

# Differential post-transcriptional control of ornithine decarboxylase and spermidine-spermine $N^1$ -acetyltransferase by polyamines

Mirjana Fogel-Petrovic, Slavoljub Vujcic, John Miller, Carl W. Porter\*

Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

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**Abstract** Ornithine decarboxylase (ODC) and spermidine/spermine  $N^1$ -acetyltransferase (SSAT) are short-lived polyamine enzymes with rate-limiting roles in controlling polyamine biosynthesis and catabolism, respectively. We have found that treatment of MALME-3M human melanoma cells for 6 h with 10  $\mu$ g/ml cycloheximide (CHX) increases ODC and SSAT mRNA 6–9-fold. When cells containing CHX-induced SSAT mRNA were washed and post-incubated for an additional 6 h in drug free media, enzyme activity increased only 2-fold above that in untreated cells despite the >6-fold increase in accumulated mRNA. Inclusion of 10  $\mu$ M spermine or spermidine in the post-incubation medium increased SSAT activity ~7-fold without further elevating SSAT mRNA levels. This indicates post-transcriptional regulation which, due to the similarity between polyamine-mediated increases in SSAT activity and available mRNA, probably occurs at the level of mRNA translation. In contrast to the SSAT response, polyamines markedly reduced ODC activity (but not mRNA) to one sixth that in cells not exposed to polyamines. The findings illustrate how via post-transcriptional mechanisms, shifts in intracellular polyamine pools can simultaneously and differentially regulate polyamine biosynthesis and catabolism. It is hypothesized that these post-transcriptional responses enable cells to rapidly and sensitively control intracellular spermidine and spermine pools.

**Key words:** Ornithine decarboxylase; Polyamines; Post-transcriptional regulation; Spermidine; Spermine; Spermidine/spermine  $N^1$ -acetyltransferase

## 1. Introduction

Intracellular polyamine pools are known to be sensitively maintained by the combined action of polyamine-specific effector systems [1,2]. Designated polyamine homeostasis [3,4], this process is comprised of three major effectors: ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase which control polyamine biosynthesis, spermidine/spermine  $N^1$ -acetyltransferase (SSAT) which controls polyamine catabolism and excretion, and a putative polyamine transport apparatus which controls polyamine uptake. These effectors are short-lived and differentially regulated by intracellular polyamine pools [3]. Exposure to exogenous polyamines causes polyamine biosynthesis (reviewed in [5,6]) and uptake [7–9] to decrease and polyamine catabolism to increase

[4,10,11]. Lowering intracellular polyamine pools brings about the opposite effects. The mechanisms underlying polyamine regulation of ODC have been fairly well characterized (reviewed in [5,6]) while those involved in control of SSAT and particularly uptake are less understood.

We have investigated induction of SSAT by natural polyamines and an exaggeration of this response by certain polyamine analogs in MALME-3M human melanoma cells [12–14], a cell line found to be exceptionally responsive in this regard [3]. Analogs of spermine (SPM) such as  $N^1,N^{11}$ -bis(ethyl)norspermine (DENSPM) increase enzyme protein up to several hundred-fold and produce a 10–20-fold accumulation of SSAT mRNA due to increased gene transcription and mRNA stabilization [12,13]. Because the mRNA response is much less than the increase in SSAT activity, post-transcriptional mechanisms such as stabilization of SSAT protein and enhanced translation have also been implicated [15–18]. By contrast, the natural polyamine SPM brings about a ~7-fold increase in SSAT protein (or activity) which is accompanied by a low level increase in SSAT mRNA [13,14]. Due to this relatively modest mRNA response, transcriptional and/or post-transcriptional regulation of SSAT by the natural polyamines has not been as well defined as with the analogs.

We recently observed that SSAT mRNA can be profoundly induced by inhibitors of protein synthesis [13], a phenomenon previously recognized in other gene systems [19,20]. Treatment with cycloheximide (CHX) for only 6 h, for example, resulted in SSAT mRNA levels comparable to those produced by DENSPM, the most potent known inducer of this enzyme. Since ODC is also among the genes which are induced by such inhibitors [21–23], CHX treatment can be used to simultaneously induce ODC and SSAT mRNA. This combined response provides an opportunity to study post-transcriptional regulation of these two related genes in the context of the same intact cells. Portions of this work were recently published in abstract form [24].

## 2. Materials and methods

MALME-3M human melanoma cells (provided by Dr. R. Shoemaker, National Cancer Tumor Testing Laboratory, Frederick, MD) were maintained as monolayer cultures growing in RPMI 1640 medium containing 2% HEPES/MOPS as a buffer (Sigma, St. Louis, MO), 1 mM aminoguanidine as a serum oxidase inhibitor and 10% NuSerum as a semidefined serum substitute (Collaborative Research Products, Bedford, MA). Cells were seeded at  $5 \times 10^6$  cell per 150 mm Petri dish and incubated 24 h before treatment with polyamines, analog or inhibitors of protein synthesis. On the basis of earlier studies [13], a 6 h treatment with CHX was found to be sufficient to achieve near-maximal induction of SSAT mRNA without causing cytotoxicity. Thus, in a typical experiment, MALME-3M cells were treated for 6 h with 10  $\mu$ g/ml CHX (Sigma, St. Louis, MO), washed twice with media and incubated for an additional 6 h in the

\*Corresponding author. Fax: (1) (716) 845-8857.  
E-mail: Porter@sc3101.med.buffalo.edu

**Abbreviations:** CHX, cycloheximide; DENSPM,  $N^1,N^{11}$ -diethylnorspermine (also known as BENSPM,  $N^1,N^{11}$ -bis(ethyl)norspermine); DRB, dichlorobenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ODC, ornithine decarboxylase; SPD, spermidine; SPM, spermine; SSAT, spermidine/spermine  $N^1$ -acetyltransferase

presence or absence of 10  $\mu$ M SPD, SPM or DENSPM (provided by Dr. W. Leopold, Parke-Davis, Ann Arbor, MI). It should be noted that during the 6 h CHX treatment, incorporation of [ $^{35}$ S]methionine decreased to <5% of that seen in control cells (data not shown). Following inhibitor wash-out and post-incubation in drug free media, methionine incorporation recovered to at least 85% that seen in control cells by 30 min. The presence of either 10  $\mu$ M SPD or SPM during the post-incubation had no effect on the recovery of protein synthesis.

Following treatment, the polyamine enzymes SSAT and ODC were extracted from MALME-3M cells and assayed as described previously [25]. Intracellular polyamine and acetylated polyamine pools were determined on an acid extract of cells using a high performance liquid chromatography system described elsewhere [26].

SSAT and ODC mRNA was analyzed by Northern blotting. RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation as previously described by Fogel-Petrovic et al. [12]. Samples of total RNA (10  $\mu$ g for SSAT mRNA or 30  $\mu$ g for ODC mRNA) were separated on 1.5% agarose/formaldehyde gels, transferred to Duralon nylon membrane (Stratagene, La Jolla, CA) and hybridized to  $^{32}$ P-labeled cDNA encoding human SSAT ([27]; provided by Dr. R. Casero, Johns Hopkins Oncology Center, Baltimore, MD), or human ODC ([28], provided by Dr. Ölle Jänne, University of Helsinki, Finland). Following exposure to X-ray film, Northern blots were 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech Laboratories Inc., Palo Alto, CA) as an internal control for evaluating RNA lane loading.

### 3. Results

Treatment of MALME-3M cells for 6 h with 10  $\mu$ g/ml CHX increased mature SSAT mRNA (present as 1.3 and 1.5 kb bands) to a level  $\sim$ 9-fold greater than in control cells (Fig. 1). A third band (3.5 kb) containing SSAT preprocessed (heteronuclear) mRNA was also induced suggesting transcriptional activation by CHX [12,13]. The inhibitor was found to be a much more potent inducer of SSAT mRNA than either SPD or SPM. Northern blots prepared with mRNA from these same cells and probed for ODC, revealed that ODC mRNA was also increased  $\sim$ 6-fold by CHX (Fig. 2). Thus, both SSAT and ODC mRNA are rapidly induced by CHX in MALME-3M cells.

To determine whether mRNA induced by CHX could be translated into active enzyme protein, MALME-3M cells were pretreated for 6 h with 10  $\mu$ g/ml CHX, washed free of the inhibitor and then incubated an additional 6 h in inhibitor free medium. During the post-incubation, SSAT (Fig. 1) activity consistently increased only  $\sim$ 2-fold relative to control cells. Since this is far less than expected on the basis of available CHX-induced mRNA (i.e. 9-fold at the beginning of the post-incubation, 6-fold at the end) and since SSAT is known to be positively regulated by polyamines [4,12], the possibility was considered that mRNA translation may be restricted by

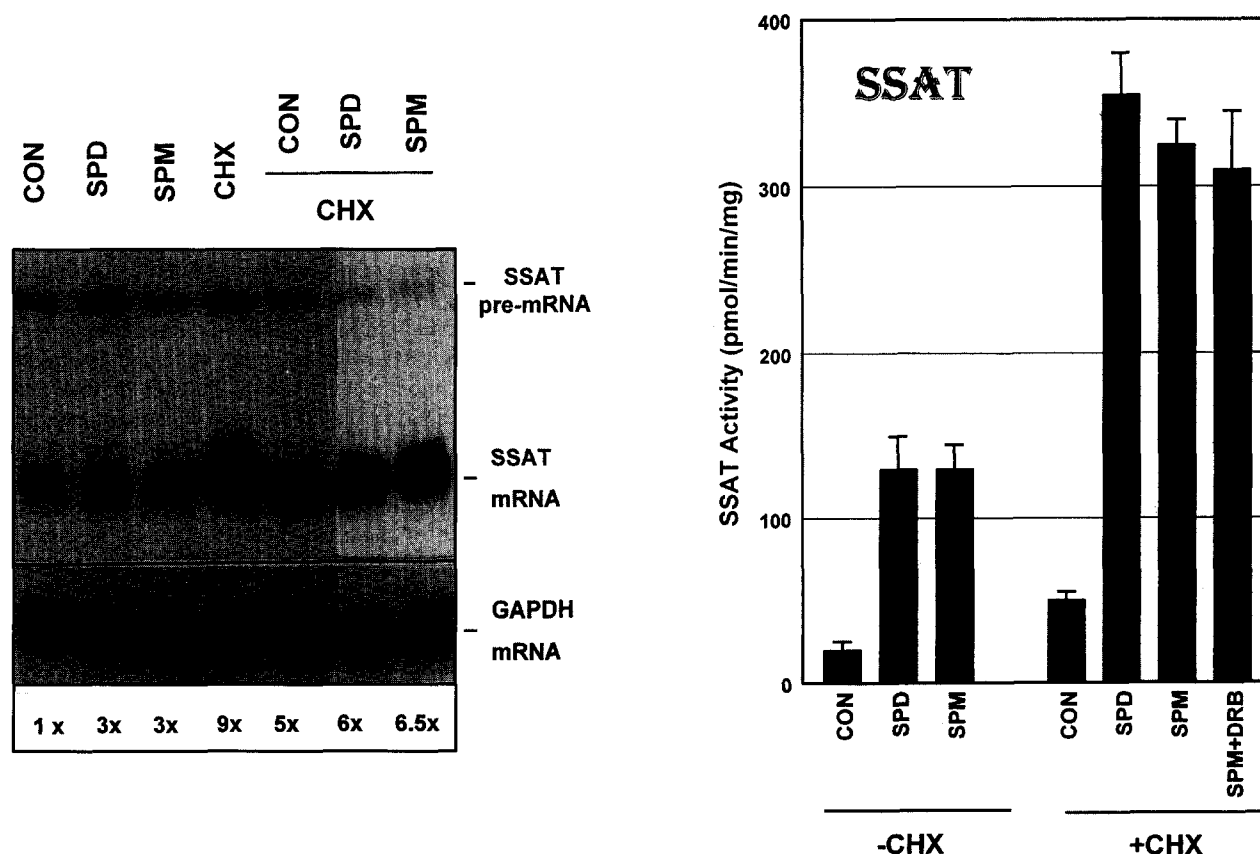


Fig. 1. Northern blot of SSAT mRNA (left) and bar graph of SSAT activity (right) from MALME-3M cells treated for 6 h in the presence or absence of CHX (at 10  $\mu$ g/ml), washed and then post-incubated for an additional 6 h in the presence or absence SPM or SPD. Incubations with SPM were also carried out with the transcription inhibitor dichlorobenzimidazole (DRB, at 25  $\mu$ g/ml). SSAT hybridizing RNA appears as a  $\sim$ 3.5 kb pre-processed or heteronuclear RNA (SSAT pre-mRNA) and as 1.3 and 1.5 kb cytoplasmic transcripts [12,13] which appeared in these experiments, as a single band. Northern blots are representative of two identical experiments; enzyme activity is based on data from four experiments. Fold increase values at the bottom of Northern blot were obtained by first quantitating SSAT mRNA relative to GAPDH mRNA and then to SSAT mRNA in control cells.

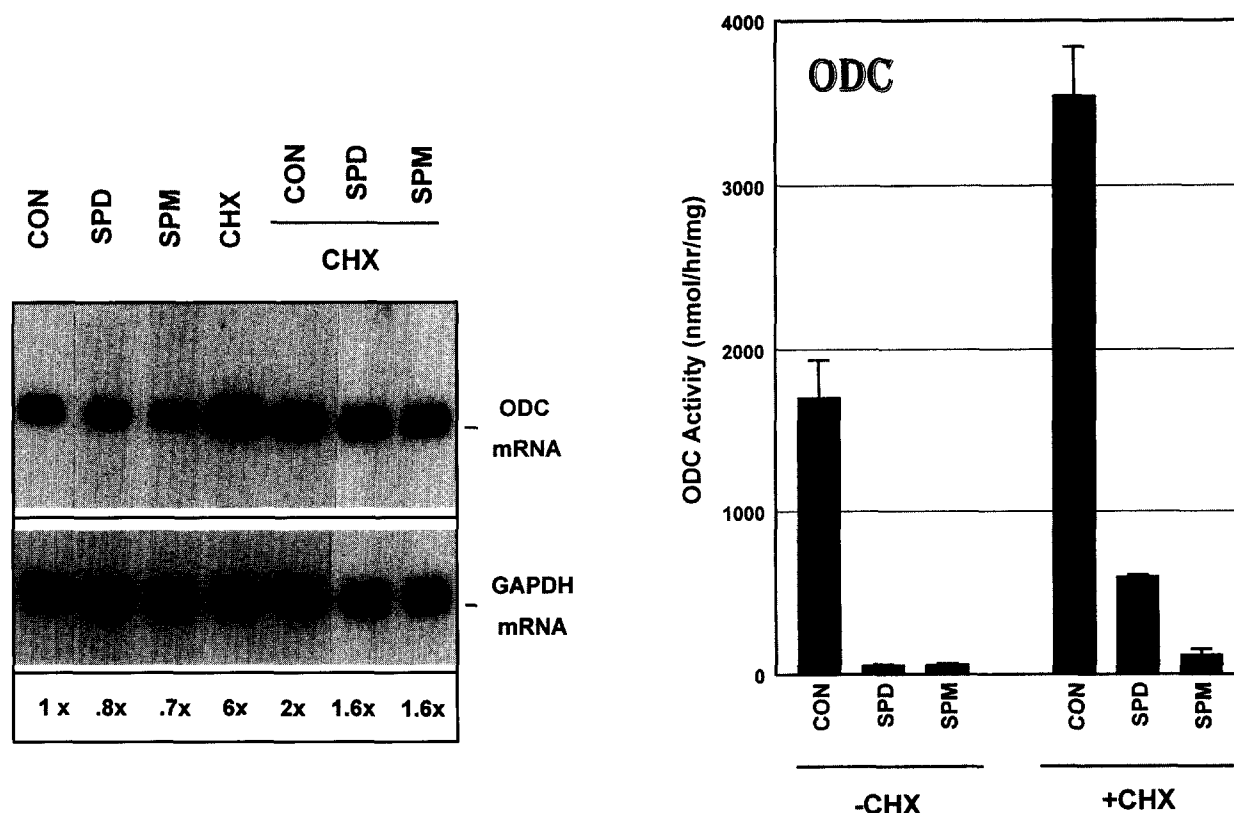


Fig. 2. Northern blot of ODC mRNA (left) and bar graph of ODC activity (right) from MALME-3M cells treated for 6 h with 10  $\mu$ M SPD, SPM or CHX (at 10  $\mu$ g/ml) or pretreated for 6 h with CHX (10  $\mu$ g/ml), washed and then post-incubated for an additional 6 h in medium in the presence or absence of SPM or SPD. Northern blots are representative of two identical experiments; enzyme activity is based on data from three experiments. Fold increase values at the bottom of Northern blot were obtained by first quantitating ODC mRNA relative to GAPDH mRNA and then to ODC mRNA in control cells.

intracellular polyamines levels. Inclusion of 10  $\mu$ M SPD or SPM in the post-incubation medium increased SSAT activity nearly 7-fold. As indicated by enzyme data presented in Table 1, SPM was more effective at 1  $\mu$ M than SPD. Since polyamines neither increased SSAT mRNA nor opposed its decline during the post-incubation (Fig. 1), the rise in SSAT activity was attributed to post-transcriptional events. This is further indicated by the finding that the inhibitor of transcription dichlorobenzimidazole DRB (at 25  $\mu$ g/ml) had no effect on SPM induction of enzyme activity (Fig. 1).

As with SSAT, pretreatment with CHX induced ODC mRNA  $\sim$ 6-fold which resulted in a 2-fold increase in ODC

activity, a level which correlated well with the 2-fold increase in mRNA following post-incubation. Whereas polyamines caused an increase in SSAT activity, they produced a sharp decrease in ODC activity. In response to either SPD or SPM, ODC fell to  $\sim$ 15% that seen in cells not exposed to polyamines. At the same time, they had no significant effect on ODC mRNA decline during the post-incubation period indicating that as with SSAT, the effect on enzyme activity was due to post-transcriptional events.

Inclusion of 10  $\mu$ M DENSEPM during the post-incubation (Fig. 3) increased SSAT activity from  $\sim$ 7-fold to  $>$ 50-fold. Because the latter represents only a modest increase over

Table 1  
Polyamine enzymes and pools of CHX pretreated MALME-3M cells treated with SPD or SPM<sup>a</sup>

Treatment <sup>b</sup> (6/6 h)	Enzyme activities		Intracellular polyamines (pmol/10 <sup>6</sup> cells)				
	ODC <sup>c</sup>	SSAT <sup>c</sup>	PUT	SPD	N <sup>1</sup> -AcSPD	SPM	NAcSPM
Control (Con)	1540	20	2340	2575	170	2490	< 10
Con/10 $\mu$ M SPD	40	80	1810	3830	240	2610	< 10
Con/10 $\mu$ M SPM	50	105	1720	1480	280	4380	< 10
CHX/medium only	3160	45	3470	3780	410	4050	20
CHX/1 $\mu$ M SPD	2455	75	2090	5655	1645	4560	30
CHX/10 $\mu$ M SPD	605	330	2015	5820	1570	3780	60
CHX/1 $\mu$ M SPM	435	205	1640	2790	920	4260	100
CHX/10 $\mu$ M SPM	145	335	1370	1755	1300	3980	160

<sup>a</sup>Data represent mean values taken from duplicate experiments in which agreement was within  $<$ 15% of the mean following normalization of control values.

<sup>b</sup>CHX at 10  $\mu$ g/ml; / = wash.

<sup>c</sup>ODC activity, pmol/h/mg; SSAT activity, pmol/min/mg.

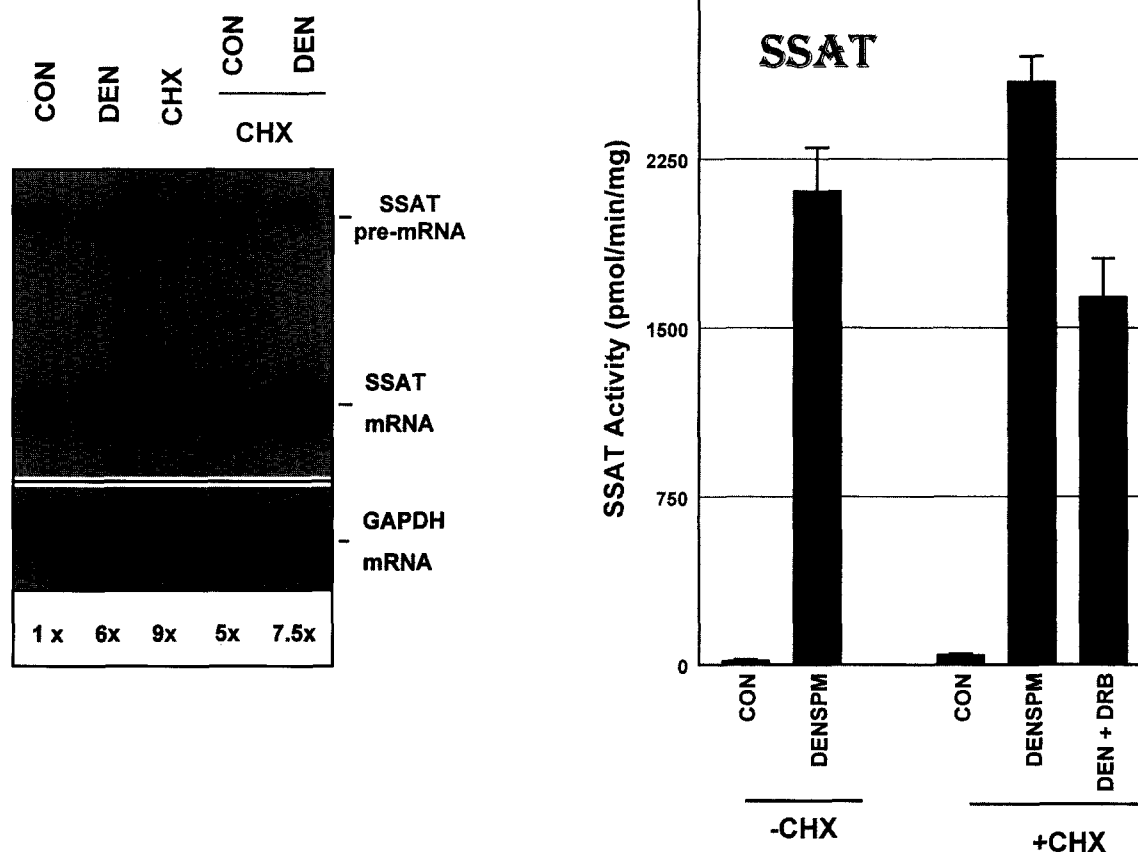


Fig. 3. Northern blot of SSAT mRNA (left) and bar graph of SSAT activity (right) from MALME-3M cells treated for 6 h in the presence or absence of CHX (at 10  $\mu$ g/ml), washed and then post-incubated for an additional 6 h in the presence or absence of DENSPM. Post-incubations were also carried out with the inhibitor of transcription, dichlorobenzimidazole (DRB, at 25  $\mu$ g/ml). SSAT hybridizing RNA appears as a  $\sim$ 3.5 kb pre-processed or heteronuclear RNA (SSAT pre-mRNA) and as 1.3 and 1.5 kb cytoplasmic transcripts [12,13] which appeared in these experiments, as a single band. Fold increase values at the bottom of Northern blot were obtained by first quantitating SSAT mRNA relative to GAPDH mRNA and then to SSAT mRNA in control cells. Note the scale difference for SSAT activity relative to Fig. 1. Data are based on four experiments performed in duplicate.

SSAT induction in cells treated with DENSPM alone, we assume that the response capabilities of gene expression are nearly saturated. Unlike with SPM, the analog partially prevented the decline of SSAT mRNA levels during the 6 h incubation as found by Northern blots (Fig. 3). The possibility that this was due to transcriptional activation was reinforced by the finding that co-treatment with DENSPM and the inhibitor of transcription DRB consistently reduced induction of SSAT activity by  $\sim$ 30% (Fig. 3). By contrast, DRB had no effect on SPM induction of SSAT activity as is consistent with data in Fig. 1. Thus, DENSPM post-transcriptional effects could not be unambiguously separated from effects on SSAT mRNA transcription. However, since the final increase in enzyme activity ( $\sim$ 50-fold) was far greater than the relative increased in available transcripts (7.5-fold), post-transcriptional effects would seem to play a more important role.

An analysis of polyamine pools is provided in Table 1. Perhaps due to rises in ODC and possibly *S*-adenosylmethionine decarboxylase activities following CHX washout, all three polyamine pools were elevated by CHX pretreatment. Exposure to SPD during the post-incubation increased SPD and SPM pools while exposure to SPM increased only the SPM pool. In correlation CHX-induced increases in SSAT,

the enzyme products *N*<sup>1</sup>-acetylSPD and *N*<sup>1</sup>-acetylSPM rose substantially following inhibitor treatment. *N*<sup>1</sup>-AcetylSPD increased even further (i.e. 3–5-fold) when CHX pretreatment was followed by a post-incubation with SPD or SPM.

#### 4. Discussion

Although the ability of polyamines to induce SSAT activity has been known for some time [10,11], the underlying mechanisms have been difficult to study due to the relatively small amounts of enzyme protein and mRNA found in cells under both basal and induced conditions. This problem was at least partially obviated by the finding that certain polyamine analogs can bring about several hundred-fold increases in enzyme protein and/or activity in some cell types [29]. The use of analogs for studying mechanisms of induction, however, is complicated by the multiplicity of their effects on SSAT gene expression [12,13,15–18] and by uncertainty whether enzyme responses are truly representative of those produced by the natural polyamines. As we have shown here, SSAT mRNA can be rapidly induced by inhibitors of protein synthesis to levels comparable to those produced by the most potent analog, DENSPM [12,13]. Since ODC mRNA is also induced, the system provides a useful means for studying

polyamine-mediated post-transcriptional control of both SSAT and ODC gene expression in the context of intact cells.

Evidence for post-transcriptional control of SSAT gene expression by polyamines is based primarily on the extent to which they increased enzyme activity relative to available mRNA. When cells were treated with CHX and washed, expression of SSAT activity during the post-incubation was only increased by ~2-fold. Although this indicated that CHX-induced mRNA could be translated, the increase was far less than expected on the basis of the 6-fold rise in SSAT mRNA. Exposure to SPD or SPM during the post-incubation increased SSAT activity ~7-fold, a level more consistent with the available mRNA. Polyamine induction of enzyme activity was further supported by the accumulation of acetylated polyamines. This also indicates that the induced enzyme activity was fully functional within the cell. Since polyamines increased enzyme activity without affecting mRNA levels, the response is attributed to post-transcriptional events. More specifically, the near 1:1 correlation between increased activity and available transcripts strongly suggests that regulation involves enhanced mRNA translation as opposed to enzyme stabilization since the latter would be expected to substantially increase this ratio. As an example, analogs such as DENSPM are known to greatly prolong enzyme protein half-life [15,16,18] and, as discussed below, produce activity to mRNA ratios in the range of 8:1. Since there is no evidence that natural polyamines can similarly stabilize SSAT protein and since they induce enzyme activity in proportion to available mRNA, translational regulation is strongly implicated.

It is interesting to consider that despite the presence of intracellular polyamines estimated to be in the millimolar range, and the availability of excess SSAT mRNA, translation is not enhanced until exogenous polyamines are imported. Presumably, this is because endogenous polyamines are compartmentalized or bound and not able to participate in this regulatory role – an arrangement that is wholly consistent with a homeostatic response mechanism to polyamine influx.

Relative to SPD and SPM, the analog DENSPM produced profound increases in SSAT activity in CHX-pretreated cells. The conversion of the 6-fold increase in SSAT mRNA to a 50-fold increase in enzyme activity is probably due mainly, but not entirely, to post-transcriptional events. Given the magnitude of the message to activity ratio (~8:1), the most significant action by the analog may be stabilization of SSAT protein, an analog effect which has been previously demonstrated by half-life determinations [15,16]. Enhanced translation may also play a role in analog post-transcriptional effects. Parry et al. [18] used transfected COS-7 cells which overexpress SSAT mRNA to show that increases in SSAT activity by  $N^1,N^{12}$ -di(ethyl)spermine was partially due to enhancement of SSAT mRNA translation [18]. Northern blot data presented here suggest that analog effects on mRNA levels must also be considered. As previously reported [12,13] and as evidenced by the prevention of SSAT mRNA decline during the post-incubation period, analogs are capable of increasing gene transcription and mRNA stabilization [12,13]. All of these various regulatory events are probably further prolonged by the inability of SSAT to acetylate DENSPM and hence, to facilitate its elimination from the cell via excretion or catabolism.

The simultaneous induction of SSAT and ODC mRNA by CHX provides a novel opportunity to compare post-transcrip-

tional regulation of both enzymes. In contrast to effects on SSAT activity, SPD and SPM sharply reduced ODC activity in CHX-pretreated cells by 6–7-fold. Down-regulation of ODC is not unexpected since it is one of the first recognized cellular events to be unequivocally attributed to exogenous polyamines [30,31]. Basically, two post-transcriptional mechanisms have been implicated: suppression of translation [5] and induction of a specific protein termed antizyme which binds to, inhibits, and contributes to the degradation of ODC (reviewed by Hayashi et al. [6]). Most likely, both mechanisms are invoked during the ODC response described here.

The novelty of the present findings resides in the translational control of SSAT activity by natural polyamines and the differential post-transcriptional regulation of two polyamine related enzymes in the context of the same intact cells. The results illustrate how polyamine catabolism as regulated by SSAT and polyamine biosynthesis as regulated by ODC can be rapidly and simultaneously controlled by intracellular pools. These responses, together with that of polyamine transport which is also known to be regulated by polyamines [7–9], function in a coordinated manner to sensitively maintain intracellular polyamine pools at a relatively constant level [3]. Such a homeostatic system would ensure an adequate supply of polyamines for cell growth while at the same time preventing cellular toxicity due to polyamine excess.

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