Rabbit hepatic lipase cDNA sequence: low activity is associated with low messenger RNA levels

Roderic J. Warren,[1] David L. Ebert, Alana Mitchell, and Philip J. Barter*

Baker Medical Research Institute, Melbourne, Australia, and Wollongong University,* Australia

Abstract We have investigated a possible mechanism for the reported low activity of hepatic lipase (HL) in the rabbit by cloning and sequencing the cDNA for rabbit HL and using the clone to quantify mRNA levels. A 1.6 kb cDNA clone was sequenced and found to encode the mature protein of 477 amino acids and 20 amino acids of the hydrophobic leader peptide. A high degree of amino acid sequence identity was demonstrated with human (81%) and rat (79%) HL. The putative active site was well conserved, and mutations reported to reduce activity in HL or lipoprotein lipase were not present in the rabbit sequence. The activity and mRNA levels were compared with those of the rat, an animal possessing relatively high HL activity. In post-heparin plasma of the rat, HL activity was nine times greater than in that of the rabbit (24.9 ± 1.6 units per ml plasma, n = 5 vs. 2.7 ± 0.1, n = 5, P = 0.0001). Comparison of mRNA levels was made by dot blot analysis of liver poly (A +) RNA obtained from each species and probed with either rabbit or rat HL cDNA, labeled to the same specific radioactivity. Specific HL mRNA levels were found to be nine times greater in the rat than in the rabbit (8.90 ± 0.11 units, n = 5 vs. 1.00 ± 0.01, n = 5, P = 0.0001). Thus, low hepatic lipase activity in the rabbit is associated with low mRNA levels, suggesting that the observed species difference in activity is due to differences in the level of mRNA. —**Warren, R. J., D. L. Ebert, A. Mitchell, and P. J. Barter.** Rabbit hepatic lipase cDNA sequence: low activity is associated with low messenger RNA levels. *J. Lipid Res.* 1991. **32:** 1333–1339.

Supplementary key words hepatic triacylglycerol lipase

Hepatic lipase is a glycoprotein, produced by liver cells, that binds to the sinusoidal surface of hepatic endothelial cells (1). It plays a fundamental role in the metabolism of lipoproteins by hydrolyzing the triacylglycerol in high density lipoproteins, intermediate density lipoproteins, and chylomicron remnants (2–4). Of the species examined, the rabbit, which shows a propensity to diet-induced atherosclerosis, has unusually low HL activity in post-heparin plasma which may account for the lipoprotein profile of rabbit plasma resembling that of human subjects with HL deficiency (5).

The cDNAs and a number of genes encoding various lipases have now been sequenced, including those of rat and human HL (6–10). These lipases share functional and primary structural homology, suggesting that HL together with lipoprotein and pancreatic lipases forms part of a gene family (11, 12).

To determine whether low HL activity in the rabbit was due to significant changes in the primary structure of the protein or to low levels of specific mRNA, the rabbit HL cDNA was cloned and sequenced. The rabbit HL clone was then used for the estimation of rabbit HL mRNA levels and comparison was made with the rat, an animal with much greater post-heparin plasma HL activity. We report that the primary structure of rabbit HL is very similar to that of rat and human HL. We also report that rabbit HL mRNA levels are low when compared to those of the rat. These findings suggest that low HL activity in the rabbit is the result of low mRNA levels.

MATERIALS AND METHODS

Restriction enzymes were purchased from Boehringer Mannheim (FRG). T_4 polynucleotide kinase was purchased from Pharmacia (USA). A rabbit liver cDNA library in λ gt10 was obtained from Clonetech (USA). T_4 DNA polymerase, T_4 ligase, and radioisotopes were from Bresatec (Australia). Amplification of DNA was performed using the polymerase chain reaction (PCR) (Amplitaq, Perkin-Elmer, USA) and Hybaid Thermal Reactor (Integrated Sciences, USA) (13). Sequencing was performed using a modified bacteriophage T7 DNA polymerase (Sequerase, United States Biochemical Corp.). United States Biochemical Corp.). All other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

Southern blot analysis

Genomic DNA was prepared from peripheral white blood cells of rabbits and rats by the method of Poncz et al. (14). Five-μg aliquots of DNA were digested with

Abbreviations: HL, hepatic lipase; kb, kilobases; mRNA, messenger RNA; PCR, polymerase chain reaction.

[1]To whom correspondence should be addressed at: Baker Medical Research Institute, Commercial Road, Prahran, Victoria, 3181, Australia.

*Eco*R1, run on 0.8% agarose gels, and transferred to Zeta probe membrane (Bio-Rad, USA) by alkali transfer in 0.4 M NaOH after depurination in 0.25 M HCl for 5 min. Membranes were prehybridized for 12 h at 65°C in 7% (w/v) sodium dodecyl sulfate (SDS) with 0.5% (w/v) skim milk powder, 2 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M sodium phosphate at pH 7.7, 1 mM EDTA) and 0.5 mg/ml salmon sperm DNA. A full-length rat HL probe was provided by Dr. M. Schotz (UCLA) (6) and a human HL clone was obtained by screening a lambda gt10 human cDNA library with the rat probe. The human probe was confirmed as full-length (7) by sequencing using the dideoxy chain termination method (15). The probes were labeled by random priming (16) with [^{32}P]α-dATP to a specific activity of 10^9 cpm/μg using a kit (Boehringer Mannheim, FRG) and hybridized with the DNA for 12 h at 62°C. Final wash conditions were 0.5 × SSC (1 × SSC: 15 mM sodium citrate, 150 mM sodium chloride, pH 7.0.), 0.1% SDS at 65°C. The membranes were then exposed to Kodak X-AR film for 24–48 h at −70°C with a Dupont Lightning Plus intensifying screen.

Northern blot analysis

Rabbit and rat RNA were isolated from liver tissues by the method of Chomczynski and Sacchi (17) and enriched for poly (A)$^+$ RNA by oligo (dT) selection (18). Ten-μg aliquots were run on a 1.0% agarose gel containing 8% formaldehyde and transferred to nitrocellulose membranes by capillary action in 20 × SSC. The membranes were baked at 80°C in a vacuum oven for 2 h and prehybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution (1 × = 1 g/l Ficoll 40, 1 g/l polyvinyl pyrollidone, 1 g/l bovine serum albumin), 0.1% SDS with 100 μg/ml denatured salmon sperm DNA, at 42°C for 12 h. Hybridization was performed at 42°C with the radiolabeled rat or human HL cDNA probe. The filters were washed finally in 0.2 × SSC, 0.1% SDS at 55°C and autoradiographed.

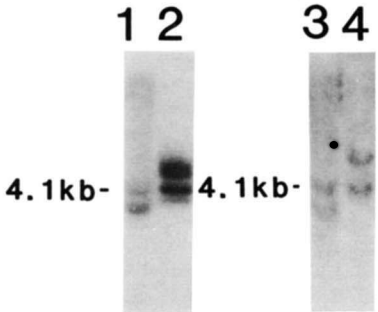

Fig. 1. Southern blot analyses with the human and rat HL cDNA probes. Rabbit (lanes 1 and 3) and rat (lanes 2 and 4) genomic DNA sample (5 μg) were digested with *Eco*R1, size-fractionated by gel electrophoresis, transferred to Zeta probe membranes, and hybridized with radioactively labeled rat (lanes 1 and 2) or human (lanes 3 and 4) HL cDNA

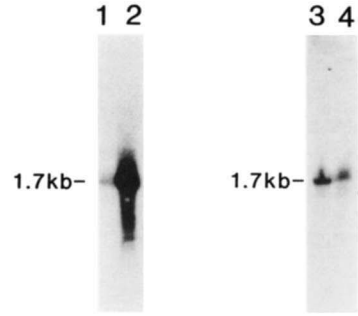

Fig. 2. Northern blot analyses with the human and rat HL cDNA probes. Ten μg aliquots of liver poly (A)$^+$ RNA from rabbit (lanes 1 and 3) and rat (lanes 2 and 4) were size-fractionated by gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with radioactively labeled rat (lanes 1 and 2) or human (lanes 3 and 4 HL) cDNA.

Cloning strategy

The human HL cDNA was used to screen 500,000 plaques of a rabbit liver cDNA library packaged in the bacteriophage λ gt10. Hybridization was performed at 42°C with the human HL probe. The membranes were washed as for Northern blots. Ten positive plaques were eluted in 100 μl of H_2O and 5 μl was amplified by PCR, using 25 base primers consisting of 17 bases complementary to the lambda sequences adjacent to the unique *Eco*R1 cloning site plus a *Bam*HI site (primer 1: CCGGATCCAGCAAGTTCAGCCTGGT), or a *Hind* III cut site (primer 2: TTAAGCTTCTTATGAGTATTTCTTC). The PCR products were run on 0.8% agarose gels, transferred to a nylon membrane (Zetaprobe, Bio-Rad, USA) and probed with the human HL cDNA. Those fragments that hybridized strongly with the human HL clone were end-filled with the Klenow fragment of DNA polymerase and ligated into pUC19 (16), to generate sufficient DNA for restriction mapping. Fragments were then subcloned into the sequencing vectors M13 mp18 and mp19 and sequenced by dideoxy chain termination (15). All of the 5' to 3' coding strand and 80% of the complementary strand were sequenced and all restriction sites used for subcloning were crossed. A second independent PCR clone was sequenced in the same manner to allow detection of any errors resulting from amplification by *Taq* polymerase. Three bases differed between the two clones. The areas containing these bases were sequenced in a third, independent PCR clone. This clone was found to differ only at these bases and the matches scoring 2/3 are shown.

Comparison of messenger RNA

Rabbit and rat liver poly (A)$^+$ RNA were prepared as described above, applied in three dilutions (0.4, 2.0, and 4.0 μg in duplicate) to nitrocellulose by dot blotting (Bio-Rad miniblot system) and crosslinked by ultraviolet light

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                    1  AGT CCC CTG TGT GTC CCC ATT TTC TTG GCT GTA TGC ATC TTG ATC CAA TCA AGT ACC CAT
                  -20  ser pro leu cys val pro ile phe leu ala val cys ile leu ile gln ser ser thr his

   61  GGA CAA AGC CTA AGA CCA GAG CCA TTC GGA AGA AGA GCT CGA GTT ACA GCA ACA AAG AAA ACA CTG CTG GAG ACA
    1  gly gln ser leu arg pro glu pro phe gly arg arg ala arg val thr ala thr lys lys thr leu leu glu thr

  136  GAA ACC AGG TTC CTG CTC TTC AAA GAC AAA GCC AAT AAG GGC TGT CAG ATT CGG CTC CAT CAC GCA GAC ACG CTT
   26  glu thr arg phe leu leu phe lys asp lys ala asn lys gly cys gln ile arg leu his his ala asp thr leu

  211  CAG GAG TGC GGC TTC AAC TCG TCG CTG CCT TTG GTG ATG ATT GTC CAC GGG TGG TCG GTG GAC GGC CTG CTG GAA
   51  gln glu cys gly phe asn ser ser leu pro leu val met ile val his gly trp ser val asp gly leu leu glu

  286  AGC TGG ATC TGG CAG ATG GTG GCG GCG CTG AAA TCA CAG CCG GCC CGG CCA GTG AAC GTG GGG CTG GTA GAC TGG
   76  ser trp ile trp gln met val ala ala leu lys ser gln pro ala arg pro val asn val gly leu val asp trp

  361  ATC TCC CTG GCA CAC AGC CAC TAT GCC GTC GCG GTG CGC AAT GCC CGC CTG GTG GGC CAG GAG GTG GCG GCT CTT
  101  ile ser leu ala his ser his tyr ala val ala val arg asn ala arg leu val gly gln glu val ala ala leu

  436  CTT CAG TGG CTG GAG GAA TCT GCT CCT TTT TCT CGA AGC AAT GTT CAC CTA ATT GGG TAC AGC CTG GGT GCT CAT
  126  leu gln trp leu glu glu ser ala pro phe ser arg ser asn val his leu ile gly tyr ser leu gly ala his

  511  GTC GCC GGA TTT GCT GGC AGC TAC ATC AGC GGA AAG CAC AAG ATT GGA AGA ATT ACA GGG CTG GAT GCT GCA GGC
  151  val ala gly phe ala gly ser tyr ile ser gly lys his lys ile gly arg ile thr gly leu asp ala ala gly

  586  CCT CTG TTC GAG GGG ACG TCC GCC AGT GAC CGT CTT TCT CCA GAT GAT GCC AAC TTT GTG GAT GCC ATT CAC ACC
  176  pro leu phe glu gly thr ser ala ser asp arg leu ser pro asp asp ala asn phe val asp ala ile his thr

  661  TTT ACC CGG GAA CAC ATG GGC CTC AGT GTG GGC ATC AAA CAG CCC GTA GGC CAC TAT GAC TTC TAC CCC AAC GGG
  201  phe thr arg glu his met gly leu ser val gly ile lys gln pro val gly his tyr asp phe tyr pro asn gly

  736  GGC TCC TTC CAG CCT GGC TGT CAC TTC CTG GAG CTC TAC AAA CAC ATC GCC CAG CAC GGC TTA AAC GCC CTC TCG
  226  gly ser phe gln pro gly cys his phe leu glu leu tyr lys his ile ala gln his gly leu asn ala leu ser

  811  CAG ACC ATC AAG TGC GCC CAC GAG CGG TCG GTG CAC CTC TTC ATT GAC TCC CTG CTG CAC CCC AGC ATG CAG AGC
  251  gln thr ile lys cys ala his glu arg ser val his leu phe ile asp ser leu leu his pro ser met gln ser

  886  ACG GCC TAC CAG TGC AGC GAC ATG GAC AGC TTC AGC CAG GGC CTG TGC CTG GGC TGC ACC AAG GGC CGC TGC AAC
  276  thr ala tyr gln cys ser asp met asp ser phe ser gln gly leu cys leu gly cys thr lys gly arg cys asn

  961  ACA CTG GGC TAC CAC ATC CGC CAG GAG CCC CTG AGC AAG GGC AAG CGG CTC TTC CTC GTG ACC CAG GCC CAG TCG
  301  thr leu gly tyr his ile arg gln glu pro leu ser lys gly lys arg leu phe leu val thr gln ala gln ser

 1036  CCC TTC AGA GTT TAT CAT TAC CAG TTC AAG ATT CAG TTC ATC AAT CAA ATT GAG AAG CCA CTG GAA CCA ACT TTT
  326  pro phe arg val tyr his tyr gln phe lys ile gln phe ile asn gln ile glu lys pro leu glu pro thr phe

 1111  ACC ATG TCA CTT CTC GGA ACA AAA GAG GAA ATG CAG AAA ATC CCC ATC ACG CTG GGC GAG GGA ATT ACT AGT AAT
  351  thr met ser leu leu gly thr lys glu glu met gln lys ile pro ile thr leu gly glu gly ile thr ser asn

 1186  AAG ACC TAT TCC TTT CTG ATC ACG CTG AAT TTG GAT ATC GGC GAG CTG ATG GTG ATC AAG TTC AAA TGG GAA AAC
  376  lys thr tyr ser phe leu ile thr leu asn leu asp ile gly glu leu met val ile lys phe lys trp glu asn

 1261  AGC GCA GTG TGG GCC AAC GTC TGG AAT ACG GTG CAG ACC ATC ATC CCC TGG GGC ATA AAG CCC CGA AAC TCG GGC
  401  ser ala val trp ala asn val trp asn thr val gln thr ile ile pro trp gly ile lys pro arg asn ser gly

 1336  CTC ATT CTG AAG ACC ATC AGA GTC AAA GCC GGA GAA ACC CAG CAA AGA ATG ACA TTT TGC TCA GAA AAC ATG GAT
  426  leu ile leu lys thr ile arg val lys ala gly glu thr gln gln arg met thr phe cys ser glu asn met asp

 1411  GAT CTG CAA CTT CAC CCG ACC CAG GAG AAA AAC TTC GTG AGA TGT GAA GTA AAC CCA AAA AAA TTG AAG CTA AAG
  451  asp leu gln leu his pro thr gln glu lys asn phe val arg cys glu val asn pro lys lys leu lys leu lys

 1486  ATC AAA TGA gttttgctgaagactcggtgtaaagaataaataaaaattcatttattccttaaaaaaaaaa
  476  ile lys
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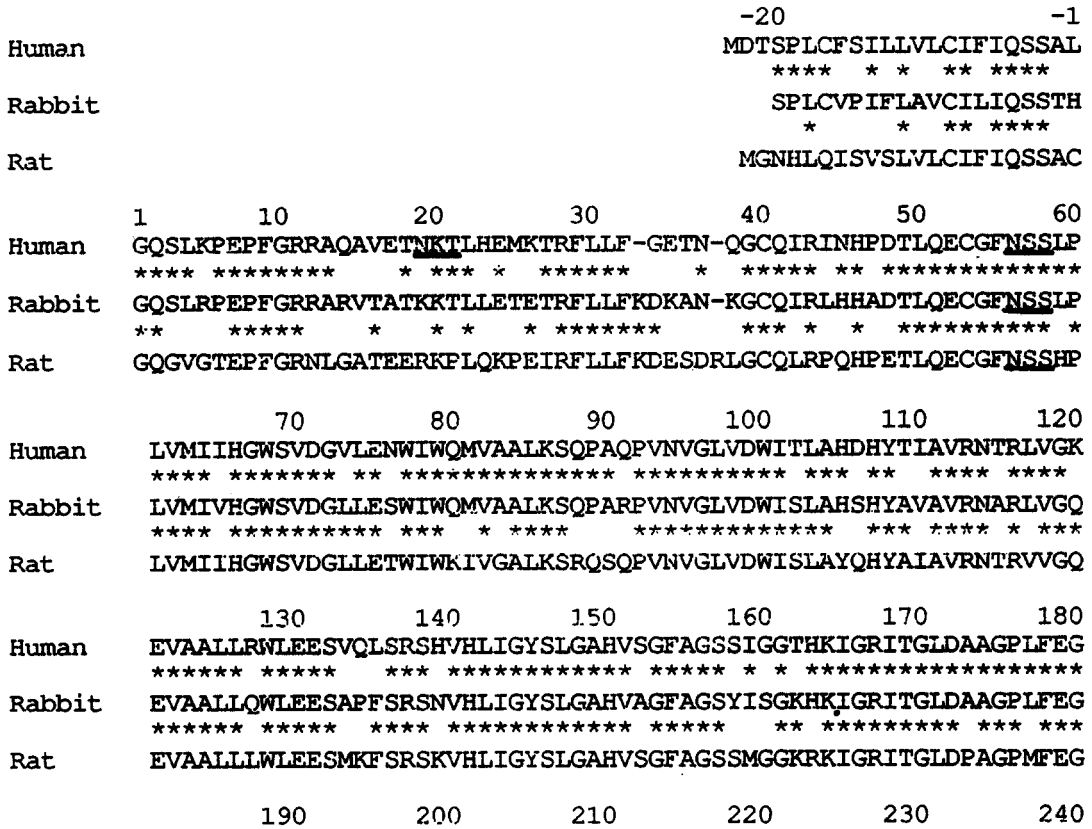
Fig. 3. The cDNA sequence for rabbit HL. The rabbit HL cDNA clone comprised 1431 nucleotides plus a poly (A) tail. The predicted amino acid sequence is shown below the DNA sequence. Positive numbering indicates the beginning of the mature protein.

(Stratalinker, Integrated Sciences, USA). The mRNA of each species was probed with the homologous cDNA clone. The probes were labeled to the same specific activity of 2.5×10^8 cpm/μg DNA and the membranes were washed as described for the Northern blot. Autoradiographs were scanned by laser densitometry (Ultrascan XL, LKB Bromma, Sweden) to quantify specific mRNA levels. Only data in the linear range of the signal response were considered. The data were normalized for mRNA content by probing the stripped filters with oligo $(dT)_{30}$, end-labeled with [^{32}P]γ-ATP. The results are expressed as units which are the corrected HL absorbances.



Hepatic lipase activity

Hepatic lipase activity in rabbits and rats was measured in post-heparin plasma collected 15 min after the injection of heparin (100 U/kg). The HL lipolytic activity was assayed using an artificial triolein emulsion in the presence of 1 M NaCl as described by Huttunen et al. (19). Activities are expressed as units per ml of post-heparin plasma (1 unit = 1 μmol free fatty acid released per h).

Statistics

Data are presented as the mean ± standard error. Comparison between groups was determined by Student's *t*-test. Significance was set at $P < 0.05$.

RESULTS

Cloning and sequencing of rabbit hepatic lipase cDNA

Southern blot analysis of rabbit and rat genomic DNA digested with *Eco*R1 and probed separately with both the rat and human HL cDNA is shown in **Fig. 1**. Both probes hybridized to the same restriction fragments in rabbit DNA or rat DNA, suggesting homology between the HL coding regions of the three species.

Fig. 2 shows the results of Northern blot analysis of rabbit and rat poly $(A)^+$ RNA probed with both the rat and human HL cDNA probes. Both probes hybridized to a discrete rabbit liver mRNA species that was slightly smaller than the 1.7 kb reported for rat HL mRNA (6). When similar quantities of rabbit poly $(A)^+$ RNA were hybridized with rat and human probes labeled to the same specific activity, the intensity of signal was greater with the human HL cDNA (Fig. 2, lane 3) than with the rat HL cDNA (Fig. 2, lane 1). The human HL probe was therefore used to screen a rabbit liver cDNA library.

Ten λ gt10 plaques that hybridized strongly to the human HL cDNA were isolated and the inserts were amplified by PCR. Two size populations of PCR product measuring 1.6 and 0.8 kilobases were identified. The 1.6 kb clones were shown to hybridize strongly to the human HL cDNA on Southern blot analysis (result not shown) and were chosen for restriction mapping and sequencing. From a consideration of the human HL sequence, initial restriction mapping was performed using *Sac* I and *Eco* RV (7). The rabbit HL restriction map resembled that of the human with common *Sac* 1 sites at 95 and 766 bases and an *Eco* RV site at 1220 bases.

The DNA sequence corresponding to the mature protein is shown in **Fig. 3**. The rabbit HL cDNA clone comprises 1431 nucleotides plus a poly(A) tail. The 3' untranslated region is 48 nucleotides and we have obtained 60 nucleotides of the 5' hydrophobic leader peptide sequence that is 69 nucleotides in the human and 66 nucleotides in the rat (6, 7). Amino acid sequence identity of rabbit HL was high when compared to human (81%) and rat (79%) HL (**Fig. 4**). The potential glycosylation sites present in rabbit HL (underlined in Fig. 4) appear to be highly conserved between species (6).

Comparison of hepatic lipase activity and mRNA levels in rabbit and rat

Post-heparin HL activity in the rabbit was low, in agreement with a previous report from this laboratory where the activity of rabbit HL was compared with that of human subjects (5). Activity from the rat was nine times greater than from the rabbit (24.9 ± 1.60 units/ml plasma, n = 5, vs. 2.66 ± 0.10, n = 5, $P = 0.0001$).

Northern blot analysis of rabbit liver poly $(A)^+$ RNA probed with the rabbit HL clone confirmed that the probe hybridized to a single discrete species of mRNA of 1.6 kb. Specific HL mRNA levels in the rat and rabbit were compared by dot blot analysis of poly $(A)^+$ liver RNA probed with their respective HL cDNA clones. A representative sample of dots is shown in **Fig. 5**. Messenger RNA levels were nine times greater in the rat than in the rabbit, which was comparable to the difference in HL activity obtained from post-heparin plasma (8.90 ± 0.11 absorbance units, n = 5, vs. 1.000 ± 0.01, n = 5, $P = 0.0001$).

DISCUSSION

We have previously shown that the rabbit has low HL activity in post-heparin plasma when compared to the activity of human subjects (5). To further investigate this low activity we have established that the rabbit genome possesses a gene that shows homology to the HL cDNA probes of human and rat. Using the human HL cDNA, we have isolated and sequenced a near full-length clone of the rabbit HL cDNA. The rabbit HL cDNA of 1.6 kb is similar in length and sequence to published information on both human and rat HL (6, 7).

Hepatic lipase shares regions of sequence, and functional homology, with lipoprotein and pancreatic lipases (12). Knowledge of the tertiary structure of human pancreatic lipase (20) confirms biochemical evidence (21) that

Fig. 4. Comparison of amino acid sequences of rabbit, human and rat HL. The rabbit HL mature protein of 477 amino acids and 20 amino acids of the hydrophobic leader peptide is represented. The degree of amino acid identity with human HL was 81% and with rat HL was 79%. Predicted N-linked glycosylation sites are underlined.

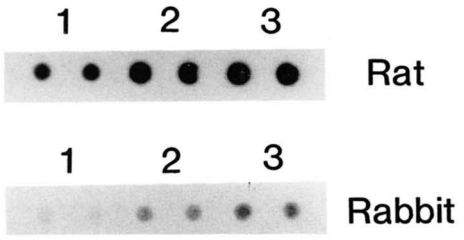


Fig. 5. Comparison of specific HL mRNA levels in the rabbit and the rat. Rabbit and rat liver poly $(A)^+$ RNA was applied in three dilutions (1 = 0.4 μg, 2 = 2.0 μg, 3 = 4.0 μg) to nitrocellulose by dot blotting and probed with their respective HL clones labeled by random priming to the same specific activity of 2.5×10^8 cpm/μg DNA. Absorbances obtained from laser scanning were normalized by reprobing with an oligo $d(T)_{30}$ probe.

Ser 152 is essential for lipolytic activity. This ser is part of a Gly-x-Ser-x-Gly motif that is present in all lipases and together with Asp 176 and His 263 comprises the catalytic domain. This domain is covered by a loop structure bounded at either end by disulfide linked Cys residues (237 and 261). Similar primary and tertiary structural features are also found in the triacylglycerol lipase from *Mucor mietei* (22).

Although there are no large deletions in the rabbit HL cDNA, low activity could result from point mutations. A number of mutations have been described in the structures of both hepatic and lipoprotein lipase that reduce or abolish activity (23–29); however, only one of the reported mutations was found within the structure of rabbit HL. The substitution of Gly for Glu at position 188 abolishes LPL activity in the human (26), but this substitution is found to occur naturally in all hepatic lipases sequenced to date. Thus, low HL activity in the rabbit does not appear to be explained by obvious amino acid differences between rabbit HL and lipases from other species.

We also examined mRNA levels in rabbit and compared them to the rat, an animal with high HL activity. Both activity and mRNA levels were about ninefold lower in the rabbit. This finding suggests that the difference between rabbit and rat HL activity may be due to low mRNA levels, implying that differences in the rate of mRNA transcription or degradation may be responsible for the observed disparity between species.

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