Role of the 5'-untranslated region of mRNA in the synthesis of S-adenosylmethionine decarboxylase and its regulation by spermine

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S-Adenosylmethionine decarboxylase (AdoMetDC), a rate-limiting enzyme in polyamine biosynthesis, is regulated by polyamines at the levels of both transcription and translation. Two unusual features of AdoMetDC mRNA are a long (320 nt) 5'-untranslated region (5'UTR), which is thought to contain extensive secondary structure, and a short (15 nt) open reading frame (ORF) within the 5'UTR. We have studied the effects of altering these elements on both the expression of AdoMetDC and its regulation by n-butyl-1,3-diaminopropane (BDAP), a spermine synthase inhibitor. Human AdoMetDC cDNAs containing alterations in the 5'UTR, as well as chimaeric constructs in which the AdoMetDC 5'UTR was inserted ahead of the luciferase-coding region, were transfected into COS-7 cells. Construct pSAM320, which contains all of the 5'UTR, the AdoMetDC protein-coding region and the 3'UTR, was expressed poorly (2-fold over the endogenous activity). Deletion of virtually the entire 5'UTR, leaving nt -12 to -1, increased expression 59-fold, suggesting that 5'UTR acts as a negative regulator. The same effect was seen when the 27 nt at the extreme

5' end were removed (pSAM293, 47-fold increase), or when the internal ORF which is present in this region was destroyed by changing the ATG to CGA (pSAM320-ATG, 38-fold increase). The expression and regulation of pSAM44 (made by deleting nt -288 to -12), which has very little predicted secondary structure, was very similar to that of pSAM320 indicating that the terminal 27 nt including the internal ORF rather than extensive secondary structure may be responsible for the low basal levels of AdoMetDC expression. These results, confirmed using luciferase constructs, suggest that the negative effect on expression is predominantly due to the internal ORF. Depletion of spermine by BDAP increased the expression from pSAM320 more than 5-fold without affecting AdoMetDC mRNA levels. Expression from pSAM293 was unchanged by spermine depletion, whereas that from pSAM320-ATG was increased 2.5-fold. These results indicate the presence of a spermine response element in the first 27 nt of the 5'UTR that may include but is not entirely due to the internal ORF.

INTRODUCTION

The polyamine-biosynthetic pathway plays an important role in the proliferation of mammalian cells and is therefore a target for the design of chemotherapeutic agents (Pegg, 1988; Seiler, 1991). Depletion of polyamine levels has been shown to lead to reduction in cellular growth and alterations in cellular differentiation. Cellular polyamine content is tightly regulated, and S-adenosylmethionine decarboxylase (AdoMetDC), which catalyses the rate-limiting step in the production of spermidine and spermine, forms an important branch point which commits S-adenosylmethionine to a role in polyamine synthesis (Tabor and Tabor, 1984; White and Morris, 1989; Pegg and McCann, 1992). AdoMetDC is synthesized as a proenzyme which then undergoes an internal processing reaction forming both the α - and β -subunits and the pyruvate prosthetic group, which is located at the N-terminus of the α -subunit. The processing and activity of AdoMetDC are increased by putrescine providing a means by which the increased availability of putrescine raises the formation of decarboxylated AdoMet which is needed for the next enzyme in the biosynthetic pathway, spermidine synthase, to convert putrescine into spermidine (Pegg, 1984; Pegg and McCann, 1992).

AdoMetDC activity increases in response to a variety of stimuli that increase cell growth. In addition, activity is altered in response to changes in polyamine levels in several cell lines as

well as in animal models (White and Morris, 1989; Heby and Persson, 1990; Pegg and McCann, 1992). It has been suggested that this regulation may occur not only at the level of mRNA synthesis or stability, but also at the translational level (Kameji and Pegg, 1987; Pajunen et al., 1988; Persson et al., 1989; White et al., 1990; Shantz et al., 1992; Stjernborg et al., 1993). Translational regulation of AdoMetDC also appears to occur during growth stimulation (Mach et al., 1986; Pajunen et al., 1988; White and Morris, 1989). An unusual feature of AdoMetDC mRNA is a long 5'-untranslated region (5'UTR) of more than 300 nucleotides (Pajunen et al., 1988; Maric et al., 1992; Hill and Morris, 1992, 1993; Pulkka et al., 1993; Waris et al., 1993), and translational regulation could be mediated through this 5'UTR (Kameji and Pegg, 1987; Hill and Morris, 1992, 1993; Suzuki et al., 1993). The stable predicted secondary structure for the AdoMetDC 5'UTR [-289 kJ (-69 kcal)] and the presence of an upstream AUG translation-initiation site within the leader sequence may interfere with the initiation of translation and may also play a role in translational regulation by polyamines. In order to examine these possibilities, we have constructed cDNA plasmids containing various truncations and alterations of the AdoMetDC 5'UTR and examined the effects of these changes on both the expression of these plasmids and their regulation by spermine using the spermine synthase inhibitor n-butyl-1,3-diaminopropane (BDAP) (Baillon et al., 1989; Pegg and Coward, 1993).

Abbreviations used: AdoMetDC, S-adenosylmethionine decarboxylase (EC 4.1.1.50); AdoMet, S-adenosylmethionine; 5'UTR, 5'-untranslated region; ORF, open reading frame; BDAP, n-butyl-1,3-diaminopropane.

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EXPERIMENTAL

Materials

The antiserum to human AdoMetDC was raised in rabbits as described previously (Shirahata and Pegg, 1985). [γ - 32 P]ATP (300 Ci/mmol) was 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. BDAP was a generous gift from Dr. J. K. Coward, Department of Chemistry, University of Michigan, Ann Arbor, MI, U.S.A. Restriction enzymes were from Stratagene, La Jolla, CA, U.S.A., Promega Corp, Madison, WI, U.S.A. and Bethesda Research Laboratories, Bethesda, MD, U.S.A. Plasmid p5UTR/34 was generously provided by Dr. O. A. Jänne, University of Helsinki, Helsinki, Finland. Other biochemical reagents used were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., Bio-Rad, Richmond, CA, U.S.A., Becton Dickinson, Cockeysville, MD, U.S.A. and Difco, Detroit, MI, U.S.A.

Plasmid construction

The plasmids constructed, all in the pEUK-C1 expression vector (Clontech, Palo Alto, CA, U.S.A.), are illustrated in Figure 1. The methods of construction were as follows. Plasmid pSAM320 was constructed by digestion of pSAMh1 (Pajunen et al., 1988) with *Pvu*II and *Bam*HI and isolation of a 1717 bp fragment containing 97 bases of the 5'UTR, the protein-coding region and the 3' end. The remainder of the 5'UTR fragment was isolated from p5UTR/34 (Shantz et al., 1992; Maric et al., 1992) after digestion with *Sac*I and *Pvu*II. All fragments were purified using the GeneClean procedure (Bio 101, La Jolla) according to the manufacturer's instructions. The final plasmid was prepared by ligation of the 1717 bp and 223 bp fragments along with the pEUK-C1 expression vector digested with *Sac*I and *Bam*HI. The ligated DNA, from this and all subsequent plasmids described, was precipitated and transformed into competent DH5 α F' cells (a *rec* A1 *end* A1, *r*_k⁻*m*_k⁺ strain derived from *Escherichia coli* strain DH5) by electroporation using a Transfecto 100 instrument (BTX, Inc., San Diego, CA, U.S.A.), followed by growth for 1 h in SOC medium [2% (w/v) bactotryptone, 0.5% (w/v) bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose] at 37 °C and 225 rev./min. Cells were plated on Luria-Bertani plates containing 50 μ g/ml ampicillin and incubated overnight at 37 °C. Colonies chosen from the plates were grown overnight in Luria-Bertani medium containing ampicillin, and the miniprep DNA was isolated using standard techniques (Maniatis et al., 1989) and checked by restriction digests for the proper insert. The presence of the required inserts was then confirmed by sequencing using Sequence 2.0 (United States Biochemical, Cleveland, OH, U.S.A.) (Stanley et al., 1989).

For construction of pSAM320-ATG, p5UTR/34 linearized with *Sac*I was used as a template for amplification by PCR using a programmable thermal cycler (Techne Inc., Princeton, NJ, U.S.A.) and *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.) by standard procedures (Higuchi, 1989). This replaced a *Csp*45I site present in the vector just before the start of the UTR sequence with an *Xho*I site. The sense primer used for this reaction was 5'-GGTACCCCTCGAGGCTTACACAGTATGGCCG-3' with the *Xho*I site underlined. The antisense primer used was a primer corresponding to the SP6 RNA polymerase promoter region of the pGEM-3Z plasmid (Promega). The entire 5'UTR insert was isolated by digestion with *Xho*I and *Xba*I and ligated into the

Bluescript SK – vector (Stratagene) for sequencing. This plasmid was designated BLUE-UTR1, and was linearized with *Sca*I and used as a template for a second PCR in which the internal ORF present from bases –309 to –289 of the AdoMetDC 5'UTR was destroyed by replacing the ATG codon with a *Sal*I site. A sense primer with the sequence 5'-GCCCCCCTCG-AGGCTTACACAGTTCGACCCGGCGAC-3', where the *Sal*I site is underlined, was used. The antisense primer used corresponded to the T3 promoter region of the Bluescript plasmid. The product of PCR was digested with *Xho*I and *Csp*45I to yield a fragment of 343 bases containing the entire 5'UTR (without the ATG codon) plus the bases coding for the first six amino acids of AdoMetDC. pSAMh1 was digested with *Csp*45I and *Bam*HI to yield a 1602 bp piece which contained the entire 3' end and the protein-coding region starting at amino acid number 7. The final plasmid was isolated after ligation of these two fragments with the pEUK-C1 vector which had been digested with *Xho*I and *Bam*HI.

Plasmid pSAM293 was made from pSAM320 (5 μ g), which was first digested with *Xho*I and *Nhe*I overnight at 37 °C. With no change of buffer, 1 μ l of the four dNTPs (Pharmacia Biotech, Piscataway, NJ, U.S.A.) at 0.5 mM each and 5 units of Sequenase 2.0 were added and the reaction mixture was incubated at 30 °C for 15 min in order to fill in the overhanging ends. This reaction was stopped by heating to 75 °C for 10 min. The DNA was precipitated and resuspended in T4 DNA ligase buffer and allowed to react with DNA ligase overnight at 16 °C.

For construction of pSAM44, plasmid pSAM320 was digested with *Xho*I and *Nhe*I and the 80 bp fragment isolated using a MERmaid kit (Bio 101) as recommended by the manufacturer. pSAMh1 was digested with *Csp*45I and *Bam*HI and the 1602 bp piece isolated. To link the *Nhe*I site to the *Csp*45I site and also restore the six amino acids lost on digestion of pSAMh1 with *Csp*45I, sense and antisense linkers were constructed, the sense linker with the sequence 5'-CTAGAGTCTCACGG-TGATGGAAGCTGCACATTTTTT-3' and the antisense linker with the sequence 3'-TCAGAGTGCCACTACCTTCG-ACGTCTAAAAAAGC-5'. The start codon is underlined in both sequences. These two linkers were included in a ligation reaction along with the *Xho*I–*Nhe*I fragment, the *Csp*45I–*Bam*HI fragment and the pEUK-C1 vector digested with *Xho*I and *Bam*HI to obtain the final plasmid of interest. This plasmid contains the same internal ORF as is present in pSAM320.

Using pSAM44 linearized with *Bam*HI as a template, PCR was used to replace the internal ATG codon with a *Sal*I site. The same sense primer as used for the construction of pSAM320-ATG and an antisense primer complementary to a region within the AdoMetDC protein-coding region were used. The product with the altered ATG sequence was isolated by digestion with *Xho*I and *Bgl*III, a unique site within the AdoMetDC protein-coding region. This resulted in a 154 bp fragment that was isolated using the MERmaid procedure. The fragment was then ligated into pSAM44 cut with the same enzymes to yield the plasmid designated pSAM44-ATG.

To construct pSAM12, pSAMh1 was digested with *Csp*45I and *Bam*HI and the 1602 bp fragment isolated. The same sense and antisense linkers used in the construction of pSAM44 were used in a ligation reaction with the *Csp*45I–*Bam*HI fragment and pEUK-C1 digested with *Xba*I and *Bam*HI (the same linkers could be used for the construction of pSAM44 as *Xba*I and *Nhe*I have complementary overhangs).

The luciferase plasmid pLuc320 was constructed by first isolating the luciferase protein-coding region after digestion of plasmid pGL2 (Promega) with *Bam*HI and *Sac*I. Using pSAM320 as a template, PCR was used to create a *Bam*HI site

Figure 1 Structure of AdoMetDC and luciferase expression vectors

The expression vector pEUK-C1 (4.9 kbp) was used to construct AdoMetDC and luciferase plasmids as described in the Experimental section. The restriction sites used are shown in each plasmid diagram. □, AdoMetDC 5'UTR (320 bases); ▨, AdoMetDC internal open reading frame (ORF) (-312 to -297); ▩, AdoMetDC protein-coding region and 3'UTR (1620 bases); ▤, luciferase-protein-coding region (1649 bases).

at the 3' end of the 5'UTR. The antisense primer used in the reaction contained the sequence 3'-AAGCGATCAGAG-TGCCCTAGGTTTCGACGTGT-5' in which the *Bam*HI site is underlined. The sense primer used corresponded to a region within the pEUK-C1 vector sequence. The product of this reaction was digested with *Xho*I and *Bam*HI and ligated together with the *Bam*HI-*Sac*I fragment and the pEUK-C1 vector digested with *Xho*I and *Sac*I. This plasmid uses the ATG codon in the luciferase-coding region.

pLuc320-ATG was prepared by first digesting pSAM320-ATG with *Nde*I, which cuts within the pEUK-C1 vector, and *Nhe*I, which cuts within the 5'UTR of AdoMetDC. A 1983 bp

piece was isolated. pLuc320 was cut with the same enzymes and a 5000 bp piece was isolated and ligated to the 1938 bp fragment.

Cell culture and transfection

COS-7 cells were grown and transfected as described previously (Shantz et al., 1992). BDAP (50 μ M) was added when the cells were plated and spermine (10 μ M) was added 3 h before transfection. All results are given as means $\pm$ S.D. When a control chloramphenicol acetyltransferase plasmid (SV40-CAT, Promega) was co-transfected to correct for transfection efficiency, the results were the same. Cell extracts were prepared for enzyme

assays, polyamine measurements, Western blots or RNA blots as described by Shantz et al. (1992).

Primer-extension analysis

Primer-extension analysis was carried out on total RNA samples isolated by the method of Chomczynski and Sacchi (1987) from COS-7 cells which had been transfected with pSAM320-ATG or pEUK-C1 as a control. Primer extension was carried out using an AMV reverse transcriptase primer-extension system (Promega) as described by the manufacturer. An oligomer complementary to bases -272 to -247 of the AdoMetDC 5' leader sequence, which was labelled at the 5' end using [γ - 32 P]ATP, was used as a primer. The extended products were analysed on an 8% denaturing polyacrylamide gel containing 7 M urea. AdoMetDC assays confirmed that cells transfected with pSAM320-ATG expressed AdoMetDC approx. 40-fold above control levels.

Western blots

SDS/PAGE was performed by the method of Laemmli (1970) on 100 μ g of cytosolic protein from COS-7 extracts. Proteins reactive with anti-AdoMetDC polyclonal antibodies were detected as previously described (Shantz et al., 1992).

Determination of RNA content

Slot-blots on to Duralon-UV (Stratagene) were performed with a Slot-Blot manifold (Schleicher and Schuell, Keene, NH, U.S.A.). A concentration range of 0.25–2.0 μ g of RNA from transfected cells was used. AdoMetDC RNA (0.5–5 ng) was used as a positive control and yeast tRNA (2.5–10 μ g) as a negative control. RNA was cross-linked to the membranes with a Stratalinker (Stratagene). SP6 RNA polymerase and digoxigenin-labelled UTP were used to prepare a cRNA probe complementary to the mRNA for human AdoMetDC by using pCM9 (Stanley et al., 1989) as a template. RNA was detected using a nucleic acid detection kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions.

Enzyme and protein assays

AdoMetDC activity was assayed by measuring the release of $^{14}\text{CO}_2$ from *S*-adenosyl[carboxy- ^{14}C]methionine (Shirahata and Pegg, 1985). One unit of enzyme activity represents the formation of 1 nmol of $^{14}\text{CO}_2$ /30 min per mg of protein. Luciferase assays were performed with a luciferase assay system (Promega) and quantified against a standard curve using purified firefly luciferase (Boehringer-Mannheim). Results are expressed as pg of luciferase per mg of protein. Protein was measured by the method of Bradford (1976).

RESULTS

Structure of the 5'UTR of AdoMetDC

The 5'UTR of human AdoMetDC mRNA is shown in Figure 2. Computer modelling of the most stable secondary structure of the leader sequence using the program FOLD (Zucker and Stiegler, 1981) results in a free energy of formation (ΔG) of -289 kJ (-69 kcal)/mol. The AUG of the internal ORF is also shown. Primer-extension analysis was used to determine the start

site for AdoMetDC transcription with total RNA from COS-7 cells transfected with either AdoMetDC plasmid pSAM320-ATG or the pEUK-C1 expression vector as a control. These results (not shown) confirmed that the transcription start site for the AdoMetDC plasmids in the pEUK-C1 expression vector was the same as that for endogenous AdoMetDC.

Effect of AdoMetDC 5'UTR on expression in COS-7 cells

In order to investigate the effects of AdoMetDC 5'UTR on the extent of expression of the protein, plasmids containing various alterations in the 5'UTR in the pEUK-C1 vector were transfected into COS-7 cells and their AdoMetDC activity measured. pSAM320, which contains the entire 5'UTR, was relatively poorly expressed, showing an increase of only 2-fold over endogenous AdoMetDC levels (Table 1). When virtually the entire 5'UTR was removed (pSAM12), AdoMetDC activity was increased almost 60-fold over control, suggesting that the 5'UTR acts as a negative regulator. In fact, removing only the first 27 bases of the 5'UTR (pSAM293) resulted in a similar increase in expression, suggesting that the negative regulatory element resides in these 27 bases.

As the internal ORF in the 5'UTR is present from bases -309 to -289, it was also deleted in construct pSAM293. In order to determine whether this ORF is involved in the negative regulation of AdoMetDC expression, plasmid pSAM320-ATG was constructed in which the internal ORF was removed by replacing the initial ATG with CGA. When pSAM320-ATG was transfected into COS-7 cells, the resulting AdoMetDC activity was almost 40-fold higher than that of mock-transfected cells. This strongly suggests that the internal ORF plays an important role in inhibiting the translation of AdoMetDC mRNA in COS-7 cells.

This result was confirmed using chimaeric constructs, which placed either the entire 5'UTR of AdoMetDC, or one in which the internal ATG was mutated to CGA, directly ahead of the gene encoding for luciferase. The construct pLuc320-ATG produced almost 100-fold more luciferase activity than pLuc320 (Table 1), confirming that the internal ORF has a powerful negative effect on expression.

Involvement of the 5'UTR in spermine regulation of AdoMetDC expression

The results above support the concept that the expression of AdoMetDC is controlled by its 5'UTR. In order to study the possibility of polyamine-mediated translational regulation of AdoMetDC involving this region, BDAP was used to deplete spermine in COS-7 cells. The effects of this depletion on AdoMetDC derived from the transfected plasmids were then measured. Intracellular polyamine levels in cells transfected with plasmid pSAM293 did not differ greatly from those in mock-transfected cells despite the large increase in AdoMetDC activity. This is probably due to the fact that only a small percentage of the cells in the population actually express the enzyme but it is also possible that additional spermidine was acetylated and excreted, as we did not measure polyamines in the culture medium. All cells treated with BDAP showed a large decrease in spermine and corresponding rise in spermidine, consistent with spermine-synthase inhibition (Baillon et al., 1989; Pegg and Coward, 1993). Putrescine content was also depressed by BDAP, possibly as the result of repression of ornithine decarboxylase (Pegg, 1988). Addition of excess spermine to the tissue culture medium resulted in the return of spermidine to near control levels and, despite the large amount of spermine added (10 μ M), only about a twofold overshoot in spermine levels (Table 2).

Figure 2 Potential secondary structure in the 5'UTR of AdoMetDC mRNA

The 5'UTR of human AdoMetDC mRNA was subjected to secondary-structure analysis using the program FOLD (Zucker and Stiegler, 1981). The AUG of the internal ORF is indicated. The predicted ΔG of the structure is -289 kJ (-69 kcal)/mol.

Table 1 Effect of AdoMetDC 5'UTR on expression of AdoMetDC or luciferase in transfected COS-7 cells

Cells were grown and transfections performed as described in the Experimental section using 15 or 20 μ g of plasmid DNA. AdoMetDC or luciferase activities were measured 48 h after the start of transfection and are the means $\pm$ S.D. of from six to nine determinations for AdoMetDC or duplicate determinations for luciferase.

Transfected with plasmid	AdoMetDC specific activity (nmol/30 min per mg of protein) or luciferase activity (pg/mg of protein)	Fold increase in activity over mock-transfected cells
None	0.14 ± 0.02	1.0
pSAM320	0.30 ± 0.04	2.1
pSAM320-ATG	5.59 ± 0.07	38
pSAM293	6.86 ± 0.95	47
pSAM12	8.66 ± 1.47	59
pLuc320	20.3	—*
pLuc320-ATG	1887	—*

* Endogenous luciferase activity is zero.

Table 2 Effect of BDAP and spermine on polyamine levels in transfected COS-7 cells

Details of transfections are described in the Experimental section. Polyamines were measured 48 h after the start of transfection and are the means of duplicate experiments.

Transfected with plasmid	Compound added	Polyamines (nmol/mg of protein)		
		Putrescine	Spermidine	Spermine
None	None	13.4	28.1	26.6
None	50 μ M BDAP	< 0.2	70.8	4.1
None	50 μ M BDAP + 10 μ M spermine	0.5	15.8	40.5
pSAM320	None	4.8	20.3	19.1
pSAM320	50 μ M BDAP	< 0.2	63.1	3.0
pSAM320	50 μ M BDAP + 10 μ M spermine	< 0.2	17.5	37.1
pSAM293	None	5.8	23.3	23.7
pSAM293	50 μ M BDAP	< 0.2	61.4	7.1
pSAM293	50 μ M BDAP + 10 μ M spermine	< 0.2	22.5	56.9

Figure 3 Effect of alterations in the AdoMetDC 5'UTR on expression and regulation of constructs transfected into COS-7 cells

COS-7 cells, either mock-transfected or transfected with the plasmids shown, were treated with BDAP or BDAP plus spermine as described in the Experimental section. After harvesting, cell extracts were assayed for AdoMetDC or luciferase activity and the values for mock-transfected cells were subtracted from identically treated cells that had been transfected with plasmid. Results are shown with the mean values printed above the bars and in (a) with the S.D. indicated. (a) AdoMetDC activity. The values for mock-transfected cells are as follows: control, 0.14 ± 0.02 nmol/30 min per mg of protein; control plus BDAP, 2.2 ± 0.23 nmol/30 min per mg of protein; control plus BDAP and spermine, 0.07 ± 0.01 nmol/30 min per mg of protein. Results are means of from six to nine experiments. Note that the results for pSAM320 are multiplied by 10. (b) Luciferase activity. Values are calculated as pg of luciferase expressed/mg of protein and are the means of duplicate estimations. Plasmid GL2 (Promega) is a control plasmid for luciferase expression that does not contain any AdoMetDC sequences and was used to control for any non-specific effects of either BDAP or spermine. The results for pLuc320-ATG, which is efficiently expressed, are divided by 100. ■, Control; ▨, + BDAP; ▩, + BDAP and spermine; □, + spermine.

After the addition of BDAP, expression of pSAM320 was increased 5-fold (Figure 3a). This increase was completely reversed by the addition of 10 μ M spermine. In contrast, expression of pSAM293 was changed only slightly by the presence of BDAP or spermine, either alone or in combination, providing evidence that any region in the 5'UTR that responds to changes in spermine levels is in the first 27 bases. [The slight increase in AdoMetDC activity seen in the presence of BDAP can probably be explained by the fact that this compound stabilizes the enzyme

against intracellular degradation by increasing the half-life of endogenous AdoMetDC from about 50 min to about 3 h in COS-7 cells (results not shown).]

Although the 27 bases at the extreme 5' terminus were needed for repression of translation by spermine, removal of the internal ORF did not completely abolish the effect. Expression of AdoMetDC from pSAM320-ATG was increased significantly after BDAP treatment (2.5-fold increase, $P < 0.001$) and this increase was reversed by adding back spermine (Figure 3a). These experiments suggest the presence of a spermine response element between bases -320 and -293 of the 5'UTR that may act in combination with the internal ORF to regulate AdoMetDC expression.

Despite the changes in AdoMetDC activity, addition of BDAP or BDAP and spermine had no significant effect on RNA levels, as measured by slot-blots, in either mock-transfected cells or those transfected with the SAM constructs (results not shown). We have demonstrated previously that alterations in endogenous AdoMetDC activity in response to BDAP and spermine were accompanied by parallel changes in the amount of enzyme protein (Shantz et al., 1992). Similar studies using densitometric scanning of Western blots in the present experiments showed that addition of BDAP increased the amount of AdoMetDC in both mock-transfected cells and cells transfected with either pSAM320 or pSAM293 (results not shown).

Similar experiments using BDAP to manipulate cellular spermine levels and examine the effect on protein expression were also performed using the luciferase constructs pLuc320 and pLuc320-ATG (Figure 3b). The production of luciferase from pLuc320-ATG was not affected by the presence of BDAP or BDAP and spermine. Expression of luciferase from pLuc320 was increased in the presence of BDAP, and this increase was abolished by spermine, suggesting that the plasmid is regulated by polyamines. However, less than 2-fold induction was obtained compared with 5-fold for AdoMetDC production from pSAM320. Thus part but not all of the response to spermine appears to be mediated exclusively via the 5'UTR. These results, taken with those above, suggest a possible interaction of bases -320 to -293 with an area within either the protein-coding region or 3'UTR of AdoMetDC, as these elements are not present in the luciferase plasmids.

Importance of the location of bases -320 to -293

The experiments described above show that the regulation of AdoMetDC expression occurs on several levels. The presence of the internal ORF appears to inhibit translation of the AdoMetDC mRNA. These low levels of translation can be increased by the depletion of spermine, and some of the bases contained between -320 and -293 of the AdoMetDC mRNA 5'UTR are necessary for this increase to occur. Plasmid pSAM44 was constructed to test the importance of the location of bases -320 to -293 in this response to spermine depletion. In this plasmid, bases -288 to -12 are deleted, leaving the 12 bases preceding the protein-coding region [which are known to be important in translation (Kozak, 1991)] intact, as well as the internal ORF. In addition, plasmid pSAM44-ATG was constructed in which, analogous to pSAM320-ATG, the ATG codon of the internal ORF was replaced by CGA. When pSAM44 was transfected into COS-7 cells, its expression was similar to that of pSAM320 (Figure 4), indicating that the internal ORF could inhibit translation even within this shortened 5'UTR. This result was confirmed by the transfection of pSAM44-ATG, which expressed 10 times more AdoMetDC activity than pSAM44.

Plasmid pSAM44 was also similar to pSAM320 in the effect of

Figure 4 Importance of the location of bases -320 to -293 on the expression and regulation of AdoMetDC

COS-7 cells transfected with either $15 \mu\text{g}$ of pSAM44-ATG or $20 \mu\text{g}$ of pSAM44 were harvested 48 h after transfection and analysed. Results are presented as described in the legend to Figure 3. ■, Control; ■, + BDAP; ▨, + BDAP and spermine.

spermine on AdoMetDC expression. Addition of $50 \mu\text{M}$ BDAP increased the expression from pSAM44 5-fold, and this increase could be completely reversed by the addition of spermine. The expression of pSAM44-ATG was also regulated by changes in spermine content, although less dramatically (Figure 4). Thus the 27-base sequence at the 5' end and the 12 bases at the 3' end of the 5'UTR appear to be sufficient to inhibit translation and for response to spermine depletion.

DISCUSSION

AdoMetDC activity shows striking changes in response to factors affecting cell growth and to changes in polyamine levels. With the exception of the stimulation of enzyme activity by putrescine, which is brought about by a conformational change in the protein lowering its K_m for the substrate, it appears that all of these changes are accomplished by an alteration in the amount of the enzyme protein present. Numerous mechanisms influencing this amount are known to occur and to be subject to physiological regulation. These include the rate of proenzyme processing to the mature enzyme, the rate of enzyme degradation and the transcription of AdoMetDC mRNA [reviewed by Pegg et al. (1988), White and Morris (1989) and Pegg and McCann (1992)]. However, in many cases, changes in these factors are not sufficient to account for the alteration in AdoMetDC activity, suggesting that translational regulation of the synthesis of the protein also occurs. Experimental evidence consistent with translational regulation has been reported for a number of published experiments showing that: (a) an increased synthesis of AdoMetDC occurs under conditions in which the mRNA content does not change (Stjernborg et al., 1993); (b) the content of AdoMetDC mRNA in polysomes is increased when growth is stimulated or polyamines depleted (White et al., 1990; Hill and Morris, 1992);

and (c) the translation of AdoMetDC mRNA in reticulocyte lysates is inhibited by polyamines (Kameji and Pegg, 1987; Suzuki et al., 1993).

The present experiments confirm and extend these earlier studies supporting translational regulation and identify regions of the 5'UTR involved. Our results show clearly that, in COS-7 cells, the intact 5'UTR of AdoMetDC is strongly inhibitory to translation of the mRNA and that this inhibition is located in the 27-base sequence at the extreme 5' end of the 5'UTR. This sequence contains an ORF in a good sequence context for translation (Kozak, 1991) which could code for a hexapeptide. A mutation that changed the initiation codon of this sequence abolished the inhibitory activity. Thus the internal ORF rather than extensive secondary structure appears to be responsible for the low expression of AdoMetDC, as the 5'UTR of pSAM44 is predicted to have a free energy of only -24.3 kJ (-5.8 kcal)/mol and is expressed to a similar extent to the wild-type pSAM320. The importance of the internal ORF in regulating AdoMetDC expression has also been demonstrated using a cell line that overexpresses initiation factor eIF-4E. Even in the presence of high levels of this factor, which is thought to melt mRNA secondary structure, the expression of AdoMetDC from pSAM320 was not affected (Shantz and Pegg, 1994). It is likely that the internal ORF is involved in the inhibition of translation perhaps by causing the ribosomes to dissociate from the mRNA before reaching the correct initiation codon. This is consistent with other studies in which upstream AUG codons in yeast and mammalian transcripts have a major inhibitory effect on translation of downstream protein-coding sequences (Hinnebusch, 1990; Hershey, 1991; Kozak, 1991, 1992). Our results provide strong evidence in favour of the idea that initial binding of ribosomes to AdoMetDC mRNA does occur at the cap structure and is affected by the presence of the ORF located very close to the 5' end of the mRNA. The extent to which the 40 S subunit can remain attached to the mRNA after the termination codon of this ORF may regulate the translation of AdoMetDC mRNA. As discussed by Kozak (1991), the resumption of scanning and reinitiation does occur in mammalian mRNAs particularly when the first 5'-ORF is short. However, this reinitiation can be very inefficient particularly when the second ORF is located at a considerable distance from the 5' cap. This may be due to a loss of initiation factors from the 40 S complex. The structure of AdoMetDC mRNA is unusual in that the first short ORF is located very close to the cap structure and the second ORF coding for AdoMetDC is separated from it by 288 nt. The results with pSAM44 show that the internal 281 nt between the 27 bases at the 5' end and the 12 bases at the 3' end of the 5'UTR are not needed for the inhibition in the sense that they can be deleted completely and inhibition still occurs. However, it is quite possible that the secondary structure in this region promotes the dissociation of the 40 S particle or prevents the reattachment of ribosomal subunits or initiation factors and that the insertion of a different sequence without these properties could overcome the inhibition. Our results are in agreement with the recent report by Suzuki et al. (1993) showing that removal of 80 nucleotides from the 5' end of the 5'UTR of the mouse AdoMetDC mRNA increased its translation in reticulocyte lysates.

Our results confirm the conclusions of Hill and Morris (1992, 1993) that the sequence forming the internal ORF in the AdoMetDC 5'UTR is important in the regulation of translation, but their experimental system, results and conclusions differ from ours in several respects. They studied the polysomal distribution of either AdoMetDC mRNA or a reporter mRNA made up of the coding sequence of growth hormone and the 5'UTR from AdoMetDC and found that, depending on the cell

type, the ORF in the 5'UTR had a major effect on translation or growth-hormone production. They reported that the ORF in the 5'UTR of bovine AdoMetDC (which is identical with that in the human sequence) negatively regulates the synthesis of the protein in T-lymphocytes and T-cell lines but not in non-lymphoid cell lines. Furthermore, they found that the actual composition of the putative hexapeptide may be important in blocking translation in T-cells (Hill and Morris, 1993). This work also suggests that this ORF is important in the cellular regulation of AdoMetDC but there is an unexplained discrepancy between their results and ours in that COS-7 cells are also non-lymphoid, yet we see a very large inhibitory effect of the 5'UTR on AdoMetDC expression in these cells. It is possible that this difference is related to the expression vectors and reporter genes used in the two studies. Another explanation could be that differences in the spermine content of the different cell types alter the efficiency of mRNA translation as described below.

The powerful inhibitory effect of the AdoMetDC 5'UTR on translation is likely to be linked to the regulation of the activity of this enzyme. Genomic and/or cDNA clones for the proenzyme of AdoMetDC have now been obtained from several mammalian species including human, bovine, rat, mouse and hamster (Mach et al., 1986; Pajunen et al., 1988; Hill and Morris, 1992; Maric et al., 1992; Pulkka et al., 1993; Tekwani et al., 1992; Waris et al., 1993). The nucleotide sequences of the mRNAs predicted by these sequences are extremely similar, with more than 90% identity in both the amino acid-coding region and in the 5'UTR, which varies in length from 310 to 327 nt. The similarity in the amino acid-coding regions where there are only eight amino acid residues that differ between the human and the rodent enzymes may reflect the multiple functions of the protein including proenzyme processing, putrescine activation, enzyme activity and rapid turnover. However, the fact that the nucleotide sequence of 5'UTR is equally well conserved suggests that it has an essential function in the correct level of expression of the protein. As described above, the studies by Hill and Morris (1992, 1993) suggest that this may be related to the cell-specific level of expression of AdoMetDC. However, our results favour an alternative explanation which is that the sequence is involved in the regulation of AdoMetDC by polyamines. The apparent cell-specific expression of AdoMetDC may then be related to the different intracellular polyamine contents.

Our results showing that the sequence that responds to changes in intracellular spermine levels is located in the 27 nt at the 5' end of the 5'UTR is in agreement with previous work in which it was shown that AdoMetDC expression from a plasmid lacking the first 72 nt of the 5'UTR (−320 to −248) was not regulated by spermine (Shantz et al., 1992). It is also consistent with the finding that removal of the 5'-terminal 80 nucleotides from the mouse AdoMetDC mRNA reduced the inhibitory effect of polyamines on its translation in a reticulocyte lysate (Suzuki et al., 1993). Our findings do not provide any support for the suggestion by Waris et al. (1993) that a sequence located at the 3' end of the 5'UTR of mouse AdoMetDC, which binds to a 39 kDa protein in the presence of spermine, is involved in the translational regulation of AdoMetDC synthesis. However, the binding of this protein with a sequence corresponding to nucleotides −50 to +19 was demonstrated. As the luciferase reporter constructs that contained nt −320 to +1 showed only a partial

response to spermine in our experiments, it is possible that there is some interaction between the nucleotides at the start of coding region and the 27 nt sequence at the extreme 5' end of the 5'UTR. Further study of the sequences required to confer the full degree of spermine regulation is needed. The identification of a sequence that affects mRNA translation in a spermine-dependent manner may provide a useful research tool because cellular spermine levels can be altered significantly by exposure to inhibitors or spermine without affecting cell growth (Pegg, 1988; Pegg and Coward, 1993).

This research was supported by grant CA-18138 from the National Institutes of Health. Some equipment was provided by grants from the Alcoa Foundation. We thank Anne Stanley for the synthesis of the oligonucleotides used in plasmid constructions.

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