Amino Acids (2012) 42:485–493
DOI 10.1007/s00726-011-1027-7

ORIGINAL ARTICLE

Tissue-specific alternative splicing of spermidine/spermine N^1-acetyltransferase

Mervi T. Hyvönen · Anne Uimari · Jouko Vepsäläinen · Alex R. Khomutov · Tuomo A. Keinänen · Leena Alhonen

Received: 11 March 2011 / Accepted: 2 June 2011 / Published online: 2 August 2011

Abstract The polyamines, spermidine and spermine, are abundant organic cations participating in many important cellular processes. We have previously shown that the rate-limiting enzyme of polyamine catabolism, spermidine/spermine N^1-acetyltransferase (SSAT), has an alternative mRNA splice variant (SSATX) which undergoes degradation via nonsense-mediated mRNA decay (NMD) pathway, and that the intracellular polyamine level regulates the ratio of the SSATX and SSAT splice variants. The aim of this study was to investigate the effect of SSATX level manipulation on SSAT activity in cell culture, and to examine the in vivo expression levels of SSATX and SSAT mRNA. Silencing SSATX expression with small interfering RNA led to increased SSAT activity. Furthermore, transfection of SSAT-deficient cells with mutated SSAT gene (which produced only trace amount of SSATX) yielded higher SSAT activity than transfection with natural SSAT gene (which produced both SSAT and SSATX). Blocking NMD in vivo by protein synthesis inhibitor cycloheximide resulted in accumulation of SSATX mRNA, and like in cell culture, the increase of SSATX mRNA was prevented by administration of polyamine analog N^I,N^{II}-diethylnorspermine. Although SSATX/total SSAT mRNA ratio did not correlate with polyamine levels or SSAT activity between different tissues, increasing polyamine levels in a given tissue led to decreased SSATX/total SSAT mRNA ratio and vice versa. Taken together, the regulated unproductive splicing and translation of SSAT has a physiological relevance in modulating SSAT activity. However, in addition to polyamine level there seems to be additional factors regulating tissue-specific alternative splicing of SSAT.

Keywords Polyamines · Polyamine analogs · Transgenic animals · Regulated unproductive splicing and translation · Nonsense-mediated mRNA decay · Cycloheximide · Alternative splicing

Abbreviations

APAO	Acetylpolyamine oxidase
AZ	Antizyme
DENSpm	N^1,N^{11}-diethylnorspermine
MeSpd	1-Methylspermidine
N^1-AcSpd	N^1-acetylspermidine
NMD	Nonsense-mediated mRNA decay
ODC	Ornithine decarboxylase
Put	Putrescine
RUST	Regulated unproductive splicing and translation
siRNA	Small interfering RNA
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N^1-acetyltransferase

M. T. Hyvönen (✉) · A. Uimari · L. Alhonen
A. I. Virtanen Institute for Molecular Sciences, Biocenter Kuopio, University of Eastern Finland, Yliopistonranta 1E, 70210 Kuopio, Finland
e-mail: mervi.hyvonen@uef.fi

J. Vepsäläinen · T. A. Keinänen
Laboratory of Chemistry, Department of Biosciences, Biocenter Kuopio, University of Eastern Finland, Yliopistonranta 1E, 70210 Kuopio, Finland

A. R. Khomutov
Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilova street 32, Moscow 119991, Russia

Introduction

The polyamines, spermidine and spermine, are organic cations present at millimolar concentrations in eukaryotic cells (Pegg 2009). Due to their flexible carbon backbone and cationic nature, the polyamines can interact with various negatively charged molecules such as DNA, RNA, phospholipids and acidic protein structures, and they can also be covalently bound to proteins. The polyamines play important roles in many cellular processes, such as proliferation, differentiation and apoptosis, and their cellular levels are kept in tight control via multiple regulated pathways. In particular, targeting polyamine metabolism has been attractive therapeutic approach for cancer and parasitic diseases (Bachrach 2004; Clark et al. 2010; Heby et al. 2007).

The expression of the rate-limiting enzymes of polyamine metabolism is regulated by mechanisms involving transcriptional, posttranscriptional, translational and post-translational level (Wang 2007; Pegg 2008). The expression of spermidine/spermine N^1-acetyltransferase (SSAT), the enzyme acetylating and facilitating the N^1-acetylpolyamine oxidase (APAO)-mediated conversion of spermine to spermidine and spermidine to putrescine, is also highly regulated at many levels of gene expression. The transcription of SSAT is induced by polyamines via binding of polyamine-modulated factor 1 together with Nrf-2 to SSAT promoter region (Wang et al. 1999, 2001). Furthermore, polyamines stabilize SSAT mRNA and accelerate its translation (Pegg 2008; Butcher et al. 2007), and SSAT enzyme protein is protected from degradation by polyamine-induced conformational change (Coleman et al. 1995). We showed previously that in addition to other mechanisms, SSAT expression is regulated by a process called regulated unproductive splicing and translation (RUST) (Hyvönen et al. 2006). In RUST, there is production of an alternative mRNA splice variant, which contains premature stop codon targeting the mRNA to rapid degradation via nonsense-mediated mRNA decay (NMD) pathway (Wagner and Lykke-Andersen 2002). In the case of SSAT, our findings indicated that the intracellular polyamine level regulates the alternative splicing of SSAT, high polyamine levels promoting the production of normal SSAT mRNA, and low levels the generation of an unproductive mRNA splice variant (SSATX) (Hyvönen et al. 2006) which is targeted to NMD (Fig. 1). Our findings also suggested that polyamines could regulate the alternative splicing of some other genes, such as cdc2-like kinase 1.

Since NMD-targeted splice variants exist in low level for majority of genes, it has been suggested that many of them do not have regulatory role but merely exist as insignificant background (Pan et al. 2006). By contrast, other reports point out to an important regulatory role of RUST in gene expression (Green et al. 2003). Based on mutagenesis and knockdown experiments, our present results indicate that RUST contributes to the regulation of SSAT activity. In addition the data indicates that although the exact expression levels of SSAT and SSATX mRNA in different tissues are regulated not only by polyamine levels but also by other tissue-specific factor(s), the alteration of polyamine level in a particular tissue changes SSATX/total SSAT mRNA ratio. Thus, RUST is a part of complex regulatory system which has evolved to fine-tune SSAT activity and polyamine metabolism.

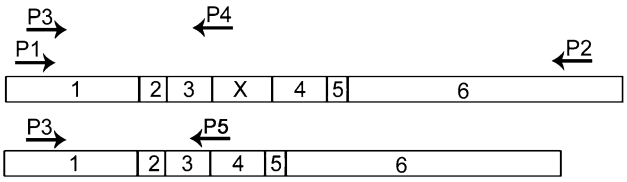

Fig. 1 Exon organization of SSATX and SSAT mRNAs and the primers used in this study

Materials and methods

Materials

DENSpm was synthesized essentially as described earlier (Rehse et al. 1990), and 1-MeSpd as described in (Järvinen et al. 2006). Zinc sulfate heptahydrate was obtained from Sigma-Aldrich.

Animal experiments

Syngenic mice or those overexpressing SSAT under the control of mouse metallothionein I promoter (MT) were generated earlier (Suppola et al. 1999). Mice were injected intraperitoneally with DENSpm (125 mg/kg i.p. in saline, 24 h before killing) alone or with CHX (15 mg/kg in saline, 16 h before killing). Syngenic or MT-SSAT transgenic Wistar rats were generated earlier (Alhonen et al. 2000). The animals were injected intraperitoneally with 1-MeSpd (100 mg/kg i.p. in saline) or zinc sulfate (10 mg^{Zn}/kg i.p. in H_2O) and euthanized 6 h later. Tissues were harvested, frozen immediately with liquid nitrogen and stored at −70°C until analyses. Two-month-old animals were used in the experiments. The animals were housed in a 12-h-light/dark-cycle facility with free access to food and water. The Institutional Animal Care and Use Committee of the University of Eastern Finland and the Provincial Government approved the animal experiments.

Construction of the plasmids

The wild-type SSAT gene expressing plasmid was constructed as follows. The genomic SSAT gene of size 3,096 bp, containing all the exons and introns, was PCR-amplified from the 129 SVJ mouse genomic DNA with the primers 5′-TACGTCGACGTCTTGCCACTTCTTAGC-3′ and 5′-CTAGCGGCCGCACCACCTTGTTCTTCATC-3′ (restriction enzyme digestion sites for *Sal*I and *Not*I, respectively, are underlined) and inserted as a *Sal*I–*Not*I fragment into the vector pCMV/myc/cyto (Invitrogen) from which the *Nco*I restriction enzyme site was deleted. The plasmid containing the SSAT gene from which the acceptor and donor codons from both sides of exon X were mutated was constructed by using the PCR-based mutagenesis according to (Ko and Ma 2005). Shortly, the forward and reverse PCR primers containing an *Ear*I restriction enzyme digestion site followed by the introduced mutation were designed for the exon–intron boundaries of exon X. PCR primers located upstream and downstream of the exon X passing the positions of *Bgl*II and *Axy*I restriction enzyme digestion sites of SSAT sequence, respectively, were used together with the acceptor and donor codon-mutated primers to amplify the genomic SSAT gene. For the donor codon mutation the plasmid containing the wild-type SSAT gene (described above) was used as a template for PCR. The amplified PCR-products were digested with *Ear*I and ligated. The obtained mutated fragment was digested with *Bgl*II and *Axy*I and inserted into the wild-type SSAT gene containing plasmid from which the corresponding *Bgl*II–*Azy*I region of SSAT gene was removed. The obtained plasmid containing the mutated donor codon was used as a template for PCR to introduce the acceptor codon mutations into the SSAT gene. The PCR and cloning was done as described above for the donor codon mutation and yielded a plasmid carrying the genomic SSAT gene with both donor and acceptor codons of exon X mutated. The used primers are listed below. Upstream SSAT: 5′-ATCTGTTTCCTGGCA CCT-3′; downstream SSAT: 5′-AGTAACCAGAAACAG GTG-3′; donor codon mutation: (forward) 5′-TTCACTC TTCA**CTC**TAAGGTCCTATTATCGCAC-3′, (reverse) 5′-ATCACTCTTCA**GAG**CATTTATCATGATCATCTAC-3′; acceptor codon mutation: (forward) 5′-TTCACTCTTCA**C TC**TTACAGTCTCTAGCTTCG-3′, (reverse) 5′-ATCAC TCTTCA**GAG**GAAGATTGGGACAAATTAAAG-3′. *Ear*I restriction enzyme digestion site is underlined and the introduced mutation is marked as bold.

Cell culture

Primary fetal fibroblasts from SSAT-deficient (Niiranen et al. 2006) or MT-SSAT transgenic mice were prepared as described previously (Alhonen et al. 1998). Cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 50 μg/ml gentamycin (Sigma-Aldrich), and incubated in humidified atmosphere at +37°C, 10% CO_2. The cells were harvested by trypsinization, washed with PBS, pelleted and stored at −70°C until analyses. The cell number was measured electronically with Coulter Counter model Z1 (Coulter Electronics).

Transfections

SSAT-deficient cells were plated onto 6-well plates (0.2×10^6 cells/well) and grown overnight. The cells were transiently transfected with 1 μg of plasmid using standard calcium phosphate precipitation technique. After 4 h, fresh medium was changed (supplemented with 15 μM MG132 or 10 μM DENSpm), and cells harvested for analyses 24 h after.

siRNA-mediated knockdown of SSATX

Primary fetal fibroblasts were plated onto 6-well plates (0.2×10^6 cells/well) and grown overnight. The cells were transiently transfected with 100 nM SSATX siRNA (5′-AAGGTTACAGTCTCTAGCTTC-3′, Ambion) or Silencer negative control siRNA (cat.no. #AM4635, Ambion) for 24 h using siPORT NeoFX (Ambion) as transfection agent. RNA was extracted and processed as described below. Samples were also collected for SSAT activity analysis.

Polyamine samples and SSAT activity

Cell pellets were lysed on ice in lysis buffer (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol). Tissue samples were homogenized to 2–3 × vol of the same buffer without Triton X-100. Samples for polyamine measurement were taken and mixed in a ratio of 9:1 with 50% sulphosalicylic acid containing 100 μM diaminoheptane (cells) or in a ratio of 1:9 with 5% sulphosalicylic acid containing 10 μM diaminoheptane (tissues). Intracellular polyamines and polyamine analogs were measured with HPLC according to the published method (Hyvönen et al. 1992). SSAT activity was measured as described earlier (Libby 1978). The amount of DNA was measured using PicoGreen reagent (Invitrogen) according to manufacturer's instructions.

Alternative splicing of SSAT

RNA was extracted with TRI-reagent (Sigma-Aldrich) according to manufacturer's instructions. DNase-treatment,

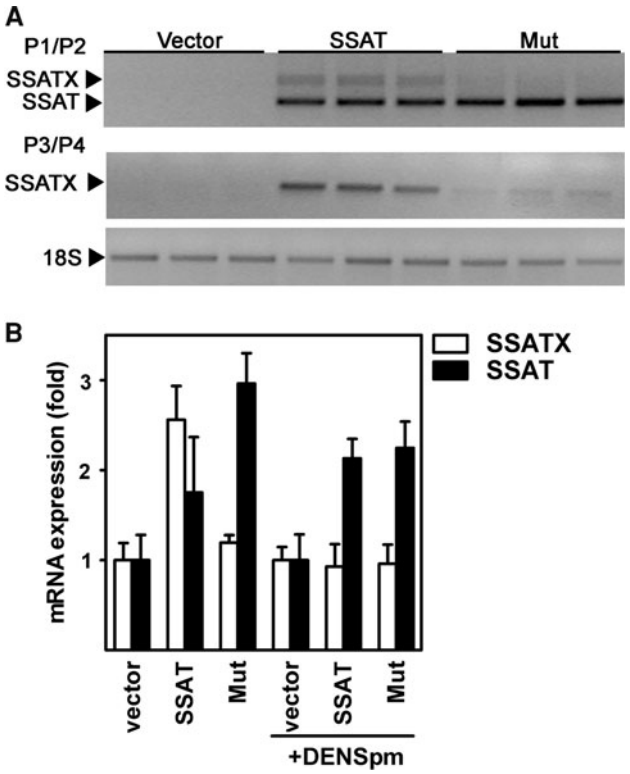

Fig. 2 Transfection of mutated SSAT gene (Mut) produces only trace amount SSATX mRNA. SSAT-deficient cells were transfected with empty vector, genomic SSAT or mutated genomic SSAT for 48 h, in the presence or absence of 10 μM DENSpm for the last 24 h. **a** Expression of SSATX and SSAT mRNA (*upper panel*), SSATX mRNA only (*middle panel*) and 18S rRNA control (*lower panel*). *Each lane* represents individual cell sample. **b** Quantitation of SSATX and SSAT mRNA expression by real-time quantitative RT-PCR. 18S rRNA was used as control. Results are mean ± SD, $n = 3$

cDNA synthesis and RT-PCR or quantitative RT-PCR were carried out as previously published (Hyvönen et al. 2006).

Statistical analysis

Values are mean ± SD. One-way analysis of variance (ANOVA) with Tuckey's post hoc test was used for multiple comparisons with the aid of a software package, GraphPad Prism 4.03 (GraphPad Software Inc.). *, ** and *** refer to p values of <0.05, <0.01 and <0.001, respectively.

Results and discussion

Transfection of mutated SSAT gene

Mouse full SSAT gene was cloned and acceptor and donor codons from both sides of exon X were mutated to generate mutated SSAT gene. Then, plasmids were transfected to SSAT-deficient cells and the levels of SSAT and SSATX mRNA were analyzed by RT-PCR (Fig. 2a) or real-time quantitative RT-PCR (Fig. 2b). As shown in Fig. 2, mutated SSAT construct generated only trace amount of SSATX variant, while the amount of SSAT mRNA produced was about twofold as compared to normal SSAT construct. Thus, cells with mutated SSAT showed lower SSATX/SSAT mRNA ratio than cells with normal SSAT. As reported earlier (Hyvönen et al. 2006), increasing polyamine level by DENSpm-treatment led to the decreased production of SSATX mRNA from normal SSAT gene. Figure 3 shows that cells transfected with mutated SSAT showed higher SSAT activity than those transfected with normal SSAT or empty vector. Since SSAT is degraded by the proteasome and has very short half-life, the difference between normal SSAT and mutated SSAT was even more prominent when the degradation of SSAT protein was inhibited with the proteasomal inhibitor MG132. Thus, lowering SSATX/SSAT mRNA ratio

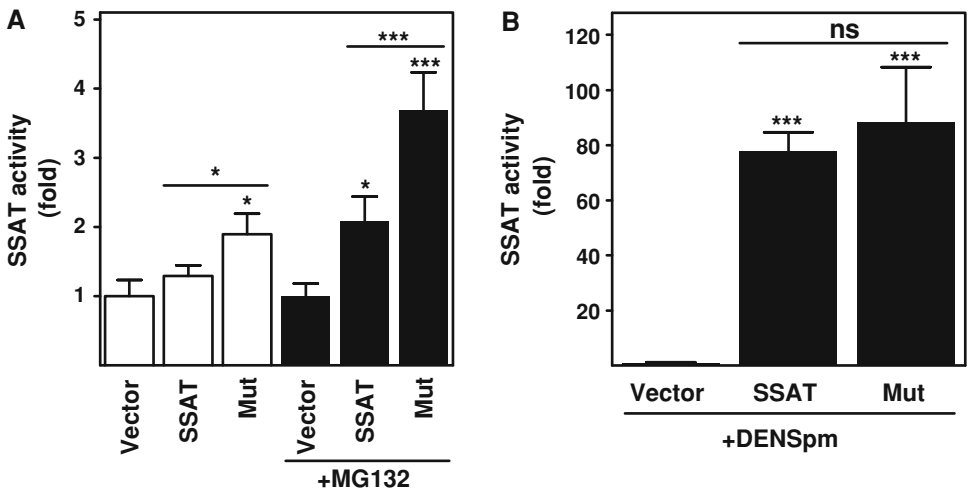

Fig. 3 Transfection of mutated SSAT gene (Mut) results in higher SSAT activity than transfection of normal SSAT gene. SSAT activity in SSAT-deficient cells transfected with empty vector, genomic SSAT or mutated genomic SSAT (48 h), and treated with **a** proteasomal inhibitor MG132, or **b** 10 μM DENSpm for the last 24 h. Results are mean ± SD, $n = 3$. *ns* not statistically significant

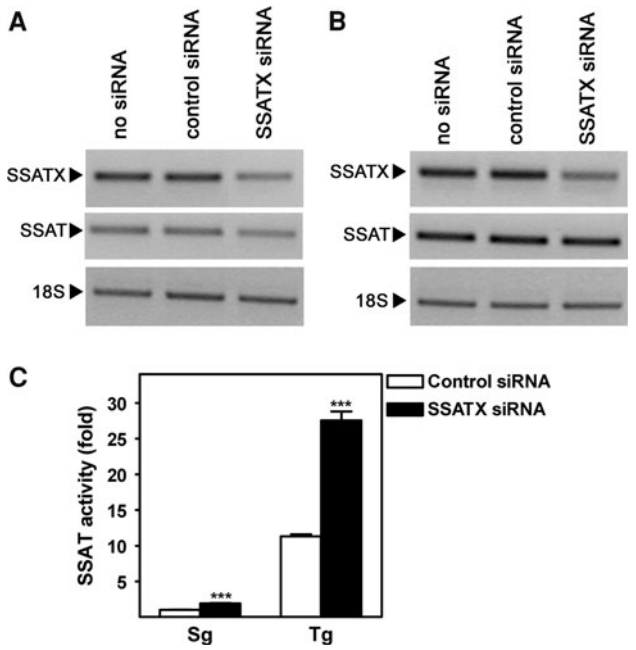

Fig. 4 Knockdown of SSATX mRNA in fetal fibroblasts increases SSAT activity. SSATX and SSAT mRNA expression in **a** syngenic, and **b** MT-SSAT transgenic cells transfected with negative control or SSATX siRNA (24 h). *Each lane* represents a pooled sample from triplicate cell samples. SSATX primers P3/P4: Sg 30 cycles, Tg 25 cycles, SSAT primers P3/P5. 18S rRNA was used as control. **c** SSAT activities. Results are mean ± SD, $n = 3$

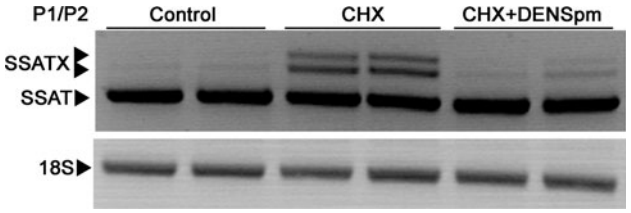

Fig. 5 Cycloheximide increases hepatic SSATX mRNA level in mice. Syngenic mice were injected with protein synthesis inhibitor CHX (15 mg/kg i.p. in saline, 16 h before killing) alone, or with DENSpm (125 mg/kg i.p. in saline, 24 h before killing), and livers were harvested for RNA extraction and RT-PCR analysis. Expression of SSAT and SSATX mRNA was analyzed with RT-PCR using primers P1/P2. 18S rRNA was used as control. *Each lane* represents individual animal

resulted in increased SSAT activity. By contrast, no significant difference in SSAT activity between mutated SSAT and normal SSAT was observed when the cells were treated with DENSpm (Fig. 3), since they showed similar SSATX and SSAT mRNA levels (Fig. 2b).

Silencing of SSATX mRNA

In the next experiment, SSATX mRNA was silenced in syngenic or MT-SSAT transgenic mouse primary fetal fibroblasts using siRNA targeted to the junction of exons 3/X. Transfection of SSATX siRNA significantly decreased the amount of SSATX mRNA in both genotypes while the amount of SSAT mRNA remained practically unchanged (Fig. 4a, b). As indicated in Fig. 4c, SSATX silencing resulted in increased SSAT activity in both genotypes as compared to negative control siRNA. Lowering SSATX/SSAT mRNA ratio by SSATX siRNA had similar effect on SSAT activity as transfection of mutated SSAT gene, although SSAT mRNA was increased only in mutagenesis experiment. Upon siRNA administration, MT-SSAT transgenic cells displayed higher increase in SSAT activity than syngenic cells, which was probably due to the higher amount of SSAT mRNA present, and thus lower SSATX/SSAT mRNA ratio. Taken together, the cell culture data collectively demonstrates that the SSATX/SSAT mRNA ratio, and not the amount of SSAT mRNA alone, regulates SSAT activity. Although SSATX mRNA is rapidly degraded and it does not produce protein, it is translated by the ribosomes until they reach NMD-triggering stop codon after which the mRNA and the nascent protein are degraded. Therefore, the level of SSATX mRNA regulates SSAT activity apparently by competing with SSAT mRNA for translation.

Accumulation of SSATX mRNA in vivo in response to CHX

To investigate whether SSATX is degraded via NMD also in vivo, syngenic mice were injected with translation inhibitor cycloheximide (15 mg/kg i.p., 16 h before killing). Since NMD requires ongoing translation to function, inhibition of protein synthesis will block also NMD and lead to the accumulation of NMD-targeted mRNAs. Indeed, animals treated with CHX showed dramatic increase in hepatic SSATX steady-state level and a single injection of DENSpm (125 mg/kg i.p., 24 h before killing) decreased SSATX mRNA almost back to control level (Fig. 5). Note that in Fig. 5 the lower band of the doublet is a hybrid containing both SSATX and SSAT mRNAs, and it is formed due to the similar structure of the two mRNAs when using primers P1/P2 (Hyvönen et al. 2006). The same doublet is also visible to some extent in Fig. 2a upper panel.

SSATX/SSAT mRNA expression levels in vivo

Next, SSATX and SSAT mRNA expression levels and their correlation with SSAT activity and polyamine pools were studied in different tissues of syngenic and MT-SSAT transgenic rats. As indicated in Table 1, the ratio of SSATX to SSAT mRNA in syngenic rats was high (>1.0) in lung, kidney, brown adipose tissue and large

Table 1 SSATX and SSAT mRNA expression levels, SSAT activities and polyamine pools in syngenic and MT-SSAT transgenic rat tissues

Genotype and tissue	SSATX/SSAT mRNA (ratio)	SSATX mRNA/18S rRNA (copies/ng RNA)	SSAT mRNA/18S rRNA (copies/ng RNA)	SSAT activity (pmol/h/mg tissue)	Put (pmol/mg tissue)	Spd (pmol/mg tissue)	Spm (pmol/mg tissue)	N^1-AcSpd (pmol/mg tissue)	Spd + Spm
Syngenic									
Lung	2.57	18 ± 4	7 ± 3	4 ± 1	50 ± 12	540 ± 161	225 ± 84	47 ± 43	765
BAT	1.62	110 ± 28	68 ± 41	n.d.	n.d.	411 ± 87	626 ± 27	665 ± 66	1,037
Kidney	1.60	121 ± 12	76 ± 36	18 ± 6	26 ± 19	314 ± 14	476 ± 43	n.d.	790
Large intestine	1.06	85 ± 11	80 ± 15	20 ± 25	83 ± 83	702 ± 197	543 ± 52	74 ± 45	1,245
Heart	0.93	28 ± 4	30 ± 7	1 ± 0	38 ± 23	421 ± 72	423 ± 55	80 ± 19	844
Skin	0.81	44 ± 21	54 ± 26	8 ± 2	332 ± 158	825 ± 325	211 ± 75	n.d.	1,036
Pancreas	0.67	6 ± 1	9 ± 2	20 ± 7	66 ± 10	6,018 ± 1,317	869 ± 231	n.d.	6,887
Small intestine	0.66	39 ± 10	59 ± 15	20 ± 14	225 ± 74	1,208 ± 132	625 ± 61	n.d.	1,833
Testis	0.51	55 ± 6	107 ± 12	14 ± 2	26 ± 15	328 ± 25	528 ± 57	46 ± 26	856
Spleen	0.41	14 ± 5	34 ± 7	29 ± 5	61 ± 12	1,236 ± 223	507 ± 40	n.d.	1,743
WAT	0.38	16 ± 6	42 ± 26	2 ± 1	50 ± 23	978 ± 461	421 ± 215	n.d.	1,399
Liver	0.24	5 ± 0	21 ± 7	2 ± 1	n.d.	994 ± 195	599 ± 65	n.d.	1,593
MT-SSAT transgenic									
Kidney	0.58	121 ± 5	210 ± 8	20 ± 4	39 ± 14	250 ± 19	490 ± 42	n.d.	740
Large intestine	0.30	902 ± 365	3,032 ± 1,410	12 ± 4	89 ± 27	572 ± 31	484 ± 40	37 ± 54	1,056
Spleen	0.27	12 ± 3	45 ± 11	27 ± 2	142 ± 30	1,214 ± 135	544 ± 43	21 ± 30	1,758
Pancreas	0.26	1,120 ± 193	4,362 ± 223	144 ± 68	3,184 ± 1,739	6,066 ± 1,245	711 ± 92	125 ± 84	6,777
Small intestine	0.25	105 ± 50	419 ± 275	31 ± 14	344 ± 76	1,157 ± 489	554 ± 83	n.d.	1,711
Heart	0.20	235 ± 121	1,151 ± 249	2 ± 1	103 ± 19	422 ± 35	485 ± 21	126 ± 35	907
Testis	0.19	370 ± 116	1,995 ± 548	30 ± 2	45 ± 12	352 ± 46	433 ± 74	60 ± 10	785
Liver	0.15	3,952 ± 68	26,613 ± 713	21 ± 6	1,210 ± 173	858 ± 127	135 ± 25	89 ± 35	993
WAT	0.13	69 ± 35	550 ± 231	3 ± 1	213 ± 65	975 ± 201	505 ± 104	n.d.	1,480
Skin	0.13	170 ± 12	1,294 ± 57	16 ± 5	626 ± 211	972 ± 208	252 ± 37	n.d.	1,124
BAT	0.12	3,864 ± 1,579	32,396 ± 2,017	4 ± 1	390 ± 64	557 ± 75	692 ± 117	822 ± 128	1,249
Lung	0.11	321 ± 61	2,847 ± 260	6 ± 1	128 ± 32	460 ± 86	231 ± 54	63 ± 37	691

Data are mean ± SD, $n = 5$

n.d. not detectable

Table 2 SSATX/total SSAT mRNA ratio, SSAT activity and polyamine levels in syngenic and MT-SSAT transgenic rats treated with zinc or MeSpd for 6 h

Tissue and treatment	SSATX/total SSAT mRNA (ratio)	SSAT activity (pmol/h/mg protein)	Put (pmol/mg protein)	Spd (pmol/mg protein)	Spm (pmol/mg protein)	N^1-AcSpd (pmol/mg protein)	MeSpd (pmol/mg protein)	Spd + Spm + MeSpd
Syngenic								
Liver								
Control	0.67 ± 0.07	0.1 ± 0.1	n.d.	2,834 ± 370	4,826 ± 321	<300		7,660 ± 460
Zn	0.42 ± 0.06	0.2 ± 0.2	495 ± 71	5,654 ± 1,284	4,203 ± 544	n.d.		9,857 ± 743
MeSpd	0.28 ± 0.09	0.9 ± 0.6	507 ± 175	2,672 ± 414	4,141 ± 526	<300	5,212 ± 945	12,026 ± 811
Pancreas								
Control	0.24 ± 0.03	14 ± 6	501 ± 148	29,198 ± 4,132	3,952 ± 942	n.d		33,150 ± 4,602
Zn	0.23 ± 0.01	20 ± 4	1,027 ± 559	27,506 ± 2,735	3,400 ± 655	n.d.		30,905 ± 3,293
MeSpd	0.17 ± 0.04	14 ± 6	1,511 ± 488	22,422 ± 4,921	5,433 ± 1,103	404 ± 145	11,284 ± 3,612	39,139 ± 7,426
WAT								
Control	0.23 ± 0.05	3 ± 1	1,321 ± 267	3,753 ± 347	3,103 ± 63	n.d.		6,856 ± 376
Zn	0.30 ± 0.03	2 ± 1	740 ± 325	3,261 ± 942	2,182 ± 229	n.d.		5,443 ± 970
MeSpd	0.14 ± 0.02	8 ± 3	n.d.	2,101 ± 924	1,933 ± 145	n.d.	3,221 ± 1,089	7,255 ± 2,030
MT-SSAT transgenic								
Liver								
Control	0.02 ± 0.00	11 ± 2	3,298 ± 918	5,919 ± 149	1,443 ± 97	825 ± 88		7,362 ± 231
Zn	0.10 ± 0.01	45 ± 13	6,598 ± 1,520	848 ± 205	542 ± 154	301 ± 47		1,390 ± 195
Pancreas								
Control	0.05 ± 0.01	25 ± 8	8,448 ± 5,697	32,403 ± 2,548	2,715 ± 299	n.d.		35,118 ± 2,734
Zn	0.24 ± 0.05	2733 ± 1633	32,049 ± 11,554	3,337 ± 1,441	2,099 ± 351	700 ± 470		5,436 ± 1,620
WAT								
Control	0.06 ± 0.01	5 ± 1	766 ± 110	2,191 ± 771	1,743 ± 606	n.d.		3,934 ± 1,273
Zn	0.09 ± 0.02	37 ± 9	1,598 ± 532	2,323 ± 685	1,559 ± 433	n.d.		3,882 ± 1,096

Data are mean ± SD. $n = 3$–6

n.d. not detectable

intestine, whereas low ratios (<0.6) were found in liver, white adipose tissue (WAT), spleen and testis. MT-SSAT transgenic rats displayed generally much lower SSATX/SSAT mRNA ratios than syngenic rats in all tissues, apparently due to the fact that the transgene construct contains SSAT cDNA (without exon X), and the amount of SSATX mRNA relies only on the compensatory induction of endogenous SSATX. Our previous cell culture studies have indicated that SSATX/total SSAT mRNA ratio correlates with the total higher polyamine level (excluding putrescine and acetylated polyamines) (Hyvönen et al. 2006; Weisell et al. 2010). Here, irrespective of the genotype, SSATX/total SSAT mRNA ratio did not correlate with polyamine levels or SSAT activity between different tissues (Table 1, and data not shown). However, when polyamine levels were altered in a given tissue either by injection of 1-MeSpd or zinc sulfate (Zn), the change in SSATX/total SSAT mRNA ratio correlated with the degree of alteration of higher polyamine pool (Table 2). When polyamine pools were increased, SSATX/total SSAT ratio was decreased, and when polyamine pools were decreased, SSATX/total SSAT ratio was increased. In transgenic animals, injection of Zn rapidly induced MT promoter and the production of SSAT mRNA, as expected (not shown). Induction of the SSAT transgene was accompanied by decreased Spd and Spm levels and compensatory induction of endogenous SSATX mRNA (not shown). Of several tissues examined, syngenic animals displayed decreased polyamine levels in response to zinc only in WAT, and that was accompanied by increased SSATX/total SSAT ratio.

Taken together, our data indicates that the higher polyamine level regulates the alternative splicing of SSAT in vivo, but there are also other factor(s) present which contribute to the regulation of the tissue-specific mRNA expression levels. The fact that endogenous SSATX was markedly increased in MT-SSAT transgenic rats (which do not harbor exon X in their transgene) when polyamine pools were depleted further supports the notion that SSATX mRNA is not only present at low level background, but its level is dynamically regulated in response to polyamine level alterations. Thus, RUST participates in the fine-tuning of SSAT expression. Further studies are needed to elucidate the exact mechanism how polyamines affect the alternative splicing process.

Acknowledgments This work was supported by grants from the Academy of Finland and by the strategic funding of the University of Eastern Finland. The authors thank Ms. Marita Heikkinen, Ms. Anne Karppinen, Ms. Arja Korhonen and Ms. Tuula Reponen for skillful technical assistance.

References

Alhonen L, Karppinen A, Uusi-Oukari M, Vujcic S, Korhonen VP, Halmekytö M, Kramer DL, Hines R, Jänne J, Porter CW (1998) Correlation of polyamine and growth responses to N^1,N^{11}-diethylnorspermine in primary fetal fibroblasts derived from transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase. J Biol Chem 273:1964–1969

Alhonen L, Parkkinen JJ, Keinänen T, Sinervirta R, Herzig KH, Jänne J (2000) Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. Proc Natl Acad Sci USA 97:8290–8295

Bachrach U (2004) Polyamines and cancer: minireview article. Amino Acids 26:307–309

Butcher NJ, Broadhurst GM, Minchin RF (2007) Polyamine-dependent regulation of spermidine-spermine N^1-acetyltransferase mRNA translation. J Biol Chem 282:28530–28539

Clark K, Niemand J, Reeksting S, Smit S, van Brummelen AC, Williams M, Louw AI, Birkholtz L (2010) Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*. Amino Acids 38:633–644

Coleman CS, Huang H, Pegg AE (1995) Role of the carboxyl terminal MATEE sequence of spermidine/spermine N^1-acetyltransferase in the activity and stabilization by the polyamine analog N^1,N^{12}-bis(ethyl)spermine. Biochemistry 34:13423–13430

Green RE, Lewis BP, Hillman RT, Blanchette M, Lareau LF, Garnett AT, Rio DC, Brenner SE (2003) Widespread predicted nonsense-mediated mRNA decay of alternatively-spliced transcripts of human normal and disease genes. Bioinformatics 19:118–121

Heby O, Persson L, Rentala M (2007) Targeting the polyamine biosynthetic enzymes: a promising approach to therapy of African sleeping sickness, Chagas' disease, and leishmaniasis. Amino Acids 33:359–366

Hyvönen T, Keinänen TA, Khomutov AR, Khomutov RM, Eloranta TO (1992) Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. J Chromatogr 574:17–21

Hyvönen MT, Uimari A, Keinänen TA, Heikkinen S, Pellinen R, Wahlfors T, Korhonen A, Närvänen A, Wahlfors J, Alhonen L, Jänne J (2006) Polyamine-regulated unproductive splicing and translation of spermidine/spermine N^1-acetyltransferase. RNA 12:1569–1582

Järvinen AJ, Cerrada-Gimenez M, Grigorenko NA, Khomutov AR, Vepsäläinen JJ, Sinervirta RM, Keinänen TA, Alhonen LI, Jänne JE (2006) Alpha-methyl polyamines: efficient synthesis and tolerance studies in vivo and in vitro. First evidence for dormant stereospecificity of polyamine oxidase. J Med Chem 49:399–406

Ko JK, Ma J (2005) A rapid and efficient PCR-based mutagenesis method applicable to cell physiology study. Am J Physiol Cell Physiol 288:1273–1278

Libby PR (1978) Calf liver nuclear *N*-acetyltransferases. Purification and properties of two enzymes with both spermidine acetyltransferase and histone acetyltransferase activities. J Biol Chem 253:233–237

Niiranen K, Keinänen TA, Pirinen E, Heikkinen S, Tusa M, Fatrai S, Suppola S, Pietilä M, Uimari A, Laakso M, Alhonen L, Jänne J (2006) Mice with targeted disruption of spermidine/spermine N^1-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but show signs of insulin resistance upon aging. J Cell Mol Med 10:933–945

Pan Q, Saltzman AL, Kim YK, Misquitta C, Shai O, Maquat LE, Frey BJ, Blencowe BJ (2006) Quantitative microarray profiling provides evidence against widespread coupling of alternative

splicing with nonsense-mediated mRNA decay to control gene expression. Genes Dev 20:153–158

Pegg AE (2008) Spermidine/spermine-*N*(1)-acetyltransferase: a key metabolic regulator. Am J Physiol Endocrinol Metab 294:995–1010

Pegg AE (2009) Mammalian polyamine metabolism and function. IUBMB Life 61:880–894

Rehse K, Puchert E, Leissring S (1990) Antiaggregatory and anticoagulant effects of oligoamines. 12. Alkyl- and arylalkyl-derivatives of putrescine, spermidine and spermine. Arch Pharm 323:287–294

Suppola S, Pietilä M, Parkkinen JJ, Korhonen VP, Alhonen L, Halmekytö M, Porter CW, Jänne J (1999) Overexpression of spermidine/spermine N^1-acetyltransferase under the control of mouse metallothionein I promoter in transgenic mice: evidence for a striking post-transcriptional regulation of transgene expression by a polyamine analogue. Biochem J 338:311–316

Wagner E, Lykke-Andersen J (2002) mRNA surveillance: the perfect persist. J Cell Sci 115:3033–3038

Wang JY (2007) Polyamines and mRNA stability in regulation of intestinal mucosal growth. Amino Acids 33:241–252

Wang Y, Devereux W, Stewart TM, Casero RA Jr (1999) Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine *N*(1)-acetyltransferase gene. J Biol Chem 274:22095–22101

Wang Y, Devereux W, Stewart TM, Casero RA Jr (2001) Characterization of the interaction between the transcription factors human polyamine modulated factor (PMF-1) and NF-E2-related factor 2 (Nrf-2) in the transcriptional regulation of the spermidine/spermine N^1-acetyltransferase (SSAT) gene. Biochem J 355:45–49

Weisell J, Hyvönen MT, Häkkinen MR, Grigorenko NA, Pietilä M, Lampinen A, Kochetkov SN, Alhonen L, Vepsäläinen J, Keinänen TA, Khomutov AR (2010) Synthesis and biological characterization of novel charge-deficient spermine analogues. J Med Chem 53:5738–5748