Hepatic lipase gene is transcribed in rat adrenals into a truncated mRNA

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Abstract Rat adrenals contain a lipase activity that is indistinguishable from hepatic lipase (HL) present in liver. Expression of HL mRNA in adrenals was studied using the method of reverse transcription-polymerase chain reaction (RT-PCR). A 596-bp fragment of HL cDNA spanning exons 5 to 8 was amplified when using total RNA from rat adrenals and liver, but not from heart or kidney. The abundance of HL mRNA was quantified by competitive RT-PCR using a standard RNA that was generated in vitro by transcription from a deleted HL cDNA construct. Adrenals contained 0.4 attomoles of HL mRNA per μg of total RNA, compared to 16 attomoles in liver. In hypertrophic adrenals isolated from corticotrophin-treated rats, the abundance also amounted to 0.4 attomoles of mRNA per μg of total RNA. However, amplification of full-length cDNA from either control or hypertrophic adrenals was never observed. Detailed analysis by PCR using different combinations of primers indicated that exons 3 to 9 including the 3'-untranslated region were expressed in adrenal RNA, but not the first two coding exons. The upstream part of the adrenal lipase mRNA was cloned after rapid amplification of cDNA ends (RACE). The resulting clones showed a unique 126-bp sequence 5' of the exon 2-exon 3 junction. This sequence contained multiple termination codons in all three reading frames but lacked a potential start codon. RT-PCR using an HL-specific primer and an oligonucleotide directed against this 5'-sequence showed that it is not only expressed in RNA from adrenals but also in liver. Pulse-labeling of freshly isolated adrenocortical cells with [^{35}S]methionine followed by immunoprecipitation with anti-HL antibodies failed to show synthesis of mature HL, but indicated the synthesis of immunoreactive proteins in the 40-45 kDa range that remained mainly intracellular. Hence, the HL gene is transcribed in adrenals but results in an mRNA species with a unique 5'-end. Translation from an internal start site may produce an intracellular HL isoform that differs markedly from the liver-type lipase previously identified in adrenals.—**Verhoeven, A. J. M., D. Carling, and H. Jansen.** Hepatic lipase gene is transcribed in rat adrenals into a truncated mRNA. *J. Lipid Res.* 1994. **35:** 966-975.

Supplementary key words reverse transcription • polymerase chain reaction • liver-type lipases • hypertrophic adrenals

Hepatic lipase (HL; E.C. 3.1.1.34) is present in the liver of most vertebrates at the endothelial lining of blood sinusoids (1). The enzyme plays a central role in lipoprotein metabolism. Notably, HL is shown to facilitate uptake by liver cells of cholesterol from high density lipoproteins (HDL) (1-3). A highly similar enzyme activity (liver-type lipase) has been detected in rat, human, hamster, and bovine adrenal cortex (4-6) and in rat ovaries (6-8). At least in the rat, these organs depend largely on HDL cholesterol as the source for steroidogenesis (9, 10). The liver-type lipase activity is almost exclusively localized in the zona fasciculata and the corpora lutea (8, 11, 12), which are most active in steroid hormone production. In addition, the enzyme activity in these organs varies in parallel with steroid output (7, 13, 14), suggestive of an important role in cholesterol supply.

The liver-type lipases from rat adrenals and ovaries closely resemble HL from the liver. These enzymes share the properties of salt-resistance and alkaline pH-optimum, all three are released from the respective organs by heparin infusion, and show similar affinities for Sepharose-heparin (6, 15). Moreover, all three enzymes are equally recognized by polyclonal and monoclonal antibodies prepared against rat HL (6, 16). Finally, the peptide map of adrenal lipase made by digestion with V8-protease is similar to that of HL (17). These observations strongly suggest that all three lipases represent a single gene product.

HL is synthesized and subsequently secreted by hepatocytes. The origin of the liver-type lipases in adrenals and ovaries remains unknown. Attempts to detect local de novo synthesis in unstimulated rat adrenals and highly luteinized ovaries by [^{35}S]methionine incorporation into immunoreactive protein have been unsuccessful (17, 18). HL mRNA has never been observed in

Abbreviations: ACTH, adrenocorticotrophic hormone; bp, base pairs; BSA, bovine serum albumin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDL, high density lipoproteins; HL, hepatic lipase; nt, nucleotide; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TBE, Tris-borate-EDTA buffer; TE, Tris-EDTA buffer.

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adrenals and ovaries by classical Northern blotting (17–20), or in ovaries by application of the polymerase chain reaction (18). This led to the hypothesis that the liver-type lipases are synthesized exclusively in the liver and subsequently transported to the adrenals and ovaries. However, in initial experiments we found that part of the HL mRNA could be detected by RT-PCR in RNA from adrenals, ovaries, and testes, but not in heart or kidney (21). In the present study, we elaborated on this finding and studied the abundance, nature, and possible translation product of the HL gene transcript in the rat adrenal gland.

MATERIALS AND METHODS

RNA isolation

Male Wistar rats (250–300 g) were killed by decapitation, and the blood was allowed to drain. Then, organs were quickly excised. Adrenals were collected directly in liquid nitrogen; liver, heart, and kidney were cut into pieces and also dropped in nitrogen. The tissues were stored at −70°C until use. Hypertrophic adrenals were obtained from animals after 5–9 daily injections with 50 μg of a synthetic corticotrophin analogue (Synacthen, Ciba-Geigy N.V., Belgium), as detailed previously (13). Total RNA was isolated from individual adrenals, or from 0.25–0.50 g of tissue by the acid–guanidinium thiocyanate–phenol method (22). RNA concentrations were determined by spectrophotometry at 260 nm (23). The quality of the RNA preparations was judged from the ratio of 28S over 18S ribosomal RNA after denaturation and electrophoresis in a 1% agarose/TBE gel (23). Alternatively, 1 μg of denatured RNA was analyzed by Northern blotting using a ^{32}P-labeled 18S ribosomal RNA antisense oligonucleotide (5'-ACG GTA TCT GAT CGT CTT CG-3', nt 1070–1051, ref. 24) as probe.

Oligonucleotide primers

Oligonucleotides (cf. Fig. 3A) were synthesized with the PCR-Mate 391 DNA synthesizer (Applied Biosystems). HL-1 (5'-GTG GGC ATC AAA CAG CCC-3', nt 712–729) and HL-2 (5'-CAG ACA TTG GCC CAC ACT G-3', nt 1307–1289; numbering according to the rat cDNA sequence, ref. 19) were used for quantitative RT-PCR. According to the genomic DNA sequence of human HL, this fragment extends from exon 5 into exon 8 with more than 14 kb intron sequence (25, 26). Full-length HL cDNA was amplified using HL-4 (5'-GGT AAG ACG AGA GAC ATG GG-3', nt 1–20) and HL-5 (5'-CCT TTG TGT AAT TTG GCT TCC G-3', nt 1602–1581). Other HL-specific primers mentioned in the text were HL-3 (5'-CGG GGG CTC CTT CCA GCC TGG-3', nt 741–761), HL-7 (5'-CAC TAT GCT ATT GCC GTG C-3', nt 403–421), HL-12 (5'-TGG CTT GCT AGA AAC CTG G-3', nt 297–315), HL-13 (5'-TGT CAT GAT CAT CCA CGG G-3', nt 267–285) and AHL-13 (5'-CCC GTG GAT GAT CAT GAC-3', nt 285–268). For PCR of GAPDH cDNA (27), the oligonucleotides GAPDH-1 (5'-CTC TCT TGC TCT CAG TAT CC-3', nt 1120–1101) and GAPDH-2 (5'-TCT TCT TGT GCA GTG CCA GC-3', nt 35–54), which span the entire coding sequence, were used. Combinations of primers employed in RT-PCR always framed at least one intron of genomic DNA.

RT-PCR

First-strand cDNA synthesis and subsequent amplification by PCR was performed in a single tube starting with 1 μg of total RNA. Immediately before use, the RNA was heated for 3 min at 70°C. First-strand synthesis was performed in a total volume of 20 μl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM of each dNTP, 50 pmol random hexamers, 20 U RNAsin, and 50 U of M-MLV reverse transcriptase (Perkin-Elmer Cetus). Incubations were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 10 min at room temperature, followed by 30 min at 42°C and 5 min at 99°C. Thereafter, the total incubation volume was adjusted to 100 μl by adding pre-mixed components, and overlaid with 50 μl of light mineral oil (Sigma, St. Louis, MO); final concentrations were 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 15 pmol each of an upstream and downstream primer, and 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus). After incubation for 2 min at 94°C, amplification was performed in 35 cycles of, successively, 1 min 94°C, 1 min 55°C, and 1 min 72°C, unless otherwise stated. Possible carry-over of PCR products or contamination with cDNA-containing plasmids was assessed by performing parallel incubations in the absence of reverse transcriptase. A no-template control was included in each experiment. The PCR products were analyzed by separating 10-μl aliquots on a 3% agarose/TBE gel (Nusieve:Seakem = 3:1; FMC Bioproducts, Vallensbæk Strand, Denmark).

Semi-quantitative RT-PCR

Quantitation of HL mRNA was done by competitive RT-PCR (28). In this assay, 0.5 μg of tissue RNA was mixed with 0.5 μg of yeast RNA containing increasing amounts of competitor RNA, and then reverse transcribed and amplified as described. Semi-quantitation was done visually by comparing the intensities of the ethidium bromide-stained bands on agarose gels. The competitor RNA was generated in vitro from an HL cDNA construct in which an 80-bp Sau3A fragment (nt 1009–1088) was deleted. Liver cDNA was amplified using the primers HL-1 and HL-2, and the resulting 596 bp DNA product was partially digested with Sau3A. The 296 bp and 219 bp restriction fragments were gel-purified, religated and re-amplified with HL-1 and HL-2. The 516 bp

product was TA-cloned (29) into pBluescript (Stratagene, La Jolla, CA). A recombinant plasmid was selected that contained the insert in the sense orientation with respect to the T7 promoter. The plasmid was linearized by digestion with PvuII, which cuts the vector at about 300 bp downstream of the insert, thereby providing a 3'-tail for random priming comparable in size to that in the target mRNA. After linearization, the DNA template (approx. 0.5 μg) was transcribed for 2 h at 40°C with T7 RNA polymerase (400 U/ml; Promega) in the presence of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 5 mM NaCl, 100 μg/ml of BSA, 0.5 mM of each ribonucleotide, and 4000 U/ml of RNAsin. Thereafter, the DNA was digested with 400 U/ml of RNAse-free DNAse I (Boehringer Mannheim, Germany) for 30 min at 37°C. The RNA was extracted with phenol–chloroform and ethanol-precipitated. After resuspension in TE, the RNA concentration was measured by spectrophotometry at 260 nm, and then the RNA was serially diluted into TE containing 0.5 μg/ml of yeast RNA.

Rapid amplification of 5'-cDNA (5'-RACE)

5'-RACE was performed in principle according to Frohman, Dush, and Martin (30). First-strand cDNA was synthesized from 4 μg adrenal total RNA using oligo$(dT)_{16}$ as primer. After ultrafiltration through Centricon-100 (Amicon), the cDNA was G-tailed by incubation with 5 μM dGTP and 25 units of terminal deoxynucleotidyl-transferase (Pharmacia, Uppsala, Sweden) for 30 min at 37°C in a final volume of 20 μl. After inactivation of the enzyme for 5 min at 65°C, the volume was brought to 150 μl with TE. Then, 10 μl of this solution was subjected to nested PCR (30 cycles; 1 min 94°C, 1 min 58°C, 1 min 72°C; 100 μl final volume) using primers against the G-tail and various HL-specific oligonucleotides. In the first round of PCR, a mixture of 20 pmol of a hybrid dC17-adapter primer (5'-AAG GAT CCG TCG ACA TCG ATA ATA CGA CTC ACT ATA AGG GAC CCC CCC CCC CCC CCC C-3') and 80 pmoles of the adapter primer (5'-GAT AAT ACG ACT CAC TAT AAG GGA-3') were used, whereas subsequent rounds of PCR were performed with 60 pmol of the adapter primer. Two strategies of nested PCR were followed using 60 pmol of different HL-specific primers. *i)* After the first round using HL-2, the mixture was diluted 10-fold into a fresh incubation and PCR was performed with HL-14; *ii)* HL-5 was used in the first round, HL-7 in the second, and HL-14 in the third round. After gel-purification of the PCR products and TA-cloning into pBluescript, double-strand sequencing was performed by the dideoxy termination method (31) using vector-specific oligonucleotides as primers.

Pulse-labeling with [^{35}S]methionine

Adrenocortical cells were freshly isolated from the adrenals of five rats that had been treated with ACTH for 5–6 days, as described above. After removing the surrounding fat, the adrenals were minced and then shaken for 50 min at 37°C with collagenase (Sigma, type I; 3 mg/ml). Intact adrenocortical cells were isolated by centrifugation (10 *g*, room temperature, 5 min) through a layer of 2% BSA in Krebs-Ringer solution. The cells were divided into six vials, and incubated for the times indicated at 37°C with 100 μCi Tran^{35}S label (ICN, Irvine, CA) in methionine-free Ham's F10 medium containing 20% lipoprotein-free human serum (after dialysis), 10 U/ml heparin and 0.6 mU/ml ACTH. The incubations were stopped on ice, and cold methionine (1 mM, final concentration) was added. The cells were separated from the extracellular medium by centrifugation (1 min, 10,000 *g*, 4°C), and then resuspended and lysed in 0.5 ml PBS containing 1% Triton X-100, 0.1% SDS, 1 mM methionine, and protease inhibitors (100 U/ml Trasylol, 5 μg/ml leupeptin, 1 mM PMSF, 100 μg/ml benzamidine, and 1 mM EDTA); after 30 min on ice, the lysates were centrifuged (5 min, 10,000 *g*, 4°C) and the supernates were used for immunoabsorption. Protease inhibitors were added to the extracellular media in the same final concentrations. In parallel, rat hepatocytes were freshly isolated by collagenase perfusion (32), and pulse-labeled with Tran^{35}S label as described above.

^{35}S-labeled HL and HL-related proteins were collected from cell lysates and extracellular media by immunoabsorption to Sepharose-coupled goat polyclonal anti-HL IgGs. The samples were first treated for 3 h at 4°C with 50 μl of a 50% slurry of Sepharose coated with goat IgGs prepared from nonimmune serum. After centrifugation (30 sec, 10,000 *g*, 4°C), the supernates were incubated overnight at 4°C with 50 μl of a 50% slurry of anti-HL coated Sepharose. Thereafter, the beads were collected by centrifugation, and washed twice with 1 ml of, successively, 1% Triton X-100 in PBS, PBS, 2 M NaCl in PBS, and finally PBS. The absorbed proteins were released by 10-min boiling in 15 μl of Laemmli's sample buffer, and separated by electrophoresis through an SDS-containing 10%-polyacrylamide gel. Radioactive bands were visualized according to standard procedures by fluorography using Amplify (Amersham). The immunoglobulins used, as well as their coupling to Sepharose, have been described elsewhere (32).

Alternatively, HL-related proteins were collected by immunoprecipitation with a mixture of five different monoclonal anti-HL IgGs (16). After pre-clearing with protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden), the cell lysates and extracellular media were incubated for 1 h at 4°C with 25 μl each of five different hybridoma supernatants, followed by a 1-h incubation with 10 μl of whole rabbit anti-mouse IgG antibodies (1 mg/ml; Nordic Immunology, Tilburg, The Netherlands). Thereafter, 50 μl of a 50% slurry of protein A-Sepharose 4B was added,

and the incubation was continued for 16 h at 4°C. The beads were collected, and treated further as described above.

RESULTS

Detection of HL mRNA in rat adrenals

RT-PCR of total liver RNA using the HL-specific oligonucleotides HL-1 and HL-2 yielded one major product with the expected size of 596-bp (**Fig. 1A**). A similarly sized product was obtained with RNA isolated from whole adrenals. No such product was generated in the absence of reverse transcriptase. Identity of this PCR product with part of the HL cDNA was confirmed by restriction mapping and internal re-amplification (21). Identical results were observed when oligo$(dT)_{16}$ was used to prime cDNA synthesis (21), which might indicate that the RNA target contained a poly-A tail. Amplification was observed with each of twelve independent RNA preparations from different adrenals. Under the same conditions RT-PCR on heart and kidney RNA remained negative. In contrast, the full-length cDNA of GAPDH was amplified with RNA from these tissues as well as liver and adrenals (Fig. 1B). Control experiments showed that the RNA preparations used all contained comparable amounts of 18S and 28S ribosomal RNA, indicating that the quality of these preparations was highly similar. These results demonstrate that at least part of the HL gene is transcribed in rat adrenals, but not in heart and kidney.

Quantitation of HL mRNA

To assess the abundance of HL mRNA in adrenals relative to liver, RT-PCR was performed as described above except that product formation was monitored after successive rounds of amplification. With 1 μg of adrenal RNA, the 596-bp DNA band became UV-detectable after 28–30 cycles, whereas with 1 μg of liver RNA this band was already observed after 20 cycles. Then, liver RNA was serially diluted into yeast RNA and subjected to RT-PCR in parallel with undiluted adrenal RNA. With 10–20 ng of liver RNA, the RT-PCR product became apparent after a similar number of cycles as with 1 μg of adrenal RNA (not shown). This indicated that the abundance of HL mRNA in whole adrenals is about 1–2% of that in liver.

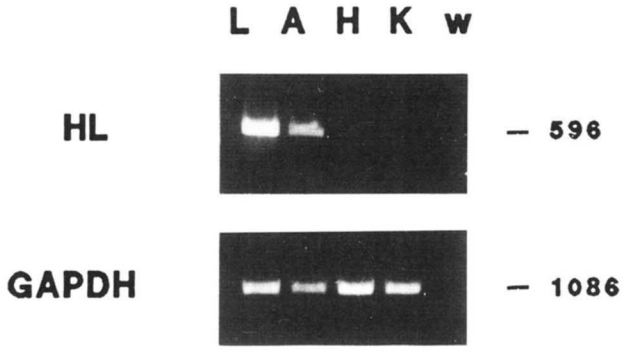


Fig. 1. Detection of HL mRNA by RT-PCR in rat adrenals. RT-PCR was performed with RNA from liver (L), adrenal (A), heart (H), and kidney (K), and a no-template control (w). After first-strand cDNA synthesis, the incubation mixture was split into two aliquots for amplification of the HL cDNA (upper panel) and almost full-length cDNA encoding for GAPDH (nt 35–1120; lower panel). Amplification was for 35 (HL mRNA) or 25 cycles (GAPDH mRNA).

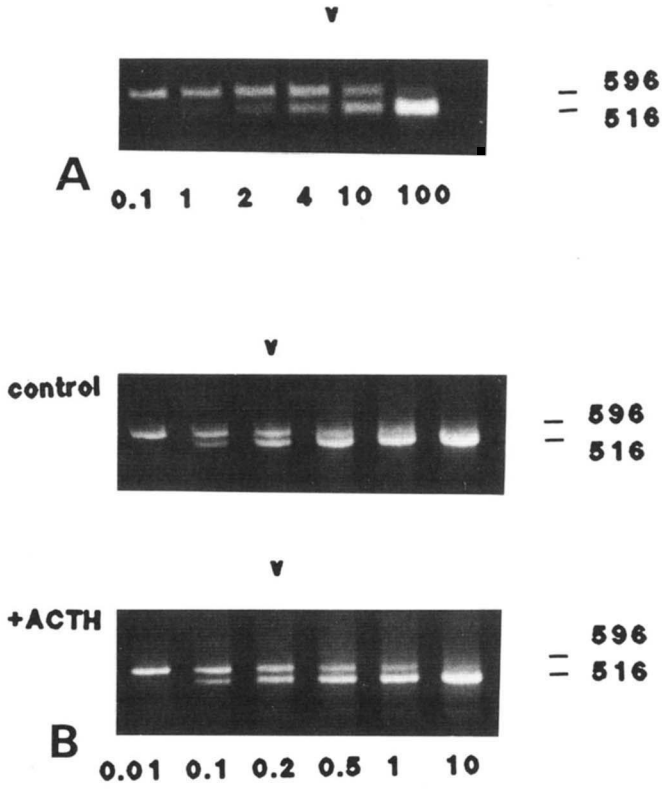


Fig. 2. Quantitation of HL mRNA in liver and adrenals. Competitive RT-PCR was performed on liver RNA (A), and on adrenal RNA (B) isolated from either untreated (upper panel) or ACTH-treated rats (lower panel). To 0.5 μg of tissue RNA, the indicated amounts (in attomoles) of the deleted HL RNA competitor were added. Amplification was for 25 cycles (liver) or 35 cycles (adrenals). The arrowheads indicate the lanes where the 596 and 516-bp bands show equal intensities under UV.

Next, the amount of HL mRNA in liver and adrenals was quantitated by competitive RT-PCR. An internal standard RNA was generated that contained the HL sequence framed by the primers HL-1 and HL-2 except for an internal 80-bp fragment. Known amounts of competitor HL RNA lacking an internal 80-bp fragment were mixed with 0.5 μg of tissue RNA and then subjected to RT-PCR. When the competitor RNA was increased from 0.01 to 100 attomoles, the amount of the 516-bp PCR product increased whereas the 596-bp PCR product from the endogenous HL mRNA gradually disappeared (**Fig. 2**). By interpolation, both PCR products showed equal

signal intensities when liver and adrenal RNA were mixed with 8 and 0.2 attomoles of standard RNA, respectively. These results indicate that liver contains approximately 16 attomoles of HL mRNA per μg total RNA. The abundance in adrenals is 40-fold less at 0.4 attomoles per μg of total RNA. Identical results were obtained for at least one other liver and adrenal.

Expression of HL mRNA in hypertrophic adrenals

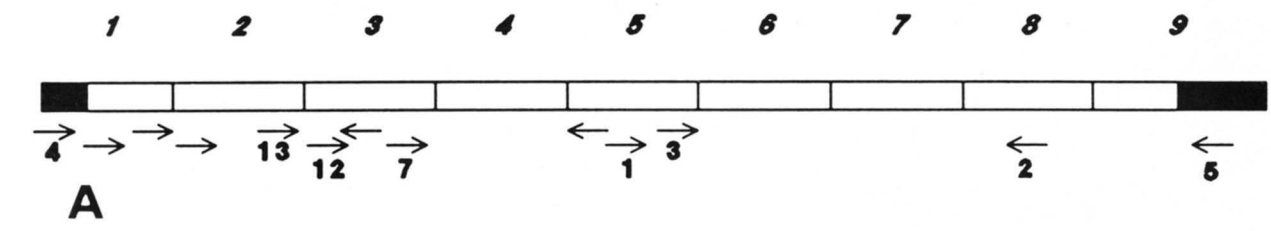
Treatment of rats with ACTH resulted in a marked increase in adrenal volume. Accordingly, the amount of total RNA isolated from these organs increased 3-fold from 43 ± 10 μg (n = 3) to 132 ± 31 (n = 6) μg per adrenal (means ± SD). By competitive RT-PCR on adrenals from two different rats, 0.4 attomoles of mRNA was found per 1 μg of total RNA, similar to the number found for untreated adrenals (Fig. 2B). This indicates that, within the limits of detection, the HL gene transcript is not specifically induced by ACTH treatment. Rather, the total number of HL mRNA molecules per adrenal increases in parallel with the increase in tissue volume and total RNA.

Is the entire HL mRNA expressed in adrenals?

Full-length cDNA (nt 1–1602) was readily amplified from a liver RNA preparation using the oligonucleotides HL-4 and HL-5 as primers. In contrast, several attempts to detect the entire mRNA in adrenals from either untreated or ACTH-treated rats by RT-PCR remained negative. Using HL-1 and HL-5, the expected RT-PCR product of 891-bp was obtained with adrenal as well as liver RNA, demonstrating the presence of the 3'-end of the mRNA including the 3'-untranslated region (**Fig. 3**). RT-PCR was then performed using HL-5 as the downstream primer, and a number of different sense primers. Amplification was obtained with HL-7 and HL-12. Upon Southern blotting, these PCR products hybridized to an internal oligonucleotide, HL-3, demonstrating the specificity of the reaction. With HL-13, which anneals to HL cDNA only 30 bp upstream of HL-12, however, the expected RT-PCR product of 1336-bp was obtained with liver RNA, but not with adrenal RNA. Using HL-4 or any of three other primers upstream of HL-13, RT-PCR on adrenal RNA remained negative (data not shown). In addition, amplification of the sequence between HL-13 and one of two other anti-sense primers upstream of HL-2 was never observed. Moreover, the combination of HL-4 and AHL-13, an oligonucleotide antisense to HL-13, failed to give a PCR product. Identical results were obtained with RNA isolated from control and hypertrophic adrenals. In all cases, a DNA product of expected size was generated by parallel RT-PCR on liver RNA, which served as a positive control. These observations strongly suggest that the HL mRNA found in liver is also expressed in normal and hypertrophic adrenals except for the 5'-end upstream of HL-12.

Identification of the 5'-end of adrenal lipase mRNA

5'-RACE was performed on adrenal RNA using two different strategies of nested PCR with HL-specific primers. Both strategies yielded a visible DNA product of approximately 250-bp, that hybridized with HL-12 but not with HL-13. Both products were cloned and sequenced. The two independent PCR products were almost identical, except that clone 1 extended 18 bp fur-



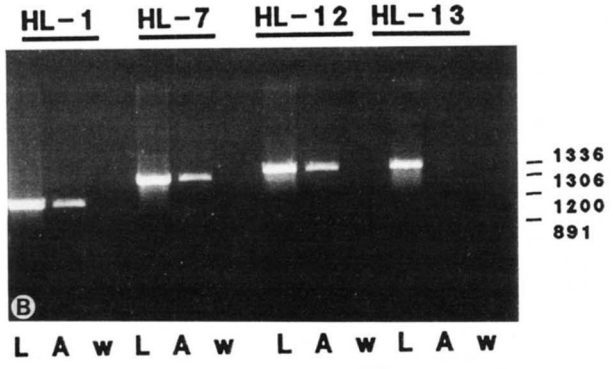


Fig. 3. Adrenal HL mRNA does not contain the 5'-end of the liver counterpart. A: Depicts the exon organization of rat HL cDNA, which is inferred from the one established for human HL cDNA (24, 25). The open bar indicates the coding sequence with the exon numbers given above. The closed bars represent the untranslated regions. The arrows define the positions of all the oligonucleotides used in RT-PCR on adrenal RNA with the arrowheads pointing in the 3'-direction; the names of only those primers that are mentioned in the text are given. B: RT-PCR was performed on liver (L) and adrenal (A) RNA, and a parallel water control (w) using HL-5 as the downstream primer. After first-strand cDNA synthesis, the incubation was split into four aliquots for amplification with different upstream primers, as indicated on top of the lanes.

ther upstream than clone 2, and had a slightly shorter oligo(dC) stretch at its 5'-end reflecting a shorter G-tail of first-strand cDNA (**Fig. 4**). In addition, four single base differences were apparent along the sequence, probably due to the infidelity of the Taq DNA polymerase reaction; no attempts were made to resolve these ambiguities. The 3'-ends of both sequences were identical to part of the HL cDNA corresponding to exon 3 (nt 292–313, numbering according to ref. 19). The homology with HL cDNA, however, was completely lost upstream of base 292, where exon 3 is supposedly joined to exon 2 according to the organization of the human HL gene (25, 26). Instead, 126 bp of novel sequence were found upstream of this junction.

Closer inspection of this novel sequence revealed the complete absence of an ATG triplet. Moreover, multiple termination codons are present in all three reading frames. The dinucleotide AG at positions −2 and −1 combined with the pyrimidine-rich stretch between −17 and −3 conforms to the consensus sequence for intron-exon boundaries (33). Taken together, this novel sequence has intron-like features, and might represent the intron upstream of exon 3 in the HL gene. Unfortunately, to the best of our knowledge, the sequence of the rat HL gene at this splice junction has not yet been determined.

A sense oligonucleotide (INT) was made according to the sequence of clone 1 (position −112 to −93), and used in RT-PCR on adrenal and liver RNA in combination with HL-9, which recognizes the HL gene in exon 5 (25, 26, 34, 35). A band of the expected 481-bp was produced with adrenal RNA from control as well as from ACTH-treated rats (**Fig. 5B**). Surprisingly, RT-PCR on liver RNA gave a similar product. Formation of these PCR products was only seen when reverse transcriptase was included in the initial incubation mixture; even after Southern blotting, no hybridizing band was observed with ^{32}P-labeled HL-12 in the −RT controls (not shown). Hence, these results are not due to contamination with genomic or any other DNA sequences. Similar results were obtained with HL-2 as the anti-sense primer, which is targeted at exon 8 of the HL gene (not shown). These data confirm that the new sequence is expressed in the RNA contiguous with HL mRNA. Moreover, the possibility that this RNA species represents immature, unspliced HL RNA seems unlikely because several introns are spanned by the primer combinations used, and the observed product sizes fit perfectly with that of mature, fully spliced HL cDNA.

Apparently, the truncated HL mRNA species initially

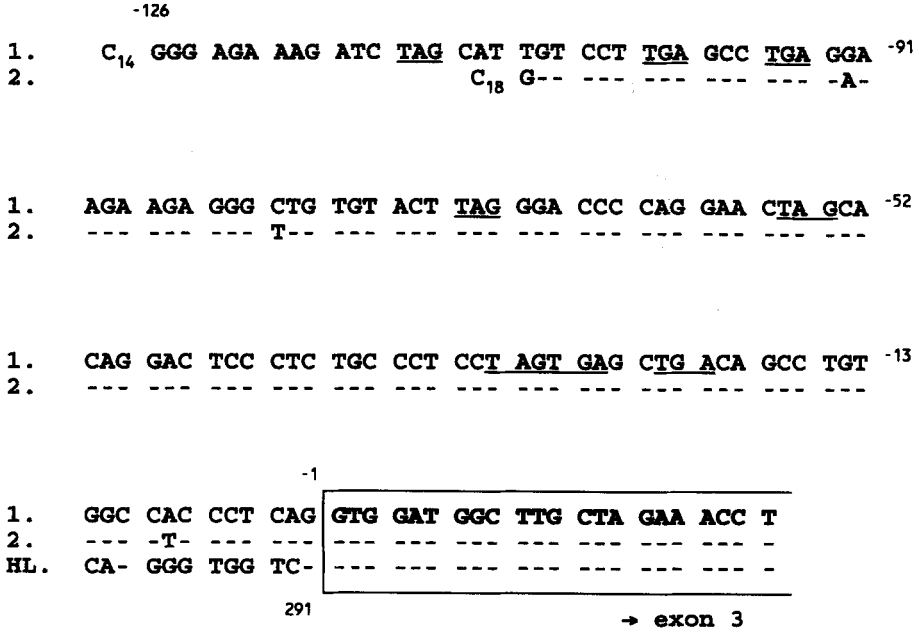


Fig. 4. 5'-RACE of the adrenal HL gene transcript. Two independent clones were obtained by 5'-RACE using two different strategies of nested PCR with HL-specific primers. Both strands of each insert were sequenced using vector-specific oligonucleotides; only part of the sequence between the adapter primer and HL-14 that were used in the last round of nested PCR is given here. The sequence of clone 2 is compared with that of clone 1, and with part of HL cDNA comprising the junction between exon 2 and 3. Bases that are identical to the clone 1 sequence are indicated by hyphens. The sequence corresponding to exon 3 is boxed, whereas the possible termination codons are underlined. The numbering above the nucleotide sequence indicates the position upstream of the exon 3 sequence, whereas the number beneath the HL sequence indicates the position in the cDNA sequence (19).

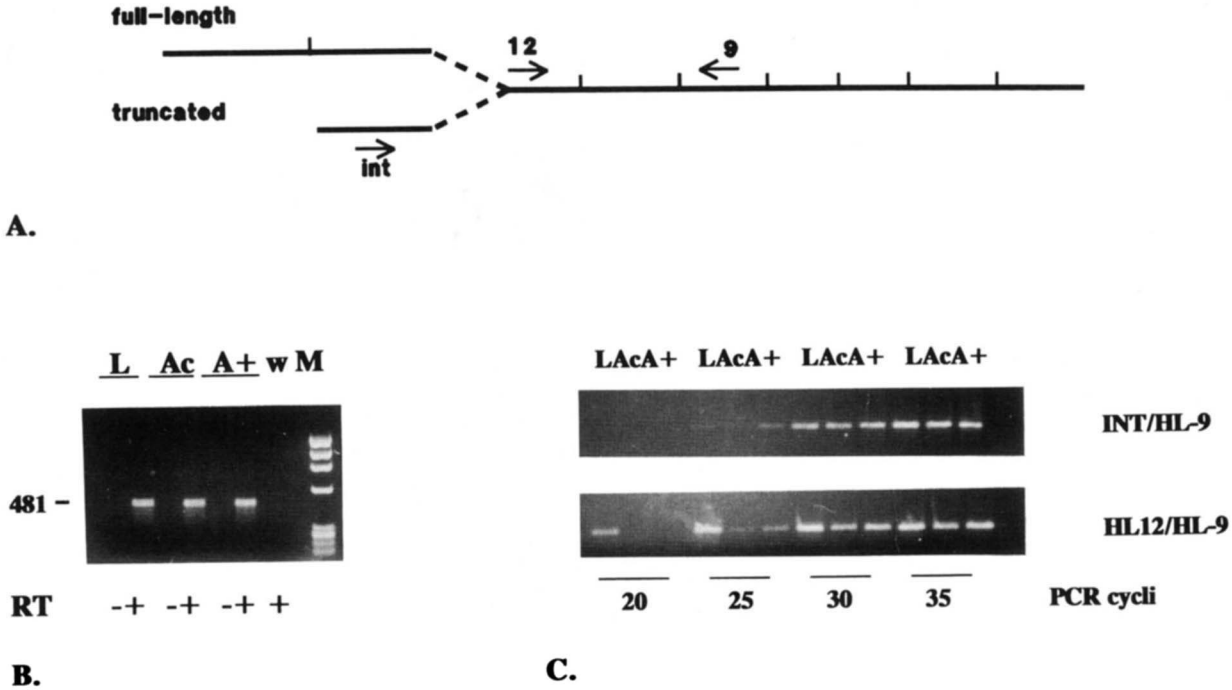


Fig. 5. Expression of the truncated HL mRNA in adrenals and liver. RT-PCR was performed on RNA from liver (L), control adrenals (Ac) and ACTH-stimulated adrenals (A+) using HL-9, and either INT, which recognizes the novel sequence identified by 5'-RACE, or HL-12, which recognizes both full-length and truncated HL cDNA, as indicated schematically in panel A. In panel B, PCR was performed for 35 cycles with INT and HL-9 with (+) or without (–) inclusion of reverse transcriptase in the RT-step. A no-template control (w) was also included. In lane M the ϕX174-HaeIII molecular size markers are run. In panel C, PCR was performed for the indicated number of cycles using INT and HL-9 (upper part) or HL-12 and HL-9 (lower part).

found in adrenals is also present in liver (Fig. 5A). Using INT and HL-9, a UV-detectable RT-PCR product of the truncated message was observed after 30 cycles of amplification with liver RNA, similar to adrenal RNA (Fig. 5C). In contrast, using HL-12 and HL-9, the product of both the truncated plus full-length message was seen after 20 cycles with liver RNA opposed to 30 cycles with adrenal RNA. This finding suggests that, in liver, the truncated message is only a minor component compared to the full-length one, but represents the major, if not only, HL gene transcript in the adrenals.

Pulse-labeling of adrenocortical cells

Freshly isolated adrenocortical cells were incubated in suspension with [^{35}S]methionine. At the end of the pulse, cells and media were separated and the radiolabeled proteins were immunoprecipitated with anti-HL antibodies. **Fig. 6** shows the results obtained with a polyclonal IgG preparation; highly similar results were obtained with a mixture of five different monoclonals (not shown). No radiolabeled protein, either in the extracellular media or in cell lysates, was detected in the 55–60 kDa range corresponding to mature HL secreted by hepatocytes. In the cell lysates, however, two major protein bands were observed in the 40–45 kDa range. Even after 2 h labeling with [^{35}S]methionine, these bands were hardly detectable in the extracellular medium, indicating that these proteins remained mainly intracellular.

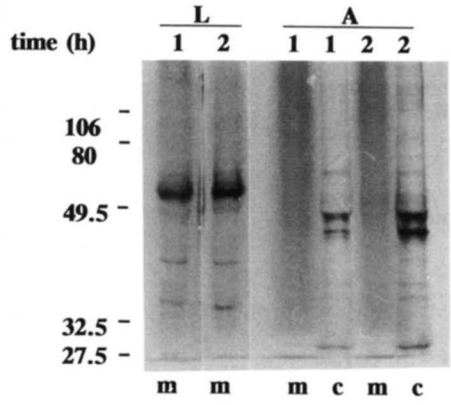


Fig. 6. Pulse-labeling of adrenocortical cells and liver cells with [^{35}S]methionine. Freshly isolated liver parenchymal cells (L) and adrenocortical cells (A) were incubated for the indicated time with [^{35}S]methionine. After pulse-labeling, the cell-free media (m) and cell lysates (c) were incubated with polyclonal anti-HL IgGs immobilized on Sepharose beads. The immunoabsorbed proteins were separated by SDS-PAGE on a 10% gel and visualized by fluorography. The positions of the pre-stained molecular weight markers are indicated.

DISCUSSION

The presence of an HL-like activity in the adrenal glands from rat and other mammals has long been recognized. In the present study, we show that the HL gene is transcribed in rat adrenals. An HL gene transcript was also detected in human adrenals, and in rat ovaries and testes, but not in heart and kidney (21). These findings suggest that expression of the HL gene is confined to steroidogenic organs, at the levels of both mRNA and enzyme activity. However, the transcript found in adrenals differs considerably from the full-length mRNA in liver. If this truncated message is translated, the resulting protein would be quite distinct from HL as well as from the liver-type lipase found in adrenals. The predicted protein would lack the N-terminal part of HL, but would have the same C-terminal part. Its molecular mass would be 45 kDa at the most, depending on whether the first ATG in the truncated message (codon 134, according to the sequence in ref. 19) is used as the translational start site, which is much smaller than the 58 kDa of mature HL. Moreover, since the signal sequence is missing in the truncated message, the translation product would remain in the cytosol. In fact, candidate proteins that meet these criteria were found upon immunoprecipitation from pulse-labeled adrenocortical cells (Fig. 6). Hence, the liver-type lipase found in adrenals cannot possibly be the product of the HL gene transcript described here. This study, therefore, provides strong evidence in favor of the hypothesis that the liver-type lipase found in adrenals is synthesized elsewhere, notably in liver (17, 19, 20). Preliminary data indicate that the HL gene transcript in rat ovaries also lacks the first two exons (D. Vieira, A. J. M. Verhoeven, and H. Jansen, in preparation), which might indicate that this also holds for the liver-type lipase found in ovaries (18). How hepatic lipase is transported to adrenals and ovaries remains unknown at present. In analogy to lipoprotein lipase (36, 37), HL may be displaced from the liver by binding to lipoproteins, which then target the enzyme to these steroidogenic organs.

The finding of a novel HL gene transcript in liver, adrenals, and possibly ovaries, but not in heart and kidney, and the detection of candidate translation products in the 40–45 kDa range in adrenocortical cells suggests this is a unique feature of steroidogenic organs. The intracellular translation product may have an important, though hitherto unknown, function in steroidogenesis. Possibly, the truncated HL mRNA translates into a protein with enzymatic properties different from HL. If the 40–45 kDa proteins found in adrenocortical cells indeed represent the HL gene product, translation must have started from the ATG at codon 134, which is upstream of the sequence coding for the catalytic triade (19). Hence, this protein may possess serine protease activity but possibly towards different substrates. The exon 2–exon 3 junction that would be missing from the adrenal product codes for the β5-loop as identified in the model for closely related pancreatic lipase (38). This loop is proposed to interact with the surface loop that covers the catalytic pocket (34). For pancreatic lipase and lipoprotein lipase, this loop serves an important role in determining substrate specificity (39, 40).

The truncated mRNA found as the major HL gene transcript in adrenals, and as a small fraction of total HL gene transcripts in the liver, starts to diverge from the full-length message at base 291 (cf. ref. 19). In human HL cDNA this position marks the junction between the exons 2 and 3 (25, 26). Unfortunately, the genomic organization of the rat gene is unknown, but the nucleotide sequences surrounding all the exon junctions identified for the human cDNA are highly conserved in the rat (19). It is probably safe to assume that the change from exon 2 to exon 3 in the rat cDNA occurs at the same position. Therefore, we hypothesize that the truncated and full-length messages represent alternate splice forms from a single, initial gene transcript. Alternatively, both species may originate from different transcriptional start sites. The possibility that the two mRNA species are derived from different genes seems less likely. First, evidence for the existence of more than one HL gene in the rat or human genome has not been reported. Second, both transcripts are recognized by HL-5, which anneals to the 3'-untranslated region. This part of the sequence most likely differs among multiple genes, as, in the absence of any selective pressure, noncoding sequences would have diverged much faster than coding sequences.

In conclusion, we have shown here that the HL gene is transcribed in adrenals as well as in liver, but not in nonsteroidogenic organs. This suggests an important role of this gene in cholesterol handling in these organs. In liver, this gene transcribes into two mRNA species, possibly as the result of alternative splicing or an additional transcription start site. Only one of these splice forms is found in the adrenals. Apparently, the splicing or the transcription start site that results in full-length HL mRNA is completely silenced in adrenals. A similarly strict tissue-specificity has been reported for alternative splicing of several other genes such as for mouse α-amylase (41). How this tissue-specific expression is effected remains to be elucidated. The finding of a candidate protein product of the truncated mRNA in adrenals opens the possibility that the HL gene codes for two different proteins, an extracellular enzyme produced in liver and transported to the steroidogenic organs and an intracellular enzyme. Both products may be important to steroidogenesis in these organs.

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REFERENCES

1. Jansen, H., and W. C. Hülsmann. 1985. Enzymology and physiological role of hepatic lipase. *Biochem. Soc. Trans.* **13:** 24–26.
2. Bamberger, M., M. Glick, and G. H. Rothblat. 1983. Hepatic lipase stimulates the uptake of HDL cholesterol by hepatoma cells. *J. Lipid Res.* **24:** 869–876.
3. Collet, X., C. Vieu, H. Chap, and B. P. Perret. 1991. Behaviour of phospholipase-modified HDL towards cultured hepatocytes. II. Increased cell cholesterol storage and bile acid synthesis. *Biochim. Biophys. Acta.* **1081:** 211–219.
4. Jansen, H., and J. C. Birkenhäger. 1981. Liver lipase-like activity in human and hamster adrenocortical tissue. *Metabolism.* **30:** 428–430.
5. Cordle, S. R., S. J. Yeaman, and R. A. Clegg. 1983. Salt-resistant (hepatic) lipase: evidence for its presence in bovine liver and adrenal cortex. *Biochim. Biophys. Acta.* **753:** 213–219.
6. Jansen, H., and W. J. de Greef. 1981. Heparin-releasable lipase activity of rat adrenals, ovaries and testes. *Biochem. J.* **196:** 739–745.
7. Gåfvels, M., G. Bengtsson-Olivecrona, and T. Olivecrona. 1989. Correlation of plasma progesterone concentration to ovarian H-type lipase activity during pseudopregnancy in the rat. *J. Reprod. Fertil.* **86:** 589–598.
8. Hixenbaugh, E. A., and L. G. Paavola. 1991. Heterogeneity among ovarian blood vessels: endogenous hepatic lipase is concentrated in blood vessels of rat corpora lutea. *Anat. Rec.* **230:** 291–306.
9. Andersen, J. M., and J. M. Dietschy. 1978. Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J. Biol. Chem.* **253:** 9024–9032.
10. Gwynne, J. T., and J. F. Strauss, III. 1982. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr. Rev.* **3:** 299–329.
11. Persoon, N. L. M., W. C. Hülsmann, and H. Jansen. 1986. Localization of the salt-resistant heparin-releasable lipase in rat liver, adrenal and ovary. *Eur. J. Cell Biol.* **41:** 134–137.
12. Jansen, H., W. J. de Greef, and J. T. J. Uilenbroek. 1985. Localization of liver-type lipase in rat ovaries and its activity during the estrous cycle and lactation. *Mol. Cell. Endocrinol.* **42:** 253–258.
13. Jansen, H., G. C. Schoonderwoerd, M. G. A. Baggen, and W. J. de Greef. 1983. The effect of corticotrophin on liver-type lipase activity in adrenals and liver and high density lipoprotein subfractions in the rat. *Biochim. Biophys. Acta.* **753:** 205–212.
14. Jansen, H., and W. J. de Greef. 1988. L-type lipase activity in ovaries of superovulated rats. *Mol. Cell. Endocrinol.* **57:** 7–15.
15. Jansen, H., C. Kalkman, J. C. Birkenhäger, and W. C. Hülsmann. 1980. Demonstration of a heparin-releasable liver lipase-like activity in rat adrenals. *FEBS Lett.* **112:** 30–34.
16. Persoon, N. L. M., H. J. Sips, W. C. Hülsmann, and H. Jansen. 1986. Monoclonal antibodies against salt-resistant rat liver lipase. Cross-reactivity with lipases from rat adrenals and ovaries. *Biochim. Biophys. Acta.* **875:** 286–292.
17. Doolittle, M. H., H. Wong, R. C. Davis, and M. C. Schotz. 1987. Synthesis of hepatic lipase in liver and extrahepatic tissues. *J. Lipid Res.* **28:** 1326–1334.
18. Hixenbaugh, E. A., T. R. Sullivan, Jr., J. F. Strauss, III, E. A. Laposata, M. Komaromy, and L. G. Paavola. 1989. Hepatic lipase in the rat ovary. Ovaries cannot synthesize hepatic lipase but accumulate it from the circulation. *J. Biol. Chem.* **264:** 4222–4230.
19. Komaromy, M. C., and M. C. Schotz. 1987. Cloning of rat hepatic lipase cDNA: evidence for a lipase gene family. *Proc. Natl. Acad. Sci. USA.* **84:** 1526–1530.
20. Semenkovich, C. F., S-H. Chen, M. Wims, C-C. Luo, W-H. Li, and L. Chan. 1989. Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J. Lipid Res.* **30:** 423–431.
21. Verhoeven, A. J. M., and H. Jansen. 1994. Hepatic lipase mRNA is expressed in rat and human steroidogenic organs. *Biochim. Biophys. Acta.* **1121:** 121–124.
22. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, a Laboratory Manual. 2nd Ed. Cold-spring Harbor Laboratory Press, New York.
24. Chan, Y. L., R. Gutell, H. F. Noller, and I. G. Wool. 1984. The nucleotide sequence of a rat 18S ribosomal ribonucleic acid gene and a proposal for the secondary structure of 18S ribosomal ribonucleic acid. *J. Biol. Chem.* **259:** 224–230.
25. Ameis, D., G. Stahnke, J. Kobayashi, J. McLean, G. Lee, M. Büscher, M. C. Schotz, and H. Will. 1990. Isolation and characterization of the human hepatic lipase gene. *J. Biol. Chem.* **265:** 6552–6555.
26. Cai, S. J., D. M. Wong, S. M. Chen, and L. Chan. 1989. Structure of the human hepatic triglyceride lipase gene. *Biochemistry.* **28:** 8966–8971.
27. Fort, P., L. Marty, M. Piechaczyk, S. El Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.* **13:** 1431–1442.
28. Gilliland, G., S. Perrin, K. Blanchard, and H. F. Bunn. 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA.* **87:** 2725–2729.
29. Marchuk, D., M. Drumm, A. Saulino, and F. S. Collins. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19:** 1154.
30. Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* **85:** 8998–9002.
31. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
32. Verhoeven, A. J. M., and H. Jansen. 1990. Secretion of rat hepatic lipase is blocked by inhibition of oligosaccharide processing at the stage of glucosidase I. *J. Lipid Res.* **31:** 1883–1893.

33. Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acid Res.* **10:** 459–472.
34. Stahnke, G., R. Sprengel, J. Augustin, and H. Will. 1987. Human hepatic triglyceride lipase: cDNA cloning, amino acid sequence and expression in a cultured cell line. *Differentiation.* **35:** 45–52.
35. Martin, G. A., S. J. Busch, G. D. Meredith, A. D. Cardin, D. T. Blankenship, S. J. T. Mao, A. E. Rechtin, C. W. Woods, M. M. Racke, M. P. Schafer, M. C. Fitzgerald, D. M. Burke, M. A. Flanagan, and R. L. Jackson. 1988. Isolation and cDNA sequence of human postheparin plasma hepatic triglyceride lipase. *J. Biol. Chem.* **263:** 10907–10914.
36. Vilella, E., J. Joven, M. Fernández, S. Vilaró, J. D. Brunzell, T. Olivecrona, and G. Bengtsson-Olivecrona. 1993. Lipoprotein lipase in human plasma is mainly inactive and associated with cholesterol-rich lipoproteins. *J. Lipid Res.* **34:** 1555–1564.
37. Eisenberg, S., E. Sehayek, T. Olivecrona, and I. Vlodavsky. 1992. Lipoprotein lipase enhances binding of lipoproteins to heparan sulfate on cell surfaces and extracellular matrix. *J. Clin. Invest.* **90:** 2013–2021.
38. Van Tilbeurgh, H., M-P. Egloff, C. Martinez, N. Rugani, R. Verger, and C. Cambillau. 1993. Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature.* **362:** 814–820.
39. Dugi, K. A., H. L. Dichek, G. D. Talley, H. B. Brewer, Jr., and S. Santamarina-Fojo. 1992. Human lipoprotein lipase: the loop covering the catalytic site is essential for interaction with lipid substrates. *J. Biol. Chem.* **267:** 25086–25091.
40. Faustinella, F., L. C. Smith, and L. Chan. 1992. Functional topology of a surface loop shielding the catalytic center in lipoprotein lipase. *Biochemistry.* **31:** 7219–7223.
41. Breitbart, R. E., A. Andreadis, and B. Nadal-Ginard. 1987. Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. *Annu. Rev. Biochem.* **56:** 467–495.