Altered Levels of Growth-related and Novel Gene Transcripts in Reproductive and Other Tissues of Female Mice Overexpressing Spermidine/Spermine N\textsuperscript{1}-Acetyltransferase (SSAT)*

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Overexpression of SSAT (polyamine catabolic enzyme) in female mice results in impaired ovarian folliculogenesis and uterine hypoplasia. To identify the molecular basis for this, the gene expression profiles in uterus and ovary and for comparison, liver and kidney, from non-transgenic (NT) and SSAT transgenic (ST) mice were compared. The mRNA abundance for lipoprotein lipase and glyceraldehyde-3-phosphate dehydrogenase was elevated in all four ST (>NT) tissues. The translation initiation factor-3 subunit 5 mRNA, and transcripts related to endogenous murine leukemia provirus (MLV-related) and murine retrovirus-related sequences (MuRRS) were decreased in ST tissues. A novel calmodulin-related mRNA was strongly induced in ST liver and kidney. SSAT overexpression was associated with increased levels of IGFBP-related 3 mRNA levels in the uterus. Exogenous spermidine and spermine elevated endogenous IGFBP-2 and SSAT mRNA abundance, whereas, putrescine stimulated IGFBP-2 mRNA abundance and transcribed IGFBP-2 gene promoter activity in human (Hec-1-A) uterine cells. Sp1 and BTEB1 mRNAs that encode transcription factors for the IGFBP-2 gene also were induced in some ST tissues. The data suggest that SSAT and polyamines are important for the control of molecular pathways underlying reproductive tract tissue growth, phenotype, and function.

The polyamines putrescine, spermidine, and spermine are ubiquitous components of cells. Although many of their specific functions are still unclear, these polycationic molecules are essential for cell proliferation and differentiation (1, 2). The intracellular levels of polyamines are tightly regulated by the cells’ growth status (3), which in turn, is dependent on metabolic pathways that mediate their cell synthesis, degradation, and/or excretion. Ornithine decarboxylase (ODC) is the first rate-limiting enzyme in polyamine biosynthesis and has been the subject of intense scrutiny in the last decade, due to its possible involvement in proliferative disorders including cancer. Development of several drugs, notably difluoromethyl ornithine, which inhibits ODC, results in a depletion of the cellular polyamine pool and a decrease in cell proliferation (4, 5). Although inhibitors of polyamine synthesis are potential candidates for cancer chemotherapy, the results of clinical trials have not always met expectations. Furthermore, recent studies with transgenic mice have shown that life-long overexpression of ODC or other polyamine biosynthetic enzymes does not increase the incidence of spontaneous tumors (6–8). The absence of marked phenotypic changes in these mice may be attributable to the relatively minor changes observed in higher polyamine pools, although an accumulation of putrescine in tissues was observed.

The increase in putrescine in transgenic mice overexpressing ODC or other polyamine anabolic enzymes suggests that a counter-regulatory mechanism, such as activation of the catabolic pathway, may maintain polyamine homeostasis in vivo. Spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT) is the rate-limiting enzyme in polyamine catabolism that, together with polyamine oxidase, back-converts spermine and spermidine ultimately to putrescine, a function that is presumed to prevent cellular toxicity due to polyamine excess (9). Recently, there has been growing interest in this pathway as a target for manipulating polyamine pools to control cell proliferation. Indeed, several polyamine analogues, particularly the compound N,N\textsuperscript{-}bis(ethyl) putrescine, have been developed and shown to down-regulate ODC, and more importantly, to up-regulate SSAT. In a number of cell lines (10–12), the induction of SSAT is closely associated with the anti-proliferative action of these drugs. This linkage appears to be mediated by the depletion of spermidine and spermine, as facilitated by SSAT, together with the inability of analogues to substitute for the depleted natural polyamines in functions associated with cell proliferation.

Although polyamine analogues may provide an effective way to regulate SSAT and hence, cell growth processes, application of these drugs in an in vivo context is complicated, partly...
because of their interference with the polyamine synthetic pathway and transport (13, 14). Recently, the Porter and Jänne laboratories (15) generated SSAT overexpressing transgenic mice to further define a role for SSAT in polyamine metabolism in vivo. As expected, these mice exhibit profound changes in tissue polyamine pools, including a large accumulation of putrescine, the appearance of N\(^{\text{3}}\)-acetylsperrmidine, and in certain tissues a decrease in spermidine and spermine. These changes occur despite simultaneous increases in ODC and other polyamine anabolic enzyme activities. The magnitude of changes in the polyamine levels was much more prominent than those observed in transgenic mice overexpressing ODC, further emphasizing the prominent role of SSAT in maintaining polyamine homeostasis. Disturbances in polyamine pools due to overexpression of SSAT lead to marked phenotypic changes as well, including permanent hair loss at an early age, skin wrinkling, loss of subcutaneous fat, and in females, an underdeveloped uterus and abnormal ovaries. Interestingly, the latter two tissues of normal and SSAT transgenic mice did not differ with respect to tissue spermidine and spermine contents, whereas, the SSAT overexpressors had markedly elevated putrescine content in female reproductive organs. These collective results demonstrated the utility of SSAT overexpression as a means to modulate polyamine pools in tissues, for the purposes of unraveling the role(s) of polyamines in normal and abnormal cellular proliferation, differentiation, and apoptosis.

Although transgenic mice provide a useful model for identifying metabolic and biological consequences of altered polyamine pools, molecular mechanisms underlying these changes remain elusive. Given the fact that polyamines are polycations at physiological pH and thus, can interact with negatively charged molecules such as DNA and RNA, it is highly likely that deregulation of polyamine pools may affect expression of multiple genes which could explain in part, the phenotypic and morphological changes observed in the female reproductive tract tissues of SSAT transgenic mice. In the present study, the gene expression profiles in uterus and ovary, and for comparison, in liver and kidney, of SSAT overexpressing mice were evaluated relative to those of their normal, non-transgenic counterparts. A number of distinct genes, some of which are known to be associated with growth regulation (IGFBP-2, IGFBP-3, and Krüppel-like (KLF) transcription factors) and some of which are novel, were identified to exhibit marked alterations in mRNA levels during SSAT overexpression, suggesting that SSAT and/or polyamines are crucial for the control of molecular pathways underlying reproductive tract tissue growth, phenotype and function.

**Experimental Procedures**

**Animals and Tissue Collection**—Transgenic mice systemically overexpressing the polyamine catabolic enzyme SSAT were previously generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15). Members of the UKU165b line were generated using standard pronuclear microinjection techniques as described in detail elsewhere (15).

**DNA Extraction**—Total cellular RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturers recommendations. RNA samples were freed of contaminating DNA by treatment with DNase I.

**mRNA Differential Display** (ddRT-PCR) — Differential display reagents and primers (HIEROGLYPHTM) were purchased from GENO-MYX Corp., Foster City, CA. An equal amount of RNA from the uterus, ovary, liver, and kidney of ST (n = 4) and NT (n = 4) mice were pooled within tissue but kept separate between groups. DNA-free total RNA (2 μg/tissue sample) was subjected to reverse transcription using anchored 3′ oligo(dT) primer sets (5′-T,NM-3′, where NM = GA, GA, GG, GT, CA, CC, or CG, primers 1–7, respectively). Following reverse transcription, one-tenth of this reaction (2 μl) was used in a PCR amplification reaction (20 μl) containing 400 μM dNTP of each dNTP, 2.5 μCi of [γ-\(^{33}\)P]dATP, and two primers: 4 μM T, G oligo(dT) (anneal at 94°C for 1 min and 4 μM of arbitrary primer, M13r, CAGCTCCAG-T(3′) or M13r-AP2 (5′-GCTAGCATG-G-3′). These reactions also contained 1 unit of AmpliTaq DNA polymerase (PerkinElmer Life Sciences, Norwalk, CT). The PCR was performed with 25 cycles of 15 s denaturation at 92°C, 30 s annealing at 46°C, and 2 min extension at 72°C.

**Northern Blot Analysis**—Total cellular RNA (20 μg) was fractionated in a 1.5% formaldehyde-agarose gel and transferred to a Biotrans nylon membrane by downward capillary transfer using the TurboBlotting system (Schleicher and Schuell, Keene, NH). Nylon membranes were cross-linked by exposure to a UV light source for 1.5 min and then baked at 85°C for 25 min. Blots were pre-hybridized in ULTRAlhybTM (Ambion, Austin, TX) at 42°C for 2 h. Hybridization was carried out overnight in the same buffer containing a cDNA fragment that was labeled with [\(^{33}\)P]dCTP by nick translation (Amersham Biosciences, Inc., Piscataway, NJ). The same procedure was used for labeling of cDNA inserts (or PCR products) representing porcine SSAT, porcine IGF-I, rat IGF-II, rat IGFBP-2, human IGFBP-3, rat acid labile subunit, human Sp1, human BTEB1 (KL9F), and human BTEB2 (KLFS) mRNAs. The membranes were washed twice at 42°C for 15 min with 2 × SSC, 0.1% SDS, then twice with 0.1 × SSC, 0.1% SDS using the same conditions. After a final wash, the membrane was subjected to autoradiography using intensifying screens at -80°C. The relative changes in mRNA levels were quantified by use of a Gel Documentation & Analysis System (Alpha Innotech Corp., San Leandro, CA). The bands were stripped of radioactive probe between hybridizations by washing twice for 45 min in 1% SDS at 95°C and were stored at -20°C until further use.

**DNA Sequence Analysis**—Sequencing of cloned cDNA fragments was carried out by the DNA Sequencing Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

**Cell Culture**—Human Hec-1-A endometrial carcinoma cell line (American Type Culture Collection, Manassas, VA) was cultured in McCoy’s 5A medium with 10% fetal bovine serum and maintained at 37°C in an atmosphere of 5% CO\(_2\), 95% air. For polyamine treatments,
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Hec-1-A cells were plated into 6-well plates (~200,000 cells/ml) and allowed to grow in serum-containing medium until confluent. Cells were then incubated in serum-free medium for 24 h, at which time they received fresh serum-free medium also containing 400 μM putrescine, spermidine, or spermine. RNA was extracted 24 h after polyamine addition.

**IGFBP-2 Promoter Reporter Construct**—An IGFBP-2 promoter construct containing 1397 bp of DNA S to the translation initiation codon (the latter located within exon 1) of the porcine IGFBP-2 gene fused to the luciferase reporter gene was used in transient transfection experiments.

**SSAT Mammalian Expression Vector**—The entire coding region of porcine SSAT (17) was subcloned into the EcoRI site of the pInd vector (Invitrogen Corp.). The orientation (sense or antisense) of the resulting constructs was confirmed by restriction digestion and nucleotide sequence analysis. Plasmid DNAs were purified using the Qiagen Maxi Kit (Qiagen, Chatsworth, CA).

**Transient Transfection and Luciferase Assays**—Hec-1-A cells were plated in 6-well plates and grown until 60–70% confluent. The IGFBP-2 promoter luciferase reporter construct was co-transfected with the SSAT sense or antisense expression vectors and their effects on IGFBP-2 promoter activity determined under serum-free conditions. Transfections were performed using LipofectAMINE (Invitrogen, Rockville, MD), following the manufacturers suggested procedure. Cells were harvested 48 h after transfection and whole cell extracts were analyzed for luciferase activity in an Autolumat Luminometer (EG&G, Berthold, Germany). Results from transfection analysis were normalized for protein content of cellular extracts, expressed as LSM ± S.E.M., and compared using the GLM procedures of the SAS statistical package. Similar statistical analyses were performed on Northern blot data, which were first corrected for loading by use of corresponding 18S ribosomal RNA intensity.

**RESULTS**

**Differential Display Analysis of Gene Expression**—Total cellular RNA was isolated from the uterus, ovaries, liver, and kidneys from each of four SSAT-transgenic (ST) and four non-transgenic (NT) control mice. Equal amounts of RNA from each tissue of ST or NT groups were pooled for all subsequent RNA analyses (ST, +lanes; NT, −lanes). Prior to ddRT-PCR, the levels of SSAT mRNAs were examined by Northern blot to confirm their differential expression in tissues of ST and NT littermates, using porcine SSAT cDNA previously cloned in these laboratories (17) as hybridization probe. Two expected RNA transcripts, a major species of ~1.3 kb and a minor species of ~3.5 kb, were abundantly expressed in all four tissues of ST mice, whereas these were barely or non-detectable in corresponding tissues of the NT mice (Fig. 1). The sizes of these two transcripts are identical to those previously reported for the SSAT gene of other mammalian species (17).

A representative portion of a typical differential display gel, which illustrates how candidate SSAT-regulated cDNAs/mRNAs were identified, is shown in Fig. 2. All combinations of two arbitrary primers and seven anchored primers, the sum total of which theoretically covers ~7% of the total mRNA population (Technical Bulletin, GENOMYX Corp.), were used to examine gene expression changes in the four tissues of ST and NT mice. Visual inspection of resultant autoradiograms revealed bands that were of similar intensity between a particular tissue type of ST and NT mice as well as a smaller percentage of bands whose intensities differed between the mouse groups. Interestingly, the majority of these putative differentially expressed transcripts were confined to uterus and ovary, tissues with marked observable phenotypes due to the presence of the transgene (15).

**Confirmation of Differentially Expressed Genes and Determination of Identities**—Twenty-five of the cDNA fragments noted above were excised from gels and subjected to a second round of PCR, using the original combination of primers for each. Of the twenty-five bands identified on the original gels, 19 were successfully re-amplified by PCR and subcloned. All 19 were subjected to Northern blot analysis to confirm differential mRNA expression. Fourteen of these exhibited changes in gene expression that confirmed the original differential-display patterns. Of these, one pair and one set of three had identical mRNA expression patterns and the same transcript sizes (data not shown), suggesting that those within a group were derived from the same mRNA(s), despite having distinct migration positions on the original differential display gels. This was confirmed when differential display products were sequenced in their entirety and subjected to computer analysis. The identities of the final resultant 11 different cDNAs are summarized in Table I, and their corresponding Northern blots presented in Figs. 3 and 4, respectively. Many of these RNAs appear to be novel with respect to function. One (O 1-4-5) has 100% identity to mouse lipoprotein lipase (LPL). Another (L 1-2-3) has 99% similarity to a mouse EST reported in GenBankTM. This cDNA fragment has a complete open reading frame encoding a protein with strongest sequence relatedness (although not identical) to the calcium binding, signal transducing protein, calmodulin, and is designated here as CALM-Rel (Fig. 5) (accession number AY061807). Another dRT-PCR product (U 2-6-5) is the mouse homolog of the mRNA/cDNA encoding human subunit 5 (ε, 47 kDa) of eukaryotic translation initiation factor-3 (eIF-3) (Fig. 6) (accession number AY061808). The U 1-3-7 and 1-3-10 ddRT-PCR products had strong similarity but were not identical to each other and to the endogenous murine leukemia provirus (MLV) genome/transcripts and are here designated as MLV-Rel1 (accession number AY061810) and MLV-Rel2 (accession number AY061809). Sequence analysis of MLV-Rel2 indicated the interesting possibility that this defective
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| Summary of cDNA clones identified by differential display analysis of uterus, ovary, liver, and kidney of non-transgenic and SSAT-transgenic mice |
|---|
| ddRT-PCR product | Accession number of closest match | Size (bp) of cDNA fragment | Identity (perfect match or similarity similar but not identical to) |
| U 1–1–5 | X67279 | 629 | Mouse hepatitis viral receptor/biliary glycoprotein |
| U 1–1–11 | AA277455 | 722 | Unknown function |
| L 1–2–3 | AI18547 | 688 | CALM-Rel |
| U 1–3–7 | K032930 | 640 | MLV-Rel1 |
| U 1–3–10 | M17326 | 708 | MLV-Rel1* |
| U 1–3–15 | BF020640 | 489 | Unknown function |
| O 1–4–5 | NM008509 | 605 | Lipoprotein lipase |
| U 1–4–4 | X02487 | 650 | MuRRS* |
| U 2–3–0 | AI527208 | 674 | Unknown function |
| U 2–7–1 | AI604532 | 697 | Unknown function |
| U 2–6–5 | AW471978 | 654 | eIF-3s5 |

a Similar but not identical.

FIG. 4. Northern blots of endogenous retrovirus–related transcripts in tissues of non-transgenic (−) and transgenic (+) mice. All panels are autoradiograms of the Northern blots with the exception of the lowermost panel which is the ethidium bromide-stained gel prior to blotting. The size(s) of the major transcript(s) for each gene is indicated to the right.

FIG. 3. Northern analysis of “candidate” differentially expressed mRNAs in tissues of non-transgenic (−) and transgenic (+) mice. All panels are autoradiograms of the Northern blots with the exception of the lowermost panel which is the ethidium bromide-stained gel prior to blotting. The size(s) of the major transcript(s) for each gene is indicated to the right.

A retroviral transcript encodes amino-terminal and carboxyl-terminal truncated viral envelope (Env) proteins in normal mouse tissues (Fig. 7). The U 1–1–5 sequence is an identical match with the mouse hepatitis virus receptor which is also found in GenBank™ under the designation of biliary glycoprotein. U 1–4–4 appears to be a new member of the mouse retrovirus-related sequence (MuRRS) transcript family (accession number AY061811).

Differentially Expressed Genes across Tissues—Of the cloned ddRT-PCR products, a few of the corresponding mRNAs were up-regulated and many more were down-regulated in the relevant tissues of ST mice, although some of these changes clearly were tissue-specific. To account for possible variations in RNA loading and gel transfer during Northern analysis, blots were hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe since this is conventionally used as the loading control. Surprisingly, the GAPDH probe itself yielded a substantially higher mRNA signal in all SSAT-transgenic relative to control, tissues (Fig. 3), although ethidium bromide staining of 28 and 18S rRNAs showed comparable amounts of rRNA in each lane and subsequent hybridization with other candidate gene probes, in some tissues, indicated no changes between mouse groups (see later results).

As a result, all hybridization results were corrected for changes in the intensities of ethidium bromide-stained 28 S and 18 S rRNA bands.

The LPL mRNA, a single transcript of ~3.8 kb, was barely detectable in the uterus and ovaries, but was abundantly expressed in the kidneys of NT mice. Levels of this transcript were up-regulated in uterus (~2.3-fold) and in ovaries (~1.9-fold) of ST mice, with no corresponding changes observed in those of the kidney (Fig. 3). This transcript, however, was not expressed at detectable levels in the liver of ST or NT mice. The calmodulin-related (CALM-Rel) transcript also was induced in a tissue-dependent manner upon overexpression of SSAT. This ~1.3-kb transcript, while undetectable in all four tissues of NT mice, was readily apparent in liver and kidneys of ST mice. In contrast, no detectable levels of CALM-Rel mRNA were observed in uterus and ovaries of the ST mice.

Expression of eIF-3s5 mRNA, a single transcript of ~1.5 kb, was high in all four tissues of NT mice. However, this level of expression was dramatically reduced in uterus and ovaries of ST mice, with a similar downward trend, albeit of lesser magnitude, observed for liver and kidney. Murine leukemia provirus–related (MLV-Rel1) transcripts exhibit three distinctive sizes, one major species of 5.3 kb and two minor species of ~8.2 and 3.5 kb, respectively (Fig. 4). The highly related MLV-Rel2 sequence showed a similar pattern of three transcripts (7.2, 5.4, and 3.5 kb), with the 5.4-kb transcript being the major species and the minor species being only weakly discernible (Fig. 4 and data not shown). MLV-Rel1 and -Rel2 transcripts were abundantly expressed in all four tissues of NT mice, with highest expression in ovaries. However, expression of these transcripts was reduced to nearly undetectable levels in all four corresponding tissues of ST mice. Expression of yet another retrovirus–related RNA sequence, a new member of the MuRRS family, also was similarly altered in tissues of ST mice. At least five distinct MuRRS transcripts were detected, four minor species of ~13, 7.7, 3.9, and 2.6 kb, and one major species of 5.1 kb. The major MuRRS transcript was most abundantly expressed in ovary > uterus = kidney > liver, of NT mice and expression of this transcript was suppressed to undetectable levels in the
corresponding tissues of ST mice. The four minor transcripts also appeared to behave in a similar manner. In contrast to the above, the murine hepatitis viral receptor cDNA clone has two transcript sizes of 4.2 and 3.8 kb, respectively. The major transcript in kidney is 3.8 kb whereas the major transcript in liver and uterus is the 4.2-kb variant. Both transcripts were up-regulated in the uterus of ST mice, whereas the reverse was true for liver. There were no alterations in the expression levels of these transcripts in ovary and kidney between the two mouse groups.

Abundance of two other transcript classes (U 1-3-15 and U 1-1-11) was altered in a tissue-selective manner upon SSAT overexpression (Fig. 3). U 1-3-15 transcripts (2 and 1.1 kb) were present in the uterus and ovaries of NT mice, but only the smaller transcript was detected in the liver and kidney. Expression of the larger transcript was nearly undetectable in uterus and ovaries of ST mice, while that of the smaller transcript was not significantly altered in these same tissues. In liver and kidney, however, the expression of the 1.1-kb transcript was increased in ST relative to NT littersmates. The expression of the 1.4-kb U 1-1-11 transcript was down-regulated in uterus and ovaries, but was up-regulated in liver and kidney of ST relative to NT, mice. The U 2-3-0 and U 2-7-1 RNAs also exhibited tissue-specific increases or decreases in the ST versus NT mice (Fig. 3).

Expression of IGFs and Their Binding Proteins (IGFBPs)—In conjunction with the arbitrary ddRT-PCR approach, the expression of IGFs and their binding proteins also was examined in the NT and ST tissues, since corresponding proteins for these genes have been shown to play important role(s) in cell growth and differentiation of multiple tissues, including those of the female reproductive tract (18). Northern blot analysis for IGF-I mRNA revealed three transcripts, two major species of 7.5 and 0.9 kb and one minor species of 1.3 kb, respectively, in ovary, uterus, and liver, but not kidney, of NT mice (Fig. 8). No changes in the levels of any of these transcripts were observed between corresponding tissues of ST and NT mice. Similarly, the expression levels of IGF-II mRNA were not altered in tissues of ST mice, compared with those of NT mice (data not shown). By contrast, the expression of the IGFBP-2 and -3 were

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**Fig. 5.** DNA sequence and open reading frame of ddRT-PCR product (panel A), and corresponding protein relatedness to murine calmodulins (panel B), for the novel calmodulin-related (CALM-Rel) gene transcript induced in liver and kidney of SSAT-transgenic mice. Shown for comparison are the sequences of the protein products of the mouse calmodulin-1, -2, and -3 genes.
dramatically altered in a tissue-specific manner with SSAT overexpression (Fig. 8). The levels of IGFBP-2 mRNA (a single transcript of 1.4 kb) were undetectable in uterus and ovary of NT mice, but were robustly induced in corresponding tissues of ST mice (Fig. 8, Table II). Conversely, expression of this mRNA, which was already low in liver and kidney of NT mice, was further reduced in corresponding ST mouse tissues. The expression of IGFBP-3 mRNA (2.4 kb) was most abundant in the uterus of normal mice; however, this was significantly reduced with SSAT overexpression (Fig. 8, Table II). In the other tissues examined, the levels of IGFBP-3 mRNA were not correspondingly altered in ST mice. The dramatic changes in IGFBP-2 and -3 mRNA levels in relevant tissue(s) were observed at the level of their respective proteins (Fig. 9). IGFBP-3 protein levels were diminished (130%) in the uterus, while those of IGFBP-2 were induced (96%) in the uterus and to a lesser extent (58%) in ovary, by SSAT overexpression. Uterine and ovarian expression of the IGFBP-3 acid-labile subunit mRNA was undetectable in NT and ST mice. In contrast, this mRNA was observed in liver and kidney, although this was unaffected by SSAT overexpression.

**Fig. 6.** DNA sequence and open reading frame of ddRT-PCR product (panel A), and corresponding protein relatedness to the carboxyl terminus of human translation initiation factor 3, subunit 5 (panel B), for the RNA transcript suppressed in uterus, ovary, liver, and kidney of SSAT-transgenic mice. This cDNA/mRNA represents the mouse homolog of the human eIF-3α5 protein. The upper line is the deduced mouse amino acid sequence; the corresponding human sequence is compared (third line) to this, with conserved residues indicated on the second line. The 5′ end of the mouse cDNA clone corresponds to amino acid 210 of the human protein.

**A. Mouse Partial eIF-3α5 cDNA and Protein Sequences**

| Protein | Accession | Description |
|---------|-----------|-------------|
| eIF-3α5 | NM_001072 | Human translation initiation factor 3, subunit 5, mRNA |
| eIF-3α5 | NM_001072 | Human translation initiation factor 3, subunit 5, protein |

**B. Partial eIF-3α5 Protein Sequence**

Mouse cDNA clone

| Accession | Description |
|-----------|-------------|
| NM_001072 | Human translation initiation factor 3, subunit 5, mRNA |
| NM_001072 | Human translation initiation factor 3, subunit 5, protein |

**DISCUSSION**

Previous studies of SSAT-transgenic mice have shown that overexpression of SSAT leads to changes in intracellular polyamine pools, including an accumulation of putrescine (15). Since these alterations are accompanied by a distinctive phenotype including hair loss, reduced subcutaneous fat, hypoplasia of the uterus (both endometrial (stroma and glands) and
myometrial compartments are affected) and ovarian dysfunc-
tion (reduced folliculogenesis and absence of corpora lutea) (15), it is reasonable to speculate that some or all of these effects may reflect changes in gene expression profiles as a consequence of chronically altered or aberrant intracellular polyamine levels and/or pool composition. In this initial attempt to identify genes whose expression, at the level of mRNA, are altered in response to changed polyamine homeostasis, we used the differential display technique as well as the candidate gene approach to compare mRNA populations from uterus, ovaries, liver, and kidneys of ST mice with those of NT litter-
mates. Uterus and ovary were selected based upon their pro-
nounced morphological and functional changes in the trans-
genic line. The present results clearly demonstrate that
dysregulation of polyamine pools (i.e. increased putrescine, N\textsubscript{1}-acetylspermine and N\textsubscript{1}-acetylspermidine in vivo, 15) via long-
term overexpression of SSAT leads to marked changes in gene
expression profiles in these and other tissues.

Of the differentially expressed genes evaluated here, two
(LPL and GAPDH) are involved in energy metabolism and were
induced in the ST transgenic mice, albeit in a tissue-dependent
manner. Although LPL and GAPDH represent but a small
fraction of genes involved in energy metabolism, their identifi-
cation as potentially polyamine-regulated suggests an impor-
tant role for these polycations in the regulation of energy me-
tabolism. Indeed, this supposition is consistent with an earlier
study of rat adipocytes (19), where exogenous polyamines
(spermine, spermidine, and putrescine) significantly inhibited
adenosine deaminase-stimulated lipolysis. The physiological
implications of these observed changes remain unclear at pres-
ent, but such alterations may be partly responsible for the
phenotypic changes (i.e. reduced adiposity) associated with
SSAT overexpression. LPL is a key regulatory enzyme respon-
sible for hydrolysis of triglycerides in plasma lipoprotein, gen-
erating free fatty acids (FFA) and cholesterol. The intracellular
metabolism of FFA differs in various tissues subsequent to
cellular uptake. In adipose tissue, FFA is re-esterified and
deposited as lipid droplets for storage. In contrast, FFAs are
mainly utilized for \textsuperscript{1}H\textsubscript{2}oxidation and energy production in non-
adipose tissues such as muscle. The marked up-regulation of
LPL mRNA expression in uterus and ovaries of transgenic
mice, therefore, suggests increased FFA and cholesterol uptake

\begin{tabular}{l}
\textbf{Frame +2 Env (amino terminal) peptide} \\
275 atggaaggtccagcttcctaaacccccttaagataagattacaac \\
320 cggctgggccccttaatagctctgggtctttgaagggcagga \\
365 gtacgcttgtaaaggtcacgctctccatcaggtctttcaagtlacc \\
410 vavqsdsphqvfntw \\
455 tccctctcgggacaaatgcgtacgctctttcgtcagctttgt \\
500 aaaaagacgagatctttcggtgtttcagccgtctttgcccaaca \\
545 gtataccacactttaaa559 \\
\end{tabular}

\begin{tabular}{l}
\textbf{Frame +3 Env (carboxy terminal) peptide} \\
477 atgccttttctctgtcagttgtaaagacgagatctttcggtgtta \\
522 cagggctcctgttctcgaccaacagttatccacaactttacattata \\
567 gatccagaaaaagtggaaactcaggtgtataa 596 \\
\end{tabular}
by both tissues in response to increased SSAT. However, increased levels of LPL expression are not always beneficial since such changes inevitably lead to increased \( \beta \)-oxidation rate, which may in turn lead to cell death. Recent studies with transgenic mouse lines have shown that muscle-specific overexpression of LPL causes a severe myopathy (20, 21). Upregulation of LPL gene expression may be directly responsible in part, for the morphological and functional changes observed in the uterus (i.e. myopathy of the myometrium) and ovaries of ST mice, possibly occurring via similar mechanisms.

The up-regulation of GAPDH mRNA levels in tissues of ST mice is an interesting and novel observation, especially since oxidative stress has been shown to increase levels of GAPDH in a rodent cell line (22). Thus, increased GAPDH mRNA levels in the present study may be indicative of increased oxidative stress in tissues of SSAT-transgenics, a linkage that has been previously documented in unrelated studies with a human non-small cell line (23) and human breast cancer cell lines (24), where polyamine analogue-induced programmed cell death was shown to be a consequence in part, of the oxidative stress resulting from generation of \( \text{H}_2\text{O}_2 \). Induction of GAPDH expression has been suggested to constitute a defense mechanism for protection of cells against environmental stresses, including oxidative stress (24, 25), however, increased levels of GAPDH also induced apoptosis in a number of cell types, particularly neuronal cells (26). In COS-7 cells, overexpression of GAPDH induced apoptosis (27), while its suppression by antisense technology led to a subsequent attenuation of apoptosis in cerebellar granule cells (28, 29). Therefore, any significant increase in GAPDH gene expression may have a detrimental effect on cell viability. Surprisingly, morphologic and functional alterations in the present study were only apparent in uterus and ovary, but not in liver and kidney, despite the greater increase in expression of this gene occurring in the latter tissues with SSAT overexpression. Whatever the cause and consequence (if any) of GAPDH induction in the current animal model, these results differ from those obtained with an in vitro model of acute SSAT induction in MCF-7 cells (30), where no change in GAPDH mRNA expression was observed upon altered SSAT or polyamines.

Another novel finding was that chronically altered polyamine pools in SSAT transgenic mice were correlated with massive reductions in the mRNA levels of endogenous virus-related genes (MLV-Rel1, -Rel2, and MuRRS), all of which were highly expressed in the corresponding tissues of normal mice. Interestingly, this marked suppression was most apparent for uterus and ovary, which exhibited higher basal expression of these transcripts than kidney and liver. The abundant expression of these transcripts in reproductive tissues suggests their integration into or near uterine/ovarian genetic loci, although their specific functions in reproductive and other processes are totally unknown. A similar pattern of tissue MLV proviral-related RNA (related to but not identical in sequence to the MLV-Rel1 and -Rel2 transcripts reported here) expression was previously reported in another study with the C57BL/6 mouse strain, where a single transcript of 5.2 kb was predominantly expressed in the reproductive tissues of both sexes (31). It is well recognized that a number of retroviruses, including those highly related to the transcripts identified in the present study, are capable of transforming normal cells into neoplastic types via activation of proto-oncogenes (32). The involvement of retrovirus expression in oncogenesis is best illustrated by the MMTV induction of mouse mammary tumors (33), whereby retrovirus integration into preferred sites of the genome alters transcriptional mechanisms in cis and leads to the activation of an adjacent proto-oncogene (e.g. \( \text{wnt} \)). Although the majority of the retrovirus-related sequences in the mouse germ line are defective and therefore, incapable of producing viral particles, one of the multiple MLV-Rel transcripts (8.3 kb) detected in the present study appears to be the non-defective, infectious MLV proviral RNA, based on its size (31). The expression of this transcript is very low compared with those of the defective MLV transcripts, nevertheless, the presence of a full-length transcript suggests the capability of synthesizing viral protein. Moreover, the MLV-Rel2 transcript described in the present study has two open reading frames potentially encoding trun-
FIG. 10. A and B, effects of polyamines on SSAT and IGFBP-2 mRNA abundance in Hec-1-A cells. Cells were incubated in serum-free medium (CONT, control) containing 400 μM polyamine (PUT, putrescine; SPD, spermidine; SPM, spermine) for 24 h. Total cellular RNA was isolated and analyzed (25 μg/lane) by Northern hybridization, using labeled cDNA probes. B, Northern blot band intensities were obtained from two independent experiments (two or three replicate cell/RNA preparations per treatment per experiment as in A) and statistically analyzed after correction for 18 S ribosomal RNA intensity. C, activity of transfected IGFBP-2 gene promoter (5 μg/well) is increased by 100 μM putrescine in serum-free medium (CONT). D, activity of transfected IGFBP-2 gene promoter (5 μg/well) is positively associated with SSAT expression. Shown is luciferase activity after co-transfection with SSAT sense (S) or antisense (As) expression vectors (1 μg of DNA/well) in the absence of serum in the medium. Panels C and D each represent results of three to four independent experiments; data in B–D are expressed as LSM ± S.E.M., with asterisks indicating statistical differences (p < 0.05).
**Fig. 11.** *Sp1* and *BTEB1* but not *BTEB2* gene transcripts are induced in some tissues of SSAT-transgenic mice. Shown are the autoradiograms of the Northern blots. The sizes of the corresponding transcripts are indicated to the right.

**Note:**
- In vivo study, in which a complex of IGFBP-3 and IGF-I enhanced protein synthesis under conditions of semistarvation (46). This was not observed when free IGF-I alone was administered. Therefore, the combination of increased expression of IGFBP-2 and the simultaneous decreased IGFBP-3 expression is predicted to lead to a substantial overall decrease in the availability/delivery of IGF-I and IGF-II to target tissues, possibly resulting in the observed uterine hypoplasia and ovarian hypofunction in ST mice. Interestingly, these phenotypes are somewhat mimicked by IGF-I null mutant female mice (47), further suggesting a possible overlap in reproductive functions of SSAT and the IGF system. We capitalized on the observed positive relationship of SSAT and IGFBP-2 in mouse uterus to examine whether SSAT and polynamines directly affect IGFBP-2 gene expression or alternatively, whether the observed in vivo phenomenon might be a secondary effect of altered SSAT expression. In the absence of any continuous uterine cell lines of murine origin, we used the well-characterized human Hec-1-A uterine cell line to attempt to link the polynamines and/or SSAT with altered IGFBP-2 gene activity. This was based on the previous observation that uteri and ovaries of the SSAT transgenics did not differ from non-transgenics with regard to such linkages. Subunit 5 (eIF3-5) of this complex is a member of the Mov-34 family of protein synthesis initiation factors, are under way to clarify these possibilities.
- Chronic overexpression of SSAT was accompanied by decreased eIF3 subunit 5 (e, 47 kDa) mRNA abundance, an effect most apparent for the uterus and ovaries. Eukaryotic translation initiation factor 3 is a large, structurally complex, 10-subunit complex that has a central role in the initiation of translation. This complex binds to 40S ribosomal subunits in the absence of other initiation factors and helps to maintain 40 and 80S ribosomal subunits in a dissociated state. The eIF3 complex also stabilizes initiator methionyl-tRNA binding to 40 S subunits and is absolutely required for mRNA binding (49). Several of the other eIF3 protein subunits have been previously implicated in normal and abnormal cellular growth (50–53), although subunit 5 has evidently not been previously implicated in such linkages. Subunit 5 (eIF3p47) of this complex is a member of the Mov-34 family of eukaryotic proteins (54). As inferred from the studies of other subunits, an observed down-regulation of the eIF3 s5 gene would lead to an inhibition of protein synthesis and cell growth. Similarly, other initiation factors have been tied to cell death. eIF-5 has been implicated as a major trigger in the apoptosis of a hepatoma cell line DH23A upon induced accumulation of
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putrescine (55). A similar role has been proposed for eIF4GII (56) and eIF-2α (57). Although a role for eIF3 has not been directly examined, the dramatic down-regulation of expression of this gene in the uterus and ovary, with accompanying alterations in phenotype or function, suggest a physiological linkage of eIF-3 with altered SSAT levels, cell proliferation, and/or apoptosis.

Chronic overexpression of SSAT resulted in altered expression levels of other genes whose identities are currently unknown. Interestingly, the transcripts for these genes were, for the most part, down-regulated in ST mice, in a tissue-selective manner. These observations may represent a general phenomenon for tissues overexpressing SSAT, although this could also simply reflect preferential amplification of certain cDNA fragments due to the primer sets utilized. However, if indeed there is a general inhibition of gene expression associated with SSAT overexpression, this differs from a recent study in which treatment of Rat-2 cells with difluoromethyl ornithine, a specific inhibitor of polyamine biosynthesis, caused the induction of 26 of 35 differentially expressed mRNAs, including that for GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58). Nevertheless, in the same report, kidneys of transgenic mice overexpressing ODC and GAPDH (58).

In summary, the present study has demonstrated that the chronic overexpression of SSAT leads to marked changes in gene expression in reproductive and non-reproductive tissues of female mice. Of the differentially expressed genes examined here, the number was found to be disproportionately higher in the uterus and ovary than in kidney and liver, consistent with the greater phenotypic and functional changes observed in the former tissues with SSAT overexpression. Although further studies are required to define the functionality of many of the genes identified on the SSAT phenotypes observed, our findings suggest that functional changes associated with SSAT overexpression are mediated at least in part, via the long-term effects of polyamines (e.g. putrescine, spermidine, and/or spermine) on expression of genes encoding metabolic enzymes, endogenous retroviral transcripts, IGF-binding proteins, and certain members of the KLF transcription factor family.

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Altered Levels of Growth-related and Novel Gene Transcripts in Reproductive and Other Tissues of Female Mice Overexpressing Spermidine/Spermine \(N^1\)-Acetyltransferase (SSAT)  
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