S-Adenosylmethionine decarboxylase gene expression in rat hepatoma cells: regulation by insulin and by inhibition of protein synthesis

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We have investigated expression of the *S*-adenosylmethionine decarboxylase (AdoMetDC) gene in H4-II-E rat hepatoma cells treated with growth factors (epidermal growth factor and transforming growth factor β1) and inducers (cAMP and insulin). Treatment with insulin caused a marked increase in both RNA level and enzyme activity. The stability of AdoMetDC mRNA was not altered by insulin treatment: the accumulation of mRNA in hepatoma cells therefore seems to be due to an increase in the transcription rate. Cycloheximide was found to be a strong inducer of AdoMetDC mRNA transcription and the effects of insulin and cycloheximide were additive, suggesting that they increase expression by separate mechanisms. Chloramphenicol acetyltransferase assays in rat hepatoma cells using 5′ flanking regions of different lengths revealed that the promoter region extending 337 bp upstream from the transcription start site contains elements involved in insulin response.

INTRODUCTION

Polyamines seem to be essential for mammalian cell proliferation [1–3]. The two key regulatory enzymes in the biosynthesis of polyamines are ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50). The product of AdoMetDC, decarboxylated AdoMet, serves as an aminopropyl donor in spermidine and spermine synthesis [2,3]. The mammalian enzyme is synthesized as an inactive precursor of approx. 38 kDa, which is converted to the active enzyme with subunits of approx. 31 and 8 kDa by an autocatalytic mechanism. The larger subunit contains a covalently bound pyruvate prosthetic group that is formed during cleavage by a serinolysis reaction [4]. Three classes of regulatory phenomena apparently underlie changes in AdoMetDC activity. These include changes (1) in the amount of AdoMetDC mRNA either by increasing the transcription rate or by a stabilization of the mRNA [5–7], (2) in the translation efficiency of the mRNA [8,9] and (3) in the intracellular stability of the enzyme itself [10,11]. One or more of these steps is negatively regulated by the cellular concentrations of spermidine and spermine [11]. In spite of the fact that there is a large repertoire of AdoMetDC inducers, transcriptional regulation of the AdoMetDC gene is poorly understood. To study the regulation of AdoMetDC expression we have previously characterized the rat gene (AMD1B) coding for AdoMetDC [12]. This gene consists of eight exons spanning more than 16 kb and encodes two transcripts (2.1 and 3.4 kb) differing in the lengths of their 3′ untranslated region [5]. In rapidly proliferating cells, AdoMetDC activity and the level of polyamines are much higher than in non-proliferating cells, and AdoMetDC activity can be induced in quiescent mammalian cells by several proliferative stimuli such as hormones [13–15], tumour promoters [16] and growth factors [6]. The use of specific inhibitors of polyamine-synthesizing enzymes has shown that synthesis of polyamines is, in fact, a prerequisite for cell proliferation [17].

One approach to dissecting the pathways of transcriptional regulation has been through the use of protein synthesis inhibitors [18,19]. AdoMetDC induction by mitogens has been shown to require protein synthesis *de novo* but to precede cell division [20]. Therefore its gene can be classified as an early growth response gene [21]. The very short physiological half-life of the enzyme enables a new level of enzyme protein to be reached very rapidly after the application of a vast array of stimuli that lead to cell growth. To study the molecular events involved in the growth-related regulation of AdoMetDC gene expression, we have investigated AdoMetDC induction in the rat hepatoma cell line, H4-II-E, after treatment with agents known to influence cell proliferation. This cell line expresses many hepatic proteins and although AdoMetDC constitutes only a small fraction of the intracellular proteins in tissues, liver is one of the richest enzyme sources. Therefore the H4-II-E hepatoma cell line is a useful model for studies of the regulation of AdoMetDC gene expression. It has been widely used to study the effects of insulin and cAMP on gene expression because of its responsiveness to these reagents. For example, insulin stimulates the transcription of the p33, glyceraldehyde 3-phosphate dehydrogenase and c-*fos* genes [22–24] whereas it inhibits the transcription of the phosphoenolpyruvate carboxykinase gene [25,26]. In contrast, cAMP increases phosphoenolpyruvate carboxykinase gene transcription in rat hepatoma cells. There are several other examples where both cAMP and insulin increase gene transcription, such as the prolactin [27] and acetyl-CoA carboxylase [28] genes. Growth factors such as the multifunctional transforming growth factor β1 (TGF-β1) and epidermal growth factor (EGF) influence cell proliferation and differentiation in a wide range of cell types including hepatoma cells. The multimodal nature of TGF-β1 is seen in its ability to either stimulate or inhibit cell proliferation depending on the origin of a cell line.

In the present study we examined the effect of insulin, cAMP, TGF-β1 and EGF on AdoMetDC gene expression in the rat hepatoma cell line H4-II-E. It is known that insulin stimulates AdoMetDC activity in its target tissues [29,30] but little is known about whether this response is achieved at the transcriptional or post-transcriptional level. We therefore also examined the effect of cycloheximide on insulin induction of AdoMetDC gene

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase; Bt_2cAMP, dibutyryl cAMP; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; TGF-β1, transforming growth factor β1.

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The AMD1B sequence will appear in the EMBL Nucleotide Sequence Database under the accession numbers Z15109, Z15122 and Z15123.

expression to determine whether protein synthesis *de novo* is required.

EXPERIMENTAL

Materials

Avian myeloblastosis virus reverse transcriptase, the pCAT-Basic Vector and the pCAT-Control Vector were obtained from Promega and the pTKGH-plasmid from Nichols Diagnostics Institute (San Juan Capistrano, CA, U.S.A.). Thermostable DNA polymerase (Dynazyme®) was from Finnzymes Inc. (Espoo, Finland). Restriction endonucleases, DNA-modifying enzymes, acrylamide and deoxyribonucleoside triphosphates were from Pharmacia Biotech or Boehringer Mannheim. Other reagents were of molecular biology grade and purchased from Sigma. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Cell cultures and their treatment with mitogens

The rat hepatoma cell line H4-II-E (American Type Culture Collection, Rockville, MD, U.S.A.) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % (v/v) fetal bovine serum (FBS). Cells were grown at 37 °C in a humidified atmosphere of air/CO_2 (19:1). The insulin-degrading activity frequently found in FBS was inactivated by heating at 55 °C for 30 min [31]. The rat hepatoma cells were grown in serum-free medium for 16 h before additions of insulin (1.74, 17.4 or 174 nM), dibutyryl cAMP (Bt_2cAMP) (1 mM), EGF (5 ng/ml) or TGF-β1 (10 ng/ml).

Assay of AdoMetDC activity

AdoMetDC was assayed by measuring the release of $^{14}CO_2$ from [*carboxy*-^{14}C]AdoMet as described previously [32,33]. Protein concentration was determined by the method of Lowry et al. [34].

RNA extraction and analysis

Total RNA was extracted from cell monolayers by the method of Chomczynski and Sacchi [35] and fractionated (25 μg per lane) on a 1 % (w/v) agarose gel containing formaldehyde [36], blotted onto nitrocellulose and hybridized with a nick-translated cDNA probe (1013 bp *Pst*I–*Pvu*II fragment of pSAMr1 [5]). The relative changes in mRNA levels were determined from Northern blots by using a Molecular Dynamics computing laser densitometer (model 300A), and the data were analysed with the Image Quant program.

Construction of promoter–chloramphenicol acetyltransferase (CAT) fusion genes

The rat AMD1B promoter fragment ($-669/+16$ bp) was synthesized by PCR with the o611 clone [12] as a template. The 5′ and 3′ primers were the nucleotides $-669/-650$ and $-7/+16$ respectively of the rat AMD1B sequence. Similarly, the promoter fragment ($-337/+16$) was synthesized from nucleotides $-337/-318$ and $-7/+16$ as the 5′ and 3′ primers respectively. *Hin*dIII restriction sites were added to all primers. The fragments were ligated into a *Hin*dIII-restricted pCAT-Basic vector. These two constructs, pCAT-669 and pCAT-337, contained 669 bp and 337 bp of the 5′-flanking sequence of the rat AMD1B gene. The orientations of DNA fragments in the two constructs were confirmed by DNA sequencing.

DNA transfections and CAT assay

The pCAT–AMD1B plasmids (pCAT-337 and pCAT-669) were purified by two successive equilibrium centrifugations in CsCl/ethidium bromide gradients [37]. H4-II-E cells were grown until 60–70 % confluent and each plate was co-transfected with 13 μg of pCAT–AMD1B or pCAT-Control (simian virus 40–CAT) plasmid and 3 μg of pTKGH-plasmid using Lipofectamine reagent in accordance with procedures recommended by the supplier (Gibco BRL). After transfection, the cells were washed twice with PBS and the medium was replaced with 10 ml of fresh medium containing 10 % FBS for 24 h. Thereafter the cells were grown in serum-free medium for 16 h before treatment with insulin. The cells were harvested 48–72 h after the transfections and CAT activities were measured as described [38]. The transfection efficiency was assayed by measuring the levels of secreted growth hormone in the medium by radioimmunoassay as described by the supplier (Pharmacia). Each plasmid construct was tested in duplicate in at least 13 independent experiments with different DNA preparations.

Statistical analysis

Differences between means were compared by using Student's *t*-test. Statistical significance was inferred when $P < 0.05$.

RESULTS AND DISCUSSION

Because it is known that the activity of AdoMetDC is induced by various agents related to cell growth, we investigated the effects of cAMP, insulin, EGF and TGF-β1 on the expression of the AdoMetDC gene in rat hepatoma cells. After 6 h of incubation with these reagents, the level of AdoMetDC mRNA was increased approx. 3-fold by insulin treatment but did not change significantly after treatment with cAMP, EGF or TGF-β1 (Figure 1, Table 1). Correspondingly, the enzyme activity was markedly increased (8.3-fold compared with the control) by insulin treatment in the rat hepatoma cells (Table 1). AdoMetDC activity increased 2.8-fold after cAMP treatment, whereas neither EGF nor TGF-β1 had any significant effect on the enzyme activity. To determine the optimal concentrations for the maximal insulin response, the effects of various concentrations of insulin on

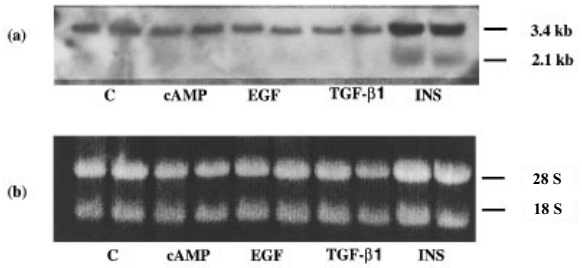

Figure 1 Expression of AdoMetDC mRNA after incubation with various reagents

H4-II-E cells were grown to confluence in standard DMEM containing 10% FBS, then incubated in serum-free DMEM for 16 h. Bt_2cAMP (1 mM), EGF (5 ng/ml), TGF-β1 (10 ng/ml) and insulin (INS; 17.4 nM) was then added to the medium, and after a 6 h incubation period cells were harvested for RNA isolation. Samples of total RNA (25 μg per lane) as duplicates were analysed by Northern blotting (**a**) as described in the Experimental section. Ethidium bromide staining shows a comparable amount of RNA in each lane (**b**). C, control.

Table 1 Effect of Bt_2cAMP, EGF, TGF-β1 and insulin on the amount of AdoMetDC activity and mRNA in H4-II-E cells

To estimate the relative changes in mRNA (2.1 and 3.4 kb), the Northern blots (Figure 1) were scanned with a densitometer and the mRNA level of the control was set at 1. To determine AdoMetDC activity, cells were cultured in the presence or absence of the reagents as described in the legend to Figure 1. AdoMetDC activity (expressed in nmol of CO_2 per 30 min per mg of protein) was assayed as described in the Experimental section. Data are means ± S.D. for the number of experiments shown in parentheses. Statistical significance for the differences between the values for treated cells and control cells: *, $P < 0.001$.

Cell treatment	AdoMetDC activity	mRNA
Control	0.12 ± 0.04 (6)	1.00 (4)
Bt_2cAMP	0.33 ± 0.09* (6)	0.91 ± 0.26 (4)
EGF	0.14 ± 0.06 (6)	0.81 ± 0.41 (4)
TGF-β1	0.11 ± 0.04 (6)	0.63 ± 0.20 (4)
Insulin	0.99 ± 0.08* (6)	3.13 ± 1.04 (4)

Table 2 Dose response of AdoMetDC induction by insulin in H4-II-E cells

H4-II-E cells were grown to confluence in DMEM containing 10% FBS, then incubated in serum-free medium for 16 h. Insulin was added to serum-deprived cells for 4 h at the indicated concentrations. AdoMetDC activity is expressed in nmol of CO_2 per 30 min per mg of protein. Data represent the means ± S.D. for duplicate determinations from four plates of cells. Statistical significance compared with control cells: *, $P < 0.003$; **, $P < 0.001$.

	AdoMetDC activity
Control	0.23 ± 0.08
1.74 nM insulin	0.64 ± 0.15*
17.4 nM insulin	0.96 ± 0.08**
174 nM insulin	0.39 ± 0.23

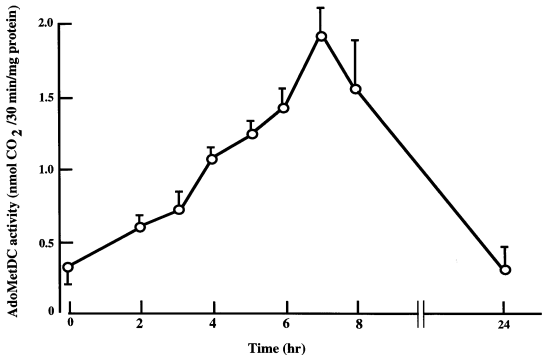

Figure 2 Time course of AdoMetDC induction by insulin in H4-II-E cells

Cells were grown in DMEM containing 10% FBS, then incubated in serum-free medium for 16 h. Insulin (17.4 nM) was added to serum-deprived cells for the indicated times. Each point represents the mean ± S.D. for triplicate experiments.

AdoMetDC activity were investigated (Table 2). Enzyme activity was increased maximally by treatment with 17.4 nM insulin, whereas 174 nM insulin had a slightly inhibitory effect. To determine the optimal incubation time with insulin, the activity of AdoMetDC was assayed at various times after the addition of insulin (Figure 2). The activity gradually increased up to 7 h and decreased thereafter, reaching the basal level within 24 h.

The levels at which insulin controls the activation or inactivation of a number of enzymes include transcription, translation and covalent protein modification [39–42]. To examine whether the effect of insulin on AdoMetDC is mediated at the transcriptional or post-transcriptional level, H4-II-E cells were pretreated with actinomycin D or cycloheximide. As shown in Table 3, insulin (17.4 nM for 7 h) stimulated AdoMetDC activity approx. 4-fold in serum-deprived cells. This induction was abolished by pretreatment with actinomycin D or cycloheximide, indicating that AdoMetDC stimulation is mainly due to the increased transcription of the AdoMetDC gene. The accumulation of AdoMetDC mRNA was also prevented by actinomycin D pretreatment, confirming further that the regulation of the AdoMetDC activity by insulin occurs at the transcriptional level (Figure 3 and Table 3). However, treatment with cycloheximide

Table 3 Effect of cycloheximide and actinomycin D pretreatment on insulin stimulation of AdoMetDC activity and mRNA level in H4-II-E cells

Confluent serum-deprived cells were pretreated with either 4 μM actinomycin D for 15 min or 0.1 mM cycloheximide for 1 h before exposure to insulin (17.4 nM) for 7 h. Results represent the means ± S.D. for four plates individually assayed for AdoMetDC activity (expressed in nmol of CO_2 per 30 min per mg of protein). To estimate the relative changes in mRNA, the Northern blots [eight independent blots, one of which is shown in Figure 3(a)] were scanned with a densitometer and the mRNA level of the control was set at 1. Statistical significance: *, $P < 0.001$ compared with untreated cells; **, $P < 0.001$ compared with insulin-treated cells; ***, $P < 0.003$ compared with cycloheximide-treated cells.

Addition	AdoMetDC activity	mRNA
None	0.32 ± 0.12	1.00
Insulin	1.28 ± 0.21*	2.76 ± 0.78
Actinomycin	0.12 ± 0.06	1.09 ± 0.46
Insulin + actinomycin	0.15 ± 0.04**	0.84 ± 0.35
Cycloheximide	0.06 ± 0.04	5.44 ± 0.47
Insulin + cycloheximide	0.08 ± 0.05**	8.34 ± 1.36**/***

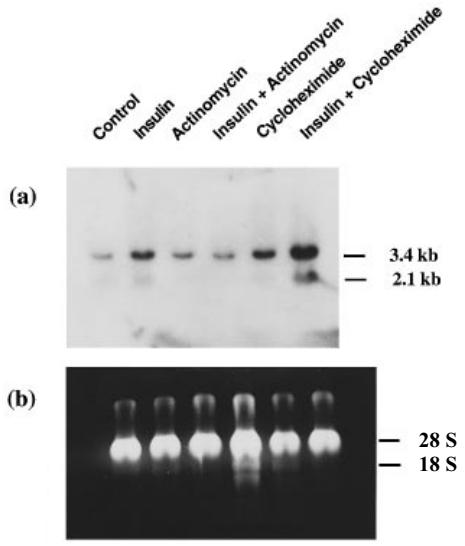

Figure 3 Effect of actinomycin D and cycloheximide on insulin-stimulated AdoMetDC mRNA accumulation

Serum-deprived H4-II-E cells (five plates per condition) were pretreated with either 4 μM actinomycin D for 15 min or 0.1 mM cycloheximide for 1 h before exposure to insulin (17.4 nM) for 7 h. Equal amounts (25 μg) of total RNA isolated from the cells were analysed for AdoMetDC mRNA content by Northern blotting (**a**). Ethidium bromide staining shows a comparable amount of RNA in each lane (**b**).

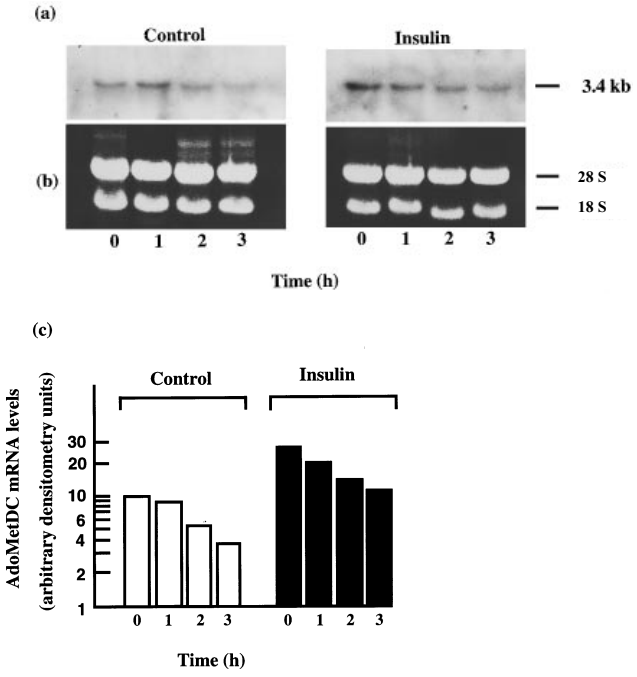

Figure 4 Effect of insulin on the turnover of AdoMetDC mRNA

Confluent serum-deprived H4-II-E cells were pretreated with 17.4 nM insulin for 7 h before exposure to actinomycin D (4 μM). Total RNA was harvested 0, 1, 2 and 3 h after addition of actinomycin D and analysed by Northern blot hybridization (**a**). Ethidium bromide staining shows a comparable amount of RNA in each lane (**b**). The Northern blots were scanned with a densitometer and the mRNA level of the control at time 0 h was set at 10 (**c**).

caused a marked increase in the level of AdoMetDC mRNA. In the absence of insulin, cycloheximide stimulated an approx. 5.5-fold increase in the AdoMetDC mRNA level and insulin treatment further increased the level to approx. 8.5-fold. Thus the effects of insulin and cycloheximide were approximately additive, suggesting that they increase the expression of the AdoMetDC messenger by two separate mechanisms. There are several mechanisms by which cycloheximide could superinduce AdoMetDC mRNA. Superinduction could result from a decrease in a negative regulator of transcription. The inhibition of protein synthesis could decrease the concentration of a labile protein that acts to inhibit expression of the AdoMetDC gene. A decrease in this putative inhibitory protein would increase the rate of transcription. In this case, AdoMetDC gene expression would reflect a balance between positive and negative regulation of transcription. A second general model for superinduction postulates that the degradation of AdoMetDC mRNA is dependent on a labile protein and the inhibition of protein synthesis stabilizes the mRNA.

To confirm that insulin induction of AdoMetDC activity is mediated by an increase in transcription and not by stabilization of the mRNA, the half-life of the mRNA was assayed in insulin-treated and non-treated hepatoma cells (Figure 4). In both cases the half-life remained the same, 1.7 h, indicating that insulin regulation of AdoMetDC activity occurs at the transcriptional level.

To localize the promoter region necessary for the insulin response, chimaeric constructs containing 337 or 669 bp of the 5′-flanking region of the AMD1B gene were ligated to the CAT reporter gene and transfected into H4-II-E cells (Table 4). Treatment with insulin increased CAT activity approx. 1.5-fold

Table 4 Insulin modulation of CAT activity in transiently transfected H4-II-E cells

H4-II-E cells were co-transfected with 3 μg of pTKGH-plasmid and 13 μg of pCAT-Control, pCAT-669 or pCAT-337 constructs using the Lipofectamine reagent as described in the Experimental section. Transfected cells were preincubated for 16 h in serum-free medium, then grown in the presence (17.4 nM) or absence of insulin for 6 h. The cells were then harvested and assayed for CAT activity and the level of secreted growth hormone in the medium. The ratio of CAT activity to growth hormone level served as a measure for normalized CAT activity. The changes in CAT activity are presented. Values are means $\pm$ S.E.M. for the numbers of separate experiments indicated in parentheses. Statistical significance: *, $P < 0.01$ compared with pCAT-669; **, $P < 0.001$ compared with pCAT-Control; †, no significant difference from pCAT-Control.

Plasmid construct	Change in CAT activity (fold)
pCAT-Control	1.01 $\pm$ 0.07 (15)
pCAT-337	1.49 $\pm$ 0.08*/** (13)
pCAT-669	1.14 $\pm$ 0.10† (13)

in rat hepatoma cells transfected with the pCAT-337 plasmids but did not change CAT activity in cells transfected with pCAT-669 plasmids. This may be due to the presence of repressor elements in the extended promoter region. Taken together, these data show that insulin induction of AdoMetDC activity occurs at the transcriptional level and that the promoter region extending 337 bp from the transcription start site contains elements that can increase AMD1B gene transcription in response to insulin. This region, however, does not contain any of the insulin response elements characterized so far [27,28,43,44]. Precise identification of the promoter elements and their binding factors remains to be made. Ongoing studies analysing proteins binding to the insulin-responsive promoter region of the AMD1B gene may ultimately lead to the identification of the insulin response factor involved in AdoMetDC induction by the hormone.

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