Polyamine-dependent Regulation of Spermidine-Spermine N\(^1\)-Acetyltransferase mRNA Translation*\(^a\)

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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\(^{11}\)-diethyl-norspermine (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 cytosolic 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\(^{11}\)-diethyl-norspermine (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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\(^b\) 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\(^{11}\)-diethyl-norspermine; 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 Nhel 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 rLucSSAT510. 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 rLucSSAT510, rLucSSAT533, rLucSSAT166, and rLucSSAT68 (shown in Fig. 1B).

To make FLAG-tagged constructs, the coding region of SSAT was amplified using rLucSSAT510 as template and primers F3 and R6. Reverse primer R6 added the terminal lysine and stop codon missing from rLucSSAT510. The entire SSAT coding region was cloned into the HindIII and BamHI sites of the p3XFLAG-CMV-7.1 expression vector (Sigma) yielding FLAG-SSAT513. 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-SSAT504, FLAG-SSAT498, FLAG-SSAT492, and FLAG-SSAT486. 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-SSAT513, FLAG-SSAT513, FLAG-SSAT45-513, and FLAG-SSAT45-513.

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. 5B. 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% CO2 in air.

For luciferase studies, cells were seeded at a density of 1 × 10⁶ cells/well (6-well plate) and co-transfected with 1 μg of pcDNA3-HA-ubiquitin and 2 μg of either FLAG-SSAT513 or FLAG-SSAT504 plasmid. After an overnight incubation, cells were co-transfected with 10 μM DENSpm and 5 μM MG132 (Calbiochem) or vehicle (Me2SO) 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 × 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 × 10⁶ cells/well (6-well plate) and co-transfected with 1 μg of pcDNA3-HA-ubiquitin and 2 μg of either FLAG-SSAT513 or FLAG-SSAT504 plasmid. After an overnight incubation, cells were co-transfected with 10 μM DENSpm and 5 μM MG132 (Calbiochem) or vehicle (Me2SO) 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 × 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 × 10⁶ cells/well (6-well plate) and transfected with 25 μg of FLAG-SSAT513 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 1 min. Quantification was performed using the ΔΔCt method (25).
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for 30 s. A melting curve was obtained to verify specificity of the PCR. Samples were analyzed by the comparative \( C_{\text{T}} \) method. In Vivo Transcription/Translation—Reaction mixtures contained 1 \( \mu \text{g} \) of rLucSSAT\(_{510}\) DNA and 40 \( \mu \text{g} \) of rabbit reticulocyte lysate (TNT Quick Coupled Transcription/Translation System, Promega), in a total volume of 50 \( \mu \text{l} \). 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 \( \mu \text{g/ml} \) to stop the transcription. Reactions were divided into two aliquots containing equal amounts of mRNA, and DENSPM (50 \( \mu \text{M} \)) or an equal volume of vehicle was added to each aliquot. The paired reactions were pre-warmed, and protein synthesis was initiated by the addition of 20 \( \mu \text{M} \) methionine. Aliquots (5 \( \mu \text{l} \)) were removed at 0, 5, 10, 15, and 30 min and boiled immediately in Laemmli buffer. Samples were then separated on 12% SDS-polycrylamide 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 di-thiothreitol) containing protease inhibitor mixture (Sigma). 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 [\( \alpha^{\text{32P}} \)]UTP (3000 Ci/mmol, GE Healthcare) as described elsewhere (17). Full-length transcripts were isolated on 6% urea/acylamide 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 \( \mu \text{l} \)) contained 10 \( \mu \text{g} \) of HeLa cytoplasmic extract and 100,000 cpm of \( ^{32P} \)-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 \( \mu \text{g/ml} \) 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 \( \mu \text{M} \)).

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 \( \mu \text{M} \) (Fig. 1A). A series of SSAT ORF deletion reporter constructs was generated (Fig. 1B) 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. 1C). 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. 1C). 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.

DENSMP 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 \( \mu \text{g/ml} \)) was added to stop the transcription, and the reaction was then divided into two equal aliquots, one treated with 50 \( \mu \text{M} \) 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 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
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-SSAT513) 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. 3A). 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. 3A). 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. 3B). By contrast, when cells were treated with the protein synthesis inhibitor cycloheximide (10 μg/ml) and DENSPM, induction was completely abolished (Fig. 3B).

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. 3C). 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

### TABLE 1

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Fusion protein half-life</th>
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<tr>
<td></td>
<td>−DENSPM (+DENSPM)</td>
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<tr>
<td>rLuc-SSAT510</td>
<td>1.17 ± 0.04 (1.29 ± 0.14)</td>
</tr>
<tr>
<td>rLuc-SSAT333</td>
<td>1.29 ± 0.11 (1.31 ± 0.12)</td>
</tr>
<tr>
<td>rLuc-SSAT166</td>
<td>1.12 ± 0.10 ND*</td>
</tr>
<tr>
<td>rLuc-SSAT68</td>
<td>1.91 ± 0.10 ND</td>
</tr>
<tr>
<td>rLuc-SSATUTR</td>
<td>3.46 ± 0.11 ND</td>
</tr>
<tr>
<td>rLuc</td>
<td>2.34 ± 0.19 ND</td>
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*ND, not determined.**

FIGURE 1. DENSPM induction of SSAT-luciferase fusion proteins. A, DENSPM dose-response curve. HeLa cells were transiently transfected with rLucSSAT510 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.

FIGURE 2. In vitro translation of rLucSSAT fusion protein. Reactions containing rabbit reticulocyte lysate and rLucSSAT510 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-SSAT513) 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. 3A). 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. 3A). 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. 3B). By contrast, when cells were treated with the protein synthesis inhibitor cycloheximide (10 μg/ml) and DENSPM, induction was completely abolished (Fig. 3B). 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. 3C). In fact, transcript levels were reduced in the presence of DENSPM, which is consistent with increased translation and translation-dependent mRNA degradation (18).
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).

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-SSAT513) or a truncated construct (FLAG-SSAT504), 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-SSAT513 or FLAG-SSAT504 was observed (Fig. 4A). When MG132 was added, a small amount of FLAG513 was observed in the absence of DENSPM (Fig. 4B). These data indicate that the lack of FLAG-SSAT protein observed in the absence of DENSPM was not because of rapid protein degradation.
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 on transiently transfected HeLa cells (Fig. 5A). Because SSAT-luciferase fusion protein studies indicated that the 3’ third of the ORF was important, initial deletions were of this region. The FLAG-SSAT504 deletion had the same inducibility as full-length SSAT (Fig. 5B). The next shortest construct, FLAG-SSAT492, was DENSPM-responsive but showed some leakiness with a small amount of FLAG-SSAT protein being produced in the absence of DENSPM (Fig. 5A). 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-SSAT498s, FLAG-SSAT492s, and FLAG-SSAT486s produce similar mRNAs to that of the inducible construct FLAG-SSAT504 but yield proteins identical to constructs FLAG-SSAT498, FLAG-SSAT492, and FLAG-SSAT486 respectively (Fig. 5B). As observed with FLAG-SSAT486, FLAG-SSAT498s, and FLAG-SSAT492s was not inducible by DENSPM, even though the putative instability element was present in its mRNA sequence (Fig. 5B). Two other constructs, FLAG-SSAT504s, and FLAG-SSAT504s, contain silent mutations that alter the mRNA sequence but yield identical protein to the DENSPM-inducible FLAG-SSAT504. Both mutated constructs were DENSPM-responsive (Fig. 5B). 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.

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 stem-loops were predicted when only 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. 6A). 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 45 bases of the SSAT coding region as well as the bases between 492 and 504 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. 6A) remained but the structure of SL1 was altered. However, when 45 bases was removed, a single stem-loop was predicted that

\[
\text{DENSPM inducibility of FLAG-SSAT deletion proteins.}\]
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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. 7C). 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. 7D). 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 P333–513 was subjected to REMSA analysis. A single band was observed (Fig. 7E, *first lane*) that represented specific binding, because it was completely competed by unlabeled P333–513 and only slightly competed by excess nonspecific RNAs (Fig. 7E). In addition, the specific binding was competed by unlabeled P333–513 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 P1–165, DENSPM inhibited protein binding to P333–513 in a concentration-dependent manner with 10 μM decreasing the intensity of specific binding by 80% (Fig. 7F). 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-SSAT922), the ability of a shorter oligonucleotide competitor equivalent, P333–492, to compete protein binding to the P333–513 Riboprobe in REMSA was assessed. Both unlabeled P333–513 and P333–492 (~100× molar excess) inhibited specific protein binding to the labeled P333–513 Riboprobe (Fig. 8A). In addition, labeled P333–492 Riboprobe showed specific protein binding similar to that observed for labeled P333–513 (Fig. 8B). 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 P1–165 specific binding was competed not only by unlabeled P1–165 but also by P333–513. In a similar manner, binding to labeled P333–513 was competed by both unlabeled P333–513 and P1–165 (Fig. 8C). 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
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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 stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels. If endogenous polyamines regulate SSAT translation, the presence of SSAT mRNA in a translationally repressed state means that the cell can respond quickly to stimuli that increase SSAT transcription would not alter SSAT levels.
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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). 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, 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-protein complex 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-displaceable 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 DENSPP. 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.

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REFERENCES
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