Species-specific alternative splicing generates a catalytically inactive form of human hormone-sensitive lipase

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Hormone-sensitive lipase (HSL) catalyses the rate-limiting step of adipose tissue lipolysis. The enzyme is also expressed in steroidogenic tissues, mammary gland, muscle tissues and macrophages. A novel HSL mRNA termed hHSL-S, 228 bp shorter than the full-length HSL mRNA, was detected in human adipocytes. hHSL-S mRNA results from the in-frame skipping of exon 6, which encodes the serine residue of the catalytic triad. The corresponding 80 kDa protein was identified in human adipocytes after immunoprecipitation. The truncated protein expressed in COS cells showed neither lipase nor esterase activity but was phosphorylated by cAMP-dependent protein kinase. hHSL-S mRNA was found in all human tissues expressing HSL,

except brown adipose tissue from newborns. It represented approx. 20% of total HSL transcripts in human subcutaneous adipocytes. No alternative splicing was detected in other mammals. Human and mouse three-exon HSL minigenes transfected into primate and rodent cell lines reproduced the splicing pattern of the endogenous HSL genes. Analysis of hybrid human/mouse minigenes transfected into human cell lines showed that *cis*-acting elements responsible for the skipping of human exon 6 were restricted to a 247 bp region including exon 6 and the first 19 nt of intron 6. Moreover, divergence in exonic splicing elements between mouse and human was shown to be critical for the species-specific alternative splicing.

INTRODUCTION

In mammals, long-term imbalances between energy intake and expenditure are translated into changes in the body's store of lipid. Hydrolysis of the triacylglycerol stored in adipose tissue is the major supply of energy through the release of non-esterified fatty acids into the plasma. The rate-limiting step of adipose tissue lipolysis is the hydrolysis of triacylglycerol into diacylglycerol by hormone-sensitive lipase (HSL). HSL is thus one of the enzymes determining whole-body fuel availability and it accounts for most of the detectable lipolysis in the post-absorptive state [1,2]. HSL is under acute neural and hormonal control. The enzyme is activated by catecholamines through phosphorylation by cAMP-dependent protein kinase. This phosphorylation is believed to occur on a single serine residue, called the regulatory site. The ability of insulin to antagonize hormone-induced lipolysis can to a large extent be explained by its ability to decrease cAMP levels via activation of phosphodiesterase 3B [3,4]. Another phosphorylation site adjacent to the regulatory site seems to be phosphorylated to a high extent in unstimulated adipocytes. Phosphorylation of this serine residue, called the basal site, does not directly affect enzyme activity [5]. Because phosphorylation of the two sites is mutually exclusive, it has been proposed that phosphorylation of the basal site has an anti-lipolytic role [6].

The human HSL gene is composed of nine exons encoding the adipose tissue enzyme [7]. Exon 6 encodes the serine residue of the catalytic triad, whereas the phosphorylation sites are encoded by exon 8. HSL has some unique features among mammalian lipases. The enzyme activity is controlled through reversible phosphorylation. HSL shows a relatively high catalytic activity at low temperature, a property that could be of importance in hibernating animals [7]. In addition, HSL has the same catalytic

activity towards cholesterol ester and triacylglycerol. The cholesterol ester hydrolase activity could be of importance in steroidogenic tissues and macrophages [8].

Although acute regulation of HSL activity through reversible phosphorylation is well documented for adipose tissue, few studies have been devoted to variations of HSL expression in human. Recently, blunted HSL expression and low lipolytic capacity in adipose tissue was reported in normal-weight subjects with a family tendency to obesity and in patients with familial combined hyperlipidaemia [9,10]. In view of the key role of HSL in energy metabolism and its possible involvement in disorders of lipid metabolism, we investigated mechanisms that control HSL gene expression.

Alternative splicing has an important role in the regulation of gene expression. We report here the identification of an alternatively spliced form of human HSL. In-frame skipping of exon 6 generates a catalytically inactive HSL called hHSL-S, which includes the phosphorylation site module. hHSL-S mRNA and protein were characterized. The relative abundance of hHSL-S and of the full-length transcript (hHSL-L) mRNA species was studied in different tissues from human and other mammalian species. The molecular basis of the alternative splicing was investigated with HSL minigenes transfected into primate and rodent cell lines.

MATERIALS AND METHODS

RNA analyses

Human fat biopsies and animal tissues were collected in accordance with INSERM's and Toulouse University Hospital's guidelines. Human and animal adipocytes were isolated as reported previously [11]. Cell lines were maintained by standard cell culture techniques. Total RNA was prepared by a single-step

Abbreviations used: HSL, hormone-sensitive lipase; RT-PCR, reverse transcription-PCR.

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guanidinium thiocyanate/phenol/chloroform extraction [12]. Total cytoplasmic RNA from HT29 cells was purified after non-ionic detergent lysis of plasma membranes and removal of nuclei with a standard procedure [13]. To eliminate residual genomic DNA, total and cytoplasmic RNA species were treated with amplification grade DNase I (Gibco-BRL). Poly(A)⁺ RNA was prepared from DNase I-treated total and cytoplasmic RNA species with a Dynabeads mRNA purification kit (Dyna). Poly(A)⁺ RNA species from other adult human tissues were from Clontech. Human brown-adipose-tissue cDNA was from a pool of biopsies from two newborns (gift from Dr. Daniel Ricquier, CNRS). Reverse transcriptase-PCR (RT-PCR) was started by treatment of poly(A)⁺ RNA and total RNA in the presence or absence of 200 units of Superscript reverse transcriptase (Gibco-BRL) in a buffer containing each dNTP at 0.5 mM, oligo(dT) (100 ng) and RNase inhibitor (2 units/ μ l; Pharmacia). cDNA was heated for 2 min at 94 °C and subjected to 30 or 35 cycles of amplification (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min) followed by 7 min of extension at 72 °C with *Thermus aquaticus* polymerase (Promega) in a thermal cycler (Biometra). Southern blotting experiments were performed with a standard procedure [13]. RNase protection analysis was performed as described earlier [11]. Briefly, a 1.3 kb human HSL cDNA clone [7] was digested with *Apa*I and *Eco*RI (present in the vector multiple cloning site). The 453 bp fragment, which comprises sequences corresponding to exon 6, exon 7 and the beginning of exon 8, was subcloned into pBluescript (Stratagene). The vector was linearized with *Bam*HI, and cRNA probe was produced with T3 RNA polymerase (Promega). Signals were quantified with digital imaging (Molecular Dynamics 445SI PhosphorImager).

Protein analyses

Human adipocyte 110000 g fat-depleted supernatants (HSL activity approx. 30 nmol of non-esterified fatty acid/min) and homogenates of COS-7 cells expressing hHSL-S (80 μ g of protein) and hHSL-L (HSL activity approx. 30 nmol of non-esterified fatty acid/min) were immunoprecipitated with affinity-purified polyclonal chicken antibody raised against recombinant rat HSL [14]. To minimize proteolytic activity, protease inhibitors were included as follows: 40 μ g/ml leupeptin, 4 μ g/ml antipain and 2 μ g/ml pepstatin. Aliquots were incubated for 2 h at room temperature with anti-HSL antibody, followed by incubation with immobilized anti-(chicken IgY) (Promega) for 1 h at 0 °C. Precipitates were washed five times with 1 ml of 10 mM Tris/HCl (pH 7.4)/0.9% NaCl, then subjected to SDS/PAGE and electroblotted to nitrocellulose membranes. Western blot analysis was performed with the enhanced chemiluminescence system (Amersham) with chicken anti-HSL as primary antibody and horseradish-peroxidase-conjugated anti-(chicken IgY) (Promega) as secondary antibody. For phosphorylation experiments, aliquots of 14000 g supernatants of transfected COS-7 cell homogenates were incubated with 5 mM MgCl₂/0.3 mM [γ -³²P]ATP (more than 3000 c.p.m./pmol of ATP)/1.2 mM dithioerythritol/0.5 μ M okadaic acid/0.2 unit/ μ l catalytic subunit of cAMP-dependent protein kinase (Sigma) for 30 min at 37 °C. In control experiments, cAMP-dependent protein kinase was omitted and the specific peptide inhibitor of cAMP-dependent protein kinase (Sigma) was included at 3 μ M. After the incubation period, EDTA was added to 10 mM and the samples were subjected to a two-cycle immunoprecipitation procedure [15]. After the first precipitation performed as above, the precipitates were denatured with SDS; after the addition of Triton X-100 the second immunoprecipitation was performed overnight at 4 °C. The final immunoprecipitates were subjected to SDS/PAGE and

analysed by digital imaging (Fujix BAS 2000). In some experiments the gels were subjected to Western blot analysis as described above. Mock-transfected cells were used as controls. Lipase and esterase activities were determined with 1(3)-mono-[³H]oleoyl-2-oleylglycerol and *p*-nitrophenolbutyrate respectively as substrates [7,16].

Constructs and transfection

To construct hHSL expression vectors, a full-length human HSL cDNA (hHSL-L) was obtained by screening a human fat-cell λ gt10 cDNA library (Clontech) with a 489 bp cDNA probe derived from exon 1. hHSL-L cDNA was subcloned into the expression vector pcDNA3 (InVitrogen). To obtain the hHSL-S expression vector, RT-PCR was performed on human adipocyte poly(A)⁺ RNA with primers derived from sequences in exons 1 (5'-GGACAACATAGCCTTCTTCTCGA-3') and 8 (5'-GTCTCAGCTGACAGCGACATCT-3'). The low-molecular-mass PCR product was purified and cut with *Sfi*I (site located in exon 5) and *Aat*II (site located in exon 8). hHSL-S cDNA was created by ligating the digested PCR product into an *Sfi*I and *Aat*II-cut pBluescript containing hHSL-L cDNA. hHSL-S cDNA was released with *Bam*HI and *Eco*RV present in the polylinker of pBluescript and cloned into *Bam*HI and *Eco*RV-cut pcDNA3. Identity with hHSL cDNA sequence was checked by dideoxy sequencing. To construct mouse and human HSL minigenes, the region between exons 5 and 7 was amplified from 3T3-F442A cell genomic DNA and human cosmid clone III1 [7] respectively. DNA was heated for 2 min at 94 °C and subjected to 35 cycles of amplification (94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min) followed by 7 min of extension at 72 °C with high-fidelity *pfu* DNA polymerase (Stratagene) and a primer derived from exon 5 (5'-TCTCCATCGACTACTCCCTGGCC-3') and a T7 primer (5'-TAATACGACTCACTATAGGG-3') in a thermal cycler (Biometra). PCR products were purified and subcloned into the *Srf*I site of pCRScript (Stratagene). Human and mouse HSL constructs were inserted in the sense orientation into the expression vector pSVSport (Gibco-BRL). Hybrid human/mouse HSL minigenes were constructed from human and mouse minigenes by a two-step PCR overlap extension method [17] and 40-mer hybrid primers composed of human and mouse sequences or primers, which sequences were identical between mouse and human. The same technique was used to generate a human minigene with a 475 bp deletion within intron 6. The 40-mer gene-specific primers were identical with target sequences on both edges of the deleted sequence. The establishment of the identity of all constructs with published sequences and the determination of intronic sequences of the human and mouse HSL genes [7,18] were performed by automatic DNA sequencing (Applied Biosystems 373A). Introns 5 and 6 of the rat HSL gene were amplified from genomic DNA with a mix of 98% *Taq* DNA polymerase (Appligene) and 2% *pwo* DNA polymerase (Boehringer-Mannheim). Two products from independent amplifications were sequenced. Splice site predictions were performed with the HSPL program (Baylor College of Medicine Search Launcher) [19], PC gene software (Intelligenetics) [20] and neural network [21]. Cells were transfected with the calcium phosphate co-precipitation technique or the lipofectin (Gibco-BRL) procedure. RNA was extracted 48 h (calcium phosphate technique) or 72 h (lipofectin technique) after transfection. For cells transfected with expression vectors, protein analyses were performed on cells harvested 72 h after transfection. For minigene analyses, cDNA was synthesized in a final volume of 20 μ l from 2 μ g of DNase I-treated total RNA as described in the RNA analyses section. A 50 μ l PCR mixture contained 10 mM Tris/HCl,

pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, each dNTP at 200 μ M, each primer at 0.2 μ M and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer). PCR cycle conditions performed in a GeneAmp 2400 PCR system (Perkin Elmer) were as follows: denaturation for 2 min at 94 °C, followed by 25 to 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The number of cycles was optimized for each construct and transfection experiment so as to remain in the exponential range of amplification. A 10 μ l sample of each reaction mixture was run on a 1.5% (w/v) agarose gel and detected after staining with ethidium bromide by using a low-light-level camera and a gel documentation system 5000 (UVP). Southern blotting was used to confirm the results obtained with the ethidium bromide-stained gel by standard procedures [13].

RESULTS

Characterization of hHSL-S mRNA and protein

RT-PCR amplification of human adipocyte poly(A)⁺ and total RNA was performed with various combinations of primers derived from sequences in exons 1 to 9. Products of sizes consistent with the exonic structure of the gene were obtained. However, a second shorter fragment was consistently observed when the sequence to be amplified included exon 6. The difference in size was approx. 230 bp, i.e. the size of exon 6 (228 bp). Sequencing of PCR fragments derived from three different individuals showed an exclusion of exon 6 from the short transcript. No frameshift occurred because the glycine residue encoded by the 3' end of exon 6 and the 5' end of exon 7 is reconstituted between exons 5 and 7.

To show that the alternatively spliced HSL was not an artifact and was present in the fully processed mRNA fraction, RT-PCR was performed on RNA from the colon cancer cell line HT29, which has been shown to express human HSL [22]. The ratio between the full-length transcript and the alternatively spliced form was similar in total RNA, poly(A)⁺ RNA and cytosolic poly(A)⁺ RNA (results not shown). Furthermore hHSL-S mRNA was detected by a RNase protection assay, showing that the short transcript was not an artifact of reverse transcription owing to mRNA secondary structure and that it was relatively abundant in human adipocytes (Figure 1). We next searched for a novel HSL protein in human adipocytes. Fat-depleted super-

Figure 1 RNase protection analysis of hHSL transcripts

The hHSL cRNA probe corresponded to sequences starting 17 nt from the beginning of exon 6 to 50 nt in exon 8. The overall length of the undigested probe was 494 nt (438 bp from hHSL mRNA and 56 nt from the vector). The probe was uniformly labelled with [³²P]UTP. RNA samples were digested with a mixture of RNase A and RNase T1 (Promega), and resistant hybrids were separated on a 5% (w/v) polyacrylamide/7 M urea gel. The protected bands corresponding to hHSL-L and hHSL-S transcripts were 438 nt long and 210 nt long respectively. Protected products are shown for synthetic RNA (lane +); 10 μ g of yeast tRNA (lane -); 30 μ g of total RNA from COS cells transfected with hHSL-L expression vector (lanes 1 and 2); or with hHSL-S expression vector (lanes 3 and 4) and 30 μ g of total RNA from human adipocytes (lane 5). Densitometric analysis of the hHSL-L and hHSL-S bands in lane 5 showed that hHSL-S mRNA represented 40% of total HSL transcripts expressed in human adipocytes of this subject.

Figure 2 Detection of hHSL-related proteins

Human adipocyte infranatants (lipase activity approx. 30 nmol of non-esterified fatty acid/min) from four individuals (lanes 1–4) and COS cell homogenates were subjected to immunoprecipitation with an anti-HSL antibody. The immunoprecipitates were analysed by SDS/PAGE [8% (w/v) gel] followed by Western blot analysis with the enhanced chemiluminescence system (Amersham), with chicken anti-HSL as primary antibody and horseradish peroxidase-conjugated anti-chicken IgY as secondary antibody. COS cells were transfected with hHSL-L expression vector (lanes 5 and 6; lipase activity approx. 30 nmol of non-esterified fatty acid/min), hHSL-S expression vector (lanes 7 and 8; 80 μ g of protein) or the vector alone (lanes 9 and 10; 80 μ g of protein). The prestained molecular mass markers (Sigma) were α_2 -macroglobulin (193 kDa), β -galactosidase (112 kDa), fructose-6-phosphate kinase (86 kDa), pyruvate kinase (70 kDa), fumarate (56 kDa), lactic dehydrogenase (39 kDa) and triose phosphate isomerase (36 kDa). Recombinant rat HSL (84 kDa) was also used as a molecular mass standard.

Figure 3 Phosphorylation of hHSL-L and hHSL-S by cAMP-dependent protein kinase

Expression vectors for hHSL-L (lane 1) and hHSL-S (lanes 2 and 3) were transfected into COS cells. An aliquot of cell homogenate (approx. 1 mg of protein) was incubated with the catalytic subunit of cAMP-dependent protein kinase (0.2 unit/ μ l) and 0.3 mM [γ -³²P]ATP (lanes 1 and 2) or with the cAMP-dependent protein kinase peptide inhibitor (2 μ M) and 0.3 mM [γ -³²P]ATP (lane 3) as described in the Materials and methods section. After incubation, homogenates were subjected to immunoprecipitation with an anti-HSL antibody; immunoprecipitates were analysed by SDS/PAGE [8% (w/v) gel] and digital imaging.

natants were subjected to immunoprecipitation with an antibody directed against recombinant rat HSL. Western blot analysis identified two HSL-related proteins (Figure 2). The major band had a molecular mass of 88 kDa, corresponding to the full-length protein. A second form of 80 kDa was detected. The 8 kDa difference is consistent with the lack of the 76 residues encoded by exon 6. Moreover, an 80 kDa protein was also detected in extracts of COS cells transfected with an expression vector for hHSL-S (Figure 2).

Functional analysis of hHSL-S

hHSL-L and hHSL-S expression was studied in COS cells. mRNA and proteins corresponding to both forms were produced (Figures 1 and 2). In cells expressing hHSL-S and in cells transfected with the empty vector, lipase [2.4 ± 0.9 (mean $\pm$ S.D.) compared with 2.8 ± 0.9 nmol of non-esterified fatty acid/min per mg of protein; $n = 5$] and esterase (42 ± 3 compared with 39 ± 2 nmol of *p*-nitrophenol/min per mg of protein; $n = 3$) activities were not different. In cells expressing hHSL-L, lipase and esterase activities were 23 ± 1 nmol of non-esterified fatty acid/min per mg of protein ($n = 5$) and 203 ± 12 nmol *p*-nitrophenol/min per mg of protein ($n = 3$) respectively. Phosphorylation experiments showed that hHSL-S was phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase (Figure 3). No band was detected in mock-transfected cells. By a comparison of Western

Figure 4 Tissue distribution of the hHSL-L and hHSL-S mRNA species in man and expression of HSL transcripts in various species

(A) Poly(A)⁺ RNA (500 ng) from various human tissues and 2 µg of human white-adipocyte total RNA were subjected to reverse transcription. Half of the cDNA pool was subjected to PCR with primers derived from sequences located in exons 3 and 7. Samples (20 µl) of the 50 µl PCR reactions were fractionated in a 2% (w/v) agarose gel. Products corresponding to hHSL-L and hHSL-S mRNA species are 886 and 658 bp long respectively. A negative of an ethidium bromide-stained gel is shown. Abbreviations: Test., testis; Adr., adrenal glands; M.G., mammary gland; Plac., placenta; Musc., skeletal muscle; W.A.T., white adipose tissue; B.A.T., brown adipose tissue. (B) Total RNA (2 µg) from various tissues was subjected to reverse transcription. Half of the cDNA pool was subjected to PCR with primers derived from sequences located in exons 3 and 7. Samples (20 µl) of the 50 µl PCR reactions were fractionated in a 2% (w/v) agarose gel. Products corresponding to hHSL-L and hHSL-S mRNA species are 886 and 658 bp long respectively. A negative of an ethidium bromide-stained gel is shown. Abbreviations: W.A.T., white adipose tissue; Test., testis; 3T3, 3T3-F442A mouse adipocytes; Hum., human; Rab., rabbit.

blots with the phosphoimage analysis, the stoichiometries of the phosphorylations of HSL-L and HSL-S were found to be of the same order of magnitude. Thus hHSL-S is a catalytically inactive phosphorylatable protein.

Tissue distribution of hHSL-S

To analyse the tissue distribution of the mRNA species encoding the two isoforms, poly(A)⁺ RNA from human tissues was subjected to RT-PCR. Two PCR fragments corresponding to hHSL-L and hHSL-S were detected in tissues that have been shown to express HSL in the rat with the noticeable exception of brown adipose tissue of newborn infants, which expressed only hHSL-L (Figure 4A). The identity of the amplified products was checked by Southern blot analysis with a human HSL cDNA probe. For human adipose tissue samples, the quantity of total RNA available allowed the use of the quantitative RNase protection assay. In one subject, hHSL-S transcripts represented 40% of total hHSL transcripts (Figure 1). Analysis of subcutaneous adipocytes of 24 women showed that hHSL-S transcripts represented 19 ± 10% of total HSL transcripts (range 8–40%). The presence of hHSL-S mRNA was next studied in tissues from various mammals. Two primers derived from sequences in exons 3 (sense primer) and 5 (anti-sense primer) were selected on the basis of a total identity between human, rat and mouse HSL cDNA sequences [7,8,18]. RT-PCR experiments showed no band corresponding to hHSL-S in species other than human (Figure 4B).

Species-specific splicing of HSL minigene pre-mRNA species

The molecular basis of species-specific alternative splicing is poorly understood. An analysis of all the splice sites of the human HSL gene with three computer packages revealed that the acceptor site of intron 5 was the weakest splice site predicted by the programs (Figure 5). In the mouse HSL gene the 3' splice site of intron 5 was as weak as its human counterpart, suggesting that other factors account for the difference in splicing between the two species. Therefore we wished to determine the importance of the flanking sequences and of the cellular environment in the regulation of exon 6 splicing. Three-exon human and mouse HSL minigenes were used to study exon 6 splicing (Figure 6). After transfection of the minigenes into various cell types, cDNA species were amplified with primers derived from sequences located in exons 5 and 7 and a primer from an adjacent vector

hHSL	ceagttctec	cagettgagt	tectecctgc	cctcctccgc	aggcctctgc	cctctgccagg	ttgtcaccac	ccaccctctc	cccaaacagg	90
rHSL	gcattctcgt	tgatcttggg	ac-ttgcaga	cctcagct--	gagagcccag	ttct-cctgc	tcattg--g	cctctctctc	ccacaactag	83
mHSL	gcattctcgt	tggtctttaga	at-tt-cgta	cctcactc--	gagagcctgc	tcgtgccagg	ctatgactgg	gctctctctc	ccacaactag	86
	* * *	*	* * *	**** *	* * *	* * *	* *	* * *	* * *	
hHSL	GCTCAACAGG	GGAACGAATC	TGCTTTCGG	GGGACAGTGC	AGGCGGGAAC	CTCTGCTTCA	TCGGGCAGCA	GCCTACGGGG		180
rHSL	GTTCAACCGG	AGAGCGGATA	TGCTTTCGG	GGGACAGCGC	AGGTGGGAAC	CTCTGCATCA	TCGGGCAGCA	GCCTATGGGG		173
mHSL	GTTCAACTGG	AGAGCGGATA	TGCTTTCAG	GGGACAGTGC	AGGTGGGAAT	CTCTGCATCA	TCGGGCAGCA	GCCTATGGAG		176
	* * * * *	* * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *		
hHSL	TGCGGGTGCC	AGATGGCATC	ATGGCAGCCT	ACCCGGCCAC	AATGCTGCAG	CCTGCCGCCT	CTCCCTCCCG	CCTGCTGAGC	CTCATGGACC	270
rHSL	TGAGGGTGCC	AGATGGCATC	ATGGCAGCCT	ACCCAGTTAC	CACCTCTGAG	TCCTCTGCTT	CTCCCTCTCG	TCTGCTGAGC	CTCATGGATC	263
mHSL	TGAGGGTGCC	AGATGGCATC	ATGGCAGCCT	ACCCAGTTAC	CACCTCTGAG	TCCTCTGCTT	CTCCCTCTCG	TCTGCTGAGC	CTCATGGACC	266
	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *	* * * * *		
hHSL	CCTTGCTGCC	CCTCAGTGTG	CTCTCCAAGT	GTGTGAGCGC	CTATGCTGgt	gaggcccaca	cctcagcag	ggaacacaga	ctgcattgat	360
rHSL	CTCTTCTACC	ACTGAGCGTA	CTCTCCAAGT	GTGTGAGCGC	CTATTCAGgt	aag-accaag	cctcag--	-----	-----	329
mHSL	CTCTTCTACC	ACTGAGCGTA	CTCTCCAAGT	GTGTGAGCGC	CTATTCAGgt	aaggtccatg	ccctcag--	-----	-----	333
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hHSL	gtctgactcc	tgccagg//intron 6 (420bp)	tttatcc	cttttgaagc	gattggatat	tctcgggtcc	ctggggaccac	agatgectga		852
rHSL	-----	-----	-----	-----	agtggtggcag	-ctggaggcc	tgcatgctcc	agatactcca		368
mHSL	-----	-----	-----	-----	agtatggcag	cggtaatgcc	tgcatgctcc	agatactcca		363
	* * *				* * *	* * *	* * *	* * *		
hHSL	agttctgggc	gttaaaactg	ggcccagaaa	ggggaaaaca	actcagctac	ccacatata	tctgcaggTG	CAAGACGGA	GGACCACTCC	942
rHSL	ag-tctgtgt	gttagaactg	ggcctaaaaa	-gagacacgc	acttggcctc	ccacttgcat	tctgcaggGA	CAGAGACGA	GGACCAATTT	456
mHSL	ag-tctgtgt	gttagaactg	ggcctaaaaa	agagagacaa	actcagcctc	ccacatcggt	tctgcaggGA	CAGAGGAGA	GGACCAATTT	452
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hHSL	AACTCAGACC	AGAAGCCCT	CGGCATGATG	GGGCTGGTGC	GGCGGGACAC	AGCCCTGCTC	CTCCGAGACT	TCCGCTGGG	TGCTCTCTCA	1032
rHSL	GACTCAGACC	AGAAGCCATT	GGGCTGATG	GGGCTGGTGC	AGAGAGACAC	GTCCTGTGTC	CTCAGAGACC	TCCGCTGGG	CGCTCTCTCA	546
mHSL	GACTCAGACC	AGAAGCCACT	AGGCTGATG	GGGCTGGTGC	AGAGAGACAC	TTCGCTGTTC	CTCAGAGACC	TCCGCTGGG	TGCTCTCTCA	542
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Figure 5 Genomic sequence of the region containing exon 6 of human (h), rat (r) and mouse (m) HSL

The 3' region of intron 5, exon 6, intron 6 and the 5' region of exon 7 are shown. Lower-case letters indicate intronic sequences; capital letters indicate exonic sequences. The GT/AG consensus sequences for splice junctions are underlined.

Figure 6 Analysis of pre-mRNA splicing from human (H) and mouse (M) HSL minigenes in primate and rodent cells

(A) Schematic representation of the minigenes. Adjacent vector sequences (pSVSport; Gibco-BRL) are shown as black areas. Exons and introns are represented as open boxes and solid lines, respectively. The positions of the primers are represented by horizontal arrows. (B) Cells were transfected with 5 μ g of DNA; 2 μ g of total RNA was reverse-transcribed into cDNA. One-quarter of the cDNA pool was PCR amplified with a primer derived from sequence in exon 5 and a T7 primer. Samples (10 μ l) of the 50 μ l PCR reactions were fractionated in a 1.5% (w/v) agarose gel. Products corresponding to HSL-L and HSL-S mRNA species are 540 and 310 bp long respectively. A negative of an ethidium bromide-stained gel is shown. The species origins of the cell lines are as follows: HepG2 and HeLa, human; COS-7, monkey; 3T3-F442A and L929, mouse; CHO, hamster.

sequence. The use of the vector-derived primer made possible the distinction between minigene mRNA species and the low level of endogenous HSL gene transcription occurring in some cell lines (L929, 3T3-F442A and Chinese hamster ovary cells). The minigene pre-mRNA species were correctly spliced in all cell lines, indicating that the minigenes reproduced a splicing pattern identical with the endogenous HSL genes. A single mRNA species was obtained from the mouse HSL pre-mRNA, whereas two mRNA species with or without an exon 6 sequence were detected in cells transfected with the human HSL minigene. The identities of the PCR products were confirmed by Southern blot analysis with human and mouse HSL cDNA probes. The splicing pattern did not differ with the origin (rodent or primate) of the transfected cells. These results showed that *cis* elements are critical for the human-specific skipping of exon 6.

Because mouse (125 bp) and rat (122 bp) introns 6 are much shorter than their human counterpart (599 bp), it was hypothesized that *cis*-acting sequences within the human intron could be of importance to promote alternative splicing. Comparison of human, mouse and rat intron 6 sequences showed that the rodent introns were highly similar to the 5' and 3' regions of the human intron (Figure 5). The 472 bp insertion at position 20 in human intron 6 was composed of two monomers of a dinucleotide repeat and an *Alu*-like sequence. A human HSL minigene (Δ i6) lacking the entire human-specific sequence in intron 6 was transfected into HepG2 and HeLa cells (Figure 7). Two mRNA species were obtained, indicating that the minisatellite was not responsible for the alternative splicing.

To identify which sequences in the human HSL gene are involved in exon 6 skipping in a human cellular context, we generated hybrid constructs between the human and mouse HSL

Figure 7 Analysis of pre-mRNA splicing from a truncated human (Δ i6) and chimaeric human/mouse HSL minigenes

(A) Schematic representation of HSL minigenes composed of three exons (5, 6 and 7) and two introns (5 and 6). Vector sequences (pSVSport; Gibco-BRL) are not shown. Human and mouse sequences are represented as open and filled rectangles respectively; exons and introns are represented as wide and narrow rectangles respectively. The number of base pairs in each intronic segment is shown above each intron. The minigenes are named in accordance with the nomenclature described in the Results section. (B) Cells were transfected with 5 μ g of DNA into HeLa cells; 2 μ g of total RNA was reverse-transcribed into cDNA. One-quarter of the cDNA pool was PCR amplified with a primer derived from sequence in exon 5 and a T7 primer. Samples (10 μ l) of the 50 μ l PCR reactions were fractionated in a 1.5% (w/v) agarose gel. Products corresponding to HSL-L and HSL-S mRNA species are 540 and 310 bp long respectively. A negative of an ethidium bromide-stained gel is shown.

minigenes and transfected them into HeLa and HepG2 cells. Segments of the human minigene were replaced by analogous murine regions. This strategy was preferred to deletion-based analysis, which can produce artifacts caused by the disruption of *cis*-acting elements and the modification of RNA secondary structure. Most of the constructs were named by mouse sequence localization in the hybrid minigene (L and R for left or right of the central exon; see Figure 7) and chimaera junction position (i5, e6 and i6 for junctions in intron 5, exon 6 and intron 6, and i5/e6 and e6/i6 for junctions at the boundaries between intron 5 and exon 6 and between exon 6 and intron 6 respectively). In the e6H $\rightarrow$ M minigene, human exon 6 was replaced by mouse exon 6. Re6 and Le6 exhibited no exon skipping. Because the sequences of the primers used for these constructions were identical between mouse and human, there was no disruption of potential *cis*-acting sequences. The results therefore suggested that several intronic and/or exonic sequences contributed to the human-specific alternative splicing. Two mRNA species were detected in cells transfected with Li5 and Li5/e6, indicating that sequence

divergence in exon 5 and intron 5 between the mouse and human HSL genes was not responsible for the species-specific alternative splicing. The 3' region of intron 6 and exon 7 were not involved because Ri6 and Li5Ri6 showed exon skipping similar to the wild-type human HSL minigene. Divergence in the first 19 nt of intron 6 is of importance because no alternative splicing was observed in cells transfected with Re6/i6. Replacement of human exon 6 by mouse exon 6 (construct e6H → M) led to a loss of exon skipping, showing the existence of exon splicing elements involved in alternative splicing. Similar results were obtained in HepG2 cells (results not shown).

DISCUSSION

The present findings demonstrate the existence of a novel form of human HSL produced by alternative splicing of the pre-mRNA. Skipping of exon 6 generates a protein devoid of both lipase and esterase activity but containing the phosphorylation sites.

Alternative splicing of pre-mRNA is a general mechanism that provides a versatile means of regulating gene expression. It has been shown that alternative splicing often occurs in developmentally regulated and tissue-specific patterns. Whereas all adult tissues expressed the short transcript, hHSL-S mRNA is apparently not expressed in brown adipocytes from newborns. Moreover, studies of adipose tissue samples from different subjects revealed variable levels of hHSL-S transcripts. These results suggest a regulation of the alternative splicing that could provide a means for fine-tuning HSL expression and activity in adipose tissues. The amount of hHSL-L is thought to be the rate-limiting factor in adipocyte lipolysis [23,24]. Regulation of alternative splicing could have a direct consequence on the lipolytic capacity of adipose tissue through modulation of the ratio of hHSL-L mRNA to hHSL-S mRNA. In that respect, the decreased HSL activity levels in normal-weight subjects with a family tendency to obesity could be the result of an increase in the proportion of hHSL-S mRNA because the total level of hHSL mRNA determined by solution hybridization was not different from that of control patients [10]. In man, study of the molecular mechanisms underlying variations of HSL expression should now include the determination of the level of hHSL-S transcripts.

Alternative splicing can also serve as a way of producing variant protein isoforms with differing functions. A puzzling feature of this alternative splicing is the conservation of the open reading frame in hHSL-S, leading to a protein identical with hHSL-L in its N-terminal and C-terminal regions. The absence of enzymic activity of hHSL-S is expected because of the absence of Ser⁴²³, which has been shown by site-directed mutagenesis to be necessary for the catalytic activity of rat HSL [25]. More recently, the building of a three-dimensional model for the catalytic domain of HSL proposed that Ser⁴²³, together with Asp⁷⁰³ and His⁷³³ (numbering from rat HSL), form the catalytic triad of HSL [26]. In full agreement with this proposal, site-directed mutagenesis experiments showed that both Asp⁷⁰³ and His⁷³³ are essential for lipase and esterase activity [27]. The overall topology of the catalytic domain is an α/β -hydrolase fold, as shown for all lipases with a known three-dimensional structure. The core of the α/β -hydrolase fold is encoded by exons 5, 6 and 9 and the last residues of exon 8, whereas exon 7 and most of exon 8 encode an external module that contains the phosphorylation sites. Exon 6 encodes residues forming two β -strands and an intervening α -helix ($\alpha_{5,6}$) that are centrally located in the α/β -hydrolase fold. Removal of these structural elements is therefore likely to have major consequences on the overall structure of the protein. However, hHSL-S was produced in an

apparently stable form in both *in vitro* translation (results not shown) and cell transfection experiments. Moreover an immunoreactive protein corresponding to hHSL-S was detected in human adipocytes (Figure 2). The fact that the exon/intron boundaries 5/6 and 6/7 correspond to connections between, rather than within, structural elements probably allows proper folding into a stable protein. From the three-dimensional model one would presume that, in HSL-S, α -helix $\alpha_{4,5}^2$, which follows β -strand 4, connects directly to the first α -helix ($\alpha_{6,7}^1$) of the regulatory module.

The role of hHSL-S is quite elusive at the moment. Studies of the functional domains of lipases have shown that, in some cases, catalytic activity and lipid binding reside in different domains. We have recently proposed that the N-terminal domain of HSL, encoded by exons 1 to 4, either alone or through interaction with the catalytic domain, interacts with the lipid droplet [14]. The N-terminal domain is present in HSL-S, suggesting that the truncated protein could bind to lipid droplets and interfere with hHSL-L. Phosphorylation of HSL by cAMP-dependent protein kinase in rat adipocytes was shown to promote HSL translocation to the lipid droplet, where it hydrolyses triglycerides [28]. Because hHSL-S seems to be phosphorylated by cAMP-dependent protein kinase, it could compete with hHSL-L during lipolytic stimulation, altering hHSL-L access to the substrate. Alternatively one cannot exclude the possibility that hHSL-S has a new, still unknown, function.

Few examples of species-specific exon skipping have been reported. One such case is the alternative splicing of exon 4 in erythroid 5-aminolaevulinate synthase, an enzyme catalysing the rate-limiting step in haem biosynthesis, which is found in man but not in mouse and dog [29]. Species-specific exon inclusion has been reported for the gene encoding aggrecan, an abundant non-collagenous component of the extracellular matrix of cartilage [30]. To our knowledge, the molecular basis for species-specific alternative splicing has never been investigated. The contribution of several splicing signals was recently shown in the analysis of the class-specific (chicken compared with rat) splicing of an alternative exon of β -tropomyosin [31,32]. To achieve correct splicing, specific *cis* sequence elements at intron boundaries are required. The degree of similarity to the consensus sequences seems to be correlated with the affinity for associated splicing factors [33]. In that respect, the weak consensus sequence at the 3' end of intron 5 is likely to have a role in exon 6 skipping in the human HSL pre-mRNA. However, replacement of human intron 5 by its mouse counterpart did not abolish exon skipping, and sequencing of the 3' end of mouse HSL gene intron 5 showed, as in the human gene, a weak acceptor site. This suggested that either other differences in *cis*-acting elements between the two genes or splicing factors preferentially expressed in human tissues could promote exon 6 skipping. Transfection of human and mouse HSL minigenes in primate and rodent cells resulted in the maintenance of a species-specific mRNA processing pattern, showing that regulatory information was confined to *cis*-acting elements. Human intron 6 is much longer than its rat and mouse counterparts (Figure 5). An analysis of the additional sequence revealed that it was composed of a microsatellite and an *Alu* sequence. In some cases, retroposition of *Alu* repeats within the human sequence leads to discernable phenotypes [34]. Therefore we hypothesized that human intron 6 could contain specific *cis* sequence elements important for alternative splicing. Transfection of a truncated minigene (construct Δ i6; Figure 7) showed that the part of intron 6 specific to the human gene does not promote exon 6 skipping. These results do not support a role for *Alu* sequences in alternative human HSL pre-mRNA splicing.

Subsequent analysis of several human/mouse chimaeric mini-genes showed that sequences within a 247 nt region encompassing exon 6 and the 5' splice site of intron 6 have a role in the human-specific exon skipping (Figure 7). Intron 5 and most of intron 6 including the 3' splice site do not contribute to the alternative splicing. The results are in agreement with an exonic perspective of splice site recognition [35]. The exon definition model postulates that the exon is recognized as an entity, and this requires coupled definition of the 5' and 3' splice sites. A role for the exon sequence in splice site selection has been documented for a number of genes. Exon splicing elements frequently lie within alternatively spliced exons, where they can act as activating [36] or inhibitory [37] sequences. Splicing factors that belong to the serine/arginine-rich (SR) protein family bind to exon sequences and make protein-protein contacts with the protein complex U2AF bound near the 3' splice site and U1 small nuclear ribonucleoproteins bound to the 5' splice site [35,38]. Replacement of the human by the mouse exon (construct e6H → M; Figure 6) abolished exon skipping, demonstrating the role of exon splicing elements in the alternative splicing of human HSL pre-mRNA. As shown in Figure 5, 34 nt are specific to human exon 6 when compared with the mouse and rat sequences. In alternative splicing, one or two mutations within exon sequences can alter full recognition of the exon [39]. In our model it is likely that the exon 6 sequence, along with suboptimal splice sites (especially at the 3' end of intron 5) and divergence in the first 19 nt of intron 6, mediate the species-specific difference in exon skipping. The results establish a system that will be useful in deciphering the molecular basis of species-specific alternative splicing and in characterizing exon sequences involved in splice site selection.

In conclusion, we have described a novel product of the HSL gene characterized by species- and tissue-specific expression. Our results show that the exon 6 sequence contributes to the human-specific alternative splicing and provide a new model for the study of splice site selection. The alternative splicing generates, in human tissues, a short transcript that is translated into a catalytically inactive but phosphorylatable protein. Variation of the level of the short transcript could represent a mechanism of regulation with the potential to affect the lipolytic capacity of human fat cells.

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