Properties and Regulation of Human Spermidine/Spermine
N\textsuperscript{1}-Acetyltransferase Stably Expressed in Chinese Hamster
Ovary Cells

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Spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT) appears to be the rate-limiting enzyme of polyamine catabolism, yet studies of its regulation have been limited by the low amounts of SSAT in uninduced cells. A system for studying SSAT was established by stably transfecting Chinese hamster ovary cells with a construct where SSAT cDNA was under control of the cytomegalovirus promoter. Thirteen of 44 clones expressed significantly increased SSAT activity (650–1900 compared with 24 pmol/min/mg protein in control cells). SSAT activity was directly proportional to SSAT protein, which turned over very rapidly (t\textsubscript{1/2} of 29 min) and was degraded through the ubiquitin/proteasomal pathway. The increased SSAT activity caused perturbations in polyamine homeostasis and led to a reduction in the rate of growth under clonal conditions. N\textsuperscript{1},N\textsuperscript{12}-bis(ethyl)spermine greatly increased SSAT activity in controls and SSAT transfected clones (to about 10 and 60 nmol/min/mg protein, respectively). N\textsuperscript{1},N\textsuperscript{12}-Bis(ethyl)spermine caused an increase in the SSAT half-life and a slight increase in SSAT mRNA, but these changes were insufficient to account for the increase in SSAT protein suggesting that translational regulation of SSAT must also occur.

Spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT)\textsuperscript{1} is the catabolic enzyme primarily responsible for regulation of intracellular polyamine concentrations in mamalian cells (1, 2). SSAT converts spermine and spermidine to their N\textsuperscript{1}-acetyl derivatives, which can be excreted from the cell or further metabolized, by polyamine oxidase, ultimately to putrescine. SSAT activity is highly regulated and is induced in response to high intracellular levels of natural polyamines (3, 4). Additionally, SSAT activity can be induced by a number of other stimuli, including hormones, physiological stimuli, drugs, and toxic agents (1, 2). Cellular levels of SSAT are usually very low, and therefore, it has been difficult to study the regulation of this enzyme under cellular conditions where it is not induced. Studies of SSAT under conditions where the enzyme has been induced have provided evidence of SSAT regulation at the levels of transcription (5, 6), mRNA stabilization (6, 7), mRNA translation (8), and protein degradation (8–11).

Structural polyamine analogues of the bis(ethyl)polyamine type, which are currently of interest as cancer chemotherapeutic agents because of antineoplastic effects against a variety of tumor types, are the most potent inducers of SSAT activity identified to date (1, 12–14). It should be noted that although these analogues are quite similar in structure to the natural polyamines and can induce SSAT activity, they are not themselves substrates of the acetyltransferase because of their N\textsubscript{1} terminal substituents. The bis(ethyl)polyamine analogues accumulate intracellularly, induce SSAT activity, and deplete intracellular polyamine pools. Correlations have been demonstrated between the cytotoxicity of these analogues and the SSAT induction, which in some cell types can reach greater than 1000-fold. However, it is not yet clear whether SSAT induction is integral to analogue cytotoxicity. How the various levels of SSAT regulation combine to produce the rapid and high induction of enzyme activity, which results from exposure to these analogues is not yet fully understood.

The current studies were therefore carried out to obtain a greater understanding of the natural cellular regulation of the SSAT enzyme in its noninduced state and to examine SSAT regulation during induction of the enzyme by the polyamine analogue N\textsuperscript{1},N\textsuperscript{12}-bis(ethyl)spermine (BE 3-4-3). We now report the establishment of a CHO cell line stably transfected with human SSAT and which expresses levels of SSAT activity which have allowed investigation of cellular properties and regulation of the enzyme in its noninduced state. Induction of SSAT by BE 3-4-3 was used to provide a more detailed understanding of SSAT regulation by contrasting the properties of SSAT in the presence of the analogue to those in the noninduced state.

**EXPERIMENTAL PROCEDURES**

.Materials—[1-\textsuperscript{14}C]acetyl-CoA (50 or 63 Ci/mol) was obtained from ICN Biochemicals (Costa Mesa, CA). The proteasome inhibitor MG 132 was purchased from Calbiochem. Cycloheximide was obtained from Sigma. LipofectAMINE and Genetecin were purchased from Life Technologies, Inc. BE 3-4-3 was kindly provided by Dr. Raymond Bergeron (University of Florida, Gainesville, FL).

.Cell Culture—CHO cells were maintained in minimum essential α-medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA), 100 units/ml penicillin, and 100 units/ml streptomycin (CHO medium). Cultures were incubated at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere and were passaged every 5–7 days to maintain exponential growth. To assess cell growth over time, cells were plated at 2 × 10\textsuperscript{3} cells/cm\textsuperscript{2} in 24-well plates in 2 ml of medium and incubated at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere. Cells from triplicate wells were harvested at appropriate times and counted using a model Z\textsubscript{3} Coulter Counter (Coulter Electronics, Hialeah, FL).
Assay of Colony Forming Ability—To assess the colony forming ability of the transfected cell lines, single cell suspensions of each cell line were plated in triplicate in 60-mm culture dishes, at densities of 25, 50, 100, and 150 cells/dish, in 6 ml of CHO medium supplemented with 0.5 mg/ml Geneticin (selective medium). Cells were grown for 5 or 10 days and then stained with crystal violet (w/v, 0.9% ethanol) to visualize colonies. Colonies of 50 or more cells were counted.

Construction of pCMV-SSAT—The plasmid pSAT9.3 containing the SSAT cDNA in the Bluescript vector (15) was used as a template for polymerase chain reaction to introduce a BamHI restriction site upstream of the initiation codon and to change the existing BamHI site downstream of the stop codon to a unique NheI site. The resulting product contained the entire SSAT coding region flanked by BamHI and NheI fragments. Sequencing was performed on both strands by the chain reaction-induced mutations and ligated into the same sites of a pCMV-Neo-Bam vector generously provided by Dr. B. Vogelstein (The Johns Hopkins University, Baltimore MD) in which the multiple cloning site had been expanded to include unique AflII and NheI sites. The resulting plasmid was termed pCMV-SSAT.

Transfection of pCMV-SSAT into CHO Cells—Exponentially growing CHO cells were plated at a density of 1 × 10^7 cells/cm^2 and 12–18 h allowed for adherence to the culture plate. For transfection, cells were incubated with LipofectAMINE (20 μg/plate) and 2 μg of plasmid DNA for 6 h in humidified 5% CO2 atmosphere. After 6 h, medium was aspirated, fresh medium (minimum essential medium supplemented with 10% fetal bovine serum) was added, and cultures were incubated for an additional 48 h. Cells were then replated at low density in selective medium and incubated for 12–16 days, at which time individual colonies were selected and transferred to individual wells of a 24-well plate.

Analysis of SSAT Activity and Determination of SSAT Half-life—Exponentially growing cells were plated in triplicate at 5–6 × 10^4 cells/cm^2. Following attachment, the medium was changed, and the cells were incubated for 48 h, unless otherwise indicated. SSAT activity was determined in cellular extracts by an assay which measures the amount of incorporation of radioactivity from [1-14C]acetyl-CoA into [1-14C]acetylspermidine and the resulting product contained the entire SSAT coding region flanked by 31 and 123 noncoding sequences, respectively. The BamHI/NheI fragment was sequenced to confirm the absence of polymerase chain reaction-induced mutations and ligated into the same sites of a pCMV-Neo-Bam vector generously provided by Dr. B. Vogelstein (The Johns Hopkins University, Baltimore MD) in which the multiple cloning site had been expanded to include unique AflII and NheI sites.

RESULTS

Expression of wtSSAT in CHO Cells—In order to establish a cell line that would express increased levels of SSAT activity, CHO cells were stably transfected with pCMV-SSAT, a plasmid coding for human wtSSAT under control of the CMV promoter. Forty-four clones were selected and screened for SSAT activity levels in the absence and presence of BE 3-4-3, which was used to determine whether the SSAT activity of the clone was inducible by a polyamine analogue. Of the 44 clones selected, 13 exhibited significantly greater basal SSAT activity (650–1900 pmol/min/mg protein) than control cells transfected with empty vector (24 pmol/min/mg protein) (Fig. 1). SSAT activity of the same 13 clones was also induced, after 48 h in the presence of 0.5 or 1 μM BE 3-4-3, to levels significantly higher than those induced in control cells under the same conditions (Fig. 1). The clones were numbered sequentially at the time that each was transferred from its initial culture well when confluence had been reached. Therefore, the numbering reflects the relative initial growth rates of the individual clones with clone 1 the fastest and clone 44 the slowest. Twelve of the 13 clones that expressed increased basal levels of SSAT were among the clones numbered 22–44, suggesting that the increased SSAT levels may have had an effect on the initial growth of these clones and slowed the establishment of thriving colonies. The wtSSAT clone 43, which exhibited increased basal SSAT expression both in the absence and presence of the inducing agent, was selected for further studies and is the clonal cell line referred to unless otherwise stated.

Growth Characteristics of the wtSSAT Clone—Since one objective of these studies was to determine whether increased SSAT activity has an effect on cell growth and viability, the colony forming ability and population growth characteristics of the wtSSAT clone were compared with those of control cells containing empty vector. Assessment of the ability to form colonies from single cells revealed that the size of the colonies resulting from growth of the wtSSAT clone, either 5 days (Fig. 2) or 10 days following plating, was considerably smaller than that of colonies formed from control cells. Visual inspection of cell size and cell number per colony indicated that this difference in colony size was a result of cell number per colony and cannot be attributed to differences in the sizes of individual cells between the wtSSAT clone and the control. This indicates a difference in the rate of growth between the two cell lines with growth of the wtSSAT cells slower than the vector control under these clonal conditions. The percent of colonies formed, however, was quite similar for the wtSSAT clone (48 ± 3 at day 5 and 51 ± 8 at day 10) and the vector control (50 ± 8 at day 5 and 55 ± 10 at day 10).

Despite the slower rate of growth of the wtSSAT cells observed in the colony forming experiments described above, there was no detrimental effect of SSAT expression on the growth of exponentially growing cultures of the wtSSAT cells. Populations of untreated wtSSAT clone cells and vector control cells were similarly, with doubling times of 19.2 ± 1.6 and 17.5 ± 0.6 h, respectively and a plateau density for each of 4–5 × 10^5 cell/cm^2.

The presence of higher levels of SSAT also had little effect on cell growth in the presence of 1 μM BE 3-4-3, with cessation of exponential growth between 48 and 72 h and a final density 8–10 times the original cell number for the vector control and
14–17 times the original cell number for the wtSSAT clone. IC<sub>50</sub> values determined following 120-h incubation with BE 3-4-3 were 2\times10^{-10} M and 9\times10^{-9} M for the wtSSAT clone and vector control, respectively, indicating a slight decrease in the sensitivity of the wtSSAT clone to this polyamine analogue.

Two other wtSSAT clones (clones 29 and 41) that had increased levels of basal SSAT activity similar to clone 43 were also tested for colony forming ability, population growth, and sensitivity to BE 3-4-3. Results were similar to those reported for wtSSAT clone 43 (data not shown). These results suggest that an increase in basal SSAT activity from 24 to ~1600 pmol/min/mg protein may have an effect on initial cell growth rates from a single cell but does not adversely affect the growth of the exponentially growing cell populations. In fact, the results suggest that the increased SSAT activity may even render these cells marginally less sensitive to the growth inhibitory effects of the polyamine analogue BE 3-4-3.

Effects of Increased SSAT Expression on Polyamine-related Enzyme Activities and Intracellular Polyamine Levels—To assess the effects of the increased basal SSAT levels on polyamine homeostasis in the wtSSAT clone, intracellular polyamine pools, SSAT activity, and activity of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), two of the polyamine synthetic enzymes, were measured over time (Table I). The SSAT activity of the wtSSAT clone was initially higher than control and rose throughout the 48-h period of the measurements, while that of the control cells remained comparatively low at all time points. ODC activity increased in both the control and wtSSAT clone over time, but was higher in the wtSSAT clone at all times. AdoMetDC activity was initially higher in the wtSSAT clone and increased to a greater extent over time than that of the control. Distinct polyamine pool perturbations were observed at all time points in wtSSAT cells, where putrescine levels were highly increased, spermidine levels were moderately decreased, and...

**Fig. 1.** SSAT activity of CHO clones stably transfected with human wtSSAT. CHO cells were stably transfected with pCMV SSAT and assayed for SSAT activity as described under “Experimental Procedures.” Shown is the SSAT activity following 48-h incubation in the absence of drug (black) or in the presence of 0.5 μM BE 3-4-3 (light gray) or 1 μM BE 3-4-3 (dark gray). V indicates control cells transfected with empty vector. Values represent averages of triplicate determinations.

**Fig. 2.** Colony forming ability of control and wtSSAT cells. To assess colony forming ability, cells were plated in single cell suspensions, incubated for 5 days, and stained to visualize colonies as described under “Experimental Procedures.” Shown is a photograph of representative plates of colonies resulting from growth of control cells transfected with empty vector (left) and wtSSAT cells (right).
TABLE I
Polyamine pools and SSAT, ODC, and AdoMetDC activities in wtSSAT and vector control cells

| Time (h) | Putrescine (nmol/mg protein) | Spermidine (nmol/mg protein) | Spermine (nmol/mg protein) | N³-acetylspermidine (nmol/mg protein) | N⁶-acetylspermidine (nmol/mg protein) |
|---------|-----------------------------|-------------------------------|-----------------------------|--------------------------------------|--------------------------------------|
| Control | 0.85 ± 0.50                 | 0.72 ± 0.40                   | 0.67 ± 0.30                 | 0.06 ± 0.04                          | 0.04 ± 0.03                          |
|         | 24  1.25 ± 0.85             | 1.09 ± 0.72                   | 1.01 ± 0.65                 | 0.88 ± 0.40                          | 0.75 ± 0.30                          |
| wtSSAT  | 48  2.06 ± 1.50             | 1.91 ± 1.30                   | 1.83 ± 1.25                 | 1.71 ± 0.88                          | 1.58 ± 0.80                          |

*ND, not detected.*

Properties and Regulation of Human SSAT

Intracellular polyamine levels, and the activity of SSAT, ODC, and AdoMetDC, were determined as described under “Experimental Procedures,” using cell extracts of exponentially growing control or wtSSAT clone cells harvested at 0, 24, and 48 h. Values are averages ± S.D. of measurements from triplicate cultures of a representative experiment.

**SSAT Half-life and Degradation**—The level of SSAT activity measured in the wtSSAT clone under normal growth conditions was sufficiently high to allow detailed investigation of the degradation of the SSAT protein and its intracellular half-life. Cells were incubated for 48 h, treated with cycloheximide to prevent new protein synthesis, and then harvested for SSAT activity measurements and Western blot analysis to quantitate the amount of SSAT protein. From the assay results (Fig. 3), the SSAT activity half-life was determined to be 29 min and the SSAT protein half-life was 26 min. These data indicate that, in the noninduced state, the SSAT protein is short-lived and that decreases in protein are responsible for and directly proportional to reductions in enzyme activity.

Preliminary indications that SSAT may be degraded via the ubiquitin/proteasomal pathway have been published based on in vitro studies of the breakdown of SSAT protein synthesized in a coupled transcription/translation system in reticulocyte lysates (19). We have now found that a specific inhibitor of the 26 S proteasome, MG 132 (20), has a profound effect on SSAT degradation in this system (Fig. 4). In the absence of the proteasomal inhibitor, SSAT was rapidly degraded and higher molecular weight bands of labeled protein, consistent with ubiquitination of SSAT, appeared at 15 min and remained present through 1 h. In the presence of 50 μM MG 132, the rate of degradation of SSAT was reduced and the SSAT-ubiquitin bands were visible throughout the 3-h period of the assay (Fig. 4).

To confirm that intracellular degradation of SSAT occurs through the proteasome complex as suggested by the in vitro data, two types of experiments were carried out using the transfected CHO cells. First, SSAT activity and SSAT protein amounts were assessed in the presence of 25 μM MG 132 added concurrently with cycloheximide so that the effect of the proteasomal inhibitor on the intracellular half-life of SSAT could be determined (Fig. 5). In the presence of MG 132, the SSAT activity and protein amounts were directly proportional (Fig. 5), as they were in the absence of the inhibitor (Fig. 3). The SSAT activity and SSAT protein half-lives were each extended significantly from 29 min to >300 min in the presence of the proteasomal inhibitor.

Second, the cellular SSAT activity was assessed at the beginning and end of a 4 h incubation in the absence or presence of 10, 25, and 50 μM MG 132. With no drug present, SSAT activity increased only slightly from 1338 ± 140 to 1587 ± 140

N³-acetylspermidine and N⁶-acetylspermidine were present in measurable quantities.

**Fig. 3.** SSAT activity and SSAT protein levels in the absence of new protein synthesis. Cells of the wtSSAT clone were grown for 48 h, 200 μM cycloheximide was added to stop protein synthesis, and cells were harvested at the times shown for assay of SSAT activity (A) and Western analysis (B) as described under “Experimental Procedures.” In A, the circles represent SSAT activity, and the squares represent relative amounts of SSAT protein as determined from the Western analysis. SSAT activity values represent mean ± S.D. (n = 15). For the Western analysis, 150 μg of protein were loaded in each lane.
pmol/min/mg protein after 4 h. In contrast, SSAT activity was increased significantly to 4805 ± 383, 4668 ± 113, and 4182 ± 430 pmol/min/mg protein, following 4-h exposure to 10, 25, or 50 μM MG 132, respectively. These results correlate with the reduced protein degradation in the presence of MG 132 observed with the SSAT half-life determination. Together, these data indicate that cellular SSAT degradation is occurring through proteasomal action with loss of protein and enzyme activity occurring simultaneously.

Effects of BE 3-4-3 on SSAT Expression and Activity—Having established SSAT parameters under noninducing conditions, it was possible to investigate changes in SSAT regulation during induction by the polyamine analogue BE 3-4-3, one of the most potent SSAT-inducing agents known. SSAT activity and protein expression were assessed in crude extracts of the wtSSAT clone and vector control following 48-h incubation with BE 3-4-3 concentrations ranging from 0 to 25 μM. Cells of the wtSSAT clone exhibited basal SSAT activity of ~700 pmol/min/mg protein, a 2-fold increase in SSAT activity was noted at 0.1 μM BE 3-4-3, the lowest concentration tested, and there was a steep concentration-dependent increase of activity to a maximum of ~60,000 pmol/min/mg protein reached after exposure to 5 μM BE 3-4-3 (Fig. 6A). Western blot analysis indicated a concentration-dependent increase of SSAT protein, which correlated directly with the observed SSAT activity increase (Fig. 6B). Although the SSAT activity was much lower in the control CHO cells (basal SSAT activity of ~25 pmol/min/mg protein), similar effects of BE 3-4-3 were observed. There were SSAT activity increases of 1.5-, 2-, and 7-fold following exposure to 0.1, 0.25, and 0.5 μM BE 3-4-3, respectively. The SSAT activity increase was much greater after exposure to 1 μM BE 3-4-3, and the maximum activity of ~10,000 pmol/min/mg protein was reached at 2 μM BE 3-4-3 (Fig. 6A). The SSAT protein of the control was detectable only in extracts from cells exposed to ≥1 μM BE 3-4-3 where a concentration-dependent increase of SSAT protein directly correlating with the SSAT activity increase was observed (Fig. 6B). These data indicate that the effects of BE 3-4-3 on SSAT regulation are similar for the control and the wtSSAT cells.

Effect of BE 3-4-3 on SSAT Half-life and mRNA Levels—The rapid and large increase of SSAT protein and activity in the presence of BE 3-4-3 may result from the effects of the analogue at one or more levels of SSAT regulation. To investigate whether this polyamine analogue was exerting an effect on the longevity of the SSAT protein, the half-life of SSAT in the presence of BE 3-4-3 was determined using two different protocols. First, cells of the wtSSAT clone were incubated in the presence of 0.5 μM BE 3-4-3 for 48 h, at which time cycloheximide was added and cells were harvested at several time points for SSAT activity measurements and half-life determination. Second, cells were incubated in drug-free conditions for 48 h, at which time 0.5 μM BE 3-4-3 was added concurrently with cycloheximide, and cells were harvested at several times for
determination of SSAT activity and half-life. The presence of BE 3-4-3 for 48 h prior to the addition of cycloheximide resulted in significant lengthening of the SSAT half-life to 200 min as compared with 29 min in the absence of the polyamine analogue (Fig. 7). Concurrent addition of the analogue with the inhibitor of new protein synthesis also resulted in significant lengthening of the SSAT half-life from 29 to 85 min (Fig. 7). These data indicate that interaction between BE 3-4-3 and existing protein prevents degradation of the enzyme and prolongs its period of activity.

To investigate the regulation of SSAT at the level of SSAT message, steady state SSAT mRNA levels were determined in control and wtSSAT cultures which had been exposed either to no drug or varying concentrations of BE 3-4-3 for 48 h. In the absence of the inducing agent, the SSAT message level of the wtSSAT clone was 5.7 \( \pm \) 2.8 (\( n = 9 \)) times greater than that of the control cells, indicating increased SSAT transcription with the CMV promoter. Thus, in the noninduced state an \(-6\)-fold increase in SSAT mRNA (described above) resulted in a \(-28\)-fold SSAT activity increase (Fig. 6). The effect of BE 3-4-3 on the SSAT message was similar in the two cell lines with SSAT mRNA increased only slightly over that in each cell line in the absence of the polyamine analogue. At BE 3-4-3 concentrations of 1, 5, and 10 \( \mu M \), in a representative experiment, the SSAT message increases were 1.6-, 2.9-, and 2.4-fold, respectively, for the control, and 1.2-, 2.1-, and 2.0-fold, respectively, for the wtSSAT clone. Therefore these data indicate that the \(-3\)-fold increases in steady state levels of SSAT mRNA in the presence of BE 3-4-3 are far too small to fully account for the \(\approx90\)-fold concentration-dependent induction of SSAT activity mediated by BE 3-4-3 in either the control or the wtSSAT clone.

**DISCUSSION**

Although there is abundant evidence that SSAT plays a very important role in polyamine metabolism in mammalian cells (1, 7, 21), details of the mechanisms of SSAT regulation are not well understood. One of the difficulties inherent to the study of SSAT has been that the very low cellular levels of this enzyme have prevented accurate assessment of its properties under normal cellular conditions. Information related to SSAT properties and regulation has come largely from studies of the enzyme following exposure to agents that induce its activity, often quite dramatically, and that may alter its regulation. Additionally, effects on cell growth and survival often result from the use of inducing agents, and it has been difficult to assess which effects are directly attributable to the increased SSAT activity. The SSAT protein has been expressed at high levels in bacterial systems (22); however, stable overexpression in mammalian cells has not previously been achieved. The successful stable transfection and overexpression of the SSAT
protein in CHO cells reported here has now enabled study of the properties and regulation of SSAT without the use of an inducing agent. Furthermore, the SSAT activity of all of the clones exhibiting increased basal levels of SSAT was highly inducible by BE 3-4-3, a characteristic that provided the basis for assessing changes in SSAT regulation during its induction.

The basal level of SSAT activity achieved in the clones transfected with SSAT cDNA, while substantially greater than the endogenous level in CHO cells, was still modest compared with SSAT activity levels observed in many cell lines in the presence of an inducing agent. However, it should be noted that many of the potent SSAT inducers are either substrates or inhibitors of the enzyme. Therefore, the extent to which such induction alters SSAT activity toward endogenous polyamines is not clear.

The increased basal level of SSAT in the transfected CHO cells (from 24 to ~1600 pmol/min/mg protein) clearly caused altered polyamine homeostasis with perturbations in the polyamine distribution that were consistent with increased flux through the catabolic pathways. Putrescine levels were highly increased, spermidine was decreased, and N1-acetylspermidine accumulated. The polyamine synthetic enzymes ODC and AdoMetDC are responsible for maintaining intracellular spermidine and spermine levels, and the observed increases in the activities of these enzymes would function to restore the polyamine pools depleted by SSAT mediated polyamine catabolism. These compensatory actions prevent depletion of the polyamine pools and may partially explain why there is little effect on growth of the wtSSAT clone. Polyamine depletion has been implicated in cytostasis and cytotoxicity resulting from polyamine analogues, even when SSAT activity is only moderately induced (14, 21, 24). However, in those instances, ODC was also down-regulated by the analogue and therefore no increase in polyamine synthesis occurred to replenish the pools. There was also an increase in the total intracellular polyamine content in the wtSSAT clones, a cellular parameter that is usually tightly controlled by the actions of the polyamine metabolic enzymes. The production of detectable levels of N9-acetylspermidine, which is unlikely to be produced directly by SSAT since SSAT is highly specific for the formation of N1-acetylspermidine (25), may be a cellular response to limit the total polyamine level since the only known fate of N9-acetylspermidine is de-acetylation or excretion from the cell.

The profound effects on cell growth brought about by exposure to polyamine analogues that are powerful inducers of SSAT has raised the question of whether increased SSAT activity is in itself detrimental to cell growth and survival. The current data suggest that increased SSAT activity may at least retard cell growth from the single cell stage, since the majority of the clones that exhibited increased SSAT activity were among the slowest growing of all the clones selected, no clones exhibiting extremely high levels of SSAT activity were isolated, and the wtSSAT clone 43 grew more slowly under clonal conditions. The fact that the population growth characteristics of the clones expressing increased SSAT activity were similar to the control cells suggests that the observed increase of SSAT activity does not adversely affect the growth of exponentially growing cell populations. This latter observation is consistent with the recent findings of Alhonen et al. (21) that primary fetal fibroblasts, derived from transgenic mice overexpressing SSAT and which express a 20-fold increase in SSAT activity, grew similarly to primary fibroblasts derived from nontransgenic counterparts. The current finding that the wtSSAT clone was actually marginally less sensitive to growth inhibition by BE 3-4-3 than the control cells, however, differs from the observation in that study where the SSAT overexpressing fetal fibroblasts displayed an increased sensitivity to a polyamine analogue.

The increase in SSAT expression in the transfected CHO cells was clearly due to an increase in mRNA content. There was an approximately 6-fold increase in mRNA levels in the uninduced state. This increase is probably due to the greater strength of the CMV promoter compared with the endogenous SSAT promoter, but an increased gene copy number in the transfected cells may also contribute. The observation that the magnitude of the SSAT activity increase is greater than the SSAT mRNA increase suggests translational regulation of SSAT. Regulation at the level of translation has been demonstrated for both of the polyamine synthetic enzymes ODC and AdoMetDC (26), and this may be a common feature of the polyamine-related enzymes. Since the SSAT derived from the transfected plasmid construct does not contain the complete 5'- and 3'-UTR regions of the endogenous gene, yet the data suggest translational regulation of SSAT, we can conclude that these regions are not factors in regulation of SSAT translation in the noninduced state. Parry et al. (8) have previously reported that in COS-7 cells, neither the 5'- nor 3'-UTR of SSAT mRNA was important for translational regulation of SSAT by BE 3-4-3.

We have now provided data that substantiate previous esti-
mates (8–10) that, under conditions where the enzyme is not induced, SSAT is a short-lived protein. Our results indicate that under normal cellular growth conditions the SSAT protein has a half-life of 26–29 min and SSAT degradation occurs through the proteasomal complex. SSAT activity is directly proportional to the amount of SSAT protein.

When BE 3-4-3 is present, the degradation of SSAT is inhibited, and this stabilization contributes to the increases in SSAT protein but is far less than is needed to account for the total increase observed. The lengthened SSAT half-life in the presence of the analogue, even in the absence of new protein synthesis, suggests that a direct stabilizing interaction between BE 3-4-3 and the SSAT protein may be one factor contributing to the increased SSAT activity. The results indicate that the SSAT half-life in the presence of BE 3-4-3 is increased from 29 min to greater than 200 min.

The presence of BE 3-4-3 also slightly increases the amount of SSAT mRNA in a concentration-dependent manner, but this increase is also far too small to account for the massive increase in SSAT activity. Therefore, we can conclude that transcriptional regulation is not a major factor in the observed SSAT induction. The small increase in SSAT message observed may result from stabilization of the SSAT mRNA and a lengthening of the SSAT mRNA half-life. Such an effect has been observed by Fogel-Petrovic et al. (6), where they reported an increase of SSAT message half-life from 17 to 64 h in the presence of BE 3-4-3. The similarity of the SSAT message increase for both the control and the wtSSAT clone would indicate that if such stabilization is occurring, then it does not involve the portions of the 5'- and 3'-UTRs, which are not present in the wtSSAT clone.

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