Post-transcriptional regulation of the content of spermidine/spermine *N*¹-acetyltransferase by *N*¹*N*¹²-bis(ethyl)spermine

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Spermidine/spermine *N*¹-acetyltransferase (SSAT) is the rate-limiting enzyme for the degradation and excretion of polyamines in mammalian cells, and its activity is known to be increased enormously on exposure to polyamines and polyamine analogues. The mechanism by which such an analogue, BESM [*N*¹*N*¹²-bis(ethyl)spermine], increases the content of SSAT was investigated by transfecting COS-7 cells with plasmids containing SSAT cDNA in the pEUK expression vector. Despite a large increase in mRNA production, there was only a very small increase in SSAT activity in the transfected cells. When BESM was added at 36 h after transfection, there was a large and very rapid increase in SSAT protein amounting to 380-fold in 12 h without any increase in the mRNA. SSAT protein turned over very rapidly, with a half-life of about 20 min. In the presence of BESM, this turnover was greatly reduced, and the half-life increased to more than 13 h. However, this increase was not sufficient to account for all of the increase in SSAT protein,

suggesting that there is also regulation of the translation of the mRNA by BESM. Further evidence for such translation regulation was obtained by studying the polysomal distribution of the SSAT mRNA. In the absence of BESM, most of the mRNA was present in fractions which sedimented more slowly than the monoribosome peak. In BESM-treated cells, a significant proportion of the SSAT mRNA was moved into the small-polysome region of the gradient. The expression of SSAT and the effects of BESM on the polysomal distribution of SSAT mRNA were not affected by the 5'- or 3'-untranslated regions of the mRNA, since constructs which lacked all of these regions gave similar results to constructs containing the entire mRNA sequence. These results show that the increased transcription of the SSAT gene that occurs in the presence of polyamine analogues such as BESM is not sufficient for SSAT expression and that post-transcriptional regulation is critical for the control of SSAT content.

INTRODUCTION

Polyamine levels in mammalian cells are regulated not only by changes in the rate of biosynthesis but also by excretion and degradation [1–4]. Spermidine/spermine *N*¹-acetyltransferase (SSAT) plays a key role in these processes. Its products, *N*¹-acetylspermidine and *N*¹-acetylspermine, are rapidly acted upon by polyamine oxidase and converted into putrescine. Both putrescine and *N*¹-acetylspermidine are excreted from the cell. Therefore induction of SSAT leads to a rapid loss of polyamines. SSAT is induced by many hormones, drugs and physiological stimuli (reviewed in [5,6]). Polyamines themselves are good inducers of SSAT [7,8], and it has been suggested that a common feature of the myriad of inducers is a rise in the free intracellular polyamine level [3]. Certain polyamine analogues, including *N*¹*N*¹²-bis(ethyl)spermine (BESM) and *N*¹*N*¹¹-bis(ethyl)norspermine (BENSM), are extremely powerful inducers of SSAT and increase the activity by more than 100-fold within a few hours [9–15]. Treatment of tumour cells with these inducers leads to an inhibition of growth and cell death, and these analogues are currently undergoing trials as antitumour agents [16–19].

The mechanism of induction of SSAT by polyamines and by analogues is not well understood. Studies with specific antibodies to SSAT have shown that the increased enzyme activity is

brought about by an increase in protein [11,20]. An increased mRNA content was found in cells treated with BESM, leading to suggestions that polyamines regulate SSAT transcriptionally; however, in some cases the rise in SSAT mRNA was less than the increase in protein [8,13,21–24]. SSAT has a very short half-life [7,20,25], and stabilization of the protein by binding to the analogues could also contribute to increasing the content of the protein [11,12,26].

In the experiments presented here we have inserted the SSAT cDNA into a mammalian cell expression vector containing the simian-virus-40 (SV40) late promoter and the SV40 origin of replication and have studied the effects of BESM on the production and content of SSAT in COS-7 cells. The results show that expression of SSAT mRNA alone has little effect on the content of SSAT protein. Addition of BESM to cells containing SSAT mRNA rapidly led to a large accumulation of SSAT protein. Addition of BESM stimulated the translation of SSAT mRNA and also greatly retarded the degradation of the SSAT protein. The combination of the effects on translation and protein stabilization led to a several-hundred-fold increase in SSAT within 12 h without any increase in mRNA in the transfected COS-7 cells. These results indicate that the increased transcription of SSAT mRNA occurring in cells exposed to BESM and related compounds may be necessary for SSAT

Abbreviations used: SSAT, spermidine/spermine *N*¹-acetyltransferase; BESM, *N*¹*N*¹²-bis(ethyl)spermine; BENSM, *N*¹*N*¹¹-bis(ethyl)norspermine; UTR, untranslated region; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40; CHO, Chinese-hamster ovary; MEM, minimal essential medium; G418, geneticin.

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expression, but is not sufficient for this to occur and emphasize the critical nature of post-transcriptional regulation of SSAT activity.

EXPERIMENTAL

Materials

[1-¹⁴C]Acetyl-CoA (55 Ci/mol) was purchased from ICN Biochemicals, Costa Mesa, CA, U.S.A. Geneticin (G418) and α -minimal essential medium (α -MEM) were purchased from Gibco, Grand Island, NY, U.S.A. The RNA isolation kit (no. 200345), Duralose-UV nitrocellulose and Bluescript vector were from Stratagene, La Jolla, CA, U.S.A. The nucleic acid detection kit (Genius 3, no. 1175 041) was from Boehringer Mannheim Corp., Indianapolis, IN, U.S.A. Poly(vinylidene difluoride) membrane (0.22 μ m thickness) filter units were purchased from Millipore Corp, Bedford, MA, U.S.A. Plasmid pHbAPr-1-neo [27] was generously provided by Dr. L. Kedes, Department of Biochemistry, University of Southern California, Pasadena, CA, U.S.A. Plasmids pEUKC1 [29] and pMAMneo [30] were purchased from Clontech, Palo Alto, CA, U.S.A.

Plasmid constructions

Plasmids pAP3/F7, pSATH1 and pSAT9.3, containing the SSAT cDNA in the Bluescript vector [22,23], were used to isolate SSAT cDNA fragments. For stable transfections, two constructions were used. For one, pSATH1 was digested with *Sa*I and *Bam*HI, and the resulting 972-bp fragment was inserted into pHbAPr-1-neo [27], cut with the same enzymes to produce pHbAPr-SSAT. For the second, pSAT9.3 was digested with *Xho*I and *Xba*I, and the resulting 784-bp fragment was inserted into the pMAMneo-Blue vector cut with the same enzymes.

For transient transfections, fragments from the Bluescript plasmids containing SSAT cDNA were inserted into pEUKC1 to form pEUKSAT1-4. The pEUKC1 vector was cut with *Xho*I and *Bam*HI, and the fragments containing SSAT cDNA isolated from pSATAP3/F7, pSATH1 and pSAT9.3 digested with the same enzymes were inserted to generate pEUKSAT1, pEUKSAT2 and pEUKSAT3 respectively. Plasmid pEUKSAT4 was constructed using an insert isolated by PCR. A sense primer corresponding to the T7 promoter region of the vector and a 27-base antisense primer (3'-CCTCACTCCTCCCTAGGACATCTACTG-5') complimentary to bases 510-541 (containing three mismatches, shown in **bold** type) was used for PCR extension of pSATH1 to introduce a *Bam*HI site five bases 3' of the termination codon sequence. The PCR product was digested with *Xho*I and *Bam*HI, and the resulting 545-bp fragment was ligated into the 4.9 kb fragment obtained from the digestion of pEUKSAT2 with the same enzymes.

Assay of SSAT activity

SAT activity was measured by assaying the conversion of [1-¹⁴C]acetyl CoA into [1-¹⁴C]acetylspermidine [30] using an assay medium containing 50 mM Tris/HCl, pH 7.8, 3 mM spermidine and 15 μ M [1-¹⁴C]acetyl-CoA (53 μ Ci/ μ mol) in a total volume of 0.1 ml and incubating for 10 min at 30 °C. Results were expressed as nmol of acetylspermidine produced/min per mg of protein extract used. Protein was determined by the method of Bradford [31], with BSA as a standard.

Cell culture and transfection

Chinese-hamster ovary (CHO) cells were grown in monolayers in

α -MEM containing 10 % fetal-bovine serum, 20 mM NaHCO₃ and 12 μ g/ml each of penicillin and streptomycin. Cells were seeded at 8×10^5 cells/100 mm dish for experimentation. COS-7 cells were grown in monolayer in Dulbecco's MEM supplemented with 10 % fetal-bovine serum, 20 mM NaHCO₃, 12 μ g/ml each of penicillin and streptomycin plus 2 mM glutamate. For experimentation, cells were seeded at $(2-2.5) \times 10^5$ cells/60 mm dish in 5 ml of medium. Both cell lines were kept under 5 % CO₂ with 95 % relative humidity at 37 °C and passaged once weekly.

The calcium phosphate method for transfection was used as described by Gorman [32]. Cells were plated at their respective densities and grown overnight. On the second day, medium was replaced 3 h before transfection. Prior to addition to cells, 10-40 μ g of DNA was precipitated with calcium in a Hepes-buffered solution (21 mM Hepes/138 mM NaCl/5 mM KCl/1.5 mM Na₂HPO₄/5.5 mM dextrose, pH 7.12), containing 0.125 mM CaCl₂. The calcium phosphate-DNA precipitate was added to cells 3 h after the addition of fresh medium. For transient transfections, cells received a shock at 4 h after transfection using 15 % glycerol for 3 min. During experimentation, drug additions were made either 1 h prior to transfection or 36 h after transfection as specified. Cells were harvested at 48 h after transfection unless stated otherwise. Stable transformants were identified by survival following successive additions of 800 μ g/ml G418 at days 4, 7, 10 and 13 after transfection. Surviving cells were diluted twice for the isolation of colonies derived from single cells. After 30 days, cells were used for experimentation. Drug additions were made at 24 h after plating, and cells were harvested 24-96 h later.

Determination of SSAT mRNA

Total RNA was prepared from cells using the guanidinium thiocyanate method [33] and the RNA isolation kit (Stratagene no. 200345) according to the manufacturer's instructions. For Northern blots, the RNA was suspended in sample buffer (formamide/formaldehyde/sodium phosphate buffer, 76:21:3, by vol.), heated to 55 °C for 15 min, and then immediately placed on ice. A small volume of loading buffer [40 % (w/v) sucrose/60 mM EDTA/0.25 % Bromophenol Blue] plus 1 mg/ml ethidium bromide was added, and 10 μ g per lane of RNA was loaded on to a 0.66 M formaldehyde/5.25 % acrylamide gel. Gels were run for 5 h at 100 V. RNA from the gel was transferred on to wet Duralose-UV membrane by electroblotting at 10 V overnight, followed by an additional 3.5 h at 30 V. RNA was u.v.-cross-linked to the membrane using a Stratalinker and detected with antisense RNA probes as described below. For the determination of SSAT mRNA using slot blots, RNA samples (0.1-2.0 μ g) was diluted in 30 % 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 20 % formaldehyde and heat-treated for 10 min at 65 °C, followed by 10 min on ice. RNA was loaded on to a slot-blot apparatus holding dry Duralose-UV membrane, and a vacuum was applied. Wells were washed once with 10 $\times$ SSC. RNA was then u.v.-cross-linked using the Stratalinker and detected with an antisense RNA probes as described below.

Digoxigenin-conjugated non-radioactive sense and antisense RNA probes were prepared as follows using Bluescript plasmids containing cDNA inserts SSAT, actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [15,22]. Linearized DNA was incubated with a NTP mixture containing 10 mM CTP, GTP and ATP, 6.5 mM UTP and 3.5 mM digoxigenin-conjugated UTP in the presence of T7 or SP6 RNA polymerase for 2 h at 37 °C. The reaction was terminated by heating to 65 °C for 10 min. RNA was precipitated at -20 °C with 0.1 vol. of 4 M

LiCl and 3 vol. of cold ethanol. RNA was dissolved in RNAase-free sterile water, its content measured spectrophotometrically, and the solution stored frozen at -80°C .

For RNA detection using these probes, membranes were prehybridized with a solution of 50% formamide, $5\times\text{SSC}$, 0.1% *N*-dodecanoylsarcosine, 0.02% SDS and 2% blocking reagent for 1 h at 68°C in a rotating hybridization incubator. Hybridization was performed in the same solution at 68°C overnight using 100 ng/ml heat-denatured non-radioactive anti-sense RNA probe. Membranes were washed twice each with $2\times\text{SSC}$ and 0.1% SDS at room temperature and then $0.1\times\text{SSC}$ and 0.1% SDS at 68°C . Membranes were then equilibrated at room temperature in a buffer containing 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, and blocked in a 1% blocking solution (Boehringer Mannheim Genius kit 3) prepared in the same buffer. Alkaline-phosphatase-conjugated anti-digoxigenin antibody was added for 30 min. The membranes were washed, and a colorimetric reaction was performed using 75 mg/ml Nitroblue Tetrazolium salt plus 50 mg/ml 5-bromo-4-chloroindol-3-yl phosphate toluidinium salt in 70% (w/v) dimethylformamide. The reaction was allowed to proceed without shaking and in complete darkness for approx. 2 h and was terminated using a solution of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA. Wet membranes were scanned, and band densities were determined densitometrically. The equivalent sense probe was used to confirm the absence of any signal from the plasmid DNA contaminating the RNA preparation.

Polysomal profile analysis

Analysis of mRNA distribution in polysomes [34] was carried out using a published procedure [35]. Prior to harvesting, cells were treated for 5 min at 37°C with 0.1 mg/ml cycloheximide. All harvesting solutions contained 0.1 mg/ml cycloheximide. Cells were washed with PBS (0.14 M NaCl/2.7 mM KCl/10 mM Na_2HPO_4 /1.8 mM KH_2PO_4 , pH 7.0) and detached from the plate using 0.25% trypsin solution at 37°C . PBS containing 1 mM phenylmethanesulphonyl fluoride at 4°C was then added to inhibit trypsin. Cells were pelleted by centrifugation at 700 *g* for 10 min at 4°C , and cytoplasmic extracts were prepared by treatment in a Dounce homogenizer with low-salt buffer [20 mM Tris/HCl (pH 7.4)/10 mM NaCl/3 mM MgCl_2]. The extract was centrifuged immediately at 27000 *g* for 30 s to remove nuclei, and 10 mg/ml heparin and 1.5 M NaCl were added to the supernatant. Extracts were applied to a 0.5–2.0 M linear sucrose gradient and centrifuged for 115 min at 36000 rev./min at 4°C using a Beckman SW41 rotor. Gradients were fractionated and the absorbance of fractions was monitored at 254 nm. Fractions (0.5 ml each) were collected, and total RNA was isolated from each fraction as follows. Samples were incubated with 0.5% SDS and 0.1 mg/ml proteinase K for 30 min at 37°C . Protein was extracted twice with an equal volume of phenol/chloroform (1:1, v/v), followed by an extraction with an equal volume of chloroform. The RNA was precipitated with 20 μg /ml glycogen and 2.5 vol. of ethanol at -20°C overnight and analysed for SSAT, actin and GAPDH mRNAs as described above.

RESULTS

Attempts to express SSAT in CHO cells by stable transfection were unsuccessful. The SSAT cDNA was inserted into the constitutive expression vector pHbApr-1-neo or the glucocorticoid-inducible vector pMAMneo. These plasmids, which both also express the neomycin-resistance gene, were used to transfect CHO cells, and clones of cells that had incorporated the

plasmid DNA were selected using G418. None of the 14 clones from cells transfected with the SSAT in pHbApr-1-neo expressed SSAT activity above control levels, despite the presence of plasmid-derived SSAT mRNA. (The extent to which the mRNA was increased was not determined accurately, since the basal level of SSAT mRNA in the non-transfected CHO cells was barely detectable in the absence of BESM.) When these cells containing pHbApr-1-neo with an SSAT insert were treated with 5 μM BESM, SSAT activity rose by more than 100-fold, but a similar rise was obtained in the control CHO clones selected from cells transfected with the vector plasmid alone. SSAT cDNA inserted into the pMAMneo vector was used to test whether the reason for these negative results was that constitutive expression of SSAT was detrimental to the cell. CHO cells were transfected with this plasmid, and 23 neomycin-resistant colonies were isolated, grown up and exposed to 1 mM dexamethasone. There was no detectable increase in SSAT expression in any of these clones.

These results suggested that the increase in SSAT levels by BESM may not be due solely to increased transcription of the SSAT gene. In order to obtain more information on the factors needed for SSAT expression, a transient system using COS-7 cells was used. The basal level of SSAT in COS-7 cells is comparable with that in CHO cells, and SSAT can be induced with a 24 h exposure to BESM in COS-7 cells to values about half of those seen in CHO cells. Various cDNAs for SSAT that all contained the coding sequence, but differed in the amount of 5'- and 3'-untranslated regions (UTRs), were cloned into the pEUKC-1 vector and used to transfect the COS-7 cells.

In the first experiments, COS-7 cells were treated with BESM or spermidine or both. After 1 h the cells were transfected with pEUKSAT1 or pEUKSAT2, and SSAT activity was determined after 48 h (Table 1). There was little, if any, increase in SSAT activity in response to the pEUKSAT plasmids alone or given with spermidine. Cells treated with BESM alone showed a large rise in the basal level of SSAT. There was a slightly higher SSAT activity in those also receiving the pEUKSAT plasmids, but the extent of increase was hard to estimate, because of the high SSAT activity in the controls. However, in cells treated with both spermidine and BESM, the endogenous SSAT activity was increased only slightly, but there was a much larger increase in the SSAT activity in those cells also receiving the pEUKSAT plasmids (Table 1).

The exogenous spermidine was given in these experiments to compensate for the possible loss of polyamines in response to BESM treatment, since such loss might affect the replication of plasmid DNA and synthesis of RNA and protein needed for expression. However, measurement of the intracellular polyamines indicated that there was little loss of polyamines under these conditions, and spermidine produced only 30% rise in spermidine and an equivalent loss in spermine. However, the uptake of BESM was decreased 5-fold by the addition of spermidine. This is in agreement with published studies showing that BESM competes with spermidine for the spermidine-transport carrier [17,36,37]. The protocol was therefore changed to reduce the time period of exposure to BESM and test whether spermidine addition was needed (Table 2 and Figure 1). As shown in Table 2, a 12 h exposure to 10 μM BESM given at 36 h after transfection led to a more-than-100-fold increase in SSAT activity derived from pEUKSAT2 and increased the endogenous SSAT less than 3-fold. Co-addition of spermidine with the BESM reduced the increase in plasmid-derived activity slightly, and spermidine was not used in subsequent experiments.

The time course for SSAT activity in COS-7 cells transfected with pEUKSAT1 or mock-transfected, treated with BESM 36 h

Table 1 Effect of BESM and spermidine on SSAT activity in COS-7 cells transfected with pEUKSAT

COS-7 cells were treated with 5 μ M BESM or 20 μ M spermidine 1 h prior to transfection with no plasmid, pEUKSAT1 or pEUKSAT2 as indicated. Cells were harvested at 48 h after transfection and SSAT activity was measured. Results are means $\pm$ S.D.

Addition	SSAT activity (nmol/min per mg) in cells transfected with:		
	No plasmid	pEUKSAT 2	pEUKSAT1
None	0.2 $\pm$ 0.04	0.3 $\pm$ 0.04	0.2 $\pm$ 0.03
20 μ M Spermidine	0.3 $\pm$ 0.01	0.6 $\pm$ 0.1	0.4 $\pm$ 0.05
5 μ M BESM	21.1 $\pm$ 4.3	23.4 $\pm$ 2.6	28.9 $\pm$ 3.9
5 μ M BESM + 20 μ M spermidine	0.8 $\pm$ 0.1	12.0 $\pm$ 2.3	8.7 $\pm$ 0.9

Table 2 Effect of BESM on SSAT activity in COS-7 cells transfected with pEUKSAT

COS-7 cells were transfected with pEUKSAT2 or mock-transfected (with no DNA) and 36 h later, 10 μ M BESM $\pm$ 20 μ M spermidine was added as shown. Cells were harvested 12 h later and SSAT activity was measured. Results are the means of duplicate samples which agreed within $\pm$ 10%.

Addition	SSAT activity (nmol/min per mg)		
	Activity in cells transfected with:		Increase due to plasmid (nmol/min per mg)
	No plasmid	pEUKSAT2	
None	0.6	0.7	0.1
10 μ M BESM	1.7	30.9	29.2
10 μ M BESM + 20 μ M spermidine	0.8	19.3	18.5

Figure 1 Time course of induction of SSAT activity by BESM in COS-7 cells transfected with pEUKSAT1

Control (no DNA) (circles) and pEUKSAT3 (squares) transfected COS-7 cells were exposed to 0 (open symbols) or 10 μ M BESM (filled symbols) 36 h later. Acetyltransferase activity (main Figure) and immunoprecipitable SAT activity (inset) was measured at times indicated following the addition of drug.

later and monitored for a further 24 h is shown in Figure 1. There was no significant increase in SSAT in response to pEUKSAT1 alone, but when 10 μ M BESM was added to these cells, there was a very rapid rise in SSAT which peaked 12 h later. In contrast, the SSAT activity in the mock-transfected cells increased much more slowly and to a much lesser extent.

The assay used for SSAT in these experiments is non-specific, measuring the spermidine-dependent incorporation of label from

[14 C]acetyl-CoA into material that binds to cellulose phosphate. The measured values are therefore likely to include contributions from other acetyltransferases and non-enzymic acetylation of spermidine. These components contribute much more to the low basal activity, and the specific induction of SSAT is therefore underestimated in these measurements. In order to determine more accurately the specific induction of SSAT, cell extracts were immunoprecipitated with an antibody to SSAT [20]. The results (shown in the inset to Figure 1) indicated that, within 3 h of exposure to BESM, greater than 95% of the activity in the pEUKSAT1-treated cells was precipitable with antibody, whereas less than 15% was precipitable in the cells not treated with drug. When these values were used to correct the measured activities, it was calculated that, after 12 h exposure to BESM, cells transfected with pEUKSAT1 showed a 400-fold increase in true SSAT, with less than 5% representing endogenous (non-plasmid derived) activity. After 24 h of exposure, the increase was 800-fold, of which 20% was endogenous activity. Therefore, the actual rise in plasmid-derived SSAT activity was approx. 380-fold at 12 h and 600-fold at 24 h after addition of BESM.

Since the transcription of RNA from the pEUKSAT plasmids is driven from an SV40 promoter, it was unlikely that the addition of BESM would affect transcription of the SSAT mRNA, but it was possible that some effect on RNA processing or stabilization contributed to the large increase in SSAT expression. However, the content of mRNA measured at 12 h after BESM was added actually declined slightly in response to BESM, even though there was the expected large increase in SSAT activity (Table 3). A time-course study of the content of SSAT mRNA in cells transfected with pEUKSAT1 showed that the RNA content declined over the period from 42 to 60 h after transfection, and this decline was greater when BESM was present (results not shown).

Table 3 Effect of BESM on SSAT activity and mRNA content in COS-7 cells transfected with pEUKSAT

COS-7 cells were treated as described in the legend to Figure 2 and harvested 12 h after addition of BESM for the determination of SSAT activity or SSAT mRNA content. The levels of SSAT-specific RNA were determined by Northern-blot analysis using an antisense RNA probe. Results are the means of triplicate estimations. Abbreviation: ND, not detected.

Plasmid used	SSAT activity (nmol/min per mg)		SSAT mRNA (densitometer units)	
	No addition	+ 10 μ M BESM	No addition	+ 10 μ M BESM
None	0.5	0.6	ND ^a	ND
pEUKSAT2	0.3	29.4	1.7	1
pEUKSAT4	0.7	33.4	2.7	1

Table 4 Effect of the 5'- and 3'-UTR on the expression of SSAT activity in COS-7 cells

COS-7 cells were transfected as shown, and 10 μ M BESM was added where indicated 36 h later. Cells were harvested 12 h later and SSAT activity measured. Results are the mean $\pm$ S.D. or the means of duplicate samples.

Plasmid	Length of SSAT cDNA sequences		SSAT activity (nmol/min per mg of protein)	
	5'-UTR	3'-UTR	No addition	+ 10 μ M BESM
None	—	—	0.3 $\pm$ 0.02	1 $\pm$ 0.1
pEUKSAT1	165	383	0.5 $\pm$ 0.05	23 $\pm$ 1.9
pEUKSAT2	5	148	0.6	18
pEUKSAT3	145	123	0.6	17
pEUK SAT4	5	5	0.4 $\pm$ 0.05	24 $\pm$ 2.3

It can be seen from the results in Tables 1–3 and Figure 1 that the constructs pEUKSAT1, pEUKSAT2 and pEUKSAT3 all gave rise to a high level of SSAT expression when BESM was added. These constructs differ in the length of the 5'- and 3'-UTRs, and pEUKSAT2 has virtually all of the 5'-UTR deleted (Table 4). This suggests that the synthesis of SSAT is not regulated by BESM by interactions with the 5'-UTR. In order to determine if the 3'-UTR is involved in BESM-dependent regulation of SSAT, pEUKSAT4, which lacks all but five bases on either side of the 513-base coding region, was constructed. As shown in Table 4, there was no difference between the response of this plasmid to BESM and that of pEUKSAT4, which contains the entire cDNA with both the complete 5'- and 3'-UTRs. Northern blots (not shown) of SSAT mRNA isolated from cells treated with the various pEUKSATs containing different amounts of the 5'- and 3'-ends confirmed that the transcriptional products were of the expected size and that BESM treatment did not increase the SSAT mRNA content.

These results show clearly that SSAT induction by BESM occurs by either translational or post-translational mechanisms. Since polyamines and analogues have been reported to stabilize SSAT, this possibility was investigated in cells transfected with pEUKSAT3 by measuring the decline of SSAT activity after protein synthesis was stopped with cycloheximide. Because much of the acetylase activity in the cells that did not receive BESM was not SSAT (Figure 1), immunoprecipitable SSAT was measured in this experiment. In the absence of BESM, the half-life of SSAT was approx. 20 $\pm$ 5 min, and in the presence of BESM this value was increased substantially to about 13 h (Figure 2). This half-life is too long to be estimated accurately over the 6 h period in which the cells could be kept in cycloheximide without toxic effects, but it is clear that SSAT protein is appreciably stabilized by BESM. Although the value could not be determined precisely, the best estimate is that it amounted to about a 40-fold increase in stability. However, this stabilization

Figure 2 Effect of BESM on the half-life of SSAT in transfected COS-7 cells

The COS-7 cells were transfected with pEUKSAT3 treated with 0 or 10 μ M BESM 36 h later. After a further 12 h, cycloheximide (50 μ g/ml) was added and samples removed at the time shown and the immunoprecipitable SSAT activity determined. The results were then expressed as a percentage of the SSAT activity at 12 h. Results are shown as means $\pm$ S.D. for three to six estimations.

cannot account for the entire increase in SSAT in response to BESM. Analysis using the computer program model Stella II k_s/k_d [38] was used to determine the extent to which the observed 380-fold in SSAT activity could be accounted for by stabilization

Figure 3 Calculation of relationship between SSAT stability and synthesis rate to account for increased activity

The increase in SSAT synthesis rate needed to obtain a 380-fold increase in protein over 12 h was calculated using the program Stella II k_s/k_d [38] for a range of increases in SSAT stability from 40 to 10^6 -fold.

Figure 4 Effect of BESM on distribution of SSAT, GAPDH and actin mRNAs

COS-7 cells were transfected with pEUKSAT3 (panels a–c) or pEUKSAT4 (d and e) and 10 μ M BESM added after 36 h (open circles) or no addition made (filled circles). At 48 h, cells were harvested, and lysed and the polysomes were separated by sucrose gradient centrifugation. Ten fractions were collected from the experiment with pEUKSAT3 and 12 fractions from the experiment with pEUKSAT4. The 80 S monosome peak was present in fraction 3 (a–c) or fraction 4 (d and e). The content of SSAT mRNA (a and d), GAPDH mRNA (b and e) and actin mRNA (c) was then measured in each fraction.

of the protein (Figure 3). With a 40-fold increase in stability, the rate of synthesis would need to be increased by 20-fold. Even if the stabilization is much greater than this, a 15-fold increase in synthesis would be needed. These results provide strong evidence that the translation of SSAT mRNA is increased dramatically by BESM.

More direct evidence for an effect on mRNA translation was obtained by studying the polysomal distribution of the SSAT mRNA. Cell lysates were prepared from COS-7 cells transfected with pEUKSAT1 or pEUKSAT4 and then exposed to BESM or no addition. The lysates were then separated on ribonuclease-

free linear sucrose gradients. RNA was extracted from individual fractions from these gradients and the content of SSAT mRNA determined (Figure 4). The content of actin and GAPDH mRNA was also measured. (The sensitivity of SSAT mRNA detection was not sufficient for the measurement of SSAT mRNA in the non-transfected COS-7 cells.) The distribution of GAPDH and actin mRNA was not affected by transfection.

In the absence of BESM, most of the SSAT mRNA was found near the top of the gradient in fractions sedimenting more slowly than the monosome peak. On treatment with BESM, there was a shift of the SSAT mRNA into the polysome fractions. The distribution of GAPDH and actin mRNAs were not altered by BESM, and these mRNAs were present in the polysome fractions in all cases. The size of the polysomes containing SSAT mRNA was quite small, showing a peak corresponding to monomers and dimers even in the presence of BESM. BESM caused a similar shift in the distribution of SSAT mRNA, even when it was derived from pEUKSAT4, which contains only five bases from each of the 5'-UTR and the 3'-UTR. This indicates that only the coding region of the mRNA is required for the polysomal distribution of SSAT mRNA to be influenced by BESM.

DISCUSSION

Mammalian SSAT is a tetramer with four identical subunits of M_r 20000. There is no evidence for any post-translational modifications of the protein that alter activity [6]. The probable absence of such modifications is supported by the fact that the recombinant human SSAT expressed in *Escherichia coli* can be purified in good yield to a specific activity similar to that of SSAT isolated from H157 lung tumour cells (L. Parry, J. Lopez-Ballister, L. Wiest and A. E. Pegg, unpublished work). SSAT activity appears to be regulated by changes in the amount of enzyme protein [20,21,39]. SSAT is one of the most inducible proteins present in mammalian cells. Increases in SSAT activity in certain tumour cells in response to the most powerful inducers such as BESM and BENSM can amount to more than 1000-fold within a 24–48 h period [9,11–13,19].

An increased content of SSAT mRNA has been observed in many human tumour cell lines after treatment with BESM, BENSM and related compounds [8,11–15,22–24]. The increased transcription of the SSAT gene in response to BESM or BENSM may be necessary for part of the increase in synthesis, but the present experiments show that an increase in SSAT mRNA is not sufficient for this purpose. Although all cell lines examined respond to the bis(ethyl)polyamines with a rise in SSAT, the extent of this rise varies strikingly between cell lines. It remains to be determined to what extent alterations in post-transcriptional steps rather than alterations in transcription account for this cell specificity in SSAT induction. This is an important point, since the ability of BESM and BENSM to kill tumour cells may be related to the increased level of SSAT [6,9,12,13,15,19,40]. The prior determination of tumour sensitivity and SSAT inducibility and the use of conditions maximizing sensitivity may be essential for the successful clinical use of these agents.

Our results indicate that the translation of SSAT mRNA derived from the pEUK vector requires BESM. Although the transcribed mRNA contains sequences derived from the vector, it is inherently unlikely that these would contribute to alterations in expression in response to BESM that resemble those occurring physiologically. Furthermore, the expression of luciferase from constructs in the pEUK vector was not altered by BESM, and the fraction of ornithine decarboxylase or *S*-adenosylmethionine decarboxylase mRNAs expressed from this vector that were

present in polysomes was not altered by BESM (L. M. Shantz and A. E. Pegg, unpublished work).

The change in polysomal distribution of SSAT mRNA after addition of BESM provides convincing evidence for its translational regulation. In the absence of BESM, the SSAT mRNA sedimented more slowly than the 80 S monosome peak, indicating that it was not associated with the protein-synthetic machinery. After addition of BESM, a significant proportion of the SSAT mRNA was associated with polysomes. The size of these polysomes was, however, quite small, even for a protein of the size of SSAT. On the basis of the absorbance profile of the gradients and the location of actin mRNA, which is known to be associated with pentasomes [34], the SSAT mRNA is mainly associated with mono- and di-meric polysomes. This finding suggests that initiation or translation of the region at the 5' end of the mRNA is very slow compared with the elongation.

Thus it appears that SSAT is one of a growing number of eukaryotic proteins whose synthesis is strongly regulated at the level of translation. Several mechanisms have been identified for translational regulation, including the presence of extensive secondary structure in the 5'-UTR, the presence of short open reading frames in the 5'-UTR, alterations in phosphorylation status and activity of initiation factors and the binding of specific proteins to the mRNA [41–48]. Some evidence exists indicating that sequences in the 3'-UTR of certain mRNAs may also be involved in translational regulation [49,50]. However, our results suggest that neither the 5'- nor the 3'-UTRs are involved in the regulation of SSAT mRNA translation. The finding that the 5'-UTR of SSAT is not involved in its translational regulation is quite surprising, since this region is extremely well conserved in mammalian SSAT sequences, with 97% identity between the hamster and the human cDNAs [6,51]. However, deletion of virtually all of the 5'-UTR and 3'-UTR derived from the SSAT cDNA did not allow translation in the absence of BESM, ruling out the possibility that any secondary structure or open reading frame in the regions of the mRNA 5' to the initiation codon are responsible for the inhibition. Furthermore, the SSAT mRNA is translated reasonably effectively in reticulocyte lysates [11,22,51] and SSAT is readily expressed in *E. coli* when cloned into the pIN-III vector (L. Parry, J. Lopez-Ballister, L. Wiest and A. E. Pegg, unpublished work). Both in reticulocyte lysates and in *E. coli*, BESM has little if any direct effect on SSAT synthesis [51]. Although a theoretical analysis of secondary structure of the SSAT mRNA revealed a loop structure with 60% base-pairing formed by the first 76 bases of the coding region, the free energy associated with this structure was only -117 kJ (-28 kcal)/mol. This structure would be insufficient to block translation unless it was close to the message cap site, and even very stable structures [-250 kJ (-60 kcal/mol)] do not block translation when the loop is 3' of the initiating AUG codon [52].

These considerations render it unlikely that the effects of BESM on SSAT translation are mediated via a polyamine analogue directly altering the secondary structure present in the mRNA. A more likely explanation is that a protein inhibitory to SSAT synthesis is present in the cells and that BESM acts to relieve this inhibition. This type of regulation occurs with ferritin mRNA, but the sequence interacting with the repressor protein is in the 5'-UTR ([47,48] and references cited therein). In the case of SSAT, it appears that the key regulatory sequence, if it exists, must be in the coding region. Attempts to identify this sequence and modify it to allow SSAT expression in the absence of BESM or other polyamine inducers are in progress. Another possibility is that part of the N-terminus of the newly synthesized SSAT protein interferes with translation unless it is bound to BESM.

Although our experiments relate only to the induction of

SSAT by BESM and BENSM, it is likely that post-transcriptional regulation is a major means of regulation of SSAT. The polyamine analogues are likely to act on SSAT by mimicking the effects of the polyamine themselves, and many other factors that influence SSAT may do so by altering the free polyamine pool [6,53]. Recently, the induction of SSAT by heat shock was shown to occur in the absence of any change in the mRNA [53]. Although Shappell et al. [8] have reported that SSAT mRNA levels were affected by intracellular polyamines in a manner parallel with changes in SSAT activity, the actual magnitude of the alterations of mRNA was much less than the alterations in SSAT activity which, in any event, were underestimated because they did not distinguish immunologically between SSAT and other acetylases in the basal state.

Our results do not support the contention that BENSM affects SSAT mRNA levels by increasing the stability of the mRNA [24]. In contrast, the content of SSAT mRNA in the transfected COS-7 cells was clearly reduced by BESM. It is possible that the changes in stability of SSAT mRNA reported to occur in human melanoma cells [24] involve forms of the mRNA not present in the transcripts derived from the pEUKSAT vectors, but this is unlikely, since pEUKSAT1 contains all of the 5'- and 3'-UTR. The enhanced degradation of SSAT mRNA after BESM addition is consistent with the observed effects on translation, since the transfer of the mRNA from an untranslated pool to the polyribosomes may render it more available for degradation. Degradation of RNA in association with active protein synthesis is a well known regulatory mechanism [54,55].

Finally, our results indicate that the stabilization of SSAT protein by BESM [10–12] plays a very significant role in the extent to which SSAT protein is increased by polyamine analogues. The very rapid turnover of SSAT protein in cells was first reported on the basis of activity measurements by Matsui and Pegg [25] and confirmed using antibodies [20], but an exact half-life for this protein is difficult to measure accurately because of the very small amount of SSAT and the presence of interfering acetyltransferases in uninduced cells. However, in L6 cells treated with spermidine, the half-life of SSAT was estimated to be 9 min [7]. The value of 20 ± 5 min obtained in the present study for cells not treated with BESM is in reasonable agreement with this, and it is clear that BESM greatly increases the half-life of SSAT by at least 40-fold. It is likely that BESM brings about this stabilization by binding to the active site of the enzyme, since it is known to be a competitive inhibitor of the activity [11,56]. This effect of BESM resembles that of methylglyoxal bis(guanylhydrazine), which increases SSAT substantially by stabilizing the protein against intracellular degradation [20,57].

Thus BESM affects SSAT levels in cells not only by increasing mRNA levels, but by facilitating the translation of the mRNA and by preventing the rapid degradation of the protein. These factors combine to bring about a very large induction of SSAT. The striking differences in the abilities of quite closely related bis(ethyl)polyamines to induce SSAT [6,12–19] may be due to differences in the ability to affect any of these steps.

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