

# Alternative 5'-untranslated regions of mouse GH receptor/binding protein messenger RNA are derived from sequences adjacent to the major L2 promoter

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## Abstract

Heterogeneity of 5' untranslated region (5'UTR) sequences is a common feature of growth hormone receptor/binding protein (GHR/BP) mRNA from a number of species. Two major 5'UTR sequences (designated L1 and L2 in the mouse) have been cloned from rodents, ruminants and primates, and are known to correspond to transcripts generated from independently regulated promoters. A variable number of other 5'UTRs with diverse sequences have been cloned from rat, human and bovine tissues. To characterize alternative 5'UTR usage in mouse GHR/BP mRNA, we carried out 5' rapid amplification of cDNA ends using RNA from non-pregnant mouse liver and adipose tissue. Three novel 5'UTR sequences were

obtained. Sequencing of genomic DNA revealed that exons corresponding to these three sequences are clustered within 1 kb downstream of the exon encoding 5'UTR L2, and the associated L2 promoter. The novel 5'UTRs are present at very low levels relative to the total pool of GHR/BP mRNA in liver, fat, kidney, and mammary gland as determined by ribonuclease protection assays. On the basis of these data, we propose that these 5'UTR sequences may result from the use of cryptic transcription start sites and splice donor sites under the influence of the adjacent L2 promoter/enhancer region.

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## Introduction

The metabolic and somatogenic actions of growth hormone (GH) are mediated by the GH receptor (GHR) and modulated by serum GH-binding protein (GHBP). Both of these proteins are products of the growth hormone/binding protein (GHR/BP) gene. They are generated by alternative splicing of a common pre-mRNA in rodents, or by proteolytic shedding of the extracellular domain of the GHR protein in other mammals (Edens & Talamantes 1998).

GH acts on a large number of target organs. Therefore expression of the GHR/BP gene takes place at varying levels in many tissues and cell types. To enable differential tissue-specific expression, GHR/BP transcription is controlled by at least two independently regulated promoters (reviewed in Edens & Talamantes 1998, Schwartzbauer & Menon 1998). These promoters are located relatively far apart (>10 kb), upstream of the first coding exon of the gene (exon 2). Transcription initiated at each of the alternative promoters generates a transcript with a distinct 5' sequence. These first exons are spliced to exon 2, resulting in mRNAs with alternative 5' untranslated regions (5'UTRs). The presence of multiple alternative

5'UTRs has been found to be a common feature of GHR/BP mRNA from a number of species, including mice, rats, humans, cattle, sheep and rabbits (reviewed in Edens & Talamantes 1998).

Two major promoters and 5'UTRs have been identified in a number of species. One of these 5'UTR sequences is predominant in extrahepatic tissues (Adams 1995, Domene *et al.* 1995, Lucy *et al.* 1998, Moffat *et al.* 1999a). This 5'UTR – designated L2 in the mouse (Southard *et al.* 1995), 1B in ruminants (Adams 1995, Heap *et al.* 1996), V2 in human (Pekhletsy *et al.* 1992) and GHR<sub>2</sub>/V1 in the rat (Baumbach & Bingham 1995, Domene *et al.* 1995) – has a highly conserved GC-rich sequence. The pattern of expression in mouse tissues shows that the associated promoter is responsible for the general expression of the mouse GHR/BP gene (Moffat *et al.* 1999a, Yu *et al.* 1999), and this is probably also the case in other species. The other 5'UTR, which is liver-specific, is designated L1 in the mouse (Southard *et al.* 1995), 1A in ruminants (O'Mahoney *et al.* 1994, Heap *et al.* 1995), V1 in the human and the baboon (Godowski *et al.* 1989, Pekhletsy *et al.* 1992, Zogopoulos *et al.* 1999) and GHR<sub>1</sub>/V2 in the rat (Baumbach *et al.* 1989, Domene *et al.* 1995). The relative level of expression of this 5'UTR

has been assessed in mouse (Ilkbahar *et al.* 1999, Moffat *et al.* 1999a), rat (Baumbach & Bingham 1995), bovine (Lucy *et al.* 1998) and ovine (Li *et al.* 1999) liver. This 5'UTR variant constitutes a variable proportion of the total pool of hepatic GHR/BP mRNA, ranging from <5% to >50%, depending on the species, gender and developmental or physiological state of the animal. The L1 promoter in the mouse and the homologous promoters in other species are, therefore, probably responsible for developmental and hormone-responsive regulation of hepatic GHR/BP synthesis (Ahlgren *et al.* 1995, Baumbach & Bingham 1995, Southard *et al.* 1995, Zogopoulos *et al.* 1996, Li *et al.* 1999).

In addition to these two common 5'UTR forms there is considerable variety in the number and sequence of alternative 5'UTRs found to be associated with GHR/BP mRNA. Seven other 5'UTRs, V3–V9, have been reported for human GHR/BP cDNA (Godowski *et al.* 1989, Pekhletsky *et al.* 1992, Goodyer *et al.* 1999). In the rat, alternative 5'UTRs (V3–V5) that have no homology with the human 5'UTRs have been cloned (Frick & Goodman 1992, Domene *et al.* 1995). Bovine GHR 5'UTR variants designated V3–V8 and 1C have been sequenced (GenBank accession numbers AF036290–AF036297; Jiang *et al.* 1999); one of these (1C) has homology to rat 5'UTR V4. Two factors contribute to uncertainty regarding the functional significance of these mRNA variants. First, most of the sequences show little or no conservation between species. Secondly, the level of expression of 5'UTR variants, other than the two major forms, relative to the total amount of GHR/BP mRNA has been determined only for rat 5'UTR V4 (Domene *et al.* 1995) and bovine 1C (Jiang *et al.* 1999).

The mouse GHR/BP cDNAs cloned to date have contained only the L1 and L2 5'UTR variants (Smith *et al.* 1989, Southard *et al.* 1995). In order to determine whether these are the only 5'UTR variants present in the mouse GHR/BP gene, we have carried out a more thorough search for 5'UTR variants, using 5' rapid amplification of cDNA ends (RACE) with RNA from liver and fat from non-pregnant mice. Here, we report the detection and characterization of three new GHR/BP 5'UTR variants. We discuss their relationship to GHR/BP 5'UTR variants from other species, we have determined their levels of expression in several GHR/BP-expressing tissues, and we have determined the locations of the exons encoding several of these sequences. On the basis of these analyses, we suggest a hypothesis to explain the origin of these minor alternative 5'UTR variants.

## Materials and Methods

### Animals

Female Swiss Webster mice (8 weeks old) were purchased from Simonsen Laboratories (Gilroy, CA, USA). All pro-

cedures for the care and use of animals were approved by the Chancellor's Animal Research Committee of the University of California, Santa Cruz.

### RNA isolation

Total RNA was isolated using a modification of the guanidine-phenol-chloroform method (Chomczynski & Sacchi 1987). After phenol-chloroform extraction and repeated precipitations with isopropanol and ethanol, RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), re-extracted with phenol and chloroform, precipitated with LiCl and then redissolved in nuclease-free H<sub>2</sub>O.

### 5'RACE

Amplification of 5' ends of GHR/BP cDNA was carried out using the 5'RACE system (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Total RNA from liver or adipose tissue from non-pregnant female mice was reverse-transcribed using a GHR/BP-specific reverse primer (GHR-exon5R, CAG GTT GCA CTA TTT CGT CAA C, complementary to bases 459–481 relative to the 5' boundary of exon 2). First-strand cDNA was tailed with terminal transferase and dCTP. Tailed cDNA was used as the template for two rounds of a semi-nested polymerase chain reaction (PCR). The reverse transcription primer was used in conjunction with the anchor primer (Life Technologies) for initial amplification. PCR products were re-amplified with a nested GHR/BP-specific reverse primer (GHR-exon2R, TGG TGA CGC CAG TGC CAA GGT TA, complementary to bases 31–55 relative to the 5' boundary of exon 2).

### Screening and sequencing of cloned RACE products

PCR products were ligated with plasmid pGEM-Teasy (Promega) according to the manufacturer's instructions. White colonies were picked and replica-plated for further screening. Colonies were transferred to nylon membranes (MagnaLift, MSI, Westborough, MA, USA) and prepared for hybridization (Sambrook *et al.* 1989). An oligonucleotide probe specific for 5'UTR L2 (L2-F, GGC TTC TGT CTC CCG AGG CGA AAC T) was end-labeled with <sup>32</sup>P using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Membranes were hybridized with probe as described (Sambrook *et al.* 1989), washed in 6 × SSPE at 55 °C and exposed to film overnight. Colonies that did not hybridize to the L2 probe were identified and recovered from the replica plate. To determine the size of the insert in each plasmid, a PCR was carried out directly on lysed colonies by using primers (T7 and SP6) that flank the site of insertion. A small portion of the bacterial colony was suspended in 10 µl

reaction mix containing 1 × PCR buffer (Promega), 0.2 U *Taq* polymerase (Promega), 2.5 mM MgCl<sub>2</sub> and 0.2 μM each primer. Amplification was carried out for 25 cycles and samples were analyzed on 2% agarose gels. Colonies containing plasmids with inserts of the size expected for RACE products with the 5'UTR sequence were selected for sequencing. Plasmid DNA from selected colonies was isolated (Quantum miniprep kit, Bio-Rad, Foster City, CA, USA) and sequenced using T7 and/or SP6 primers. Sequencing was carried out using ABI Prism Bigdye terminator reagents (Perkin Elmer, Foster City, CA, USA) and an ABI 310 sequence analyzer.

#### Analysis of genomic DNA

A genomic fragment containing the 5'UTR L2 exon was isolated from a lambda library as previously reported (Moffat *et al.* 1999a). A 3.2 kb XbaI fragment extending downstream from the L2 exon was subcloned into pBlue-script. Sequencing was carried out using primers designed to extend outwards from known sequences.

#### Ribonuclease protection assay

To construct a 5'UTR-L4 variant-specific riboprobe template, mouse-liver RNA (1 μg) was first reverse-transcribed using the Exon5-R primer and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Primers L4-F (CTG CAG AGT CTT GAG AGC TG) and GHR-3R (CTG CCA GTG CCA AGG TTA, complementary to bases 121–139 numbered from the 5' boundary of exon 2) were used to amplify a fragment of 5'UTR L4-variant mRNA. The PCR was carried out using 1 μl 1/100 dilution of reverse transcription product as template in a 20 μl reaction in 1 × PCR buffer (Promega) containing 0.5 U *Taq* polymerase (Promega), 2.5 mM MgCl<sub>2</sub> and 0.2 μM each primer. Amplification was carried out for 30 cycles. The PCR product was cloned into pGEM-T-easy as described above.

Antisense RNA probe labeled with α[<sup>32</sup>P]UTP was generated by T7 polymerase transcription (Ambion, Austin, TX, USA) using the cloned 5'UTR L4 reverse transcriptase/PCR (RT-PCR) product as the template, and gel-purified. Total RNA (10 μg) from the indicated tissues was annealed with 5 × 10<sup>4</sup> c.p.m. probe; ribonuclease protection assays (RPAs) were carried out using the Hybspeed RPA kit (Ambion) according to the manufacturer's instructions. Products were analyzed by electrophoresis on a 6% sequencing gel alongside size markers generated using Century marker templates (Ambion).

## Results

#### Identification of novel 5'UTR sequences

In our previous studies, in which the mGHR/BP cDNAs were cloned from the livers of pregnant mice, the alter-

**Table 1** Numbers of 5'RACE product clones containing each of the alternative 5'UTR sequences

	Tissue	
	Liver	Adipose
Clones screened	200	450
5'UTR		
L1	6	2
L2	96	398
L3	4	3
L4	6	3
L5	4	1
5' truncated <sup>1</sup>	84	43

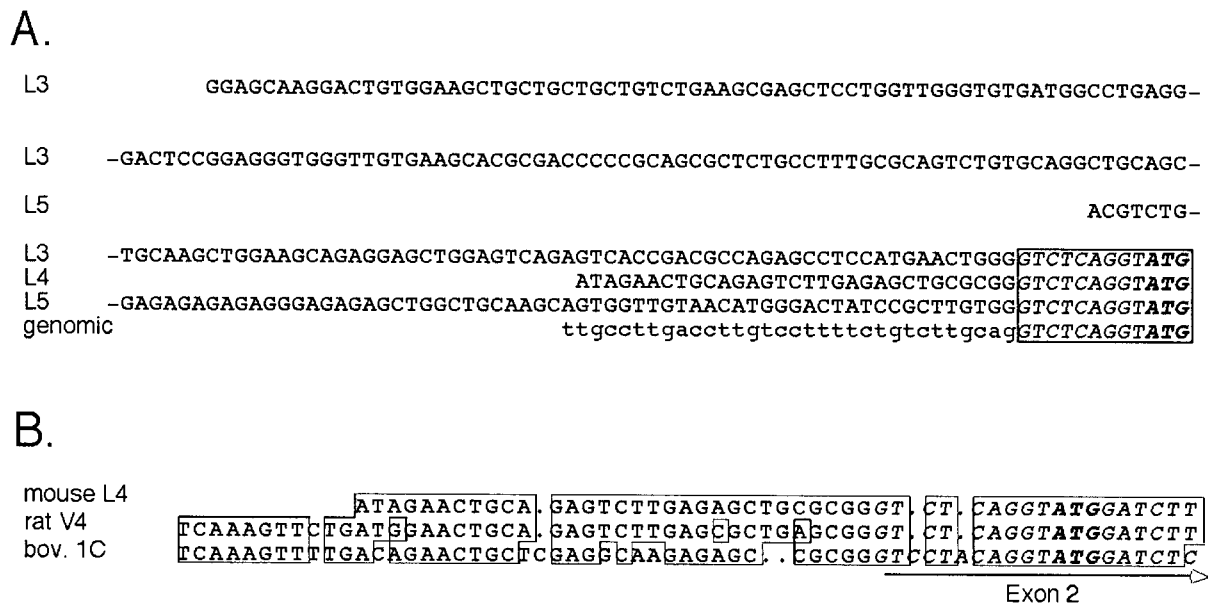
<sup>1</sup>No. of clones lacking the 5'UTR sequence.

native 5'UTRs L1 and L2 were identified (Smith *et al.* 1989, Southard *et al.* 1995). To extend the search for 5'UTR variants, we analyzed mRNA from liver and adipose tissue from non-pregnant mice, since these tissues have relatively high levels of GHR/BP expression without the high levels of 5'UTR L1 induced in the liver during pregnancy.

5'RACE was carried out on liver and adipose total RNA, using nested GHR/BP-specific primers. The resulting pools of RACE products were cloned into a plasmid vector. In order to isolate low-abundance clones corresponding to minor species, we first identified and eliminated clones that contained the L2 sequence. Colony hybridization analysis was carried out using an L2-specific oligonucleotide probe. As expected, most of the clones were eliminated in this manner (Table 1). Clones that did not contain the 5'UTR L2 sequence were tested for insert size by PCRs using primers flanking the cloning site in the plasmid. The insert size of a number of clones, shown as 'truncated' in Table 1, was found to be less than that expected for a RACE product extending into the 5'UTR sequence. Plasmids were isolated from clones remaining after these screens and were sequenced.

The results of this screen are summarized in Table 1. Three novel 5'UTR sequences were identified. In all cases, these sequences contained the exon 2 sequence as expected, and diverged from the genomic sequence at the same position as L1 and L2, 9 bases upstream of the start codon. This position corresponds to the 5' splice acceptor site of exon 2 – a strong indication that all of the novel sequences represent genuine 5'UTRs generated by alternative splicing. These 5'UTRs were designated L3, L4 and L5.

As shown in Fig. 1, the shortest and most frequently cloned sequence, 5'UTR L4, was highly related to the rat 5'UTR V4/adipo4 sequence (Domene *et al.* 1995, Frick & Goodman 1992), and to the 3' end of bovine 5'UTR-1C1, 1C2 and 1C3 sequences (Jiang *et al.* 1999). The mouse sequence was shorter than its homologs, so we carried out a further 5'RACE amplification using an



**Figure 1** Novel 5'UTRs cloned by 5'RACE. (A) Sequences of cloned 5'RACE products and the genomic sequence at the 5' flank of exon 2. The common exon 2 sequence is boxed and the initiation codon of the GHR/BP open reading frame is shown in bold. The intron sequence is in lower-case lettering. (B) Alignment of the L4 5'UTR sequence with homologous sequences from rat and bovine GHR/BP. Positions of identity are boxed. Exon 2 is shown in italics and the initiation codon is shown in bold.

L4-specific reverse primer to determine if the original RACE clones were products of incomplete reverse transcription. However, all clones obtained had an identical 5' end, 31 nucleotides (nt) upstream of the junction with exon 2. 5'UTR L3 also showed relatedness to part of the bovine 1C sequence (see Fig. 2), but had no detectable similarity to any of the rat or human 5'UTR sequences. The L5 sequence had no detectable homology with known 5'UTR sequences.

#### Genomic organization of exons encoding novel 5'UTRs

Knowledge of the location of the exons giving rise to the novel 5'UTRs, and their flanking sequences, is important in determining the origin and possible regulatory significance of the novel 5'UTRs. In the bovine GHR/BP gene, it has recently been found that the exon corresponding to the 5'UTRs 1C1, 1C2 and 1C3, which have homology to mouse 5'UTRs L3 and L4, lies downstream of the 5'UTR 1B exon (Jiang *et al.* 1999). The bovine 1B sequence is homologous to the mouse L2 5'UTR and 5' flanking region. We therefore sequenced the region downstream of the L2 exon, using a genomic clone described previously (Moffat *et al.* 1999a).

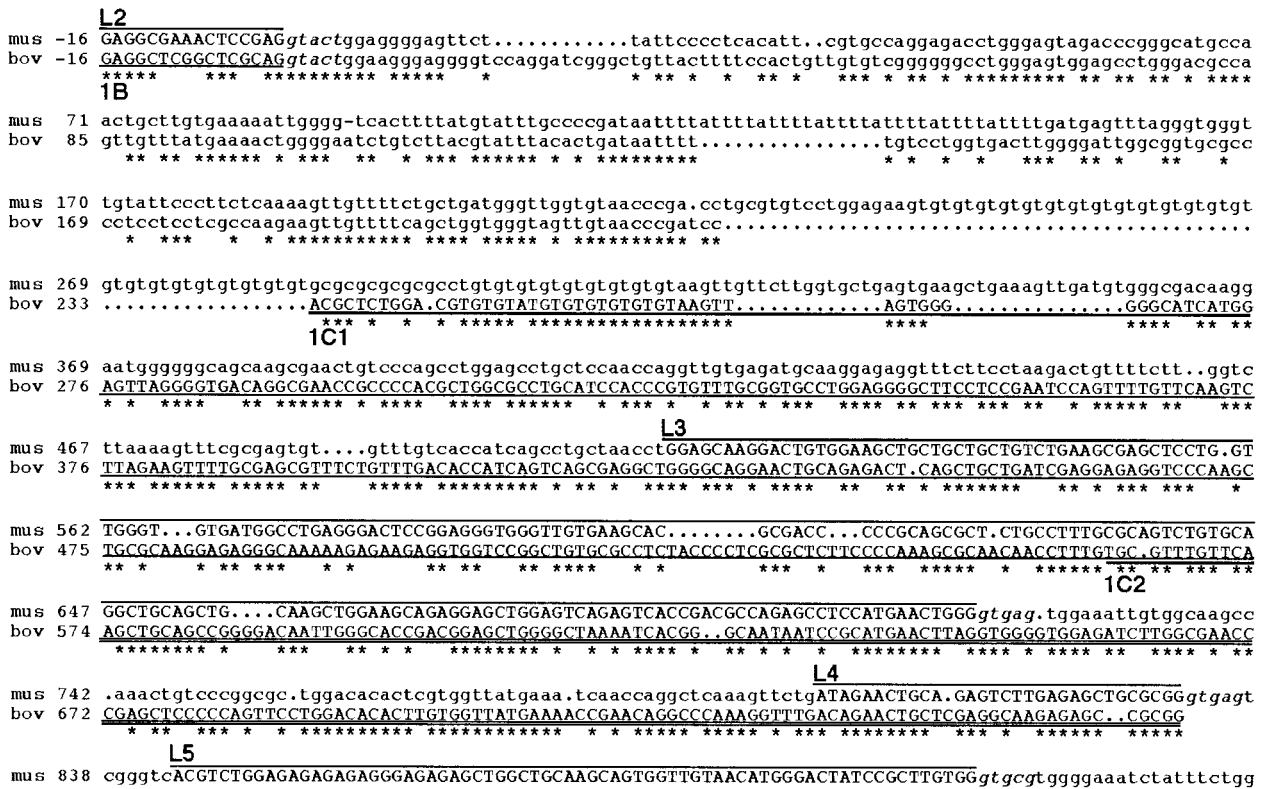
The 900 bp region 3' to exon L2 was found to contain sequences identical to 5'UTRs L3, L4 and L5. Flanking the 3' end of each of these sequences was a sequence similar to the splice donor consensus GTPuPuGT. We conclude, therefore, that these are exons that give rise to the 5'UTR sequences.

An optimized sequence alignment was carried out on the mouse and bovine sequences extending downstream from the L2 and 1B exons respectively. This alignment is shown in Fig. 2. These sequences show significant homology, with the exception of a larger region of GT and GC dinucleotide repeats in the mouse sequence. The locations of the 5'UTR-encoding exons within these sequences are shown in the figure. The locations of the L3 and L4 sequences are similar to their homologous regions in the bovine 1C exon. The L5 exon lies immediately downstream of L4; the sequence of the bovine gene for this region is unavailable.

#### Novel 5'UTRs are expressed at low levels

To obtain direct measurements of the relative abundance of each of the 5'UTR variants compared with the total pool of GHR/BP mRNA, we used an RPA. As the L4 variant was the more abundant 5'RACE product cloned, and as the bovine and rat homologs have been shown to be expressed at significant levels (Domene *et al.* 1995, Jiang *et al.* 1999), analysis of this variant is presented here.

An antisense RNA probe stretching from the 5' UTR sequence to a site corresponding to the 3' end of exon 3 was produced. This probe was expected to yield two possible protected fragments: one would be a 169 nt fragment corresponding to mRNA containing the 5'UTR sequence, and the other would be a 138 nt fragment spanning only exons 2 and 3, which would represent all



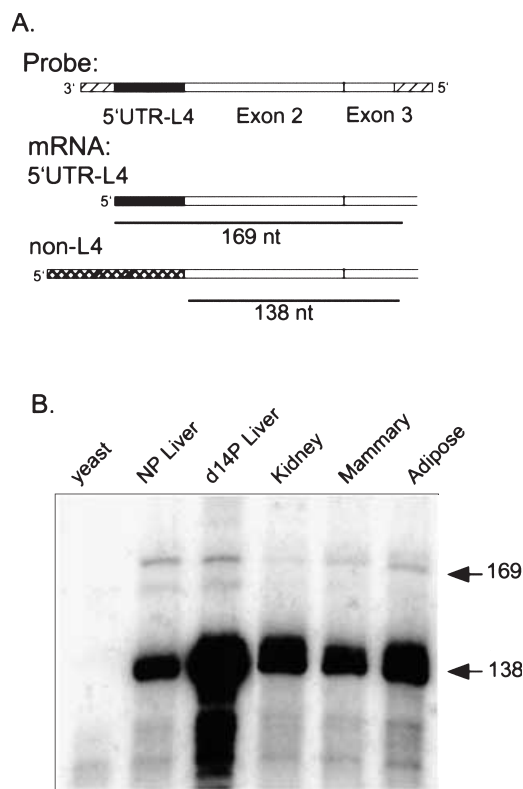
**Figure 2** Genomic organization of exons giving rise to alternative 5'UTRs. The sequence of genomic DNA containing L2, L3, L4 and L5 exons is aligned to the bovine GHR/BP exon 1B/1C region. Sequences corresponding to the alternative 5'UTRs are in upper-case letters, underlined and labeled; lower-case letters indicate intervening introns. Positions of nucleotide identity are shown by asterisks. Splice donor sites are in italics. Numbering is relative to the 3' end of exon L2/1B. This sequence has been appended to GenBank accession number AF120480.

other variants. RNA isolated from tissues known to express a relatively high level of GHR/BP mRNA (liver and adipose from pregnant female mice, and liver, kidney, mammary gland and adipose tissue from non-pregnant female mice) was assayed for the presence of 5'UTR L4 variant mRNA, the results are shown in Fig. 3. GHR/BP mRNA was readily detectable, as indicated by the strong 138 nt band corresponding to the exon 2–exon 3 fragment. A band of 169 nt, representing the 5'UTR L4-containing mRNA, was also detectable, but at very low levels relative to the shorter product. Quantification of the band intensities, with correction for the differing specific activities of the fragments, showed that the 5'UTR L4 mRNA constitutes less than 1% of the total GHR/BP mRNA in any of the tissues examined. The L3 and L5 variants were also detectable at the limit of sensitivity in similar assays (data not shown). As Fig. 3 shows, the amount of L4-containing mRNA remained at approximately the same proportion relative to the non-L4 band in all samples except the liver RNA from pregnant mice. We previously established that this increase in hepatic

GHR/BP mRNA during pregnancy in the mouse is entirely due to transcription of the 5'UTR L1 variant (Ilkbahar *et al.* 1999).

### Discussion

Heterogeneity of 5'UTRs has been reported as a common feature of GHR/BP mRNA from a number of species, but the causes and significance of this diversity are not completely understood. The two 5'UTRs that show the highest level of expression and the greatest conservation of sequence between species have been definitively shown to be controlled by different promoter regions. These alternative promoters enable differential tissue specificity and hormone-responsiveness of transcription. Furthermore, evidence for a post-transcriptional regulatory role for alternative GHR/BP 5'UTRs comes from our finding that the 5'UTR L2 sequence inhibits translation of a reporter gene (Moffat *et al.* 1999b). However, the number and sequence of the other 5'UTRs reported to date



**Figure 3** Expression of 5'UTR L4. (A) The design of the antisense riboprobe used to differentiate 5'UTR variant mRNAs. Hatched bars represent vector-derived sequence. Sizes of fragments protected by 5'UTR-L4 variant mRNA, and mRNA with any other 5'UTR sequence ('non-L4'), are shown by the horizontal lines. B. RPAs carried out using the 5'UTR L4 probe. The probe was hybridized with 10  $\mu$ g total RNA from the indicated adult female mouse tissues. 'NP Liver' and 'd14P Liver' indicate liver RNA from non-pregnant and pregnant (day 14) mice respectively. Bands corresponding to the 169-base L4-containing fragment, and the 138-base exon 2–exon 3 fragment are arrowed.

show considerable variation between species. Given the sensitivity and tendency to over-represent rare sequences that is inherent in the PCR technique (Mathieu-Daudé *et al.* 1996), the ability to clone a 5'UTR variant is not necessarily an indication that it makes a significant contribution to the total pool of GHR/BP mRNA. For example, GHR/BP 5'UTRs V3 and V4 have been detected but not quantified by RT-PCR in human and baboon tissues (Zogopoulos *et al.* 1996, 1999), whereas these and most of the other human 5'UTRs were undetectable by Northern blotting (Zou *et al.* 1997).

In this report we show that the novel mouse variants, though readily amplifiable by RT-PCR, are very rare compared with the total GHR/BP mRNA pool in tissues expressing high levels of GHR/BP. This is consistent with our previously published data, in which we showed that,

with the exception of the liver during pregnancy, the L2 variant constitutes >95% of GHR/BP mRNA not only in the tissues tested here, but also in the heart, muscle, testes, ovary, placenta, pancreas, spleen, thymus, brain and fetal liver (Moffat *et al.* 1999a).

Thus, questions arise as to the origin and function of these minor variants. On the basis of the cDNA sequences, it is assumed that alternative 5'UTRs are generated from separate exons with distinct transcription start sites, since the alternative possible mechanism, 5' truncation, does not occur in eukaryotic pre-mRNA processing. Such transcription start sites could each be associated with a distinct promoter and enhancer, as is the case with L1 and L2 and their homologs in other species. Alternatively, they could arise by the selection of alternative transcription start sites and downstream splice donor sites under the regulation of a common regulatory region.

We propose that these transcripts are not the products of independently acting promoters, but perhaps arise from promiscuous transcription initiation at cryptic sites within the region of influence of the strong L2 enhancer elements. Primary transcripts arising from these events would then be spliced at the first efficient splice donor site occurring downstream from the start of transcription. Direct mapping of transcription start sites for the newly cloned 5'UTR sequences was precluded by the low levels of expression of these mRNAs. However, in the case of 5'UTR, L4 repeated attempts to clone longer 5'RACE products resulted only in the sequence shown here, strongly suggesting that the 5' end of the RACE product represents the transcription start site.

The very low levels of expression of these variants makes it impractical to disprove the existence of multiple independent promoters by experimental means. Nevertheless, several points support our speculation that transcription is a consequence of proximity to the L2 promoter/enhancer. The first finding is the location of these exons relative to the L2 promoter and the rest of the GHR gene. Between the L2 exon and exon 2 there is at least 25 kb of intronic sequence available for alternative promoters, and the other major promoter, L1, is located more than 10 kb downstream of L2 (Moffat *et al.* 1999a). Nevertheless, the three newly cloned 5'UTR exons, like their bovine homologs (Jiang *et al.* 1999), are clustered in a small region immediately downstream of the L2 promoter (or 1B in the bovine gene). The second finding is the significant variation in transcription start sites and splice donor sites selected out of an overall partially conserved sequence when this region of the mouse GHR/BP gene is compared with the homologous sequences from other species.

Although the applicability of this mechanism to other alternative GHR/BP 5'UTRs in other species is not proved, it is noteworthy that, in addition to the bovine gene, a similar clustering of exons encoding alternative 5'UTR sequences has been found in the human GHR

gene, (Zou *et al.* 1997, Goodyer *et al.* 1999). This pattern suggests that, in this case, transcription from multiple start sites might also be under the control of a common enhancer region. This model offers a simple explanation for at least some the diversity of 5'UTRs detected in GHR/BP mRNAs, in comparison to a mechanism by which every 5'UTR variant arises from an independent promoter.

In either case, the physiological significance of the multiplicity of GHR/BP 5'UTRs remains to be established. It is known that the 5'UTR sequence can have profound effects on the translation and stability of a mRNA (Gray & Wickens 1998, Frischmeyer & Dietz 1999); consequently, we can also speculate that the GHR/BP alternative 5'UTRs may have regulatory significance even in the absence of independent transcriptional control. The data presented here will contribute to further studies designed to resolve these questions.

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