The Testicular Form of Hormone-sensitive Lipase HSL\textsubscript{tes} Confers Rescue of Male Infertility in HSL-deficient Mice*

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Inactivation of the hormone-sensitive lipase gene (HSL) confers male sterility with a major defect in spermatogenesis. Several forms of HSL are expressed in testis. HSL\textsubscript{tes} mRNA and protein are found in early and elongated spermatid, respectively. The other forms are expressed in diploid germ cells and interstitial cells of the testis. To determine whether the absence of the testis-specific form of HSL, HSL\textsubscript{tes}, was responsible for the infertility in HSL-null mice, we generated transgenic mice expressing HSL\textsubscript{tes} under the control of its own promoter. The transgenic animals were crossed with HSL-null mice to produce mice deficient in HSL in non-gonadal tissues but expressing HSL\textsubscript{tes} in haploid germ cells. Cholesteryl ester hydrolase activity was almost completely blunted in HSL-deficient testes. Mice with one allele of the transgene showed an increase in enzymatic activity and a small elevation in the production of spermatozoa. The few fertile hemizygous male mice produced litters of very small to small size. The presence of the two alleles led to a doubling in cholesteryl ester hydrolase activity, which represented 25% of the wild type values associated with a qualitatively normal spermatogenesis and a partial restoration of sperm reserves. The fertility of these mice was totally restored with normal litter sizes. In line with the importance of the esterase activity, HSL\textsubscript{tes} transgene expression reversed the cholesteryl ester accumulation observed in HSL-null mice. Therefore, expression of HSL\textsubscript{tes} and cognate cholesteryl ester hydrolase activity leads to a rescue of the infertility observed in HSL-deficient male mice.

Hormone-sensitive lipase (HSL)\textsuperscript{1} is an intracellular enzyme with a broad substrate specificity. HSL catalyzes the hydrolysis of tri-, di-, and monoacylglycerols, cholesteryl, and retinyl esters, as well as other lipid and water-soluble molecules (1). A testicular form of the enzyme HSL\textsubscript{tes} is expressed in rodent and human testis (2). HSL\textsubscript{tes} mRNA expression is high in early spermatids (3). Immunolocalization of the protein in human and rodent seminiferous tubules showed that the highest level of expression occurred in elongated spermatids. Other forms of HSL are expressed elsewhere in the testis. HSL-like immunoreactivity is observed in the cytoplasm of type B spermatogonia (human and mouse), primary spermatocytes (human and mouse), and Sertoli cells (human) (3, 4).

The 3.9-kb human HSL\textsubscript{tes} mRNA is translated into 1068- and 1076-amino acid proteins in rat and humans, respectively (2). HSL\textsubscript{tes} contains a unique NH\textsubscript{2}-terminal domain in addition to the 775 amino acids common to all forms of HSL (5, 6). This additional domain is encoded by a testis-specific exon located 15 kb upstream of the first of the 9 exons common to all known HSL isoforms. The 5′-region of HSL\textsubscript{tes} mRNA is distinct from that of the other human HSL mRNA expressed in testicular diploid cells. The genomic organization of HSL\textsubscript{tes} suggested, as often seen when a gene is expressed in somatic cells and in haploid germ cells, the use of different promoters to govern tissue-specific expression. Our transgenic studies of the 5′-flanking region of the human HSL\textsubscript{tes}-specific exon revealed that 95 bp upstream of the transcription start site is sufficient for expression of a reporter gene in mouse testis but not in other HSL-expressing tissues (3, 7). Therefore, a short proromoter is essential for testis expression of HSL\textsubscript{tes}.

The role of HSL in testis was revealed by the phenotype of HSL-deficient mice (8, 9). Male mice homozygous for the mutant allele (HSL\textsuperscript{−/−}) are sterile. Abnormalities in spermatogenesis result in profound alterations of spermatid maturation and oligospermatia. To determine the importance of HSL\textsubscript{tes} in male sterility, we generated a transgenic line (HSL\textsubscript{tes}/HSL\textsuperscript{−/−}) expressing the human HSL\textsubscript{tes} under the control of its own promoter and, through intercross with HSL\textsuperscript{−/−} mice, produced animals deficient for HSL in all tissues but haploid germ cells (HSL\textsuperscript{−/−} HSL\textsubscript{tes}/HSL\textsuperscript{−/−} and HSL\textsuperscript{−/−} HSL\textsubscript{tes}/HSL\textsuperscript{−/−}). Our data show that HSL\textsubscript{tes} is the sole HSL form responsible for impaired spermatogenesis. A comparison of HSL\textsuperscript{−/−} HSL\textsubscript{tes}/HSL\textsuperscript{−/−} and HSL\textsuperscript{−/−} HSL\textsubscript{tes}/HSL\textsuperscript{−/−} animals revealed the rate-limiting role of HSL\textsubscript{tes} and the importance of cholesteryl ester hydrolase (CEH) activity in the action of HSL on spermatogenesis and more specifically on spermiogenesis.

EXPERIMENTAL PROCEDURES

HSL\textsubscript{tes} Transgenic and HSL-null Mice—The studies followed the INSERM and Louis Buguard Institute Animal Facility guidelines and were approved by the local Animal Ethics Committee in Lund, Sweden.

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To obtain rescued male mice, the double heterozygous mice were either
containing the testis-specific exon and part of exon 1, a 2.6-kb HkzNotI fragment containing the 3'-part of exon 1, intron 1, exon 2, and intron 2, the 5'-end of exon 3, and a 2.3-kb NotI/EcoRI human HSL<sup>+</sup> cDNA piece containing exons 3–9 and the 3'-untranslated region (Fig. 1). The vector pLS301 (Invitrogen, Carlsbad, CA) containing the HSL<sub>rec</sub> construct was linearized with HindIII and EcoRI. Transgenic mice were generated after microinjection of this construct in fertilized oocytes of female from the B6D2F1 strain (Elevage Janvier). Screening of the founders was made, after tail genomic DNA extraction, by PCR analysis with the following conditions: sense primer (5'-GCAAGACGCGACCA-
TGCTTGACAGC-3') and antisense primer (5'-GTGTTGCGCCATTGTTTATGTAAG-3') with 56°C as annealing temperature. The size of the amplification was 277 bp. Two transgenic lines (lines 29 and 32) were established. Line 29 was used in this study. HSL-null mice were generated by targeted disruption of the HSL gene in 129SV-derived embryonic stem cells by standard procedures as described elsewhere (10, 11). In brief, the cDNA encoding the Aequorea victoria green fluorescent protein was inserted in-frame into exon 5 of the HSL gene, followed by a neomycin resistance gene, thereby disrupting the catalytic domain. Two recombination embryonic stem cell colonies were used for generation of two independent HSL-null mouse lines. There was no phenotypic difference between the two lines. A single line was used for subsequent studies. We developed a PCR assay with three primers in order to distinguish HSL<sup>+</sup>/H9262/H11003 from HSL<sup>−/−</sup> or HSL<sup>+/−</sup> mice. The sense primer, 5'-ACTGCA-
CAGCCTGGCAAAAT-3', was common to both alleles; the antisense primers used to discriminate the wild type (WT) and targeted alleles were, respectively: 5'-AGGCTCAGCTGGTCTAGCC-3' and 5'-GCTG-
AATCTGTCGCTGTTTAATGTAAG-3'. Primers were used at 500 pm (sense) and 250 pm (antisense) with 2.5 mm MgCl<sub>2</sub>. The denaturation step was at 94°C for 30 s, the annealing step at 52°C for 30 s, and the elongation step at 72°C for 30 s. PCR product sizes were, respectively, 290 and 353 bp.

**Intercross of HSL<sub>rec</sub> Transgenic Mice with HSL-null Mice**—First we mated transgenic animals of line 29 to obtain male mice homozygous for the transgene. Two males were selected and mated with HSL-null female mice because male null mice are sterile. All pups were heterozygous both for the testis-specific transgene and for the invalidation gene. To obtain rescued male mice, the double heterozygous mice were either mated together or double heterozygous male mice were mated with female-null mice. We therefore obtained male mice with one or two alleles of the HSL<sub>rec</sub> transgene that were genotyped using quantitative PCR on tail genomic DNA. From experiments to build standard curves, 2.5 ng of DNA was chosen for the genotyping of animals using a Taqman chemistry-based human HSL<sub>rec</sub>-specific assay-on-demand and a GeneAmp 7000 sequence detection system (Applied Biosystems, Courtaboeuf, France). The murine hypoxanthine ribosyltransferase (Hprt) gene was amplified as the reference gene using SYBR green chemistry (Applied Biosystems) with the following primers: mHPRT-S (5'-CGACGCAGCAACAGCAGCA-3') and mHPRT-AS (5'-GGACGCAGCAACAGCAGCA-3'). One unit of hydrolase activity is equivalent to 1 μmol of fatty acid released per minute at 37°C.

**Measurement of Neutral Lipid Molecular Species by Gas-Liquid Chromatography**—Ethyl acetate, chloroform, and methanol were purchased from Carlo Erba. Diacylglycerol-1,3-dimyristoyl, stigmasterol, cholesteryl heptadecanoate, and triheptadecanoylglycerol were obtained from Sigma. Half of the testis was weighted and crushed using a Potter homogenizer with the following solution: methanol/chloroform/water containing 5 mM EDTA (1.1/0.5/5.5 ml). Lipids from an equivalent of 10 mg of tissue were extracted with 1 ml of methanol/chloroform: water (2:1:1). The suspension was centrifuged for 10 min at 1500 rpm to pellet the proteins. Supernatant was transferred into an 8-ml Teflon-sealed vial and two internal standards: 3 μg of stigmastanol, 2 μg of diacylglycerol-1,3-dimyristoyl, 14.2 μg of cholesteryl heptadecanoate, and 3 μg of triheptadecanoylglycerol. Double phase was generated by adding 2.25 ml of chloroform, 2 ml of methanol, and 1.75 ml of water according to Bligh and Dyer (13). The chloroform phase was filtered over glass wool, evaporated to dryness, and dissolved in 50 μl of ethyl acetate. 4 μl of lipid extract was analyzed by gas-liquid chromatography on a 4890 Hewlett Packard system using an Ultra1 Hewlett Packard fused silica capillary column (5 μ x 0.31 mm i.d.) coated with cross-linked dimethylsiloxane (14). Oven temperature was programmed from 200 to 340°C at a rate of 6°C per min, and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 315 and 345°C, respectively. Proteins were determined by enhanced chemiluminescence reagent (Amersham). One unit of hydrolase activity is equivalent to 1 μmol of fatty acid released per minute at 37°C.

**Statistical Evaluation**—Values are expressed as mean ± S.E. Group means were compared with the control mean using the Mann-Whitney test (SAS software, version 8.0, SAS Institute Inc., Cary, NC).

**RESULTS**

**Creation of Transgenic HSL<sub>rec</sub> Mice**—To produce transgenic mice with expression of human HSL<sub>rec</sub> in testis, we generated a 7.6-kb fragment containing 1.4 kb of the testis-specific promoter that has been shown in transgenic mice to drive expression of a reporter gene in spermatids (7) (Fig. 1). Only haploid germ cells express significant amounts of human and mouse HSL<sub>rec</sub> (3, 4). To enhance transgene expression, two endogenous introns were added in the construct (16). Two transgenic founders, 29 and 32, were obtained and bred to establish trans-
iggic lines. Lines 29 and 32 had 5–10 and 2–5 transgene copies, respectively. Western blot analysis was performed on testis protein extracts to determine the expression of human HSLtes.

An antibody directed against the testis-specific part of human HSLtes recognized proteins between 110 and 130 kDa in testes of transgenic animals (Fig. 2A). No reactive band was detected in adipose tissue. Because of the low homology between human and mouse testis-specific sequences (2), the antibody did not recognize the endogenous murine HSLtes. An antibody directed against all forms of HSL detected human and murine testicular forms of HSL and the 82-kDa adipose tissue murine HSL (Fig. 2B).

Intercross with HSL-null Mice—As human HSLtes expression was higher in line 29 than in line 32 (Fig. 2A and data not shown), line 29 was used for an intercross with HSL-null mice. A PCR assay was set up on tail genomic DNA to genotype the mice. Normalization of the murine hypoxanthine ribosyltransferase gene, transgene quantities were 0.164 ± 0.014 (0.13–0.19; n = 5) for HSL−/− HSLtes+/- mice and 0.348 ± 0.058 (0.23–0.47; n = 4) for HSL−/− HSLtes+/- mice. The validity of our assay was confirmed by genotyping the offspring. Transmission of the transgene to offspring followed Mendelian rules. Intercrossing of HSL−/− HSLtes+/- female mice with HSL+/- HSLtes+/- male mice gave the expected 25% HSL−/− HSLtes+/- male mice. Intercross between HSL−/− HSLtes+/- mice produced 6.25% HSL−/− HSLtes+/- and 3.12% HSL+/- HSLtes+/- male mice, respectively.

Body and Testis Weights—The body weights of genetically modified mice with various genotypes were not different from that of the WT mice except that of the HSL−/− HSLtes+/-, which was slightly lower (−11%, p < 0.05, Table I). The testis weights of HSL−/− and HSL−/− HSLtes+/- mice were much lower than that of WT mice (−40%, p < 0.001, and −42%, p < 0.001, respectively). The weight of this organ was partially restored in HSL−/− HSLtes+/- male mice (18% compared with HSL−/−, p < 0.05, and −29% compared with WT, p < 0.01).

Histological Analysis—Histological observations reveal that a low number of early and late spermatids and no spermatozoa were found in the seminiferous epithelium of the HSL−/− mice (Fig. 3, B versus A). Furthermore, in these mice, numerous large vacuoles were present in the seminiferous tubules. These vacuoles were absent in the HSL−/− HSLtes+/- mice (Fig. 3C). In these animals, the spermatid pool had not increased, and rare spermatozoa could be observed. In contrast, numerous spermatozoa were seen in HSL−/− HSLtes+/- mice, and spermatogenesis generally appeared qualitatively normal (Fig. 3D).

In the different transgenic lines, there were no apparent abnormalities in the interstitium. In accordance with our data showing that spermatogenesis was deficient in HSL-null mice, no spermatozoa was seen in the epididymides, which instead were packed with cell debris and immature round germ cells generally identified as spermatids, a number of which were multinucleated (Fig. 3, insert B versus insert A). The epididymal lumen of the HSL−/− HSLtes+/- mice also contained numerous immature germ cells in most instances with a single nucleus and a few spermatozoa (Fig. 3C, insert). By contrast, the epididymis of HSL−/− HSLtes+/- mice was filled with numerous spermatozoa. Only a few immature germ cells were present (Fig. 3D, insert).

Sperm Reserves—Sperm reserves in HSL-deficient mice had decreased by more than 99% when compared with WT mice (Fig. 4). Sperm reserves of the HSL−/− HSLtes+/- animals were markedly increased when compared with those of the HSL−/− mice (20×, p < 0.05) but represented only 2.5% of the WT mice values. In HSL−/− HSLtes+/- mice, sperm reserves were increased about 10-fold when compared with the reserves of the HSL−/− HSLtes+/- animals to reach 26% of that of the WT.

Fertility Studies—The libido (mating behavior) of HSL−/−, HSL−/− HSLtes+/-, and HSL−/− HSLtes+/- male mice was normal as judged by the time necessary for recovery of all copulatory plugs (10, 8, and 3 days, respectively) when compared with WT mice (15 days). The mating trials show that HSL−/− male mice were sterile (Figs. 4 and 5). In contrast, the fertile (12%) HSL−/− HSLtes+/- male produced litters with a lower number of pups compared with WT male mice. The presence of the two HSLtes alleles totally restored fertility. The average litter size sired by these mice was normal.

Human HSLtes mRNA Levels, Enzymatic Activities, and Neutral Lipid Analysis—We wished to determine whether there was a relationship between the number of transgene alleles and HSLtes mRNA expression and enzymatic activities. Human HSLtes mRNA level was two times higher in the testes of HSL−/− HSLtes+/- mice compared with HSL−/− HSLtes+/- mice (69 ± 5, n = 3 versus 28 ± 3, n = 4). HSL possesses both CEH and diacylglycerol lipase activities (1). We found that hydrolysis of cholesteryl esters was extremely low in HSL−/− testis, whereas the diacylglycerol lipase activity represented 15% of the WT levels (Fig. 6). The presence of HSLtes on one allele...
restored CEH activity to 10% of WT values. This activity was doubled and represented 25% of WT values in homozygous HSLtes transgenic animals. Expression of the transgene promoter leads to the rescue of infertility observed in HSL-deficient mice. The data point to the unique role of HSL in Leydig cells suggests that the enzyme may play a role in spermatogenesis with an increased vacuolization of the spermatids (arrowheads) and rare spermatids (arrowheads) but were filled with multinucleated early spermatids (fine arrows) and cell debris. In contrast, the epididymis of HSLtes mice (insert in D) were packed with numerous spermatozoa (arrowheads) and less early spermatids, among which many fewer multinucleated cells were present (fine arrows).

DISCUSSION

We have shown that expression of HSLtes under the control of its own promoter leads to the rescue of infertility observed in HSL-deficient male mice. The means ± S.E. p < 0.05 (a), p < 0.01 (b), and p < 0.001 (c) compared with the wild type values and p < 0.05 (d), p < 0.01 (e), and p < 0.001 (f) compared with the HSL-/- group values.

FIG. 3. Histology of the testis and epididymis from WT, HSL+/H11002, HSL+/- HSLtes, and HSL-/- HSLtes mice. Compared with WT mice (A), HSL-/- mice (B) presented an altered spermatogenesis with an increased vacuolization of the seminiferous epithelium (thick arrows) and no spermatozoa in the lumen (arrowheads). C, HSL-/- HSLtes mice animals presented only a few small vacuoles as well as a few late spermatids and rare spermatozoa (arrowheads). D, in HSL-/- HSLtes mice, numerous spermatozoa were observed (arrowheads), and spermatogenesis appeared qualitatively normal. Compared with WT mice (insert in A), the lumen of the caput epididymis of HSL-/- (insert in B) and HSL-/- HSLtes (insert in C) mice contained no or very few spermatozoa (arrowheads) but were filled with multinucleated early spermatids (fine arrows) and cell debris. In contrast, the epididymis of HSL-/- HSLtes mice (insert in D) were packed with numerous spermatozoa (arrowheads) and less early spermatids, among which many fewer multinucleated cells were present (fine arrows).

FIG. 4. Sperm reserve and number of pups according to paternal genotypes. Sperm reserves (n = 4–11) and pups per vaginal plugs (n = 9–30) were measured for mice with different genotypes. Values are the means ± S.E. p < 0.05 (a), p < 0.01 (b), and p < 0.001 (c) compared with the wild type values and p < 0.05 (d), p < 0.01 (e), and p < 0.001 (f) compared with the HSL-/- group values.

FIG. 5. Fertility assessment. Males with indicated genotypes were mated with B6D2/F1 female. Vaginal plugs were verified daily, and positive female were isolated. Born pups were counted.
mone have been reported in HSL-null mice (8). Consistent with a lack of influence on endocrine status, the libido of HSL-deficient male mice was normal, as shown by the production of a normal rate of copulatory plugs. Rescue of the infertility of HSL<sup>-/-</sup> male mice by expression of HSL<sub>tes</sub> shows that, among the various forms expressed in testis, it plays a predominant role in testicular physiology, as expression of HSL in cell types other than germ cells does not seem critical for a qualitatively normal spermatogenesis.

Studies in rodents and humans have shown that HSL<sub>tes</sub> mRNA is strongly expressed in early spermatids, whereas the protein accumulates in elongated spermatids (3, 7). This lag between mRNA and protein appearance is characteristic of many genes expressed in haploid germ cells. To evaluate the role of HSL<sub>tes</sub> in these defects, we used the HSL<sub>tes</sub> promoter to get a spatial and temporal transcription of the transgene similar to the endogenous gene. The sterility of HSL-deficient male mice associated with an alteration of the spermatogenetic process, and more specifically of spermiogenesis (spermatid differentiation) observed here, is in agreement with previous data on HSL<sup>-/-</sup> mice (23), whereas diacylglycerol lipase activity is markedly decreased. However, there is a substantial residual activity that suggests the existence of other lipases that hydrolyze diglycerides in testis. No relationship was found in the various transgenic lines between diacylglycerol lipase activity and fertility, and there was no difference in triglyceride and diglyceride contents between WT and HSL<sup>-/-</sup> mice. However, a relationship was found between CEH activity, cholesteryl ester level, and fertility. HSL-deficient testes have very low levels of CEH activity and show accumulation of cholesteryl esters as reported previously (8). The doubling of HSL<sub>tes</sub> mRNA levels and CEH activity observed in HSL<sup>-/-</sup> HSL<sub>tes</sub> mice compared with HSL<sup>-/-</sup> HSL<sub>tes</sub> mice is related to a doubling of cholesteryl ester content in the testis. A comparison of enzymatic activities with fertility data revealed that a threshold of esterase activity around 0.1 milliunit/mg protein is necessary to restore sperm reserves to levels sufficient to totally restore fertility.

The CEH activity mediated by HSL<sub>tes</sub> in haploid germ cells is therefore essential for spermiogenesis. HSL hydrolyzes various kinds of substrates including triglycerides, diglycerides, cholesteryl esters, and retinyl esters (1, 24). The role for retinoic acid has been illustrated in the dysfunction of spermatogenesis observed in vitamin A-deficient mice and mice with a targeted disruption of the retinoic acid nuclear receptor, RAR<sub>α</sub> (25, 26). However, the defects observed with impairment of retinoic acid production or signaling occur during the early stages of spermatogenesis. It is therefore likely that other proteins are involved besides HSL<sub>tes</sub>. The involvement of HSL<sub>tes</sub> in cholesterol metabolism may be crucial, as shown by CEH activity and testicular cholesteryl ester level data. During development of the guinea pig, there is a positive relationship between HSL expression in seminiferous tubules and the ratio of free to esterified cholesterol (19). Abnormal formation of membranous intercellular bridges between germ cells in HSL-null mice suggests a role for HSL in membrane stabilization and integrity (9). Cholesterol level determines membrane fluidity, and its distribution is highly organized in spermatid membranes (27, 28), which probably explains the presence of a great number of multinucleated early spermatids in the epididymis of the HSL<sup>-/-</sup> mouse. HSL<sub>tes</sub> may therefore control the subcellular deposition of cholesterol in specific membrane domains. The anchoring to membranes may be provided by the NH<sub>2</sub>-terminal domain, which is found only in HSL<sub>tes</sub>. This domain adds 301 amino acids to the protein expressed in adipose tissue (2). Its enrichment in proline may indicate an involvement in protein-protein interactions but this hypothesis has yet to be tested.

Taken together, our results indicate that male sterility in HSL-deficient mice is due to the lack of expression in spermatids of the testicular form, HSL<sub>tes</sub>, and suggests that it is mediated by impairment of CEH activity. The dramatic effect of a lack of HSL<sub>tes</sub> leads to the question of whether mutations in the HSL gene may be the cause of male infertility in humans. Moreover, the synthesis of specific HSL<sub>tes</sub>...
inhibitors may constitute a strategy for the development of a male contraceptive.

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