THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 39, pp. 28530–28539, September 28, 2007

Polyamine-dependent Regulation of Spermidine-Spermine N^1-Acetyltransferase mRNA Translation*[S]

Received for publication, February 12, 2007, and in revised form, August 9, 2007 Published, JBC Papers in Press, August 9, 2007, DOI 10.1074/jbc.M701265200

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Spermidine-spermine N^1-acetyltransferase (SSAT) is induced in response to an elevation in intracellular polyamine pools. The increased enzyme activity is the result of an increase in gene transcription, mRNA translation, and protein stability. Induction of SSAT by polyamine analogues can lead to intracellular polyamine depletion and apoptosis. The mechanism by which polyamines alter the translational efficiency of SSAT mRNA is not well understood. In this study, we investigated the regulation of SSAT translation by the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM). DENSPM induced expression of both FLAG-tagged SSAT and SSAT fused to *Renilla* luciferase in a time- and concentration-dependent manner. This effect was not inhibited by actinomycin D indicating that changes in gene transcription did not explain the enhanced expression in the presence of DENSPM. Furthermore, because FLAG-SSAT did not contain the 5′- or 3′-untranslated regions of SSAT, translational regulation involved the coding sequence only. By contrast, cycloheximide completely inhibited induction by DENSPM, indicating a requirement for new protein synthesis. Deletion constructs identified two regions of the SSAT protein-coding RNA sequence that conferred polyamine responsiveness. Using these regions as probes in RNA electrophoretic mobility shift assays, we observed specific binding of a cytoplasmic protein. In addition, we found that the interaction between the RNA probes and the binding protein could be inhibited by DENSPM in a concentration-dependent manner. These results suggest that polyamines regulate SSAT mRNA translational efficiency by inhibiting a repressor protein from binding to regions of the coding sequence of the SSAT transcript.

Spermidine-spermine N^1-acetyltransferase (SSAT)[2] is a key enzyme in the degradation of the essential polyamines spermidine and spermine (1, 2). Normally, the intracellular level of SSAT is low, but it can be rapidly induced by elevating intracellular polyamine concentrations (3) or by treating cells with polyamine mimetics such as N^1,N^{11}-diethylnorspermine (DENSPM) or N^1,N^{12}-bis(ethyl)spermine (4, 5). SSAT expression is regulated at several different levels (5, 6). Gene transcription is increased by polyamines via an Nrf-2-dependent pathway, leading to an increase in SSAT mRNA (7). Moreover, polyamines stabilize SSAT mRNA (6) and increase translational efficiency (5). Finally, elevated polyamine levels can lead to a stabilization of the SSAT protein by inhibiting its polyubiquitination and targeting to the proteasome (8). N-terminal substituted polyamine analogues are not substrates for SSAT but appear to mimic the endogenous polyamine and cause an increase in intracellular SSAT activity that can be >1000-fold higher than that in untreated cells. The elevation in SSAT levels leads to a depletion of intracellular polyamines and induction of apoptosis (4). The candidate drug DENSPM is currently under development as an anti-cancer agent (9, 10).

The regulation of SSAT mRNA translation by polyamines is not fully understood. Fogel-Petrovic and coworkers (11) showed that cycloheximide can increase SSAT mRNA in Malme-3 M cells, but removal of the protein synthesis inhibitor was not accompanied by an increase in enzyme activity. However, addition of polyamines led to an increased rate of SSAT translation, suggesting that protein synthesis required polyamines. Similarly, Parry and coworkers (5) reported that N^1,N^{12}-bis(ethyl)spermine increased the amount of SSAT mRNA associated with the protein-synthesizing 80 S monosomes. They also concluded from deletion studies of an SSAT expression construct that the region of the mRNA responsive to N^1,N^{12}-bis(ethyl)spermine was located within the protein coding sequence. Thus, SSAT translation appears to be inhibited at low polyamine concentration, and this inhibition is released when polyamine levels are increased.

SSAT activity is induced by a number of physiological stimuli, including oxidative stress (12), x-ray irradiation (13), insulin-like growth factor-I (14), cytotoxins (15), and heat shock (16). Some of these stimuli do not appear to alter mRNA levels, suggesting that co- and/or post-translational mechanisms predominate. In the present study, we have investigated the translational regulation of the SSAT mRNA by DENSPM using two models, FLAG-tagged SSAT and SSAT fused to luciferase. Sequences located within the protein-coding region, and in close proximity to both the start and stop codons, are essential for the translational induction of SSAT transcript. We provide evidence for the binding of a cytosolic protein to the SSAT mRNA that can be displaced by DENSPM.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[2] The abbreviations used are: SSAT, spermidine-spermine N^1-acetyltransferase; DENSPM, N^1,N^{11}-diethylnorspermine; REMSA, RNA electrophoretic mobility shift assay; UTR, untranslated region; CMV, cytomegalovirus; HRP, horseradish peroxidase; ORF, open reading frame.

EXPERIMENTAL PROCEDURES

Cloning of SSAT 5′-UTR and Coding Region—The SSAT 5′-UTR and various lengths of the coding sequence were cloned upstream and in-frame with the *Renilla* luciferase gene located in the XhoI/XbaI sites of pcDNA3.1 (rLuc). Initially, the SSAT 5′-UTR and the entire coding region except the terminal lysine and stop codon were amplified from a human breast cancer cDNA library (Prof. Peter Leedman, Western Australian Institute for Medical Research) using primers F1 and R1 (see supplemental Table S1 for oligonucleotides used in this study) and cloned into the NheI and BamHI sites of the vector pGL3 (Promega, Madison, WI). It was then removed by digestion with KpnI/BamHI and cloned into the same sites in rLuc such that the SSAT and luciferase sequences were in-frame to generate $rLucSSAT_{510}$. Deletions of the coding region were achieved by amplifying SSAT using a common forward primer F2 and different reverse primers (R2, R3, R4, and R5), which were designed to generate products that contained the entire 5′-UTR only or together with 333, 166, or 68 bases of the coding region, respectively. The resulting constructs were named $rLucSSAT_{UTR}$, $rLucSSAT_{333}$, $rLucSSAT_{166}$, and $rLucSSAT_{68}$ (shown in Fig. 1*B*).

To make FLAG-tagged constructs, the coding region of SSAT was amplified using $rLucSSAT_{510}$ as template and primers F3 and R6. Reverse primer R6 added the terminal lysine and stop codon missing from $rLucSSAT_{510}$. The entire SSAT coding region was cloned into the HindIII and BamHI sites of p3XFLAG-CMV-7.1 expression vector (Sigma) yielding FLAG-$SSAT_{513}$. A series of 3′ deletion constructs was made using the common forward primer F3 and various reverse primers (R7, R8, R9, and R10), which produced products containing the first 504, 498, 482, or 486 bases of the SSAT coding region, respectively. These constructs were named FLAG-$SSAT_{504}$, FLAG-$SSAT_{498}$, FLAG-$SSAT_{492}$, and FLAG-$SSAT_{486}$. A series of 5′ deletion constructs was made using various forward primers (F4, F5, and F6) with the common reverse primer R6, which produced products missing the first 15, 30, or 45 bases of the coding region, respectively. These constructs were named FLAG-$SSAT_{15–513}$, FLAG-$SSAT_{30–513}$, and FLAG-$SSAT_{45–513}$. In addition, FLAG-tagged SSAT constructs were made that contained premature stop codons or base mutations. The primers used to make these constructs are shown in supplemental Table S1, and the location of premature stop codons and base mutations are shown in Fig. 5*B*. Constructs were verified by DNA sequencing.

Cell Culture and Transient Transfections—HeLa (human cervical adenocarcinoma) cells were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 37 °C in an atmosphere of 5% CO_2 in air.

For luciferase studies, cells were seeded at a density of 2.5×10^5 cells/well (24-well plate) and incubated overnight at 37 °C. Cells were cotransfected in the absence of fetal bovine serum with 1 μg of rLucSSAT plasmid and 0.1 μg of the internal control plasmid pGL3-basic (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were treated with up to 100 μM DENSPM (a kind gift from Dr. Carl Porter, Roswell Park Cancer Institute, Buffalo) for a further 24 h. Cells were then washed and assayed for luciferase activity using a Dual Luciferase Kit (Promega). For FLAG studies, cells were seeded and transfected as above with 1 μg of FLAG-SSAT plasmids, unless stated otherwise. Following transfection, cells were incubated overnight at 37 °C, and then treated with 10 μM DENSPM or vehicle for 4 h. In some experiments, cells were treated with actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml). Cells were washed with phosphate-buffered saline and harvested by scraping into 0.4 ml of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM dithiothreitol. Cells were then disrupted on ice by sonication, and the supernatants were cleared by centrifugation for 10 min at $16,000 \times g$ (4 °C). Protein concentrations were determined by the method of Bradford (Bio-Rad), and equal amounts (10 μg) of each sample were separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted using anti-FLAG-M2 HRP monoclonal antibody (Sigma).

MG132 Treatment and Immunoprecipitation—HeLa cells were seeded at a density of 1×10^6 cells/well (6-well plate) and co-transfected with 1 μg of pcDNA3-HA-ubiquitin and 2 μg of either FLAG-$SSAT_{513}$ or FLAG-$SSAT_{504}$ plasmid. After an overnight incubation, cells were co-treated with 10 μM DENSPM and 5 μM MG132 (Calbiochem) or vehicle (Me_2SO) and incubated for a further 16 h. Cells were washed twice with phosphate-buffered saline and harvested by scraping into 0.8 ml of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM dithiothreitol. Cells were then disrupted on ice by sonication, and the supernatants were cleared by centrifugation for 10 min at $16,000 \times g$ (4 °C). Anti-FLAG-M2 monoclonal antibody (10 μg, F 3165, Sigma) was added, and the supernatants were rotated for 2 h at 4 °C. Protein G-Sepharose 4B (P 3296, Sigma) was then added, and the lysates were rotated for a further 1 h at 4 °C. Immunoprecipitates were collected by centrifugation, and the beads were washed three times with phosphate-buffered saline. The recovered proteins were separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted using anti-HA antibody (H 6908, Sigma) according to the manufacturer's instructions.

RNA Extraction, Reverse Transcription, and Real-time PCR—For RNA studies, HeLa cells were seeded at 1×10^6 cells/well (6-well plate) and transfected with 2.5 μg of FLAG-$SSAT_{513}$ plasmid. Cells were then incubated for 4 h in the absence or presence of 10 μM DENSPM, and total RNA was extracted using TRIzol reagent (Invitrogen) as outlined in the manufacturer's instructions. First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) as per the manufacturer's protocol. Reactions lacking reverse transcriptase also were performed to ensure no plasmid DNA contamination. Expression levels of FLAG-SSAT mRNA were quantified using the iCycler iQ Real-time PCR Detection System (Bio-Rad). First strand cDNA was amplified using specific primers for FLAG-SSAT (F10 and R7) or β-actin (F11 and R21). Reactions contained iQ Supermix (Bio-Rad), 6 pmol of each primer, and 1 μl of cDNA in a final volume of 25 μl. Samples were amplified using the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 15 s, and extension at 72 °C

for 30 s. A melting curve was obtained to verify specificity of the PCR. Samples were analyzed by the comparative C_T method.

In Vitro Transcription/Translation—Reaction mixtures contained 1 μg of rLucSSAT$_{510}$ DNA and 40 μl of rabbit reticulocyte lysate (TNT Quick Coupled Transcription/Translation System, Promega), in a total volume of 50 μl. Initially, reactions were incubated at 30 °C for 30 min in the absence of methionine to synthesize transcript. Then, actinomycin D was added to a final concentration of 5 μg/ml to stop the transcription. Reactions were divided into two aliquots containing equal amounts of mRNA, and DENSPM (50 μM) or an equal volume of vehicle was added to each aliquot. The paired reactions were prewarmed, and protein synthesis was initiated by the addition of 20 μM methionine. Aliquots (5 μl) were removed at 0, 5, 10, 15, and 30 min and boiled immediately in Laemmli buffer. Samples were then separated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, immunoblotted using anti-*Renilla* luciferase monoclonal antibody (MAB4410, Chemicon), and quantified by densitometry (Quantity One software, Bio-Rad).

Preparation of Cytoplasmic Extracts—HeLa cells were washed with cold phosphate-buffered saline and scraped into cytoplasmic extraction buffer (10 mM HEPES (pH 7.2), 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 0.2% Nonidet P-40, 1 mM dithiothreitol) containing protease inhibitor mixture (Sigma). Cell extracts were incubated on ice for 20 min and then centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C, and the supernatant was retained. Extract was used immediately or snap-frozen in liquid nitrogen and stored at −80 °C until required. Protein concentrations were determined by the Bradford method (Bio-Rad).

Preparation of RNA Transcripts—Fragments of the SSAT 5′ coding region were amplified by PCR using rlucSSAT$_{510}$ as template and the common forward primer F7 and the reverse primers R16, R17, and R18. PCR products were digested with BamHI and HindIII and cloned into the same sites of pBluescript II KS$^+$ (Stratagene) in the T7 sense orientation, giving the constructs P_{1-42}, P_{1-77}, and P_{1-165}, where the number indicates the number of bases of the SSAT insert (beginning at the start codon). Fragments of the SSAT 3′ coding region were also amplified using the common reverse primer R19 and the forward primers F8 and F9, giving the constructs $P_{433-513}$ and $P_{333-513}$. $P_{333-492}$ was made using forward primer F9 and reverse primer R20. All constructs were then linearized with HindIII and used as templates in *in vitro* transcription reactions. Linearized templates were transcribed using T7 RNA polymerase (Invitrogen) in reactions containing [α-^{32}P]UTP (3000 Ci/mmol, GE Healthcare) as described elsewhere (17). Full-length transcripts were isolated on 6% urea/acrylamide gels, eluted for 3 h at 22 °C in 0.5 M ammonium acetate/1 mM EDTA, and ethanol-precipitated to recover the RNA. Unlabeled RNA transcripts were synthesized as above but with 2.5 mM UTP.

REMSAs—Binding reactions (10 μl) contained 10 μg of HeLa cytoplasmic extract and 100,000 cpm of ^{32}P-Riboprobe (P_{1-42}, P_{1-77}, P_{1-165}, or $P_{333-513}$) and were performed as described elsewhere (17). Briefly, reactions were incubated for 30 min at 22 °C, 0.3 unit of RNase T1 (Roche Applied Science) was added for 10 min, and the followed by heparin (Sigma, final concentration of 5 μg/μl unless stated otherwise) for 10 min. Samples were then electrophoresed on 5% native acrylamide gels, dried, and analyzed with a Personal Molecular Imager FX (Bio-Rad). In some assays, cell extracts were preincubated for 10 min at 22 °C with nonspecific or specific competitor unlabeled RNA (~100× molar excess) or with DENSPM (0–50 μM).

RESULTS

SSAT-luciferase Fusion Protein Is Induced by DENSPM—To determine whether SSAT fused to a reporter protein was inducible by DENSPM treatment, human cervical HeLa cells were transfected with rLucSSAT$_{510}$ and then treated with increasing concentrations of drug. The SSAT-luciferase fusion protein showed almost a 10-fold increase in expression, with an EC_{50} of 2.7 ± 0.4 μM (Fig. 1*A*). A series of SSAT ORF deletion reporter constructs was generated (Fig. 1*B*) in an attempt to locate the region of SSAT that was responsive to DENSPM. The basal expression levels of the longer fusion proteins rLucSSAT$_{333}$ and rLucSSAT$_{166}$ were not different from rLucSSAT$_{510}$, whereas those of rLucSSAT$_{68}$ and rLucSSAT$_{UTR}$ were significantly greater than rLucSSAT$_{510}$ (Fig. 1*C*). The observed differences in basal expression levels were a result of differing fusion protein half-lives (Table 1). None of the C-terminal rLucSSAT deletion proteins were induced by DENSPM, including the control rLuc vector (Fig. 1*C*). Similar results were obtained using the human melanoma cell line MM2058 (data not shown). These results suggest that the induction of SSAT is dependent on the presence of the 3′ third of the coding region. To investigate the effect of DENSPM on the stability of SSAT-luciferase proteins, the inducible rLucSSAT$_{510}$ and the noninducible rLucSSAT$_{333}$ proteins were treated with cycloheximide, and their half-lives were determined in the absence or presence of DENSPM. DENSPM had no significant effect on the half-life of either fusion protein (Table 1).

DENSPM Enhances the Translation of SSAT-luciferase Fusion Protein in Vitro—The effect of DENSPM on the translation of rLucSSAT$_{510}$ was investigated using an *in vitro* transcription/translation system. First, mRNA was synthesized from rLucSSAT$_{510}$ plasmid in a reaction containing rabbit reticulocyte lysate, but lacking methionine. Actinomycin D (5 μg/ml) was added to stop the transcription, and the reaction was then divided into two equal aliquots, one treated with 50 μM DENSPM and the other with an equal volume of vehicle. Protein synthesis was initiated by the addition of methionine and the rate of protein synthesis measured over 30 min. Aliquots taken from each paired reaction at 0, 5, 10, 15, and 30 min were boiled immediately in Laemmli buffer and subjected to Western blot using an anti-*Renilla* luciferase antibody. The blots were quantified by densitometry, and the slope of the linear part of the curves was used to measure the rate of rLucSSAT protein synthesis (Fig. 2). The rate of protein synthesis in the presence of DENSPM was significantly greater than that in its absence (74.4 ± 3.4 and 19.8 ± 2.1 density units/min, respectively).

Induction of FLAG-tagged SSAT by DENSPM Is via Translational Regulation—An inherent problem with the luciferase fusion proteins used in the first part of this study is the possibility of direct interference with the luciferase enzyme activity by the attached SSAT fragments. For that reason, FLAG-tagged

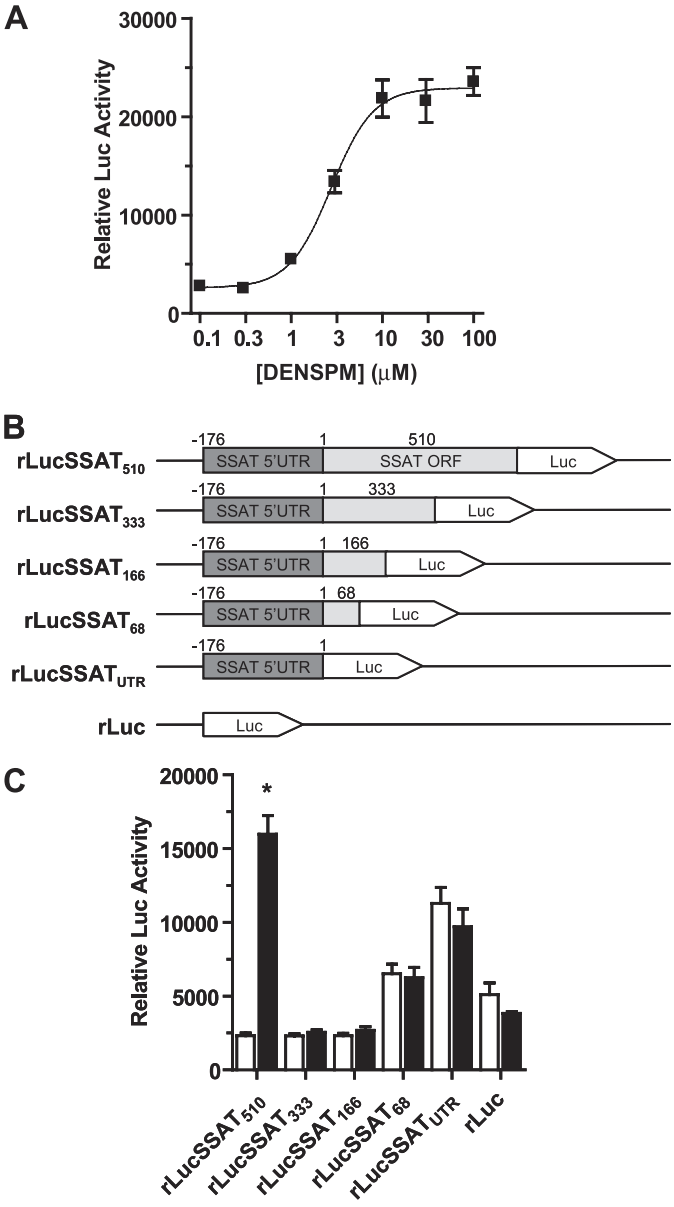

FIGURE 1. **DENSPM induction of SSAT-luciferase fusion proteins.** *A*, DENSPM dose-response curve. HeLa cells were transiently transfected with rLucSSAT$_{510}$ and then treated with increasing amounts of DENSPM for 24 h. Data are expressed as mean ± S.E., $n = 4$. *B*, schematic view of rLucSSAT deletion constructs. *C*, effect of DENSPM on truncated SSAT-luciferase fusion proteins. HeLa cells were transfected with the various rLucSSAT deletion constructs and then treated with either vehicle (*open bars*) or 10 μM DENSPM (*closed bars*) for 24 h. Data are expressed as mean ± S.E., $n = 4$. *, indicates significantly different ($p < 0.05$) from untreated controls.

TABLE 1
Stability of SSAT-luciferase fusion proteins in HeLa cells

Constructs	Fusion protein half-life	
	−DENSPM	+DENSPM
	h (mean ± S.E.)	
rLuc-SSAT$_{510}$	1.17 ± 0.04	1.29 ± 0.14
rLuc-SSAT$_{333}$	1.29 ± 0.11	1.31 ± 0.12
rLuc-SSAT$_{166}$	1.12 ± 0.10	ND[a]
rLuc-SSAT$_{68}$	1.91 ± 0.10	ND
rLuc-SSAT$_{UTR}$	3.46 ± 0.11	ND
rLuc	2.34 ± 0.19	ND

[a] ND, not determined.

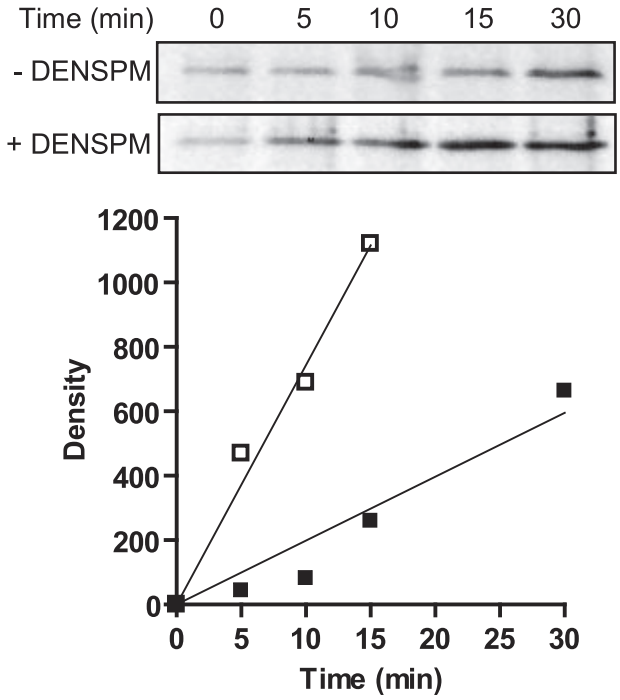

FIGURE 2. ***In vitro* translation of rLucSSAT fusion protein.** Reactions containing rabbit reticulocyte lysate and rLucSSAT$_{510}$ plasmid were incubated at 30 °C for 30 min to synthesize mRNA. Reactions were then treated with actinomycin D (5 μg/ml) and divided into two aliquots containing equal amounts of mRNA. One aliquot was treated with 50 μM DENSPM (*open squares*), whereas the other served as an untreated control (*closed squares*). Protein synthesis was initiated by adding methionine to the prewarmed reactions. Aliquots of each reaction were removed at 0, 5, 10, 15, and 30 min and immediately boiled in Laemmli buffer. rLucSSAT fusion protein was detected by Western blot using anti-*Renilla* Luciferase antibody and quantified by densitometry (Quantity One software, Bio-Rad). The zero time points were subtracted from subsequent samples.

SSAT was used as a model for the remainder of the study, and protein levels rather than enzyme activities were measured. Full-length SSAT was cloned into a FLAG vector (FLAG-SSAT$_{513}$) and expressed in HeLa cells to investigate the mechanism by which DENSPM induces SSAT expression. In the absence of DENSPM treatment, no FLAG-SSAT protein was detectable by Western blot using an anti-FLAG HRP antibody (Fig. 3*A*). Upon DENSPM (10 μM) treatment there was a time-dependent increase in FLAG-SSAT protein production, with near maximal levels occurring by 8 h (Fig. 3*A*). To determine whether the induction of FLAG-SSAT required new mRNA synthesis, cells were treated with actinomycin D (5 μg/ml) for 10 min, and then with 10 μM DENSPM for 4 h. Induction of FLAG-SSAT by DENSPM was not affected by the presence of actinomycin D (Fig. 3*B*). By contrast, when cells were treated with the protein synthesis inhibitor cycloheximide (10 μg/ml) and DENSPM, induction was completely abolished (Fig. 3*B*). To determine if FLAG-SSAT mRNA was present in the absence of DENSPM, total RNA was extracted from control cells and cells that had been treated with 10 μM DENSPM for 4 h. Following reverse transcription, specific primers were used to quantify FLAG-SSAT transcript levels by real-time PCR. Transcript levels were not increased in the presence of DENSPM (Fig. 3*C*). In fact, transcript levels were reduced in the presence of DENSPM, which is consistent with increased translation and translation-dependent mRNA degradation (18). A previous

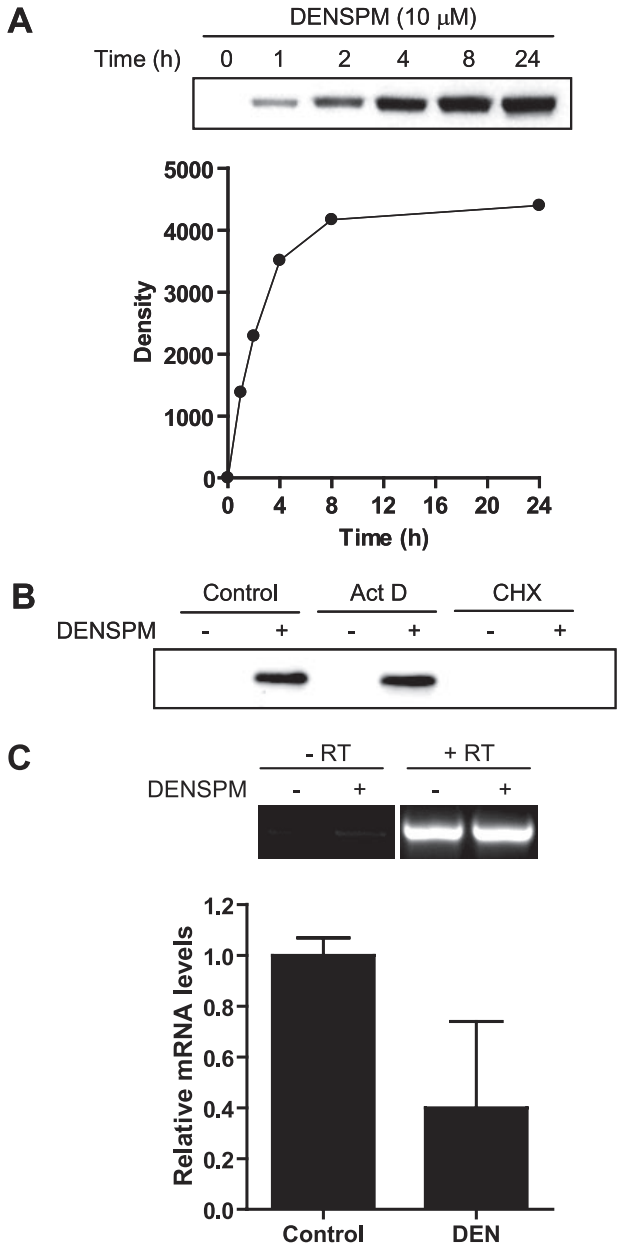

FIGURE 3. **Expression of FLAG-SSAT in HeLa cells.** *A*, HeLa cells were transiently transfected with pFLAG-SSAT$_{513}$ plasmid, incubated for 24 h, and then treated with DENSPM (10 μM) for up to 24 h. At the indicated times, cells were lysed, and FLAG-SSAT detected by Western blot using anti-FLAG-M2 HRP antibody. Densitometry was performed using Quantity One software (Bio-Rad). *B*, HeLa cells were transiently transfected with pFLAG-SSAT$_{513}$ plasmid and incubated for 24 h. Cells were then treated with either actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml), or left untreated. After 10 min, cells were treated with DENSPM (10 μM) or vehicle for 4 h, and then lysed and FLAG-SSAT detected by Western blot. *C*, HeLa cells were transiently transfected with pFLAG-SSAT$_{513}$ plasmid, incubated for 24 h, and then treated with DENSPM (10 μM) for 4 h. Total RNA was extracted and reverse transcribed, and FLAG-SSAT mRNA was quantified by real-time PCR. Data were normalized to β-actin and expressed relative to control.

study by Parry and coworkers (5) also observed a decrease in SSAT mRNA levels following polyamine analogue treatment. This indicates that expression of FLAG-SSAT mRNA has little effect on SSAT protein levels. Combined, these results indicate that translation of FLAG-SSAT mRNA is blocked in the absence of DENSPM. In addition, because the SSAT 5′- and 3′-UTRs were not present in the plasmid, the translational control of SSAT by DENSPM is mediated by the protein coding region, which is in agreement with a previous study (5).

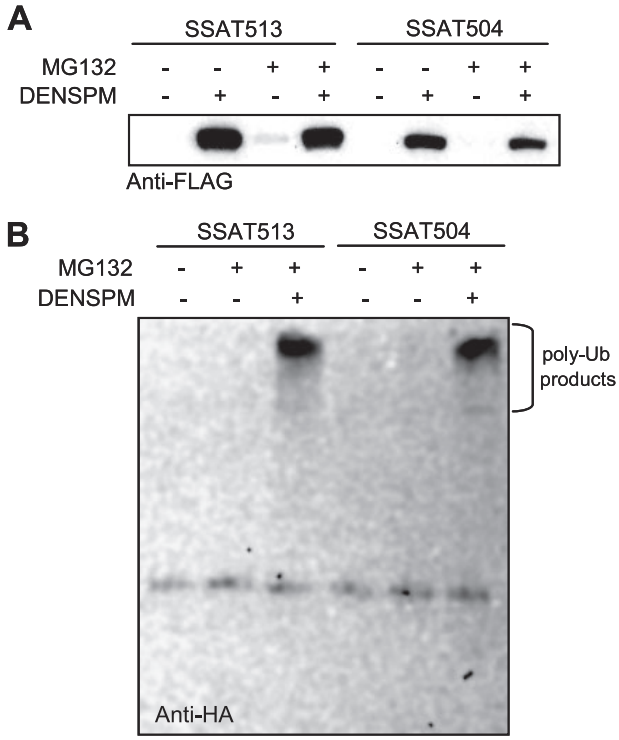

FIGURE 4. **Effect of DENSPM on the stability of FLAG-SSAT proteins.** *A*, HeLa cells were transiently transfected with either pFLAG-SSAT$_{513}$ or pFLAG-SSAT$_{504}$ plasmids, incubated for 24 h, and then treated with MG132 (20 μM) in the presence and absence of DENSPM (10 μM) for 16 h. FLAG-SSAT proteins were detected using anti-FLAG-M2 HRP antibody. *B*, HeLa cells were transiently transfected with HA-ubiquitin and either pFLAG-SSAT$_{513}$ or pFLAG-SSAT$_{504}$ plasmids, incubated for 24 h, and then treated with MG132 (20 μM) in the presence and absence of DENSPM (10 μM) for 16 h. Cells were lysed and immunoprecipitated with anti-FLAG antibody, Western blotted, and probed with anti-HA antibody. Polyubiquitinated products are shown.

Induction of FLAG-SSAT by DENSPM Is Not Due to Protein Stabilization—SSAT is degraded via the ubiquitin/26 S proteasome pathway (19), and it has been demonstrated that the C-terminal MATEE amino acid motif plays a critical role in polyamine-mediated stabilization of the protein (20). As a result, the lack of FLAG-SSAT protein detected in cells not treated with DENSPM may be due to rapid turnover of the protein. To address this possibility, HeLa cells were transiently transfected with plasmid containing either full-length SSAT (FLAG-SSAT$_{513}$) or a truncated construct (FLAG-SSAT$_{504}$), which produces a protein that is reported to be stabilized in the absence of polyamine analogue (19). Cells were then treated with 10 μM DENSPM in the absence or presence of the proteasomal inhibitor MG132 (20 μM). In cells not treated with DENSPM or MG132, no expression of either FLAG-SSAT$_{513}$ or FLAG-SSAT$_{504}$ was observed (Fig. 4*A*). When MG132 was added, a small amount of FLAG$_{513}$ was observed in the absence of DENSPM. This low level of translation may be due to the presence of endogenous polyamines. In addition, no polyubiquitinated products were observed in lysates from cells treated with MG132 in the absence of DENSPM (Fig. 4*B*). These data indicate that the lack of FLAG-SSAT protein observed in the absence of DENSPM was not because of rapid protein degradation.

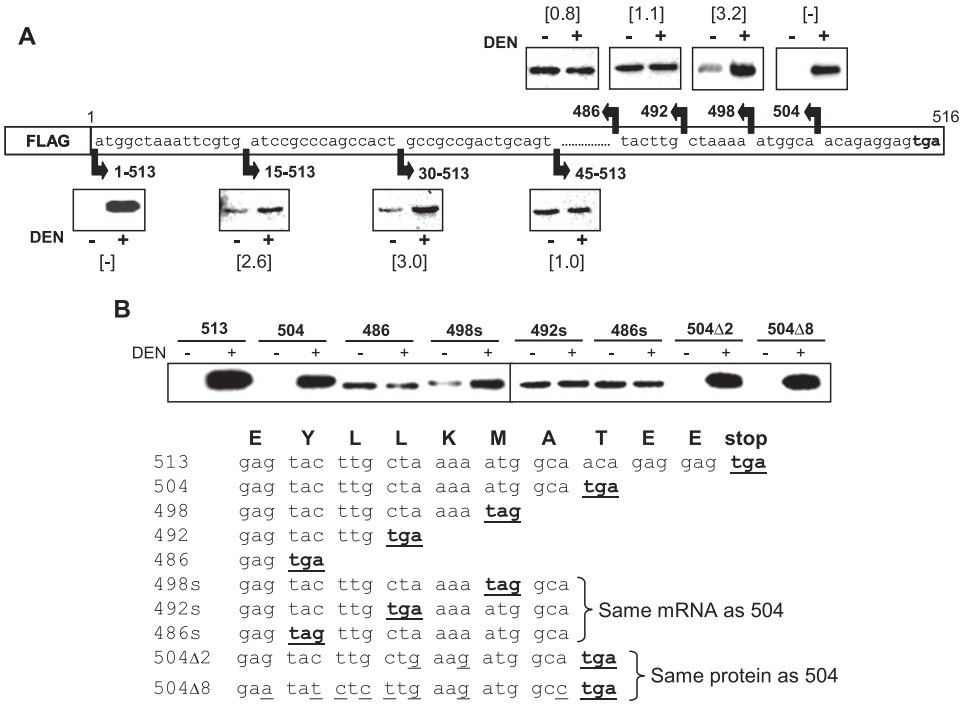

FIGURE 5. **DENSPM inducibility of FLAG-SSAT deletion proteins.** *A*, HeLa cells were transiently transfected with plasmids that produce FLAG-tagged full-length SSAT protein (FLAG-SSAT$_{513}$), N-terminal (FLAG-SSAT$_{15-513}$, FLAG-SSAT$_{30-513}$, and FLAG-SSAT$_{45-513}$), or C-terminal (FLAG-SSAT$_{504}$, FLAG-SSAT$_{498}$, FLAG-SSAT$_{492}$, and FLAG-SSAT$_{486}$) truncated SSAT proteins. After transfection, cells were treated with DENSPM (10 μM) or vehicle for 4 h, and cell lysates were prepared and Western blotted using anti-FLAG HRP antibody. Densitometry was performed using Quantity One software (Bio-Rad), and –fold induction is presented above each blot in parentheses. *B*, HeLa cells were transiently transfected with FLAG-SSAT plasmids containing premature stop codons (FLAG-SSAT$_{498s}$, FLAG-SSAT$_{492s}$, and FLAG-SSAT$_{486s}$) that produce truncated proteins but have similar mRNAs to FLAG-SSAT$_{504}$, or plasmids containing base changes in the SSAT mRNA (FLAG-SSAT$_{504\Delta2}$, FLAG-SSAT$_{504\Delta8}$) but have the same protein sequence as FLAG-SSAT$_{504}$. After transfection, cells were treated with DENSPM (10 μM) or vehicle for 4 h, and cell lysates prepared and Western blotted using anti-FLAG HRP antibody.

(Fig. 5*A*). These results show that the first 45 bases of the SSAT coding region as well as the bases between 492 and 504 are required for DENSPM responsiveness.

Functional mRNA instability elements have been detected within the coding regions of several mRNAs (21). The bases located between 492 and 504 of the SSAT coding region bear resemblance to an mRNA instability element identified in plasminogen activator inhibitor type 2 as well as in five other mRNAs (21). To investigate the possibility that the involvement of this sequence in DENSPM responsiveness is due to inducible stability of the SSAT transcript, a number of different constructs were made that contained in-frame premature stop codons. These constructs were transiently transfected into HeLa cells, and the effect on DENSPM inducibility was examined. FLAG-SSAT$_{498s}$, FLAG-SSAT$_{492s}$, and FLAG-SSAT$_{486s}$ produce similar mRNAs to that of the inducible construct FLAG-SSAT$_{504}$ but yield proteins identical to constructs FLAG-SSAT$_{498}$, FLAG-SSAT$_{492}$, and FLAG-SSAT$_{486}$, respectively (Fig. 5*B*). As observed with FLAG-SSAT$_{486}$, FLAG-SSAT$_{486s}$ was not inducible by DENSPM, even though the putative instability element was present in its mRNA sequence (Fig. 5*B*). Two other constructs, FLAG-SSAT$_{504\Delta2}$ and FLAG-SSAT$_{504\Delta8}$, contain silent mutations that alter the mRNA sequence but yield identical protein to the DENSPM-inducible FLAG-SSAT$_{504}$. Both mutated constructs were DENSPM-responsive (Fig. 5*B*). Together, these results do not support the concept that the sequence between 492 and 504 of the SSAT coding region is an inducible stability element that confers responsiveness to DENSPM.

Sequences Essential for DENSPM Induction of SSAT Are Located Both at the 5′- and 3′-Ends of the mRNA—To identify the region of the SSAT mRNA involved in SSAT translational induction, we constructed a series of FLAG-SSAT deletion mutants and determined the effect of DENSPM in transiently transfected HeLa cells (Fig. 5*A*). Because SSAT-luciferase fusion protein studies indicated that the 3′ third of the ORF was important, initial deletions were of this region. The FLAG-SSAT$_{504}$ deletion had the same inducibility as full-length FLAG-SSAT$_{513}$ (Fig. 5*A*). The next shortest construct, FLAG-SSAT$_{498}$, was DENSPM-responsive but showed some leakiness with a small amount of FLAG-SSAT protein being produced in the absence of DENSPM. The FLAG-SSAT$_{492}$ and FLAG-SSAT$_{486}$ constructs were not inducible by DENSPM with similar amounts of FLAG-SSAT protein translated in the absence and presence of DENSPM (Fig. 5*A*). These results suggest that the coding sequence located between bases 492 and 504, relative to the SSAT start codon, serves to repress translation in the absence of DENSPM. Deletions also were performed on the 5′-end of the SSAT ORF. FLAG-SSAT$_{15-513}$ and FLAG-SSAT$_{30-513}$ constructs were inducible by DENSPM, but showed some leakiness with a small amount of FLAG-SSAT protein being translated in the absence of DENSPM (Fig. 5*A*). The FLAG-SSAT$_{45-513}$ construct was not responsive to DENSPM, and there were similar levels of protein translated in the absence and presence of DENSPM

DENSPM-displaceable RNA-binding Proteins Interact with the Coding Region of SSAT mRNA—Folding analysis of the SSAT mRNA using the modified method of Zucker (MFold version 3.2) (22) predicted a number of stem-loops, including 2 in the first 165 bases of the protein coding region, and both stem-loops were still predicted when only the first 165 bases were folded separately (Fig. 6*A*). The start codon is located at the base of the first stem-loop, and the entire folding of this region resulted in a predicted change of free energy (ΔG) of −42.1 kcal/mol. Folding analysis of this region where the first 15, 30, or 45 bases were missing resulted in a marked increase in ΔG values to −30.6, −24.4, and −17.2 kcal/mol, respectively. When 15 or 30 bases were removed, SL1 and SL2 (see Fig. 6*A*) remained but the structure of SL1 was altered. However, when 45 bases was removed, a single stem-loop was predicted that

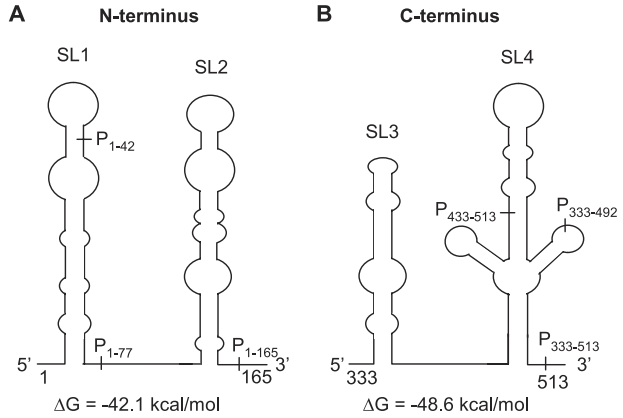

FIGURE 6. **Predicted RNA folding of SSAT.** *A*, the secondary structure for the first 165 nucleotides of the SSAT coding sequence was predicted using MFold (Ver. 3.2) (22) and contains two stem-loop structures, SL1 ($\Delta G = -30.5$ kcal/mol) and SL2 ($\Delta G = -9.8$ kcal/mol). SL1 has the start AUG at its base (5′). The probes used in REMSAs are shown as P_{1-42}, P_{1-77}, and P_{1-165}. *B*, the predicted secondary structure for the last 180 nucleotides of the SSAT coding sequence also contains two stem-loops, SL3 ($\Delta G = -18.9$ kcal/mol) and SL4 ($\Delta G = -27.9$ kcal/mol). SL4 has the stop UGA at its base (3′). The probes used in REMSAs are shown as $P_{433-513}$, $P_{333-513}$, and $P_{333-492}$.

bore no resemblance to either SL1 or SL2. Together with the FLAG-SSAT 5′ deletion data, this suggests that RNA secondary structure may play an important role in the repression of SSAT translation. Folding analysis of the SSAT mRNA also predicted the presence of two stem-loops in the final 180 bases of the protein coding sequence, and the entire folding of this region resulted in a ΔG of −48.6 kcal/mol (Fig. 6*B*). Progressive deletion of the last 9, 15, or 21 bases from the 3′-end resulted in only minor changes in ΔG values (−47.6, −43.4, and −42.7 kcal/mol, respectively). However, the secondary structure changed considerably when 21 bases were removed, and SL4 (see Fig. 6*B*) was not predicted. When 15 bases were removed, SL4 was predicted but with slight structural alterations, and when only 9 bases were removed, SL4 was predicted and had the same structure as that predicted for the full-length sequence. Together with the FLAG-SSAT 3′ deletion data, this analysis supports a role for RNA secondary structure in the regulation of SSAT mRNA translation.

RNA stem-loops often form sites for interaction with RNA-binding proteins that can regulate translational efficiency. To determine whether the first 165 bases of the SSAT coding region bound cellular protein(s), REMSAs were performed using three different Riboprobes; P_{1-42}, P_{1-77}, and P_{1-165}, consisting of the first 42, 77, and 165 bases of the mRNA relative to the translational start site, respectively (shown in Fig. 6*A*). Fig. 7*A* (*right panel*) shows a major band for the P_{1-165} probe that remained evident as the stringency of the assay was increased. By contrast, the P_{1-42} probe showed no specific binding (Fig. 7*A*, *left panel*), whereas the P_{1-77} probe appeared to bind cellular proteins at low stringency only (Fig. 7*A*, *center panel*) suggesting that the affinity of the proteins for this probe was less. The specificity of the RNA binding to the P_{1-165} probe was demonstrated by inhibition with unlabeled P_{1-165} probe (Fig. 7*B*). Excess nonspecific RNA (Bluescript or tRNA) only decreased binding slightly, and unlabeled P_{1-42} and P_{1-77} did not inhibit the P_{1-165} shift to any greater extent than nonspecific RNA (Fig. 7*B*).

To test whether one or more of the specific binding proteins were affected by DENSPM, a dose-response study was undertaken. DENSPM inhibited binding in a concentration-dependent manner with 10 μM decreasing the intensity of the specific binding by 60% (Fig. 7*C*). Because stringency was controlled by the amount of heparin added to the REMSA, we examined whether nonspecific interactions between the cationic DENSPM and the anionic heparin could account for these observations. For this, a probe from the 3′-UTR of the androgen receptor (23) was used in a separate REMSA (Fig. 7*D*). DENSPM up to 10 μM had no effect on the resulting shift of the probe. Moreover, if DENSPM did interact significantly with the heparin, a decrease in stringency would result and a concomitant increase in nonspecific binding would be expected, but was not observed.

To assess whether the 3′ region of the SSAT coding region bound cellular protein(s), the Riboprobe $P_{333-513}$ was subjected to REMSA analysis. A single band was observed (Fig. 7*E*, *first lane*) that represented specific binding, because it was completely competed by unlabeled $P_{333-513}$ and only slightly competed by excess nonspecific RNAs (Fig. 7*E*). In addition, the specific binding was competed by unlabeled $P_{433-513}$, which consists of the last 80 bases of the SSAT ORF. This suggests that protein binding only requires the last 80 bases of the SSAT mRNA coding region. As was the case for P_{1-165}, DENSPM inhibited protein binding to $P_{333-513}$ in a concentration-dependent manner with 10 μM decreasing the intensity of specific binding by 80% (Fig. 7*F*). Concentrations of DENSPM of >10 μM resulted in an apparent increase in specific binding, most likely caused by DENSPM interactions with heparin, resulting in a decrease in assay stringency.

Because DENSPM was unable to induce FLAG-SSAT when the last 21 bases of the coding region were deleted (FLAG-$SSAT_{492}$), the ability of a shorter oligonucleotide competitor equivalent, $P_{333-492}$, to compete protein binding to the $P_{333-513}$ Riboprobe in REMSA was assessed. Both unlabeled $P_{333-513}$ and $P_{333-492}$ (~100× molar excess) inhibited specific protein binding to the labeled $P_{333-513}$ Riboprobe (Fig. 8*A*). In addition, labeled $P_{333-492}$ Riboprobe showed specific protein binding similar to that observed for labeled $P_{333-513}$ (Fig. 8*B*). These data suggest that the binding of protein to the 3′-end of the coding sequence is not, by itself, sufficient to block translation. However, because changes in either the 5′ or 3′ coding sequences increased DENSPM-independent translation, it appears that these two regions may interact *in vivo*. The possibility that the same RNA-binding protein interacts with both the 5′- and 3′-ends of the SSAT ORF was investigated. In REMSA using labeled P_{1-165}, specific binding was competed not only by unlabeled P_{1-165} but also by $P_{333-513}$. In a similar manner, binding to labeled $P_{333-513}$ was competed by both unlabeled $P_{333-513}$ and P_{1-165} (Fig. 8*C*). This suggests that the same, or similar, protein may bind to both the 5′ and 3′-ends of the SSAT coding sequence.

DISCUSSION

Polyamines play a critical role in cellular processes such as growth, differentiation, and apoptosis. As a result, their intracellular concentrations are tightly regulated. In the cell, the

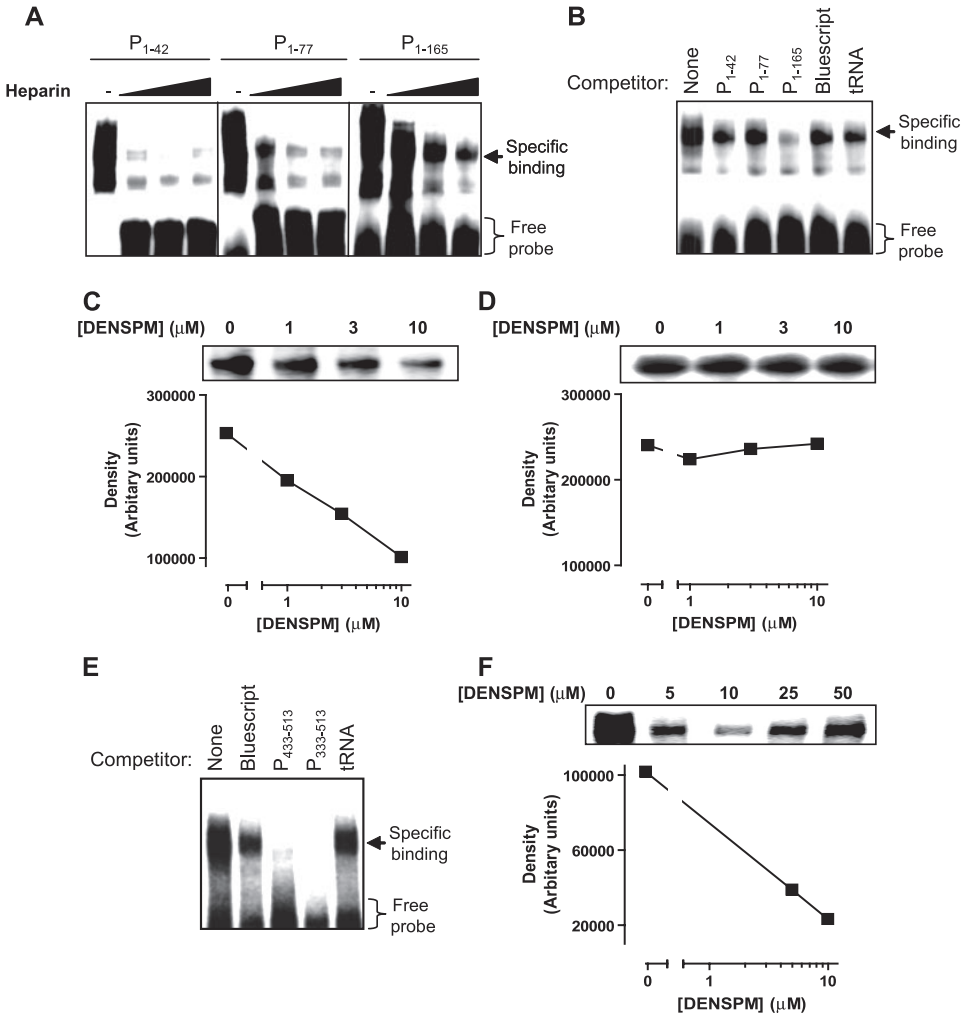

FIGURE 7. **Proteins bind specifically to the coding region of SSAT mRNA.** *A*, REMSAs were performed using 5 μg of cytoplasmic extract and 100,000 cpm of ^{32}P-labeled P_{1-42}, P_{1-77}, or P_{1-165} transcripts, which contain the first 42, 77, or 165 bases of the protein coding region of SSAT mRNA, respectively. Increasing amounts of heparin (0, 1.25, 2.5, and 5 μg/μl) were added to REMSAs to increase the stringency of the assay. *B*, competition studies were performed by addition of unlabeled RNA (~100× molar excess) to the cytoplasmic extract prior to incubation with ^{32}P-labeled P_{1-165} Riboprobe. *C*, effect of DENSPM on the specific interaction of P_{1-165} Riboprobe with cytoplasmic extract. REMSAs were quantified by densitometry (Quantity One software) and shown graphically. *D*, effect of DENSPM on the specific interaction of an androgen receptor Riboprobe with cytoplasmic extract. *E*, REMSAs were performed using a ^{32}P-labeled Riboprobe consisting of the last 180 bases of the protein coding region of SSAT mRNA ($P_{333-513}$). Competition studies were performed by addition of unlabeled RNA (~1000× molar excess) to the cytoplasmic extracts prior to incubation with Riboprobe. $P_{433-513}$ contained the last 80 bases of the coding region of SSAT transcript. *F*, effect of DENSPM on the specific interaction of $P_{333-513}$ Riboprobe with cytoplasmic extract.

ously reported that the response of different cell types to DENSPM is quite variable. Some cells show high SSAT induction, and cytotoxicity, at concentrations as low as 1 μM (27), whereas others require a drug concentration at least an order of magnitude higher for induction (28, 29). SSAT expression is regulated at several levels, including translation (5, 11), which requires elevated polyamine levels for optimum protein production. This may explain the synergy between polyamine analogs and some conventional chemotherapies, such as oxaliplatin, that increase SSAT mRNA (30).

Little is known about the molecular mechanisms that underlie the increase in translational efficiency of SSAT mRNA in the presence of polyamines. In the present study, we have used SSAT-luciferase fusion proteins and FLAG-tagged SSAT proteins to characterize and investigate the polyamine-dependent translational regulation of SSAT mRNA. Initial experiments using SSAT-luciferase fusion proteins suggested that a motif located within the 3′ third of the coding region serves to repress translation in the absence of polyamines. The induction of $rLucSSAT_{510}$ fusion protein by DENSPM was not due to protein stabilization, because the half-life of the fusion protein was not altered by the presence of DENSPM. In addition, DENSPM enhanced the translation of SSAT-luciferase fusion protein in an *in vitro* translation system.

In a study that addressed the role of the 5′- and 3′-UTRs of the mRNA, Parry and coworkers showed that neither of these regions of the transcript conferred sensitivity to the analogue N^1,N^{12}-bis(ethyl)spermine (5). This lack of involvement of the UTRs in the translational regulation of SSAT was confirmed in the present study using FLAG-tagged SSAT and DENSPM. When the coding region of SSAT was cloned into a FLAG vector, no detectable protein was expressed in the absence of DENSPM, even though considerable mRNA was present. Upon DENSPM treatment, FLAG-SSAT protein was induced, but mRNA levels actually decreased. This is consistent with increased translational activity. In addition, actinomycin D had no effect on the level of FLAG-SSAT protein following DENSPM treatment. By contrast, induction was completely abolished by the presence of the protein synthesis inhibitor cycloheximide, confirming a

ability of endogenous polyamines such as spermine to regulate the translation of SSAT and control its expression would provide an efficient mechanism for maintaining polyamine homeostasis. The presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to restore polyamine homeostasis following an elevation in their levels. If endogenous polyamines regulate SSAT translation, stimuli that increase SSAT transcription would not alter SSAT expression unless polyamine levels were also elevated.

Because SSAT induction can induce growth arrest as well as apoptosis, it is a novel potential target for anticancer therapy (24). Several polyamine analogues have been identified as potent inducers of SSAT, including DENSPM. This drug has progressed to Phase II clinical trials in renal cell carcinoma and advanced breast cancer (25, 26). However, it has been previ-

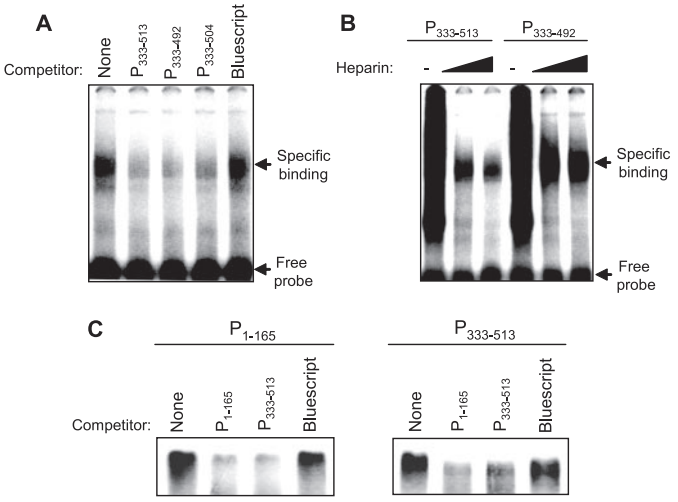

FIGURE 8. **Deletion of the last 21 bases of the SSAT coding region does not affect protein binding in REMSA.** *A*, competition studies were performed by addition of unlabeled RNA (~100× molar excess) to the cytoplasmic extract prior to incubation with 100,000 cpm of ^{32}P-labeled $P_{333–513}$ Riboprobe. *B*, REMSAs were performed using 5 μg of cytoplasmic extract and 100,000 cpm of ^{32}P-labeled $P_{333–513}$ or $P_{333–492}$. Increasing amounts of heparin (0, 2.5, and 5 μg/μl) were added to REMSAs to increase the stringency of the assay. *C*, to determine if the same RNA-binding protein interacts with both the 5′- and 3′-ends of SSAT, competition studies were performed by addition of unlabeled RNA (~100× molar excess) to the cytoplasmic extract prior to incubation with 100,000 cpm of ^{32}P-labeled $P_{1–165}$ or $P_{333–513}$ Riboprobes.

requirement for new protein synthesis. The ability of cycloheximide to prevent the induction of FLAG-SSAT by DENSPM suggests that induction is not a result of protein stabilization, but rather a release of a translational block. The lack of involvement of protein stabilization is further supported by experiments using the proteasomal inhibitor MG132. SSAT is degraded via the ubiquitin/26 S proteasome pathway (19). If, in the absence of DENSPM, the lack of FLAG-SSAT protein is due to rapid protein degradation, then the addition of MG132 should result in the accumulation of polyubiquitinated products, and this was not observed. Furthermore, FLAG-$SSAT_{504}$, which reportedly produces a protein with enhanced stability (19), is not present in the absence of DENSPM. Once DENSPM has relieved the translational block and SSAT protein has been synthesized, DENSPM may then function to stabilize the protein as has been reported in previous *in vitro* studies (8, 19).

Using a series of deletion constructs, we have identified two polyamine-responsive regions within the SSAT coding mRNA. Deletion of the first 45 bases of the coding region resulted in the total loss of translational block and any induction by DENSPM. Deletion of the last 21 bases of the coding region had the same effect, indicating that both regions are essential for translational repression and DENSPM induction of SSAT. When the folding of the first 165 bases was modeled, two stem-loops were predicted. The first stem-loop contained the first 76 bases of the coding sequence and the second contained bases +89 to +163. The first stem-loop was independent of flanking sequences and contained the translation start site at its base. Interestingly, this region shows high homology between mammalian species (84%) with most of the base variation occurring in bulges or non-paired regions of the two predicted stem-loops. This suggests that the secondary structure of the mRNA is conserved. The moderately stable stem-loop structures present in the regions of the SSAT coding mRNA important for DENSPM responsiveness would not be sufficient to prevent translation (5, 31), whereas a RNA-protein complex could conceivably block translation.

The formation of stem-loops in mRNA can promote the interaction with regulatory proteins that modulate translational efficiency as well as RNA stability (32). Using the 165-base fragment from SSAT mRNA that contained both predicted stem-loops, we identified specific protein interactions by REMSA. When the RNA fragment was truncated to include only the first stem-loop, specific binding was still evident, but the apparent binding affinity was considerably diminished, suggesting that the second putative stem-loop is crucial. Specific binding also was evident using the 3′ region of the coding SSAT mRNA in REMSA. Interestingly, deletion of the last 21 bases of the 3′ Riboprobe did not alter specific protein binding in REMSAs. This was in contrast to FLAG-SSAT expression studies where removal of the last 21 bases resulted in translation in the absence of DENSPM. This implies that, in isolation, protein binding to the 3′ polyamine-responsive region is not solely responsible for the translational block. Importantly, the interactions between the cytoplasmic components and both the 5′ and 3′ regions of the coding mRNA were inhibited by DENSPM. In addition, REMSA cold competition studies indicated that the same RNA-binding protein was interacting with both the 5′ and 3′ regions. These results suggest that both of these regions of the SSAT coding mRNA bind to a repressor protein that can be displaced by polyamines resulting in an increase in translational efficiency. The *in vitro* translation studies suggest that the protein involved in the repression of SSAT translation is present in rabbit reticulocyte lysate, which could provide a convenient source for its isolation and identification. Although there are numerous examples of translational regulation by RNA-binding proteins, most interact with the 5′-UTR or 3′-UTR of their target gene (32). Dihydrofolate reductase binds to its own mRNA within the coding sequence and suppresses translation (33). Moreover, repression can be reversed by the folate analogue methotrexate. Cytoplasmic proteins also have been shown to interact with translational control elements located in the coding region of proopiomelanocortin (34) and thymidylate synthase (35) mRNAs. We hypothesize that a polyamine-displacable RNA-binding protein interacts with structural motifs in both the 5′- and 3′-ends of the SSAT coding mRNA and represses translation. The possibility of cross-talk between the 5′- and 3′-ends of the coding sequence via the RNA-binding protein is plausible and could explain how removal of either the 5′- or 3′-end of the sequence allows translation to proceed in the absence of DENSPM. Previous studies have shown that cross-talk between motifs in the 3′-UTR and events far upstream is possible, occurring directly or via protein-protein interactions (36, 37).

In summary, the current study has identified two regions of the SSAT protein coding sequence that are involved in the translational repression of SSAT in the absence of elevated polyamine levels. It appears that a RNA-binding protein that possibly interacts with stem-loop structures and blocks translation can be displaced by DENSPM, allowing translation to proceed. It also appears that the 5′ and 3′ polyamine-responsive

regions interact with each other *in vivo*, as changes in either region results in translation in the absence of DENSPM. Further studies are required to identify the RNA-binding protein involved in the translational repression of SSAT and to characterize its interaction with each region of the transcript. This work contributes to our growing understanding of the regulation of SSAT expression, which is an emerging target for anticancer drug development.

Acknowledgments—We thank Peter Leedman, Dianne Beveridge, and Michael Epis (Western Australian Institute for Medical Research, Perth, Western Australia, Australia) for assistance with the RNA electrophoretic mobility shift assays.

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Polyamine-dependent Regulation of Spermidine-Spermine N^1-Acetyltransferase mRNA Translation

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J. Biol. Chem. 2007, 282:28530-28539.
doi: 10.1074/jbc.M701265200 originally published online August 9, 2007
