Characterization of the promoter of human adipocyte hormone-sensitive lipase

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Hormone-sensitive lipase (HSL) catalyses the rate-limiting step of adipose tissue lipolysis. The human HSL gene is composed of nine exons encoding the adipocyte form and a testis-specific coding exon. Northern blot analyses showed that human adipocytes express a 2.8 kb HSL mRNA, suggesting the presence of a short (20–150 bp) 5′ untranslated region (5′-UTR). A single 5′-UTR of approx. 70 nt was detected in RNase H mapping experiments. Two 5′-UTRs of 70 and 170 nt respectively were obtained by rapid amplification of cDNA ends and cDNA library screenings. RNase protection experiments, with probes derived from the two products, showed that human adipocyte HSL mRNA contains only the 70 nt product. Primer extension analysis mapped the transcriptional start site 74 nt upstream of the start codon. In HT29, a human cell line expressing HSL, the presence of the short or the long 5′-UTR is mutually exclusive. The short and long 5′-UTR exons were located 1.5 and approx. 13 kb respectively upstream of the first coding exon. Various portions of the 5′-flanking region upstream of the short product exon were linked to the luciferase gene and transfected into cells that express HSL (HT29 cells and rat adipocytes) and do not express HSL (HeLa cells). High luciferase activity was found for constructs containing the sequence between nt −2400 and −86, but not for shorter constructs. An analysis of 14 kb of genomic sequence revealed the presence of five DNase I hypersensitive sites associated with active gene transcription. Three of the sites are located in the vicinity of the transcriptional start site and could be linked to the minimal promoter activity. Two of the sites are located downstream of the exon containing the start codon, suggesting the presence of intronic regulatory elements.

INTRODUCTION

In mammals, hydrolysis of the triacylglycerol stored in adipose tissue is the major supply of energy through the release of free 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 cAMP-dependent phosphorylation, whereas insulin prevents this phosphorylation through the hydrolysis of cAMP.

As well as being expressed in adipose tissue, HSL is also expressed in steroidogenic tissues, mammary gland and muscle tissues [3–5]. Two isoforms of human HSL have been characterized. Human adipocytes express an 88 kDa form, whereas human testes express a 120 kDa form [5,6]. On comparison with rat HSL mRNA, a 3.3 kb human HSL mRNA was found in adipose tissue [7]. Subsequently, a long 5′ untranslated region (5′-UTR) was characterized in human adipocytes [5]. We deduced from these results that the full-length human adipocyte HSL cDNA was 3250 nt long. The 120 kDa HSL protein in human testis is encoded by a 3.9 kb mRNA [6]. Analysis of coding sequences revealed that adipocyte and testis HSL are 775 and 1076 residues long respectively. The difference in size is due to a testis-specific N-terminal region. Elucidation of the HSL gene organization showed that nine coding exons are common to both forms [5,6]. The additional sequence in testis HSL is encoded by a 1.2 kb exon located 16 kb upstream of the first exon encoding the adipocyte form. The complex organization of the gene suggested the existence of several tissue-specific promoters.

The amount of HSL is considered to be the rate-limiting factor in adipocyte lipolysis. Any variation in HSL expression will therefore modulate the extent of adipose tissue lipid mobilization. Although acute regulation of HSL activity through reversible phosphorylation is well documented in adipose tissue, few studies have been devoted to variations in HSL expression in man. 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 [8,9]. Adaptation to a new physiological state can also modify HSL expression. Prolonged calorie restriction leads to an increase in adipocyte lipolysis and in HSL activity and protein levels [10]. Weight loss or calorie restriction causes a decrease in basal sympathetic activity [11,12]. A role for changes of sympathetic tone in the modulation of HSL expression was proposed [10]. This hypothesis is supported by the inhibitory action of cAMP on HSL gene expression observed in 3T3-F442A and BFC-1 adipocytes [13].

In view of the key role of HSL in energy metabolism and variations in HSL expression seen in physiological and pathological states, it is important to acquire detailed knowledge about the mechanisms regulating human HSL gene transcription. We

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; HSL, hormone-sensitive lipase; HSS, DNase I hypersensitive sites; RACE, rapid amplification of cDNA ends; 5′-UTR, 5′ untranslated region.

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have investigated HSL expression in human adipocytes and a colon cancer cell line, HT29, that has been shown to express human HSL [14]. The size and relative abundance of HSL mRNA 5′-UTRs were determined by Northern blot analysis, RNase H mapping, RNase protection assay and various cDNA cloning protocols. Two novel exons corresponding to 5′-UTRs were characterized. The adipocyte-specific transcriptional start site was mapped by primer extension analysis, and the promoter activity was analysed in HT29 cells, rat adipocytes and HeLa cells by using a sensitive reporter gene assay. Finally, DNase I hypersensitive sites (HSS) associated with active gene transcription were mapped in the human HSL gene.

MATERIALS AND METHODS

RNA preparation

Total RNA was prepared from isolated human adipocytes or HT29 cells by using a single-step guanidium thiocyanate/phenol/chloroform extraction [15]. Poly(A)$^+$ RNA was prepared from total RNA with a Dynabeads mRNA purification kit (Dynal). Briefly, 100 μg of total RNA was heated at 65 °C for 5 min, hybridized to $(dT)_{25}$-linked magnetic beads at room temperature for 10 min, cleaned twice with washing solution [10 mM Tris/HCl (pH 7.5)/0.15 M LiCl/1 mM EDTA] and eluted with 20 μl of 2 mM EDTA at 65 °C. Poly(A)$^+$ RNA was used immediately.

Northern blot analyses

Human adipocyte total RNA and poly(A)$^+$ RNA, RNA transcribed *in vitro* from human HSL cDNA (2888 and 2660 bp) and RNA size markers obtained by transcription *in vitro* of different fragments of λ DNA (Promega) were electrophoresed in a 1% (w/v) agarose/2.2 M formaldehyde gel, transferred to nylon membrane (Hybond N; Amersham) and cross-linked with UV [16]. The blots were hybridized for 16 h at 65 °C in 0.5 M Na_2HPO_4/1 mM EDTA/7% (w/v) SDS/1% (w/v) BSA with a 585 bp human HSL cDNA probe (nt 101–685 downstream of ATG) generated by PCR with the oligonucleotide 5′-AGGCGTTTTTGCCGGTGTAC-3′ as the sense primer and 5′-TGTGATCCGCTCAAACTCAG-3′ as the anti-sense primer. RNA size markers were hybridized with ^{32}P-labelled λ DNA/*Hin*dIII fragments. Blots were washed at a final stringency of 15 mM NaCl/1.5 mM citric acid/0.1% SDS at 65 °C and subjected to digital imaging (Molecular Dynamics).

RNase H mapping

Human adipocyte total RNA (30 μg) was freeze-dried and resuspended in 10 μl of RNase H buffer [20 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/100 mM KCl/0.1 mM dithiothreitol/5% (w/v) sucrose] containing 10 pmol of the single-stranded anti-sense oligonucleotide 5′-GTACACCGGCAAAAACGCCTGACAG-3′ (nt 120–96 downstream of ATG). After 10 min at 70 °C, hybridization was performed for 30 min at 37 °C. Then 40 μl of RNase H buffer containing 7 units of RNase H (Amersham) were added and digestion was performed for 45 min [17]. After precipitation with ethanol, the digestion products were separated on a polyacrylamide/urea gel. The gel was washed twice in 7% (v/v) formaldehyde/9 mM Tris/borate/0.2 mM EDTA and RNA was passively transferred to a nylon membrane. Hybridization was performed as described above with ^{32}P-labelled oligonucleotide (nt 63–38 downstream of ATG) and cDNA probe (the most 5′ 50 nt of product B; see below).

cDNA library screening

Two human fat-cell cDNA libraries (Clontech) were screened with the ^{32}P-labelled 585 bp human HSL cDNA probe. Hybridization was performed under standard conditions [5,16]. Stringent washes were performed at 60 °C in 30 mM NaCl/3 mM citric acid/0.1% SDS. Positive clones were subcloned into pBluescript KS (Stratagene) and sequenced by dideoxy sequencing.

Rapid amplification of cDNA ends (RACE)

The Marathon RACE kit (Clontech) was used to amplify the 5′ ends of human HSL cDNA species in accordance with the manufacturer's protocol. Briefly, 500 ng of poly(A)$^+$ RNA from human adipocytes and HT29 cells were reverse-transcribed with oligo(dT). After synthesis of the complementary strand, a modified anchor primer was ligated to the 5′ end of the double-stranded cDNA with T4 DNA ligase. Amplification of the 5′ ends was performed with a sense primer derived from the anchor primer and a gene-specific anti-sense primer (nt 120–96 downstream of ATG). RACE products were subcloned into pBluescript KS and sequenced by dideoxy sequencing.

RNase protection assay

Two RACE products were used as probes. Probes A and B contained 170 and 70 bp respectively of the 5′ UTR. Anti-sense RNA species produced with T7 RNA polymerase for probe A and T3 RNA polymerase for probe B were labelled by incorporating [^{32}P]UTP into the reaction mixture [18]. The DNA template was eliminated by RQ1 DNase (Promega) digestion for 15 min at 37 °C. The quality and integrity of labelled probes were checked on a 5% (w/v) polyacrylamide/7 M urea gel. Hybridizations were performed by mixing the labelled cRNA probe in 30 μl of hybridization buffer [80% deionized formamide/0.4 M NaCl/1 mM EDTA/40 mM Pipes (pH 6.7)] with the appropriate RNA (30 μg of human adipocyte RNA or 200 μg of HT29 RNA). The mixture was heated to 95 °C for 5 min and transferred immediately to a 55 °C heating block for 14–18 h. Thereafter 0.3 ml of 300 mM NaCl/5 mM EDTA/10 mM Tris/HCl (pH 7.5) containing 20 μg of RNase A and 1 μg of RNase T1 were added. After 1 h at 37 °C, digestion was stopped by the addition of 50 μg of proteinase K, and the samples were incubated for a further 15 min at 37 °C. Guanidinium thiocyanate (4 M, 1 vol.)/25 mM sodium acetate (pH 7)/0.5% sarcosyl/0.1 M β-mercaptoethanol and 10 μg of yeast tRNA were added, followed by precipitation with propan-2-ol. The RNA pellets were washed with 70% (v/v) ethanol, dried, dissolved in 10 μl of sample buffer [97% (v/v) deionized formamide/0.1% SDS/10 mM Tris/HCl (pH 7.0)], and loaded on a 5% (w/v) polyacrylamide/7 M urea gel. The gel was analysed by digital imaging (Molecular Dynamics).

Primer extension analysis

A 24-mer oligonucleotide 5′-CTCCTAGGCATCTTCCGAGCTTCC-3′ complementary to nt 29–42 in exon B (see the Results section) was labelled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The ^{32}P-labelled primer was hybridized with 500 ng of human adipocyte poly(A)$^+$ RNA in 150 mM KCl/10 mM Tris/HCl (pH 8.3)/1 mM EDTA for 90 min at 65 °C and cooled slowly to room temperature [16]. The hybridized primer–RNA complex was extended in a 45 μl volume containing 20 mM Tris/HCl, pH 8.3, 10 mM $MgCl_2$, 5.5 mM dithiothreitol, 0.15 mM dNTP, 50 units of RNase inhibitor (RNA Guard; Pharmacia) and 200 units of reverse transcriptase (Superscript II; Gibco-BRL) for 90 min at 42 °C. The reaction was stopped

by adding 105 μl of RNase mix (10 mM Tris/HCl (pH 7.5)/1 mM EDTA/100 mM NaCl containing 20 μg/ml RNase A and 100 μg/ml salmon sperm DNA) for 15 min at 37 °C. After extraction with phenol/chloroform (v/v) and precipitation with ethanol, the extension products were separated on a 6 % (w/v) polyacrylamide/8 M urea sequencing gel and detected by autoradiography. The corresponding HSL genomic fragment was sequenced with the same primer and run in parallel.

Plasmid constructions, cell transfection and luciferase analyses

A 2.6 kb DNA genomic fragment containing the transcription start site was subcloned and used as a template in the PCR-based generation of different deletions of the promoter region. PCR reactions were performed with a proof-reading DNA polymerase (*Pfu* DNA polymerase; Stratagene) and a common anti-sense primer that ended 38 bp downstream of the transcription start site. Nine fragments (−2400, −1000, −431, −367, −290, −187, −86, −57 and −31 bp) were cloned in a sense orientation upstream of the firefly luciferase-coding gene into the pGL3-basic vector (Promega). Two fragments (+1000 and +431 bp) were cloned in the anti-sense orientation. Transfections into HT29 cells were performed with cationic lipids (TFX-50; Promega) and 2 μg of each construct in 1.9 cm² plates. Transfection efficiencies were normalized by co-transfecting the different constructs with 20 ng of pRL-CMV vector containing the cDNA encoding *Renilia* luciferase (Promega). HeLa cells in 9.4 cm² plates were transfected with lipofectin (Gibco-BRL) plus 2 μg of each construct and 100 ng of pRL-CMV vector. HT29 and HeLa cells were harvested 48 h after transfection for the determination of luciferase activity. Isolated rat adipocytes were prepared by collagenase digestion of epididymal fat pads [18]. Cells were washed three times in Dulbecco's modified Eagle's medium (DMEM). Packed cells (0.2 ml) supplemented with 0.2 ml of DMEM were distributed in electroporation cuvettes with a 0.4 cm gap (Eurogentec) containing 20 μg of each construct and 1 μg of pRL-CMV vector. After preliminary experiments to determine optimal transfection conditions, adipocyte electroporation [19] was performed at 200 V and 900 μF (Celljet; Eurogentec). Cells were immediately transferred to 5 ml tubes containing 1.5 ml of DMEM supplemented with 10 % (v/v) fetal calf serum (Gibco-BRL) and incubated at 37 °C for 24 h. Firefly and *Renilia* luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Luminoskan; Labsystem).

Nuclei preparation and digestion with DNase I

Nuclei preparation was done by the method of Richard-Foy and Hager [20], with modifications. HT29 and Caco2 cells were grown in DMEM supplemented with 5 % (v/v) fetal calf serum (HT29) or 10 % (v/v) fetal calf serum (Caco2). Cells were grown to confluence, washed with PBS, scraped with a rubber 'policeman' in PBS and centrifuged for 5 min at 1700 ***g*** at 4 °C. The cell pellet was resuspended in homogenization buffer [10 mM Tris/HCl (pH 7.4)/15 mM NaCl/60 mM KCl/0.15 mM spermine/0.5 mM spermidine/1 mM EDTA/0.1 mM EGTA/0.2 % Nonidet P40/5 % (w/v) sucrose] and homogenized in a Dounce homogenizer (ten strokes with pestle A). The homogenate was centrifuged for 20 min at 1600 ***g*** through a 10 % (w/v) sucrose cushion in homogenization buffer. The pellets were resuspended in washing buffer [10 mM Tris/HCl (pH 7.4)/15 mM NaCl/60 mM KCl/0.15 mM spermine/0.5 mM spermidine], pelleted again and pooled in 3 ml of washing buffer. The amount of DNA in the nuclear suspension was estimated by measuring the absorbance at 260 nm of a fraction of the nuclear suspension lysed in 1 % (w/v) SDS. The integrity of nuclei was checked by Trypan Blue coloration. The cold nuclear suspension was divided into 1 ml fractions, adjusted with 10 μl of 50 mM $CaCl_2$ and 20 μl of 50 mM $MgCl_2$. The reaction was initiated by additions of various amounts of 1 mg/ml bovine pancreatic DNase I (approx. 2000 units/mg; Worthington). The tubes were incubated for 10 min at 20 °C. The reaction was stopped by addition of 1 ml of stop buffer [25 mM EDTA/2 % (w/v) SDS/200 μg/ml proteinase K] and incubated overnight at 37 °C. The samples were first extracted with phenol, then with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and finally three times with chloroform/3-methylbutan-1-ol (24:1, v/v) and precipitated with ethanol. Pellets were resuspended in 10 mM Tris/HCl (pH 7.4)/2.5 mM EDTA. For each DNase I digestion point, 40 μg of purified DNA was digested to completion with *Eco*RI or *Xho*I (5 units/μg of DNA). Samples were precipitated in the presence of 0.01 vol. of tRNA (10 mg/ml), resuspended in 10 mM Tris/HCl (pH 7.4)/1 mM EDTA, and electrophoresed on a 0.8 % agarose gel for 12–16 h. The DNA was transferred passively to nylon membrane (Hybond N; Amersham). Probes (20–30 ng) were labelled with [^{32}P]dCTP (3000 Ci/mmol) with the use of a random priming kit (Pharmacia). Hybridization was performed under standard conditions [16]. Blots were washed at a final stringency of 30 mM NaCl/3 mM citric acid/0.1 % SDS. Blots were autoradiographed for 2–7 days.

RESULTS

Size of HSL mRNA and 5′ non-coding region

Precise analysis of human adipocyte total and poly(A)$^+$ RNA showed that the size of human HSL mRNA was between 2.75 and 2.8 kb (Figure 1) and not 3.3 kb as previously reported [7]. This result consequently indicates that the 5′-UTR(s) are much shorter (between 20 and 150 bp assuming a poly(A) tail between 50 and 150 nt) than initially reported (632 nt) [5].

To determine accurately the number and length of 5′-UTR(s), we developed an RNase H mapping test. Adipocyte total RNA

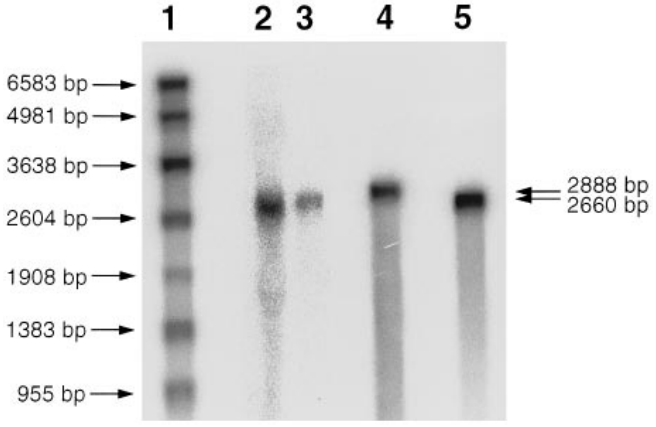

Figure 1 Northern blot analysis of human HSL mRNA size in adipocytes

Total and poly(A)$^+$ RNA were prepared from isolated human adipocytes as described in the Materials and methods section. RNA size markers (500 ng) (lane 1), 30 μg of adipocyte total RNA (lane 2), 250 ng of adipocyte poly(A)$^+$ RNA (lane 3), 2888 bp human HSL RNA transcribed *in vitro* (lane 4) and 2660 bp human HSL RNA transcribed *in vitro* (lane 5) were electrophoresed on a 1 % (w/v) agarose/2.2 M formaldehyde gel and transferred to nylon membrane. The blot was hybridized with ^{32}P-labelled HSL cDNA and λ DNA/*Hin*dIII probes. The digital picture is representative of three independent experiments that gave similar results.

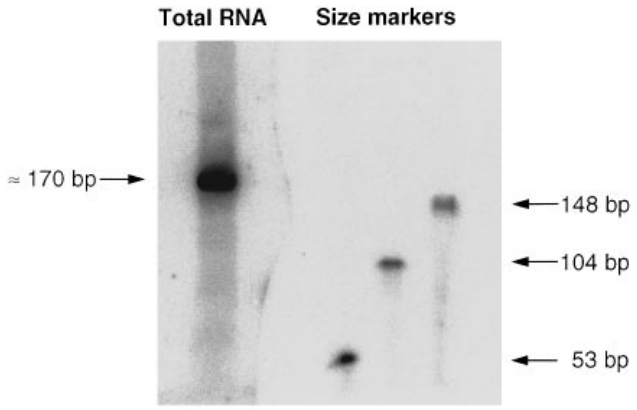

Figure 2 RNase H mapping analysis of human HSL mRNA 5′-UTR

Adipocyte total RNA (30 μg) was digested by RNase H as described in the Materials and methods section, then denatured and electrophoresed on a polyacrylamide/urea gel. The blot was hybridized with a ^{32}P-labelled oligonucleotide located in the coding sequence (nt 63–38 downstream of ATG). RNA size markers were produced by transcription *in vitro* and labelled by including [^{32}P]UTP in the reaction mixture. The digital picture is representative of five independent experiments that gave similar results.

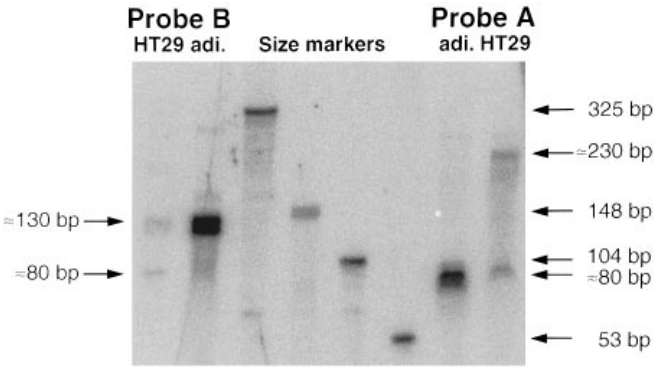

Figure 3 Abundance of 5′-UTRs in human HSL transcripts determined by RNase protection assay

RACE products A and B were used as cRNA probes (see the Results section). Uniformly ^{32}P-labelled probes were hybridized with 30 μg of adipocyte (adi.) total RNA and 200 μg of HT29 cell total RNA. Samples were digested with a mixture of RNase A and RNase T1, and resistant hybrids were analysed by electrophoresis on a polyacrylamide/urea gel. RNA size markers were produced by transcription *in vitro* and labelled by including [^{32}P]UTP in the reaction mixture. The digital picture is representative of four independent experiments that gave similar results.

was annealed to a single-stranded oligonucleotide located in the coding region of human HSL from nt 120–96. RNA hybridized to DNA was digested with RNase H and the products of digestion were electrophoresed in a denaturing polyacrylamide gel. After blotting, the 5′-fragment(s) of the HSL mRNAs were revealed by hybridization to a labelled oligonucleotide located in the coding region between nt 63 and 38. A single band was detected, showing the presence of a single major 5′-UTR in the HSL mRNA of human adipocytes (Figure 2). The 5′-UTR was approx. 70 bp long, which is in agreement with results from Northern blot analysis.

Cloning of 5′ non-coding regions

Two independent strategies were used to clone the 5′ non-coding regions of HSL transcripts in human adipocytes and HT29, the only human cell line known to express an HSL protein similar in size to the adipocyte isoform [14]. 5′-RACE experiments were performed on poly(A)$^+$ RNA from human adipocytes and HT29 cells. Two major products, named A and B, were cloned and sequenced. The A and B sequences did not correspond to the sequence of the previously published 5′-UTR [5]. In adipocytes, many more clones (more than 80 %) corresponding to the B product than to the A product were obtained. A and B contained 170 and 70 bp respectively of 5′-UTRs. The 20 nt immediately upstream of the start codon were common to the two products. In one RACE experiment, a single PCR product corresponding to the 5′ end of the previously reported 5′-UTR [5] was obtained. This result indicated that HSL mRNA species with a 5′-UTR more than 600 nt long, corresponding to a genomic sequence contiguous to exon 1, are rare in human adipocytes. Accordingly no hybridization was seen in Northern blot experiments containing human adipocyte total RNA with a probe derived from that region (nt 128–502 of the ATG) (results not shown). Two human adipose tissue cDNA libraries were screened with a cDNA probe (nt 101–593 downstream of ATG). The clones containing 5′-UTRs were sequenced. They corresponded to products A and B. Again, B was the more abundant product.

Abundance of the 5′ non-coding regions in HSL transcripts

Because RACE and library screening are not quantitative methods, the respective abundance of 5′-UTRs in HSL transcripts was determined with an RNase protection assay. Products A and B were used as cRNA probes on adipocyte and HT29 cell total RNA (Figure 3). In adipocytes, resistant hybrids were 80 bp (probe A) and 130 bp (probe B). The hybrid obtained with probe A corresponded to the protection of 63 nt of coding region and 20 nt of the 5′-UTR immediately upstream of the start codon, indicating that the 5′-UTR specific to probe A was not present in significant amounts in adipocytes. Probe B, in contrast, was entirely protected. These results show that the 5′-UTR specific to probe B is the major 5′-UTR in human adipocyte HSL transcripts. The presence of a second band of slightly shorter size than the approx. 130 bp band (Figure 3) was observed in two of four experiments. This resistant hybrid might result from the formation of an intramolecular loop at one edge of the cRNA probe that precludes hybridization with the entire target mRNA. The RNase protection assay results were confirmed in RNase H mapping experiments. A single band corresponding to a 5′-UTR of approx. 70 bp was detected by using the most 5′ 50 nt of product B as a probe. When product A-specific sequence was used as a probe, no signal was detected (results not shown). In HT29 cells, RNase protection assay showed that protected products were 230 and 80 bp with probe A, and 130 and 80 bp with probe B. The 80 bp resistant hybrid corresponded to 63 nt of coding region and 20 nt of 5′-UTR as described above for adipocytes. The 230 and 130 bp bands corresponded to the protection of product A- and product B-specific 5′-UTRs respectively. These results show the presence of two types of HSL transcript in HT29 cells containing either product A- or product B-specific 5′-UTRs. These 5′-UTRs were mutually exclusive because only two protected bands were observed (Figure 3).

Genomic localization of two novel exons

Four cosmids containing overlapping DNA sequences, which have been mapped to chromosome 19centq13.1–13.2 and shown

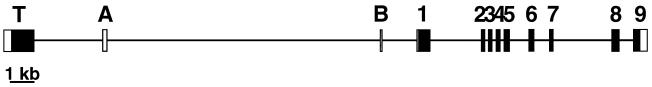

Figure 4 Organization of the human HSL gene

Coding sequences are shown as filled boxes and UTRs as open boxes. Exon T is the testis-specific exon; the newly characterized exons are exons A and B.

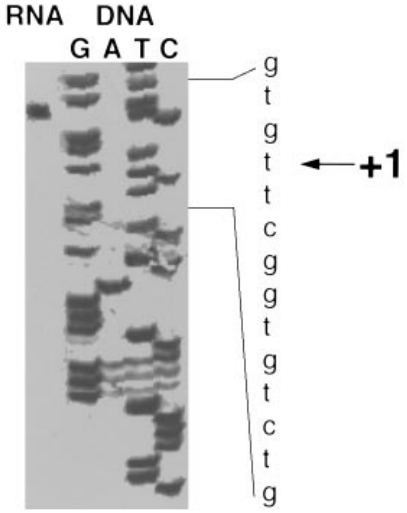

Figure 5 Determination of the human HSL gene transcriptional start site with the use of primer extension analysis

A 24-mer oligonucleotide complementary to RACE product B-specific 5′-UTR was ^{32}P-labelled and hybridized to 500 ng adipocyte poly(A)$^+$ RNA. Reverse transcription was performed as described in the Materials and methods section. Genomic sequencing was performed with the same 24-mer oligonucleotide. The extension and sequencing products were separated on a 6% (w/v) polyacrylamide/8 M urea gel. The autoradiogram is representative of two independent experiments that gave similar results.

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-778  gggggcgtgc atatcttagc tggggagaag gcaactgggg agtacctctt
-728  ggcaagtgac aagctgaggc ccagacaagg aatctgtgga atgacacggg
-678  tgaatgacat gggtgaggaa tctctctctc tgagcctctg tttccctgtg
-628  tgaggtggga tgccagtcac agcagggtgg gctgagaggg gtaagtggca
-578  ggtccaggtt acccagtgtg gtcacaccac ccagtaggac cgacaggcag
-528  ctcaggcccc tagctgagcc tggcactgcc cttaatcggc ttttgtcctt
-478  ccaggaggga caggcagggc tggctacctc ctctctcccc ccaacagctg
-428  gggctggctg tgccagggcc agactaggag gcgggagctg agatcactgg
-378  gcctttggct ccctgagtcc tctctgggga cagagagggc agcctggctg
-328  ggtgagaggg gacggtttgt tcaggactgg ggctaggact cctgggtcct
-278  gaaggatgaa ggggctgggg gcctggactc ctgggtctga gggaggaggg
-228  gctatgggtg tggactcctg ggttctaagg cagaggagcc tgtaacagga
-178  ctaccagttg agctgagtct gggagcaggt ggggggcaga gcagggagct

                                                        CAC
-128  gagccctcta ctctgtttac agcacgtggt cctcactgat ctttctgggt

      box                                               GC box
-78   gggaggtggc ttgtgcggct acaccctggg caggccagcc ccgcccccgg

      A+T rich
-28   gtttattgcc ccaggctgct actggcacAA GCCACAGACC AGCAGTCCCA
+23   GCCCAGGGAA GCTCGGAAGA TGCCTAGGAG GGctgagtgt cc--intron
      (1508 bp)- acctgcccac agCCTCAAGG CTCATCCACA ACATGGACCT
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Figure 6 Genomic sequence of the region containing exon B

Lower-case letters indicate promoter and intronic sequences; capital letters show exonic sequences. The transcriptional start site is indicated as +1. The GT/AG consensus sequences for splice junctions are underlined. Putative transcription factor binding sites are overlined. The start codon for the adipocyte form of human HSL is shown in italics.

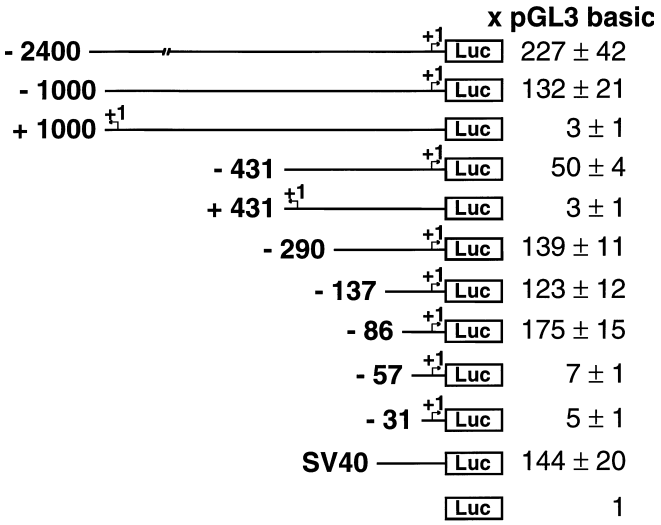

Figure 7 Deletion analysis of human HSL promoter activity in HT29 cells

HT29 cells were transiently transfected with HSL promoter–luciferase gene fusion constructs and pRL-CMV as control of transfection efficiency, as described in the Materials and methods section. The data are means ± S.E.M. for nine experiments. Activities are normalized for transfection efficiency and expressed relative to the activity of the promoterless pGL3 basic vector. HSL promoter activities were compared with the activity of the viral promoter SV40. The transcriptional start site is indicated as +1.

Table 1 Deletion analyses of human HSL promoter activity in rat adipocytes and HeLa cells

Rat adipocytes and HeLa cells were transiently transfected with HSL promoter–luciferase gene fusion constructs and pRL-CMV as control of transfection efficiency as described in the Materials and methods section. 5′-Flanking regions of various lengths were generated by PCR with sense primers located at various distances from the transcriptional start site and a common anti-sense primer located at +38. The data are means ± S.E.M. for three independent experiments performed in duplicate. Activities are normalized for transfection efficiency and expressed relative to the activity of the promoterless pGL3 basic vector. HSL promoter activities were compared with the activity of the viral promoter SV40.

Construct	Rat adipocytes	HeLa cells
−2400	36 ± 6	54 ± 4
−1000	63 ± 15	79 ± 3
−431	50 ± 10	68 ± 9
−86	56 ± 9	83 ± 5
−57	2 ± 1	6 ± 1
pGL3 basic	1	1
SV40	60 ± 3	47 ± 2

to contain the HSL gene [21], were used in Southern blot experiments. Products A and B hybridized to a 7 kb *Bam*HI fragment and a 6.2 kb *Eco*RI fragment respectively. Sequencing of the genomic fragments revealed that the 5′-UTRs were contained in single exons. Genomic and cDNA sequences were identical. Exon A was located approx. 2.8 kb downstream of the testis-specific exon (exon T) and exon B was located 1508 bp upstream of exon 1 (Figure 4). Consensus sequences for 5′ and 3′ splice junctions were found at the 3′ ends of exons A and B and at the 5′ end of exon 1, 20 nt upstream of the translation initiation codon.

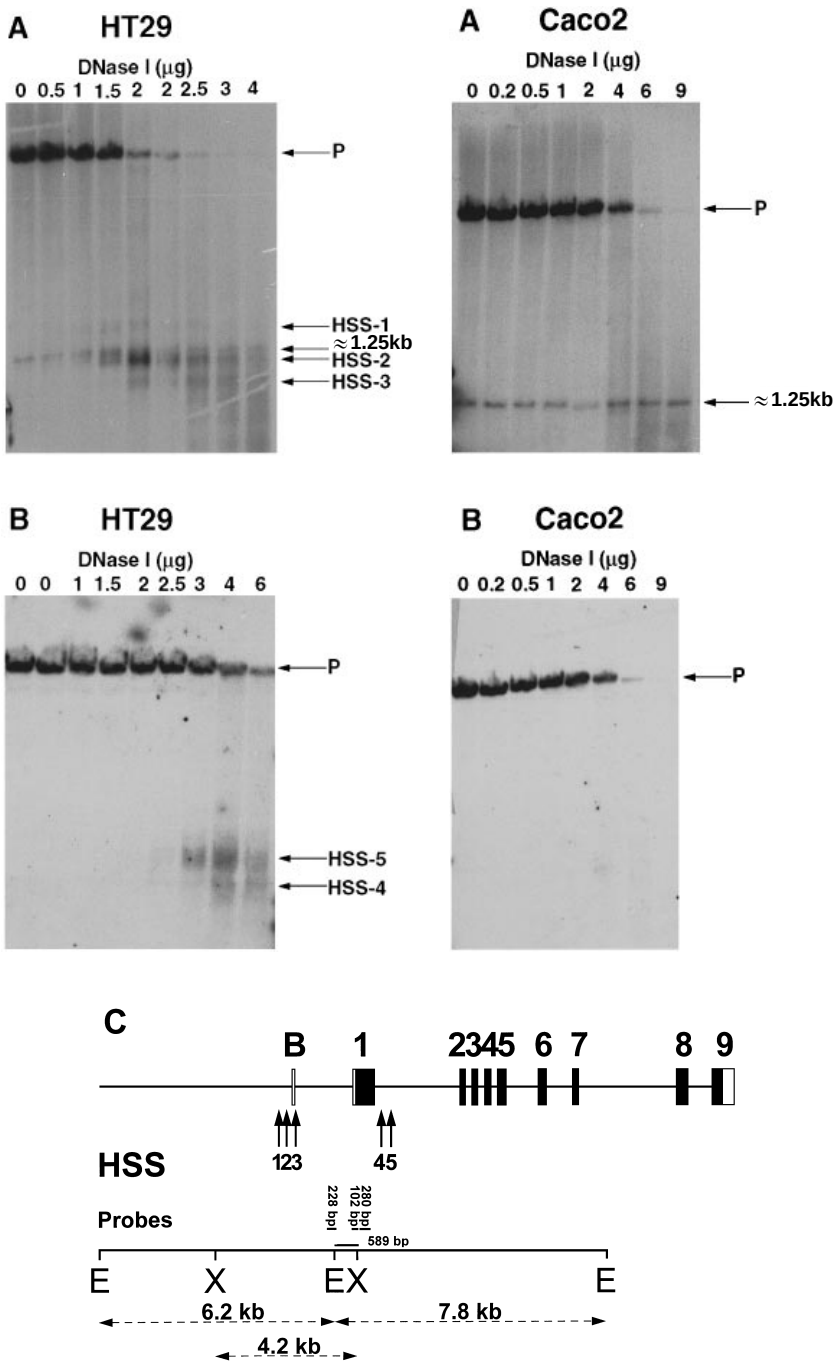

Figure 8 Localization of HSS associated with the human HSL gene

HT29 and Caco2 cell nuclei were digested with increasing amounts of DNase I as indicated. DNA isolated from DNase I-digested and undigested nuclei was digested with *Eco*RI, electrophoresed and transferred to nylon membrane as described in the Materials and methods section. The blot was hybridized with a 228 bp (**A**) or a 280 bp (**B**) ^{32}P-labelled probe. The autoradiogram is representative of three independent experiments that gave similar results. P represents the parental band undigested by DNase I. (**C**) Restriction map (E, *Eco*RI; X, *Xho*I) and the localization of the probes and the HSS in the human HSL gene.

Determination of the adipocyte-specific transcriptional start site

Primer extension analysis with human adipocyte poly(A)$^+$ RNA and an anti-sense oligonucleotide derived from the exon B sequence showed that the transcriptional start site was located 54 bp upstream of the 3′ splice site (Figure 5). The mapping was confirmed by Northern blot analyses of adipocyte total RNA with labelled oligonucleotides. Hybridization was seen with oligonucleotides derived from the exon B sequence but not with oligonucleotides derived from upstream genomic sequences (results not shown). Computer-based analysis [22] with the TSSG program (Baylor College of Medicine, Houston, TX, U.S.A.) of 2360 bp upstream of the translation initiation codon predicted one promoter with a transcriptional start site corresponding

exactly to the nucleotide determined in primer extension analysis (Figure 6). No CCAAT and TATA boxes were found. A GC box was found between −33 and −38 bp.

Deletion analysis of HSL promoter activity

To test whether the 5′-flanking region upstream of exon B had promoter activity, a series of 5′ deletions within the first 2.4 kb were generated and ligated to the luciferase reporter gene. These deletions shared a common 3′ end at position +38. Plasmids were transiently transfected into HT29 cells (Figure 7). The −2400 nt construct resulted in higher reporter gene activity than the SV40 promoter. High luciferase activity was observed for constructs containing the sequence between nt −2400 and −86. A decrease in activity was observed between nt −2400 and −431, suggesting the presence of *cis*-acting elements in that region. 5′-Flanking regions in inverted orientations (nt +1000 and +431) showed very low luciferase activities. Further deletion of sequences between nt −86 and −57 resulted in a marked decrease of luciferase activity, suggesting that *cis*-acting sequences beween nt −86 and −57 were necessary for HSL promoter activity. To determine whether the promoter was active in an adipocyte cell context, plasmids were transiently transfected into rat adipocytes by electroporation [19]. High luciferase activity was found beween nt −2400 and −86 (Table 1). The nt −431 construct did not show lower activity than the other constructs. A similar pattern was observed in HeLa, a human cell line that does not express HSL. These results suggest that the molecular determinants of adipocyte specificity are not present in the 2400 bp upstream of the transcriptional start site.

Mapping of HSS

To map HSS associated with the transcription of the human HSL gene, control and DNase I-treated nuclei were prepared from HT29 and Caco2, two human colon cancer cell lines that respectively express and do not express HSL. DNA was purified and digested with *Eco*RI or *Xho*I. After gel electrophoresis and transfer to nitrocellulose membrane, fragmented DNA was hybridized to various probes labelled by random priming. *Eco*RI-digested genomic DNA was first hybridized to a PCR-generated probe corresponding to the most 3′ 228 bp of the 6.2 kb *Eco*RI fragment (Figures 8A and 8C). This fragment contained exon B and its 5′ flanking sequence. In the absence of DNase I, two bands were detected in HT29 and Caco2 cells, the undigested *Eco*RI piece and a band of approx. 1.25 kb. With increasing amounts of DNase I, three bands, named HSS-1, HSS-2 and HSS-3, were generated from HT29 nuclei. These bands were not detected in Caco2 nuclei. HSS-1, a weak but reproducible signal, was located approx. 1.5 kb upstream of the *Eco*RI site. HSS-2 and HSS-3 were located at approx. 1.2 and 0.9 kb respectively upstream of the *Eco*RI site. To confirm the presence of these HSS in HT29 cells and to determine whether the approx. 1.25 kb band was specific, genomic DNA was digested with *Xho*I, which yielded a 4.2 kb band in undigested nuclei and hybridized with a *Xho*I–*Stu*I 102 bp probe located at the 3′ extremity of the *Xho*I fragment. The approx. 1.25 kb band was not detected in undigested nuclei. With increasing concentrations of DNase I, two bands were detected approx. 1.6 kb and 1.8 kb respectively upstream of the *Xho*I site that most probably correspond to HSS-2 and HSS-3 (results not shown). In experiments with *Eco*RI and *Xho*I, the HSS-2 band was more intense and appeared at lower DNase I concentrations than HSS-3. A 7.8 kb *Eco*RI fragment containing the first seven exons encoding the adipose form of HSL was also probed to locate hypersensitive sites (Figures 8B and 8C). First, a 589 bp *Eco*RI–*Xho*I piece was used as probe. It hybridized to multiple bands ranging from 3 to 20 kb on *Eco*RI-cut genomic DNA from nuclei not digested by DNase I (results not shown). Sequencing analysis revealed that this piece contained repetitive elements that are specific to this region of chromosome 19 [23]. A second probe corresponding to a 280 bp *Acc*I–*Bst*I fragment yielded two bands in HT29 cells but none in Caco2 cells. These sites, named HSS-4 and HSS-5, were located downstream of exon 1, approx. 1.4 and 1.9 kb from the *Eco*RI site.

DISCUSSION

The present paper describes the first characterization of a human HSL promoter. We establish that the human HSL transcripts expressed in adipose tissue are 2.7–2.8 kb long and contain a single 5′-UTR of 74 nt. The first 54 nt of this 5′-UTR are found, in the genomic sequence, in exon B located 1.5 kb upstream of exon 1 (Figure 4). In HT29 cells, two mutually exclusive 5′-UTRs are present in HSL transcripts. One corresponds to exon B and the other (exon A) was mapped in the genomic sequence approx. 2.8 kb downstream of the testis-specific exon. The 5′-flanking region of exon B functions as a strong promoter. Cell-type-specific HSS were found in the vicinity of the transcriptional start site of exon B and in the intron downstream of exon 1.

The human HSL gene shows a complex organization with the expression of at least three alternative 5′ termini in HSL transcripts. The exons containing the various 5′ ends are spread over a large genomic region because the distance from exon T to exon B is approx. 15 kb, in contrast with the distance between exons 1 and 9 (approx. 11 kb). An unusual feature of the HSL gene is the use of coding (exon T) and non-coding (exons A and B) 5′ alternative exons. Splicing of pre-mRNA sequences corresponding to these exons occurs at the same position in exon 1, 20 nt upstream of the translation initiation codon. Our results strongly suggest the use of alternative promoters in a tissue-specific way. Exon T is specific to the testis and not used in adipose tissue [6]. Exon B is used in adipocytes and HT29 cells but not in testis. A compilation of the results obtained with various techniques indicates that exon A-containing transcripts are found in significant amounts in HT29 cells and at very low levels in adipocytes. Furthermore the results show that the previously identified 5′-UTR of 632 nt contiguous to exon 1 is not present to a significant extent in adipocytes. The possibility that the relative abundance of the various 5′-UTRs in human adipose tissue is subject to regulation cannot, however, be ruled out. A multiplicity of mRNA species with different 5′ termini has been shown for several genes. A good example is the γ-glutamyltransferase gene, which codes for six mRNA species from at least five different promoters [24,25]. The use of alternative promoters underlies the tissue specificity of expression in a number of genes such as the human aromatase cytochrome *P*-450 gene [26,27].

This study provides the first functional characterization of a human HSL promoter; 5′ deletion constructs from nt −2400 to −86 showed high luciferase activity in two cell types expressing HSL, i.e. rat adipocytes and HT29 cells, and in HeLa cells, which do not express HSL. These results show that the first 2.4 kb of the 5′-flanking region contain strong positive regulatory sequences. However, the sequences responsible for the tissue-specific expression of HSL are not present in this region because the pattern of promoter activity was similar in HeLa cells, adipocytes and HT29 cells. Adipose-specific enhancer can be located far upstream of the transcriptional start site as shown for the aP2

fat-specific enhancer located between −4.9 and −5.4 kb [28]. Deletion of HSL promoter sequences from nt −86 to −57 resulted in a marked decrease in luciferase activity. These results indicate that the 5′-end border of the minimal promoter is located between nt −86 and −57. This region contains a CAC sequence in a C-rich environment that could bind Sp1 and related transcription factors and participates in the *trans*-activation of the human HSL promoter [22,29]. An interesting feature of the promoter is the lack of consensus sequences for CAAT and TATA boxes. CAAT and TATA-less promoters initially described for housekeeping genes have now been shown for a number of genes with restricted tissue expression. Several features characterize this type of promoter. GC boxes are often present upstream of the transcriptional start site, which is true of the HSL promoter between nt −33 and −38. Binding of Sp1 to the GC box seems in this context to be required for the stabilization of the initiation complex [30]. Moreover, in some TATA-less promoters, the TATA box is replaced by an (A+T)-rich sequence that differs from the consensus TATA sequence but is nevertheless a functional promoter element. In the HSL promoter, the (A+T)-rich sequence located between nt −22 and −27 could have this role. Another feature of TATA-less promoters is the presence of an initiator element that encompasses the transcriptional start site. Analysis of DNA sequence requirements for transcriptional initiator activity revealed the approximate consensus sequence $YYCA_{+1}NWYY$ [31]. However, the corresponding sequence in the HSL gene ($CACA_{+1}AGCC$) differs from the consensus sequence at positions −2 and +3. This divergence apparently does not impair the promoter activity because the minimal promoter (−86 bp construct) is as strong as the SV40 promoter (Figure 7 and Table 1). Moreover, unlike many TATA-less promoters that are characterized by numerous transcriptional start sites, a single transcriptional start site was detected in the HSL gene. This indicates that the proximal elements [e.g. the GC box, the (A+T)-rich sequence and the initiator-like element] allow precise positioning of the RNA polymerase II complex.

DNase I HSS mapping is a convenient technique for scanning relatively large genomic regions for potential regulatory sequences. HSS sites are nucleosome-free regions that seem to be an essential feature of chromatin structure in eukaryotes [32]. The formation of HSS accompanies gene activation, and known regulatory elements reside within regions of DNase I hypersensitivity. Moreover, precise nucleosome positioning can facilitate or preclude the accessibility of *cis*-acting elements to *trans*-acting factors [20]. HSS-2 and HSS-3 are located in the vicinity of the human HSL gene minimal promoter (Figure 8C). These HSS were related to active gene transcription because they were not present in Caco2 cells. HSS-2 and HSS-3 could represent the binding sites of nuclear factors that participate in the formation and stabilization of the transcription initiation complex. The position of HSS-2 could correspond to the region between nt −86 and −57 that is necessary to drive high reporter gene activity. In agreement with an important role for that region, HSS-2 was more hypersensitive than the other HSS, regardless of the restriction enzyme used. These results are consistent with the binding of *trans*-acting factors essential for minimal promoter activity. Furthermore we analysed a region containing the first seven exons encoding adipocyte HSL. Two HSS sites were found in the intron downstream of exon 1 in HT29 cells but not in Caco2 cells. The presence of these sites suggests the existence of enhancers linked to active HSL gene transcription. The presence of intronic HSS has been shown for a number of genes. Intronic HSS sites linked to gene expression have been characterized in fatty acid synthase, another key enzyme of lipid metabolism [33]. This site corresponds to a *cis*-acting element that confers glucose responsiveness.

Through the precise mapping of the adipocyte-specific transcriptional start site, functional characterization of the minimal promoter and chromatin structure analysis, we provide a foundation for future studies aimed at understanding the transcriptional regulation of the human HSL gene and its tissue-specific expression in white adipocytes.

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