 16.7 ± 3.1 | 24.2 ± 3.5 |
| | 5 mM-DFMO | 13.0 ± 1.1 | < 0.5 | < 0.5 | 26.8 ± 4.5 |
| | 5 mM-DFMO + 10 μM-spermidine | 1.9 ± 0.2 | < 0.5 | 18.5 ± 2.8 | 19.4 ± 4.7 |
| CHO | None | 4.0 | 13.6 | 36.3 | 15.1 |
| | 5 mM-DFMO | 28.4 | 1.8 | 9.4 | 16.7 |
| | 5 mM-DFMO + 10 μM-spermidine | 11.6 | < 0.5 | 31.2 | 10.8 |
| COS-7 | None | 1.2 ± 0.2 | 4.4 ± 0.1 | 12.5 ± 2.1 | 21.8 ± 1.4 |
| | 5 mM-DFMO | 40.4 ± 9.8 | < 0.5 | < 0.5 | 15.5 ± 0.9 |
| | 5 mM-DFMO + 10 μM-spermidine | 0.9 ± 0.2 | < 0.5 | 23.9 | 20.2 |

**Table 3. Effect of BDAP on AdoMetDC and polyamines in HT29 and COS-7 cells**

Cells were grown and harvested as described in Table 2. Both BDAP and spermine were added at the time the cells were plated. Results shown are the means ± S.D. of three or four separate experiments or the mean of two experiments: N.D., not done.

| Cell line | Treatment | AdoMetDC activity (units/mg) | Putrescine (nmol/mg) | Spermidine (nmol/mg) | Spermine (nmol/mg) |
|---|---|---|---|---|---|
| HT29 | None | 2.4 ± 0.3 | 9.0 ± 2.2 | 16.7 ± 3.1 | 24.2 ± 3.5 |
| | 50 μM-BDAP | 9.8 ± 0.9 | 0.7 ± 0.2 | 43.3 ± 6.1 | 5.6 ± 0.2 |
| | 50 μM-BDAP + 10 μM-spermine | 0.7 ± 0.2 | N.D. | N.D. | N.D. |
| COS-7 | None | 1.2 ± 0.2 | 4.4 ± 0.1 | 12.5 ± 2.1 | 21.8 ± 1.4 |
| | 50 μM-BDAP | 20.5 ± 3.7 | < 0.5 | 31.7 ± 4.3 | 4.3 ± 0.4 |
| | 50 μM-BDAP + 10 μM-spermine | 1.2 ± 0.5 | N.D. | N.D. | N.D. |

these cells and human colon tumour HT29 cells (Table 2). In all cell lines tested, exposure to DFMO gave rise to a substantial increase in AdoMetDC activity. COS-7 cells were particularly responsive, with a 34-fold rise, whereas the AdoMetDC in HT29 cells and CHO cells increased about 7-fold. The exaggerated response in COS-7 cells is even greater than the rise reported in Swiss 3T3 fibroblasts, in which AdoMetDC activity was elevated 19-fold after treatment with DFMO for 72 h (White *et al.*, 1990). Administration of spermidine completely reversed the effects of DFMO in HT29 cells and COS-7 cells and partially reversed the effect in CHO cells. The lack of complete reversal in CHO cells may be due to the fact that intracellular spermidine content was not completely restored in the cells (Table 2).

This difference in sensitivity to changes in spermidine levels may be due to the intracellular polyamine levels in the three cell lines. Spermidine levels in control CHO cells are more than twice those in control COS-7 cells (Table 2). Upon treatment with DFMO, spermidine levels were undetectable in COS-7 cells, whereas, after the same treatment, spermidine levels in CHO cells were still 9.4 nmol/mg of protein (close to control values in COS-7 cells). Likewise, addition of excess spermidine to the tissue-culture medium causes the intracellular spermidine content in COS-7 cells to overshoot to twice their control values. Despite these differences, the results in Table 2 show clearly that AdoMetDC does respond to manipulations of spermidine content in both cell lines. These cell lines can therefore be used for the studies of the regulation of AdoMetDC by spermidine.

### Effect of spermine depletion on intracellular AdoMetDC activity and polyamine levels

In order to investigate the effects of spermine on AdoMetDC expression, the spermine synthase inhibitor BDAP was used, and in this case only COS-7 cells were used as transfection recipients. The effects of BDAP on polyamines and endogenous AdoMetDC in COS-7 and HT29 cells are shown in Table 3. In both cell lines, there was a large decrease in spermine and a corresponding rise in spermidine concentration. These changes are consistent with the block at the spermine synthase step. Putrescine content was also depressed greatly by BDAP. This is likely to be due to the rise in spermidine, which is known to repress ODC (reviewed by Pegg, 1988). Treatment with BDAP greatly increased AdoMetDC levels in COS-7 cells (by 17-fold), whereas HT29 cells showed a marked but lower increase (4-fold). These changes were completely reversed by the provision of 10 μM-spermine to the culture medium. These results indicate that the COS-7 cells are suitable to study the regulation of AdoMetDC by spermine.

### Effect of polyamine depletion on AdoMetDC expression in COS-7 cells

In order to study the possibility of polyamine-mediated

**Table 4. Effect of BDAP or DFMO on AdoMetDC activity in transfected COS-7 cells**

Cells were grown and transfections performed as described in the Materials and methods section. The pSV-CAT plasmid was used as an internal control in order to confirm that the addition of exogenous DNA had no effect on intracellular AdoMetDC activity or on the action of the polyamine-biosynthesis inhibitors. The results are means ± S.D. of three to six separate estimations. The activity of AdoMetDC was determined as units/mg of protein and then expressed as an increase over that found in mock-transfected cells. In Expt. A the S-adenosylmethionine concentration was 200 μM, and in Expt. B it was 7 μM. The AdoMetDC activity in mock-transfected cells was 0.18 ± 0.04 (unit/mg) in Expt. A and 0.12 ± 0.02 in Expt. B. N.D., not done.

| Transfected with plasmid | Compound added | Change in AdoMetDC activity | |
|---|---|---|---|
| | | Expt. A | Expt. B |
| None | None | 1.0 ± 0.3 | 1.0 ± 0.2 |
| | 50 μM-BDAP | 18.5 ± 3.5 | N.D. |
| | 5 mM-DFMO | N.D. | 32.6 ± 6.0 |
| | 5 mM-DFMO + 10 μM-spermidine | N.D. | 0.7 ± 0.2 |
| pSV-CAT | None | 1.2 ± 0.1 | N.D. |
| | 50 μM-BDAP | 16.6 ± 4.5 | N.D. |
| pSAMh1 | None | 39.7 ± 9.9 | 17.8 ± 3.2 |
| | 50 μM-BDAP | 59.9 ± 13.5 | N.D. |
| | 5 mM-DFMO | N.D. | 26.3 ± 4.8 |
| | 5 mM-DFMO + 10 μM-spermidine | N.D. | 12.7 ± 2.8 |

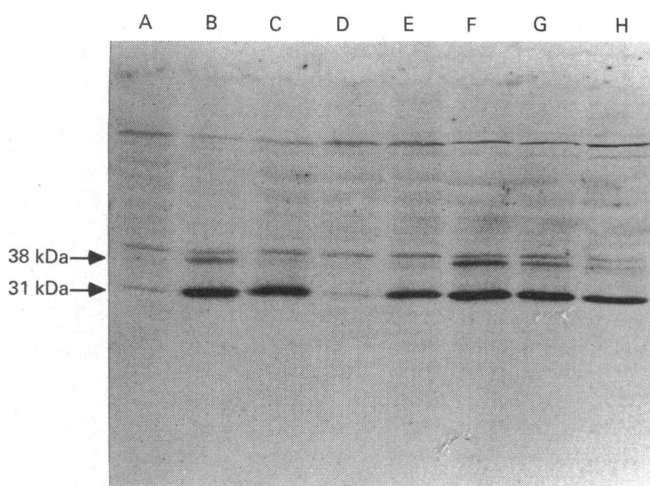


**Fig. 2. Detection of AdoMetDC protein by Western immunoblotting**

COS-7 cells, either mock-transfected or transfected with pSAMh1, were treated with BDAP, BDAP plus spermine, or DFMO as described in the Materials and methods section. After harvesting, cell extracts were assayed for AdoMetDC activity and the supernatants were separated by SDS/PAGE. Western-blot analysis was performed with polyclonal antibodies to AdoMetDC. Lanes A, mock-transfected; B, mock-transfected plus 5 mM-DFMO; C, mock-transfected plus 50 μM-BDAP; D, mock-transfected plus 50 μM-BDAP and 10 μM-spermine; E, pSAMh1; F, pSAMh1 plus 5 mM-DMFO; G, pSAMh1 plus 50 μM-BDAP; H, pSAMh1 plus 50 μM-BDAP and 10 μM-spermine.

translational regulation of AdoMetDC, the effect of BDAP or DFMO addition was examined in COS-7 cells on both the endogenous AdoMetDC expression and on AdoMetDC expression in cells that had been transfected with pSAMh1.

**Table 5. Effect of BDAP or DFMO on AdoMetDC expression in transfected COS-7 cells**

Details of transfections and addition of polyamine biosynthesis inhibitors are as described in Fig. 2 legend. Samples from the same cell extracts were divided and used for enzyme assays and Western blots. The activity of AdoMetDC was determined as units/mg of protein and then expressed as an increase over that found in the mock-transfected cells (0.09 unit/mg). AdoMetDC is formed as a 38 kDa proenzyme, which is then converted in a putrescine-dependent reaction into the 32 kDa enzyme subunit. The amounts of both of these proteins which could be detected on Western blots are shown. Units are O.D. units as quantified by a laser densitometer. BD, below the limit of detection.

| Transfected with plasmid | Compound added | Change in AdoMetDC activity (fold increase) | AdoMetDC protein (units/mg of of cell protein) | |
|---|---|---|---|---|
| | | | 38 kDa | 32 kDa |
| None | None | 1.0 | BD | 0.12 |
| | 5 mM-DFMO | 25.7 | 0.35 | 1.3 |
| | 50 μM-BDAP | 14.6 | BD | 1.16 |
| | 50 μM-BDAP + 10 μM-spermine | 1.6 | BD | 0.16 |
| pSAMh1 | None | 13.6 | BD | 0.99 |
| | 5 mM-DFMO | 34.9 | 0.61 | 1.62 |
| | 50 μM-BDAP | 34.4 | 0.33 | 1.27 |
| | 50 μM-BDAP + 10 μM-spermine | 13.9 | 0.26 | 1.14 |

An average increase in enzyme activity of almost 40-fold was observed after 48 h in cells transfected with the pSAMh1 vector, but not in mock-transfected cells or in cells transfected with a similar plasmid, pSV-CAT, containing the CAT gene, indicating that the increase in AdoMetDC activity was due to expression from the pSAMh1 vector (Table 4). Despite the large changes in AdoMetDC expression, the intracellular polyamine levels in transfected cells did not differ significantly from those in control cells (results not shown).

When cells transfected with pSAMh1 were exposed to 50 μM-BDAP in the tissue culture medium, the increase in AdoMetDC specific activity at harvest averaged about 60-fold (Table 4). In mock-transfected COS-7 cells or in COS-7 cells transfected with the control pSV-CAT vector, addition of BDAP resulted in an average 18.5-fold increase in enzyme activity. The difference between the increase in AdoMetDC activity in cells transfected with pSAMh1 with and without BDAP present is almost exactly equal to the increase seen in mock-transfected cells treated with BDAP. This indicates that addition of BDAP to COS-7 cells transfected with pSAMh1 has no effect on AdoMetDC expression from the plasmid. There was no significant effect of BDAP on the AdoMetDC mRNA content in COS-7 cells with or without transfection with pSAMh1 (results not shown).

The changes in AdoMetDC activity in response to BDAP and DFMO were due to changes in the amount of enzyme protein. Western blots showed a large increase in the bands corresponding to the enzyme or its precursor (Fig. 2). Quantification of these results is shown in Table 5. This quantification is inaccurate for the control samples, which have low levels of protein, but, within the limits of experimental accuracy, the changes in protein are sufficient to account for the changes in activity. The presence of the proenzyme form of AdoMetDC was detected in cells treated with BDAP plus pSAMh1 (Table 5) and with DFMO with or without pSAMh1 (Table 5). This is in agreement with previous reports that the processing of the proenzyme is accelerated by putrescine (Pegg *et al.*, 1988*c*), since these conditions lead to low

**Table 6. Effect of DFMO on ODC expression or CAT expression in transfected COS-7 or CHO cells**

Cells transfected with pODC were analysed by Western blotting and units quantified as in Table 5. CAT protein was measured, in cells that had been transfected with either SV40-CAT or RSV-CAT, with an e.l.i.s.a. kit as described in the Materials and methods section. N.D., not done.

| Transfected with plasmid | Compound added | ODC protein (units/mg of cell protein) or CAT protein (ng/mg of protein) | |
|---|---|---|---|
| | | COS-7 cells | CHO cells |
| pODC | None | 0.62 | N.D. |
| | 5 mM-DFMO | 0.29 | N.D. |
| | 5 mM-DFMO + 10 μM-spermidine | 0.65 | N.D. |
| SV40-CAT | None | 15.4 ± 3.2 | 10.2 |
| | 5 mM-DFMO | 6.2 ± 0.7 | 9.9 |
| | 5 mM-DFMO + 10 μM-spermidine | 17.8 ± 2.4 | 8.5 |
| RSV-CAT | None | 15.8 ± 1.2 | 8.1 |
| | 5 mM-DFMO | 17.1 ± 2.5 | 8.8 |

**Table 7. Effect of BDAP or DFMO on CAT expression in cells transfected with constructs containing regions from the AdoMetDC gene**

CAT protein was analysed in transfected COS-7 and CHO cells as shown in Table 6. All other techniques are described in the Materials and methods section. N.D., not done. [a]Significantly different from 5604-CAT + 5 mM-DFMO, $P < 0.05$. [b]Significantly different from 7662-CAT + 5 mM-DFMO, $P < 0.02$. [c]Significantly different from 5604-CAT + 5 mM-DFMO, $P < 0.01$. [d]Significantly different from 7662-CAT + 5 mM-DFMO, $P < 0.01$.

| Transfected with plasmid | Compound added | CAT protein (ng/mg of protein) | |
|---|---|---|---|
| | | COS-7 cells | CHO cells |
| 5604-CAT | None | 1.56 ± 0.47 | 1.03 ± 0.21 |
| | 50 μM-BDAP | 2.07 ± 0.77 | 1.04 ± 0.04 |
| | 50 μM-BDAP + 10 μM-spermine | N.D. | 0.97 ± 0.11 |
| 7662-CAT | None | 2.02 ± 0.65 | 1.12 ± 0.16 |
| | 50 μM-BDAP | 2.15 ± 0.26 | 1.16 ± 0.08 |
| | 50 μM-BDAP + 10 μM-spermine | N.D. | 0.80 ± 0.14 |
| 5604-CAT | None | 1.56 ± 0.47 | 1.03 ± 0.21 |
| | 5 mM-DFMO | 2.40 ± 0.53 | 1.16 ± 0.18 |
| | 5 mM-DFMO + 10 μM-spermine | 0.94 ± 0.13[a] | 0.49 ± 0.09[c] |
| 7662-CAT | None | 2.02 ± 0.65 | 1.12 ± 0.16 |
| | 5 mM-DFMO | 2.34 ± 0.42 | 1.35 ± 0.20 |
| | 5 mM-DFMO + 10 μM-spermine | 0.86 ± 0.18[b] | 0.55 ± 0.11[d] |

levels of putrescine (Tables 2 and 3). The presence of the unprocessed percursor may influence the AdoMetDC activity available in the cell, but would not affect the activity measurements in our experiments, since the cell extracts for activity assays were prepared in buffers containing putrescine. When portions of these extracts were preincubated in 2 mM-putrescine for 1 h before assay for AdoMetDC activity, to ensure that all the AdoMetDC proenzyme had been converted into the active form, there was no change in activity. Therefore, all AdoMetDC in the cell extracts was converted into the active enzyme during the harvesting procedure.

DFMO gave the expected large increase in AdoMetDC activity and protein in mock-transfected cells (Tables 4 and 5). This increase was accompanied by a 6–9-fold rise in the content of AdoMetDC mRNA (results not shown). Since the rise in AdoMetDC activity or protein is considerably larger than this, it appears that not all of the increase can be accounted for by increases in RNA levels.

There was no further increase in the AdoMetDC activity of DFMO-treated COS-7 cells when transfected with pSAMh1 (Table 4). This suggests that the expression from this plasmid is prevented by spermidine depletion. The effects of spermidine on AdoMetDC levels in this experiment were consistent with this interpretation. Addition of spermidine completely reversed the effects of DFMO and decreased AdoMetDC to control levels in mock-transfected cells, but produced only a small decrease in AdoMetDC in cells transfected with pSAMh1. The increase in AdoMetDC levels seen in response to pSAMh1 in cells treated with DFMO plus spermidine is therefore due to expression from the pSAMh1 plasmid.

**Effect of spermidine depletion on expression of other plasmids in COS-7 cells**

The inhibitory effect of spermidine depletion on the expression of pSAMh1 is not related to the nature of the inserted cDNA sequence, since expression of ODC protein (measured by Western blotting) from pCD-ODC was also blocked in DFMO-treated cells (Table 6). This plasmid contains an ODC cDNA sequence in place of the AdoMetDC in pSAMh1, but is otherwise identical. These results suggested that the decrease in spermidine content prevents expression in COS-7 cells of plasmid vectors containing the SV40 origin of replication. This was tested by examining the effect of DFMO on the production of CAT after transfections with SV40-CAT, which contains the SV40 promoter and enhancer elements as well as the SV40 origin of replication, or RSV-CAT, which contains the Rous Sarcoma Virus promoter. In COS-7 cells, there was a much lower level of CAT expression from SV40-CAT when DFMO was present, and this effect was reversed by spermidine (Table 6). In contrast, there was no effect on CAT expression from RSV-CAT. In order to test whether it is the SV40 origin of replication or the promoter/enhancer sequences which are involved, the same experiment was carried out with CHO cells, which do not express the large T antigen and do not support replication (Gluzman, 1981). As shown in Table 6, there was no effect of DFMO on the production of CAT from either SV40-CAT or RSV-CAT in CHO cells.

These results indicate that depletion of spermidine inhibits expression of plasmids in COS-7 cells by preventing replication, and that plasmids such as pSAMh1 cannot be used in this system to investigate effects of spermidine on AdoMetDC levels. Unfortunately, transfection of CHO cells with pSAMh1 gave such a small increase in AdoMetDC over basal levels that this system could also not be used for this purpose.

**Effect of polyamine depletion on expression of CAT plasmids regulated by sequences from the AdoMetDC gene**

In order to investigate the possible influence of polyamines on transcription of the AdoMetDC gene, two AdoMetDC-promoter-containing plasmids with the CAT reporter gene were used. One of these contained 1505 nucleotides of the 5′ flanking region of the AdoMetDC gene (5604-CAT) and the other had only the 173 nucleotides of the promoter (7662-CAT). Both constructs included about 100 nucleotides of the 5′-untranslated region of AdoMetDC mRNA. RSV-CAT was used as a control. Transfections of both COS-7 cells and CHO cells showed that

these constructs did have promoter activity, although it was only 10–15% of that observed with RSV-CAT, which contains the strong RSV promoter (results not shown).

The addition of BDAP or spermine resulted in no significant change in CAT expression (Table 7). Although it is possible that the constructs do not contain the needed portions of the AdoMetDC gene, it appears that the stimulatory effect of BDAP on AdoMetDC expression cannot be explained by transcriptional activation, since the AdoMetDC promoter does not respond to the presence of BDAP. This is in agreement with the results described above showing that spermine does not alter the content of AdoMetDC mRNA.

The effects of DFMO and spermidine treatment on the AdoMetDC-promoter-driven CAT expression are also shown in Table 7. In all cases, there was an increase in expression in response to DFMO and a marked decrease in expression in response to spermidine. Although these effects were quite small, they were reproducible and are in contrast with the lack of effect of DFMO on RSV-CAT expression shown in Table 6. Slot-blot analysis confirmed that the amount of CAT mRNA present was decreased in cells which were treated with DFMO plus spermidine as compared with cells treated with DFMO alone (results not shown).

These results are in agreement with the finding that the increase in AdoMetDC activity brought about by treatment with DFMO is accompanied by an increase in mRNA content. They indicate that at least part of this effect may be produced by a change in the transcription of the AdoMetDC gene.

## DISCUSSION

Variations in AdoMetDC activity are very important in the regulation of cellular polyamine levels. Our results confirm and extend previous studies showing that agents causing a decrease in polyamines lead to an increased level of AdoMetDC activity or protein (reviewed by Pegg, 1984; Pegg *et al.*, 1988*b*; see also Persson *et al.*, 1989*a*,*b*; Holm *et al.*, 1989; White *et al.*, 1990; Autelli *et al.*, 1991). Our findings provide further evidence that both spermidine and spermine can independently influence the level of this enzyme by affecting its rate of synthesis. Furthermore, the sites of regulation are different. Spermine affects primarily the translation of the AdoMetDC mRNA, whereas spermidine affects the content of this mRNA.

It is apparent from the results of studies in which exogenous spermine is added to cells (Table 1 and Fig. 1) that spermine is a very powerful repressor of AdoMetDC, and that this effect does not involve any significant change in AdoMetDC mRNA content. The results with the spermine synthase inhibitor BDAP are in agreement with this. Although there was a large increase in the amount of AdoMetDC protein, there was no increase in mRNA content in response to BDAP, nor was there an increase in expression of the CAT gene driven by AdoMetDC promoter regions. These results show that the effect of spermine is at a post-transcriptional stage. Since the expression of AdoMetDC from pSAMh1 in COS-7 cells was not increased by BDAP, it is unlikely that stabilization of the protein against degradation can play a major role. It therefore appears that the control by spermine occurs at a translational step that is not involved in the expression from pSAMh1. This plasmid contains, in the pCD expression vector, the entire protein-coding region of human AdoMetDC mRNA, all of the 3′-non-coding region of 557 nucleotides before the start of the poly(A) segment and 248 bases of the 5′-non-coding region. This is not the entire 5′ end, which is 320 bases in length (Maric *et al.*, 1992). It therefore seems likely that the 72 bases at the 5′ end of the mRNA are involved in the translational regulation of AdoMetDC. The rat and the human AdoMetDC mRNA sequences are very similar, with more than 94% identity in the 5′ untranslated regions (Pulkka *et al.*, 1991; Maric *et al.*, 1992). This is compatible with the sequences having some regulatory importance.

The results shown in Tables 1 and 3 also indicate that spermine is more active than spermidine in decreasing AdoMetDC content. Direct addition of spermine has a more pronounced effect than does addition of the same amount of spermidine. More convincingly, the exposure to BDAP that greatly increases AdoMetDC content does not decrease the total polyamine amount, because there is an increase in spermidine as spermine is decreased. These results provide clear evidence that spermine does regulate AdoMetDC and that an inactivator of spermine synthase leads to a substantial increase in the content of AdoMetDC. A previous report (Pegg *et al.*, 1987) that treatment of cells with the spermine synthase inhibitor *S*-methyl-5′-methylthioadenosine led to an increase in AdoMetDC was also interpreted to indicate an effect of spermine on the regulation of AdoMetDC, but these results are less clear, since this inhibitor lacks specificity. In particular, *S*-methyl-5′-methylthioadenosine is known to interact directly with the AdoMetDC protein (Kolb *et al.*, 1982) and to stabilize it against degradation (Holm *et al.*, 1989).

It is clear that spermidine also influences AdoMetDC content. Many studies have now shown that addition of DFMO, which decreases spermidine without decreasing spermine, increases the level of AdoMetDC protein and its mRNA (Shirahata & Pegg, 1986; Pajunen *et al.*, 1988; Pegg *et al.*, 1988*b*; Persson *et al.*, 1989*b*; White *et al.*, 1990). One explanation for this finding would be that there is an effect of spermidine on transcription of the AdoMetDC gene, and the results shown in Table 7 provide the first direct evidence for this. Regions from the AdoMetDC promoter, when linked to a CAT reporter gene, showed an activity which was inversely related to the spermidine content. This suggests that both the short and the long promoter regions in these constructs contain an element which can respond to excess spermidine. Since the expression of the 172-nucleotide-long promoter construct (7662-CAT) was inhibited by spermidine, use of deletion mutants within this region may help to locate the DFMO-responsive element. It should be noted that it is not yet clear whether all of the promoter region is contained within even the longer construct (5604-CAT) (Pulkka *et al.*, 1991; Maric *et al.*, 1992).

Even in DFMO-treated cells, the rise in mRNA content is not sufficient to account for all of the increase in AdoMetDC protein, suggesting some regulation at the level of either translation or protein turnover. Treatment for 48 h with DFMO increases the half-life of AdoMetDC in COS-7 cells from about 45 min to over 4 h (L. M. Shantz & A. E. Pegg., unpublished work) which is consistent with previous work suggesting that the turnover of AdoMetDC is influenced by polyamines (Pegg, 1984; Shirahata & Pegg, 1986; Pegg *et al.*, 1987; Autelli *et al.*, 1991). This may account for the difference in protein and mRNA levels. It is also possible that spermidine does influence the translation of AdoMetDC mRNA, albeit more weakly than spermine. However, this could not be tested by using pSAMh1, because the replication of this plasmid in COS-7 cells was spermidine-dependent (see below). The high level of transient expression obtained in these cells is needed to study AdoMetDC expression from plasmids, since there is no mammalian cell line deficient in AdoMetDC. Expression in cell lines such as CHO cells is too low to provide an adequate increase over the background endogenous level.

The results shown in Table 6 indicate clearly that in COS-7 cells the efficient expression of proteins from plasmid vectors containing the SV40 origin of replication is dependent on

spermidine. The most likely explanation for this is that spermidine is needed for plasmid replication. This replication occurs in COS-7 cells because they contain the large T-antigen, and is responsible for the very high transient expression in these cells (Gluzman, 1981). There are a number of well-documented cases in which viral replication has been found to be polyamine-dependent and blocked by inhibitors of ODC (reviewed by Tyms & Williamson, 1987).

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