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

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We recently generated a transgenic mouse line overexpressing spermidine/spermine N^1-acetyltransferase (*SSAT*) gene under its own promoter. The tissue polyamine pools of these animals were profoundly affected and the mice were hairless from early age. We have now generated another transgenic-mouse line overexpressing the *SSAT* gene under the control of a heavy-metal-inducible mouse metallothionein I (MT) promoter. Even in the absence of heavy metals, changes in the tissue polyamine pools indicated that a marked activation of polyamine catabolism had occurred in the transgenic animals. As with the SSAT transgenic mice generated previously, the mice of the new line (MT-SSAT) suffered permanent hair loss, but this occurred considerably later than in the previous SSAT transgenic animals. Liver was the most affected tissue in the MT–SSAT transgenic animals, revealed by putrescine overaccumulation, significant decrease in spermidine concentration and $> 90\,\%$ reduction in the spermine pool. Even though hepatic SSAT mRNA accumulated to massive levels in non-induced transgenic animals, SSAT activity was only moderately elevated. Administration of $ZnSO_4$ further elevated the level of hepatic SSAT message and induced enzyme activity, but not more than 2- to 3-fold. Treatment of the transgenic animals with the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM) resulted in an immense induction, more than 40000-fold, of enzyme activity in the liver of transgenic animals, and minor changes in the SSAT mRNA level. Liver spermidine and spermine pools were virtually depleted within 1–2 days in response to the treatment with the analogue. The treatment also resulted in a marked mortality (up to $60\,\%$) among the transgenic animals which showed ultrastructural changes in the liver, most notably mitochondrial swelling, one of the earliest signs of cell injury. These results indicated that, even without its own promoter, SSAT is powerfully induced by the polyamine analogue through a mechanism that appears to involve a direct translational and/or heterogenous nuclear RNA processing control. It is likewise significant that overexpression of SSAT renders the animals extremely sensitive to polyamine analogues.

Key words: mitochondrial swelling, ornithine decarboxylase, polyamine catabolism, putrescine.

INTRODUCTION

Spermidine/spermine N^1-acetyltransferase (SSAT) is the rate controlling enzyme in polyamine catabolism that, together with polyamine oxidase, catalyses the back-conversion of spermidine and spermine ultimately to putrescine. SSAT apparently prevents the accumulation of cytotoxic levels of spermidine and spermine by facilitating the excretion (through charge reduction) and oxidative catabolism of the higher polyamines [1,2]. This observation is supported by the fact that the expression of SSAT is powerfully induced by spermidine, spermine and their analogues [3]. The enzyme seems to be critical in maintaining normal polyamine pools, as its overexpression, even transiently, in transfected cells leads to a marked imbalance in polyamine pools, characterized by putrescine overaccumulation, the appearance of N^1-acetylspermidine and decreases in spermidine and spermine content [4]. We recently generated a transgenic founder animal and an independent transgenic mouse line overexpressing mouse SSAT under its own promoter [5]. Analysis of the tissues of these animals revealed a widespread activation of polyamine catabolism characterized by massive accumulation of putrescine, the appearance of N^1-acetylspermidine, not normally found in mouse tissues, and decreases in spermidine and/or spermine pools [5]. Phenotypic changes of the transgenic animals included permanent loss of hair at three to four weeks of age associated with extensive formation of dermal cysts, loss of subcutaneous fat, female infertility and reduced lifespan [5]. Primary fetal fibroblasts isolated from the transgenic animals showed extreme sensitivity towards the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM), which was cytotoxic to the transgenic cells at concentrations nearly two orders of magnitude lower than those required to halt the growth of non-transgenic cells [6]. Interestingly, specific inhibition of polyamine oxidase did not attenuate the growth inhibition exerted by the polyamine analogue [6], but this finding may not be applicable to all cell types [7]. Both the tissues of the transgenic animals as well as the transgenic cells appeared to accumulate large amounts of SSAT-specific mRNA, which, however, was not translated in the absence of polyamines or polyamine analogues [5,6]. The regulation of mammalian SSAT expression

Abbreviations used: SSAT, spermidine/spermine N^1-acetyltransferase; MT, metallothionein I; DENSPM, N^1,N^{11}-diethylnorspermine.

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by polyamines and their analogues is extremely complex involving enhanced transcription and stabilization of the mRNA [8,9], enhanced mRNA translation [10,11] and stabilization of the SSAT protein [12]. The experimental data obtained from the transgenic animals overexpressing SSAT appear to indicate that post-transcriptional regulation of gene expression is the most important mode of control [5].

In order to study the regulation of SSAT expression by polyamine analogues further, we have generated a transgenic mouse line expressing the *SSAT* gene under the control of mouse metallothionein I (MT) promoter. As expected, liver was the target organ for activated polyamine catabolism. The changes in liver polyamine pools were indicative of a marked activation of polyamine catabolism, even in the absence of heavy metal inducers. Even without its own promoter, the *SSAT* gene was greatly induced by DENSPM by mechanisms exclusively involving post-transcriptional control. Treatment of transgenic animals with the polyamine analogue for a few days led to complete depletion of liver spermidine and spermine pools and was associated with marked mortality among the transgenic, but not the non-transgenic, animals. Electron microscopic examination of liver samples indicated substantial mitochondrial swelling, which, in all likelihood, represents an early sign of cell injury.

MATERIALS AND METHODS

Materials

DENSPM was kindly provided by Warner Lambert Parke-Davis (Ann Arbor, MI, U.S.A.), and was administered to the animals intraperitoneally in physiological saline.

Gene construct

Mouse MT promoter was amplified from mouse genomic DNA as described previously [13], and was operationally linked to a mouse genomic SSAT coding region (6 kb), as shown in Figure 1.

Generation of transgenic mice

The transgenic mice were generated using a standard pronuclear microinjection technique [14]. Fertilized oocytes were obtained from superovulated BALBc × DBA/2 (CF2F1) female mice mated with CD2F1 males. The transgenic mouse line, harbouring about 80 copies of the MT–SSAT fusion gene, was designated UKU181.

Analytical methods

DNA was isolated by the method of Blin and Stafford [15]. Total RNA was extracted with guanidinium isothiocyanate [16]. Polyamines and their acetylated derivatives were determined using HPLC, essentially as described by Hyvönen et al. [17]. SSAT, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities were assayed using published methods [18–20]. For the Northern-blot analyses of SSAT, 15 μg of total RNA was electrophoresed in 1.2% agarose gel under denaturating conditions, hybridized with a Dig-UTP-labelled 608-bp single-stranded probe and detected by chemiluminescence. Glyceraldehyde-3-phosphate dehydrogenase mRNA was probed with a 1267-bp rat cDNA and Methylene Blue staining of RNA was used as a loading control. For the Western-blot analyses, antibody raised against the C-terminus of SSAT was used as described previously [6].

For transmission electron microscopy, mouse liver samples were fixed in 2.5% (v/v) glutaraldehyde/0.1 M sodium cacodylate (pH 7.4) at room temperature for 4 h. Post-fixation was carried out in 1% (w/v) OsO_4/0.1 M sodium cacodylate (pH 7.4) for 2 h. After dehydration in graded ethanol, the samples were embedded in LX-112 (Ladd Research Industries, Burlington, VT, U.S.A.). Thin sections (60 nm) were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined and photographed (JEOL JEM 1200EX transmission electron microscope; JEOL Ltd., Tokyo, Japan). For evaluation of mitochondrial dimensions, the negatives of photographs were digitalized using a Peltier-cooled 12-bit Photometrics CH 250 camera (Photometrics Inc., Tucson, AZ, U.S.A.). Power Macintosh 7100/80 computer and Image software were used for image analyses. The shortest and longest dimensions of mitochondria were measured.

For statistical analyses, the two-tailed Student's *t* test or Mann-Whitney U–Wilcoxon rank sum W test were used.

RESULTS

Tissue polyamine pools and enzyme activities in non-transgenic and transgenic mice

As indicated in Table 1, SSAT activity was only moderately enhanced in the six selected tissues of non-induced transgenic animals. However, the changes in polyamine pools were clearly indicative of marked activation of polyamine catabolism in transgenic animals. As expected, polyamine pools in liver were most profoundly affected by transgene expression, the putrescine pool was expanded by a factor of six and there was a marked accumulation of N^1-acetylspermidine; spermidine and spermine levels were significantly reduced, spermine by more than 90%.

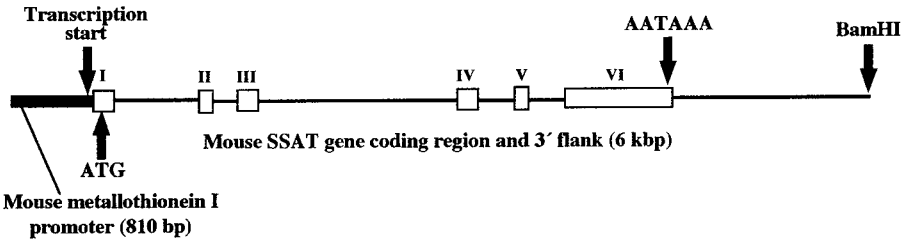

Figure 1 Mouse *MT–SSAT* fusion gene used to generate transgenic mice

The fusion gene was driven by a 810-bp MT promoter and contained all the exons and introns of the *SSAT* gene.

Table 1 Tissue polyamine metabolism in MT–SSAT transgenic mouse line 181

Data are the means ± S.E.M.; $n = 3$. Enzyme activities are expressed as pmol/10 min per mg of tissue (SSAT) and pmol/h per mg of tissue [ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (AdoMetDC)]. The concentration of polyamines is expressed as pmol/mg of tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: statistical significances between non-transgenic (tg −) and transgenic (tg +) animals.

	Enzyme activity			Polyamine pools			
Tissue	SSAT	Ornithine decarboxylase	AdoMetDC	Putrescine	N^1-Acetyl spermidine	Spermidine	Spermine
Liver tg −	0 ± 0	1.7 ± 0.7	59.0 ± 3.9	106 ± 17	< 5	1485 ± 43	1082 ± 65
Liver tg +	7.0 ± 3.0	181 ± 32**	91.0 ± 8.1*	674 ± 31***	108 ± 46	1113 ± 17**	100 ± 12***
Kidney tg −	0.9 ± 0.2	406 ± 80	14.0 ± 0.5	56 ± 15	< 5	371 ± 9	789 ± 10
Kidney tg +	1.4 ± 0.5	82 ± 28*	16.0 ± 2.8	94 ± 15	< 5	236 ± 18**	798 ± 30
Spleen tg −	7.3 ± 1.1	2.1 ± 0.9	36.0 ± 4.0	102 ± 4	< 5	1371 ± 40	917 ± 15
Spleen tg +	35.0 ± 3.3**	38 ± 25	139 ± 25**	1072 ± 110***	202 ± 15***	1442 ± 140	787 ± 42*
Heart tg −	0 ± 0	1.1 ± 0.4	11.0 ± 1.6	15.0 ± 1.5	< 5	126 ± 11	260 ± 16
Heart tg +	1.1 ± 0.3*	1.7 ± 0.4	14.0 ± 2.5	23.0 ± 6.1	< 5	79 ± 12*	271 ± 29
Brain tg −	1.9 ± 0.1	0.5 ± 0.1	29.0 ± 4.2	8.5 ± 3.0	< 5	329 ± 17	268 ± 22
Brain tg +	4.2 ± 0.1***	1.9 ± 0.6	18.0 ± 0.2	161 ± 8***	5.3 ± 0.9**	275 ± 16	289 ± 7
Testis tg −	3.7 ± 0.3	9.5 ± 0.6	15.0 ± 7.2	6.6 ± 1.5	< 5	357 ± 5.8	644 ± 8
Testis tg +	8.5 ± 0.6**	20.0 ± 3.4*	48.0 ± 4.3*	139 ± 3***	6.9 ± 2.3*	343 ± 16	522 ± 21**

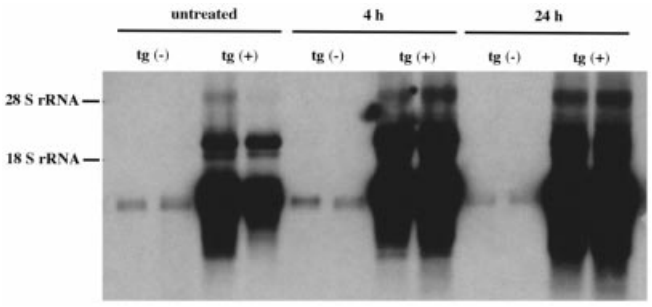

Figure 2 Effect of $ZnSO_4$ on the accumulation of SSAT-specific mRNA in liver of non-transgenic and transgenic mice

The animals received a single injection of $ZnSO_4,7H_2O$ (20 mg/kg) and were killed 4 h or 24 h later. tg (−), non-transgenic; tg (+), transgenic.

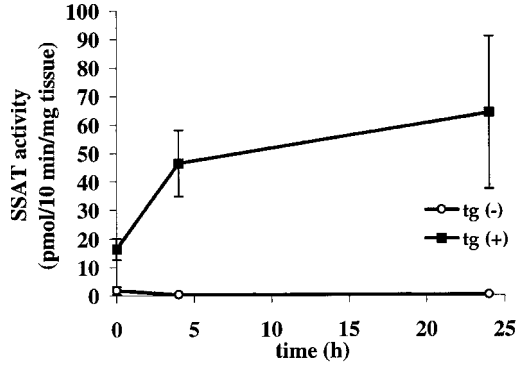

Figure 3 Effect of $ZnSO_4$ on hepatic SSAT activity in non-transgenic and transgenic animals

For experimental details see the legend for Figure 2. tg (−), non-transgenic; tg (+), transgenic. The values are means ± S.E.M. from three to four animals in each group.

Although there were small changes in the pools of the higher polyamines, the most prominent feature in the other transgenic animal tissues was a moderate to marked expansion of the putrescine pool and the appearance of N^1-acetylspermidine. Ornithine decarboxylase activity was enhanced in most of the tissues of the transgenic animals, the most marked rise (~ 100-fold) was seen in liver. Interestingly, regression analysis indicated that the increase in ornithine decarboxylase activity in tissues of the transgenic mice was inversely correlated with the concentration of spermine but not with that of spermidine, and was highly significant ($P < 0.001$). It is noteworthy that, unlike in other tissues, ornithine decarboxylase activity was substantially decreased in the kidney of transgenic mice.

Induction of hepatic *SSAT* gene expression by $ZnSO_4$

Figure 2 shows the accumulation of liver SSAT mRNA in response to a single injection of $ZnSO_4$. SSAT mRNA level in the liver of untreated transgenic animals was high in comparison with non-transgenic animals, and was even higher in zinc-treated transgenic animals. The enhanced accumulation of SSAT mRNA in transgenic animals was accompanied by a moderate (2- to 3-fold) stimulation of enzyme activity at 4 h and 24 h after zinc treatment (Figure 3), whereas SSAT activity was unchanged in the non-transgenic animals. In spite of the modest enhancement of SSAT activity in response to the metal, the spermidine pool in transgenic animals was rapidly depleted, by about 50 % (Figure 4) but the spermine pool, which was almost depleted, did not change. There was a similar transient rise in putrescine and N^1-acetylspermidine in the transgenic animals after treatment but $ZnSO_4$ had no effect on hepatic polyamine pools in the non-transgenic littermates.

Effect of DENSPM on the expression of the *SSAT* gene and hepatic polyamine pools in non-transgenic and transgenic animals

Figure 5 shows the accumulation of SSAT-specific mRNA in the livers of three non-transgenic and three transgenic animals following the administration of DENSPM (125 mg/kg body weight) for 4 days. Apart from the difference between the mRNA levels in non-transgenic and transgenic animals, the analogue appeared to have little effect on further accumulation of the

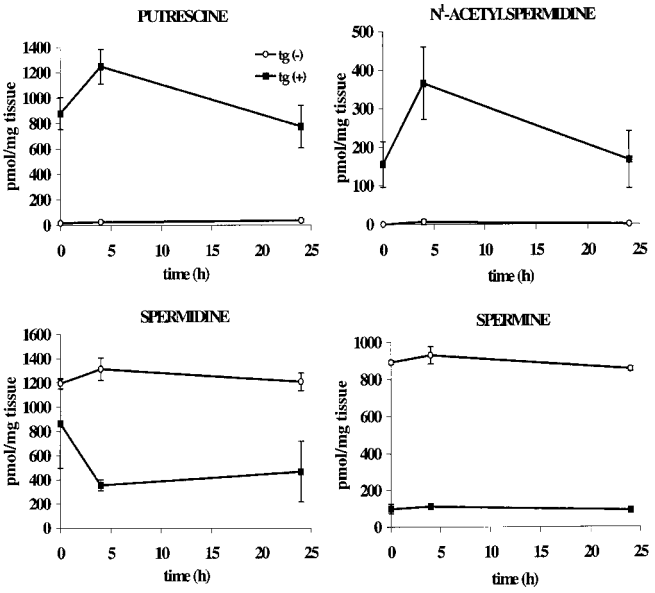

Figure 4 Effect of $ZnSO_4$ on hepatic polyamine pools in non-transgenic and transgenic animals

For experimental details see the legend for Figure 2. tg (−), non-transgenic; tg (+), transgenic. The values are means ± S.E.M. from three to four animals in each group.

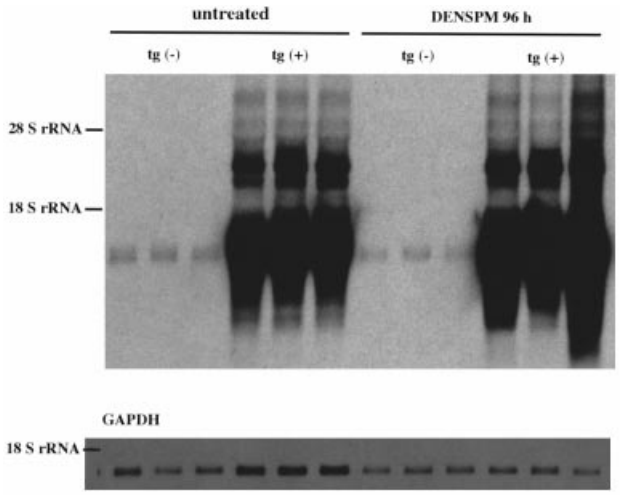

Figure 5 Effect of DENSPM on the accumulation of SSAT-specific mRNA in liver of non-transgenic and transgenic animals

The animals received 125 mg/kg body weight of DENSPM for 4 days. There were three animals in each group. tg (−), non-transgenic; tg (+), transgenic. GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a loading control.

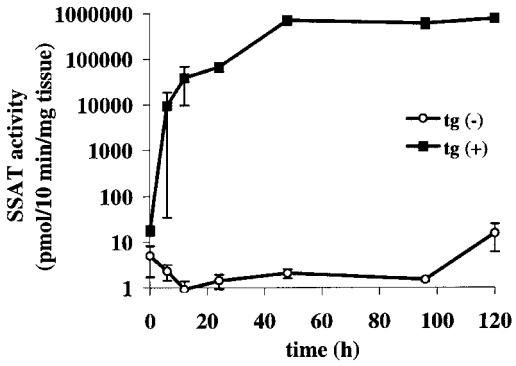

Figure 6 Effect of DENSPM on hepatic SSAT activity in non-transgenic and transgenic animals

The animals received the DENSPM (125 mg/kg body weight) for up to 120 h. There were four to six animals in each group. All the non-transgenic animals survived. The mortality among the transgenic animals was 17% at 96 h and 60% at 120 h. The values are means ± S.E.M. tg (−), non-transgenic; tg (+), transgenic. Note that the scale is logarithmic.

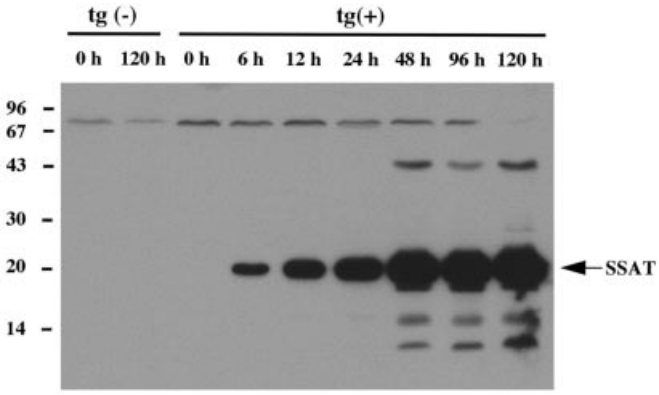

Figure 7 Effect of DENSPM on the accumulation of immunoreactive SSAT protein in the livers of non-transgenic and transgenic animals

For experimental details see the legend for Figure 6. Pooled protein samples from two to six animals. The molecular-mass markers (kDa) are shown on the left. tg (−), non-transgenic; tg (+), transgenic.

message in either of the groups. However, as indicated in Figure 6, treatment with DENSPM had a great effect on hepatic SSAT activity in the transgenic animals and enzyme activity increase by > 40000 times within 48 h in response to treatment, whereas the changes seen in non-transgenic animals were negligible (note that the scale in Figure 6 is logarithmic). Figure 7 shows a Western blot of SSAT protein after treatment with DENSPM. The accumulation of SSAT protein followed closely the changes in enzyme activity. In fact, at later time points, an apparent SSAT protein could be detected by Coomassie Blue staining, indicating that SSAT represented a major liver protein under these conditions. The treatment similarly affected polyamine pools in the transgenic animals. As depicted in Figure 8, the hepatic pool of spermidine was rapidly depleted and there was a further decrease in the already-low spermine pool in the transgenic animals; only moderate decreases were found in the pools of non-transgenic animals. This treatment was associated with overt toxicity and mortality among the transgenic animals, which was not seen in the non-transgenic animals. None of the non-transgenic animals died as a result of treatment with the analogue, 83% of the transgenic animals survived for 4 days and but only 40% survived for 5 days. A post-mortem examination of liver histology, using a light microscope, did not reveal any definite changes related to the treatment in any of the animals. However, examination by electron microscopy disclosed distinct swelling (about 2-fold) of the liver mitochondria, an early sign of cell

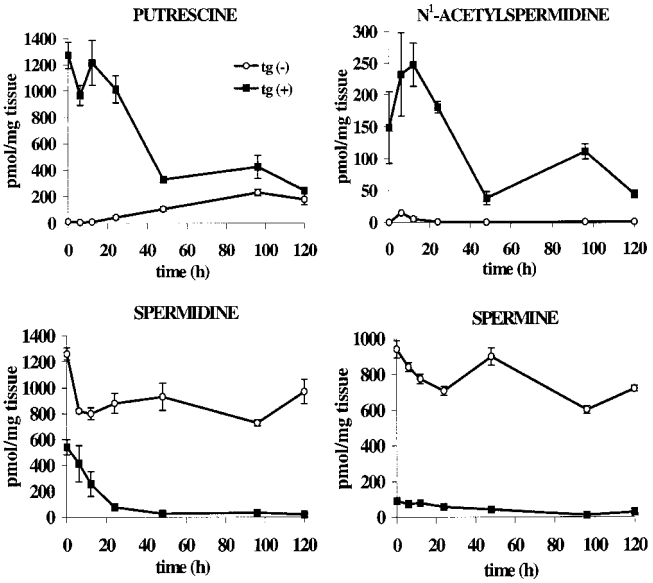

Figure 8 Effect of DENSPM on hepatic polyamine pools in non-transgenic and transgenic animals

For experimental details see the legend for Figure 6. The values are means ± S.E.M. tg (−), non-transgenic; tg (+), transgenic.

injury, in the treated transgenic group but not in the syngenic group receiving the analogue (Figure 9). Estimation of mitochondrial dimensions revealed that, in the treated non-transgenic groups, the long and short axes of the organelle were 1.03 ± 0.29 and 0.78 ± 0.19 μm (means ± S.D.) respectively, and in the treated transgenic group were 1.54 ± 0.44 and 0.99 ± 0.32 μm respectively. These differences were statistically significant ($P < 0.001$). Even without treatment, the liver mitochondria of transgenic animals appeared larger and had a peculiar round shape when compared with those of non-transgenic animals.

DISCUSSION

The transgenic animals overexpressing the *SSAT* gene under the control of the heavy-metal-inducible metallothionein promoter used in the present study resemble the *SSAT* transgenic mice overexpressing the gene under its own promoter [5]. Both lines showed the hairless phenotype, but the MT–SSAT animals lost their hair much later, which is likely to be related to a less pronounced activation of polyamine catabolism in the skin. In any event, these two independent transgenic mouse lines apparently directly link an enhanced catabolism of the polyamines to hair loss. The MT–SSAT mice displayed more conspicuous tissue changes, especially in hepatic polyamine pools. This is exemplified by the fact that, even without treatment, the liver spermine pool was reduced by more than 90 % in the transgenic animals compared with their non-transgenic littermates. As anticipated, the transcription of the transgene was induced by zinc and was accompanied by only a modest 2- to 3-fold increase in SSAT activity. Longer treatment with zinc also reduced the liver pools of spermidine and spermine. In spite of the heterologous promoter, the polyamine analogue DENSPM brought about an immense induction of the transgene with little or no sign of transcriptional activation. In fact, the steady-state level of SSAT-specific mRNA in the liver of transgenic animals was more than 100 times greater than in the non-transgenic animals; SSAT activity, however, was only marginally increased. As in cells derived from transgenic animals overexpressing SSAT from its own promoter [6], the MT–SSAT cells responded to DENSPM treatment with a profound induction of hepatic SSAT activity. This occurred despite only marginal changes in the amount of SSAT-specific mRNA. The treatment of the transgenic animals with the polyamine analogue resulted in a total depletion of liver spermidine and spermine pools within a few days, whereas the polyamine depletion seen in similarly treated non-transgenic animals was much less. Overt toxicity was associated with the

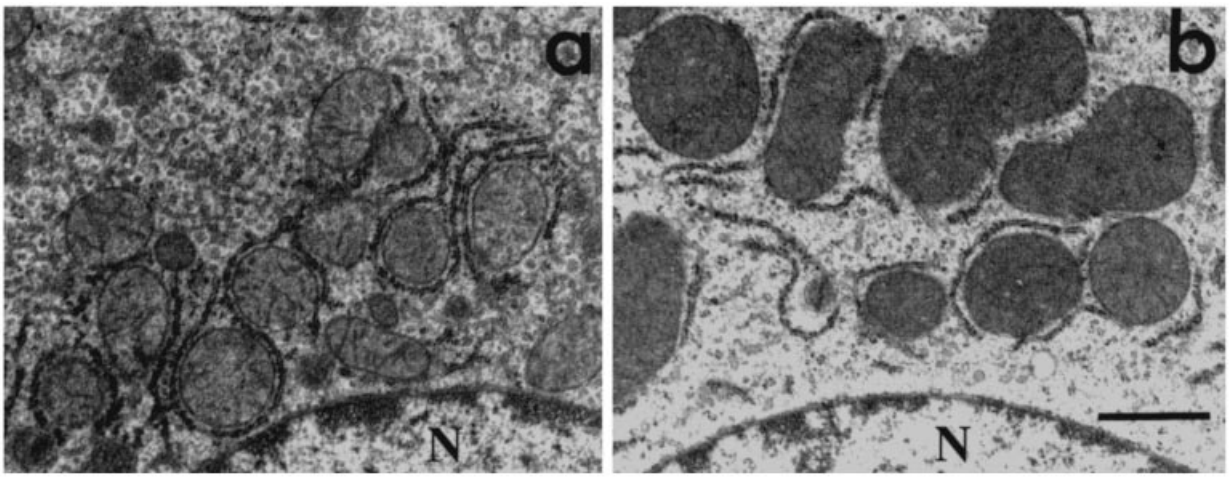

Figure 9 Transmission electron microscopy of the perinuclear area of mouse hepatocytes from non-transgenic (a) and transgenic (b) animals treated with DENSPM for three days

The samples were fixed with 2% (v/v) glutaraldehyde, post-fixed in a mixture of glutaraldehyde and OsO_4 and embedded in LX-112 resin. The sections were stained with uranyl acetate and lead citrate. In transgenic animals (**b**) the mitochondria were dilated and the bilayer membrane disappeared when compared with syngenic animals (**a**). The bar represents 1 μm. N, nucleus.

analogue treatment in transgenic, but not in non-transgenic animals. Electron microscopic examination of liver revealed that the analogue-exposed transgenic animals showed substantial mitochondrial swelling, which was not seen in the non-transgenic mice. Mitochondrial swelling is one of the earliest signs of cell injury and a wide variety of apoptotic and necrotic stimuli are known to induce progressive mitochondrial swelling [21]. Interestingly, mitochondrial integrity also appeared to be lost in 9L rat brain tumour cells depleted of polyamines by exposure to α-difluoromethylornithine [22]. Swelling of rat liver mitochondria has likewise been shown to be associated with consistent release of spermidine and spermine from the mitochondrial matrix space [23]. It is thus conceivable that the rapid and total depletion of the higher polyamines, especially spermine, brought about by the treatment with the polyamine analogue, would affect mitochondrial integrity.

The effect of DENSPM on the expression of the transgene is an interesting issue. The fact that, although the gene construct used was driven by a heterologous promoter, the polyamine analogue was able to induce SSAT activity to an extent not seen previously [6], which indicates that the promoter of the *SSAT* gene plays a minor role in the regulation of the gene expression by polyamines and their analogues. It therefore appears that the polyamine analogues exert their predominant action at some post-transcriptional level of gene expression, possibly by directly enhancing the translatability of the message. The mature SSAT mRNA seems to exist in more than one size variant. Fogel-Petrovic and co-workers [8,24] reported two mRNA species for SSAT of 1.3 kb and 1.5 kb. This size difference was apparently attributable to poly(A) tails of different length where an early induction of the 1.5-kb form by polyamine analogues was eventually replaced by the 1.3-kb form [8]. There are several potential mechanisms whereby the SSAT mRNA pattern could be changed by the polyamine analogues. Polyamines and their analogues could interact directly with either heterogenous nuclear RNA or mature mRNA. In the former case, this would result in more efficient processing of heterogenous nuclear RNA; in the latter case, the translatability of mRNA would be improved. A direct interaction is by no means excluded, as polyamines are known to directly induce ribosomal frameshifting in decoding ornithine decarboxylase antizyme [25,26]. A further possibility is the presence of antisense RNA under non-induced conditions which is then, in some way, eliminated by the polyamine analogue.

In any event, this new transgenic mouse line is not only valuable in terms of elucidating the regulation of *SSAT* gene expression by polyamines and their analogues, but it also offers a means to study the outcome of total depletion of the higher polyamines and the toxicity of polyamine analogues.

Received 21 August 1998/12 October 1998; accepted 10 December 1998

This work was supported, in part, by a Human Frontier Science Program, Academy of Finland, and by Grants CA-65942, CA-76428 and CA-22153 from the National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A. M.H. was supported by a fellowship from the Academy of Finland, Helsinki, Finland, and from the Maud Kuistila Memorial Foundation, Kuopio, Finland. S.S. was supported by a grant from the Finnish Cultural Foundation of Northern Savo, Kuopio, Finland. We gratefully acknowledge the skilled technical assistance of Ms Tuula Reponen, Mr Jukka Korhonen and Ms Sisko Juutinen.

REFERENCES

1 Seiler, N. (1987) Can. J. Physiol. Pharmacol. **65**, 2024–2035
2 Casero, Jr., R. A. and Pegg, A. E. (1993) FASEB J. **7**, 653–661
3 Shappell, N. W., Fogel-Petrovic, M. F. and Porter, C. W. (1993) FEBS Lett. **321**, 179–183
4 Vargio, C. and Persson, L. (1994) FEBS Lett. **355**, 163–165
5 Pietilä, M., Alhonen, L., Halmekytö, M., Kanter, P., Jänne, J. and Porter, C. W. (1997) J. Biol. Chem. **272**, 18746–18751
6 Alhonen, L., Karppinen, A., Uusi-Oukari, M., Vujcic, S., Korhonen, V.-P., Halmekytö, M., Kramer, D. L., Hines, R., Jänne, J. and Porter, C. W. (1998) J. Biol. Chem. **273**, 1964–1969
7 Ha, H. C., Woster, P. M., Yager, J. D. and Casero, R. A. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 11557–11562
8 Fogel-Petrovic, M., Shappell, N. W., Bergeron, R. J. and Porter, C. W. (1993) J. Biol. Chem. **268**, 19118–19125
9 Xiao, L. and Casero, R. A. (1996) Biochem. J. **313**, 691–696
10 Parry, L., Balana Fouce, R. and Pegg, A. E. (1995) Biochem. J. **305**, 451–458
11 Fogel-Petrovic, M., Vujcic, S., Miller, J. and Porter, C. W. (1996) FEBS Lett. **391**, 89–94
12 Libby, P. R., Bergeron, R. J. and Porter, C. W. (1989) Biochem. Pharmacol. **38**, 1435–1442
13 Alhonen, L., Heikkinen, S., Sinervirta, R., Halmekytö, M., Alakuijala, P. and Jänne, J. (1996) Biochem. J. **314**, 405–408
14 Hogan, B., Constantini, F. and Lacy, E. (1986) Manipulating the Mouse Embryo, pp. 1–322, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15 Blin, N. and Stafford, D. W. (1976) Nucleic Acids Res. **3**, 2303–2308
16 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
17 Hyvönen, T., Keinänen, T. A., Khomutov, A. R., Khomutov, R. M. and Eloranta, T. O. (1992) J. Chromatogr. **574**, 17–21
18 Bernacki, R. J., Oberman, E. J., Seweryniak, K. E., Atwood, A., Bergeron, R. J. and Porter, C. W. (1995) Clin. Cancer Res. **1**, 847–857
19 Jänne, J. and Williams-Ashman, H. G. (1971) J. Biol. Chem. **246**, 1725–1732
20 Jänne, J. and Williams-Ashman, H. G. (1971) Biochem. Biophys. Res. Commun. **42**, 222–229
21 Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T. and Thompson, C. B. (1997) Cell **91**, 627–637
22 Oredsson, S. M., Friend, D. S. and Marton, L. J. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 780–784
23 Tassani, V., Campagnolo, M., Toninello, A. and Siliprandi, D. (1996) Biochem. Biophys. Res. Commun. **226**, 850–854
24 Fogel-Petrovic, M., Vujcic, S., Brown, P. J., Haddox, M. K. and Porter, C. W. (1996) Biochemistry **35**, 14436–14444
25 Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F. and Hayashi, S.-I. (1995) Cell **80**, 51–60
26 Rom, E. and Kahana, C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3959–3963