Effects of Polyamines, Polyamine Analogs, and Inhibitors of Protein Synthesis on Spermidine−Spermine N[1]-Acetyltransferase Gene Expression[†]

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Received May 23, 1996; Revised Manuscript Received August 22, 1996[⊗]

ABSTRACT: The key polyamine catabolizing enzyme spermidine−spermine N[1]-acetyltransferase (SSAT) is among the few genes known to be inducible by the natural polyamines. Certain polyamine analogs markedly exaggerate this response and thus provide useful tools for studying the underlying regulatory mechanisms. As shown here, the analog which most potently induces SSAT activity, N^1,N^{11}-diethylnorspermine (DENSPM), increases SSAT mRNA in MALME-3M human melanoma cells to a maximum of >20-fold and immunodetectable SSAT protein to >300-fold. By comparison, the natural polyamine spermine is far less effective, increasing SSAT mRNA by ∼3-fold and protein by ∼7-fold. In particular, the difference in mRNA accumulation by spermine and the analog was shown to be due to differential effects on both gene transcription and mRNA stabilization. Although the analog DENSPM has been regarded as the most potent inducer of SSAT activity and mRNA, we now report that inhibitors of protein synthesis are capable of increasing SSAT mRNA to nearly comparable levels. Inhibitor-induced accumulation in SSAT mRNA was shown to involve increased gene transcription and mRNA stabilization. This suggests that, under basal conditions, SSAT gene expression is suppressed by a labile protein (or proteins). While induction of SSAT mRNA by inhibitors of protein synthesis only occurred at concentrations which blocked protein synthesis, that by DENSPM took place at concentrations which did not. The combination of either protein inhibitor with DENSPM or spermine produced an additive increase in SSAT mRNA. Taken together, these findings suggest the involvement of two separate but possibly converging pathways in the regulation of SSAT mRNA, one mediated by polyamines and their analogs and the other mediated by a labile repressor of SSAT gene transcription and/or mRNA stabilization. In addition to its apparent regulatory importance, induction of SSAT mRNA by inhibitors of protein synthesis represents a potentially useful system for studying the posttranscriptional regulation of this interesting gene.

The critical association of increased polyamine biosynthetic activity with the onset of cell proliferation is well established. In addition to being highly inducible by growth-promoting stimuli, the key polyamine biosynthetic enzyme ornithine decarboxylase (ODC)[1] is negatively regulated by intracellular polyamine pools (Pegg et al., 1994). In contrast to ODC, the polyamine catabolic enzyme spermidine−spermine N[1]-acetyltransferase (SSAT) is positively regulated by polyamines (Porter et al., 1992; Shappell et al., 1993; Fogel-Petrovic et al., 1993) and seems to function in preventing intracellular polyamine pools from reaching cytotoxic levels (Seiler, 1987; Wallace, 1987; Shappell et al., 1993). SSAT is among the few known polyamine-inducible genes and perhaps the only one in which a significant accumulation of mRNA is known to be a critical part of the response (Casero et al., 1992; Fogel-Petrovic et al., 1993).

Investigations into SSAT gene regulation have been greatly facilitated by the availability of certain N-ethylated spermidine and spermine (SPM) analogs which exaggerate the response of SSAT to natural polyamines (Casero et al., 1989; Libby et al., 1989a; Pegg et al., 1989; Porter et al., 1991; Bergeron et al., 1995). The analog N^1,N^{12}-diethylspermine [DESPM, also known as N^1,N^{12}-bis(ethyl)spermine, BESPM] has been used in the cloning of SSAT (Xiao et al., 1991) and as a tool for studying its regulation (Fogel-Petrovic et al., 1993; Xiao et al., 1996). Fogel-Petrovic et al. (1993) demonstrated in human melanoma cells that DESPM-induced accumulation of mRNA is due to combined effects on increased gene transcription and mRNA stabilization. In addition, a significant portion of the SSAT response to such analogs can be attributed to posttranscriptional mechanisms including mRNA stabilization (Fogel-Petrovic et al., 1993), enhanced mRNA translation (Parry et al., 1995) and more particularly, stabilization of the SSAT protein (Libby et al., 1989a; Coleman et al., 1996).

A structurally related homolog of DESPM, N^1,N^{11}-diethylnorspermine (DENSPM), is currently undergoing Phase I clinical evaluation as a novel anticancer agent. It is regarded as the most potent known inducer of SSAT activity,

[†] This work was supported in part by Grants CA37606 and CA13038 from the National Cancer Institute.

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[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.

[1] Abbreviations: Act-D, actinomycin D; CHX, cycloheximide; DENSPM, N^1,N^{11}-diethylnorspermine [also known as BENSPM, N^1,N^{11}-bis(ethyl)norspermine]; DESPM, N^1,N^{12}-diethylspermine (also known as BESPM); DRB, dichlorobenzimidazole riboside; ODC, ornithine decarboxylase; PUR, puromycin; SAMDC, *S*-adenosylmethionine decarboxylase; SPD, spermidine; SPM, spermine; SSAT, spermidine−spermine N[1]-acetyltransferase; TBS, Tris-buffered saline; TTBS, Tween plus Tris-buffered saline.

S0006-2960(96)01227-5 CCC: $12.00

producing increases in certain melanoma cell lines of >200-fold (Porter et al., 1991). Because previous studies by others (Olson & Spizz, 1986; Hurta et al., 1993; Hurta & Wright, 1994) demonstrated that inhibitors of protein synthesis induced mRNA of another polyamine biosynthetic enzyme, ODC, we examined whether SSAT mRNA might be similarly affected. Inhibitors such as cycloheximide (CHX) were found to induce SSAT transcripts to levels nearly comparable to those achieved with DENSPM (Fogel-Petrovic et al., 1994). This present study first characterizes and compares the effects of DENSPM and SPM on SSAT protein and mRNA and then documents the action of inhibitors of protein synthesis on SSAT mRNA alone and in combination with DENSPM and SPM. The findings provide new insights into the mechanisms underlying SSAT induction by analogs and polyamines and offers a new system for studying posttranscriptional control of SSAT gene expression. Portions of these results have been reported earlier in abstract form (Fogel-Petrovic et al., 1996a).

MATERIALS AND METHODS

Materials. The polyamine analog DENSPM was synthesized (Bergeron et al., 1988) and provided by Dr. Bergeron (University of Florida, Gainesville). Inhibitors of protein synthesis, CHX, puromycin (PUR), emenitin, and anisomycin, were purchased from Sigma (St Louis, MO). Pactamycin was a gift from the Upjohn Co. (Kalamazoo, MI). The radionucleotides [α-^{32}P]dCTP (3000 Ci/mmol) and L-[^{35}S]methionine (>1000 Ci/mmol) were both obtained from Amersham Corp. (Arlington Heights, IL). MALME-3M human melanoma cells adapted to grow in RPMI 1640 medium were donated by Dr. R. Shoemaker and colleagues at the National Cancer Tumor Testing Laboratory (Frederick, MD). Human spermidine−spermine N[1]-acetyltransferase (SSAT) cDNA (Xiao et al., 1991) was obtained from Dr. R. Casero (Johns Hopkins Oncology Center, Baltimore, MD) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, from Clontech Laboratories Inc. (Palo Alto, CA). The Western blotting kit was obtained from Amersham Corp. Duralon nylon membranes used for Northern blotting and PVDF membranes used for Western blotting were purchased from Stratagene (LaJolla, CA) and Millipore Corp. (Bedford, MA), respectively.

Cell Culture. MALME-3M human melanoma cells were maintained as monolayer cultures growing in RPMI 1640 medium containing 1 mM aminoguanidine as an inhibitor of serum oxidase and 10% NuSerum as a semidefined serum substitute (Collaborative Research Products, Bedford, MA). Cells were seeded at 5×10^6 cell/150-mm Petri dish and incubated for 24 h before treatment with polyamines, analog, or inhibitors of protein synthesis. Unless otherwise indicated, polyamines and polyamine analogs were typically used at a final concentration of 10 μM while the inhibitors of protein synthesis, CHX and PUR, were used at 10 and 100 μg/mL, respectively.

SSAT Polyclonal Antisera. Affinity-purified rabbit anti-SSAT antisera was prepared using synthetic SSAT peptides incorporated into a multiple antigen peptide system (Tam, 1988; Tam & Zavala, 1989). Three regions of SSAT were selected for peptide synthesis based on the hydrophilicity/hydrophobicity profile generated using the Kyte and Doolittle (1982) hydropathy measure. The peptides represent amino acids 22−33, 56−67, and 136−147 of the SSAT protein. They are as follows: peptide 1, K E L A K Y E Y M E D Q; peptide 2, V A E V P K E H W T P E; peptide 3, S I N F Y K R R G A S D. They were synthesized as multiple antigen peptides by Dr. Richard Cook in the Department of Medicine, Division of Immunology, Baylor College of Medicine (Houston, TX).

Male New Zealand white rabbits, lacking detectable cross-reactivity to MALME 3M and RAW264 cell proteins, were immunized with 400 μg of each multiple antigen peptide emulsified in complete Freund's adjuvant for the initial injection and subsequently boosted with the antigens in incomplete adjuvant. After three boosts at 21-day intervals, the titer was maintained by monthly injections of 200 μg of each antigen. Sera were screened against 50 ng of the individual peptides immobilized on polyvinylchloride multiwell plates using an ELISA assay (Brown & Juliano, 1986). Following binding of the primary antibody to the antigen, the reaction was visualized using goat anti-rabbit IgG coupled to horseradish peroxidase (HRP) and 2,2-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] according to the protocol supplied by the manufacturer (Bio-Rad). The sera were also screened for antibody binding to antigen immobilized on nitrocellulose in a dot blot assay using goat anti-rabbit HRP and 4-chloro-1-naphthol.

Western Blotting. MALME-3M cells (1×10^7) were treated with 10 μM SPM or DENSPM for different times, washed, precipitated by centrifugation, and lysed in 25 μL of breaking buffer (25 mM Tris, 0.1 mM EDTA, and 2.5 mM dithiothreitol, pH 7.5) in the presence of a protease inhibitor cocktail (Sigma) containing 1 μM phenylmethanesulfonyl fluoride, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 2 μg/mL soybean trypsin inhibitor, 0.5 μM benzamidine, and 25 μg/mL aprotinin and sonicated for 10 s. The cytosolic fraction was separated by centrifugation at 14 000 rpm for 20 min at 4 °C. Protein concentration was determined using a Bio-Rad protein assay kit. For Western blotting, 120−150 μg of protein was loaded onto an SDS−12% polyacrylamide gel. Following electrophoresis, proteins were transferred onto a PVDF membrane in buffer containing 20 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS (pH 8.3) using a transfer apparatus (Idea Scientific Co., Minneapolis, MN) at a constant power of 400 mA for 2 h. A blocking reaction was performed overnight in 5% nonfat dry milk in Tris-buffered saline (TBS; 137 mM NaCl and 20 mM Tris, pH 7.4). Exposure to the anti-SSAT primary antibody (diluted 1:500) was carried out for 4 h in 5% dry milk in TBS. After being washed twice in TBS containing 2.5% dry milk and 0.1% Tween 20 (TTBS) for 10 min each and then once in TBS, the membrane was incubated with a secondary antibody (goat anti-rabbit IgG peroxidase conjugate diluted 1:3000 in 2.5% dry milk in TTBS) for 1 h at room temperature. After two 10-min washes in 2.5% dry milk in TTBS and two 10-min washes in TBS, the membrane was developed using peroxidase detection reagents (ECL kit, Amersham) and exposed for 5−30 s to an X-ray film.

Immunoprecipitation. The detection and quantitation of the relatively small amounts of SSAT protein in control cells and in those induced by the polyamine SPM required immunoprecipitation using a kit obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Protein (2 mg) isolated from the cytosolic fraction of MALME 3M cells treated with 10 μM SPM was diluted in RIPA buffer (PBS,

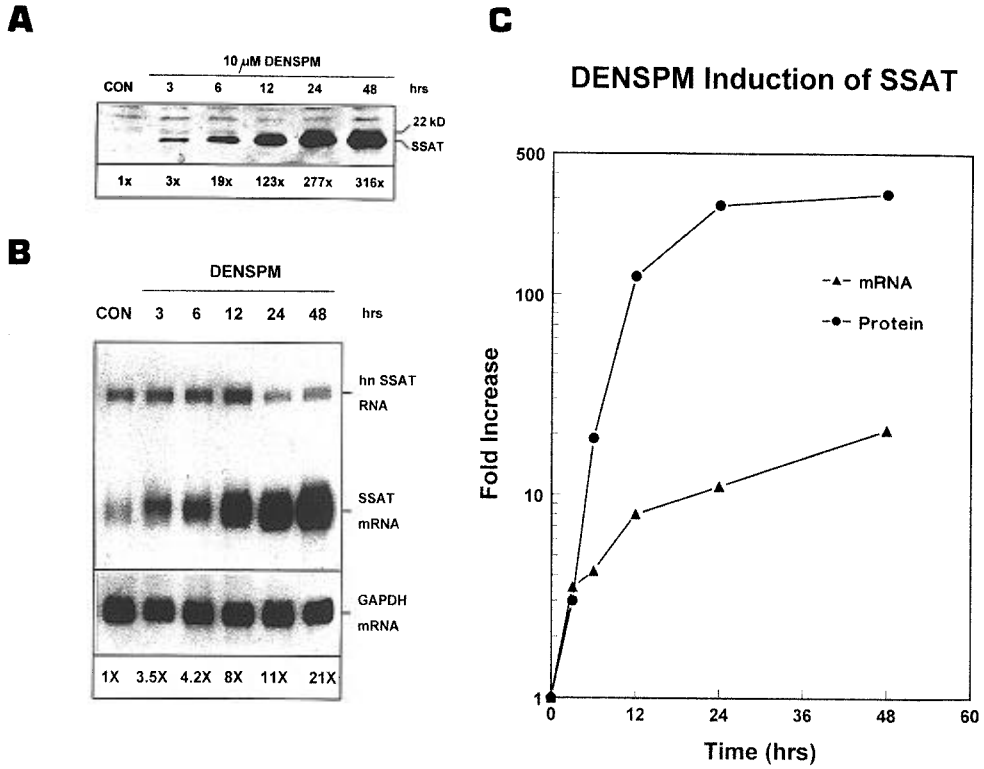

FIGURE 1: Time-dependent responses of SSAT protein detected by Western blot (A) and mRNA detected by Northern blot (B) in MALME-3M cells treated with 10 μM DENSPM. Values at the bottom represent fold increases in SSAT mRNA or protein relative to the control. A 30-kDa protein signal and GAPDH signal were used as internal controls for evaluating protein and RNA loading, respectively. These autoradiograms are representative of three experiments. (C) The graph compares the kinetics of SSAT mRNA (▲) and immunodetectable protein (●) induction. Data were taken from panels A and B. It should be noted that due to the nonlinear response of autoradiographic film, Western blot quantitation of the fold increases in SSAT protein at 24 and 48 h probably represent underestimates of the real value. hn, heteronuclear RNA.

1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) to 1 mL and immunoprecipitated for 12 h at 4 °C with anti-SSAT antibody. The protein−antibody complexes were then precipitated with 40 μL of protein A−agarose (Santa Cruz Biotechnology, Inc.), for 4 h at 4 °C, centrifuged, and washed 4 times with RIPA buffer. The resulting pellet was resuspended in electrophoresis sample buffer, boiled to dissociate protein−antibody complexes, and separated by SDS−polyacrylamide gel. The enriched SSAT protein was detected by Western blotting as described above.

Methionine Incorporation. MALME-3M cells (1×10^5) were first incubated for up to 6 h in concentrations of CHX, PUR, SPM, or DENSPM used for SSAT mRNA induction. Cells were then washed with methionine-free medium and then incubated for 30 min. in medium containing 120 μCi of L-[^{35}S]methionine, washed twice with PBS, and lysed in 100 μL of lysine buffer (0.5% sodium dodecyl sulfate in 50 mM Tris, pH 6.8). Tricloroacetic acid-precipitable radioactivity was collected on 3M filter paper which had been pretreated in 10 mM methionine and dried following the procedures of Gay et al. (1989). Triplicate filters were placed in vials with scintillation fluid and assayed in scintillation counter.

SSAT Activity Assay. The enzyme SSAT was extracted from MALME-3M cells and assayed as described previously (Libby et al., 1989a; Porter et al., 1991). It should be noted that this enzyme assay also detects other acetylase activities which, in basal measurements, may account for up to 70% of the total enzyme activity. However, in analog- or polyamine-induced samples, these activities account for less then 5% of the total activity (Porter et al., 1991).

Northern Blotting. Total RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation as previously described by Fogel-Petrovic et al. (1993). RNA samples (5−10 μg) were separated on 1.5% agarose/formaldehyde gels and transferred to nylon membrane. RNA was hybridized to ^{32}P-labeled cDNA encoding SSAT. Following exposure to X-ray film, Northern blots were then washed in stripping buffer [2 mM EDTA (pH 8.0) in 0.1% SDS] for 15−20 min at 75 °C and hybridized again with GAPDH cDNA. The GAPDH signal was used as a internal control for evaluating RNA loading. Intensity of SSAT signal on autoradiography was measured densitometrically and calculated relative to GAPDH mRNA signal in each sample and then relative to SSAT mRNA signal in control samples to determine fold increase.

RESULTS

DENSPM and SPM Induction of SSAT Protein and mRNA. Induction of SSAT protein by DENSPM and SPM was followed by Western blotting using a novel rabbit polyclonal antisera preparation derived for this purpose. With DENSPM treatment, SSAT protein accumulated steadily in a time-dependent manner to reach a >300-fold increase by 48 h (Figure 1A). By comparison, maximum increases in SSAT protein by SPM were much less (6−7-fold) and quantifiable only with immunoprecipitation (Figure 2A). Specifically, SSAT protein increased rapidly during the first 3 h of SPM treatment, then leveled off at ~7-fold by 24 h and remained constant to 48 h. Although the newly derived SSAT-directed antisera reacted with a number of cellular proteins, only one

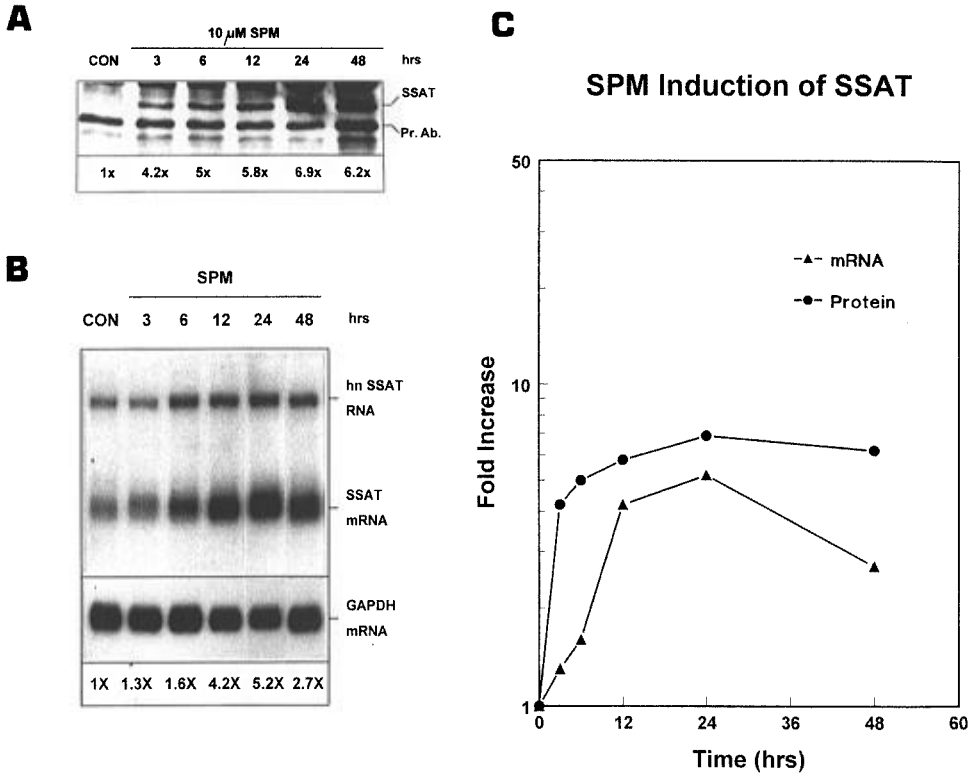

FIGURE 2: Time-dependent responses of SSAT protein as detected by Western blot (A) and mRNA as detected by Northern blot (B) in MALME-3M cells treated with 10 μM SPM. Values at the bottom represent fold increases in SSAT mRNA or protein relative to the control. Primary antibody signal (Pr. Ab.) and GAPDH signal were used as a internal controls for evaluating protein and RNA loading, respectively. hn, heteronuclear RNA. These autoradiograms are representative of two experiments. (C) The graph compares the kinetics of SSAT mRNA (▲) and immunodetectable protein (●) induction. Data were taken from panels A and B.

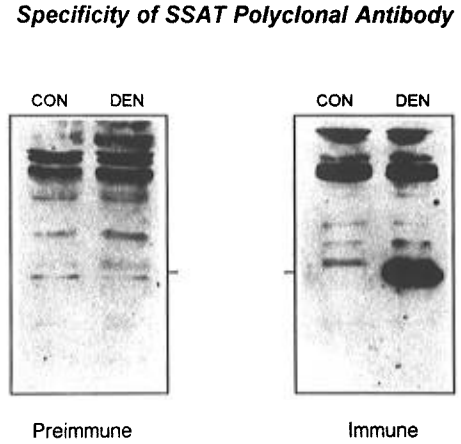

FIGURE 3: Western blots comparing SSAT protein detection by rabbit preimmune serum (left) and immune antiserum (right). Note that while the immune antiserum reacts with several proteins, only the ~20 kDa protein correlates in size with an SSAT subunit and increases with DENSPM treatment.

correlated with SSAT subunit molecular weight of ~20 kDa and increased during DENSPM or SPM treatment. Preimmune serum did not similarly react with SSAT protein in either control or DENSPM-treated cells (Figure 3).

As shown by Northern blot analysis of cells treated with 10 μM DENSPM (Figure 1B), SSAT mRNA accumulates in an almost linear fashion during the first 12 h and then increases more slowly to a maximum of ~20-fold by 24−48 h. DENSPM consistently produces the greatest accumulation of SSAT mRNA thus far seen with any polyamine analog.[2] During the first 12 h of DENSPM treatment, there was an obvious and almost immediate (3 h) increase in the 3.5-kb band of SSAT hybridizing RNA (Figure 1B). We have previously shown this band to represent preprocessed (heteronuclear) SSAT RNA which correlated with increased SSAT gene transcription as detected by nuclear run-on assays (Fogel-Petrovic et al., 1993). In contrast to DENSPM, SPM treatment (Figure 2B) resulted in a transient 5-fold increase in SSAT mRNA accumulation during the first 6−24 h, after which levels stabilized and then fell slightly to a final 2.7-fold. Heteronuclear mRNA increases at 6 h, suggesting that, like the analog, SPM also affected gene transcription but to a lesser extent.

A comparison of the kinetics for these various responses is shown in Figures 1C and 2C. With DENSPM treatment, induction of SSAT mRNA occurred at the same time as increases in SSAT protein. This is consistent with simultaneous analog effects on gene transcription as well as SSAT protein synthesis and stabilization. In contrast to analog effects, SSAT protein accumulation in SPM-treated cells increased more rapidly than mRNA accumulation, suggesting that the initial action of SPM may be at the level of translation, followed by transcriptional activation as indicated by increases in heteronuclear transcripts (3.5 kb).

Previously, we have shown that accumulation of SSAT mRNA in response to the analog N^1,N^{12}-diethylspermine is due to combined effects on SSAT mRNA stabilization and

[2] When compared with previously published results (Fogel-Petrovic et al., 1993), N^1,N^{12}-diethylspermine and DENSPM both induced SSAT mRNA to a maximum of ~20-fold in MALME-3M cells. However, when compared in the context of the same experiment, DENSPM consistently yields at least a 2-fold greater increase in SSAT mRNA than N^1,N^{12}-diethylspermine (unpublished results).

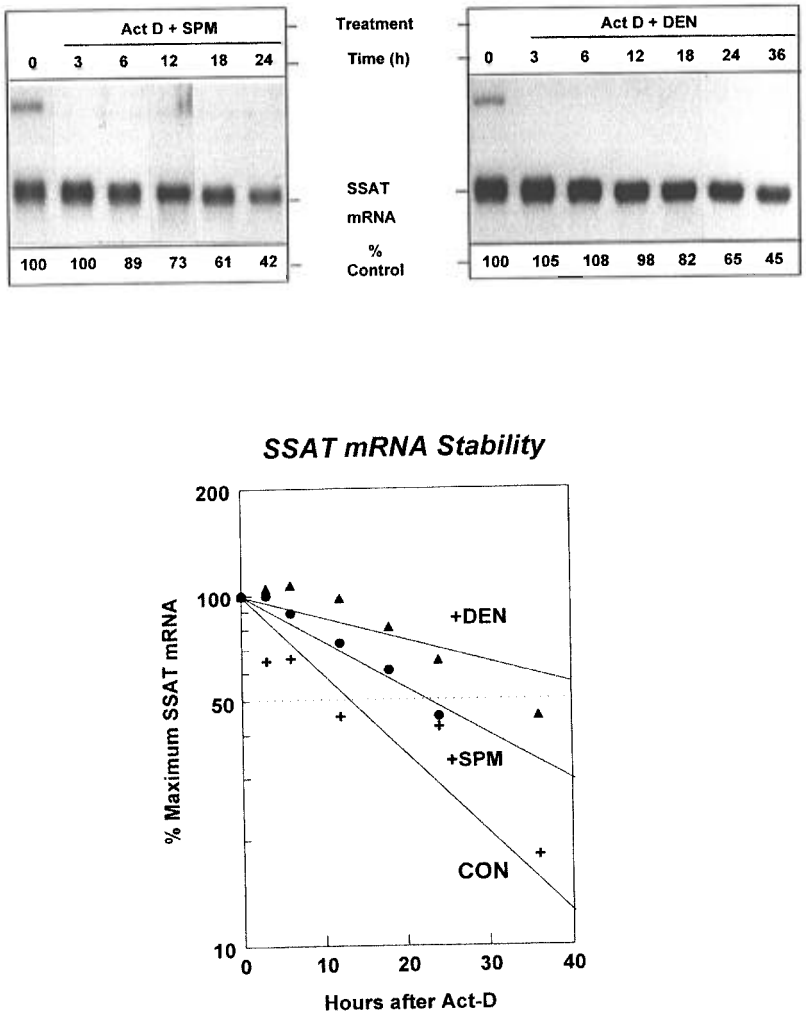

FIGURE 4: Kinetics of decay of SSAT mRNA in MALME-3M cells pretreated for 6 h with 10 μM SPM (top left panel) or DENSPM (top right panel) and then cotreated with actinomycin D (5 μg/mL) plus DENSPM (DEN) or SPM for the times indicated. Because GAPDH mRNA levels also decreased with time (not shown), quantitation of SSAT mRNA was, therefore, made relative to the control lane only. These blots are representative of findings from two experiments. The graph (bottom panel) compares decay of SSAT mRNA in control MALME-3M cells (half-life of ~14 h, data taken from Figure 6) with that in cells pretreated with SPM (half-life ~23 h) or DENSPM (half-life, >40 h).

increased SSAT gene transcription (Fogel-Petrovic et al., 1993). As shown in Figures 4 and 6, the basal half-life of SSAT mRNA in the presence of actinomycin D is ~14 h, which compares favorably to the ~17 h previously reported (Fogel-Petrovic et al., 1993). This value increased to >40 h following treatment with 10 μM DENSPM. SPM was less effective in this regard, increasing transcript half-life to ~23 h.

Induction of SSAT mRNA by Inhibitors of Protein Synthesis. We investigated whether SSAT mRNA was induced by inhibitors of protein synthesis as has been reported for ODC (Olson & Spizz, 1986; Hurta et al., 1993; Hurta & Wright, 1994). In fact, it was found to be increased to levels comparable those achieved with DENSPM over a 6-h time period. A comparison of the time-dependent induction of SSAT mRNA by CHX, PUR and DENSPM is shown in Figure 5. Results qualitatively similar to those of CHX and PUR, were also obtained with the protein inhibitors pactamycin at 2 μM, emenitin at 10 μg/mL, and anisomycin at 10 μg/mL (data not shown). As with DENSPM, both CHX and PUR increased heteronuclear SSAT mRNA, suggesting similar effects on gene transcription. Accumulation of mature SSAT mRNA continued through the 24-h period and various shifts in the 1.3- and 1.5-kb mRNA forms were observed. We have previously found that in the case of DESPM, these are due to differences in poly(A) tail length (Fogel-Petrovic et al., 1993) where an early induction of the 1.5-kb form is eventually replaced by a more predominant 1.3-kb form. PUR and CHX produced similar but more exaggerated form transitions. These form transitions are attributable to differences in poly (A) tail lengths (Fogel-Petrovic et al., 1993) and have not been reported in other cell types treated with analogs (Casero et al., 1992; Xiao et al., 1996).

As described above, the increase of heteronuclear RNA by CHX and PUR suggests that activation of SSAT gene transcription is induced by inhibitors. Results with Act-D presented in Figure 6 indicate that stabilization of message by CHX also contributes to the accumulation of SSAT mRNA by the inhibitor. As noted above, in control cells treated with Act-D (5 μg/mL), SSAT mRNA was found to decay with a half-life of ~14 h. By comparison, mRNA from cells induced for 6 h with CHX and then cotreated with CHX and Act-D was much more stable with a half-life of >40 h in the presence of the inhibitor. In both cases, heteronuclear SSAT RNA almost completely disappeared

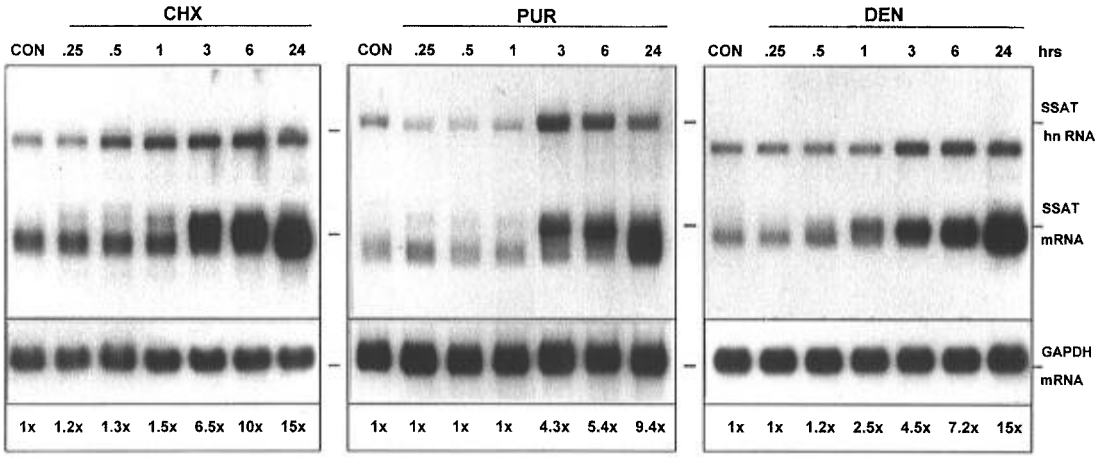

FIGURE 5: Kinetics of induction of SSAT mRNA in MALME-3M cells treated with the protein synthesis inhibitors cycloheximide (CHX; 10 μg/mL), or puromycin (PUR; 100 μg/mL) or with 10 μM DENSPM. Note that in similarity to DENSPM, both protein inhibitors increase preprocessed (heteronuclear) SSAT mRNA and induced 1.3- and 1.5-kb mature SSAT mRNA transcripts, which undergo various form transitions during treatment. Membrane blots were also probed for GAPDH mRNA as a control for equality of lane loading. These Northern blots are representative of two identical experiments. PUR and CHX were found to inhibit [^{35}S]methionine incorporation by 99−95% after only a 30-min incubation, while 10 μM DENSPM had no effect following incubations of up to 6 h.

after only 3 h as would be expected with inhibition of transcription.

Under treatment conditions used in these studies, CHX (10 μg/mL) and PUR (100 μg/mL) were found to inhibit incorporation of [^{35}S]methionine into MALME-3M cells by 95% and 99%, respectively. Previously, Mahadevan et al. (1991) observed that certain inhibitors of protein synthesis, such as anisomycin and CHX, activate transcription c-*fos* and c-*jun* genes via an interaction with signal transduction pathways and that this effect is dissociable from the inhibition of protein synthesis. To determine whether induction of SSAT gene transcription by CHX and PUR behaved similarly, we treated cells with low concentrations of CHX (50 ng/mL) or PUR (200 ng/mL). These concentrations were identical to those used by Edwards et al. (1992) in demonstrating that c-*fos* and c-*jun* transcripts could be induced without affecting protein synthesis. Under these conditions, both inhibitors failed to induce SSAT mRNA, indicating that inhibition of protein synthesis was necessary for this response. By comparison, the concentrations of DENSPM or SPM (10 μM) used to induce SSAT mRNA had no effect on [^{35}S]methionine incorporation (Fogel-Petrovic et al., 1996b). Thus, unlike with CHX and PUR, the SSAT response to DENSPM occurred via mechanisms separate from interference with protein synthesis.

In other systems, the combination of substances such as growth factors with inhibitors of protein synthesis has been shown to produce a major augmentation in the mRNA response of certain genes, a phenomenon known as a "gene superinduction" (Greenberg et al., 1986; Mahadevan & Edwards, 1991; Edwards & Mahadevan, 1992). Cotreatment of MALME-3M cells with either DENSPM or SPM plus an inhibitor of protein synthesis lead to an additive increase in SSAT mRNA (Figure 7). Following a 6-h treatment, DENSPM produced a 6-fold increase in SSAT mRNA while the inhibitors CHX and PUR induced an 8−9-fold increase. The combination of DENSPM with either inhibitor led to a ∼15-fold increase in SSAT mRNA. A similar effect was observed with SPM−inhibitor combinations (Figure 7). Additional kinetic studies examining this phenomenon over time and under various treatment sequences showed that all combinations remained in the additive range (data not shown).

DISCUSSION

The first portion of the current study provides insight into the mechanisms involved in SSAT induction by DENSPM relative to the natural polyamine SPM. By comparing the kinetics for these responses, it is apparent that DENSPM induces a more potent and sustained mRNA and protein response than SPM, reflecting its greater effects on SSAT mRNA transcription and protein stabilization. The former is indicated by increases in heteronuclear RNA (3.5-kb form), an effect which we have previously correlated with increased gene transcription rate by nuclear run-on assays (Fogel-Petrovic et al., 1993). It should be noted however, that this mRNA form has not been observed in other cell types (Casero et al., 1992; Xiao et al., 1996), suggesting the possiblity of cell-specific mechanisms. In the case of the analog, accumulation of SSAT mRNA by analogs cannot be attributed entirely to gene transcription since, as we have shown here and elsewhere (Fogel-Petrovic et al., 1993), mRNA stabilization is also critically involved, a mechanism which is less obviously utilized by SPM.

The response kinetics for SSAT mRNA and protein are consistent with a significant initial analog effect on gene transcription followed by mRNA stabilization and prolongation of protein half-life. This sequence of events differs somewhat from that seen with SPM-treated cells where increases in SSAT protein precede mRNA accumulation—the latter are accompanied by consistent elevations in heteronuclear RNA, suggesting gene activation. Thus, the initial action of SPM may be to enhance SSAT protein synthesis at the level of translation followed by a modest increase in gene transcription with no apparent effect on transcript stabilization. During the 24−48-h period, the protein to RNA ratio in DENSPM treated cells was about 10 times that seen with SPM, suggesting that either the analog is more effective than the polyamine at enhancing translation (Parry et al., 1995) or, more likely, that it causes protein stabilization, a phenomenon that has been described previously

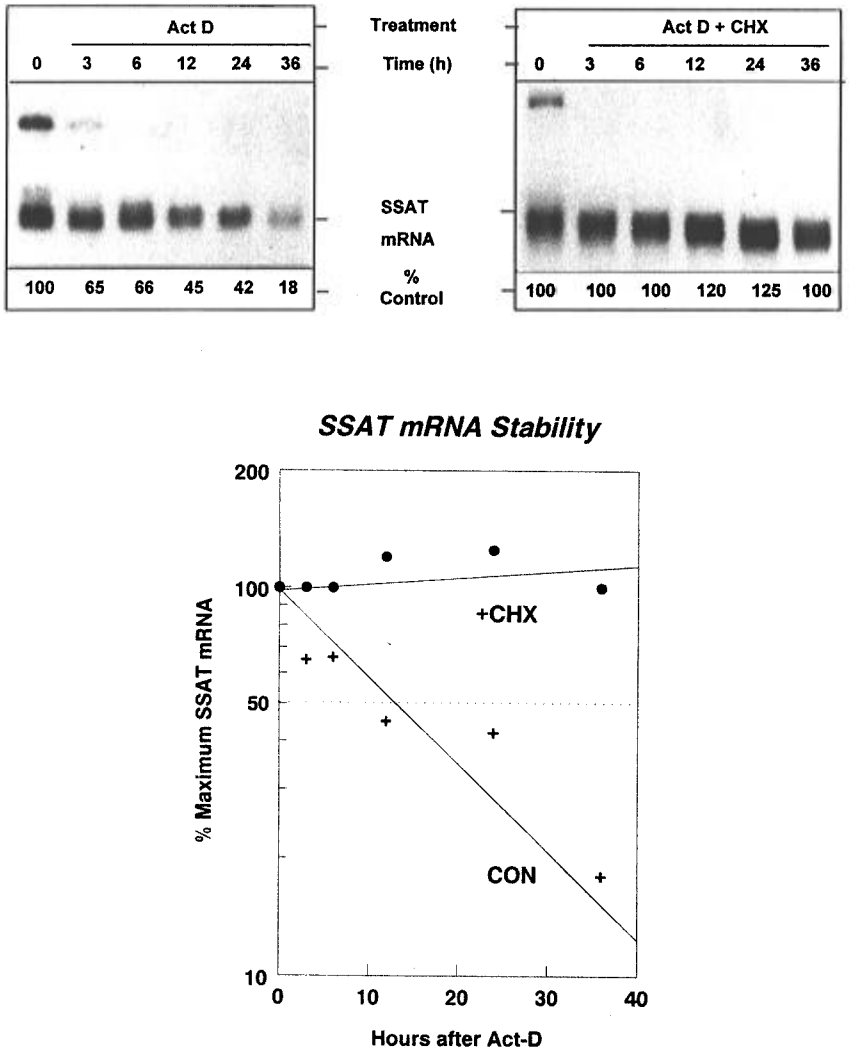

FIGURE 6: Kinetics of decay of SSAT mRNA in control MALME-3M cells (top left panel) treated with actinomycin D (5 μg/mL) and in cells pretreated for 6 h with CHX and then cotreated with Act-D and CHX (top right panel) for the times indicated. The Northern blot of cells treated with CHX was loaded with half the amount of mRNA (5 μg) used with control cells (10 μg) and exposed for less time than the blot of control cells. Quantitation was as described in Figure 3. The graph (bottom panel) compares decay of SSAT mRNA in control cells (half-life ~14 h) with that in cells pretreated with CHX (half-life >40 h).

(Libby et al., 1989a,b; Casero et al., 1992). In this regard, Coleman et al. (1995) have recently shown that the carboxy-terminal five amino acids of the human SSAT protein were critical for protein stabilization by DESPM.

It is uncertain whether the analog-mediated responses are truly different from those mediated by the natural polyamines or simply an exaggeration of the polyamine responses. For example, DENSPM has a greater effect than SPM on both gene transcription and mRNA stabilization. The analog cannot be acetylated by SSAT and therefore is not as readily degraded and/or eliminated from cells by excretion. Thus, because the analog can rapidly accumulate to very high intracellular levels (Libby et al., 1989b; Pegg et al., 1989; Porter et al., 1991), effects which appear to be analog-specific may actually represent exaggerations of those elicited by the natural polyamines. At the same time, it is possible that apparent analog effects are due to the displacement of natural polyamines. Finally, the possibility must be considered that the analog may simply interact differently from SPM at certain binding sites due to obvious structural differences between the two. A precedent for this has clearly been established with dimethyl and diethyl analogs. The former extends the half-life of SSAT activity by <6 h, and the latter, by $\gg 12$ h (Kramer et al., 1996). Although not yet reported, it is likely that similar differences in this effect exist for SPM and DENSPM.

The finding that the inhibitors of protein synthesis CHX and PUR induce mRNA accumulation is not unique to SSAT. The effect is common to a number of genes associated with (but not exclusively) the cell cycle and/or intracellular signaling. Most notably, these include the c-*fos*, c-*jun*, and c-*myc* gene families (Greenberg et al., 1986; Lau & Nathans, 1987; Chen & Allfrey, 1987; Bravo, 1990; Mahadevan & Edwards, 1991; Edwards & Mahadevan, 1992). It is also interesting that the mRNA of the polyamine biosynthetic enzyme ODC can also be induced by inhibitors of protein synthesis (Olson & Spizz, 1986; Hurta et al, 1993; Hurta & Wright, 1994). Recent studies have revealed that, depending on the gene and the inhibitor, mRNA induction can occur at inhibitor concentrations which do not affect protein synthesis (Mahadevan & Edwards, 1991; Edwards & Mahadevan, 1992) suggesting a role for mechanisms much more complex than the widely postulated loss of labile repressor proteins (Greenberg et al., 1986; Wall et al., 1986; Almendral et al.,

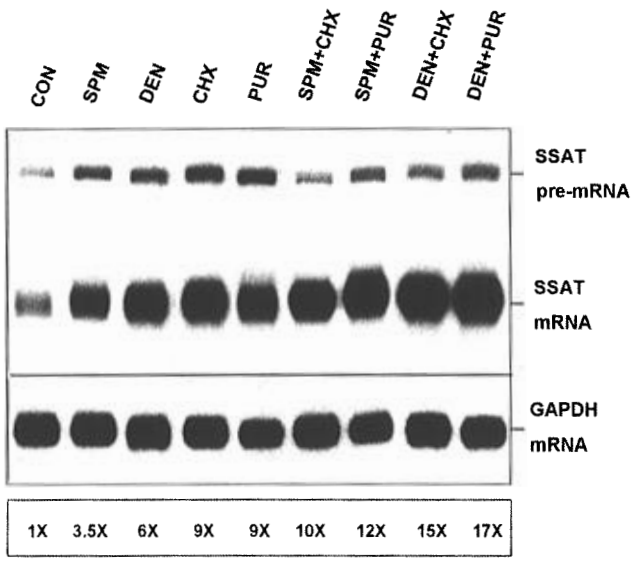

FIGURE 7: Induction of SSAT mRNA by combined treatment with protein synthesis inhibitors cycloheximide (CHX at 10 μg/mL) and puromycin (PUR at 100 μg/mL) and the polyamine analog DENSPM (DEN) or SPM. Values at the bottom of each lane represent fold increases in mature SSAT mRNA transcripts relative to control levels of SSAT mRNA after normalization to GAPDH mRNA. These Northern blots are representative of three experiments.

1988; Subramaniam et al., 1989; Morello et al., 1990). In contrast to this possibility, our present finding show that PUR and CHX fail to induce SSAT mRNA at concentrations which do not affect protein synthesis. This is consistent with the more conventional interpretation, namely, the loss of a labile nuclear repressor(s) of transcription. The data presented here suggests that such proteins control both SSAT gene transcription and mRNA degradation. Stabilization of SSAT mRNA in the presence of CHX may indicate that turnover depends upon ongoing translation of the message (Wilson & Treisman, 1988; Wisdom & Lee, 1991) or loss of unstable nucleases due to translational inhibition (Harford et al., 1990). Despite similarities in SSAT mRNA accumulation between DENSPM- and inhibitor-treated cells, it seems clear that the two act via different mechanisms. Although, with prolonged treatment, analogs of SPM are capable of interfering with protein synthesis (He et al., 1994), we have shown that both the analog and SPM induce SSAT mRNA under conditions which do not affect methionine incorporation.

It has been reported that cotreatment of cells with inhibitors of protein synthesis and various growth factors leads to an overaccumulation of transcripts for various genes. The phenomenon is known as "gene superinduction"[3] (Cochran et al, 1983; Lau & Nathans, 1987; Mahadevan & Edwards, 1991) and has been defined as "the process by which protein synthesis inhibitors accentuate and/or prolong the induction of specific genes that are normally only transiently induced in response to polypeptide growth factors, cytokines, hormones, interferons and phorbol esters" (Mahadevan & Edwards, 1991). As shown here, induction of SSAT mRNA by DENSPM and particularly SPM is clearly accentuated by either CHX or PUR. However, since the effects are additive in nature, we are reluctant to refer to the current observations as gene superinduction.[3] The involvement of labile regulatory protein(s) which control SSAT gene transcription and/or mRNA stabilization suggests that gene expression is repressed under basal conditions and released by inhibitors of protein synthesis. Because SPM and polyamine analogs such as DENSPM induce SSAT mRNA without inhibiting protein synthesis, their additive effect in combination with inhibitors of protein synthesis suggests the involvement of separate but possibly converging regulatory pathways. It seems unlikely that CHX and DENSPM or SPM are interacting through the same labile protein, since it is likely to be completely depleted by CHX and thus not able to contribute to the additive effect produced by the analog or polyamine.

We have recently exploited induction of SSAT mRNA by inhibitors of protein synthesis to an advantage. A limiting factor in studying the posttranscriptional control of the SSAT gene has been the low level of endogenous mRNA induced by the natural polyamines. While analogs can be used to exaggerate this response, it is not certain that they do so by the same mechanisms as the polyamines. In a recent study (Fogel-Petrovic et al., 1996b), CHX was used to simultaneously preinduce SSAT and ODC mRNA in MALME-3M cells. This provided a novel system to demonstrate differential posttranscriptional control of these two related genes by natural polyamines in the context of the same intact cells.

[3] Casero et al. (1994) have used the term superinduction to describe potent induction of SSAT by polyamine analogs. In the context of the present study, gene superinduction as defined in the literature (Mahadevan & Edwards, 1991) refers to the ability of inhibitors of protein synthesis to accentuate or prolong the induction of mRNA, particularly among early response genes.

ACKNOWLEDGMENT

We gratefully acknowledge the skilled technical assistance of Mr. John Miller.

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BI9612273