Characterization of transgenic mice with widespread overexpression of spermine synthase

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A widespread increase in SpmS (spermine synthase) activity has been produced in transgenic mice using a construct in which the human SpmS cDNA was placed under the control of a composite CMV-IE (cytomegalovirus immediate early gene) enhancer–chicken β -actin promoter. Four separate founder CAG/SpmS mice were studied. Transgenic expression of SpmS was found in all of the tissues examined, but the relative SpmS activities varied widely according to the founder animal and the tissue studied. Very large increases in SpmS activity were seen in many tissues. SpdS (spermidine synthase) activity was not affected. Although there was a statistically significant decline in spermidine content and increase in spermine, the alterations were small compared with the increase in SpmS activity. These results provide strong support for the concept that the levels of the higher polyamines spermidine and spermine are not determined only by the relative activities of the two aminopropyltransferases. Other factors such

as availability of the aminopropyl donor substrate decarboxylated *S*-adenosylmethionine and possibly degradation or excretion must also influence the spermidine/spermine ratio. No deleterious effects of SpmS overexpression were seen. The mice had normal growth, fertility and behaviour up to the age of 12 months. However, breeding the CAG/SpmS mice with MHC (α -myosin heavy chain)/AdoMetDC (*S*-adenosylmethionine decarboxylase) mice, which have a large increase in *S*-adenosylmethionine decarboxylase expression in heart, was lethal. In contrast, breeding the CAG/SpmS mice with MHC/ODC (*L*-ornithine decarboxylase) mice, which have a large increase in cardiac ornithine decarboxylase expression, had a protective effect in preventing the small decrease in viability of the MHC/ODC mice.

Key words: aminopropyltransferase, decarboxylase, decarboxylated *S*-adenosylmethionine, polyamine, spermidine, spermine.

INTRODUCTION

The mammalian pathway for polyamine biosynthesis from *L*-arginine and *L*-methionine is now well understood [1,2]. Putrescine is formed by ODC (ornithine decarboxylase) acting on ornithine derived from arginine by arginase. Methionine is converted into the aminopropyl donor, dcAdoMet (decarboxylated *S*-adenosylmethionine) by the sequential actions of methionine adenosyltransferase and AdoMetDC (*S*-adenosylmethionine decarboxylase). The aminopropyltransferase SpdS (spermidine synthase) uses this dcAdoMet to form spermidine from putrescine. A second and distinct aminopropyltransferase termed SpmS (spermine synthase) uses another molecule of dcAdoMet to produce spermine from spermidine. Polyamine levels are very highly regulated, and it is widely believed that this regulation occurs predominantly via alterations in the activities of ODC and AdoMetDC. An additional regulatory factor may be the activity of back-conversion reactions that convert spermine into spermidine and putrescine. The first back-conversion pathway identified involved the acetylation of the higher polyamines by SSAT (spermidine/spermine-*N*¹-acetyltransferase) and the oxidation of the acetylated products by an acetylpolyamine oxidase [3], frequently referred to as PAO (polyamine oxidase) [4]. The SSAT/PAO pathway is able to convert spermine into spermidine and spermidine into putrescine. PAO may also be inducible in some circumstances [2], but the activity of the SSAT/PAO pathway is predominantly regulated by the level of SSAT. Recently, a

spermine oxidase has been identified which converts spermine into spermidine without need for acetylation [5,6].

The extensive regulation of polyamine content suggests that polyamines must be maintained within narrow ranges for normal growth and development, but direct proof of this concept is lacking. One approach to this question is to perturb polyamine levels by transgenic deletion or overexpression of relevant proteins and examine the resulting phenotypes. Gene deletions of ODC [7] and AdoMetDC [8] are lethal at the earliest stages of embryonic development. Male Gy mice that have an X-chromosomal deletion, which includes the SpmS gene, contain no spermine and are sterile, have a greatly reduced size, multiple neurological abnormalities and a very short life span [9,10].

Transgenic mice have been generated to have either widespread or highly localized overexpression of enzymes in the polyamine pathway (reviewed in [11–13]). Large increases in ODC have been obtained by transgenic expression of a stable truncated form of ODC in the skin driven from keratin-5 or keratin-6 promoters [14], and in heart from the MHC (α -myosin heavy chain) promoter [15]. There were marked elevations in the content of putrescine and spermidine in target tissues in these animals. The keratin-5/ODC and keratin-6/ODC transgenic mice were hairless and had increased nail growth and skin wrinkling. They were more sensitive to a variety of carcinogenic stimuli. The MHC/ODC mice had a slight cardiac hypertrophy and an increased hypertrophic response to isoproterenol. The MHC promoter has also been used to produce large increases (approx. 200-fold) in

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase; CMV-IE, cytomegalovirus immediate early gene; dcAdoMet, decarboxylated *S*-adenosylmethionine; GFP, green fluorescent protein; MHC, α -myosin heavy chain; ODC, *L*-ornithine decarboxylase; PAO, polyamine oxidase; SpdS, spermidine synthase; SpmS, spermine synthase; SSAT, spermidine/spermine-*N*¹-acetyltransferase.

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AdoMetDC activity in heart [16]. This increase resulted in only a 2-fold decrease in putrescine and spermidine, and a transient 2-fold increase in spermine, but no other phenotype in the MHC/AdoMetDC mice. Much lower, but more general, levels of expression of ODC [17] and AdoMetDC [18] have been produced by the insertion of large DNA fragments that contain the gene for the relevant enzyme and flanking sequences. The increased expression in these animals is presumably due to the increased copy number, and the protein is still subject to the regulatory influences at the transcriptional, translational and post-translational levels. This may account for the relatively small increases in ODC and AdoMetDC activity and alterations in polyamine content that were obtained [18,19]. A somewhat greater level of general ODC expression was obtained using the metallothionein promoter, but the increased putrescine in these tissues was accompanied by an increase in the higher polyamines only in liver [20]. The widespread, but limited, overproduction of ODC led to male infertility and some neurobiological changes, but no general increase in spontaneous tumours or neuronal degeneration [11,21].

Mice with increased SSAT driven from the metallothionein promoter [22] or using its endogenous promoter [23] have been produced. These mice show a marked alteration in polyamine content with large increases in putrescine and declines in spermidine and spermine particularly after further induction of the transgene by exposure to zinc or to bis(ethyl)polyamines, which are known to regulate SSAT both by increasing transcription and by stabilizing the protein against degradation. The SSAT transgenic mice have a variety of phenotypic alterations, including hairlessness, loss of subcutaneous fat and female infertility. They show protection from kainate-induced neurotoxicity and reduced liver regeneration after partial hepatectomy [24].

There has been much less investigation of the effects of transgenic manipulation of aminopropyltransferase enzymes. Overexpression of SpdS in mice was described after insertion of a transgene containing the human SpdS coding sequence plus 3000 5'-flanking nucleotides and 500 3'-flanking nucleotides [25], but the level of SpdS was increased only 2–6-fold. There were no significant alterations in polyamines in these mice, and only a marginal increase in spermidine pools when they were bred with those slightly overexpressing ODC [17,25]. No genetic alterations in SpmS, except for the *Gy* gene deletion described above, have been reported previously.

In the experiments described in the present study, we have generated transgenic mice that overexpress SpmS, the last enzyme in the polyamine pathway using a composite CMV-IE (cytomegalovirus immediate early gene) enhancer–chicken β -actin promoter construct [26–28]. SpmS activity was greatly increased in many different organs, but there was only a modest decrease in spermidine and increase in the spermine content. When these mice were crossed with MHC/AdoMetDC mice or MHC/ODC mice, which produce high levels of the respective enzymes in heart, there was a significant phenotypic effect. The CAG/SpmS-MHC/AdoMetDC cross was lethal, whereas the CAG/SpmS-MHC/ODC cross had improved survival over MHC/ODC alone.

MATERIALS AND METHODS

Materials

All chemicals, unless noted, were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Oligodeoxyribonucleotides used as primers were synthesized in the Macromolecular Core Facility (Pennsylvania State University College of Medicine). The reagents used for PCR genotyping, *Taq* DNA polymerase and an Ultrapure dNTP Set, were purchased from

Promega (Madison, WI, U.S.A.) and Amersham Biosciences (Piscataway, NJ, U.S.A.) respectively. [35 S]dcAdoMet was synthesized from L-[35 S]methionine (PerkinElmer Life Sciences, Boston, MA, U.S.A.) [10].

Polyamine and enzyme analysis

Polyamine content was determined by HPLC using an ion-pair reverse-phase HPLC separation method with post-column derivatization using *o*-phthalaldehyde as described previously [29].

All enzyme assays were carried out as reported previously: ornithine decarboxylase and AdoMetDC were assayed by measuring the release of $^{14}\text{CO}_2$ from [1- ^{14}C]ornithine (PerkinElmer Life Sciences) [30] or *S*-adenosyl-L-[carboxyl- ^{14}C]methionine (Amersham Biosciences) [31] respectively. SpdS and SpmS assays were carried out by measuring the production of [^{35}S]methylthioadenosine from [^{35}S]dcAdoMet in the presence of the appropriate amine acceptor as described previously [10,32]. SSAT was measured by following the transfer of [^{14}C]acetyl groups from [^{14}C]acetyl-CoA (ICN Biochemicals, Costa Mesa, CA, U.S.A.) to spermidine [33].

Plasmid construction

In order to obtain a construct for the widespread expression of SpmS activity, the human SpmS cDNA was placed in a vector that contains a composite CMV-IE enhancer– β -actin promoter by replacing the insert encoding GFP (green fluorescent protein) in plasmid pCX-EGFP [26,27]. Plasmid pQE-hSpS, which contains the cDNA of human SpmS [34], was used as a template for PCR with sense primer 5'-ACGTATGAATTCGCCACCATGGCAGCACGG-3' and antisense primer 5'-TCGATAGAATTC-TCAGGGTTTATGC-3' (*Eco*RI sites underlined). The PCR reaction was carried out in a 0.1 ml volume containing 2.5 units of *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.), 50 ng of template DNA and 25 pmol of each of the sense and anti-sense primers under the following conditions: initial denaturation for 2 min at 92 °C, followed by 25 cycles of denaturation (at 92 °C for 30 s), annealing (at 54 °C for 30 s) and extension (at 72 °C for 1 min), with a final extension at 72 °C for 5 min. The PCR product was digested with *Eco*RI, purified on a 0.8 % (w/v) agarose gel using the Qiagen (Valencia, CA, U.S.A.) gel-extraction kit, ligated into pCX-EGFP digested with the same enzymes to remove the GFP insert and transformed into *Escherichia coli* XL1-Blue cells to form plasmid pCAG-hSpmSyn. The entire DNA insert was sequenced to ensure the correct orientation of the SpmS insert and that no secondary mutations were introduced during the plasmid construction.

Production of transgenic mice

Transgenic mice expressing SpmS (CAG/SpmS mice) were generated by DNA microinjection of fertilized B6D2F2 oocytes using standard techniques [35] in the Transgenic Core Facility (Pennsylvania State University College of Medicine). The microinjected transgene was a 3.5 kb fragment released by *Sal*I and *Bam*HI digestion from plasmid pCAG-hSpmSyn. The fragment was purified using Qiagen gel-extraction kit and DEAE Elutip-D-mini-columns (Schleicher & Schull, Keen, NH, U.S.A.). It was precipitated and resuspended in microinjection buffer (10 mM Tris/HCl, pH 7.4, containing 0.1 mM EDTA).

Genomic DNA was isolated from the tails of potential founder mice (DNeasy tissue kit; Qiagen) and subjected to PCR analysis to identify mice bearing the transgene. The 5' sense primer used was

Table 1 Spermine synthase activity in control and CAG/SpmS mice

Results are means $\pm$ S.D. Activity was measured in mice aged 1 month using three to four estimations per point, except where no S.D. is shown where only two samples were assayed. All of the increases in spermine synthase were statistically significant, except for those in line 18 in kidney and brain.

Tissue	Spermine synthase (pmol/mg per h)			
	Control	Line 8	Line 18	Line 21
Heart	18 $\pm$ 8	10 311 $\pm$ 1069	8400 $\pm$ 1986	20 997 $\pm$ 3299
Muscle	69 $\pm$ 10	18 833 $\pm$ 714	885 $\pm$ 1002	51 103 $\pm$ 14 499
Liver	3 $\pm$ 1	4655 $\pm$ 171	32 $\pm$ 23	5439 $\pm$ 966
Kidney	43 $\pm$ 14	1598 $\pm$ 237	73 $\pm$ 37	3890 $\pm$ 629
Dermis	15 $\pm$ 5	1590 $\pm$ 289	113 $\pm$ 113	3719 $\pm$ 886
Lung	17 $\pm$ 2	2340 $\pm$ 631	52 $\pm$ 19	2671 $\pm$ 580
Brain	112 $\pm$ 22	1211 $\pm$ 65	154 $\pm$ 55	2499 $\pm$ 231
Epidermis	27 $\pm$ 7	2785	356 $\pm$ 108	2261
Testis	40 $\pm$ 2	418 $\pm$ 15	181 $\pm$ 25	916 $\pm$ 253

5'-TTCGGCTTCTGGCGTGTGAC-3', which corresponds to a sequence in the actin promoter region, and the 3' antisense primer was 5'-CCAGTACTGTCCTGACTC-3', which corresponds to nt 300–317 in the human SpmS coding sequence. A 440 bp fragment was produced from the transgene and was only detected in samples from transgenic mice.

The MHC/ODC transgenic mice, which express very high levels of ODC in heart, have been described previously [15]. These mice were produced on the C57Bl/6xSJL background, but the mice used in the present experiments (line 21) were backcrossed through at least six generations to B6D2. The MHC/AdoMetDC mice were produced on the B6D2F2 background using the same murine α MHC promoter, but replacing the ODC insert with a sequence encoding human AdoMetDC [16].

RESULTS

Aminopropyltransferase activity in CAG/SpmS transgenic mice

Four separate founder CAG/SpmS mice (lines 5, 8, 18 and 21) were obtained after injection of the pCAG-hSpmSyn construct. A rapid screen of 11 tissues from the F1–F3 generations of these four lines showed that there was a widespread overproduction of SpmS, but that this varied with the founder animal and the tissue. In particular, CAG/SpmS lines 8 and 21 had the highest expression (50–1000-fold increases in many tissues), line 18 had the lowest expression and line 5 was intermediate. Three of the CAG/SpmS lines (8, 18 and 21) were studied in more detail and the results are shown in Table 1. Although there were small differences, lines 8 and 21 were generally similar, with increases in SpmS activity of more than 50-fold in many tissues. The highest activities were seen in heart and skeletal muscle with increases of 573–1167-fold, but the greatest fold increase was in liver (which has a low basal level of SpmS) where activity was increased more than 1500-fold. The increase was lowest in the brain (which has the highest basal level of expression) and testis, but was still more than 10-fold in these tissues. Line 18 had substantial increases in SpmS in heart (466-fold), but had considerably lower increases than the other lines, notably in liver and muscle where the activity was increased only 10-fold, as opposed to more than 500-fold increases in lines 8 and 21.

The alterations in SpmS in CAG/SpmS mice were maintained for at least 11 months. Measurements in heart, liver, kidney and

Table 2 Spermidine synthase activity in control and CAG/SpmS mice

Results are means $\pm$ S.D. Activity was measured in mice aged 1 month using 34 estimations per point.

Tissue	Spermidine synthase (pmol/mg per h)			
	Control	Line 8	Line 18	Line 21
Heart	519 $\pm$ 63	369 $\pm$ 18	377 $\pm$ 50	540 $\pm$ 40
Liver	2524 $\pm$ 557	2280 $\pm$ 255	1803 $\pm$ 744	2058 $\pm$ 211
Kidney	1302 $\pm$ 140	751 $\pm$ 188	1088 $\pm$ 452	764 $\pm$ 217
Brain	4079 $\pm$ 584	4226 $\pm$ 420	4095 $\pm$ 200	3543 $\pm$ 764

brain of mice of line 8 at 1, 3, 6 and 11 months showed that there was little change in the SpmS activity in these tissues in either control or transgenic mice during this period (see supplementary Table 1 at <http://www.BiochemJ.org/bj/381/bj3810701add.htm>). At 11 months, the activity was increased 173-fold in heart, 1153-fold in liver, 43-fold in kidney and 6-fold in brain. SpmS increased slightly in the control mice in heart and brain, resulting in the slightly lower fold increases in the transgenic mice compared with the values at 1 month.

The massive increase in SpmS in the CAG/SpmS mice did not result in any substantial compensatory alteration in SpdS in four tissues examined: heart, liver, kidney and brain (Table 2). SpdS activities were much higher than SpmS in control mice, particularly in liver. However, in the CAG/SpmS lines 8 and 21, SpmS activities were much larger than SpdS in heart, and in the same range as SpdS in the other tissues. Thus, in CAG/SpmS line 8 at 1 month, the ratio of SpdS/SpmS was changed from 0.03 to 28 in heart, from 0.001 to 2 in liver, from 0.03 to 0.9 in kidney and from 0.03 to 0.3 in brain.

SpdS levels declined slightly with age (particularly at 11 months in heart and liver), but there was no difference between the CAG/SpmS mice line 8 and controls in the SpdS activity over the 1–11 month period examined (see supplementary Table 2 at <http://www.BiochemJ.org/bj/381/bj3810701add.htm>). At 11 months, the ratio of spermine to SpdS was changed from 0.2 to 27 in heart, from 0.004 to 1.3 in liver, from 0.05 to 3 in kidney and from 0.05 to 0.28 in brain.

Polyamine content in CAG/SpmS mice

The polyamine content in tissues and blood of lines 8, 18 and 21 CAG/SpmS and controls at 1 month of age is shown in Table 3. Surprisingly, despite the huge increase in SpmS activity and a decrease in the ratio of SpdS/SpmS activity, the spermine content increased significantly in only a few tissues, such as liver, kidney and lung, and these increases were small. The largest change seen was in liver with lines 8 and 21, and amounted to only a 60–80 % increase. In heart, where SpmS was increased by > 500-fold in lines 8 and 21, spermine was not increased at all, and decreased slightly in line 8. In muscle, the > 300-fold rise in SpmS in these lines produced only a 21–45 % increase in spermine.

There was a statistically significant decline in spermidine in many of the tissues from the CAG/SpmS mice in which SpmS was greatly increased. Thus, in heart, muscle, liver, kidney and epidermis of lines 8 and 21, spermidine fell by approx. 50 %, and there were slightly smaller reductions in spermidine in lung, dermis and testis (Table 3). The combination of increases in spermine and a fall in spermidine resulted in a statistically significant increase in the spermine/spermidine ratio in all of the transgenic mouse tissues from lines 8 and 21 (Table 3). This ratio

Table 3 Polyamine content in control and CAG/SpmS mice

Measurements were made on the number of samples shown in parenthesis. With the exception of kidney, there was no significant difference in polyamine content between male and female mice, and the results are combined. Results are means $\pm$ S.D. Values that are statistically different from the controls are indicated: ^a $P < 0.0001$; ^b $P < 0.001$; ^c $P < 0.01$ and ^d $P < 0.05$.

(a)

Group	Tissue (n)	Putrescine	Spermidine (nmol/g of tissue)	Spermine	Spermine/spermidine ratio
Control	Heart (13)	6 $\pm$ 1	133 $\pm$ 14	252 $\pm$ 24	1.93 $\pm$ 0.09
8	Heart (9)	23 $\pm$ 4 ^a	63 $\pm$ 4 ^b	211 $\pm$ 8	3.45 $\pm$ 0.23 ^a
18	Heart (10)	12 $\pm$ 1 ^c	163 $\pm$ 10	345 $\pm$ 11 ^c	2.22 $\pm$ 0.19
21	Heart (10)	14 $\pm$ 2 ^c	60 $\pm$ 7 ^b	276 $\pm$ 31	4.56 $\pm$ 0.22 ^a
Control	Muscle (13)	17 $\pm$ 2	132 $\pm$ 11	215 $\pm$ 14	1.77 $\pm$ 0.19
8	Muscle (9)	29 $\pm$ 2 ^b	53 $\pm$ 7 ^a	311 $\pm$ 15 ^b	6.77 $\pm$ 0.91 ^b
18	Muscle (10)	14 $\pm$ 2	96 $\pm$ 10 ^d	216 $\pm$ 13	2.48 $\pm$ 0.26 ^d
21	Muscle (10)	27 $\pm$ 2 ^b	58 $\pm$ 5 ^a	261 $\pm$ 10 ^d	4.91 $\pm$ 0.49 ^a
Control	Liver (13)	13 $\pm$ 2	1194 $\pm$ 46	590 $\pm$ 16	0.50 $\pm$ 0.02
8	Liver (9)	29 $\pm$ 5 ^d	475 $\pm$ 30 ^a	964 $\pm$ 78 ^c	2.03 $\pm$ 0.14 ^a
18	Liver (10)	9 $\pm$ 1	1028 $\pm$ 17 ^c	457 $\pm$ 17 ^a	0.44 $\pm$ 0.02
21	Liver (10)	63 $\pm$ 11 ^c	493 $\pm$ 26 ^a	1090 $\pm$ 56 ^a	2.28 $\pm$ 0.21 ^a
Control	Kidney (13)	47 $\pm$ 10	318 $\pm$ 12	584 $\pm$ 15	1.86 $\pm$ 0.07
8	Kidney (9)	66 $\pm$ 8	223 $\pm$ 5 ^a	849 $\pm$ 21 ^a	3.82 $\pm$ 0.07 ^a
18	Kidney (10)	19 $\pm$ 4 ^d	259 $\pm$ 12 ^c	557 $\pm$ 24	2.18 $\pm$ 0.10 ^d
21	Kidney (10)	40 $\pm$ 4	141 $\pm$ 6 ^a	718 $\pm$ 31 ^b	5.10 $\pm$ 0.14 ^a
Control	Dermis (13)	189 $\pm$ 41	561 $\pm$ 71	190 $\pm$ 27	0.46 $\pm$ 0.12
8	Dermis (9)	376 $\pm$ 49 ^c	422 $\pm$ 24	378 $\pm$ 29 ^a	0.91 $\pm$ 0.08 ^d
18	Dermis (10)	245 $\pm$ 21	698 $\pm$ 47	216 $\pm$ 13	0.32 $\pm$ 0.03
21	Dermis (10)	257 $\pm$ 57	372 $\pm$ 64	259 $\pm$ 37	0.93 $\pm$ 0.17 ^d
Control	Lung (13)	27 $\pm$ 1	487 $\pm$ 15	377 $\pm$ 15	0.77 $\pm$ 0.02
8	Lung (9)	48 $\pm$ 6 ^c	353 $\pm$ 11 ^a	494 $\pm$ 17 ^a	1.40 $\pm$ 0.03 ^a
18	Lung (10)	35 $\pm$ 3 ^d	623 $\pm$ 33 ^c	480 $\pm$ 29 ^c	0.78 $\pm$ 0.04
21	Lung (10)	45 $\pm$ 2 ^a	277 $\pm$ 18 ^a	454 $\pm$ 17 ^c	1.67 $\pm$ 0.05 ^a
Control	Brain (13)	5 $\pm$ 1	274 $\pm$ 8	244 $\pm$ 9	0.89 $\pm$ 0.03
8	Brain (9)	9 $\pm$ 1 ^d	145 $\pm$ 9 ^a	184 $\pm$ 3 ^a	1.31 $\pm$ 0.09 ^c
18	Brain (10)	5 $\pm$ 1	213 $\pm$ 15 ^b	245 $\pm$ 8	1.20 $\pm$ 0.09 ^c
21	Brain (10)	19 $\pm$ 2 ^a	219 $\pm$ 16 ^c	267 $\pm$ 9	1.26 $\pm$ 0.08 ^c
Control	Epidermis (13)	161 $\pm$ 25	745 $\pm$ 70	225 $\pm$ 18	0.34 $\pm$ 0.04
8	Epidermis (9)	268 $\pm$ 35 ^d	318 $\pm$ 17 ^a	409 $\pm$ 24 ^a	1.30 $\pm$ 0.06 ^a
18	Epidermis (10)	238 $\pm$ 22 ^d	674 $\pm$ 45	185 $\pm$ 14	0.29 $\pm$ 0.03
21	Epidermis (10)	395 $\pm$ 80 ^d	341 $\pm$ 45 ^b	450 $\pm$ 47 ^b	1.38 $\pm$ 0.06 ^a
Control	Testis (9)	32 $\pm$ 8	345 $\pm$ 23	438 $\pm$ 38	1.26 $\pm$ 0.05
8	Testis (4)	36 $\pm$ 3	217 $\pm$ 11 ^c	568 $\pm$ 26	2.62 $\pm$ 0.07 ^a
18	Testis (5)	36 $\pm$ 2	421 $\pm$ 30	605 $\pm$ 36 ^d	1.46 $\pm$ 0.14
21	Testis (5)	44 $\pm$ 6	233 $\pm$ 41	582 $\pm$ 39	2.66 $\pm$ 0.23 ^c
Control	Ovary (3)	31 $\pm$ 11	601 $\pm$ 69	336 $\pm$ 48	0.56 $\pm$ 0.03
8	Ovary (5)	90 $\pm$ 12 ^d	462 $\pm$ 50	530 $\pm$ 42 ^d	1.16 $\pm$ 0.05 ^a
18	Ovary (5)	41 $\pm$ 7	773 $\pm$ 81	397 $\pm$ 45	0.51 $\pm$ 0.02
21	Ovary (8)	52 $\pm$ 7	398 $\pm$ 55	538 $\pm$ 83	1.35 $\pm$ 0.04 ^a

(b)

Group	Tissue (n)	Putrescine	Spermidine (nmol/g of blood protein)	Spermine	Spermine/spermidine ratio
Control	Blood (10)	20 $\pm$ 2	456 $\pm$ 68	43 $\pm$ 6	0.10
8	Blood (9)	25 $\pm$ 3	307 $\pm$ 24	35 $\pm$ 2	0.12
18	Blood (10)	19 $\pm$ 2	408 $\pm$ 32	40 $\pm$ 4	0.10
21	Blood (10)	23 $\pm$ 1	422 $\pm$ 27	49 $\pm$ 5	0.12

was increased in some, but not all tissues in line 18, reflecting the lesser increase in SpmS in this line.

Measurements of polyamine content were also made at 3, 6 and 11 months in heart, liver, kidney and brain in CAG/SpmS line 8 (see supplementary Table 3 at <http://www.BiochemJ.org/bj/381/bj3810701add.htm>). Despite some changes in the absolute polyamine levels, which are known to change with age [36], the overall pattern of a slight fall in spermidine and a

Figure 1 Body mass of CAG/SpmS line 8 mice

Results are means $\pm$ S.D. for male (squares) and female (circles) mice as indicated.

modest rise in spermine was maintained, resulting in an increased spermine/spermidine ratio of approx. 2-fold in heart and kidney, 3-fold in liver and 1.3-fold in brain. The small decline in spermine in heart in this line seen at 1 month (Table 3) was reversed at later ages, and there was a small increase of 11–30 % at the older ages, but this was very modest compared with the > 150-fold rise in SpmS at these times. The polyamine content in the blood of the CAG/SpmS mice was not significantly different from controls (Table 3).

Phenotypic changes in CAG/SpmS mice

The CAG/SpmS mice showed normal fertility and behaviour over a period of at least 1 year. The body mass of CAG/SpmS line 8 mice was actually slightly greater than littermate controls over an 11 month period, although this difference was statistically significant only in the 11-month-old female mice (Figure 1).

Crosses of CAG/SpmS with MHC/ODC and MHC/AdoMetDC mice

Transgenic mouse lines in which the activity of either AdoMetDC or ODC are increased exclusively in heart have been generated using the cardiac-specific MHC promoter [15,16]. The MHC/AdoMetDC mice have an approx. 200-fold increase in AdoMetDC activity, but have normal growth, fertility and survival. However, when MHC/AdoMetDC mice were bred with CAG/SpmS mice (lines 8 and 21), no double transgenic mice with expression of both AdoMetDC and SpmS survived to weaning (3 weeks of age) in more than 40 offspring examined. Preliminary data suggest that the lethal effect occurs at an early time, since no transgenic mice were found at 4 days (none of eight double transgenics, two of eight CAG/SpmS, four of eight MHC/AdoMetDC and two of eight controls) and at 14 days (none of 11 double transgenics, two of 11 CAG/SpmS, five of 11 MHC/AdoMetDC and four of 11 controls).

In contrast, breeding the CAG/SpmS mice with MHC/ODC mice did produce viable offspring. In 154 offspring from this cross, the number of double transgenic CAG/SpmS-MHC/ODC

Figure 2 Polyamine content and heart mass/body mass in mice resulting from crosses between CAG/SpmS with MHC/ODC transgenic mice

Measurements were made at 4 weeks of age using crosses between CAG/SpmS line 8 and MHC/ODC line 21. Results are means $\pm$ S.D.

mice was 48 (31 %), significantly more than the number of MHC/ODC mice, which was only 20 (13 %). The lower than expected frequency of MHC/ODC mice is consistent with many experiments with the MHC/ODC mice, where, with 731 mice, there were 38 % transgenic and 62 % controls at weaning in offspring of heterozygous MHC/ODC males and female controls (L. M. Shantz, A. E. Pegg and R. A. Hillary, unpublished work). The cause of this slight loss of viability in the MHC/ODC mice is not known, but may be related to the increase in putrescine, cadaverine and spermidine in heart and resulting alterations in activity of K^+ channels [15,37]. As shown in Figure 2, the cross of MHC/ODC with the CAG/SpmS mice to increase SpmS had no significant effect on the very large amount of putrescine and cadaverine in heart, but did reduce the increase in spermidine significantly and normalize the spermine/spermidine ratio (1.87 ± 0.12 in controls, 0.73 ± 0.06 in MHC-ODC and 1.68 ± 0.16 in the CAG/SpmS-MHC/ODC). The MHC/ODC mice have a slight cardiac hypertrophy, which is shown in Figure 2 as the heart mass/body mass ratio. This was not reduced in the CAG/SpmS-MHC/ODC mice, indicating that spermidine alone or the spermine/spermidine ratio is not a causative factor in this hypertrophy. There was no difference in the overexpression of ODC in the CAG/SpmS-MHC/ODC mice ($0.90 \pm 0.1 \mu\text{mol}$ of CO_2 released/mg of protein per 30 min) compared with the MHC/ODC mice ($0.95 \pm 0.2 \mu\text{mol}$ of CO_2 released/mg of protein per 30 min) or in the overexpression of SpmS in the CAG/SpmS-MHC/ODC mice ($10.8 \pm 1.1 \text{ nmol/mg per h}$) compared with the CAG/SpmS mice ($11.1 \pm 1.5 \text{ nmol/mg per h}$).

DISCUSSION

Our results confirm previous reports of the generality and strength of expression of a transgene from the composite CMV-IE enhancer/ β -actin promoter [26–28]. All four of the founder CAG/SpmS mice studied produced lines in which SpmS was elevated in multiple tissues. However, our results show clearly that the absolute level of expression is tissue dependent. Assuming that the level of endogenous expression of SpmS is not altered in the transgenic mice, the absolute levels of activity from the transgene

ranged from 378 to 10293 pmol/mg per h in line 8, from 29 to 8382 pmol/mg per h in line 18 and from 876 to 51034 pmol/mg per h in line 21 (these values were calculated by subtracting the activities given in Table 1 for non-transgenic controls from the transgenics). In previous studies of the generality of this expression, the reporter gene was a GFP that, although very convenient for examination of expression in certain cell types, does not allow for accurate quantification of expression [26–28]. The very high level of SpmS expression in heart and skeletal muscle is consistent with the transcription of the transgene coming from an actin promoter, but other factors including insertional effects must also be involved in producing the striking differences seen between the three lines studied in detail. For example, in line 18, there was only a minimal expression in liver (59 pmol/mg per h), whereas this value is >4000 pmol/mg per h in lines 8 and 21. In contrast, the expression in heart in line 18 was 8382 pmol/mg per h and only slightly less than lines 8 and 21 (10293 and 20979 pmol/mg per h respectively).

Our detailed studies of the aminopropyltransferase activities in the CAG/SpmS mice show that the high levels of SpmS were maintained over an extended period of time, but these were not accompanied by a major decline in spermidine and increase in spermine content. This cannot be explained by a concomitant increase in SpdS, since this activity did not change significantly. Our experiments therefore show clearly that the relative amounts of spermidine and spermine are not closely linked to the ratio of the two aminopropyltransferase activities. This conclusion is in agreement with the complete lack of correlation between the basal SpmS levels in various tissues and their spermine content. It is, however, quite surprising that altering the aminopropyltransferase ratio by more than two orders of magnitude does not have a more striking effect in favouring spermine accumulation, since the K_m values for dcAdoMet for spermidine and SpmS are quite similar (approx. $0.5 \mu\text{M}$) [38–40].

Only one other transgenic mouse line has been reported in which an aminopropyltransferase (in this case, SpdS) was overexpressed and only a small (2–6-fold) increase in SpdS activity was seen [13,25]. This had virtually no effect on the polyamine levels. The authors concluded that AdoMetDC controls the content of spermidine and spermine. Our results using mice in which a much larger increase in SpmS is produced in many tissues by use of the CAG promoter are generally in agreement with this conclusion. It is of particular interest in this context that the combination of the CAG/SpmS mice with MHC/AdoMetDC mice was lethal. At present, we do not know the cause of this lethality. One would not expect these transgenes to be able to increase total polyamines greatly, since production of putrescine via ODC would still be limiting, but it is possible that in the presence of unlimited dcAdoMet, virtually all of the higher polyamines in heart would be in the form of spermine. High levels of spermine could produce catastrophic effects either due to alterations in cardiac electrical activity or to apoptotic cell death. Spermine has been claimed to be more effective than spermidine in both modifications of ion-channel activity [41,42] and induction of apoptosis [43,44].

The importance of ODC in controlling the total amount of polyamines is clearly shown in the experiments in which CAG/SpmS mice were bred with MHC/ODC mice (Figure 2). Excluding cadaverine, the total polyamine content in the MHC/ODC single transgenic was 22 nmol/mg of protein compared with 9.4 nmol/mg of protein in the controls. This increase is maintained when the CAG/SpmS single transgenics (8.5 nmol/mg of protein) are compared with the CAG/SpmS-MHC/ODC double transgenics (20.3 nmol/mg of protein). Even in the presence of the increased supply of putrescine and spermidine resulting from

the ODC transgene, the huge level of transgenic SpmS in the CAG/SpmS-MHC/ODC mice led to only a 20% increase in spermine and no significant reduction in the putrescine content (Figure 2). However, the spermidine level was decreased by 44%, and this normalization may account for the better survival of the CAG/SpmS-MHC/ODC compared with the MHC/ODC mice.

It is currently unclear why the CAG/SpmS mice do not show a much larger increase in spermine and the spermine/spermidine ratio. One possible explanation of these results is that there were compensatory alterations in the activity of other enzymes in the polyamine biosynthetic and interconversion pathway. However, there was no significant decrease in either ODC or AdoMetDC in hearts of the CAG/SpmS mice. SSAT was not increased significantly and spermine oxidase activity was below the detection limit of the *in vitro* assays (results not shown). It is possible that excess spermine is made and is either degraded in some other way or excreted, but we have no evidence to support this and there was no consistent increase in the spermine content or spermine/spermidine ratio in the blood of the CAG/SpmS mice (Table 3 and supplementary Table 3 at <http://www.BiochemJ.org/bj/381/bj3810701add.htm>). We therefore conclude that the limiting factor in the regulation of spermine content is probably the supply of dcAdoMet and that this nucleoside is preferentially utilized by SpdS for the production of spermidine. How this preferential utilization occurs in the face of a massive increase in SpmS remains to be determined.

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