Transcriptional upregulation of hepatic GH receptor and GH-binding protein expression during pregnancy in the mouse

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ABSTRACT

In the mouse, GH-binding protein (GHBP) and GH receptor (GHR) are encoded by a single gene via alternative splicing. We previously demonstrated that the steady-state levels of the GHR and GHBP mRNAs are significantly elevated in mouse liver during pregnancy. Hepatic GHR and GHBP mRNAs are associated primarily with one of two different 5' untranslated regions (5' UTRs), designated 5' UTR Liver1 (L1) and Liver2 (L2). Distinct promoters associated with each of these 5' UTRs have recently been characterized. In the present study, we have investigated the role of transcriptional activation in the pregnancy-induced upregulation of GHR and GHBP mRNAs in liver. We also report on the relative contribution of the 5' UTR L1 and 5' UTR L2 promoters to the hepatic expression of the GHR/GHBP gene in the liver. Our approach was to compare, by ribonuclease protection assay (RPA), GHR/GHBP transcript levels in hepatic nuclear and total cellular RNA samples from virgin and late-pregnant mice. In these RPAs we utilized riboprobes that were complementary to the coding region of GHR/GHBP transcripts, as well as to the two noncoding, alternative first exons 5' UTR L1 and L2. When

employing the coding region probe, RPAs revealed that the gestational increase in the levels of nuclear GHR/GHBP transcripts were statistically comparable with the increase in GHR/GHBP transcript levels in total cellular RNA. This finding suggests that enhanced transcriptional activity, rather than increased cytoplasmic half-life, is responsible for the upregulation of GHR/GHBP RNA in the pregnant liver. In RPAs utilizing the noncoding region probes, both nuclear and total cellular GHR/GHBP transcripts associated with 5' UTR L1 were significantly upregulated in late-pregnant as compared with virgin mice. In contrast, the levels of both nuclear and total GHR/GHBP transcripts associated with 5' UTR L2 were comparable between nonpregnant and pregnant animals. Moreover, 5' UTR L2-containing transcripts were present at levels that were only 3–5% of the 5' UTR L1-associated transcripts in the late-pregnant liver. Thus, we conclude that the gestational upregulation of the GHR/GHBP gene in the mouse liver can be ascribed to the significantly enhanced transcriptional activity of the 5' UTR L1 promoter.

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INTRODUCTION

The many biological actions of growth hormone (GH) as an important regulator of growth and metabolism in vertebrates are mediated by two structurally related proteins: the membrane-bound GH receptor (GHR) and the soluble GH-binding protein (GHBP). In murines, these two proteins are generated by alternative splicing of a single primary

transcript of the GHR/GHBP gene (Baumbach *et al.* 1989, Edens *et al.* 1994, Zhou *et al.* 1994). The 246 amino acid extracellular, GH-binding domain of the mouse (m) GHR is identical to that of the mGHBP and is encoded by exons 2 through 7 of the GHR/GHBP gene (Smith *et al.* 1989). The transmembrane and intracellular domains encoded by exons 8, 9, and 10 of the GHR mRNA are unique to the GHR, whereas the so-called

'hydrophilic tail', a hydrophilic stretch of 27 amino acids, is unique to the GHBP and is encoded by exon 8A. The mutually exclusive splicing of exons 8 or 8A to the constitutively spliced, common exons 2 through 7 generates the GHR or GHBP mRNA respectively (Edens *et al.* 1994, Zhou *et al.* 1994).

Pregnancy is a well-established regulator of GHR and GHBP expression (Barnard & Waters 1997). The most striking changes in GHR and GHBP expression shown to date are the 30- to 50-fold increase relative to virgins in serum GHBP concentration (Cramer *et al.* 1992a,b) and the five- to sixfold increase over nonpregnant levels in hepatic GHR concentration (Camarillo *et al.* 1998) observed in the mouse during late pregnancy. This dramatic upregulation of GHR and GHBP concentrations is also reflected in the gestational profile of GHR and GHBP mRNAs in the liver, which is considered to be the major source of circulating GHBP (Baumann 1993, Barnard & Waters 1997). We previously reported that steady-state levels of hepatic GHR- and GHBP-encoding messages, as assessed by ribonuclease protection assay (RPA), increase significantly during the latter half of gestation in mice (Ilkbahar *et al.* 1995). The levels of both mRNAs start to rise on day 10 of pregnancy, continue to increase progressively until day 14, when both messages reach their maximal abundance, and plateau thereafter. GHR and GHBP mRNAs increase to a gestational maximum of about six- and twelvefold respectively, compared with their levels in nonpregnant animals. Moreover, the ratio between the two messages increases in favor of GHBP mRNA throughout pregnancy, resulting in GHBP mRNA levels that are up to four times higher than those of GHR mRNA. This suggests a gestational control mechanism of alternative splicing of the GHR/GHBP primary transcript.

The alternative splicing pathway generating GHR- and GHBP-specific mRNAs is not the only regulated splicing event involved in the expression of the mGHR/GHBP gene. Analysis of the 5' ends of GHR and GHBP mRNAs in the liver and placenta revealed two major 5' untranslated region (5' UTR) sequences, designated 5' UTR Liver1 (L1) and 5' UTR Liver2 (L2) (Southard *et al.* 1995). This sequence divergence upstream of the translation start site reflects the presence of alternative, noncoding first exons associated with different promoter regions. Northern analysis on poly(A)⁺-selected RNA revealed that the relative expression levels of 5' UTRs L1 and L2 vary between the liver and placenta, suggesting that the tissue-specific expression of GHR and GHBP is directed by differential promoter activity (Southard *et al.* 1995). It was also shown that the intensity of

hybridization signals for 5' UTR L1-associated GHR and GHBP mRNAs, as opposed to 5' UTR L2-containing GHR and GHBP mRNAs, was stronger in the late-pregnant mRNA pool compared with the nonpregnant one, where 5' UTR L1 signals were barely detectable (Southard *et al.* 1995). These findings predict a role for the promoter associated with 5' UTR L1 in the gestational regulation of mGHR/GHBP expression.

The pregnancy-induced upregulation of the steady-state levels of hepatic GHR and GHBP mRNAs summarized above could be caused by an increased rate of transcription, an increase in the stability of the two messages, or both. In this study, we set out to determine the role of transcriptional initiation in the gestational upregulation of mGHR/GHBP mRNAs by analyzing heterogeneous nuclear RNA isolated from hepatic nuclei and quantitating GHR and GHBP transcripts in virgin and late-pregnant females by RPA. Using the same approach, we also investigated the contribution of 5' UTR L1 and 5' UTR L2 promoters to the pregnancy-specific expression of the mGHR/GHBP gene.

MATERIALS AND METHODS

Extraction of hepatic nuclei

Virgin and 14-day-pregnant Swiss Webster mice (day of appearance of vaginal plug=day 0 of gestation) were purchased from Simonsen Laboratories (Gilroy, CA, USA). The mice were housed in a vivarium with a 14 h light:10 h darkness lighting cycle and were allowed free access to food and water. All procedures for the care and use of animals in this study were approved by the Chancellor's Animal Research Committee of the University of California, Santa Cruz.

Specific methods for the isolation of hepatic nuclei were based on the protocol described by Marzluff & Huang (1984). Briefly, saline-perfused livers obtained from nonpregnant and 14-day-pregnant mice, carrying 10–14 pups each, were minced and homogenized in prechilled lysis buffer (0.32 M sucrose; 5 mM CaCl₂; 3 mM magnesium acetate; 0.1 mM EDTA; 0.1% Triton X-100; 1 mM DTT; 10 mM Tris-Cl, pH=8.0) using a Dounce tissue grinder. The homogenate was layered over a cushion of prechilled centrifugation buffer (2 M sucrose; 3 mM magnesium acetate; 0.1 mM EDTA; 5 mM DTT; 50 mM Tris-Cl, pH=8.0). The nuclei were collected by centrifugation at 30 000 *g* for 45 min at 4 °C and suspended in a glycerol storage buffer (40% glycerol; 5 mM magnesium acetate; 0.1 mM EDTA; 1 mM DTT; 10 mM Tris-Cl,

pH=8.0) and frozen at -80°C until RNA extraction. An aliquot of each nuclear preparation was stained with 0.04% crystal violet and analyzed by light microscopy to assess the concentration of the nuclei and the purity of the preparation. The average yield was 4×10^7 nuclei/liver.

RNA isolation

Total cellular RNA was extracted from whole livers of virgin and 14-day-pregnant females ($n=7-8$). Nuclear RNA was purified from frozen hepatic nuclei obtained from virgin and 14-day-pregnant mice ($n=9$). Each of the tissue and nuclear preparations was individually processed for extraction and analysis of RNA. Both total cellular RNA and nuclear RNA were isolated by the guanidinium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987). RNA concentrations were determined by spectrophotometry at 260 nm.

RNA analysis

Nuclear and total hepatic RNA were analyzed by solution-hybridization RPA using Ambion's (Austin, TX, USA) RPA II kit as previously described by us (Ilkbahar *et al.* 1995). Three different [α - ^{32}P]uridine 5'-triphosphate-labeled RNA probes complementary to GHR/GHBP transcripts were generated using the MAXIScript T7 *in vitro* transcription kit (Ambion), according to the manufacturer's instructions. All three antisense probes were derived from templates consisting of pBluescript vector (Stratagene, La Jolla, CA, USA) carrying portions of mGHR and mGHBP cDNAs.

The 'coding region probe', a probe complementary to 45 bases of exon 2 and 21 bases of exon 3, was designed to detect all GHR and GHBP transcripts regardless of which 5' UTR the transcripts contain (Fig. 1A). The 125 nucleotide (nt, excluding the polylinker sequence) '5' UTR L1 probe' spanned 89 nt of alternative first exon 5' UTR L1 and 36 nt of the coding exon 2 (Fig. 1B). The riboprobe designated 5' UTR L2 was complementary to 69 nt of alternative first exon 5' UTR L2, the entire length of exon 2 (81 basepairs) and 21 bases of exon 3 (Fig. 1C).

Hepatic nuclear RNA (10–20 μg) or total cellular RNA (10–20 μg) was hybridized with excess amounts (3–4 fmol) of gel-purified GHR/GHBP-specific riboprobes. After overnight hybridization at 45°C , the samples were digested with RNase T1 (40 units/reaction) for 30 min at 37°C . The fragments protected from RNase digestion were identified on 6% polyacrylamide, denaturing gels.

Bands corresponding to probe fragments protected by hybridization to GHR/GHBP transcripts were quantitated by phosphor imaging (PhosphorImager; Molecular Dynamics, Sunnyvale, CA, USA).

Phosphor-imaging data were corrected by subtracting background values measured in each gel lane. To standardize the signal intensities obtained from different lengths of protected riboprobe fragments, pixel values were normalized for the number of uracils present in each protected fragment. The values for a given protected fragment were compared between livers of virgin and late-pregnant mice by analysis of variance followed by Fisher's Protected Least Difference Test. Differences were considered to be significant at a P value <0.05 . Due to the variability introduced by the fast rate of turnover and transient nature of unspliced, primary transcripts, a direct statistical comparison between levels of primary transcripts and spliced nuclear or total RNA was not made. Therefore, bars representing primary transcript abundance in Figs 2B, 3B, and 4C are labeled with lower case letters in contrast to bars displaying processed RNA measurements.

RESULTS

Previous, unpublished studies in our laboratory revealed that nuclear 'runoff' assays, which allow direct measurement of the rate of RNA synthesis *in situ*, were not suitable for determining the effect of pregnancy on the rate of GHR/GHBP transcription in the mouse liver. In these assays, it was necessary to normalize the labeled nuclear RNA input into hybridization reactions with respect to the number of counts from each nuclear preparation. However, the overall level of RNA synthesis in hepatic nuclei was upregulated as a function of pregnancy. Therefore, normalization of samples from nonpregnant and pregnant livers by the addition of equal counts masked the gestational increase in the transcription rate of the GHR/GHBP gene.

In order to determine the contribution of transcriptional activation to the gestational upregulation of the steady-state levels of GHR/GHBP mRNA, we devised RPAs that allowed us to quantitate spliced nuclear transcripts, unspliced primary transcripts, and total cytosolic RNA in the same experiment. The levels of these transcripts were then compared between samples from virgin and pregnant mice. In these experiments, concomitant changes in a given transcript in the nucleus and the cytosol from one physiological stage to another (virgin versus pregnant) would indicate regulation

of expression at the transcriptional level. Conversely, a higher level of a transcript in the cytosol relative to the nucleus under one physiological condition as compared with another would suggest post-transcriptional control mechanisms in the accumulation of messages in the cell. Such RPAs have been successfully employed to assess changes in the kinetics of transcription and were shown to be more sensitive than nuclear 'runoff' transcription assays (Bichell *et al.* 1992, Gronowski & Rotwein 1995). We found the most pronounced changes in GHR/GHBP expression on day 14 of pregnancy in previous studies (Ilkbahar *et al.* 1995). In the experiments described below, we have chosen this day to reflect gestational changes.

Gestational induction of mGHR/GHBP gene transcription

In both hepatic nuclear and total cellular RNA preparations, GHR/GHBP transcripts from which intron 2 was spliced out, protected the full length (66 nt excluding the polylinker sequence) of the coding region probe (Figs 1A and 2A). On the other hand, nascent nuclear transcripts containing exons 2 and 3, and the intervening intron protected only 45 bases of the riboprobe corresponding to exon 2. As expected, this 45nt band was absent from total cellular RNA samples, and was detectable only in nuclear RNA preparations, although with very weak intensity in nonpregnant hepatic nuclear RNA. The predicted 21 nt riboprobe fragment complementary to exon 3 was too small to be detectable. Thus, in this set of experiments, the 45 base-protected fragment represented primary GHR/GHBP transcripts, the 66 base band observed with nuclear RNA samples represented spliced nuclear GHR/GHBP transcripts, and the same band detected in total cellular RNA was quantitated as total GHR/GHBP RNA and reflected steady-state levels of GHR/GHBP mRNA.

The abundance of hepatic primary GHR/GHBP transcripts rose approximately four- to fivefold, that of spliced nuclear transcripts increased about tenfold, and the levels of total cellular GHR/GHBP RNA increased by six- to sevenfold in 14-day-pregnant mice relative to virgins (Fig. 2B).

Induction of 5' UTR L1-associated GHR/GHBP transcripts during pregnancy

The 5' UTR L1 probe (Fig. 1B) was designed to assess the abundance of GHR/GHBP transcripts that contain the 5' UTR L1 sequence. The two bands detectable with this probe were 89 bases and 125 bases in size, complementary to the 5' UTR L1

exon alone, and to exon 2 joined to the 5' UTR L1 exon respectively. Therefore, processed 5' UTR L1-containing GHR/GHBP transcripts, which lack the intron(s) upstream of exon 2, hybridized to the entire length of this riboprobe, giving rise to the 125 base fragment (Fig. 3A). Unspliced primary GHR/GHBP transcripts, on the other hand, were quantified using the 89 base-protected fragment. Due to its relative low molecular weight, the band complementary to 36 bases of exon 2, representing spliced RNA lacking 5' UTR L1, could not be detected.

Although levels of primary transcripts presented in Fig. 3B are associated with 5' UTR L1, these transcripts were not necessarily initiated from the promoter associated with 5' UTR L1. Studies in our laboratory have established that alternative first exon 5' UTR L2 and its promoter are located upstream of 5' UTR L1 in the mouse GHR/GHBP gene (Edens & Talamantes 1998). Therefore, primary transcripts initiated from either one of these two promoters would be detected as an 89 base-protected band by the 5' UTR L1 probe. The 125 nt band, however, accurately reflects transcripts that were initiated from the 5' UTR L1 promoter and were spliced to include alternative first exon 5' UTR L1 adjacent to exon 2. Since 5' UTR variants are spliced to exon 2 in a mutually exclusive manner, initiation from any promoter other than the L1 promoter would result in the loss of the 5' UTR L1 exon during splicing.

Processed nuclear and total GHR/GHBP transcripts associated with 5' UTR L1 (corresponding to the band of 125 bases in Fig. 3A) were almost undetectable in nonpregnant hepatic RNA samples, but their abundance was significantly increased on day 14 of pregnancy. Levels of these spliced transcripts were comparable between nuclear and total cellular RNA samples obtained from nonpregnant mice, as well as between nuclear and total cellular RNA samples from late-pregnant animals (Fig. 3B). The gestational increase in the levels of primary transcripts was not as pronounced as with the spliced transcripts (Fig. 3B).

Levels of mGHR/GHBP transcripts associated with 5' UTR L2 or other alternative first exons

The complementary RNA probe spanning 69 bases of exon 5' UTR L2 and 102 bases of exons 2 and 3 was designed to detect both GHR/GHBP transcripts that contain 5' UTR L2 and those which are associated with other 5' UTRs, and thus lack 5' UTR L2 (Fig. 1C). The full length of 171 bases (excluding the polylinker) of this probe should be protected by spliced GHR/GHBP transcripts

FIGURE 1. Design of the riboprobes for the RPA. Broken lines represent the expected lengths of the protected probe fragments. (A) 'Coding region riboprobe' used to detect transcripts containing exons 2 and 3. Intron 2 is shown as the looped segment in the unspliced primary transcript. (B) '5' UTR L1 riboprobe' used to distinguish between transcripts containing 5' UTR L1 and those not containing 5' UTR L1. (C) '5' UTR L2 riboprobe' used to distinguish between transcripts containing 5' UTR L2 and those not containing 5' UTR L2.

containing 5' UTR L2, whereas only the 102 bases corresponding to exons 2 and 3 should be protected by processed GHR/GHBP messages lacking 5' UTR L2. The expected sizes of probe fragments protected by unprocessed, primary GHR/GHBP

transcripts were 69 bases (complementary to 5' UTR L2), 81 bases (complementary to exon 2), and 21 bases (complementary to exon 3), of which the 21 nt fragment was too small to detect. Of these fragments, we were able to detect reliably only the

81 nt band, which we quantitated as primary GHR/GHBP transcripts (Fig. 4A). We were unable to detect the 69 nt band consistently, which could be due to the rapid processing and the resulting short half-life of primary transcripts initiated from the 5' UTR L2 promoter. Exon 5' UTR L2 is the farthest upstream of all exons of the mouse GHR/GHBP gene mapped to date (Edens & Talamantes 1998). It is, conceivable, therefore, that the intron immediately downstream of exon 5' UTR L2 is spliced out before any others during co-transcriptional splicing. In addition, the 5' UTR L2 sequence is extremely GC-rich (Southard *et al.* 1995), and the overall uridine content of the 69 nt complementary to exon 5' UTR L2 is only 20%, lowering the specific activity of this stretch of the riboprobe.

Quantitation of the 171 nt band revealed that the abundance of 5' UTR L2-containing GHR/GHBP transcripts in virgin hepatic nuclear RNA and total cellular RNA remained unchanged relative to late-pregnant nuclear and total cellular RNA samples respectively (Fig. 4B). However, the levels of these transcripts were significantly higher (about twofold) in total cellular RNA preparations compared with nuclear RNA pools (Fig. 4B).

In contrast, nuclear and total cellular transcripts associated with 5' UTRs other than 5' UTR L2 (based on the 102 nt band) and unspliced primary transcripts (based on the 81 nt band) were significantly upregulated by pregnancy (Fig. 4C). Profiles of GHR/GHBP transcripts lacking 5' UTR L2 (Fig. 4C) were very similar to the profiles of total GHR/GHBP transcripts assessed by hybridization to the coding region probe (Fig. 2B), and of GHR/GHBP RNA species containing 5' UTR L1 (Fig. 3B).

After correcting for the difference in size of the 102 and 171 nt bands based on uridine content, the 5' UTR L2 probe also allowed us to compare the relative amounts of 5' UTR L2-containing GHR/GHBP transcripts with those lacking 5' UTR L2. On day 14 of gestation, the predominant class of GHR/GHBP transcripts was the one not associated

with 5' UTR L2. The relative abundance of 5' UTR L2-containing transcripts was only 2.8% (nuclear RNA) to 5% (total RNA) of the amount of 5' UTR L2-lacking transcripts.

DISCUSSION

Pregnancy in the mouse has long been known to cause the most dramatic upregulation observed in GHR and GHBP expression. This is evident not only in serum mGHBP levels (Peeters & Friesen 1977, Cramer *et al.* 1992a,b) and hepatic mGHR concentrations (Camarillo *et al.* 1998), but also in the abundance of mGHR and mGHBP mRNAs in the liver (Cramer *et al.* 1992b, Ilkbahar *et al.* 1995). However, the mechanism by which this striking induction of the GHR/GHBP gene is achieved has not been elucidated. Although the presence of multiple 5' UTRs in hepatic GHR and GHBP mRNAs and their distinct expression patterns during pregnancy suggest differential promoter activity, the extent to which transcriptional activation or increased mRNA stability is responsible for this gestational upregulation has not been determined. We addressed this question by assessing the overall transcriptional activity of the GHR/GHBP gene, employing a coding region probe complementary to both GHR and GHBP transcripts (Fig. 1A). As assayed by RPA, total cellular as well as primary and processed nuclear GHR/GHBP transcripts in the liver were markedly upregulated in late-pregnant relative to nonpregnant mice. Furthermore, the range of this increase was comparable in all three pools of RNA (Fig. 2A and B). This strong correlation between the increase of total cellular and nuclear RNA levels indicates that the principal gestational regulation of GHR and GHBP expression occurs at the level of gene transcription. The magnitude of increase in the levels of nuclear GHR/GHBP transcripts is sufficient to account for the increase in the steady-state levels of total cellular GHR/GHBP RNA (Fig. 2B). Therefore, post-transcriptional processes such as increased nuclear export or cytoplasmic mRNA

FIGURE 2. Analysis of mGHR/GHBP RNA in nuclear and total cellular RNA preparations in livers obtained from nonpregnant and late-pregnant mice using the 'coding region riboprobe'. (A) Representative phosphor-image of spliced and unspliced GHR/GHBP transcripts expressed in the liver. RPAs were carried out on 20 µg of nuclear or total cellular RNA/reaction, as described in Materials and Methods. The band in the first lane corresponds to the smallest (100 bases) size marker of a RNA molecular weight standard. (B) Expression profiles of hepatic GHR/GHBP transcripts. Levels of spliced transcripts are based on the intensity of the 66 nt fragment, whereas the intensity of the 45 nt band represents levels of unspliced, primary transcripts. Each bar represents the mean±S.E.M. of individually assayed nine nuclear and eight total cellular RNA samples, each obtained from a different animal. Bars with different superscripts differ significantly ($P<0.05$). Levels of primary, unspliced transcripts (bars denoted by a and b) were not directly compared statistically with spliced nuclear or total cellular RNA (bars denoted by A and B). NP, nonpregnant; D14, day 14 of pregnancy.

FIGURE 3. Analysis of mGHR/GHBP transcripts associated with 5' UTR L1 in nuclear and total cellular RNA preparations in livers obtained from nonpregnant and late pregnant mice. (A) Representative phosphor-image of spliced and unspliced GHR/GHBP transcripts expressed in the liver. RPAs were carried out on 10 μ g of nuclear or total cellular RNA/reaction, as described in Materials and Methods. The bands in the first lane correspond to the 100 and 200 base markers of a 100 base RNA ladder. (B) Expression profiles of hepatic GHR/GHBP transcripts containing the 5' UTR L1 sequence. Abundance of primary transcripts was calculated using the 89 nt band, that of spliced transcripts using the 125 base band. Each bar represents the mean $\pm$ S.E.M. of individually assayed nine nuclear and seven total cellular RNA samples, each extracted from a different mouse. Statistical analysis was carried out as described in the legend to Fig. 2. NP, nonpregnant; D14, day 14 of pregnancy.

FIGURE 4. Analysis of mGHR/GHBP transcripts associated with 5' UTR L2 in nuclear and total cellular RNA preparations in livers obtained from nonpregnant and late-pregnant mice. (A) Representative phosphor-image of spliced and unspliced GHR/GHBP transcripts expressed in the liver. RPAs were carried out on 10 μ g of nuclear or total cellular RNA/reaction, as described in Materials and Methods. The bands in the first lane correspond to the 100 and 200 base markers of an RNA molecular weight standard. (B) Expression profiles of hepatic GHR/GHBP transcripts associated with 5' UTR L2 based on quantitation of the 171 base signal. Each bar represents the mean $\pm$ s.e.m. of nine nuclear and seven total cellular RNA samples from different mice. (C) Expression profiles of hepatic GHR/GHBP transcripts lacking the 5' UTR L2 sequence based on signal intensities of the 102 base (spliced transcripts) and 81 base (unspliced, primary transcripts) protected fragments. Each bar represents the mean $\pm$ s.e.m. of nine nuclear and seven total cellular RNA samples. Statistical analysis was carried out as described in the legend to Fig. 2. NP, nonpregnant; D14, day 14 of pregnancy.

stability can be excluded as major factors from the regulation of GHR/GHBP gene expression during pregnancy.

We previously identified two distinct 5' UTRs, L1 and L2, in hepatic and placental mRNA from late-pregnant mice, using a PCR approach to amplify cDNA ends (Southard *et al.* 1995). The complete divergence in sequence of these two 5' UTRs, their differential expression patterns under varying physiological conditions and in different

tissues (Southard *et al.* 1995), and the presence of separate transcription start sites for each (Menon *et al.* 1995, Edens 1998) predicted that 5' UTRs L1 and L2 are encoded by different alternative first exons under the control of distinct promoters. Indeed, we found GHR and GHBP mRNAs associated with 5' UTR L1 to be almost exclusively expressed in the late-pregnant liver, implicating the 5' UTR L1 promoter as the liver- and pregnancy-specific promoter (Southard *et al.* 1995).

In contrast, the 5' UTR L2 sequence was not associated exclusively with GHR and GHBP mRNAs expressed in a pregnancy- or liver-specific manner, suggesting a more constitutive mode of action for the 5' UTR L2 promoter. Extension of the analysis of divergent 5' UTR sequences to GHR and GHBP cDNAs obtained from nonpregnant mouse liver and tissues other than the liver has allowed us to isolate three additional 5' UTR variants, none of which is expressed differentially during pregnancy or at significant levels in the liver (Moffat *et al.* 1998). Taken together, these findings indicate that the two promoters mainly responsible for GHR and GHBP expression in the liver are those of 5' UTRs L1 and L2. Therefore, after establishing that transcriptional activation underlies the gestational increase in GHR and GHBP mRNAs in the mouse liver, we focused our efforts on evaluating the relative contributions of the 5' UTR L1 and L2 promoters to this increase.

Previous studies examining 5' UTR L1 and L2 expression patterns relied on Northern analysis of poly(A)-tailed RNA utilizing 5' UTR L1- or L2-specific sequences as probes. This only allowed the separate quantitation of GHR or GHBP mRNAs containing 5' UTR L1 or L2, but not the measurement of total amounts of 5' UTR L1- or 5' UTR L2-associated transcripts, which becomes crucial in the assessment of the transcriptional activity of each promoter in the overall expression of the gene. In the present study, we made use of riboprobes that detect the coding region exons in addition to either 5' UTR L1- or L2-specific sequences in an RPA. In this way, we were able to quantitate relative levels of 5' UTR L1- or L2-containing transcripts and compare these levels between total cellular and nuclear RNA samples from virgin and 14-day-pregnant mice. Our results indicate that the liver-specific transcriptional response of the mGHR/GHBP gene to pregnancy is driven by the 5' UTR L1 promoter, and not by the 5' UTR L2 promoter.

Several lines of evidence presented in this report support this conclusion. Processed GHR and GHBP transcripts containing 5' UTR L1 are almost undetectable in nuclear and total cellular RNA obtained from nonpregnant animals, but are strikingly elevated on day 14 of pregnancy (Fig. 3B). Although a gestational induction is apparent in primary transcripts as well, the magnitude of this induction does not reach that observed with processed transcripts. This may be explained by the way in which the 5' UTR L1 probe detects primary as opposed to processed transcripts. The fragment of this probe protected by primary, unspliced GHR/GHBP transcripts corresponds to exon 5'

UTR L1 (Fig. 1B). Thus, primary transcripts that have been initiated from the 5' UTR L1 promoter, as well as from the 5' UTR L2 promoter, which is located farther upstream, would hybridize to this fragment. The 5' UTR L1 riboprobe fragment detecting spliced nuclear and total cellular GHR/GHBP transcripts, however, corresponds to 5' UTR L1 joined to exon 2. Hence, only transcripts in which coding exon 2 is spliced to exon 5' UTR L1, and not to any other 5' UTR variant, would hybridize to this riboprobe fragment. Since 5' UTR exons are mutually exclusively spliced into the mature transcripts, only primary transcripts initiated from the 5' UTR L1 promoter are spliced to include exon 5' UTR L1. Therefore, the spliced GHR/GHBP transcripts detected by the 5' UTR L1 probe are derived only from the 5' UTR L1 promoter. The less pronounced upregulation of the primary transcripts, which reflect the combined activities of the 5' UTR L1 and L2 promoters, in comparison with spliced nuclear and total cellular RNA, reflecting the activity of only the 5' UTR L1 promoter (Fig. 3B), could thus be considered as additional evidence that the 5' UTR L1 promoter is primarily responsible for the hepatic induction of GHR/GHBP expression during pregnancy. This conclusion is strengthened also by our observations in RPAs employing the 5' UTR L2 probe.

Nuclear and total cellular GHR/GHBP transcripts from which exon 5' UTR L2 is absent were significantly upregulated on day 14 of pregnancy (Fig. 4C). The gestational profiles of transcripts that lack 5' UTR L2, and thus are associated with (an)other 5' UTR(s), paralleled profiles of the overall expression of GHR/GHBP transcripts (Fig. 2B) and of 5' UTR L1-associated transcripts (Fig. 3B). In contrast, the levels of 5' UTR L2-containing GHR/GHBP transcripts were comparable between nonpregnant and late-pregnant livers both in nuclear and in total cellular RNA samples (Fig. 4B). Furthermore, on day 14 of gestation, the relative amount of 5' UTR L2-lacking transcripts was many fold higher than the amount of 5' UTR L2-containing transcripts. In nonpregnant animals though, the contribution of GHR/GHBP transcripts not associated with 5' UTR L2 was much less than that of transcripts associated with 5' UTR L2. These results suggest that the 5' UTR L2 promoter is predominantly active in the nonpregnant liver, yet its contribution to the expression of the GHR/GHBP gene in the late-pregnant liver is minimal. Interestingly, unlike 5' UTR L1-associated transcripts, levels of 5' UTR L2-containing, spliced GHR/GHBP messages were lower in the nucleus than in the cytosol both in pregnant and nonpregnant animals (Fig. 4B). This

implies that association of GHR/GHBP transcripts with 5' UTR L2 increases either their rate of transport out of the nucleus or their stability in the cytoplasm. As the abundance of total, spliced GHR/GHBP transcripts remains comparable between total cellular and nuclear RNA preparations in nonpregnant as well as late-pregnant mice (Fig. 2B), such post-transcriptional processes appear to be inconsequential in the overall expression of GHR and GHBP mRNAs in the liver.

Taken together, the findings summarized above indicate that in the pregnant mouse liver GHR/GHBP transcripts lacking 5' UTR L2 are analogous to 5' UTR L1-containing transcripts and that the gestational upregulation of the GHR/GHBP gene can be attributed entirely to the increase in the levels of 5' UTR L1-associated transcripts.

The promoter of alternative first exon 5' UTR L1 has been characterized (Menon *et al.* 1995, Schwartzbauer *et al.* 1998). Sequencing of the proximal promoter region revealed two TATA boxes and putative binding sites for the common transcription factors activator protein-2 (AP-2) and CCAAT box-binding transcription factor/nuclear factor-I (CTF/NF-I). A consensus sequence for nuclear factor-interleukin 6 (NF/IL-6), a member of the CCAAT enhancer-binding protein (C/EBP) family, known to be a critical regulator of liver-specific gene expression (Johnson & Williams 1994, Poli & Ciliberto 1994), was also localized to this promoter. Transient transfection, deletional analysis, and electromobility shift assays led to the identification of two additional regulatory elements. One of these is located 3 kilobases upstream of the 5' UTR L1 exon and has been identified as an additional binding site for CTF/NF-I (Zou & Menon 1995). Analogous to the regulation of the serum albumin gene (Cereghini *et al.* 1987, Raymondjean *et al.* 1988), which is considered to be the prototypical liver-specific gene, particular interactions between C/EBP and CTF/NF-I transcription factors may underlie the liver-specific expression of 5' UTR L1-associated GHR/GHBP transcripts. A second regulatory element is 3.4 kilobases upstream of the 5' UTR L1 exon and is believed to be involved in the pregnancy-specific upregulation of the GHR/GHBP gene (Menon *et al.* 1997, Schwartzbauer *et al.* 1998). This element interacts with the single-stranded DNA-binding protein MSY-1, which functions as a repressor. A decrease in the nuclear content of MSY-1 during pregnancy is achieved by translocation of the protein into the cytoplasm (Schwartzbauer *et al.* 1998).

As would be expected of a constitutive promoter, the 5' UTR L2 proximal promoter contains putative

binding sites for ubiquitous transcription factors such as Sp1 and CTF/NF-I (Edens & Talamantes 1998).

In the rat, five 5' UTR variants of the GHR and GHBP gene have been identified (Baumbach *et al.* 1989, Domene *et al.* 1995). Among these, variants GHR₁ and GHR₂ (Baumbach & Bingham 1995) are homologous to mouse 5' UTR L1 and L2 (Southard *et al.* 1995) respectively. As in the mouse, levels of 5' GHR₁-containing GHR and GHBP mRNAs increase during pregnancy in the rat liver (Rubtsov *et al.* 1993, Baumbach & Bingham 1995). These transcripts are also expressed in a sexually dimorphic (Rubtsov *et al.* 1993, Baumbach & Bingham 1995, Gabriellson *et al.* 1995) and GH-dependent (Baumbach & Bingham 1995) fashion. The proximal promoter region (up to 608 basepairs upstream) of 5' GHR₁ does not contain any regulatory element motifs which would clarify the mechanism underlying the response of the rat GHR₁ promoter to gonadal steroids or GH. As is the case with its mouse homologue, the gestational regulation of the rat GHR₁ promoter also remains to be elucidated.

In summary, we conclude that the pregnancy-induced upregulation of steady-state levels of hepatic GHR and GHBP mRNAs can be attributed to the increased transcriptional activity of the GHR/GHBP gene. We also provide evidence that hepatic GHR/GHBP transcripts initiated from the 5' UTR L1 promoter, and not from the 5' UTR L2 promoter, are responsible for the dramatic gestational increase in GHR/GHBP gene expression. The molecular mechanism by which the 5' UTR L1 promoter is activated during pregnancy is currently under further investigation.

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