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Transient induction of a variant hepatic lipase messenger RNA by corticotropic hormone in rat adrenals

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Abstract

Hepatic lipase (HL) is present not only in liver, but also in steroidogenic organs, where it is thought to mediate cellular uptake of plasma cholesterol. In rat adrenals and ovaries, the HL gene is transcribed into a variant messenger RNA (mRNA) that lacks exons 1 and 2. Treatment of male Wistar rats with corticotropin resulted in a transient 9-fold increase in the variant HL mRNA in the adrenals, which was paralleled by synthesis of 47- to 49-kilodalton HL-related proteins. In contrast, a delayed, but sustained, 6-fold increase in adrenal HL activity was observed. This difference in time course suggests that the HL activity does not reflect HL-like proteins expressed from the variant mRNA. By Northern blotting, the variant HL mRNA was 2.6 kilobase. By screening a rat genomic library, the 5′ end of the variant HL mRNA was located in intron 2 immediately upstream of exon 3. Primer extension analysis mapped the 5′ end at nucleotide 465 upstream of exon 3. In promoter-reporter assays, the intron 2 region (−233/+350 with respect to the putative start site) showed no apparent basal activity in HepG2 hepatoma and NCI-H295R adrenocortical cells. The putative promoter in intron 2 was up-regulated in NCI-H295R human adrenocortical cells by treatment with 8-bromo-cyclic adenosine monophosphate. We conclude that intron 2 of the rat HL gene has an alternative promoter with low activity in adrenals, ovaries, and liver. In rat adrenals, this promoter is transiently activated by corticotropin.

1. Introduction

Hepatic lipase (HL; EC 3.1.1.34) is extracellularly located in the liver of most vertebrates, where it plays an important role in lipoprotein metabolism. The lipase is involved in the conversion of intermediate-density lipoprotein into low-density lipoprotein and in the uptake of very low-density lipoprotein and chylomicron remnants by the liver [1-3]. The enzyme also plays a major role in the metabolism of high-density lipoprotein (HDL). Notably, HL is known to facilitate the uptake of HDL–cholesteryl esters by liver cells [4-6]. Adrenal cortex and ovaries of a number of species including rat contain an extracellularly bound lipase activity that is indistinguishable from HL [7-9]. This lipase activity is thought to originate from liver and to be transported to the steroidogenic organs via the bloodstream [9] in association with HDL [10]. The activity in these organs varies in parallel with steroid hormone output [11-13]. At least in the rat, these organs depend largely on HDL cholesterol (HDL-C) as a source for steroid hormone production. The tissue distribution of HL is similar to that of scavenger receptor class B1 (SR-B1), which mediates delivery of HDL-C to the sites of steroidogenesis [14]. Hepatic lipase may therefore facilitate the uptake of HDL-C via SR-B1. In line with this, SR-B1 expression in adrenal glands is up-regulated when HL activity is inhibited by injection with anti-HL antibodies [15].

Synthesis and secretion of HL protein have not been detected in nonhepatic tissues, except for recent reports on mouse adrenals [16] and mouse and human macrophages [17]. In rat adrenals and ovaries, the HL gene is not expressed into the full-length HL messenger RNA (mRNA). Instead, an alternative form of HL mRNA is found in these tissues [18,19], in which the first 2 coding exons of the HL gene are replaced by an intron-like sequence of unknown origin. We designate this novel sequence as exon 1A. This variant HL mRNA is predicted to translate into a protein that lacks the N-terminal part of liver HL, including the signal sequence and the lid that covers the catalytic pocket. This variant HL mRNA is also expressed in rat liver, but at a much

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lower level than full-length HL mRNA [18,19]. In rat ovaries the amount of variant HL mRNA varied in parallel with cholesterol demand for steroidogenesis [19]. In rat adrenals and ovaries, the expression of the variant HL mRNA coincides with the de novo synthesis of 47- to 49-kilodalton (kd) proteins that are immunologically related to HL [18,19]. For rat ovaries, we have shown that the immunorelated proteins are transiently induced by gonadotropic hormones in parallel with the induction of the variant HL mRNA [19]. These HL-related proteins remained mainly intracellular. Taken together, these observations suggest a possible role for these HL-related proteins in intracellular cholesterol handling in the steroidogenic organs.

In this study, we determined the temporal relationship between HL activity, HL gene transcription, and de novo synthesis of the 47- to 49-kd HL-related proteins in rat adrenals after stimulation with corticotropin. Because the first 2 exons are not used in the expression of the variant HL mRNA, we assumed that transcription occurs from another, hitherto unidentified, promoter. We decided to identify the alternative promoter and to characterize its 5′-flanking region.

2. Materials and methods

2.1. Rat adrenal glands

Male Wistar rats (200-250 g body weight) were fed ad libitum with a standard chow diet (Hope Farm, Wilnis, Netherlands). Animals were killed by decapitation under light diethyl ether narcosis. Adrenal glands were quickly excised, and the surrounding adipose tissue was trimmed off. Hypertrophic adrenals were obtained by treating the animals for the indicated time by daily subcutaneous injection with 50 μg of a synthetic corticotrophin analogue (Synacthen, Novartis, Basel, Switzerland). For each animal, one adrenal was rapidly frozen in liquid nitrogen and used for RNA isolation or measurement of HL activity; the second adrenal was placed in medium and immediately used in pulse-labeling experiments.

2.2. Hepatic lipase activity and immunoprecipitation assays

Three adrenals from 3 different animals were pooled and homogenized in 10 volumes of phosphate-buffered saline (pH 7.4) containing 5 U/mL of heparin and 1 mmol/L benzamidine (4°C) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). After centrifugation (2 minutes, 10 000g, 4°C), the postnuclear supernatant was assayed for HL activity [7], which is defined here as the triacylglycerol hydrolase activity that is sensitive to immunoinhibition with antirat HL immunoglobulin G's (IgGs) [19]. Enzyme activities were expressed as milliunits (nanomole of free fatty acids released per minute).

Three freshly dissected adrenal glands from 3 animals were pooled and minced using a razor blade. The slices were pulse-labeled with Tran^{35}S-label (ICN, Cosa Mesa, CA) for 2 hours, and HL-related proteins were immunoprecipitated with goat antirat HL IgGs immobilized onto Sepharose 4B beads (Pharmacia, Uppsala, Switzerland), as described previously [18,19]. The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels followed by fluorography using Amplify (Amersham Biosciences, Amersham, UK).

2.3. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from 2 to 4 adrenals by the method of Chomczynski and Sacchi [20]. RNA concentrations were determined by spectrophotometry at 260 nm [21]. The quality of the isolated RNA was judged from the ratio of 28S over 18S ribosomal RNA upon electrophoresis in a 1% agarose/Tris-borate-EDTA (TBE) gel. Hepatic lipase mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined by reverse transcriptase–polymerase chain reaction (RT-PCR) starting from 1 μg total tissue RNA. After synthesis of random-primed complementary DNA (cDNA), the mixture was divided into parallel PCR incubations. Amplification was performed for 35 cycles with HL-specific primer pairs or 20 cycles with GAPDH-specific primer pairs. Hepatic lipase–specific forward primers were RHL-3 (5′-CGG GGG CTC CTT CCA GCC TGG-3′, nt 756-776; numbering according to the rat cDNA sequence [22]), RHL-12 (5′-TGG CTT GCT AGA AAC CTG G-3′, nt 297-315), or INT (5′-GCA TTG TCC TTG AGC CTG AG-3′, positions −112 to −93 upstream of exon 3 [18]), whereas RHL-2 (5′-CAG ACA TTG GCC CAC ACT-3′, nt 1307-1289) and RHL-9 (5′-GGC ATC ATC TGG AGA AAG GC-3′, nt 660-641) were used as reverse primers. The GAPDH-specific primers 5′-TCT TCT TGT GCA GTG CCA GC-3′ (nt 35-54) and 5′-CTC TCT TGC TCT CAG TAT CC-3′ (nt 1120-1101) span the entire coding sequence [23]. All RT-PCR experiments included no-template and no-RT controls.

Hepatic lipase mRNA was semiquantified by competitive RT-PCR using an internally deleted complementary RNA as competitor [18].

2.4. Genomic library screening for exon 1A

Standard molecular biology techniques were used [21]. A rat genomic library in λ DASH II (Stratagene, La Jolla, CA) was screened with the oligonucleotide INT that recognizes the sequence in the variant HL mRNA upstream of exon 3 [18]. Briefly, a total of 10^6 plaques were screened with ^{32}P-end–labeled INT. After hybridization, the final washing condition was 5 minutes at room temperature in 0.15 mol/L NaCl/0.015 mol/L sodium citrate/0.1% (wt/vol) SDS (pH 7.0). Two plaques that were positive on duplicate filters were plaque-purified 3 times [21]. The DNA from these 2 clones was analyzed by restriction mapping using the endonucleases *Bgl*II, *Hin*dIII, *Pvu*II, *Sac*I, *Sma*I, and *Xho*I (Roche, Almere, Netherlands), either alone or in combination with *Eco*RI. The digestion products were separated on a 0.7% agarose gel, followed by denaturation with 0.5 mol/L

NaOH, 1.5 mol/L NaCl, and overnight blotting to Hybond membranes. The membranes were hybridized with different ^{32}P-end–labeled HL-specific oligonucleotides: RHL-11 (5′-CTG TGG ACA AGG CGT GGG-3′, nt 78-95), RHL-13 (5′-TTG TCA TGA TCA TCC ACG GG-3′, nt 266-285), RHL-14 (5′-CAC CCA CTA TCT TCC AGA TCC-3′, nt 314-334), RHL- 8 (5′-TTA ATT GGG TAC AGC CTG GG-3′, nt 508-527), RHL-3, and INT, which recognize exons 1, 2, 3, 4, 5, and 1A, respectively. After stripping, the filters were rehybridized with a different probe. A 5.6-kilobase (kb) *Eco*RI-*Eco*RI fragment that was positive for INT was isolated from clone I and subcloned into pBluescript KS$^-$. From this construct, a 577-base pair (bp) *Hin*dIII-*Bgl*II fragment was isolated and subcloned into pBluescript KS$^-$. Sequencing of both strands was done with the Thermo-sequenase dye terminator kit (Amersham Biosciences) and the ABI 377 sequencer (Applied Biosystems, Foster City, CA).

2.5. Genomic library screening for exon 1 and the 5′-regulatory region

The same genomic library was also used for isolation of the normal HL promoter region, using an HL cDNA probe corresponding to exons 1 and 2. The probe was generated by RT-PCR on 1 μg liver RNA using the oligonucleotides 5′-GGT AAG ACG AGA GAC ATG G-3′ (nt 1-19; numbering according to Ref [22]) and 5′-CCC GTG GAT GAT CAT GAC AA-3′ (nt 266-285) as forward and reverse primers, respectively. The RT-PCR product was isolated by agarose gel electrophoresis and ^{32}P-labeled using [α^{32}-P]dCTP and the Megaprime kit from Amersham; 10^6 plaques were screened with this probe; final wash step was for 5 minutes at 65°C in 0.03 mol/L NaCl/0.003 mol/L sodium citrate/0.5% SDS (pH 7.0). Two positive clones were identified, which were plaque-purified 3 times. One of these clones was selected for further analysis. Phage DNA was isolated and digested with *Eco*RI. A 7-kb fragment [24] was cloned into pBluescript KS$^-$ and its identity with the 5′-regulatory region of the rat HL gene was verified by sequence analysis.

2.6. Southern blot analysis of genomic DNA

Rat genomic DNA was isolated from the liver of a healthy 3-month-old male Wistar rat [21]. In parallel incubations, 10 μg of this genomic DNA was digested overnight with 10 U of the restriction enzymes indicated. After size separation by agarose gel electrophoresis, the DNA fragments were transferred to a Hybond membrane and denaturated in 1.5 mol/L NaCl/0.5 mol/L NaOH. The filter was screened with 20 μg of ^{32}P-labeled *Hin*dIII-*Bgl*II 577-bp DNA fragment. The filter was finally washed for 5 minutes at 60°C in 0.03 mol/L NaCl/0.003 mol/L sodium citrate/0.5% SDS (pH 7.0).

2.7. Northern blotting

A rat multiple-tissue Northern blot (Clontech, Palo Alto, CA), which contained 2 μg poly(A)-enriched RNA from several tissues, was probed with oligonucleotide AIB (antisense to INT). After stripping, the blot was reprobed successively with ^{32}P-labeled oligonucleotide RHL-14 (specific for exon 3) and a 1.1-kb human GAPDH cDNA probe, according to the manufacturer's instructions. After hybridization with the oligonucleotides, the blot was washed for 10 minutes at 60°C in 0.3 mol/L NaCl/0.03 mol/L sodium citrate/0.5% SDS (pH 7.0), and then exposed to autoradiography film. After hybridization with the cDNA probe, the blot was washed finally for 20 minutes with 0.015 mol/L NaCl/0.0015 mol/L sodium citrate/0.5% SDS (pH 7.0) at 65°C.

2.8. Primer extension analysis

Primer extension analysis was performed according to Ref [21] using total RNA isolated from the liver of a healthy 3-month-old Wistar rat. Three different primers, corresponding to sequences located in the variant HL mRNA upstream of exon 3, were used: AIB (antisense to INT), ext-1 (5′-GAT TTC TCA ATC TCG TGC AG-3′, nt −169 to −150; numbering relative to the exon 3 sequence), and ext-2 (5′-GTC ATT GTC TGA ATC TTT CCC-3′, nt −342 to −322). Of the ^{32}P-end–labeled primers, 2×10^5 cpm was hybridized to 50 μg total RNA in the presence of annealing buffer (0.15 mol/L KCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.3). The primer-RNA mixture was treated for 2 minutes at 95°C, incubated for 5 minutes at 70°C followed by slow cooling to room temperature. After ethanol precipitation, the pellet was resuspended in 25 μL of reverse transcriptase cocktail (0.56 mmol/L of each deoxynucleotide triphosphates (dNTP), 50 μmol/L Tris [pH 8.3], 50 μmol/L KCl, 5 mmol/L dithiotreitol (DTT), 5 mmol/L $MgCl_2$, 20 U RNAsin [Promega, Madison, WI], 100 U MMLV-RT [Promega]), and primer extension was performed by incubation at 42°C for 90 minutes. After phenol-chloroform extraction and ethanol precipitation, the extended primer was resuspended in 5 μL of 10 mmol/L Tris-HCl/1 mmol/L EDTA buffer (pH 8.0). After addition of 3 μL formamide loading buffer (United States Biochemicals, Cleveland, OH) and denaturation at 70°C, the mixture was run on a 6% polyacrylamide/7 mol/L urea sequencing gel alongside a radioactive sequencing ladder. The latter was prepared from the 577-bp *Hin*dIII-*Bgl*II fragment using the same primer as in the extension reaction. DNA radioactive sequencing was performed using the Sequenase-2 kit from United States Biochemicals. After gel electrophoresis, the sequencing gel was exposed to autoradiography film.

2.9. Promoter activity

For in vitro studies, the 577-bp *Hin*dIII-*Bgl*II fragment was cloned into the pCAT-Basic reporter plasmid (Promega) to generate the intron 2 (−233/+350)–CAT construct. Similarly, the 5′-upstream regulatory region of the rat HL gene was cloned into pCAT-Basic. From the 7-kb *Eco*RI-*Eco*RI fragment containing the 5′-regulatory region of the

HL gene, the *Pst*I-*Xba*I (−437 to +9) fragment was used (HL [−437/+9]–CAT).

HepG2 hepatoma cells were cultured at 37°C and 5% CO_2 in Dulbecco modified Eagle medium (ICN) supplemented with 10% (vol/vol) fetal calf serum (Gibco, Breda, Netherlands) and penicillin/streptomycin. At 24 hours before transfection, the cells were plated in 60-mm culture dishes at 20% to 30% confluence. At 3 hours before transfection, the medium was refreshed. Transfections were performed by the calcium-phosphate coprecipitation method using 10 μg of the CAT reporter test plasmid and 0.4 μg of the RSV β-galactosidase expression plasmid (Promega), as described previously [25]. Promoter activity was expressed as picogram of CAT/nanogram of β-galactosidase to correct for differences in cell number and transfection efficiency.

NCI-H295R adrenocortical cells were cultured in Dulbecco's modified Eagle medium/Ham F12 medium (Gibco), supplemented with 2% fetal calf serum, 15 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 10 μg/mL of insulin-transferrin–sodium selenite (Roche), 10 nmol/L hydroxycortisone (Merck, Darmstadt, Germany), 10 nmol/L β-estradiol (Sigma, St Louis, MO), and penicillin/streptomycin. At 24 hours before transfection, the cells were plated in 6-well plates at 50% confluence. Transfections were performed with Lipofectamine-Plus (Invitrogen, Breda, Netherlands) according to the manufacturer's instructions, using 1.0 μg CAT reporter and 0.25 μg RSV β-galactosidase expression plasmid per well. When indicated, 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP; Sigma) was added to the medium 4 hours after transfection at a final 300 μmol/L. Incubations were continued for 48 hours, and promoter activity was determined as described above.

2.10. Data analysis

The annotated data of the rat genome project were accessed through the internet at http://www.ensembl.org/Rattus_norvegicus/. Similarly, data on the human, mouse, and dog genome projects were accessed at http://www.ensembl.org/. Sequence alignments were performed with mVista [26] through http://genome.lbl.gov/vista/. A search for potential promoter sites and transcription factor binding sites was performed with the Genomatix PromoterInspector and MatInspector computer programs at http://www.genomatix.de/, respectively.

Experimental data are expressed as mean ± SD. Differences were tested for statistical significance by Student *t* test.

3. Results

3.1. Corticotropin-induced expression of the HL gene in rat adrenals

Male adult rats were treated for several days by daily injections with synthetic corticotropin. This resulted in a gradual, up to 4-fold increase in adrenal weight after 6 days. The HL activity in the adrenals increased from 10.2 ± 0.8 to 15.4 ± 0.6 mU per 2 adrenals at day 2 of treatment (n = 3; $P < .05$). The activity continued to increase to 64.4 ± 1.1 mU per 2 adrenals at day 6 (n = 3; $P < .05$) and remained high at least until 9 days of corticotropin treatment (Fig. 1). On the basis of adrenal weight, adrenal HL activity gradually increased from 107 ± 13 mU/g in control animals to 136 ± 28 mU/g after 4 days of corticotropin treatment (n = 3, NS) and 213 ± 50 mU/g after 6 days (n = 3, $P < .05$).

Reverse transcriptase–polymerase chain reaction on RNA isolated from control adrenals using the primers RHL-2 and RHL-3 yielded the expected 552-bp product, whose identity with part of HL cDNA was confirmed by restriction mapping. Upon treatment of the rats with corticotropin, the amount of RT-PCR product transiently increased, with highest levels observed at days 1 and 2 of treatment (Fig. 1A). In contrast, the amount of PCR product

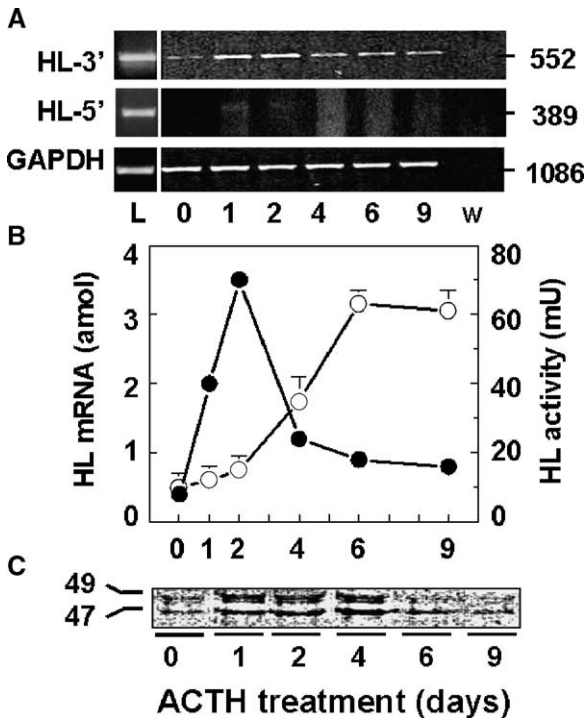

Fig. 1. Transient HL gene expression in rat adrenals induced by corticotropin treatment. Male rats were treated with corticotropin for the number of days indicated, and then the animals were killed and the adrenals dissected. Of each animal, one adrenal was used for RNA isolation, whereas the other was used for protein analysis. A, RT-PCR was performed on total RNA isolated from a pool of 3 adrenals using the primers RHL-12 and RHL-9, and RHL-2 and RHL-3, to detect the 5′ and 3′ part of HL mRNA, respectively. GAPDH mRNA was used as external standard. The numbers indicate the sizes (in bp) of the RT-PCR products. B, The time-dependent increase in the amount of adrenal HL mRNA (●; in amol/μg total RNA) is compared with adrenal HL activity (○; in mU per 2 adrenals; mean ± SD, n = 3). C, The freshly isolated adrenals of 3 animals were sliced and then pulse-labeled with [^{35}S]methionine for 2 hours. After cell lysis, HL-like proteins were immunoprecipitated from the lysates. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography. The location of the 47- and 49-kd bands in the gel is indicated. The data are representative for 3 similar experiments. L and w indicate liver total RNA and water, as positive and negative controls, respectively.

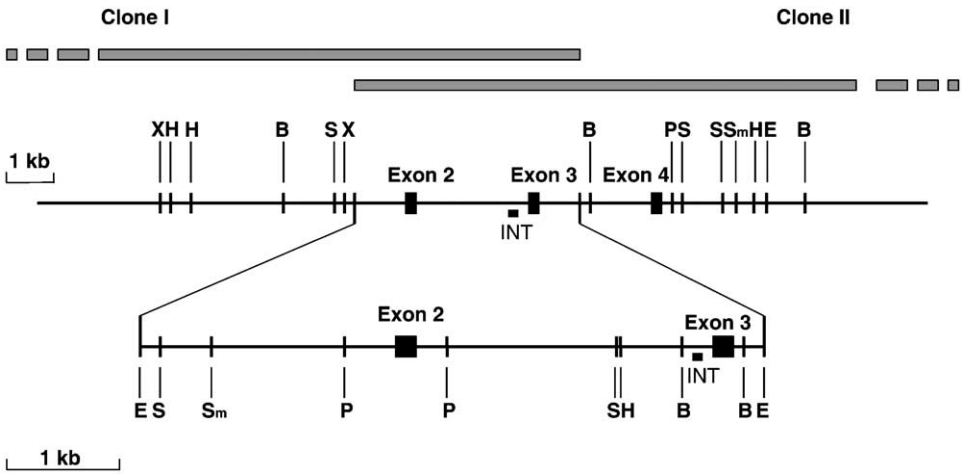

Fig. 2. Restriction map of rat genomic clones I and II. Clones I and II were isolated from the rat genomic library by hybridization to oligonucleotide INT. A partial restriction map was generated with the enzymes *Bgl*II (B), *Eco*RI (E), *Hin*dIII (H), *Pvu*II (P), *Sac*I (S), *Sma*I (Sm), and *Xho*I (X). The positions of exons 2, 3, and 4 are indicated, as well as the approximate position where the oligonucleotide INT hybridizes.

generated with GAPDH-specific primers was hardly affected by corticotropin treatment. Quantification of HL mRNA by competitive RT-PCR using an HL complementary RNA with an internal 80-nt deletion as competitor showed that control adrenals contained approximately 0.4 amol of HL mRNA per microgram of total RNA (Fig. 1B), in agreement with our previous report [18]. Upon corticotropin treatment, the amount of HL mRNA gradually increased to 3.5 amol/μg total RNA at day 2. Thereafter, the amount of HL mRNA decreased again to near-control levels after 6 to 9 days. Hence, the effect of in vivo corticotropin treatment on adrenal HL mRNA expression showed a strikingly different pattern compared with HL activity in rat adrenals.

Although part of the HL gene transcript was detected in rat adrenals, and shown to be transiently up-regulated by stimulation with corticotropin, we were unable to amplify the 5′ end of HL mRNA including exon 1 or 2, either from control [18] or from stimulated adrenals (Fig. 1A). Using the oligonucleotides INT and RHL-9, which are specific for exons 1A and 5, respectively, the expected 481-bp PCR product was obtained with all adrenal RNA preparations. The amount of PCR product generated by this primer pair was also transiently increased after 1 and 2 days of corticotropin treatment, in parallel with the PCR product generated with the primers RHL-2 and RHL-3 (data not shown). Therefore, the transient increase in the amount of HL gene transcript in rat adrenals is entirely due to up-regulation of the variant form in which exons 1 and 2 are replaced by exon 1A.

3.2. De novo synthesis of HL-related proteins

The de novo synthesis of HL-related proteins was studied by pulse-labeling with [^{35}S]methionine in whole-adrenal slices followed by immunoprecipitation with polyclonal anti-HL IgGs. With the adrenals from control rats, no immunoreactive proteins were found in the 55- to 60-kd range corresponding to full-length HL. Instead, 2 major ^{35}S-labeled protein bands with apparent molecular weight in the 47- to 49-kd region were detected (Fig. 1C). Upon in vivo stimulation of the adrenals with corticotropin, the ^{35}S incorporation into these bands increased several-fold. The incorporation of radioactivity was highest at days 2 and 4, and decreased again thereafter to near-control levels at days 6 and 9. Hence, the corticotropin-induced up-regulation of de novo synthesis of the 47- to 49-kd proteins occurred concomitantly with the up-regulation of the variant HL gene transcript in the adrenals.

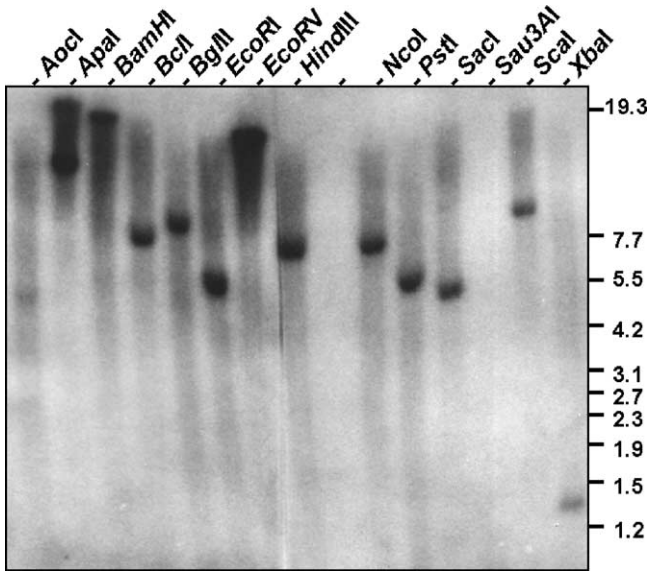

Fig. 3. Southern blot analysis of rat genomic DNA. Rat genomic DNA was digested with the endonucleases indicated. After separation by agarose gel electrophoresis, the DNA fragments were transferred to Hybond-N membrane. The membrane was hybridized with ^{32}P-labeled 577-bp *Hin*dIII-*Bgl*II fragment and exposed to autoradiography film. The migration of the molecular size markers (Roche) is indicated in kilobase.

3.3. Mapping of exon 1A to intron 2 of the rat HL gene

To determine the location of exon 1A in the rat genome, we screened a rat genomic library with oligonucleotide INT, which recognizes the known part of exon 1A. From the library, 2 positive clones (I and II) were identified and isolated. A restriction map of both clones was obtained after digestion with a number of restriction enzymes and hybridization with oligonucleotide probes specific for exons 2, 3, 4, and 1A (Fig. 2). The map reveals that the clones contained overlapping sequences of the rat HL gene. Clone I contains exons 2 to 3 and clone II contains exons 2 to 4. Neither clone hybridized with an oligonucleotide specific for exon 1 or 5. From this analysis, we deduced that intron 2 spans approximately 2.5 kb, whereas intron 3 spans approximately 3 kb. Because exon 1 is not included in clone I, and exon 5 is not included in clone II, the length of introns 1 and 4 exceed 5 and 9 kb, respectively. INT hybridized with DNA fragments that contained the intron 2 sequence

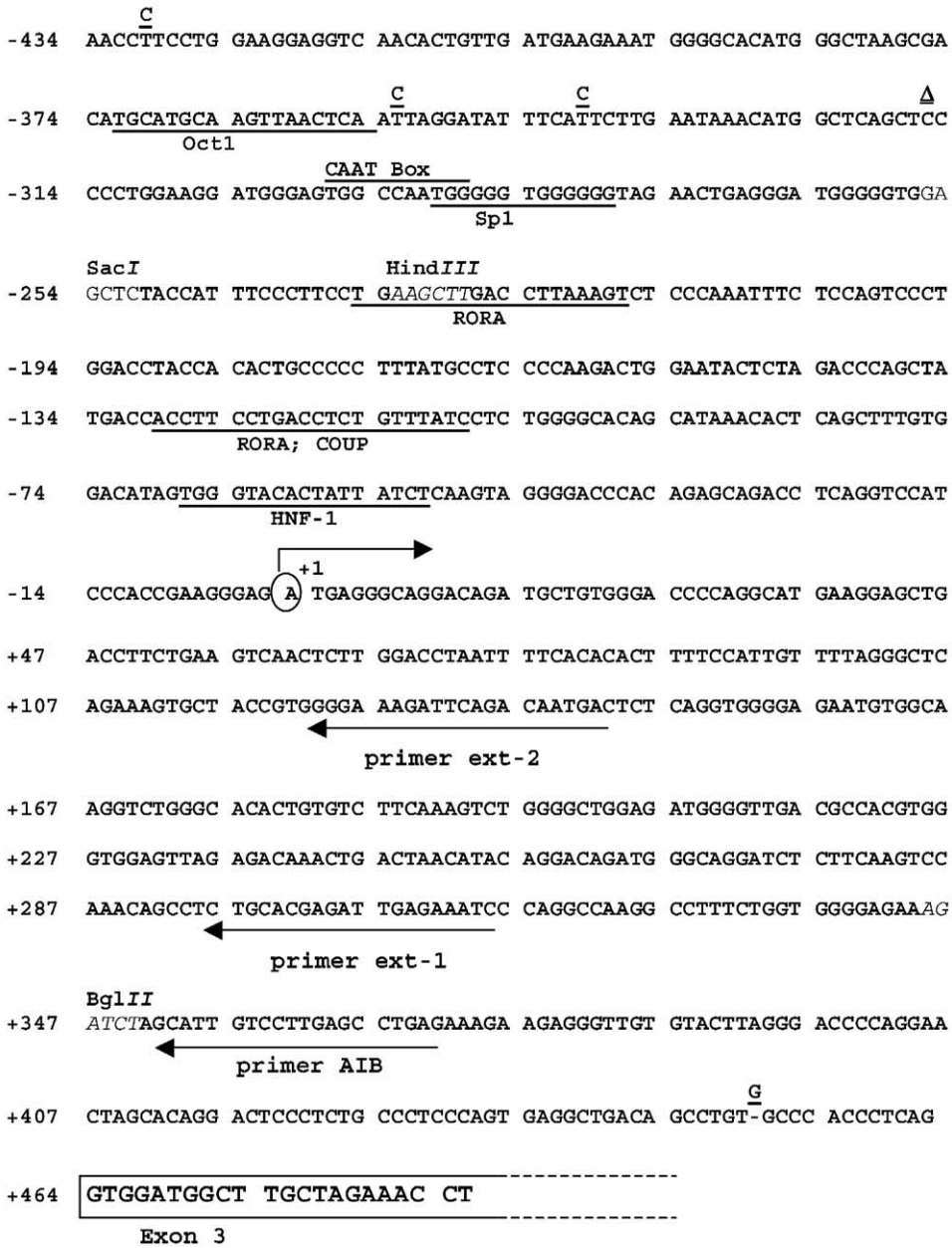

Fig. 4. Nucleotide sequence of the alternative promoter region in intron 2 of the rat HL gene. Numbering is according to the putative transcription start site (indicated by the arrow above the sequence); the first nucleotide of the transcript is denoted as +1. Exon 3 is boxed. Oligonucleotides used for primer extension are indicated by the arrows (5′→3′) underneath the sequence. The *Hin*dIII and *Bgl*II sites used for cloning into the reporter plasmid are indicated in italics. Putative transcription factor binding sites are underlined. Nucleotides that are at variance with data from the rat genome project (LIPH gene, ENSRNOG00000015747) are indicated. Δ indicates the presence of an extra nucleotide.

(Fig. 2). The location of exon 1A within intron 2 was confirmed by PCR on genomic rat DNA using primer pairs that flank intron 2. The used upstream and downstream primers were 5′-TTG TCA TGA TCA TCC ACG GG-3′ and 5′-CAC CCA CTA TCT TCC AGA TCC-3′, respectively. The resulting PCR product was approximately 2.5 kb and hybridized with the exon 1A–specific oligonucleotide INT (data not shown). Sequencing of the 3′ half of the PCR product established identity with the intron 2 sequence obtained from the isolated rat genomic clones.

To test the possibility that multiple HL-like genes exist in the rat genome, we subjected rat genomic DNA to digestion with 14 different restriction enzymes. Upon Southern blotting, the membrane was hybridized with the 577-bp *Hin*dIII-*Bgl*II fragment that contained the intron 2 sequence just upstream of exon 3. With 9 of 14 enzymes, an unambiguous single hybridizing band was found (Fig. 3). The size of the hybridizing bands obtained with *Bgl*II, *Eco*RI, *Hin*dIII, and *Sac*I were in accordance with the digestion maps of clones I and II isolated from the rat genomic library. No hybridizing bands larger than approximately 900 bp were obtained after *Sau*3AI digestion. The results with the 4 other enzymes used were not informative. These results do not indicate the presence of multiple HL-like genes in the rat genome. Therefore, it is unlikely that the alternative transcript observed in rat liver and steroidogenic organs is product of a gene distinct from the HL gene.

A 5.7-kb *Eco*RI fragment containing entire intron 2 was isolated from clone I and subcloned into pBluescript KS$^-$. Further digestion and sequencing analysis of this clone localized the exon 1A sequence in intron 2 immediately upstream of, and contiguous with, exon 3 (Fig. 4). After finishing this part of our study, the rat genome including the entire HL gene sequence has become available publicly (LIPH gene, ENSRNOG00000015747). Alignment of our sequence with that of the LIPH gene confirmed identity with intron 2 of the rat HL gene.

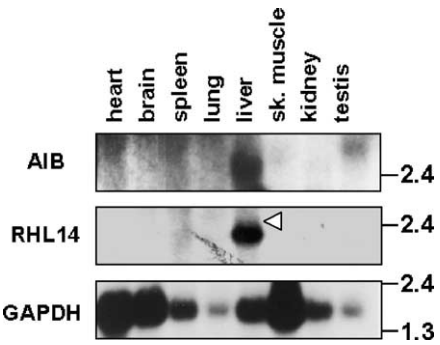

Fig. 5. Northern blot analysis. A rat multi-tissue Northern blot was probed successively with ^{32}P-labeled oligonucleotide AIB (specific for intron 2; specific activity, 0.5 × 10^9 dpm/μg; 5 × 10^6 dpm/mL), ^{32}P-labeled oligonucleotide RHL-14 (specific for exon 3; specific activity, 0.4 × 10^9 dpm/μg; 0.5 × 10^6 dpm/mL), and finally with a ^{32}P-labeled human GAPDH cDNA probe. The arrowhead in the middle panel points to a faint 2.6-kb shoulder band. Note that the differences in signal intensities with the GAPDH probe may reflect different expression levels rather than RNA loading. The migration of RNA markers (in kb) is indicated.

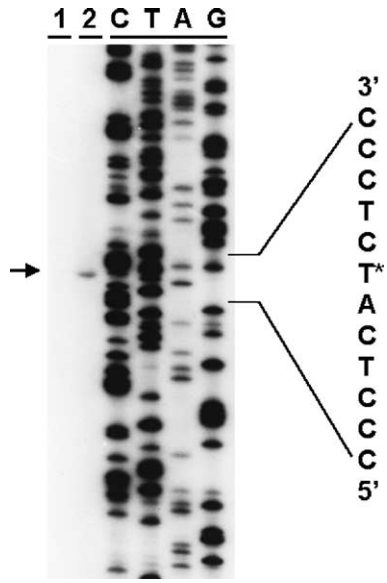

Fig. 6. Mapping of the variant HL mRNA 5′ end by primer extension analysis. The ^{32}P-labeled oligonucleotide ext-2, complementary to intron 2 sequence (Fig. 4), was hybridized to 50 μg of yeast RNA (lane 1) or rat liver RNA (lane 2). After extension of the oligonucleotide by reverse transcription, the DNA product was analyzed by polyacrylamide gel electrophoresis. The 5′ end of the transcript was determined by running in parallel DNA fragments of a sequencing reaction performed with ext-2 on the 577-bp *Hin*dIII-*Bgl*II fragment. The sequence represents the complementary strand of the intron 2 region. Asterisk indicates the transcription start site on the complementary strand.

3.4. Expression of the variant HL mRNA in rat liver

Northern blot analysis of poly(A)-rich RNA isolated from different rat tissues is shown in Fig. 5. The exon 1A–specific oligonucleotide AIB (which is antisense to INT) hybridized with a single RNA band of approximately 2.6 kb. This signal was obtained with RNA from liver, but not with RNA from any other tissue on the blot. As expected [22,27,28], the exon 3–specific oligonucleotide RHL-14 hybridized with an RNA band of approximately 1.9 kb in liver, but not in any of the other tissues tested. The additional band of about 2.6 kb was not clearly visible. These data indicate that the variant HL mRNA is 2.6 kb long and is expressed in rat liver at a much lower level compared with full-length HL mRNA.

The 5′ end of the variant HL mRNA was localized by primer extension using rat liver RNA. The primers AIB and ext-1, which recognize a sequence immediately upstream of exon 3 and the sequence at −169 to 150 nt upstream of exon 3, respectively, gave distinct products larger than 350 and 250 nt (data not shown). With primer ext-2 (−342 to −322 nt), a single product of approximately 140 nt was obtained (Fig. 6). Alignment of this product with the sequencing ladder of the 577-bp *Hin*dIII-*Bgl*II fragment pinpointed the 5′ end of the variant HL mRNA at the A residue, 465 nucleotides upstream of exon 3. These data suggest that

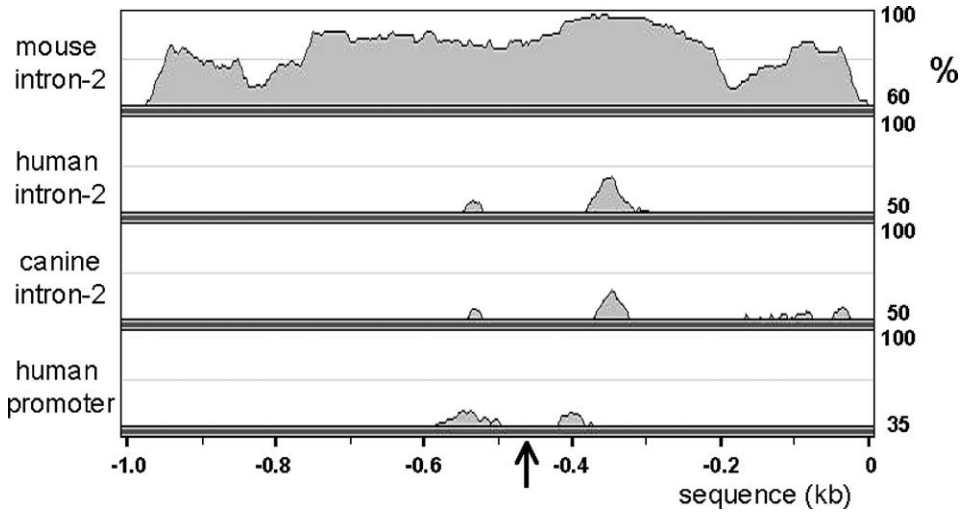

Fig. 7. Vista plot of the alignment of intron 2 from rat, mouse, human, and dog, and the human authentic HL promoter region. The −1000/+10 region of rat intron 2 (numbering relative to the start of exon 3) was aligned by the MLAGAN algorithm of the mVista program with corresponding regions of the mouse, human, and dog HL genes, and with the −500/+500 region of the human HL gene. The arrow indicates the position of the putative transcription start site in the rat intron 2 sequence.

the variant HL mRNA is transcribed from an alternative promoter within intron 2 upstream of this A-465 nucleotide.

3.5. Promoter activity

Screening the entire intron 2 sequence with the Genomatix PromoterInspector software did not identify any potential promoter region. However, this program also failed to find the authentic promoter in the rat and human HL gene when the appropriate genome sequences were submitted. Multiple alignment of the intron 2 sequences of the rat, mouse, human, and dog HL genes with mVista [26] showed little conservation except between rat and mouse (Fig. 7). However, there was a remarkable homology of two 50-bp stretches among the 4 sequences, one upstream and one downstream of the putative transcription start at the A-465 nucleotide. The former sequence was also remotely related to the −50/−100 region of the authentic human HL promoter. Using MatInspector [29], a potential HNF-1 site was identified in this homologous sequence in rat intron 2 and the authentic human HL promoter at a similar position relative to the transcriptional start site. Several potential gene regulatory elements could be identified further upstream in the rat intron 2 sequence, such as RORA, COUP, oct-1, Sp1, and CCAAT-box sites. A clear TATA box, however, was not found.

The 577-bp *Hin*dIII-*Bgl*II fragment was cloned into the reporter vector pCAT-Basic to generate intron 2-CAT (−233/+350 with respect to the putative start site) to test the transcriptional activity of this intron 2 region in liver cells. As a reference the HL (−437/+9)–CAT plasmid was used, which contained the conventional rat HL promoter region. Upon transient transfection of the HepG2 human hepatoma cell line, the activity of the conventional HL promoter was 53% ± 9% of that of the SV40 promoter (n = 4). In this assay, the intron 2 region displayed only weak promoter

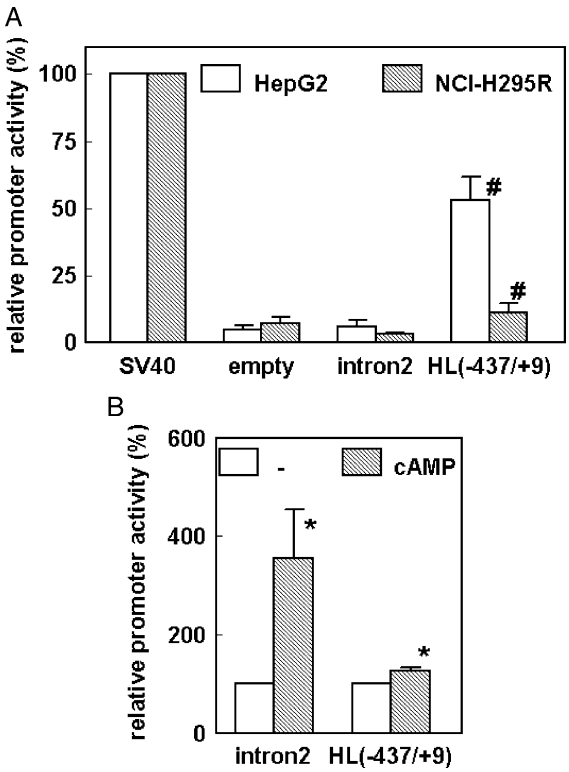

Fig. 8. Transcriptional activity of the intron 2 region in transiently transfected hepatoma and adrenocortical cells. A, Intron 2 and HL (−437/+9) CAT reporter plasmids were transfected into HepG2 (open bars) and NCI-H295R cells (hatched bars). As reference, parallel transfections with pCAT-Basic and pCAT-SV40 were performed. B, The plasmids were transfected into NCI-H295R cells, and then incubated for 44 hours without (open bars) or with 0.3 mol/L 8-Br-cAMP (hatched bars). At 48 hours posttransfection, CAT and β-galactosidase expression was determined. The results are calculated as CAT/β-galactosidase ratio, and given as percentage of the ratio obtained with pCAT-SV40 (panel A) or as a percentage of the ratio obtained without 8-Br-cAMP treatment (panel B). Data are means ± SD for 4 independent experiments. $^{\#}$and $^{*}P < .05$ relative to empty plasmid and control medium, respectively.

activity (6.9% ± 1.9%), which was not significantly different from that of pCAT-Basic (Fig. 8A). Similar results were obtained in transient transfection assays using NCI-H295R human adrenocortical cells, except that the activity of the conventional promoter (11.4% ± 3.5%) was slightly, but significantly ($P < .05$; n = 4) above the empty pCAT-Basic (7.1% ± 2.4%). Hence, the putative promoter of the variant HL appears to have low, or no, basal activity. Treatment of the adrenocortical cells with 8-Br-cAMP, which mimics the effect of corticotropin, slightly increased the activity of the conventional promoter (1.3 ± 0.1-fold; $P < .05$; n=4; Fig. 8B), whereas the activity of the intron 2 region was stimulated 3.5 ± 1.0-fold ($P < .05$; n=4).

3.6. Possible translation product

The 3 possible reading frames of the alternative transcript that consists of exon 1A, followed by exons 3 to 9, were searched for possible translation products. By far the longest open reading frame starts at an AUG within exon 4 and would result in a 38-kd protein that is identical to the C-terminal part of HL (amino acids 134-472 [22]). This open reading frame extends 243 nt further upstream of this AUG and would correspond to an HL-related protein of maximally 47 kd. Despite repetitive double-strand sequencing reactions, no potential in-frame AUG codon was found in this region of the sequence.

4. Discussion

The data we present here suggest that the rat HL gene can be transcribed from an alternative promoter within intron 2. In rat liver, this alternate promoter is much less active than the conventional promoter. In adrenals and ovaries, transcription is exclusively from the alternative promoter. Its product is a variant HL mRNA of approximately 2.6 kb, which is identical to full-length HL mRNA except that exons 1 and 2 have been substituted by a long 5′ extension of exon 3. Rat liver contains approximately 0.4 amol of the variant HL mRNA compared with 16 amol of the full-length HL mRNA per microgram of total RNA [18]. When expressed in human hepatoma HepG2 cells, the alternative promoter showed only a weak activity compared with the conventional rat HL promoter. This low promoter activity is in good agreement with the low expression levels of the variant HL mRNA compared with full-length HL mRNA in liver. The amount of the variant HL mRNA in whole rat adrenals and ovaries ranges from 0.4 to 4 amol/μg total RNA (this article) and 0.01 to 0.4 amol/μg total RNA [19], respectively, depending on the hormonal status of the animals. The expression of the variant HL mRNA in adrenals and ovaries varies in parallel with [^{35}S]methionine incorporation into 47- to 49-kd HL-immunorelated proteins (Fig. 1C; [18,19]). Sequence analysis of the variant HL transcript revealed that the longest open reading frame starts from an AUG within exon 4 (codon 134 [22]) and would translate into the C-terminal 38.5-kd part of the HL protein. The observed expression and induction of a 47- to 49-kd HL-immunorelated proteins parallel with the variant HL mRNA in both adrenals and ovaries would fit with this predicted translation product, except for its relatively large molecular mass on SDS-PAGE. Although the reading frame extends farther upstream, an in-frame AUG was not found in our sequence nor in the publicized rat genome data. Alternatively, the discrepancy in molecular size may be explained by extensive posttranslational modification. Because the variant HL proteins remain intracellular [18,19], and the predicted sequence lacks a signal peptide essential for endoplasmic reticulum–Golgi targeting and contains only one N-glycosylation consensus site [22], it is unlikely that glycosylation makes up for the discrepancy between apparent and predicted molecular size. Rat full-length HL in circulation has recently been reported to increase in apparent molecular size from 55 to 59 kd over time while losing its catalytic activity [10]. The nature of these posttranslational modifications remains unknown.

Hepatic lipase has long been thought to be exclusively synthesized and secreted from liver parenchymal cells [27]. Recently, this view has changed by the demonstration of the synthesis of HL in human and mouse macrophages [17] and in the adrenals of newborn mice [16]. Nevertheless, it is still generally accepted that most, if not all, of the HL activity present in the adrenals and ovaries of rat, mouse, human, hamster, and cow originates from the liver [9,27]. In rat adrenals and ovaries, no evidence for de novo synthesis and secretion of HL has been obtained [9,27]. Instead, we have reported the presence of a variant HL mRNA in these organs. This variant HL mRNA in adrenals and ovaries appears to be unrelated to the HL activity found in these organs because induction of HL mRNA and synthesis of the 47- to 49-kd HL-related proteins, by corticotropic or gonadotropic hormones, respectively, is only transient and precedes the long-lasting expression of HL activity. Moreover, whereas the HL activity in adrenals is heparin-releasable, and present at extracellular sites [7], the 47- to 49-kd protein that cross-reacts with anti-HL IgGs remains mainly intracellular [18,19]. The variant HL forms were only detected in in vitro pulse-labeling experiments with tissue slices from control and, particularly, corticotropin-activated adrenals. We [30], and others [10], have not been able to detect the presence of 47- to 49-kd HL-related proteins by immunoblotting in homogenates of adrenals from untreated rats. This suggests that expression of the variant HL forms in rat adrenals in vivo is very low, at least in untreated animals. As discussed previously, it is unlikely that the putative 47- to 49-kd protein product has the same catalytic activity as HL because of the lack of the N-terminus. Synthesis of variant 47- to 49-kd HL proteins, therefore, may not be functionally important.

Expression of the variant HL mRNA was transiently increased in adrenal glands upon treatment of the animals with corticotropin. Expression was maximal at days 2 and 4 of treatment. After 6 to 9 days, the amount of HL mRNA

has returned to near-normal levels as reported previously [18]. Similarly, both parameters were transiently induced in rat ovaries upon stimulation with the gonadotropins pregnant mare serum (PMS) and human chorionic gonadotropin [19]. The presence of a number of potential binding sites for regulatory transcription factors, such as Sp1, CCAAT, HNF1, RORA, COUP, and oct-1 sites, in the 5′-flanking region of the putative promoter in intron 2, opens the possibility for hormonal regulation of transcription from this promoter. Indeed, when expressed in human adrenocortical NCI-H295R cells, the alternative promoter was up-regulated more than 3-fold by treatment with cAMP.

The observation that both HL mRNA and HL-related proteins are transiently increased in rat adrenals and ovaries upon stimulation with corticotropin and gonadotropins [19], respectively, suggests that this response may be related to changes in cholesterol homeostasis of these steroid-producing cells in the early days of stimulation. This time frame overlaps with the reduction of intracellular cholesteryl ester content and parallels the increase in de novo cholesterol synthesis [31]. Therefore, the endogenous cholesterol levels may have become seriously reduced, and the need for HDL-C as exogenous source for steroidogenesis may have increased. Because the intracellular HL-related protein is probably catalytically inactive, the protein may have other functions in selective cholesterol uptake or intracellular cholesterol trafficking. Stimulation of adrenals as well as ovaries [19] results in a strong, sustained elevation of HL activity. This HL activity is not locally synthesized and therefore likely originates from circulation. Because HL in nonheparin plasma is virtually inactive [10], and the specific triglyceridase activity of HL isolated from adrenals is similar to liver HL [10], the increased adrenal HL activity is not simply due to increased entrapment of blood in the enlarged, hyperplastic glands. As HL in the circulation is mainly present on HDL [10], extracellular HL derived from liver may increase as a result of HDL trapping in the increased number of microvillar channels of the stimulated, hyperplastic adrenals [32], presumingly followed by unmasking of its catalytic activity. This increased HL activity may facilitate the influx of HDL-C in cooperation with SR-B1, which is induced in parallel with HL activity [33,34].

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