Published in final edited form as:
Biochem J. ; 422(1): 101–109. doi:10.1042/BJ20090411.

Spermine analogue-regulated expression of spermidine/spermine N1-acetyltransferase and its effects on depletion of intracellular polyamine pools in mouse fetal fibroblasts

Anne Uimari[*,1], **Tuomo A. KEINäNEN**[*], **Anne Karppinen**[*], **Patrick Woster**[†], **Pekka UIMARI Alhonen**[*,‡], **Juhani JäNNE**[*], and **Leena Alhonen**[*,1]

[*]Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, Biocenter Kuopio, University of Kuopio, FI-70211 Kuopio, Finland [†]Department of Pharmaceutical Sciences, Wayne State University, 539 Shapero Hall, Detroit, MI 48202, U.S.A. [‡]MTT, Biotechnology and Food Research, FI-31600 Jokioinen, Finland

Abstract

SSAT (Spermidine/spermine N1-acetyltransferase, also known as SAT1), the key enzyme in the catabolism of polyamines, is turned over rapidly and there is only a low amount present in the cell. In the present study, the regulation of SSAT by spermine analogues, the inducers of the enzyme, was studied in wild-type mouse fetal fibroblasts, expressing endogenous SSAT, and in the SSAT-deficient mouse fetal fibroblasts transiently expressing an SSAT–EGFP (enhanced green fluorescent protein) fusion gene. In both cell lines treatments with DENSpm (N^1,N^{11}-diethylnorspermine), CPENSpm (N^1-ethyl-N^{11}-[(cyclopropyl)-methy]-4,8-diazaundecane) and CHENSpm (N^1-ethyl-N^{11}-[(cycloheptyl)methy]-4,8-diazaundecane) led to high, moderate or low induction of SSAT activity respectively. The level of activity detected correlated with the presence of SSAT and SSAT– EGFP proteins, the latter localizing both in the cytoplasm and nucleus. RT–PCR (reverse transcription–PCR) results suggested that the analogue-affected regulation of SSAT–EGFP expression occurred, mainly, after transcription. In wild-type cells, DENSpm increased the amount of SSAT mRNA, and both DENSpm and CHENSpm affected splicing of the SSAT pre-mRNA. Depleted intracellular spermidine and spermine levels inversely correlated with detected SSAT activity. Interestingly, the analogues also reduced polyamine levels in the SSAT-deficient cells expressing the EGFP control. The results from the present study show that the distinct SSAT regulation by different analogues involves regulatory actions at multiple levels, and that the spermine analogues, in addition to inducing SSAT, lower intracellular polyamine pools by SSAT-independent mechanisms.

[1] Correspondence may be addressed to either of these authors (anne.uimari@uku.fi or leena.alhonen@uku.fi)..

AUTHOR CONTRIBUTION

Anne Uimari cloned the SSAT–EGFP fusion construct, designed and conducted most of the experiments and wrote the manuscript. Tuomo Keinäanen participated in designing the experiments, the interpretation of the results and refining the manuscript. Anne Karppinen participated in the cell culture and SSAT- and ODC-activity experiments. Patrick Woster provided CHENSpm and CPENSpm and participated in the interpretation of the results and refining the manuscript. Pekka Uimari performed statistical analyses for the protein half-life experiments. Juhani Jäanne and Leena Alhonen were the leaders of the research group (Juhani Jäanne until spring 2008) and supervised the study. Leena Alhonen has also been involved in refining the manuscript.

Keywords

enhanced green fluorescent protein (EGFP); gene expression; polyamines; spermine analogues; spermidine/spermine N^1-acetyltransferase (SSAT)

INTRODUCTION

The polyamines putrescine, spermidine and spermine are essential in central cellular processes including proliferation and differentiation [1,2]. The intracellular polyamine pools are tightly regulated by means of biosynthesis, catabolism, uptake and export. In one such catabolic pathway SSAT (spermidine/ spermine N1-acetyltransferase, also know as SAT1) catalyses the acetylation of spermine and spermidine making them suitable substrates for the next enzyme in the pathway, polyamine oxidase, or for the polyamine export machinery. Multiple factors regulate SSAT activity by controlling the gene expression at one or several levels. For example, elevated intracellular polyamine concentrations regulate SSAT by increasing gene transcription, stabilizing mRNA, enhancing mRNA translation and increasing protein stability [3]. Similarly, polyamine analogues, the mimics of natural polyamines, can effect SSAT expression at multiple levels. Several types of N-alkylated polyamine analogues have been synthesized. They vary in their ability to displace the natural polyamines in the cell, as well as to fulfil the biological functions of natural polyamines. In addition, the analogues in general are poor substrates for the polyamine catabolic enzymes. Owing to their stability, and the functional ability to regulate polyamine metabolic enzymes as well as the ability to deplete intracellular polyamine pools, the analogues produce a long-term effect which disrupts polyamine metabolism. As a consequence, disturbed cellular functions and cytotoxicity may result. The development of polyamine-based molecules for therapeutic purposes is an ongoing field of research and, for example, the N-terminally alkylated polyamine analogue DENSpm (N^1,N^{11}-diethylnorspermine) has been in clinical trials as an agent against several human solid tumours [4,5]. Antiproliferative action of DENSpm seems, at least in some cell lines, to be connected to the induction of SSAT [6–8]. DENSpm increases SSAT activity thousands of fold and affects hnRNA (heterogeneous nuclear RNA) splicing, increases SSAT mRNA level, enhances translation and affects protein stability [3]. In addition to the symmetrically substituted alkyl-polyamine DENSpm, several unsymmetrically substituted alkylpolyamine analogues such as CPENSpm (N^1-ethyl-N^{11}-[(cyclopropyl)methy]-4,8-diazaundecane) and CHENSpm (N^1-ethyl-N^{11}-[(cycloheptyl)methy]-4,8-diazaundecane) have been studied with respect to their cytotoxicity and ability to induce SSAT. Like DENSpm, CPENSpm is an effective superinducer and inhibitor of SSAT and has antitumour action *in vitro* [9,10]. Interestingly, CHENSpm is highly cytotoxic, inhibits SSAT but does not effectively elevate SSAT activity [4,11]. In the present work, the spermine analogue-mediated regulation of SSAT in non-tumour cells was studied. We produced an SSAT–EGFP (enhanced green fluorescent protein) fusion construct to facilitate the use of the detectable marker protein. The expression, localization, activity and function of SSAT–EGFP in response to spermine analogues in transiently transfected SSAT-deficient mouse fetal fibroblasts (devoid of endogenous SSAT activity) were followed. Addition of relatively stable EGFP at the C-terminus of SSAT did not abolish either the activity or the low abundance of the SSAT

enzyme. For comparison, wild-type mouse fetal fibroblasts were used to analyse the regulation of genome-encoded SSAT. The analogue-mediated changes in the activity of SSAT–EGFP and endogenous SSAT were very similar, although, as expected, the regulation of genome-encoded SSAT by the analogues was shown to be more complex. Interestingly, the levels of intracellular polyamine pools mirrored the level of SSAT activity but, as shown in the SSAT-deficient cells, were also decreased by mechanisms independent of the SSAT function.

EXPERIMENTAL

Materials

DENSpm was synthesized essentially as described in [12]. CHENSpm and CPENSpm were synthesized as described in [9]. Generation of mice with disrupted SSAT-gene has also been described previously in [13].

Syngeneic and SSAT-deficient primary fetal fibroblasts were isolated according to the method described in [14], except that the fetuses were taken on day 16 of pregnancy. The cells were seeded in six-well plates at a density of 3.5×10^5 cells per well and grown in DMEM (Dulbecco's modified Eagle's medium, Sigma–Aldrich) supplemented with 10% (v/v) FBS (fetal bovine serum) and 50 μg/ml of gentamycin at 37°C in a 7.5% CO_2 atmosphere. Polyamine analogues were used at a concentration of 10 μM.

Production of SSAT–EGFP fusion construct

The ORF (open reading frame) region of the mouse SSAT gene was amplified from cloned cDNA [15] with the primers 5'-CTAAAGCTTCAAAAGACGAAAATGGC-3' (forward, the start codon is underlined) and 5'-GATGGATCCTCCTCTGCTGCCATTTTTTAG-3' (reverse, the last codon is underlined). The PCR product was digested with BamHI and HindIII restriction enzymes and ligated in frame with the EGFP gene into the pEGFP-N1 vector (Clonetech). The structure of the constructed plasmid was confirmed by automated sequencing. Plasmid DNAs for transfection were prepared using QIAFilter Maxi kit (Qiagen) following the manufacturer's protocol.

Transfection

Cells were transfected with calcium phosphate as follows. The cells were seeded in six well plates at a density of 3.5×10^5 cells per well and incubated for 24 h. Plasmid DNA (2 μg) was mixed with 40 μl of 0.25 M $CaCl_2$. Then, 40 μl of 2 × BBS (Bes-buffered saline; 50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added and the mixture was incubated for 30 min at room temperature (22°C). The medium was removed and the cells were washed twice with DMEM. The wells were covered with 1.5 ml of DMEM and 80 μl of plasmid preparation was added into each well. The plates were kept for 2.5 h in at 37°C, after which the medium was removed and the cells were washed as described above. DMEM, with or without the analogues, was added and the cells were incubated for 24 h.

Analysis of ODC (ornithine decarboxylase) and SSAT activities and intracellular polyamine contents

The activities of ODC [16] and SSAT [17] were measured according to the published methods. Natural polyamines were determined by HPLC as described in [18]. Polyamine analogues were measured as described in [19].

Western blot analysis

Trypsinized and washed (PBS) cells were lysed in a buffer containing 25 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM dithiotreitol and 1 × complete EDTA-free protease inhibitor cocktail (Roche). Samples were centrifuged (at 10000 ***g*** for 15 min at 4°C) and protein contents from the supernatants were measured using a Bio-Rad protein assay kit. Western blot analysis was performed with standard protocols using 30 μg of protein and anti-GFP antibody (BD Living Colors® A.v. peptide antibody, Clontech) or anti-SSAT antibody (affinity-purified from the serums of rabbits injected with the *Escherichia coli*-produced recombinant mouse SSAT protein). Ponceau staining of the membrane and detection of β-actin (Sc1616, Santa Cruz Biotechnology) was used to confirm equal loading.

RT–PCR

Total RNA from the cells was extracted by TRIzol Reagent (Invitrogen) and treated with DNase I (DNA-free, Ambion) according to the manufacturer's instructions. DNase-treated RNA (1 μg) was used for first-strand cDNA synthesis using oligodT primers and AMV (avian myeloblastosis virus) reverse transcriptase (Promega) in a total volume of 25 μl. PCR reactions contained 2 μl of the first-strand cDNA, 1 × PCR buffer with $MgC1_2$ (Finnzymes), dNTP mix (200 μM each), 25 pmol forward and reverse primers and 0.5 units DyNAzyme DNA polymerase (Finnzymes) in a total volume of 50 μl. The primers used were as listed below: SSAT, 5’-TACGTCGACATGGCTAAATTTAAGATCCG-3’ (forward), 5’-TACGTCGACGATACAGCAACTTGCCAATC-3’ (reverse); EGFP, 5’-CCCTGAAGTTCATCTGCAC-3’ (forward), 5’-GTTGTACTCCAGCTTGTG-3’ (reverse); SSAT-X (alternative splice variant of SSAT), 5’-TACGTCGACATGGCTAAATTTAAGATCCG-3’ (forward), 5’-TACGTCGACCAATGCTATGTCCTCATTTAT-3’ (reverse); 18S RNA, 5’-CTACCACATCCAAGGAAG-5’ (forward), 5′-CTCAGCTAAGAGCATCGAG-3’ (reverse). The program consisted one cycle of 95°C for 3 min, 24 (18S RNA), 25 (SSAT, EGFP) or 29 (SSAT-X) cycles of 95°C for 30 s, 58°C for 40 s, 72°C for 40 s followed by one cycle of 72°C for 5 min. PCR products were separated on a 0.9% agarose gel and scanned using a Typhoon 9400 imager (GE Healthcare). The images were analysed using the Image Quant TL program (GE Healthcare).

Microscopy

To examine the fluorescence intensity, the cells were cultured on coverslips. After incubation the cells were washed with PBS and fixed with 4% (w/v) PFA (paraformaldehyde) for 30 min. After that the cells were washed twice for 10 min with PBS. For PI (propidium iodide) staining, the cells were permeabilized with 0.3% Triton X-100 (Fluka) in PBS for 15 min at room temperature and washed twice for 10 min with PBS.

Then the cells were treated with RNase (Boehringer Mannheim Biochemicals) at a concentration of 100 μg/ml in PBS for 20 min at room temperature and washed three times with PBS. The cells were counterstained with PI (Sigma–Aldrich) in PBS at a final concentration of 500 ng/ml for 5 min and washed three times with PBS. The coverslips were mounted on glass slides and examined using either a Olympus AX 70 fluorescence microscope or a Bio-Rad Radiance 2100 confocal laser scanning microscope. An argon ion laser emitting at 488 nm and 543 nm was used to excite EGFP and PI respectively. During scanning, a 40× oil immersion objective was used. Serial optical slices of 1 μm, or less, were taken.

Flow cytometry analysis

Transfected cells, in the six-well assay plates, were trypsinized, washed (PBS) and suspended in 0.5 ml of PBS. To fix the cells, 0.5 ml of 4% (v/v) PFA was added. The proportion of EGFP-positive cells was analysed by flow cytometry (FACScalibur, Becton Dickinson). EGFP expression level was obtained by dividing the mean fluorescence intensity (FL1 channel) of the EGFP-positive population by the mean fluorescence intensity of the EGFP-negative population.

Determination of SSAT and SSAT–EGFP half-lives

The syngeneic and SSAT-deficient cells were seeded in six-well plates at a density of 3.5×10^5 cells per well. SSAT-deficient cells were transfected with SSAT–EGFP as described above and both the syngeneic and transfected cells were grown with or without the analogues (10 μM) for 24 h. The medium was replaced with medium containing cycloheximide at a final concentration of 10 μg/ml with or without the analogues. Each experimental treatment was repeated three times. Samples, in triplicate, were collected from each treatment at four or five timepoints ranging from 0 min to 10 h. SSAT activity of the samples was detected as described above and mean SSAT activity values were calculated for the samples. Based on these mean activity values at different time points a linear regression line was fitted to each experiment within a treatment. The half-lives for SSAT and SSAT–EGFP were estimated from this regression line as: half-life = $-0.5 \times (a/b)$, where a is the y-intercept and b is the regression coefficient of the estimated regression line. For each treatment the mean half-life over the three experiments was calculated.

Statistical analysis

Data are expressed as mean − S.D where applicable. A Prism 4.03 (GraphPad software) GraphPad software package was used to perform one way ANOVA and Dunnett's multiple comparison test. Linear regression analyses were performed using the GLM (General linear modelling) procedures of the SAS-statistical package (v. 9.1, SAS Institute).

RESULTS

Accumulation of SSAT–EGFP in untreated and spermine analogue-treated cells

In order to study the regulation and intracellular localization of SSAT, a CMV (cytomegalovirus) promoter-driven fusion construct containing the protein coding region of SSAT at the N-terminus of EGFP was made. SSAT-deficient mouse fetal fibroblasts were

transfected with the SSAT–EGFP fusion construct or an EGFP control as described in the Experimental section. Cells were grown for 24 h either with or without 10 μM DENSpm, CPENSpm or CHENSpm. During that time, no cytotoxicity was observed (the number of cells at the end of the experiment/the number of cells at the beginning of experiment ≥ 1). The cells were fixed with PFA and studied under fluorescence microscopy. The control cells grown with or without the analogues strongly expressed EGFP and were easily seen under the microscope. In contrast, the SSAT– EGFP-transfected cells grown without the analogues, or treated with CHENSpm, contained no detectable signal. However, the intensity of fluorescence in the SSAT–EGFP-containing cells was enhanced by DENSpm and CPENSpm treatments and could be observed under fluorescence microscopy. The differences in fluorescence intensities of the samples were confirmed by measuring the values for relative fluorescence by flow cytometry. As shown in Figure 1(A), the fluorescence of EGFP-expressing control cells was decreased by the analogues, but, however, did still remain at levels severalfold higher than the fluorescence in SSAT–EGFP-expressing cells. As expected, a significant increase in the fluorescence intensity was detected in the SSAT–EGFP-expressing cells grown with DENSpm or CPENSpm compared with the cells without the analogue treatment. In contrast, fluorescence in the fusion construct-expressing cells grown with CHENSpm was undistinguishable from that of the untreated cells.

To further visualize the presence of fluorescent proteins a Western blot analysis was performed from protein samples of the cells grown under similar conditions to those described above. The EGFP control protein was easily detected in high amounts in all EGFP-transfected samples (results not shown). In contrast, in the SSAT–EGFP-transfected cells the anti-EGFP and anti-SSAT antibodies detected a very low amount of the fusion protein in the homogenates from the DENSpm-treated cells only (results not shown). For comparison, the abundance of SSAT protein in the samples of analogue-treated wild-type mouse fetal fibroblasts was analysed with a Western blot. It is known that SSAT is expressed in a very low level and, as shown in Figure 1(B), only DENSpm treatment increased the SSAT amount to a level that was detectable in the experimental set-up using this particular anti-SSAT antibody. However, the results support the view obtained from the cells expressing the SSAT–EGFP fusion protein. Thus, the intensity of the observed fluorescence correlated with the abundance of EGFP and SSAT–EGFP proteins.

Intracellular localization of SSAT–EGFP

To study the intracellular localization of SSAT–EGFP, the same samples used for fluorescence microscopy were also analysed with confocal microscopy. In the case of control EGFP-expressing cells, the fluorescent protein localized strongly both to the cytoplasm and the nucleus, as shown in the merged images of EGFP and PI-stained nuclei (Figure 2). The analogues studied had no effect on the localization of EGFP. Using higher intensity excitation light, it was also possible to visualize the SSAT– EGFP protein both in untreated and analogue-treated cells. In all samples, SSAT–EGFP was localized both in the cytoplasm and the nucleus (Figure 2). However, occasionally cells were seen in which the analogue treatment had reduced, or fragmented, nuclear localization of SSAT–EGFP (results

not shown). Whether this reflects the action of SSAT–EGFP, or analogue-induced changes in the physiology and condition of the treated cells in general, remains to be resolved.

Analogue-affected SSAT activities and intracellular polyamine levels

Spermine analogues DENSPm, CPENSpm and CHENSpm all act as inhibitors of SSAT *in vitro* [9,20]. However, only DENSpm and CPENSpm superinduce the enzyme in living cells. To study the function of SSAT–EGFP fusion protein the SSAT activity and the intracellular polyamine levels in transfected cells grown with or without the analogues was measured. As a control, EGFP-transfected cells were subjected to similar analysis. SSAT-deficient mouse fetal fibroblasts were used because they lack endogenous SSAT activity. As shown in Table 1, the presence of SSAT–EGFP led to a 9-fold induction in SSAT activity in transfected untreated cells when compared with the cells transfected with EGFP. However, the changes in intracellular polya-mine levels were only minor. In contrast, treatments with polyamine analogues caused significant changes in both SSAT activities and in intracellular polyamine levels. In DENSpm-treated cells, the activity of SSAT–EGFP was 30-fold higher than in the cells grown without the analogue and the intracellular spermidine and spermine levels were significantly depleted. CPENSpm and CHENSpm activated SSAT–EGFP by more than 16-fold and 3-fold respectively, and also significantly decreased spermidine and spermine levels. Significant differences in the accumulation of putrescine were also found, with DENSpm depleting and CHENSpm increasing the amount of putrescine. The function of SSAT–EGFP protein in transfected cells was also confirmed by the accumulation of N^1-acetylspermidine, which is considered an indicator of SSAT activity. DENSpm treatment caused the highest enzyme activity and protein accumulation of SSAT–EGFP compared with CPENSpm, and especially with CHENSpm. Thus, in the case of SSAT–EGFP, the observed SSAT activities correlated with the fluorescence and abundance of the fusion protein detected by microscopy, flow cytometry and Western blot analysis. However, we had surprising results in the SSAT-deficient cells transfected only with the EGFP gene. In all samples there was only a low background level of SSAT activity as these cells have a deficient SSAT gene. However, all the tested analogues depleted the intracellular spermidine and spermine levels significantly. In addition, DENSpm and CHENSpm significantly affected the putrescine levels. Interestingly, the changes in polyamine pools in EGFP-expressing cells were exactly the same as those in the cells transfected with SSAT– EGFP. Therefore, it seems that all tested analogues also induce a SSAT-independent mechanism to reduce intracellular polyamines in mouse fetal fibroblasts.

In order to compare the SSAT activities of the SSAT–EGFP fusion protein with the endogenous SSAT protein, as well as to study the intracellular polyamine levels, the wild-type fetal fibroblasts were subjected to similar analogue treatments as described above. As shown in Table 2, the SSAT activities and polyamine pools observed in the wild-type cells with and without the analogue treatments were very similar to the results obtained using SSAT– EGFP transfected cells deficient in endogenous SSAT.

ODC activity in spermine analogue-treated cells

To determine how spermine analogues affect the production of polyamines, the activity of ODC, the key enzyme in the polyamine biosynthetic pathway, was measured in the SSAT-

deficient and wild-type mouse fetal fibroblasts. The results are shown in Table 3. DENSpm significantly reduced ODC activity in both cell lines. Interestingly, CPENSpm significantly increased the activity in wild-type cells and significantly decreased the activity in SSAT deficient cells. However, CHENSpm did not change the ODC activity in either line. Thus the decreased intracellular polyamine pools in the analogue-treated cells do not totally correlate with the changes observed in biosynthesis.

SSAT mRNA expression in spermine analogue-treated cells

As the amount of SSAT–EGFP protein was clearly increased by DENSpm and CPENSpm, studies to analyse the steady-state level of the fusion construct mRNA were conducted. RT–PCR for the EGFP region, the SSAT region and the junction region of the fusion construct all gave similar results. Figure 3 shows the accumulation of *EGFP* and *SSAT–EGFP* mRNA-derived signals amplified with EGFP specific primers. ROD (relative optical density) of the mRNA concentration, derived from the target gene/18S rRNA ratio, indicated that CPENSpm slightly increased the transcription of EGFP in the pEGFP-N1 control plasmid-transfected cells. However, transcription of SSAT–EGFP fusion construct was not significantly affected by the analogue treatments. If anything, DENSpm slightly decreased the amount of *SSAT–EGFP* mRNA. Thus the increased amount of SSAT– EGFP protein detected in the DENSpm- and CPENSpm-treated cells was not a consequence of enhanced transcription.

It is known that DENSpm stabilizes wild-type *SSAT* mRNA and also fine-tunes the expression of *SSAT* mRNA by ‘regulated unproductive splicing and translation’ (RUST) [15]. However, the effects of CPENSpm and CHENSpm have not been published. In order to determine how the analogues affect both transcription of the genomic *SSAT* gene and splicing of the *SSAT* premRNA, RT–PCR of the analogue-treated wild-type mouse fetal fibroblasts was performed. Primers for normal *SSAT* mRNA and primers selective for an alternative splice variant, *SSAT-X*, were used. The results are shown in Figure 4. A significantly increased amount of *SSAT* mRNA was present in cells treated with DENSpm and CPENSpm. In contrast, CHENSpm did not change the amount of *SSAT* mRNA. In accordance with the previous report [15], a significant decrease in the accumulation of *SSATX*, the unproductive splice variant, in DENSpm-treated cells was observed when compared with the amount present in untreated cells. Interestingly, the unproductive splicing of *SSAT* pre-mRNA was enhanced by CHENSpm, the analogue not able to superinduce SSAT. On the other hand, CPENSpm did not affect *SSAT* premRNA splicing compared with the untreated cells. Differences in analogue-affected transcriptional regulation between endogenous *SSAT* mRNA and the transfected *SSAT–EGFP* mRNA reflect the important influence of the *SSAT* promoter and the whole genomic sequence, including introns, for the regulation and expression of the gene.

Effects of the spermine analogues on the half-life of SSAT and SSAT–EGFP proteins

In order to study how the analogues affect the stability of SSAT and SSAT–EGFP, the half-life of SSAT and SSAT–EGFP with or without the analogues was determined by analysing changes in SSAT activity after the addition of the protein synthesis inhibitor cycloheximide. The results are presented in Table 4. The half-life of SSAT and SSAT–EGFP were both

lowest in the untreated cells and were similar to each other. Both DENSpm and CPENSpm increased the half-life of SSAT and SSAT–EGFP. The extended half-lives of both proteins were also similar to each other. However, DENSpm extended the half-life more than CPENSpm. CHENSpm had only a minor effect on the SSAT half-life, but interestingly, seemed to increase the half-life of SSAT–EGFP quite extensively. Altogether, the results suggest that the wild-type SSAT and SSAT–EGFP have a similar half-life, which is extended by spermine analogues.

DISCUSSION

In the present study we generated an SSAT–EGFP fusion construct, which is expressed in low amounts but with detectable SSAT activity, in transiently transfected SSAT-deficient mouse fetal fibroblasts. The enzyme activity and intracellular accumulation of the fusion protein is significantly enhanced by spermine analogues DENSpm and CPENSpm. The spermine analogue CHENSpm only slightly increases the SSAT activity of the fusion product. All the analogues studied also cause similar changes in wild-type cells expressing endogenous SSAT. In addition, in all cases the enhanced SSAT activity correlates with decreased polyamine levels. Thus the expression of either endogenous SSAT or the transfected SSAT–EGFP can lead to similar cellular changes in mouse fetal fibroblasts. However, further study of the regulation of SSAT and SSAT–EGFP expression by spermine analogues reveals some differences. We have shown that the *SSAT–EGFP* mRNA levels in cells grown with or without the analogues are not significantly different (Figure 3). Thus transcription of the fusion construct containing only the coding region of SSAT, and not 3’ or 5’ untranslated regions, is not enhanced by the analogues and therefore increased transcription is not the reason for protein accumulation. It has been reported previously that the transcription of a FLAG–SSAT construct containing only the protein encoding sequence was not increased, but was in fact decreased, by DENSpm [22]. On the other hand, in our study a significant increase in the *SSAT* mRNA level was obtained in the wild-type mouse fetal fibroblasts, demonstrating that DENSpm induces the *SSAT* mRNA transcript production from the genomic *SSAT*. This is also the case *in vivo* since the amount of *SSAT* transcript in the liver samples isolated from DENSpm-treated mice is higher than in the samples from untreated mice [15]. In addition, we showed that the splicing of *SSAT* pre-mRNA is directed towards the variant transcript, *SSAT-X*, by CHENSpm. As reported previously [15] production of normal *SSAT* mRNA is enhanced by DENSpm. Thus the regulation of genome-encoded SSAT is differentially fine-tuned by spermine analogues. Altogether, our findings confirm that the complete regulation of endogenous *SSAT* gene expression happens at multiple levels and involves the promoter and the exonic and intronic sequences as well as the 3’ and 5’ UTRs.

Because of the low expression level, determining intracellular localization of SSAT has been difficult. Under DENSpm-induced conditions, using immunohistochemistry and immunocytochemistry, SSAT has been detected in the cytoplasm of human adenocarcinoma A549 cells and in the cytoplasm, nucleus and mitochondria of human breast cancer L56Br-C1 cells respectively [23,24]. We were not able to detect the SSAT–EGFP under the fluorescent microscope unless DENSpm or CPENSpm was added to the growth medium. However, confocal microscopy studies facilitated the detection of the fusion protein in all

conditions. SSAT–EGFP localized both in the cytoplasm and in the nucleus in untreated and analogue-treated samples. The other cell organelles were not studied in detail for the presence of SSAT– EGFP. Since EGFP is widely used to determine the subcellular localization of other proteins, SSAT–EGFP could offer a tool to follow the intracellular localization of SSAT in different types of cells, including non-tumour cells in normal growth conditions such as used in the present study.

The low basal activity of SSAT–EGFP could result from very rapid turnover of the protein, as is the case for SSAT itself [25], or it could be caused by the inefficient translation of the mRNA. In recent studies with FLAG-tagged SSAT and SSAT fused to *Renilla mullerei* luciferase, it was suggested that DENSpm regulates *SSAT* mRNA translational efficiency by inhibiting a repressor protein from binding to *SSAT* transcript [22]. In that study, the half-life of the SSAT-luciferase fusion protein in HeLa cells was not affected by DENSpm but the translation was more effective. Whether DENSpm- and CPENSpm-induced accumulation of SSAT–EGFP is affected by enhanced translation remains to be determined. On the other hand, it has been shown previously that SSAT protein stability is enhanced by the presence of DENSpm [26–28]. Studies to reveal the important regions or amino acids of SSAT protein in terms of proteosomal degradation have indicated the importance of C-terminal glutamic acid residues at positions 170 and 171 (the two last amino acids of SSAT) [27,29]. Substituting these amino acids for either glutamine or lysine, or adding two extra lysine residues at the end of the protein, rendered the SSAT protein stable [27]. In the present study, EGFP was fused to the C-terminus of the protein and therefore could influence the proteosomal degradation of the protein. However, our determination of SSAT and SSAT–EGFP half-life, based on SSAT activity, suggested that in mouse fetal fibroblasts the half-life of endogenous SSAT and plasmid-expressed SSAT–EGFP are very similar and spermine analogues DENSpm and CPENSpm stabilize the both proteins. Interestingly, CHENSpm also seemed to clearly extend the half-life of SSAT– EGFP in the transfected SSAT-deficient mouse fetal fibroblasts yet did not highly affect the half-life of SSAT protein in wild-type cells. The reason why CHENSpm does not highly induce the activity of SSAT–EGFP, although it extends the half-life of the protein, is not known. One possible explanation could be that the configuration of the fusion protein allows CHENSpm to stabilize SSAT–EGFP but at the same time the activity of SSAT–EGFP is limited. However, the total protein amounts of SSAT–EGFP and SSAT are not increased by CHENSpm as shown in this study. In addition, the production of variant transcript *SSAT-X* is increased by CHENSpm in the wild-type cells. In general, it is interesting to note that EGFP is a relatively stable protein with a half-life of 26 h [30], whereas the half-life of SSAT–EGFP is close to the half-life of the wild-type SSAT protein. This means SSAT–EGFP is an unstable variant of EGFP fusions.

Several N-alkylated polyamine analogues have been synthesized and evaluated as antitumour agents. In addition, the analogues used in this study are known to have anti-proliferative or cytotoxic effects [4]. In general, polyamine analogues inhibit cell growth in different ways. One suggested route is the superinduction of SSAT followed by the depletion of intracellular polyamines. As reported previously, the spermine analogues vary in their ability to induce SSAT [4,11]. In three different human prostate cancer cell lines

DENSpm strongly induced SSAT whereas CPENSpm induced it less than DENSpm but clearly more than CHENSpm, which gave only a low induction of SSAT activity [11]. The intracellular spermidine and spermine levels were less affected by CHENSpm in all three cell lines [11]. In the present study, the main purpose was to analyse the regulation of SSAT expression, and therefore a 24 h treatment time was used to minimize the growth inhibition or death of the cells by the analogues. However, it needs to be emphasized, that even though cytotoxicity was not detected there was reduced EGFP control protein expression in analogue-treated cells. This may reflect decreased cellular functions caused, at least partly, by a reduction of the levels of intracellular polyamines occurring within the treatment time. The reduction of spermidine and spermine pools by all analogues, and the slight accumulation of putrescine in CHENSpm- and CPENSpm-treated cells, as well as the decrease of putrescine level in DENSpm-treated samples, were similar in wild-type cells and in the SSAT– EGFP-expressing cells. Interesting results in terms of intracellular polyamine levels in response to the analogues came from the cells expressing EGFP alone. There were no changes in SSAT activity (as expected because the endogenous SSAT is disrupted) but the intracellular polyamine levels were similar to those of wild-type SSAT and SSAT–EGFP expressing cells treated with the analogues. Lower N^1-acetyl-spermidine levels in EGFP-expressing than in SSAT–EGFP-expressing cells showed that the absence of SSAT function, and in the case of DENSpm-treated EGFP transfected cells, the lower value of N^1-acetylspermidine compared with the untreated and CHENSpm- and CPENSpm-treated EGFP expressing cells, again suggested the strong ability of DENSpm to remove the intracellular polyamines. The loss of intracellular polyamines is interesting since the back-conversion of polyamines needs a functional SSAT protein. Although, spermine oxidase can convert spermine into spermidine it is not able to oxidize spermidine to putrescine [31]. Whether there are still some unknown enzymes in the catabolic pathway of polyamines able to catalyse the back-conversion remains to be seen. It has been shown previously that DENSpm depletes intracellular polyamines in mouse embryonic stem cells in which the SSAT gene is disrupted [13]. On the other hand, DENSpm treatment of several SSAT-deficient mouse tissues does not lead to depletion of higher intracellular polyamines, though the animals are more sensitive to the toxic effects of the analogue than their syngeneic littermates [32]. In addition to stimulating the catabolic enzymes, the analogues can inhibit polyamine biosynthetic enzymes, thus further lowering the intracellular polyamine pools. In this study the ODC activity was clearly reduced by DENSpm and by CPENSpm in SSAT-deficient cells. However, in wild-type cells only DENSpm decreased ODC activity and, in fact, CPENSpm increased it. We did not study the activity of AdoMetDC (S-adenosylmethionine decarboxylase) the other key enzyme in the biosynthesis of polyamines in this study. However, it has been shown previously that DENSpm and CPENSpm reduce the activity of AdoMetDC, while CHENSpm does not [4,33]. Therfore the low intracellular polyamine levels in DENSpm- and CPENSpm-treated cells could partly reflect reduced biosynthesis, but in the case of CHENSpm some additional mechanisms, including direct replacement from binding sites and the transport system, need to be considered.

In conclusion, the SSAT–EGFP protein introduced in this paper encodes a protein with many functional properties similar to SSAT and therefore is a good model system for polyamine research. In mouse fetal fibroblasts, the spermine analogues DENSpm,

CHENSpm and CPENSpm distinctly and systematically regulate the expression of SSAT at multiple levels, including the splicing of pre-mRNA. In addition, these analogues also induce SSAT-independent mechanisms to reduce intracellular polyamines levels. The results from the present study may give important information when the functions of SSAT connected to the cytotoxicity are evaluated and for the development and targeting of polyamine structure-based agents for therapeutic purposes.

Acknowledgments

We gratefully acknowledge Ms Tuula Reponen for help with the HPLC analysis and Ms Arja Korhonen and Ms Sisko Juutinen for their skilful technical assistance.

FUNDING

This work was supported by the Academy of Finland [grant number 121307].

Abbreviations used

AdoMetDC	S-adenosylmethionine decarboxylase
CHENSpm	N^1-ethyl-N^{11}-[(cycloheptyl)methy]-4,8-diazaundecane
CPENSpm	N^1-ethyl-N^{11}-[(cyclopropyl)methy]-4,8-diazaundecane
DENSpm	N^1,N^{11}-diethylnorspermine
DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
ODC	ornithine decarboxylase
PFA	paraformaldehyde
PI	propidium iodide
ROD	relative optical density
RT–PCR	reverse transcription–PCR
SSAT	spermidine/spermine N1-acetyltranferase
SSAT-X	alternative splice variant of SSAT

REFERENCES

1. Thomas T, Thomas TJ. Polyamines in cell growth and cell death: Molecular mechanisms and therapeutic applications. Cell Mol. Life Sci. 2001; 58:244–258. [PubMed: 11289306]
2. Erwin BG, Bethell DR, Pegg AE. Role of polyamines in differentiation of 3T3-L1 fibroblasts into adipocytes. Am. J. Physiol. 1984; 246:C293–C300. [PubMed: 6422770]
3. Pegg AE. Spermidine/spermine-N1-acetyltransferase; a key metabolic regulator. Am. J. Physiol. Endocrinol. Metab. 2008; 294:E995–E1010. [PubMed: 18349109]
4. Casero RA Jr, Woster PM. Terminally alkylated polyamine analogues as chemotherapeutic agents. J. Med. Chem. 2001; 44:1–26. [PubMed: 11141084]
5. Boncher T, Bi X, Varghese S, Casero RA Jr, Woster PM. Polyamine-based analogues as biochemical probes and potential therapeutics. Biochem. Soc. Trans. 2007; 35:356–363. [PubMed: 17371278]

6. Casero RA Jr, Mank AR, Xiao L, Smith J, Bergeron RJ, Celano P. Steady-state messenger RNA and activity correlates with sensitivity to N1,N12-bis(ethyl)spermine in human cell lines representing the major forms of lung cancer. Cancer Res. 1992; 52:5359–5363. [PubMed: 1327507]
7. Casero RA Jr, Pegg AE. Spermidine/spermine N1-acetyltransferase–the turning point in polyamine metabolism. FASEB J. 1993; 7:653–661. [PubMed: 8500690]
8. Jiang R, Choi W, Khan A, Hess K, Gerner EW, Casero RA Jr, Yung WK, Hamilton SR, Zhang W. Activation of polyamine catabolism by N^1,N^{11}-diethylnorspermine leads to cell death in glioblastoma. Int. J. Oncol. 2007; 31:431–440. [PubMed: 17611701]
9. Saab NH, West EE, Bieszk NC, Preuss CV, Mank AR, Casero RA Jr, Woster PM. Synthesis and evaluation of unsymmetrically substituted polyamine analogues as modulators of human spermidine/spermine-N1-acetyltransferase (SSAT) and as potential antitumor agents. J. Med. Chem. 1993; 36:2998–3004. [PubMed: 8411017]
10. Casero RA Jr, Mank AR, Saab NH, Wu R, Dyer WJ, Woster PM. Growth and biochemical effects of unsymmetrically substituted polyamine analogues in human lung tumor cells 1. Cancer Chemother. Pharmacol. 1995; 36:69–74. [PubMed: 7720179]
11. McCloskey DE, Woster PM, Casero RA Jr, Davidson NE. Effects of the polyamine analogues N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane and N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane in human prostate cancer cells. Clin. Cancer Res. 2000; 6:17–23. [PubMed: 10656427]
12. Rehse K, Puchert E, Leissring S. Antiaggregatory and anticoagulant effects of oligoamines. 12. alkyl- and arylalkyl- derivatives of putrescine, spermidine and spermine. Arch. Pharm. (Weinheim). 1990; 323:287–294. [PubMed: 2383171]
13. Niiranen K, Pietila M, Pirttila TJ, Jarvinen A, Halmekyto M, Korhonen VP, Keinanen TA, Alhonen L, Janne J. Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. effects on polyamine homeostasis and sensitivity to polyamine analogues. J. Biol. Chem. 2002; 277:25323–25328. [PubMed: 12000764]
14. Alhonen L, Karppinen A, Uusi-Oukari M, Vujcic S, Korhonen VP, Halmekyto M, Kramer DL, Hines R, Janne J, Porter CW. Correlation of polyamine and growth responses to N1,N11-diethylnorspermine in primary fetal fibroblasts derived from transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. J. Biol. Chem. 1998; 273:1964–1969. [PubMed: 9442032]
15. Hyvonen MT, Uimari A, Keinanen TA, Heikkinen S, Pellinen R, Wahlfors T, Korhonen A, Narvanen A, Wahlfors J, Alhonen L, Janne J. Polyamine-regulated unproductive splicing and translation of spermidine/spermine N1-acetyltransferase. RNA. 2006; 12:1569–1582. [PubMed: 16809818]
16. Janne J, Williams-Ashman HG. On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. J. Biol. Chem. 1971; 246:1725–1732. [PubMed: 5547701]
17. Bernacki RJ, Bergeron RJ, Porter CW. Antitumor activity of N,N′-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. Cancer Res. 1992; 52:2424–2430. [PubMed: 1568212]
18. Hyvonen T, Keinanen TA, Khomutov AR, Khomutov RM, Eloranta TO. Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. J. Chromatogr. 1992; 574:17–21. [PubMed: 1629283]
19. Kabra PM, Lee HK, Lubich WP, Marton LJ. Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: Improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J. Chromatogr. 1986; 380:19–32. [PubMed: 3745383]
20. Wu R, Saab NH, Huang H, Wiest L, Pegg AE, Casero RA Jr, Woster PM. Synthesis and evaluation of a polyamine phosphinate and phosphonamidate as transition-state analogue inhibitors of spermidine/spermine-N1-acetyltransferase. Bioorg. Med. Chem. 1996; 4:825–836. [PubMed: 8818232]
21. Reference deleted

22. Butcher NJ, Broadhurst GM, Minchin RF. Polyamine-dependent regulation of spermidine-spermine N1-acetyltransferase mRNA translation. J. Biol. Chem. 2007; 282:28530–28539. [PubMed: 17690107]

23. Casero RA Jr, Gabrielson EW, Pegg AE. Immunohistochemical staining of human spermidine/spermine N1-acetyltransferase superinduced in response to treatment with antitumor polyamine analogues. Cancer Res. 1994; 54:3955–3958. [PubMed: 8033120]

24. Holst CM, Nevsten P, Johansson F, Carlemalm E, Oredsson SM. Subcellular distribution of spermidine/spermine N1-acetyltransferase. Cell Biol. Int. 2008; 32:39–47. [PubMed: 17920945]

25. Persson L, Pegg AE. Studies of the induction of spermidine/spermine N1-acetyltransferase using a specific antiserum. J. Biol. Chem. 1984; 259:12364–12367. [PubMed: 6490617]

26. Fogel-Petrovic M, Vujcic S, Brown PJ, Haddox MK, Porter CW. Effects of polyamines, polyamine analogs, and inhibitors of protein synthesis on spermidine-spermine N1-acetyltransferase gene expression. Biochemistry. 1996; 35:14436–14444. [PubMed: 8916930]

27. Coleman CS, Pegg AE. Proteasomal degradation of spermidine/spermine N1-acetyltransferase requires the carboxyl-terminal glutamic acid residues. J. Biol. Chem. 1997; 272:12164–12169. [PubMed: 9115288]

28. McCloskey DE, Pegg AE. Altered spermidine/spermine N1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. J. Biol. Chem. 2000; 275:28708–28714. [PubMed: 10887189]

29. Coleman CS, Pegg AE. Polyamine analogues inhibit the ubiquitination of spermidine/spermine N1-acetyltransferase and prevent its targeting to the proteasome for degradation. Biochem. J. 2001; 358:137–145. [PubMed: 11485561]

30. Corish P, Tyler-Smith C. Attenuation of green fluorescent protein half-life in mammalian cells. Protein Eng. 1999; 12:1035–1040. [PubMed: 10611396]

31. Vujcic S, Liang P, Diegelman P, Kramer DL, Porter CW. Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. Biochem. J. 2003; 370:19–28. [PubMed: 12477380]

32. Niiranen K, Keinanen TA, Pirinen E, Heikkinen S, Tusa M, Fatrai S, Suppola S, Pietila M, Uimari A, Laakso, et al. Mice with targeted disruption of spermidine/spermine N1-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but show signs of insulin resistance upon aging. J. Cell. Mol. Med. 2006; 10:933–945. [PubMed: 17125596]

33. Bergeron RJ, Muller R, Huang G, McManis JS, Algee SE, Yao H, Weimar WR, Wiegand J. Synthesis and evaluation of hydroxylated polyamine analogues as antiproliferatives. J. Med. Chem. 2001; 44:2451–2459. [PubMed: 11448227]

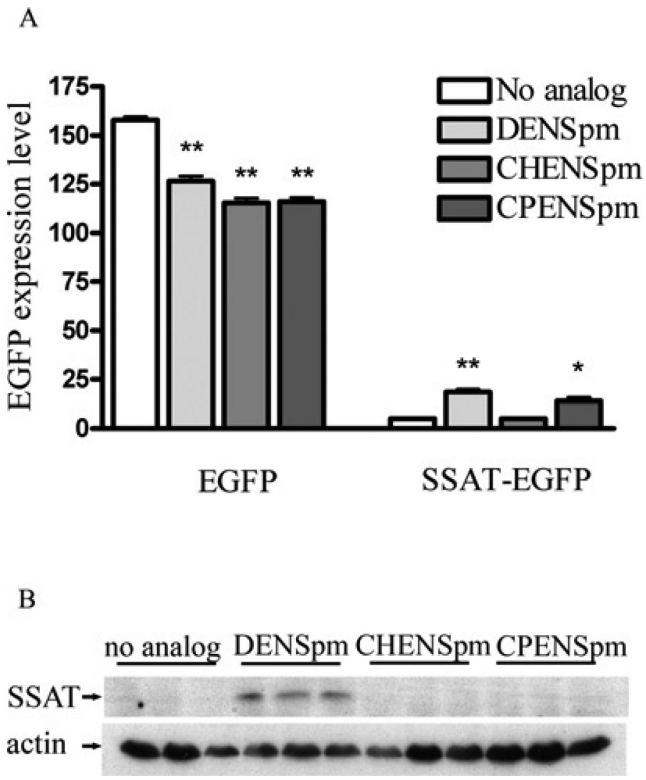

Figure 1. Effects of spermine analogues on the accumulation of SSAT protein in mouse fetal fibroblasts

(**A**) Mouse SSAT-deficient fetal fibroblasts expressing either EGFP or SSAT–EGFP were grown with or without 10 μM spermine analogues for 24 h. The proportion of positive fluorescent cells was determined with flow cytometry. The expression level was obtained by dividing the mean fluorescence intensity of the EGFP-positive population by the mean fluorescence intensity of the EGFP-negative population. One of two representative experiments is shown. Results are expressed as mean ± S.D. from three samples. *$P < 0.05$, **$P < 0.01$ compared with the corresponding untreated control groups. (**B**) The cell lysates of wild-type mouse fetal fibroblasts grown with or without 10 μM spermine analogues for 24 h were analysed for the presence of SSAT by Western blot. Three independent samples of each treatment are shown. β-Actin was used as a loading control.

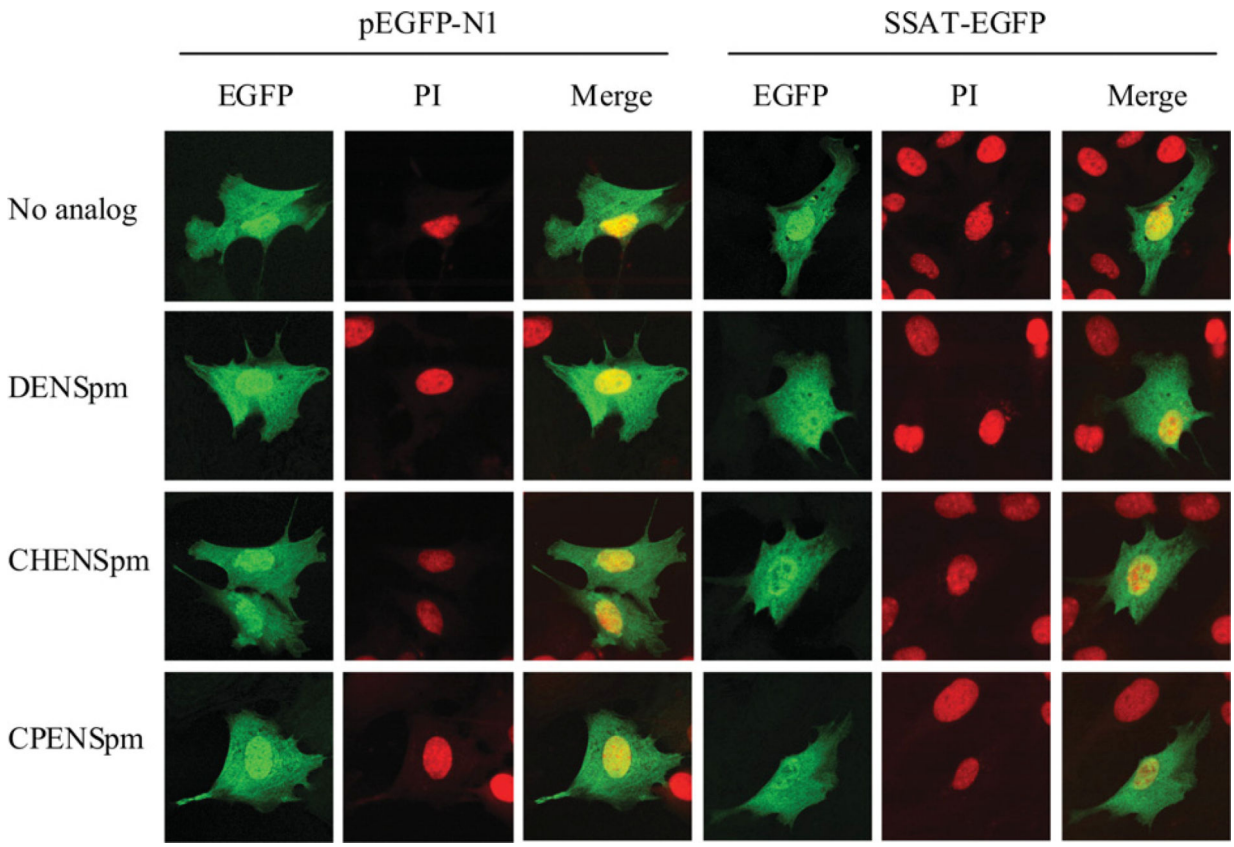

Figure 2. Intracellular localization of EGFP and SSAT–EGFP in analogue-treated cells

Control pEGFP-N1- and SSAT–EGFP-transfected cells were treated with spermine analogues for 24 h, fixed and counterstained with PI to allow nuclei to be visualized. Confocal images were obtained from the same field for detection. EGFP (green), PI (red) fluorescence. Images were merged to obtain the co-localization of the EGFP and PI (yellow). Note that the nuclei of untransformed cells are also visible.

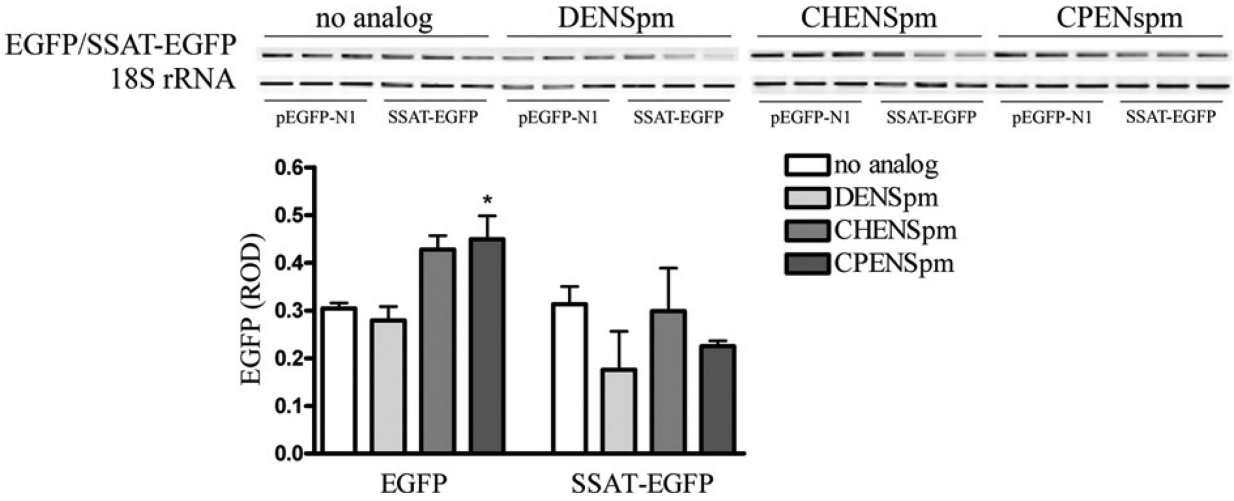

Figure 3. Transcription of SSAT–EGFP and EGFP in spermine analogue-treated cells

Upper panel: RT–PCR with EGFP derived primers recognizing EGFP and SSAT–EGFP in pEGFP-N1- or SSAT–EGFP-transfected cells respectively. Each lane represents independent samples derived from transfected cells grown with or without 10 μM spermine analogues for 24 h. 18S rRNA was amplified as an internal control. Lower panel: the intensity of the RT–PCR bands was quantified by densitometry and ROD was determined by dividing the EGFP (representing EGFP or SSAT–EGFP) value by the 18S rRNA value. Data are shown as means ± S.D. for three samples. *$P < 0.05$ compared with the corresponding untreated control group.

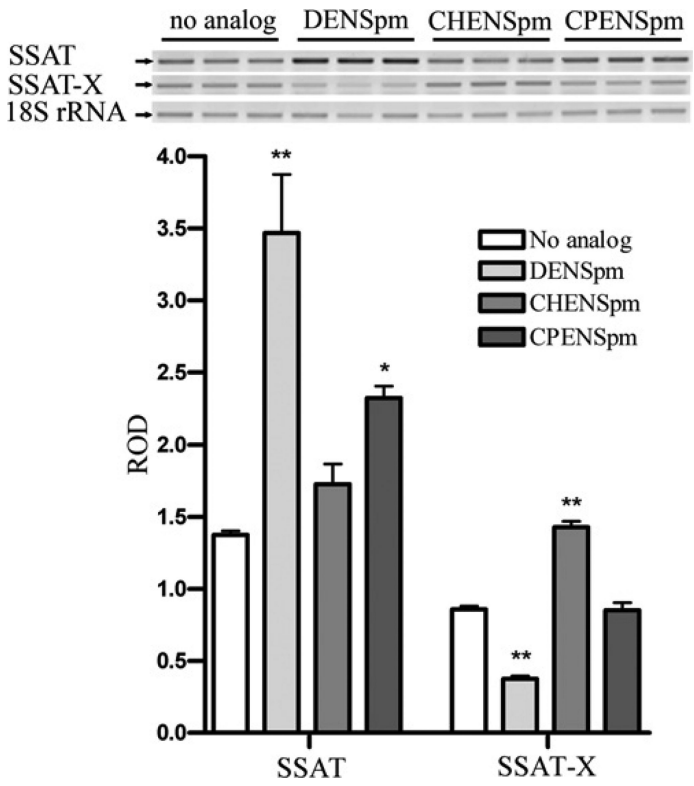

Figure 4. Accumulation of SSAT and SSAT-X mRNAs in spermine analogue-treated cells

Upper panel:SSAT andSSAT-X were amplified by RT–PCR from samples of untreated wild-type mouse fetal fibroblasts or cells treated with 10 μM spermine analogues for 24 h. 18S rRNA was amplified as an internal control. The lanes represent independent samples. Lower panel: intensity of the RT–PCR products in the gel was quantified by densitometry. ROD was determined by dividing the SSAT and SSAT-X values by the 18S rRNA value. Data are shown as means ± S.D. for three samples. *$P < 0.05$, **$P < 0.01$ compared with untreated control group.

Table 1

Intracellular polyamine pools of EGFP- and SSAT-EGFP-transfected SSAT-deficient mouse fetal fibroblasts

Treatment/Transgene	SSAT activity (pmol/10 min per mg of protein)	Polyamine pools (pmol/μg of protein)				
		Putrescine	N^1-Acetylspermidine	Spermidine	Spermine	Analogue
No analogue						
EGFP	39 ± 16	1.5 ± 0.10	0.82 ± 0.05	17.8 ± 0.42	5.6 ± 0.19	NA
SSAT-EGFP	347 ± 112	2.0 ± 0.26	0.88 ± 0.10	15.9 ± 0.16	5.0 ± 0.23	NA
DENSpm						
EGFP	19 ± 19	0.9 ± 0.08†	0.65 ± 0.09*	5.4 ± 0.38†	2.1 ± 0.17†	16.3 ± 1.15
SSAT-EGFP	10564 ± 2853†	1.1 ± 0.12†	0.95 ± 0.11	5.3 ± 0.49†	2.4 ± 0.19†	17.3 ± 0.98
CHENSpm						
EGFP	59 ± 28	2.4 ± 0.30†	0.79 ± 0.05	13.4 ± 0.22†	4.4 ± 0.02†	10.3 ± 0.92
SSAT-EGFP	1185 ± 197	2.8 ± 0.09†	1.44 ± 0.06*	12.6 ± 0.66†	4.5 ± 0.26†	10.4 ± 0.15
CPENSpm						
EGFP	45 ± 1	1.7 ± 0.07	0.79 ± 0.04	10.2 ± 0.42†	3.4 ± 0.14†	12.8 ± 1.77
SSAT-EGFP	5805 ± 51†	2.3 ± 0.06	1.05 ± 0.10	9.6 ± 0.13†	3.4 ± 0.07†	13.2 ± 1.25

Cells were grown for 24 h with or without 10 μM spermine analogues. Results shown are representative of two experiments performed in triplicate. Data are expressed as mean ± S.D. (n =3). Statistical significance of difference between no analogue- and analogue-treated cells

*P <0.05

†P <0.05. NA, not applicable.

Table 2

Intracellular polyamine pools of wild-type mouse fetal fibroblasts

Treatment	SSAT activity (pmol/10 min per mg of protein)	Polyamine pools (pmol/μg of protein)				
		Putrescine	N^1-Acetylspermidine	Spermidine	Spermine	Analogue
No analogue	153 ± 21	2.1 ± 0.12	0.33 ± 0.02	17.5 ± 0.03	5.2 ± 0.12	NA
DENSpm	24579 ± 2598†	1.2 ± 0.05†	0.55 ± 0.03†	2.1 ± 0.06†	1.9 ± 0.06†	21.8 ± 2.53
CHENSpm	355 ± 56	2.8 ± 0.06†	0.63 ± 0.08†	15.1 ± 1.20†	4.7 ± 0.28*	9.2 ± 0.49
CPENSpm	5437 ± 235†	3.0 ± 0.10†	1.11 ± 0.02†	10.4 ± 0.11†	3.5 ± 0.04†	16.1 ± 1.32

Cells were grown for 24 h with or without 10 μM spermlne analogues. Results are expressed as mean ± S.D. (n = 3). Statistical significance of difference between no analogue- and analogue-treated cells

*$P < 0.05$

†$P < 0.05$. NA, not applicable.

Table 3

ODC activity in wild-type and SSAT-cleficient mouse fetal fibroblasts

	ODC activity (pmol/30 min per mg of protein)	
Treatment	**Wild-type cells**	**SSAT-deficient cells**
No analogue	404 ± 36	393 ± 43
DENSpm	14 ± 1†	38 ± 2†
CHENSpm	449 ± 16	381 ± 31
CPENSpm	532 ± 49†	318 ± 19*

Cells were grown for 24 h with or without 10 μM spermine analogues. Results are expressed as mean ± S.D. (n = 3). Statistical significance of difference between no analogue- and analogue-treated cells

*$P < 0.05$

†$P < 0.05$. NA, not applicable.

Table 4

Half-life of SSAT and SSAT-EGFP detected in the wild-type and in the SSAT-EGFP-transfected SSAT-deficient mouse fetal fibroblasts

	Half-life (min)	
Treatment	**SSAT**	**SSAT-EGFP**
No analogue	344 ± 81	363 ± 93
DENSpm	1256 ± 287	1287 ± 570
CHENSpm	419 ± 245	1144 ± 276
CPENSpm	668 ± 154	582 ± 98

Results are expressed as means ± S.D. of three independent experiments performed in triplicates.