Suppression of Exogenous Gene Expression by Spermidine/Spermine \(N^1\)-Acetyltransferase 1 (SSAT1) Cotransfection

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Spermidine/spermine \(N^1\)-acetyltransferase 1 (SSAT1), which catalyzes the \(N^1\)-acetylation of spermidine and spermine to form acetyl derivatives, is a rate-limiting enzyme in polyamine catabolism. We now report a novel activity of transiently transfected SSAT1 in suppressing the exogenous expression of other proteins, i.e. green fluorescent protein (GFP) or GFP-eIF5A. Spermidine/spermine \(N^1\)-acetyltransferase 2 (SSAT2) or inactive SSAT1 mutant enzymes (R101A or R101K) were without effect. The loss of exogenous gene expression is not due to accelerated protein degradation, because various inhibitors of proteases, lysosome, or autophagy did not mitigate the effects. This SSAT1 effect cannot be attributed to the depletion of overall cellular polyamines or accumulation of \(N^1\)-acetylspermidine (\(N^1\)-AcSpd) because of the following: (i) addition of putrescine, spermidine, spermine, or \(N^1\)-AcSpd did not restore the expression of GFP or GFP-eIF5A; (ii) depletion of cellular polyamines with \(\alpha\)-difluoromethylornithine, an inhibitor of ornithine decarboxylase, did not inhibit exogenous gene expression; and (iii) \(N^1\),\(N^{11}\)-bis(ethyl)norspermine caused a drastic depletion of cellular polyamines through induction of endogenous SSAT1 but did not block exogenous gene expression. SSAT1 transient transfection did not affect stable expression of GFP, and stably expressed SSAT1 did not affect exogenous expression of GFP, suggesting that only transiently (episomal) expressed SSAT1 blocks exogenous (episomal) expression of other proteins. SSAT1 may regulate exogenous gene expression by blocking steps involved in transcription/translation from an episomal vector by targeting non-polyamine substrate(s) critical for this pathway.

The polyamines, putrescine, spermidine, and spermine, are naturally occurring polycations that are essential for cell proliferation (1). They regulate cellular activities at transcriptional, translational, and post-translational levels. Normally, polyamine homeostasis is maintained by highly regulated mechanisms involving control of biosynthesis, catabolism, and transport. Overaccumulation of polyamines has been associated with cell transformation or apoptosis, whereas their reduction/depletion causes inhibition of cell growth, migration, and embryonic development. Deregulation of cellular polyamines or polyamine pathway enzymes has been associated with pathological conditions, including cancer, and polyamine pathways have been targeted for cancer chemotherapy and chemoprevention.

Spermidine/spermine \(N^1\)-acetyltransferase 1 (SSAT1 or SAT1), the key enzyme in the metabolism of polyamines, catalyzes acetylation of spermidine or spermine at the \(N^1\) of the aminopropyl moiety (for a review see Ref. 2). The \(N^1\)-monooctylated spermidine or spermine can be oxidatively degraded by \(N^1\)-acetylpyrolysine oxidase to \(N^1\)-acetylaminoacetaldehyde and a smaller polyamine, reversing the biosynthetic reactions. The acetylated polyamines may also be directly excreted from cells. Thus induction/activation of SSAT1 leads to a decrease in cellular spermidine and spermine with an increase in putrescine and \(N^1\)-acetylpolypamines. The SSAT1 level is normally very low, but it can be induced rapidly by a variety of stimuli, including polyamines, polyamine analogs, toxic chemicals, certain drugs, and growth factors.

SSAT1 is highly regulated at multiple levels, including transcription, mRNA processing, mRNA translation, and protein stability/degradation (2). Polyamines and their analogs induce SSAT1 (3) by increasing the transcription rate, the stability of SSAT mRNA, and its translation (4). Furthermore, polyamines and analogs bind to the SSAT1 protein and prevent its ubiquitination and degradation by the 26 S proteosome (5–7). Thus, the half-life of SSAT1 (normally \(\leq 30 \text{ min}\)) is increased to \(\geq 12 \text{ h}\) in the presence of polyamine analogs. A polyamine analog, \(N^1\),\(N^{11}\)-bis(ethyl)norspermine (BENSpm) (8), causes a dramatic induction of SSAT1, depletion of cellular polyamines, and inhibition of growth in certain cancer cell lines (9, 10).

SSAT2 is related to SSAT1 with 46% sequence identity and 64% sequence similarity (11). Although it was initially described as SSAT2 due to its sequence similarity to SSAT1, it has a very limited ability to acetylate polyamines in vitro. The overexpression of SSAT2 neither increased acetylated polyamines nor altered cellular polyamine levels, disputing its role in the regulation of polyamines in cells. Instead, SSAT2 has high activity with thia-

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2 The abbreviations used are: BENSpm, \(N^1\),\(N^{11}\)-bis(ethyl)norspermine; \(N^1\)-AcSpd, \(N^1\)-acetylspermidine; GFP, green fluorescent protein; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase; DFMO, \(\alpha\)-difluoromethylornithine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; CHO, Chinese hamster ovary.

3 The online version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 - 4.

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lysine as a substrate and thereby was renamed thialysine acetyltransferase (11).

Studies with SSAT1 gene knock-out and transgenic animals (12) suggest a role for SSAT1 in calorie consumption, fatty acid metabolism (13–15), and in pathological conditions such as pancreatitis (16), obesity, and diabetes (13–15). SSAT1 has also been implicated in cancer, for its growth-suppressive effects through depletion of polyamines, and also for its transforming effects due to increased tissue putrescine or due to reactive oxygen species generated from oxidation of acetylpolyamines (12, 17). In addition to its role in polyamine catabolism, several other functions of SSAT1 have been proposed. SSAT1 has been proposed to be involved in integrin-mediated cell migration by its binding to the α9β1 integrin (18), which functions in conjunction with the potassium channel Kir4.2 modulated by local polyamine concentrations (19). SSAT1 was also found to be associated with a diamine exporter SLC3A2 (20) and may enhance the secretion of acetylpolyamines through this exporter. Both SSAT1 and SSAT2 (thialysine acetyltransferase) were implicated in the regulation of HIF-1 exporter. Both SSAT1 and SSAT2 (thialysine acetyltransferase) were implicated in the regulation of HIF-1α by promoting its ubiquitination and degradation by the 26S proteasome (21, 22). Although these effects seemed to be dependent on the active form of the enzyme, it is unclear whether they are consequences of acetylation of polyamines and/or other unknown substrates. Besides polyamines, SSAT1 is capable of acetylating another compound, amantadine, a polycyclic aliphatic amine drug, used in prophylaxis (23). It also acetylates its own Lys26 residue (24) and the hypusine/deoxyhypusine residue of eIF5A3, suggesting its potential role in acetylation of other target proteins.

In this study, we report a novel activity of SSAT1 in the repression of exogenous expression of other proteins, including GFP, GFP-eIF5A, deoxyhypusine synthase (DHS), and deoxyhypusine hydroxylase (DOHH) upon transient cotransfection. The effects of SSAT1 cotransfection were dependent on its acetylation activity. However, they do not appear to depend on acetylpolyamines or on general depletion of cellular polyamine levels. These findings suggest that SSAT1 exerts a thus far unknown effect, blocking exogenous gene expression from cDNA vectors, in an acetylation activity-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**

[4,5-3H]Leucine (60 Ci/mmol) and [5,6-3H]uracil (60 Ci/mmol) were purchased from PerkinElmer Life Sciences. Lipofectamine 2000, precast NuPAGE (BisTris) gels, the associated buffers, and ThermoScript reverse transcription-PCR kit were purchased from Invitrogen, and ECL Plus Western blotting detection system was from GE Healthcare. The SSAT1 antibody was purchased from Santa Cruz Biotechnology; human eIF5A-1 antibody was purchased from BD Biosciences. FLAG antibody, polyamines (putrescine, spermidine, and spermine), protease inhibitors (phenylmethylsulfonyl fluoride and EDTA), proteosome inhibitor (MG132), lysosome inhibitors (NH4Cl and bafilomycin A1), inhibitors of autophagy (3-methyladenine, wortmannin, and LY294002), nuclease inhibitor (aurintricarboxylic acid), and Jumpstart™ Redtag PCR ready mix were from Sigma. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. IQ SYBR Green Supermix was purchased from Bio-Rad. The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. Construction of mammalian expression vectors pCEFL/GFP-eIF5A, pCEFL/DHS, and pCEFL/DOHH was described previously (25–27). BEN5p was synthesized as described previously (8). The CHO cell line stably expressing SSAT1 was kindly provided by Dr. Anthony E. Pegg (Pennsylvania State University). The HeLa cell line stably expressing GFP was a gift from Dr. Mervyn J. Monteiro (University of Maryland Biotechnology Institute), and the pCMV7.1 3×FLAG/HIF-1α vector was from Dr. Gregg L. Semenza (The Johns Hopkins University).

**Methods**

**Construction of pCMV7.1 3×FLAG/SSAT1 and 3×FLAG/SSAT2 Plasmids**—A mammalian vector encoding human SSAT1 or SSAT2 with a FLAG tag at the N terminus was constructed using pCMV7.1 3×FLAG vector. The open reading frames of the human SSAT1 and SSAT2 were PCR-amplified using HeLa cDNA library as a template and using primer sets 5′-ctacagcatggatc ATG GCT AAA TTC GTG ATC CGC CCA GGC ACT GCC GCC-3′ and 5′-ctacagcatgtcgcg TCA CTC CTC TGT TGC CAC CAC CTT TAGCAA GTA CTT CTC G for SSAT1 and 5′-ctacagcatggatcgcg TCA ATG GCT TCC GTG CGG ATC CGA G-3′ and 5′-ctacagcatgtcgcg TCA CTT TCC TGC CAA CTT TCT CG-3′ for SSAT2. The SSAT1 PCR product was inserted at the EcoRI and SalI sites and SSAT2 PCR product at the NotI and SalI sites of the pCMV7.1 3×FLAG vector. The recombinant plasmid was sequenced in both the forward and reverse directions to confirm the accuracy of PCR amplification and insertion of the SSAT1 and SSAT2 open reading frames.

**Site-directed Mutagenesis**—FLAG-SSAT1 mutant constructs (R101A, R101K, and additional mutants) were generated from the pCMV7.1/FLAG-SSAT1 vector using the QuikChange site-directed mutagenesis kit and the primer sets designed for the intended mutations. The primer sets for individual mutations are as follows: R101A, 5′-GTG ATG AGT GAT TAT GCA GCC TTT GCC ATA GGA TCA-3′ and 5′-TCC TAT GCG AAA GCC TGG TGC ATC ACT CAT CAC GAA-3′; R101K, 5′-GTG ATG AGT GAT TAT AAA GCC TTT GCC ATA GGA TCA-3′ and 5′-TCC TAT GCG AAA GCC TTT ATA ATC ACT CAT CAC GAA-3′. The introduction of the correct mutation was confirmed by sequencing the entire open reading frame in both directions.

**Transfection and Fluorescence Microscopy**—The HeLa, HEK293T, COS-7, A549, mouse fibroblast NIH3T3, and CHO cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. For transient transfection, cells were transfected with the indicated expression construct using Lipofectamine 2000. Various inhibitors were added after 5 h of Lipofectamine treatment at the time of medium change. One or 2 days after transfection, GFP fluorescence was directly detected with an Axio-phot microscope (Zeiss).
Western Blotting—Transfected cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture. Proteins (~20 μg) were separated by SDS-PAGE (4–12% gel) in MES buffer and transferred to 0.2-μm nitrocellulose membrane for immunodetection using primary and secondary antibodies, and signals were detected with an ECL Plus chemiluminescence reagent.

Polyamine Analysis in Cells—Cells (60-mm dishes) were washed and harvested. 0.1 ml of cold 10% trichloroacetic acid was added to the cell pellets. 50 μl of the trichloroacetic acid supernatant was used for polyamine analysis by ion exchange chromatographic separation as described previously (28).

RESULTS

SSAT1 Suppresses Exogenous Gene Expression of GFP-eIF5A, GFP, DHS, and DOHH—In the course of a study on the acetylation of eIF5A by SSAT1, we observed a dramatic reduction in the exogenous expression of eIF5A (nontagged, FLAG-tagged, or GFP-tagged) upon transient co-transfection with an SSAT1 cDNA vector. Expression of GFP or GFP-eIF5A was markedly reduced in HeLa cells upon cotransfection with the FLAG-SSAT1 vector, as shown by the GFP fluorescence (Fig. 1A) and also by Western blot (Fig. 1B). Exogenous expression of Flag-SSAT1 itself from a cDNA vector was extremely low (Fig. 1B, bottom panel, lanes 2 and 5, upper arrow), consistent with the difficulties of overexpression of SSAT1 in human cells by transfection using a cDNA vector (9, 29). Only a moderate reduction in cellular polyamines (spermidine by ~50% and spermine by ~30%) was observed in the SSAT1-transfected cells (Table 1). It is noteworthy that such a striking inhibition of the expression of GFP or GFP-eIF5A occurred with a low level of FLAG-SSAT1 expression that caused a relatively small reduction in overall cellular polyamines.

We have compared FLAG-SSAT1 and FLAG-SSAT2 for their effects on expression of the two reporter proteins GFP and GFP-

### Table 1

| Treatment                  | Ptc (nmol/mg protein) | Spd (nmol/mg protein) | Spm (nmol/mg protein) |
|----------------------------|-----------------------|-----------------------|-----------------------|
| 1. Control                 | ND                    | ND                    | 10.27 ± 0.65          |
| 2. GFP                     | ND                    | ND                    | 10.90 ± 0.96          |
| 3. GFP-eIF5A               | ND                    | ND                    | 11.83 ± 11.11         |
| 4. GFP/SSAT1 wild type     | 6.27 ± 0.71           | 0.98 ± 0.21           | 5.30 ± 0.65           |
| 5. GFP/SSAT1 (R101A)       | 9.07 ± 0.78           | 0.32 ± 0.06           | 3.35 ± 0.39           |
| 6. GFP/SSAT2               | ND                    | ND                    | 8.03 ± 0.46           |
| 7. BENSpm                  | 0.83 ± 0.14           | ND                    | 1.53 ± 0.08           |
| 8. GFP/BENSpm              | 0.36 ± 0.05           | ND                    | 2.03 ± 0.21           |
| 9. GFP/SSAT1/BENSpm        | ND                    | ND                    | 2.03 ± 0.21           |
| 10. GFP/SSAT1/Ptc          | 9.60 ± 0.79           | 6.57 ± 0.86           | 10.53 ± 0.92          |
| 11. GFP/SSAT1/Spd          | 13.20 ± 0.99          | 0.23 ± 0.05           | 18.77 ± 1.20          |
| 12. GFP/SSAT1/Spm          | 7.53 ± 0.65           | 0.30 ± 0.08           | 14.83 ± 0.60          |
| 13. GFP/SSAT1/N2-AcSpd     | 7.33 ± 0.50           | 0.30 ± 0.08           | 12.77 ± 0.76          |
| 14. GFP/DFMO 1 day         | ND                    | ND                    | 7.73 ± 0.53           |
SSAT1 Suppresses Exogenous Gene Expression

Although SSAT2 is closely related to SSAT1 in sequence, it elicits very low acetylation activity toward polyamines in vitro (11), and its overexpression did not cause noticeable changes in the cellular polyamine pool (Table 1 (11)). In contrast to SSAT1, there was no inhibition of GFP or GFP-eIF5A expression upon cotransfection with the FLAG-SSAT2 vector. FLAG-SSAT2 itself as well as the two reporter proteins were robustly expressed in the cotransfected HeLa cells (Fig. 1, A and B), indicating that the suppressive effect is specific to SSAT1. The SSAT1 cotransfection blocked exogenous expression of not only GFP or GFP-eIF5A but also of DHS and DOHH (Fig. 1C), suggesting that it is a general phenomenon affecting all exogenously expressed proteins.

SSAT1 Activity Is Required for Suppression of GFP or GFP-eIF5A Expression—To determine whether the acetylation activity of SSAT1 is required for suppression of the reporter protein expression, we first compared the effects of the two SSAT1 mutants R101A and R101K with the wild type enzyme. From the crystal structure (24) and earlier mutagenesis study (30, 31), Arg101 is known to be important for the binding of acetyl-CoA. Substitution of this residue with Ala (or Lys) caused loss of activity to less than 5% of the wild type enzyme. There was only a small reduction in the GFP or GFP-eIF5A expression levels upon coexpression with either of the SSAT1 mutant enzymes (Fig. 2, A and B), indicating that the acetylation activity of SSAT1 is crucial for the SSAT1 effects. We further tested additional mutant enzymes, with mutation at the catalytic site (Y140A and Y140F) (24), and those with high Km values for spermidine (30), E152K, R155A, H26A, L56F (32), L56A, and E152K/R155A. None of these SSAT1 mutant enzymes effectively blocked the expression of GFP (data not shown), indicating that the SSAT1 activity is required for the effect. Interestingly, like SSAT2, the SSAT1 mutant enzymes were expressed at high levels, whereas the wild type SSAT1 protein was hardly detectable (Fig. 2B, bottom panel). The difference in the cellular levels of wild type and mutant enzymes is probably not due to differences in the stability of these enzymes per se, because no significant differences in their stability were observed in vitro when the enzymes were generated using the TnT transcription/translation system (6, 30). These data suggest that the SSAT1 activity is critical for suppression of exogenous expression of itself as well as other cotransfected genes.

There is no apparent specificity regarding the cell lines, because similar effects were observed in HeLa, HEK293T, A549, COS-7 and mouse fibroblast NIH3T3 cells (supplemental Fig. 1). The effect was not dependent on the promoter specificity, because GFP-eIF5A (under EF-1α promoter) and FLAG-eIF5A (under cytomegalovirus promoter) were equally affected by the SSAT1. Similar suppressive effects were observed using different transfection methods such as Lipofectamine, electroporation, or calcium phosphate (data not shown).

Low Expression Level of GFP or GFP-eIF5A in the SSAT1-cotransfected Cells Is Not Due to Proteolytic Degradation—The short half-life of the SSAT1 (~30 min) has been attributed to its degradation by the 26S proteosome (5). Furthermore, both SSAT1 and SSAT2 were reported to bind and promote the ubiquitination and proteosomal degradation of HIF-1α (21, 22). Therefore, we wondered whether the reduced expression level of GFP or GFP-eIF5A is due to accelerated proteolytic degradation mediated by SSAT1. Addition of the proteosome inhibitor MG132 or other protease inhibitors, including phenylmethylsulfonyl fluoride and EDTA, did not restore the expression level of GFP (data not shown) or GFP-eIF5A (Fig. 3) in the SSAT1-cotransfected cells. Neither the lysosome inhibitors (NH4Cl or bafilomycin) nor the autophagy inhibitors (3-methyladenine, LY294002, or wortmannin) showed any effect (Fig. 3). These compounds had little or no effect on GFP expression in cells not cotransfected with SSAT1 (data not shown). Thus, it is unlikely that the loss of the GFP or GFP-eIF5A expression was caused by the SSAT1-mediated proteolytic degradation.

The suppressive effect is not dependent on cotransfection of two cDNA vectors at the same time. We performed sequential transfection experiments in which SSAT1 and GFP (or GFP-eIF5A) were transfected on 2 consecutive days, and cells were observed on day 3. When SSAT1 was transfected on day 1 and GFP on day 2, GFP expression was almost completely blocked (supplemental Fig. 2). On the other hand, when GFP was transfected on day 1 and SSAT1 on day 2, SSAT1 transfection did not abolish GFP expressed prior to the SSAT1 transfection (supplemental Fig. 2). If SSAT1 were to exert its effect by enhancing proteolytic degradation of GFP, GFP levels would be diminished even when SSAT1 was transfected 1 day after GFP transfection. These findings again argue against the role of SSAT1 in proteolytic degradation of GFP, in accordance with the lack of effects of protease inhibitors (Fig. 3).

To determine whether the SSAT1-mediated suppression of GFP or GFP-eIF5A expression involves nuclease-mediated degradation of reporter genes, we tested a general nuclease inhibitor aurintricarboxylic acid. Aurintricarboxylic acid did not enhance GFP-eIF5A expression in the SSAT1-cotransfected cells (supplemental Fig. 3), suggesting that nuclease activation is probably not involved in the inhibition of exogenous gene expression.
Induction of Endogenous SSAT1 by BENSpm Does Not Block Expression of GFP and GFP-eIF5A—The polyamine analog BENSpm is a potent inducer of SSAT1 at the transcriptional and translational levels (4), and it also stabilizes SSAT1 by its binding to the enzyme and by preventing ubiquitination and degradation of SSAT1 (7). Thus, we examined the effects of BENSpm on the GFP expression and cellular polyamine levels. Consistent with previous reports, BENSpm (at 10 μM) alone caused a large induction of the endogenous SSAT1, normally not detectable by Western blotting (Fig. 4B, compare lane 5 with 1 and lane 6 with 2) and depletion of each of the cellular polyamines (Table 1) to less than 10% of the control levels. Despite a strong induction of endogenous SSAT1, BENSpm did not block expression of GFP or GFP-eIF5A (compare panel 5 with 1 and panel 6 with 2 in Fig. 4A), indicating that the suppressive effect is not simply mediated by the increased level of the SSAT1 protein in cells. Treatment of cells transfected with the FLAG-SSAT1 vector with BENSpm caused an even more dramatic increase in both the endogenous and the exogenous SSAT1 (Fig. 4B, lanes 7 and 8) and nearly complete depletion of cellular polyamines (Table 1). Despite a massive induction of both endogenous and exogenous SSAT1 in these cells, little difference in the level of GFP or GFP-eIF5A was observed (Fig. 4A, compare panel 7 with 1 and panel 8 with 2). Instead of augmenting the effect of SSAT1 transfection, BENSpm reversed the suppressive effects by restoring the level of GFP expression (Fig. 4A, compare panel 7 with 3 and panel 8 with 4).

The observed depletion of polyamines in the BENSpm-treated cells indicates that the induced SSAT1 was able to acetylate polyamines effectively in cells (Table 1). Although BENSpm may inhibit SSAT1 in cells by competing with polyamines, its affinity for SSAT1 is much lower than that of polyamines, permitting effective acetylation and depletion of intracellular polyamines. Because BENSpm did not cause suppression of the GFP expression while effectively depleting cellular polyamine pools, the loss of GFP expression cannot be attributed to the depletion of overall natural polyamines. Thus, the effects may be mediated by acetylation of a different, as yet unknown, target substrate. If this is the case, it is possible that BENSpm effectively inhibits acetylation of this non-polyamine substrate (low affinity) leading to suppression of exogenous gene expression, although not blocking acetylation of polyamines (high affinity substrate).

Suppression of Exogenous Gene Expression by SSAT1 Is Not Mediated by Depletion of Cellular Polyamines—The strong expression of GFP and GFP-eIF5A in BENSpm-treated, polyamine-depleted cells (Fig. 4) suggests that depletion of overall polyamine is not responsible for suppression of exogenous gene expression.
expression. We further investigated the potential role of polyamines and acetylpolyamines on the suppression of GFP expression. GFP expression was blocked when the expression level of FLAG-SSAT1 protein was low (Fig. 1B), and changes in overall polyamine pools were relatively small (Table 1). Addition of putrescine, spermidine, or spermine (20 μM each) to the GFP-eIF5A-transfected cells or to the SSAT1-cotransfected cells did not increase the levels of GFP-eIF5A expression (Fig. 5A). Addition of putrescine, N1-AcSpd, spermidine, or spermine did not significantly increase polyamine levels of the SSAT1-transfected cells (Table 1). N1-AcSpd, which is normally undetectable (in control cells), accumulated in the SSAT1-transfected cells at the level of ~6.3 nmol/mg protein, similar to that of spermidine. No or little accumulation of N1-AcSpd was observed in the BENSpm-treated HeLa cells, in contrast to those cells transfected with the FLAG-SSAT1 vector, possibly due to an excretion of the acetylated polyamine in the presence of intracellular BENSpm accumulation or due to its oxidation by acetylpolyamine oxidase.

To determine whether the accumulation of N1-AcSpd was responsible for suppression of the reporter protein expression, we examined the effects of accumulation of N1-AcSpd. Addition of N1-AcSpd to the medium of the GFP-transfected cells did not increase the cellular level of N1-AcSpd, probably due to its poor uptake, and did not inhibit expression of GFP. Interestingly, in cells transfected with the SSAT1 mutant R101A (with <5% activity of the wild type enzyme), reduction of cellular spermidine (to ~33% of the control level) and an increase in the N1-AcSpd (~9 nmol/mg protein) were observed (Table 1), changes even greater than in the wild type SSAT1-transfected cells (presumably due to a high level of the mutant enzyme suppression of GFP expression does not correlate with general depletion of cellular polyamines and thereby is not caused by a decrease in the overall polyamine pool.

Exogenously Expressed SSAT1 Does Not Affect Endogenous Gene Expression or Stable Expression of GFP—Whereas exogenous expression of GFP or GFP-eIF5A was consistently lost in cells cotransfected with SSAT1, no reduction in the endogenous eIF5A was observed after 48 h of SSAT1 cotransfection (Fig. 1, B and C, Fig. 2B, Fig. 3B, and Fig. 4B). Because eIF5A is a stable protein with a long half-life, we also examined the effects of SSAT1 transfection on a different endogenous protein with a short half-life, namely p53. There was no noticeable change in the level of endogenous p53 in the SSAT1-transfected cells (data not shown). In contrast to a strong suppression of the transient expression of GFP by SSAT1 cotransfection (Fig. 6A, compare panels 1 and 2), transient transfection of SSAT1 had no effect on GFP expression from chromosome-integrated GFP cDNA in a stable HeLa cell line (Fig. 6A, compare panels 3 and 4). Reciprocally, exogenous expression of GFP was not blocked in CHO cells stably overexpressing SSAT1 from a chromosome-integrated cDNA (Fig. 6B, compare panel 2 with 1 and panel 4 with 3 and Western blot signals in lanes 4 and 3). Thus, the suppressive effects seem to be limited to the episomal expression events involving the exogenously expressed SSAT1 and other exogenously expressed proteins. Moreover, the rates of synthesis of RNA and protein in HeLa cells transiently transfected or cotransfected with SSAT1 were similar to those in cells transfected with GFP or GFP-eIF5A, indicating that SSAT1 transfection does not cause global inhibition of the synthesis of endogenous RNAs and proteins (supplemental Fig. 4).
SSAT1 Suppresses Exogenous Gene Expression

Is HIF-1α Protein Level Regulated by SSAT1 in Cells?—It was recently reported that SSAT1 cotransfection reduced the level of FLAG-HIF-1α in 293T cells, and it was proposed that SSAT1 regulates HIF-1α level in cells by its binding to HIF-1α and promoting the proteosomal degradation (21). Because of our results demonstrating an inhibition of expression of cotransfected GFP, we wondered whether the reported reduction in the FLAG-HIF-1α expression level by V5-SSAT1 cotransfection follows the general pattern of suppression of cotransfected genes or is a specific event based on HIF-1α/SSAT1 specific molecular interaction as reported and whether it reflects endogenous cellular regulation. As shown in Fig. 7A, the level of FLAG-HIF-1α was markedly reduced in 293T cells upon transient cotransfection with SSAT1 (Fig. 7A, lanes 4 and 9). Treatment of the cotransfected cells with the proteosome inhibitor MG132 for 6 h prior to harvest enhanced the level of FLAG-HIF-1α in cells transfected with the FLAG-HIF-1α vector alone (Fig. 7A, compare lane 7 with 2 and lane 8 with 3). However, MG132 did not increase the level of FLAG-HIF-1α in the SSAT1 cotransfected cells (Fig. 7A, compare lane 9 with 4), suggesting that proteosome inhibition does not reverse the SSAT1 effects. Induction of endogenous SSAT1 by treatment with BENSpm did not cause significant suppression of expression of FLAG-HIF1-α (Fig. 7A, compare lane 3 with 2 and lane 8 with 7). We further examined the effects of stably overexpressed SSAT1 on FLAG-HIF-1α expression level (Fig. 7B). In this case, no reduction in FLAG-HIF-1α level was observed in CHO cells stably overexpressing SSAT1 compared with a control cell line stably transfected with an empty vector, indicating that the effect observed is confined to exogenous expression of the two genes. These results are entirely consistent with those of the GFP/SSAT1 or GFP-eIF5A/SSAT1 cotransfection studies and suggest that exogenously expressed SSAT1 affects the levels of other exog-

FIGURE 6. Comparison of the effects of transient versus stable transfection of SSAT1 and GFP. A, HeLa cells were transiently transfected with GFP vector alone (panel 1 and lane 1) or cotransfected with FLAG-SSAT1 (panel 2 and lane 2). A HeLa cell line stably expressing GFP (panel 3 and lane 3) was transiently transfected with FLAG-SSAT1 (panel 4 and lane 4). B, CHO cell line stably transfected with empty vector (EVS) was transiently transfected with GFP or GFP-eIF5A vector alone (panels 1–3) or cotransfected with FLAG-SSAT1 vector (panel and lane 5). A CHO cell line stably expressing SSAT1 was transiently transfected with GFP or GFP-eIF5A vector (panels and lanes 2 and 4). Abbreviations used are as follows: GFP, stably transfected with GFP vector; EVS, stably transfected with empty vector pCMV; SSAT1S, stably transfected with the pCMV-SSAT1.

FIGURE 7. Effects of transiently expressed FLAG-SSAT1 (A) and stably expressed SSAT1 (B) on exogenous FLAG-HIF-1α expression. A, 293T cells were transiently transfected with empty vector (lanes 1 and 6) or with FLAG-HIF-1α vector (lanes 2–5 and 7–10) alone (lane 2), cotransfected with SSAT1 (lanes 4, 5, 9, and 10). The transfected cells were treated with BENSpm (lanes 3, 5, 8, and 10) at the time of transfection. Cells were harvested at 48 h after transfection. The parallel samples were treated with MG132 for 6 h prior to harvest (lanes 6–10). B, CHO cells stably transfected with empty vector (lanes 1 and 2) or with the SSAT1 vector (lanes 3 and 4) were transiently transfected with FLAG-HIF-1α (lanes 2–4). Cells transiently transfected with FLAG-HIF-1α vector were treated with MG132 (lane 4) for 6 h prior to harvest at 24 h of transfection.
DISCUSSION

In this study, we report novel effects of SSAT1 transient transfection on the exogenous expression of cotransfected cDNAs. The effect is not limited to GFP or GFP-eIF5A and was observed for any other cotransfected genes, including those for DHS, DOHH, and HIF1-α. Only the exogenously expressed proteins, e.g. GFP-eIF5A or FLAG-eIF5A, were drastically reduced in the SSAT1-transfected cells, although the levels of endogenous eIF5A and other endogenous cellular proteins (p53 or actin) were not significantly altered. The increase in the endogenous SSAT1 by treatment with BENSpm or stable transfection of SSAT1 did not elicit inhibition of expression of GFP or GFP-eIF5A. It seems to be a general phenomenon of SSAT1 transient transfection, observed regardless of cotransfected genes, transfection methods, cell lines, or promoter of the vectors used. These findings suggest that exogenously expressed SSAT1 affects one or more steps in exogenous gene expression from cDNA plasmid transfection as follows: the transport of the plasmid to the nuclei, its transcription from the episomal vector, transport, and translation of its mRNA independently of endogenous gene expression pathways.

The SSAT1-mediated suppression of other exogenously coexpressed proteins does not appear to be due SSAT1-mediated proteolytic degradation, as was proposed for HIF-1α (21). SSAT1 itself is rapidly degraded by polyubiquitination–dependent proteosomal degradation (5). However, the proteosome inhibitor MG132 or other protease inhibitors did not enhance the level of GFP or GFP-eIF5A in the SSAT1-cotransfected cells. The SSAT1 effect is not limited to specific genes and apparently is not dependent on specific protein–protein interactions, because SSAT1 suppresses expression of any cotransfected genes, including GFP, GFP-eIF5A, DHS, DOHH, and HIF-1α. Very consistent effects of SSAT1 cotransfection were observed for all these coexpressed proteins, suggesting a general mechanism involving the exogenously expressed SSAT1 on the exogenous expression pathway. It is important to note that the previously reported studies linking SSAT1 expression and decreased HIF-1α levels were also performed as transient coexpression experiments (21). Although we observed a consistent reduction of HIF-1α upon SSAT1 cotransfection, the effect was not reversed by addition of the proteosome inhibitor MG132 (Fig. 7A). Furthermore, stably overexpressed SSAT1 did not decrease the FLAG-HIF-1α level, indicating that the observed effect is limited to that of exogenously expressed SSAT1 on the exogenous expression of HIF-1α. Thus, the effect observed by transient cotransfection, as reported for HIF-1α and SSAT1, does not necessarily reflect the in vivo regulation of endogenous HIF-1α by endogenous SSAT1.

It is intriguing that the SSAT1 effect observed is confined to the transient episomal expression of both SSAT1 and its coexpressed genes. Transiently expressed SSAT1 does not affect stable expression of chromosome-integrated GFP cDNA or any other endogenous genes. Conversely, SSAT1 stably expressed from a chromosome-integrated SSAT1 cDNA does not block transient expression of GFP. It is possible that episomal gene expression may be segregated from the chromosomal gene expression pathway and that exogenously produced proteins produced from transient transfection are processed by a separate pathway from endogenous proteins. In agreement with this notion, we have observed that exogenously produced eIF5A precursor protein is not modified by endogenous DHS and DOHH. Only upon cotransfection with all three vectors encoding FLAG-eIF5A, DHS, and DOHH was the exogenous FLAG-eIF5A modified to the hypusine form (25).

The suppressive effects are dependent on the acetylation activity specific to SSAT1, because SSAT2 did not suppress GFP expression. None of the SSAT1 mutants, including the acetyl-CoA binding site mutants (R101A and R101K) or other mutants, were effective in blocking the expression of GFP (data not shown), indicating that the SSAT1 activity is critical for the effects. Although the SSAT1 effects appear to be dependent on its enzymatic activity, the suppression of GFP or GFP-eIF5A expression did not correlate with the levels of acetylpolypeptide or cellular polyamine pools and thus cannot be attributed to a general depletion of overall cellular polyamines. DFMO treatment, which reduces cellular polyamines to a much greater extent than SSAT1 transfection, did not cause inhibition of GFP expression. Furthermore, BENSpm, which causes a massive induction of endogenous SSAT1 and effective depletion of all three natural polyamines, did not block GFP expression. Nonetheless, we cannot exclude the possibility that exogenously expressed SSAT1 associates with a nuclear transporter complex or machineries of transcription and translation specific for the episomal gene expression pathway and changes local polyamine concentrations, thereby leading to the inhibition. In this case, it is possible that BENSpm fulfills the function of natural polyamines in this pathway.

Alternatively, SSAT1 may target and alter a non-polyamine substrate critical for the exogenous gene expression pathway. Certainly, SSAT1 has been proposed to interact with other cellular proteins. The autoacetylation of its Lys26 and the acetylation of deoxyhypusine/hypusine residue of eIF5A open the possibility for other protein substrates as targets of SSAT1 that may cause a dramatic suppression of exogenous gene expression. Future efforts will be directed toward the identification of such target molecules and the elucidation of the mechanism of SSAT1 action in the exogenous gene expression pathway.

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