Differential transcription of the human spermidine/spermine N¹-acetyltransferase (SSAT) gene in human lung carcinoma cells

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The expression of spermidine/spermine N¹-acetyltransferase (SSAT), the rate-limiting enzyme in the catabolism of polyamines, is highly regulated by a number of factors including the natural polyamines and their analogues. The phenotype-specific cytotoxicity that occurs in response to a class of polyamine analogues, the diethylpolyamines, is associated with a phenotype-specific superinduction of SSAT in human non-small-cell lung carcinomas, whereas in non-responding cell types, including the small-cell lung carcinomas, the superinduction of SSAT does not occur. In this study, we have investigated the molecular basis of this phenotype-specific SSAT induction in human lung carcinoma cells in response to N¹,N¹-diethylspermine (BESpm). To facilitate the study of transcriptional regulation, we have cloned and characterized 11 kb of the human SSAT locus, including 3500 bp of the 5' promoter region. Nuclear run-on transcription studies suggest that the initial induction of SSAT results from an increase in the rate of gene transcription. Results from Northern blot analysis and ribonuclease protection assays indicate a differential expression of SSAT mRNA between the analogue-responsive H157 and non-responsive H82 cells. There is no detectable SSAT mRNA in H82 cells, even after a 24-h analogue treatment, whereas SSAT mRNA in H157 cells was detectable by Northern blot analysis and increased more than 100-fold following drug exposure. Furthermore, nuclear run-on transcription assays do not detect any active transcription of SSAT gene in either treated or untreated H82 cells. These results indicate that at least one component of the phenotype-specific induction of SSAT appears to be due to differences in transcriptional regulation of the gene. In addition, mapping of DNase I-hypersensitive sites of the SSAT gene suggest that the cell type-specific promoter/enhancer utilization may control the expression of the SSAT gene in differentially sensitive cell types in vivo.

INTRODUCTION

The polyamines spermidine and spermine are naturally occurring polyatomic components of all eukaryotic cells. Polyamines are required for cell growth and differentiation and, in some cases, cell survival [1,2]. The intracellular concentration of polyamines in mammalian cells is controlled by a combination of carefully regulated enzymic steps, including the biosynthetic enzymes ornithine decarboxylase and S-adenosylmethionine decarboxylase, and the catabolic enzyme spermidine/spermine N¹-acetyltransferase (SSAT). Polyamine content can be additionally modified by polyamine uptake and efflux mechanisms. Recently, one class of polyamine analogues, the diethylpolyamines, was developed with the intention of depleting all cellular polyamines through regulatory mechanisms, rather than direct enzymic inhibition [3,4]. These analogues have been shown to have significant anti-tumour activity in several important human solid tumour models [4–7]. We, and others, have reported that one of these polyamine analogues, N¹,N¹-diethylspermine (BESpm), is cytotoxic to a subset of cell lines representative of human non-small-cell lung carcinoma phenotypes [8] and to a few other specific cell types [6,9]. Interestingly, this cytotoxicity is accompanied by the superinduction of SSAT enzyme activity [10,11], the rate-limiting enzyme in the catabolism of polyamines. SSAT is a cytosolic protein with a very short half-life that is highly induced by a number of factors including various toxic agents, hormones, growth factors, polyamines and their analogues [12]. Recent studies using human lung carcinoma and human melanoma model systems have revealed an association between increased SSAT activity (superinduction) and phenotype-specific cytotoxicity in response to BESpm [8,13]. A pattern has emerged with the diethylpolyamines suggesting that cell types that superinduce SSAT activity (>100-fold induction within 24 h) in response to analogue exposure will die. Those cell types which only induce moderately or do not induce SSAT in response to the same compounds are generally only growth inhibited.

Studies have indicated that the phenotype-specific induction of SSAT by BESpm in responsive cell lines is accompanied by a significant increase in SSAT protein and increased steady-state mRNA levels [11,14], suggesting that the differential induction of SSAT may result from a difference in regulating the gene expression. In the present study, we have further characterized the molecular basis underlying the phenotype-specific SSAT expression in human lung carcinoma cell lines. These studies demonstrate that the transcriptional regulation of the SSAT gene plays a role in differential induction of SSAT observed between sensitive and insensitive lung carcinoma cells in response to BESpm.

MATERIALS AND METHODS

Chemicals

BESpm was synthesized as described previously [15,16] and kindly provided by the laboratory of Dr. Raymond J. Bergeron (University of Florida, Gainesville, FL, U.S.A.). This compound
was prepared as a 10 mM stock in 0.1 M HCl and diluted in medium for cell treatment to a final concentration of 10 µM [11].

**Cell culture**

Human large-cell undifferentiated lung carcinoma line (NCI H157) and the human small-cell lung carcinoma line (NCI H82) were grown in RPMI 1640 medium with 9% (v/v) calf serum, 100 units/ml penicillin and 100 units/ml streptomycin. For treatment, cells were seeded at 5 x 10^5 cells/75-cm² flask and cultures were manipulated as indicated in the Results section.

**Screening of the genomic library and sequencing of genomic clones**

A human X-chromosomal genomic library in bacteriophage vector Charon 35 (American Type Culture Collection, Bethesda, MD, U.S.A.) was screened with a 30-mer oligonucleotide 5'-GCCGCGATGTATGTCACTGAGTCGGCCGACG-3’, corresponding to the complementary sequence (+28 to +57) of the human SSAT cDNA [14]. Overlapping deletions of genomic DNA in pBluescript (Stratagene, La Jolla, CA, U.S.A.) were generated with exonuclease III and used for DNA sequencing.

**RNA isolation and Northern blot analysis**

Total cellular RNA from BESpm-treated and untreated cells was extracted using the acid phenol–guanidine isothiocyanate method [17]. A sample (10 µg) of RNA was fractionated on a denaturing 1.5% agarose gel containing 6% formaldehyde, transferred to Genescreen membrane (DuPont, Boston, MA, U.S.A.), and hybridized with a random-primer-labelled 757-bp PCR product which encompasses the coding sequence of the human SSAT cDNA [14]. Human 18 S ribosomal DNA probe was used as a loading control.

**RNase protection assay**

Templates for transcribing the RNA probes were generated by cloning the appropriate human gene fragments into pBluescript vectors. RNA probes were synthesized by in vitro RNA transcription [18]. Total RNA (10–20 µg) was lyophilized in a SpeedVac centrifuge (Savant Instruments, Farmingdale, NY, U.S.A.) and resuspended in 20 µl of 80% (v/v) formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA containing 1 x 10^6 c.p.m. of the 32P-labelled control RNA probe (human glyceraldehyde-3-phosphate dehydrogenase, GAPDH) and 2 x 10^5 c.p.m. of the 32P-labelled human SSAT RNA probe. RNA samples were hybridized overnight (>8 h) at 45 °C, followed by digestion with RNase T2 (GIBCO-BRL, Gaithersburg, MD, U.S.A.) and direct precipitation with 2 M guanidine thiocyanate and isopropanol [19]. Protected RNA fragments of 98 nt and 174 nt for SSAT and GAPDH messages, respectively, were separated by electrophoresis on a denaturing polyacrylamide gel containing 8 M urea.

**Nuclear run-on transcription**

Nuclei (1 x 10^5) in 200 µl of the run-on buffer of 5 mM Tris/HCl (pH 8.0), 2.5 mM MgCl₂, 150 mM KCl, 10 mM dithiothreitol (DTT), containing 0.5 mM each of ATP, CTP and GTP were incubated with 100 µCi of [α-^32P]UTP (800 Ci/mmol; DuPont NEN) for 30 min at 30 °C. The labelled nascent RNA transcripts were purified by the acid phenol–guanidine isothiocyanate extraction method [17,20]. The 32P-RNA (2–8 x 10^6 c.p.m.) was hybridized at 65 °C for 36-40 h to prehybridized ZetaProbe membranes (Bio-Rad, Hercules, CA, U.S.A.) containing 2 µg/slot of linearized cDNA plasmids in a solution of 0.5 M sodium phosphate (pH 7.2) containing 1 mM EDTA, 1% BSA and 7% (w/v) SDS. Membranes were then washed twice in 2 x SSC (1 x SSC: 0.15 M NaCl and 0.015 M sodium citrate)/0.5% SDS for 10 min at room temperature followed by washing twice in 0.1 x SSC/0.5% SDS for 30 min at 65 °C.

**Mapping of DNase I-hypersensitive sites (DHSs)**

Nuclei from cultured cells were prepared using a detergent-based lysis [21] with modifications. Briefly, cells were washed with ice-cold PBS and resuspended in buffer A (15 mM Tris/HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 300 mM sucrose, 5 mM MgCl₂, 0.5 mM DTT, 0.1 mM PMSF plus 0.5% Nonidet P-40) and left on ice for 10 min. The nuclei were pelleted by centrifugation (600 g, 5 min at 4 °C), resuspended in buffer A modified to contain 3 mM MgCl₂ and 0.1 mM CaCl₂ at 1 x 10^5 nuclei/ml, and digested with DNase I (ranging from 0 to 80 units/ml) on ice for 10 min. DNase I digestion was terminated by adding an equal volume of 20 mM EDTA/1% SDS. Nuclei were then digested with 50 µg/ml RNase A at 37 °C for 60 min, and 50 µg/ml proteinase K at 37 °C overnight. The genomic DNA was purified by phenol/chloroform extraction and ethanol precipitation. Aliquots of 30-40 µg of DNA were completely digested with restriction enzymes, separated on 1% agarose gels, transferred to ZetaProbe membranes [22], and hybridized with the 32P-labelled probe using a random primer oligolabelling technique [23].

**RESULTS**

**Differential expression of the SSAT mRNA in BESpm-treated human lung carcinoma cells**

Studies in our laboratory have established that the induction of the SSAT enzyme activity in human lung carcinoma cells in response to a class of polyamine analogues, the diethyl- polyamines, is phenotype-specific and appears to be due almost entirely to new protein synthesis, which is, in part, based primarily on new mRNA synthesis [5,11,14]. To investigate the molecular basis of this phenotype-specific induction by a more sensitive assay, three human lung tumour cell lines which exhibit different levels of induction of SSAT activity in response to BESpm treatment [11] were used to examine the change in the steady-state level of the SSAT mRNA by RNase protection assay (Figure 1). The cell lines used were the SSAT superinducing human large-cell lung carcinoma line (NCI H157), the SSAT uninducible human small-cell lung carcinoma line (NCI H82) and the SSAT moderately inducing human adenocarcinoma line (A549). After 24-h exposure to BESpm, a significant increase in the SSAT mRNA was found in H157 cells, and a 2-3-fold increase in A549 cells. In addition, the steady-state level of SSAT mRNA was detected in untreated H157 and A549 cells, albeit at a very low level in A549 cells. In H82 cells, however, BESpm treatment did not result in any detectable SSAT mRNA expression.

To define further the kinetics of BESpm-induced increases in the steady-state SSAT mRNA, time-course studies were performed in H157 cells. Cells were exposed to 10 µM BESpm for increasing times, up to 24 h. The total RNA was isolated at selected time points for Northern blot analysis. The results (Figure 2) indicate a rapid increase (2–3-fold) in steady-state level of the SSAT mRNA at the 30-min time point which continued to more than 100-fold after 24-h exposure to the analogue. The rapid increase in the SSAT mRNA by BESpm,
The steady-state level of SSAT mRNA in H157 cells is due to transcriptional control, nuclear run-on transcription experiments were performed. The results (Figure 3A) showed a 4–5-fold increase in the transcription rate of the SSAT gene in BESpm-treated H157 cells over a period of 30–60 min, which is consistent with the results from RNA analyses. However, after 12 h of treatment with BESpm, the SSAT transcription rate returned to levels observed in untreated cells. It appears that the initial response to analogue treatment in the steady-state level of SSAT mRNA occurs at the transcription level, whereas the late-stage accumulation of the SSAT mRNA appears to involve mechanisms other than transcriptional control [24].

The SSAT gene appears to be transcriptionally inactive in H82 cells

To determine whether the inability to detect expression of the SSAT mRNA in H82 cells was due to transcriptional inactivation of the gene, we performed nuclear run-on transcription analyses. As shown in Figure 3(B), the SSAT mRNA was transcribed only in H157 cells. By comparison, the human β-actin transcript, which served as a positive control, was equally detected in both H157 and H82 cells. Time-course studies of nuclear run-on transcription on BESpm-treated H82 cells did not detect any transcripts of the SSAT gene (results not shown). In addition, as would be expected, a much higher level of transcription on the human c-myc gene was found in c-myc-overexpressing H82 cells compared with that in H157 cells.

Nucleotide sequences of the human SSAT genomic clones

The human SSAT gene has been localized on the human X chromosome and is under the control of a TATA-less promoter [25]. However, nucleotide sequences of the SSAT genomic clone, especially the 5′-regulatory region, had not been completely sequenced. By screening a human X-chromosomal genomic library, two overlapping genomic clones were isolated which define ~11 kb of the SSAT locus (sequence has been submitted to GenBank). The overlapping clones define the 3.5 kb 5′-regulatory region, the 3.0 kb exon–intron gene body, and >6 kb of the 3′-untranslated region. We have previously reported that the human SSAT gene may contain one major and one minor transcriptional start sites at ~180 and ~160 bases relative to the translation start site respectively [25]. To confirm the authentic 5′-terminus of the SSAT mRNA, an RNase protection assay was performed using a 32P-labelled antisense RNA probe. Total cellular RNA from BESpm-treated and untreated H157 cells were hybridized with a 32P-labelled single-stranded 624-base transcript complementary to the nucleotide ~539 bp to +85 bp relative to the translation start site of the gene. Protection assays demonstrated that only one protected fragment of 245 nucleotides (Figure 4) was detected, which corresponds to the major transcriptional start site defined previously by primer extension at base pair ~180 relative to the translation start site. There was no change of the protected fragment between treated and untreated cells. These results suggest that the major site shown in primer extension analysis [24,25] is the authentic transcriptional start site, whereas the minor site appears to result from premature termination due to the secondary structure in that region.

Characterization of the human SSAT gene has revealed an interesting structural feature of the 5′-untranslated region. Two long-stretch alternating purine-pyrimidine sequences with extremely high A-T contents (>80%) are found in regions between ~3200 bp and ~2690 bp relative to the transcriptional start site. A particularly interesting sequence motif in these alternating
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Figure 3 Nuclear run-on transcription analysis

(A) Nuclei from H157 cells were isolated after exposure to 10 µM BESpm for the indicated time. The nascent RNA transcripts were labelled with [α-32P]UTP and purified from nuclei. Equal amounts of the RNA per blot were hybridized to 2 µg of plasmids of SSAT cDNA, GAPDH cDNA, and the pBlueScript vector immobilized on ZetaProbe-membranes. pBlueScript and GAPDH were used as negative and positive controls, respectively. The values from PhosphoImage analysis shown at the bottom of the blot represent the fold increase of the SSAT transcription rate relative to 0 min treatment normalized to GAPDH. (B) The 32P-labelled RNA transcripts from untreated H157, and control H82 (−), and 24 h-BESpm-treated H82 (+) nuclei were hybridized to immobilized plasmids as indicated. pSSAT-H6 contains entire exon–intron sequences of the human SSAT gene. pExon1 contains the 624-bp SacI fragment of the SSAT genomic sequences including the intact first exon and partial 5′-flanking region. pM5 contains the first exon of the human c-myc genomic sequences. pActin is the human β-actin cDNA.

purine-pyrimidine regions is one that contains four identical repeats of the sequence 5′-ATATATGTTGATATATAT-3′, which has been identified in the genome of many species, including human, mouse, chicken, Drosophila and yeast.

Mapping nuclear DHSs

In eukaryotes, key regulatory regions of active and inducible genes are maintained in an accessible configuration and generation and/or maintenance of nucleosome-free DHSs can be an important step in allowing assembly of a transcription complex for gene activation. Many studies indicate that formation of tissue- or cell-specific DHSs precedes or accompanies tissue- or cell-specific gene expression [26]. The identification of DHSs in cellular chromatin is useful as a marker of non-nucleosomal organization that could also reflect an underlying sequence-specific protein-DNA interaction. To compare the chromatin structure of the SSAT gene between responsive H157 and unresponsive H82 cells and determine potential cis-regulatory regions that might control the phenotype-specific expression of the SSAT gene, nuclei prepared from these cell types were incubated with increasing amounts of DNase I. The DNA from each cell type was then isolated and resolved by Southern blot analysis using appropriate restriction endonucleases and probes.

To map DHSs in the 5′-flanking region and the body of the SSAT gene, DNA purified from DNase I-treated nuclei was digested with HindIII and hybridized with a 766-bp NcoI–HindIII probe spanning from within the third intron into the sixth exon of the SSAT gene. As shown in Figure 5, four DHSs were detected (a–d) in H157 nuclei. DHS b was detected in both H157 and H82 nuclei, which maps to ~0.1 kb from the transcriptional start site of the SSAT gene. DHSs a, c and d were only apparent in H157 nuclei and appear to be absent in H82 nuclei. Relative to the transcriptional start site, DHS a maps to ~1.5 kb; c and d sites map to about 1.4 kb and 1.9 kb respectively. DHSs a and b are positioned in the 5′-flanking region, whereas DHSs c and d are present in the third intron. A DHS unique to the H82 cells was detected and localized in the second intron, ~0.6 kb relative to the transcriptional start site. Mapping DHSs in the 3′ region of the gene using a 245-bp HindIII–EcoRI probe downstream from the HindIII site to the end of the sixth (last) exon revealed no detectable DHS in the 3′-untranscribed region of the gene in either type of nuclei. It is important to note that the observed DHSs in the H157 cells are not altered by treatment with BESpm and no new sites are observed after treatment (results not shown).

DISCUSSION

The induction of SSAT by polyamine analogues varies among different cell lines, as well as different phenotypes originating from the same tissue [8,13,27]. The differential induction of SSAT and differential sensitivity to the analogues among the human lung cancer [5] and other human solid tumour lines
Figure 5  Cell-type-specific DNase I cleavage pattern of the human SSAT gene

Purified nuclei from H157 and H82 cells were digested with DNase I at the following concentrations (units/ml): 0 (lane 1), 20 (lane 2), 40 (lane 3), and 60 (lane 4). DNA was isolated and digested to completion with HindIII. Probes used for Southern blotting were a 766-bp SSAT genomic DNA upstream from the HindIII site (A) and a 245-bp SSAT cDNA downstream from the HindIII site (B) for 5' and 3' mapping relative to the HindIII site, respectively. Six exons are numbered and represented by filled boxes. Filled arrows represent the three specific DHSs (a, c and d) found only in H157 cells. The open arrow indicates the hypersensitive site (b) found in H157 and H82 cell lines. The H82-specific DHS is indicated by the asterisk in intron 1. Molecular size markers are indicated on the left and the right in kilobase pairs.

[9,28,29] appears to be a functional difference between the responding and non-responding cells. A phenotype-specific superinduction of SSAT activity in human lung cancer cell lines has been demonstrated to be the result of new protein synthesis, which in turn is based primarily on new mRNA synthesis [8,11]. It is known that the differential response is not based on differences in drug accumulation between responsive and non-responsive phenotypes. However, the molecular mechanisms underlying this phenotype-specific induction of SSAT are not fully understood. In this study, we examined the relationship between the induction of the SSAT activity and accumulation of the steady-state mRNA in three human lung cancer cell lines. Our results indicate that the basal level and BESpm-induced accumulation of SSAT-specific mRNA differ among cell types. Two responding phenotypes (H157 and A549 cells) express basal levels of SSAT mRNA, which increased when cells were exposed to BESpm. That the non-responding phenotype (H82 cells), on the other hand, does not express SSAT mRNA at a level sufficient to be detected by Northern blot, RNase protection, or nuclear run-on analyses, suggests that in these cells the SSAT gene may be transcriptionally inactive. Although the basis of this inactivation is not known, the DNase I hypersensitivity discussed below suggests that chromatin structure may play an important role. Overall, these observations suggest that differential expression of SSAT between H157 and H82 cells is initially at the level of transcription.

The rapid increase in the SSAT mRNA level in response to BESpm in H157 cells appears to be initially controlled at the level of transcription. It was also found that the steady-state SSAT mRNA continues to increase significantly through 24 h of treatment in the absence of continuing increases in transcription rate, suggesting that post-transcriptional mechanisms are involved in the accumulation of the mRNA. Fogel-Petrovic et al. reported that in addition to increasing transcriptional rate, BESpm has effects on the stabilization of the SSAT mRNA in MALME-3M human melanoma cells [24]. It appears that increases in steady-state SSAT mRNA are the result of a combination of the increase in gene transcription and an increase in the message half-life. It is interesting to note that treatment with BESpm for 30 min in H157 cells results in a 4-fold induction of SSAT enzyme activity, which directly corresponds to the increase in the transcription rate. Additionally, studies in human lung cancer melanoma models indicate that the analogue-induced accumulation of SSAT-specific mRNA does not account for the entire increase in SSAT activity resulting from analogue exposure. These data suggest that during early treatment (30–60 min), the induction of SSAT is controlled primarily at the level of gene transcription; later, however, post-transcriptional regulation, such as mRNA processing and translational/post-translational control, may play critical roles in the great accumulation of both the SSAT mRNA and protein. It is likely that the initial increase in the rate of gene transcription is
necessary for the downstream events of the SSAT superinduction to occur.

The susceptibility of chromatin to DNase I cleavage has been associated with actively expressed genes [26]. Our studies of DNase I hypersensitivity of the human SSAT gene have revealed multiple cell type-specific DHSs that exist in the responsive cell line H157, are absent in the non-responsive cell line H82 and which do not change in response to BESpm treatment.

Three H157 cell-specific DHSs have been located in the 5′-flanking region (DHS a) and the third intron (DHSs c and d) of the human SSAT gene. Preliminary results from transient transfection studies suggest that the region around DHS a may be important for the BESpm-mediated transcriptional activation, but has no direct role in regulation of the phenotype-specific SSAT expression [30]. DHSs c and d appear to be related to DNA methylation differences between the two cell types. It was found that several methylation-sensitive restriction sites around these DHSs were partially methylated in H82 cells, but completely unmethylated in H157 cells [31]. It seems that the presence or absence of methylation in this region reflects an alternative chromatin conformation. Open chromatin conformation around DHSs c and d may be essential prerequisites for the SSAT transcription in H157 cells, although this remains to be determined by functional analyses. The coincidence between the methylation status and the SSAT expression suggest that the cell type-specific enhancer utilization in the third intron may result in differential expression of the SSAT gene in vivo. However, it should be noted that the apparent transcriptional inactivity of SSAT in H82 cells is not a result of hypermethylation of the CpG island located in the 5′ promoter region as has been described for several other genes [31,32]. It should also be noted that the existence of a unique DHS, specific to the H82 cells, suggests the possibility of a repressor binding site mediating transcriptional repression of the SSAT gene in the H82 cells. However, further investigation will be required to determine whether repression is involved in the lack of SSAT expression in H82 cells.

Our current data provide evidence for the importance of the initial expression and transcriptional regulation of the gene in phenotype-specific SSAT induction in human lung carcinoma cells in response to the polyamine analogue. The apparent transcriptional inactivation of the SSAT gene in H82 cells suggests the possibility that SSAT activity is not required for cell survival. Further studies will be necessary to determine whether the inactivation of the SSAT gene plays a role in protecting cells from the cytotoxic activity of BESpm. Previous studies have indicated that changes in intracellular polyamine levels could result in the alteration of gene expression at the transcriptional level [33,34]. The natural polyamine spermine has been shown to produce an increase in the steady-state level of SSAT mRNA [35]. BESpm has structural and functional similarities to spermine [4]. It is possible that the BESpm-induced up-regulation of the SSAT transcription is due to the displacement of natural polyamines. Therefore, the mechanism underlying the BESpm-mediated SSAT transcription is not only useful for understanding the response to the diethylpolyamine analogues, but may also provide useful information to understand at the molecular level how polyamines can affect specific gene expression.

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