

# Growth Hormone Stimulates Hepatic Expression of Bovine Growth Hormone Receptor Messenger Ribonucleic Acid through Signal Transducer and Activator of Transcription 5 Activation of a Major Growth Hormone Receptor Gene Promoter

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The objective of this study was to determine whether and how GH regulates hepatic expression of GH receptor (GHR) mRNA in cattle. Ribonuclease protection assays revealed that injection of GH in a slow-release formula increased both hepatic GHR and IGF-I mRNAs 1 wk after the injection. The increases in GHR and IGF-I mRNAs were highly correlated. Western blot analysis showed that the injection also increased liver GHR protein level. In cattle and other mammals, hepatic GHR mRNA is expressed as variants that differ in the 5'-untranslated region due to the use of different promoters in transcription and/or alternative splicing. We found that GH increased the expression of the liver-specific GHR mRNA variant GHR1A without affecting the other two major GHR mRNA variants in the bovine liver, GHR1B and GHR1C. In transient transfection analyses, GH could robustly activate reporter gene expression from a 2.7-kb GHR1A promoter, suggesting that GH augmentation of GHR1A mRNA expression in

the liver is at least partially mediated at the transcriptional level. Additional transfection analyses of serially 5'-truncated fragments of this promoter narrowed the GH-responsive sequence element down to a 210-bp region that contained a putative signal transducer and activator of transcription 5 (STAT5) binding site. EMSAs demonstrated that this putative STAT5 binding site was able to bind to STAT5b protein. In cotransfection assays, deletion of this putative STAT5 binding site abolished most of the GH response of the GHR1A promoter. Like 1-wk GH action, 6-h (*i.e.* short-term) GH action also increased liver expression of GHR1A and total GHR mRNAs in cattle. These observations together suggest that GH directly stimulates the expression of one GHR mRNA variant, GHR1A, through binding STAT5 to its promoter, thereby increasing GHR mRNA and protein expression in the bovine liver. (*Endocrinology* 148: 3307-3315, 2007)

GH IS A MAJOR REGULATOR of animal growth and metabolism. At the cellular level, GH initiates its action by binding to a cell membrane receptor, the GH receptor (GHR) (1) and by activating multiple intracellular signaling pathways from the receptor (2). The magnitude and duration of GH action in a tissue is therefore affected by the GHR expression level in that tissue. GHR is widely distributed in the body, with the greatest expression in the liver (1). A well-established GH action in the liver is increased production of IGF-I (3), mediated primarily by the Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) pathway (4, 5). Liver IGF-I constitutes more than 75% of IGF-I in the blood (6) and plays an important role in regulating bone growth and pituitary secretion of GH (6, 7). Many factors influence GHR expression in the liver (8, 9). Liver GHR level is low in prenatal animals but rises quickly

after birth, and this postnatal increase in GHR expression may be one of the reasons that GH stimulates postnatal but not prenatal growth (9). Liver expression of GHR is reduced in undernutrition, type 1 diabetes (8, 9), and cirrhosis (10) and is believed to contribute to GH resistance associated with those situations (8-10).

Liver GHR expression is also regulated by GH (8, 9). Liver GHR number was decreased by hypophysectomy in rabbits and sheep (11). Liver GHR number was increased by continuous infusion of GH in hypophysectomized or pituitary-intact rats (12, 13) and by repeated injection of GH in pituitary-intact pigs (14) and pituitary-intact sheep (15). Liver GHR number was also increased in hypersomatotropic rats bearing pituitary tumors (16). These observations suggest that chronic action of GH up-regulates GHR expression in the liver. Acute action of GH, however, appears to down-regulate GHR expression in the liver because liver GHR number was decreased by 50% in rats 6 h after a single injection of GH (17). The down-regulatory effect of acute GH on GHR is perhaps due to increased receptor internalization (17). The up-regulatory effect of chronic GH on liver GHR expression likely involves increased expression of GHR mRNA, but the studies on this potential mechanism have generated conflicting results. Mathews *et al.* (18) and Frick *et al.* (19) reported

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GHR, GH receptor; HNF, hepatocyte nuclear factor; JAK2, Janus kinase 2; RPA, ribonuclease protection assay; STAT5, signal transducer and activator of transcription 5; UTR, untranslated region.

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no effect of hypophysectomy or continuous injection of GH on liver GHR mRNA in rats, but a more recent study by Baumbach and Bingham (20) revealed that hypophysectomy decreased liver GHR mRNA variant GHR1 in rats and that continuous infusion of GH restored its expression. Mathews *et al.* (18) also reported that liver GHR mRNA was not different between GH-deficient *lit/lit* and wild-type mice, but Iida *et al.* (21, 22) detected a decrease in liver GHR mRNA in *lit/lit* mice and an increase in liver GHR mRNA in transgenic mice overexpressing bovine GH. Currie *et al.* (23) and Bassett *et al.* (24) reported that twice-daily injection of bovine GH for 7 or 5 d did not alter hepatic GHR mRNA abundance in pregnant or lactating sheep, respectively. Therefore, whether chronic action of GH can increase GHR mRNA expression, thereby increasing GHR number in the liver, needs further clarification.

The objective of this study was to determine whether and how chronic GH affects hepatic expression of GHR mRNA and protein in cattle. In cattle and several other mammals, GHR mRNA is expressed as variants that differ in the 5'-untranslated region (5'-UTR), due to transcription initiation from different leader exons of GHR gene and/or alternative splicing of GHR pre-mRNA (25). The major GHR mRNA variants expressed in the bovine liver are GHR1A, -1B, and -1C mRNAs, which are transcribed from leader exons 1A, 1B, and 1C, respectively, and which normally represent more than 90% of total GHR mRNA in a bovine liver (26–28). The expression of GHR1A mRNA is liver and postnatal stage specific (27). Our results from this study indicate that injection of GH in a slow-release formula increases liver GHR mRNA and GHR protein expression and that the increase in GHR mRNA is due to increased expression of GHR1A mRNA. Our results also suggest that GH stimulates GHR1A mRNA expression mainly through binding of STAT5 to a response element in the GHR1A promoter.

## Materials and Methods

### Animal experiment

Liver biopsy was taken from 10 nonlactating and nonpregnant cows 1 d before and 1 wk after receiving a sc injection of 500 mg recombinant bovine GH in a slow-release formula (Monsanto Co., St. Louis, MO), and from five cows 6 h after a similar GH or saline injection. The liver biopsy was taken through the skin between the 11th and 12th ribs, as described previously (29). Once taken, the liver samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . All cows had free access to feed and water during the experiment. The animal-related protocol was approved by the Virginia Tech Animal Care Committee.

### RNA extraction and ribonuclease protection assay

Total RNA from liver samples was extracted using TRI reagent (MRC, Cincinnati, OH), according to the manufacturer's instructions. The concentration and integrity of the extracted RNA were determined by spectrophotometry and gel electrophoresis, respectively.

Ribonuclease protection assay (RPA) was used to measure the abundance of total GHR, GHR1A, GHR1B, GHR1C, IGF-I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. The riboprobe for total GHR mRNA was transcribed from a 317-bp bovine cDNA composed of GHR exons 2–5, the exons that are present in all GHR mRNA variants. The probe for GHR1A mRNA was synthesized from a 312-bp GHR cDNA fragment that was composed of 191-bp exon 1A, 81-bp exon 2, and 40-bp exon 3, and this probe hybridized with GHR1A mRNA at exons 1A, 2, and 3 and with other GHR mRNA variants (*i.e.* non-1A GHR mRNA) at exons 2 and 3. The probe for GHR1B mRNA was transcribed

from a 96-bp GHR cDNA fragment that contained 44-bp exon 1B and 52-bp exon 2. The probe for GHR1C mRNA was synthesized from a GHR cDNA fragment that contained 533-bp exon 1C and 52-bp exon 2. Similar to the GHR1A probe, the GHR1B and -1C probes hybridized not only with GHR1B and -1C mRNAs, respectively, but also with non-1B and non-1C GHR mRNA variants at the 52-bp exon 2 region. The probes for total IGF-I and GAPDH mRNAs were synthesized as described previously (30). All probes were synthesized by *in vitro* transcription in the presence of [ $\alpha$ - $^{32}\text{P}$ ]CTP, using the Riboprobe Combination Systems kit (Promega, Madison, WI), as described previously (30).

The RPA was done using the RPAII kit (Ambion, Austin, TX). Briefly, 20  $\mu\text{g}$  total RNA was hybridized with the probe for total GHR, GHR1A, GHR1B, GHR1C, or IGF-I mRNA and also the probe for GAPDH mRNA (loading control) in 20  $\mu\text{l}$  hybridization buffer for about 16 h at  $42^{\circ}\text{C}$ . The hybridization was then digested with 200  $\mu\text{l}$  of 1:100 diluted ribonucleases A and  $\text{T}_1$  at  $37^{\circ}\text{C}$  for 40 min. The ribonuclease-protected RNA fragments were precipitated and resolved on 6% polyacrylamide gels containing 7 M urea. The gels were dried, exposed to phosphorscreens, and scanned on a Molecular Imager FX System (Bio-Rad, Hercules, CA). The intensities of the ribonuclease-protected probe fragments, which reflected the abundance of their corresponding mRNAs, were measured using ImageJ (<http://rsb.info.nih.gov/ij>). The measured abundance of GHR or IGF-I mRNA in a sample was divided by that of the GAPDH mRNA in the same sample to normalize potential variation in starting amount of RNA and in performing RPA. Based on a previous study (31), liver GAPDH mRNA expression is not affected by GH treatment in cattle.

### Liver total protein extraction and Western blot analysis

About 200 mg liver tissue was homogenized in 2.5 ml ice-cold buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.6), 5 mM EGTA, 150 mM NaCl, 1 mM  $\text{Na}_2\text{VO}_4$ , 1 mM Na pyrophosphate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mg/liter aprotinin, and 10 mg/liter leupeptin. The homogenates were centrifuged at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and the supernatants were collected and stored at  $-80^{\circ}\text{C}$  until use. Protein concentrations were determined using a Bio-Rad protein assay kit according to the manufacturer's instructions.

For Western blot analysis, 40  $\mu\text{g}$  liver protein was separated by electrophoresis in an 8% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes (Bio-Rad). After blocking with 5% nonfat dried milk in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% Tween 20 for 1 h, the upper half of the membrane was incubated with anti-GHR<sub>CYTAL47</sub> (1:1000 dilution), a rabbit antibody against the human GHR intracellular domain (32) that is known to cross-react with the bovine GHR (33), at  $4^{\circ}\text{C}$  overnight; the lower half of the membrane was incubated with a rabbit antibody against  $\beta$ -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 dilution. After being washed three times in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% Tween 20, the membrane was incubated with a horseradish peroxidase-conjugated goat antirabbit IgG antibody (Santa Cruz Biotechnology) at 1:2000 dilution for 2 h at room temperature. The membrane was subsequently incubated in SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescent signals were detected by exposure to x-ray films. The intensities of GHR and  $\beta$ -actin bands were measured using ImageJ, and the measured band intensity of GHR protein in a sample was divided by that of  $\beta$ -actin in the same sample to normalize variation in protein loading and/or transfer.

### Promoter-reporter plasmid construction

A 2730-bp and a 1719-bp GHR1A promoter were cloned into the promoter-less luciferase reporter vector pGL2b (Promega) at *SacI* and *NheI* sites in a previous study (34), and these two plasmids were renamed 1A-2730 and 1A-1719, respectively, in this study. Five 5'-truncated GHR1A promoter-luciferase gene constructs, designated 1A-2200, 1A-1250, 1A-510, 1A-300, and 1A-170, were derived from 1A-2730 by standard PCR and cloning. The forward primers for these PCR were 1A2200F, 1A1250F, 1A510F, 1A300F, and 1A170F, respectively, which all contained a *SacI* restriction site linker (Table 1). The reverse primers for these PCR were 1APromoterR, which contained a *NheI* linker (Table 1).

From 1A-510, the putative STAT5 binding site between  $-380$  and  $-388$  (relative to the transcription start site for GHR1A mRNA, num-

**TABLE 1.** Sequences of oligonucleotides used in this study

Name	Sequence (5' to 3')
1A2200F	GAGAGAGCTCCATAGTGATTGTGCCAATTT
1A1250F	GAGAGAGCTCCACCTCATTAGAACTGATTC
1A510F	GAGAGAGCTCGGTGGGTCTCCAGAATGTT
1A300F	GAGAGAGCTCATTCTAATACGGCCCTTCTC
1A170F	GAGAGAGCTCATGTGAGGCAATGCGTTGTG
1APromoterR	TTCCGCTAGCAGATTCCTGGTCTTGCTGCT
1ASTAT5MF	AGCTTCCGCGGCCGCACTCCTTAGCTGTGGGAT
1ASTAT5MR	TAAGGAGTGC GGCCGCGGAAGCTCCAGGAGGGC
1ASTAT5F	TCCTTCCTTGAATC
1ASTAT5R	GAGTTCAAGGAAGGA

bered +1), was replaced by a *NotI* site to generate a mutant construct designated 1A-510M. This mutation was made by three rounds of PCR, using primer pairs 1A2200F and 1ASTAT5MR, 1ASTAT5MF and 1APromoterR, and 1A2200F and 1APromoterR, respectively (Table 1). The inserts in all new plasmids and the mutation to the STAT5 binding site were verified by sequencing at the Virginia Bioinformatics Institute (Blacksburg, VA).

#### Cell culture and transient transfection analysis

Chinese hamster ovary-derived CHO cells (ATCC, Manassas, VA) were cultured in 24-well plates in MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The CHO cells at approximately 50% confluency were transfected with 0.5 µg GHR promoter-reporter plasmid, 0.2 µg GHR expression plasmid (35), 0.2 µg STAT5b expression plasmid (35), and 1 ng *renilla* luciferase reporter construct pRL-CMV (serving as transfection efficiency control) per well, using FuGENE6 (Roche, Indianapolis, IN). Twenty-four hours after transfection, the medium was replaced with serum-free MEM, and the cells were cultured for another 16 h. The cells were then treated with 500 ng/ml recombinant bovine GH (provided by the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) for 8 h before being lysed for luciferase assay. The luciferase assay was done using the Dual-Luciferase Reporter Assay System (Promega), essentially following the manufacturer's instructions.

#### Nuclear protein extraction and EMSA

CHO cells were transfected with a constitutively active STAT5b mutant (N642H) expression plasmid (5) or vector plasmid pcDNA3.1 (Invitrogen). Forty-eight hours after transfection, nuclear proteins were isolated from the cells as previously described (35). Nuclear proteins were also isolated from CHO cells transfected with a wild-type STAT5b and a GHR expression plasmid (as described above) and treated with 500 ng/ml GH for 30 min. Protein concentrations of the nuclear extracts were determined as described for liver protein extracts. Double-stranded oligonucleotides (Table 1) corresponding to the putative STAT5 binding site in the GHR1A promoter were end-labeled using T4 polynucleotide kinase and [<sup>32</sup>P-γ]ATP. Approximately 1 ng <sup>32</sup>P-labeled oligonucleotide probe was mixed with 10 µg nuclear proteins and 2 µg poly d(IC) (Sigma) in a buffer containing 20% glycerol, 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA. The mixture was incubated at 4°C for 1 h and subsequently added with 1 µg anti-STAT5 antibody (Abcam Inc., Cambridge, MA) or 1 µg normal rabbit serum, and the incubation was continued overnight at 4°C. The mixture was resolved in a 6% polyacrylamide gel. The DNA-protein complexes were visualized by phosphorimaging as described for RPA.

#### Statistical analysis

The firefly luciferase activity from a GHR promoter-reporter construct was divided by the *renilla* luciferase activity from pRL-CMV in the same well to normalize variation in transfection efficiency. These luciferase data were analyzed by *t* test to compare two means or by one-factor ANOVA followed by the Tukey test to compare multiple means. All data are expressed as mean ± SE of the mean. Differences at *P* < 0.05 are considered significant.

## Results

### *GH injection increased liver expression of GHR and IGF-I mRNAs and GHR protein*

To determine whether chronic action of GH increases GHR mRNA expression in the bovine liver, cows were treated with recombinant bovine GH in a slow-release formula. One week after the injection, liver expression of total GHR mRNA, as measured by RPA using a probe recognizing all GHR mRNA variants (Fig. 1A), was increased 2-fold (*P* < 0.05) compared with that before the GH treatment (Fig. 1C). The GH injection also increased (*P* < 0.01) liver IGF-I mRNA expression, and the magnitude of this increase was similar to that in GHR mRNA (Fig. 1, A and C). Liver expression of GHR mRNA was highly correlated (*P* < 0.01) with that of IGF-I mRNA before and after the GH injection (Fig. 1D).

Western blotting analysis of total protein extracts from the liver, using an antibody specific for the intracellular domain of the GHR protein (32), detected two GHR protein bands (Fig. 1B). The two bands corresponded to mature (the more slowly migrating band) and precursor GHR protein, respectively (32). Based on this analysis (Fig. 1B) and a Western blotting analysis of microsomal membrane extracts (image not shown), the abundance of GHR protein in the liver 1 wk after GH injection was 2-fold (*P* < 0.05) that before GH injection (Fig. 1, B and C). This difference in GHR protein was comparable to that in total GHR mRNA (Fig. 1C), suggesting that the increase in GHR protein was due to increased expression of GHR mRNA.

### *GH injection increased GHR1A mRNA without affecting other major GHR mRNA variants in the liver*

To determine whether the increase in GHR mRNA expression after GH injection was due to increased expression of a specific GHR mRNA variant, liver RNA from another five cows before and 1 wk after GH injection were analyzed by RPA using probes specific for GHR1A, -1B, and -1C mRNAs, the three major GHR mRNA variants in the bovine liver (26). The probe for GHR1A mRNA generated two major ribonuclease-protected bands, one corresponding to GHR1A mRNA and the other one to all other GHR mRNA variants, or non-1A GHR mRNAs (Fig. 2A). Based on this RPA, GH injection increased (*P* < 0.01) liver GHR1A mRNA abundance by about 75%, whereas it had no effect on the abundance of non-1A GHR mRNA variants (Fig. 2, A and B). The RPA using an antisense probe to GHR1B or GHR1C mRNA (Fig. 2A) showed that GH injection did not affect the expression of either of these two mRNAs (Fig. 2B), whereas it increased (*P* < 0.05) the abundance of non-1B or non-1C GHR mRNAs (Fig. 2B). The magnitude of GH-induced increase in non-1B or non-1C GHR mRNA was also comparable to that in total GHR mRNA or GHR1A mRNA (Fig. 2B). These analyses together indicated that GH increased the expression of GHR1A mRNA without affecting that of GHR1B and GHR1C mRNAs in the bovine liver.

### *GH increased reporter gene expression from the GHR1A promoter*

To determine whether GH-stimulated expression of GHR1A mRNA in the liver was due to increased transcrip-

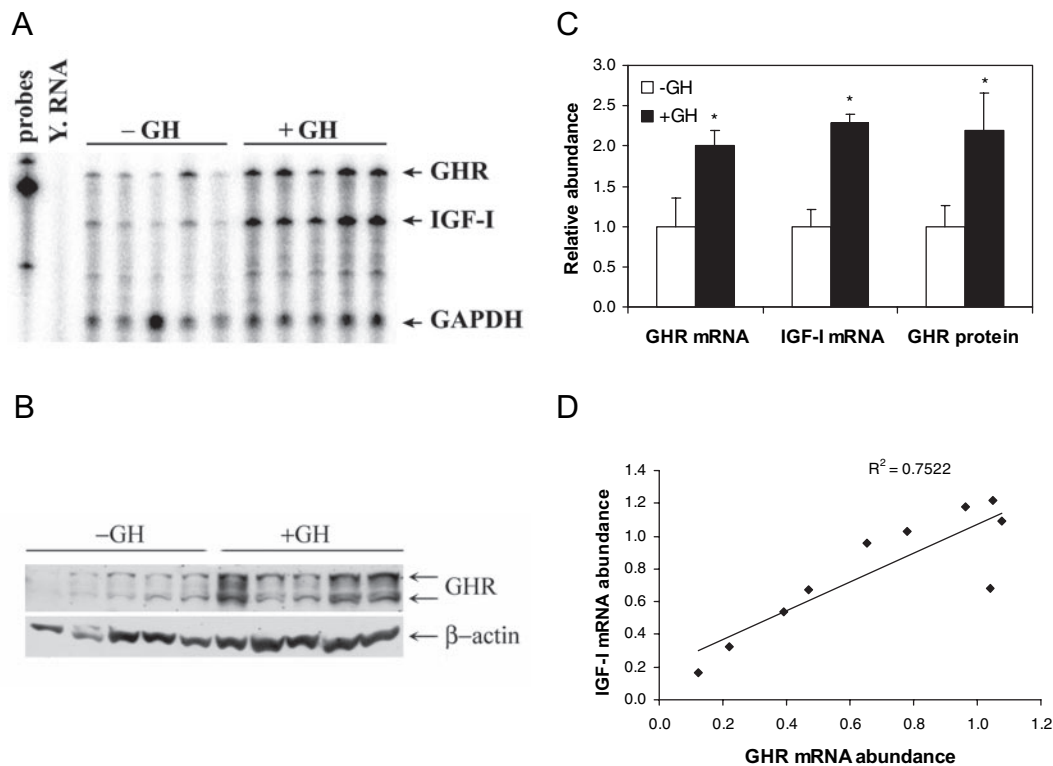


FIG. 1. One-week GH injection increased GHR and IGF-I mRNAs and GHR protein in the bovine liver. A, RPA of liver GHR and IGF-I mRNAs in cows ( $n = 5$ ) 1 d before ( $-GH$ ) and 1 wk after GH injection ( $+GH$ ). Total GHR and IGF-I mRNAs and GAPDH mRNA (loading control) were quantified simultaneously. Yeast (Y) RNA served as a negative control. Arrowheads point to ribonuclease-protected bands corresponding to GHR, IGF-I, and GAPDH mRNAs. B, Western blotting analysis of GHR protein in the bovine liver. The GHR antibody used in this analysis was raised against the intracellular domain of the human GHR (32). Two bands corresponded to the mature (the top band) and the precursor (the bottom band) GHR, respectively.  $\beta$ -Actin protein served as a loading control. C, Densitometric analysis of RNA and protein bands in A and B, respectively. The density of the band corresponding to GHR mRNA, IGF-I mRNA, or GHR protein was normalized to that of GAPDH mRNA or  $\beta$ -actin protein, respectively, in the same sample. \*,  $P < 0.05$  compared with  $-GH$  for the same mRNA or protein. D, Correlation between liver GHR mRNA and IGF-I mRNA before and after GH injection. The correlation is significant ( $P < 0.01$ ).

tion of GHR1A mRNA, a 2730-bp GHR1A promoter was tested for its response to GH in CHO cells that were made GH responsive by cotransfection of GHR and STAT5b expression plasmids. As shown in Fig. 3, the luciferase activity expressed from this GHR1A promoter was 16-fold ( $P < 0.01$ ) higher in response to GH than to PBS, the vehicle for GH. The GH-induced increase in luciferase expression from the GHR1A promoter was dependent on the inclusion of both the GHR and STAT5b expression plasmids in the transfection (data not shown). In the same CHO cells, luciferase expression from a 1000-bp GHR1B promoter, cloned in a previous study (36), was not affected by GH (Fig. 3). These results suggest that GH may stimulate GHR1A promoter activity through STAT5, a well-established component of the GHR signaling pathway, thereby increasing GHR1A mRNA expression in the bovine liver.

#### A 210-bp DNA region mediated most of the GH response of the 2730-bp GHR1A promoter

To identify the GH-responsive DNA element in the 2730-bp GHR1A promoter, 5'-deletion constructs were made from this promoter and their GH responses were compared. As shown in Fig. 4A, deletion of the region from  $-2730$  to  $-1250$  (relative to the major transcription start site for

GHR1A mRNA, numbered +1) had no effect on the response of the GHR1A promoter to GH (Fig. 4A). Deletion of the region from  $-1250$  to  $-510$  caused a 35% decrease ( $P < 0.05$ ) in GH response of the 1250-bp GHR1A promoter (Fig. 4A). Deletion of the region from  $-510$  to  $-300$  decreased ( $P < 0.01$ ) the GH response of the 510-bp GHR1A promoter by 80% (Fig. 4A). Additional deletion of the region from  $-300$  to  $-170$  had no effect on GH-induced luciferase expression from the promoter, and the 300-bp or the 170-bp GHR1A promoter remained an approximately 2-fold GH response (Fig. 4A). These observations indicated that both the DNA region between  $-510$  and  $-300$  and the 170-bp proximal promoter mediate the GH response of the 2730-bp GHR1A promoter with the former region mediating most of the response.

#### The GHR1A promoter contained a STAT5 binding site at 380 bp upstream from the transcription start site

Because the GHR1A promoter region between  $-510$  and  $-300$  appeared to mediate most of the GH response of the 2730-bp GHR1A promoter, we focused on this region for the STAT5 binding site. A candidate STAT5 binding site was located at  $-380$  of the GHR1A promoter (Fig. 4B), based on its sequence being 100% identical to the consensus sequence

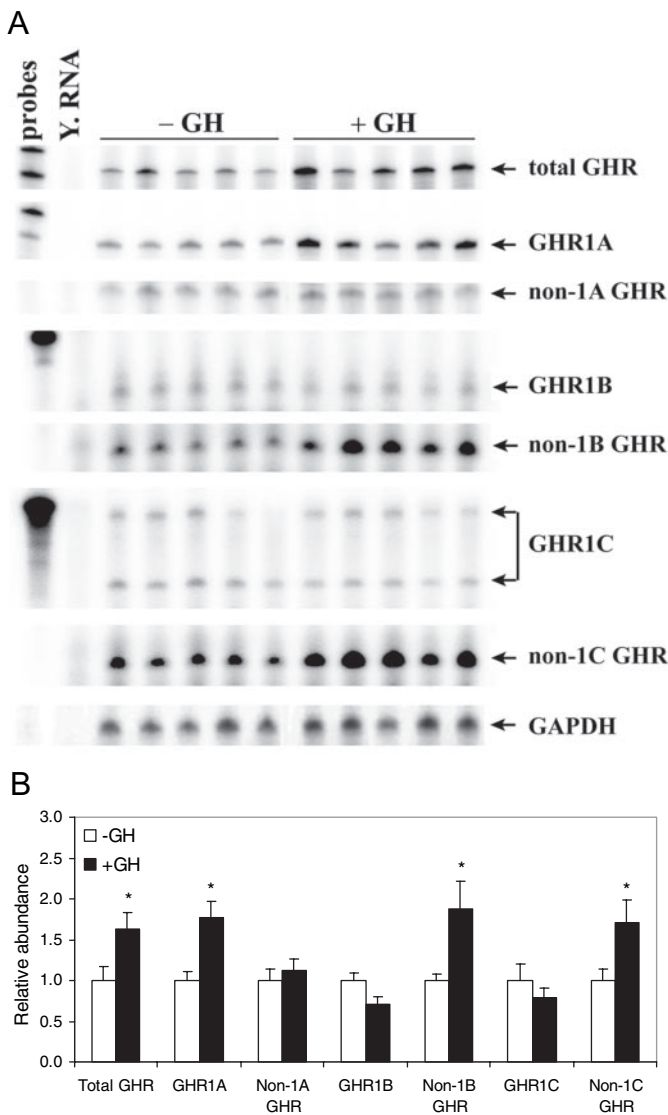


FIG. 2. One-week GH injection increased GHR1A mRNA without affecting GHR1B and GHR1C mRNAs in the bovine liver. A, RPA of liver total GHR, GHR1A, GHR1B, and GHR1C mRNAs in cows 1 d before (–GH) and 1 wk after GH injection (+GH). The probe for GHR1A mRNA hybridized with GHR1A mRNA and non-1A GHR mRNAs at different regions, generating ribonuclease-protected bands with different sizes. Similarly, the probes for GHR1B and GHR1C mRNAs generated ribonuclease-protected bands corresponding to GHR1B and non-1B GHR, and GHR1C and non-1C GHR mRNAs, respectively. The two GHR1C bands corresponded to GHR1C mRNA variants transcribed from two different start sites at GHR exon 1C (28). A probe for GAPDH mRNA was included in each RPA of total GHR, GHR1A, GHR1B, or GHR1C mRNA to control for variation in RNA loading. The GAPDH band patterns from the four RPAs were similar, and the GAPDH image from the RPA of total GHR mRNA was shown as a representative. B, Densitometric analysis of bands in A. The density of each GHR mRNA band was normalized to that of GAPDH mRNA in the same sample. \*,  $P < 0.05$ , compared with –GH for the same mRNA species.

of STAT5 binding sites: TTCNNGAA, where N is A, G, T, or C (37, 38). The ability of this potential STAT5 binding site to bind STAT5 was validated by EMSA. A double-stranded oligonucleotide corresponding to this putative STAT5 binding site formed a DNA-protein complex with nuclear pro-

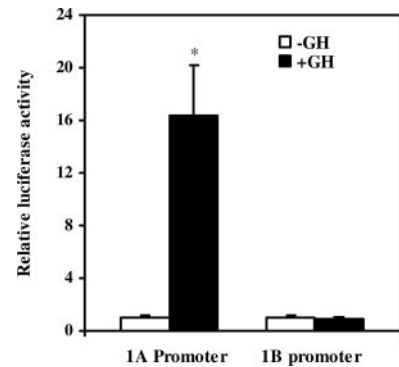


FIG. 3. GH increased luciferase expression from the GHR1A promoter but not the GHR1B promoter. A 2730-bp GHR1A promoter or a 1000-bp GHR1B promoter (36) linked 5' to the luciferase reporter gene in the promoterless vector pGL2b was cotransfected into CHO cells with a GHR expression plasmid, a STAT5b expression plasmid, and the *renilla* luciferase plasmid pRL-CMV (transfection efficiency control). The transfected cells were treated with 500 ng/ml recombinant bovine GH (+GH) or PBS (–GH) for 16 h before the luciferase assay. The experiment was repeated four times. The luciferase activity from the GHR promoter construct was divided by that from pRL-CMV to normalize variation in transfection efficiency. \*,  $P < 0.01$ , compared with –GH for the GHR1A promoter.

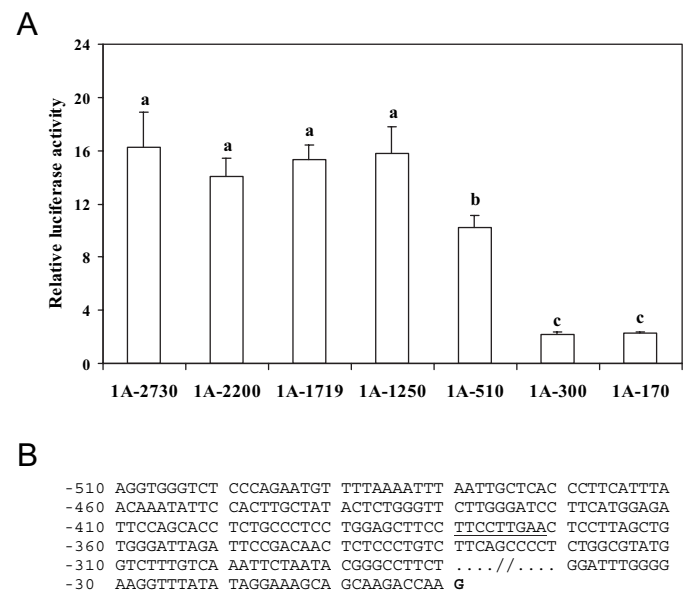


FIG. 4. A 210-bp DNA region containing a potential STAT5 binding site mediated most of the GH response of the 2730-bp GHR1A promoter. A, The 210-bp region between –510 and –300 mediated most of the GH response of the GHR1A promoter. The GH responses of 5'-truncated GHR1A promoters were tested by cotransfection analysis as described in Fig. 3. The construct 1A-2730 contained the longest GHR1A promoter, 2730 bp; the construct 1A-170 contained the shortest GHR1A promoter, 170 bp. The analysis was repeated four times. The bars labeled with different letters are different ( $P < 0.05$ ) from each other. B, The GHR1A promoter sequence contained a potential STAT5 binding site at –380. The potential STAT5 binding site, underlined, is identical to the consensus sequence (TTCNNGAA, where N is any nucleotide) for STAT5 binding sites. The nucleotide numbered +1 is the major transcription start site for GHR1A mRNA. The sequence between –280 and –40 is omitted.

teins from CHO cells expressing a constitutively active STAT5b mutant (5), whereas the same complex was not formed when the oligonucleotide was incubated with nu-

clear proteins from the CHO cells transfected with vector plasmid pcDNA3.1 (Fig. 5A). Similarly, the oligonucleotide formed a specific DNA-protein complex with nuclear proteins from CHO cells transfected with GHR and wild-type STAT5b expression plasmids and treated with GH (Fig. 5B). In both assays, the DNA-protein complex could be supershifted by an antibody recognizing STAT5b (Fig. 5, A and B), confirming the presence of STAT5b in the complexes. These gel-shift assays demonstrated that the putative STAT5 binding site at  $-380$  of the GHR1A promoter (Fig. 5B) can bind to STAT5b protein.

*The STAT5 binding site at  $-380$  of the GHR1A promoter was essential for the promoter to respond to GH*

To determine whether the STAT5 binding site at  $-380$  of the GHR1A promoter is required for the promoter to be activated by GH, this binding site was mutated in a 510-bp GHR1A promoter and the effect of the mutation on the ability of the 510-bp GHR1A promoter to respond to GH was tested by transfection assay. As shown in Fig. 6, the GH-induced luciferase activity from the mutant promoter, 1A-510M, was only 20% ( $P < 0.01$ ) of that from the STAT5 site-intact promoter, 1A-510, whereas basal luciferase expression from the two promoters was not different. These data indicated that the STAT5 binding site at  $-380$  was important for the GHR1A promoter to respond to GH-induced STAT5 activation. However, deletion of this STAT5 binding site did not completely block the GH response of the promoter (Fig. 6),

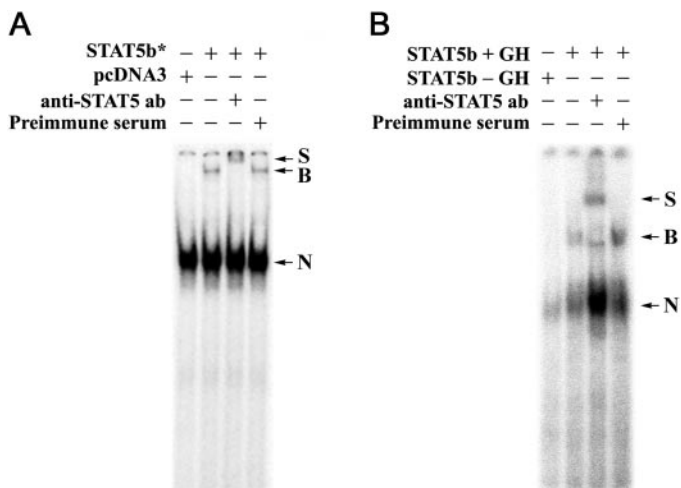


FIG. 5. EMSA of the putative STAT5 binding site at  $-380$  of the GHR1A promoter. A, A  $^{32}$ P-labeled oligonucleotide corresponding to the putative STAT5 binding site was incubated with nuclear proteins from CHO cells transfected with a constitutively active STAT5b (STAT5b\*) expression plasmid or with the cloning vector pcDNA3, in the presence (+) or absence (-) of a rabbit anti-STAT5 antibody (Ab) or rabbit preimmune serum. The arrowhead B points to a DNA-protein complex containing STAT5b and the arrowhead S indicates a supershift of this complex caused by the anti-STAT5 antibody. The arrowhead N indicates a nonspecific DNA-protein complex. The free probe has run off the gel. B, The same oligonucleotide probe in A was incubated with nuclear proteins from CHO cells transfected with wild-type STAT5b and GHR expression plasmids and treated with GH (STAT5b + GH) or without GH (STAT5b - GH). The gel was run longer than the gel in A.

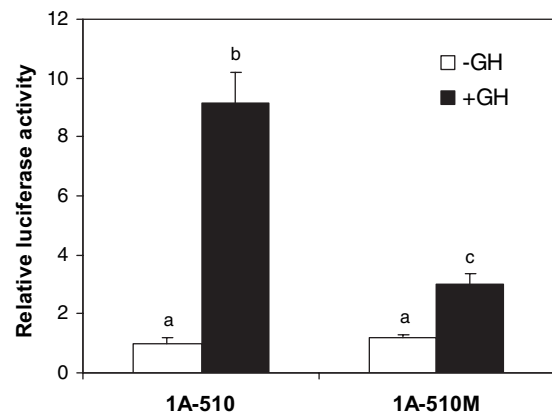


FIG. 6. The STAT5 binding site at  $-380$  was required for the GHR1A promoter to respond to GH. The GH response of a 510-bp GHR1A promoter (1A-510) containing the STAT5 binding site and the GH response of the same GHR promoter (1A-510M) with a mutated STAT5 binding site were compared by transfection analysis as described in Fig. 3. The experiment was repeated four times. The means labeled with different letters are different ( $P < 0.05$ ) from each other.

suggesting that additional DNA regions contribute to the GH response of the promoter.

*Short-term GH action also increased GHR1A and total GHR mRNAs in the liver*

To further determine whether GH can directly stimulate GHR1A mRNA and hence total GHR mRNA expression in the liver, we measured the short-term effect of GH injection on the expression levels of these mRNAs in cows. As shown in Fig. 7, A and B, cows 6 h after receiving GH injection had about 70% more ( $P < 0.05$ ) total GHR and GHR1A mRNAs in the liver than control cows receiving saline injection. As expected, the cows receiving GH injection also expressed more ( $P < 0.05$ ) IGF-I mRNA in the liver than the control cows (Fig. 7, A and B). Unexpectedly, the cows receiving GH injection also expressed more ( $P < 0.05$ ) non-1A GHR mRNAs in the liver than the control cows (Fig. 7, A and B). The biological significance of this increase in non-1A GHR mRNAs is not clear because it was a small change ( $\sim 20\%$ ) and it did not appear to sustain (Fig. 2).

## Discussion

In this study, injection of GH in a slow-release formula increased liver GHR mRNA in cattle both 6 h and 1 wk after the injection. This result is in line with the observations that continuous infusion of bovine GH increased hepatic GHR mRNA in hypophysectomized rats (20) and that hepatic GHR mRNA was increased in transgenic mice overexpressing bovine GH (21, 22). Therefore, continuous action of exogenous GH stimulates liver GHR mRNA expression at least in three species. The data from this study also showed that GH injection caused a nearly equivalent increase in GHR mRNA and protein, suggesting that GH probably does not affect the translation efficiency of GHR mRNA in the bovine liver, whereas it increases its expression. Because the increase in GHR protein likely leads to an increase in GHR number, GH stimulation of GHR mRNA expression appears to be one of the mechanisms by which continuous action of GH in-

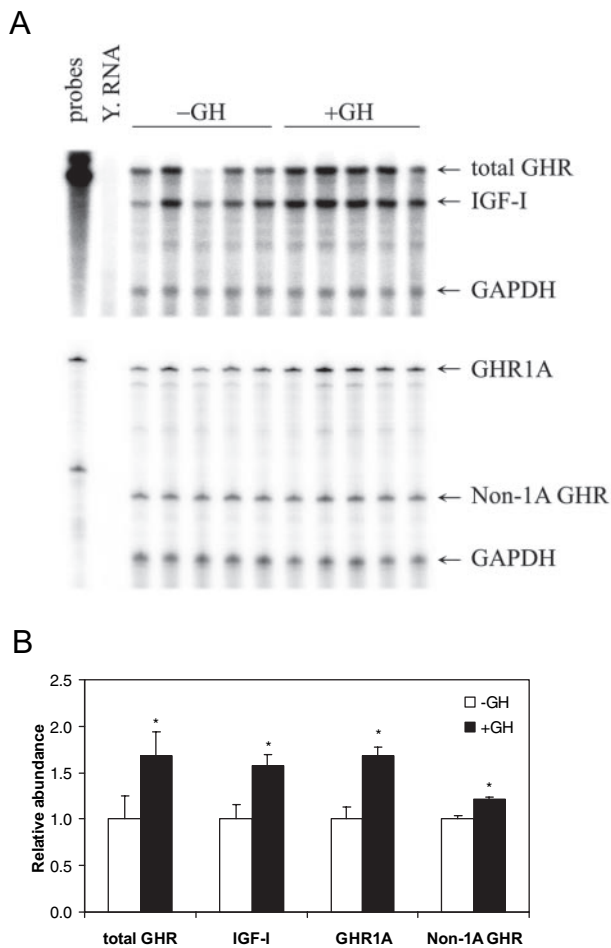


FIG. 7. Short-term GH action also increased GHR, IGF-I, and GHR1A mRNAs in the bovine liver. A, RPA of liver total GHR, GHR1A and IGF-I mRNAs in cows 6 h after GH (+GH) or saline (-GH) injection. The RPAs were performed and the ribonuclease-protected bands labeled as described in Fig. 1. B, Densitometric analysis of the ribonuclease-protected bands in A. The density of each GHR mRNA band was normalized to that of GAPDH mRNA in the same sample. \*,  $P < 0.05$ , compared with -GH for the same mRNA species.

creases GHR number in the liver, a finding that has been made in several species (11–16).

In this study, we found that GH injection significantly increased the GHR 5'-UTR variant GHR1A mRNA expression in the liver, whereas it did not alter other major GHR mRNA 5'-UTR variants. These results suggest that GH-induced GHR mRNA expression in the bovine liver is largely, if not totally, due to increased expression of GHR1A mRNA. The GHR mRNA in other mammals, such as humans, rats, mice, and sheep, is also expressed as 5'-UTR variants (25). The mouse GHR mRNA variant L1, rat GHR1, human V1, and ovine 1A are similar in sequence and liver-specific expression to the bovine GHR1A mRNA (25). GHR1 mRNA had been shown to be up-regulated by continuous infusion of GH in the hypophysectomized rats (20). Thus, GH stimulates the expression of the liver-specific GHR mRNA variant in both cattle and rats. It would be interesting to know whether GH also stimulates the expression of the mouse L1, ovine 1A, and human V1 in the liver. In this study, we did

not analyze GHR mRNA expression in extrahepatic tissues. But because GH injection did not change liver expression of GHR1B and GHR1C mRNAs, GH probably does not affect extrahepatic expression of GHR mRNA because more than 90% of the GHR mRNA in extrahepatic tissues are ubiquitously expressed GHR1B and GHR1C mRNAs (26).

GH regulates hundreds of genes in the liver (39–41). A major signaling pathway mediating this action of GH is the JAK2-STAT5 pathway from the GHR (42, 43). In this study, GH was able to markedly increase reporter gene expression from the GHR1A promoter; the GHR1A promoter was demonstrated to contain a STAT5 consensus sequence that did bind to STAT5b, the predominant STAT5 protein in the liver (44); and the STAT5 binding site was shown to be responsible for most of the GH response of the GHR1A promoter. These results together strongly suggest that GH-increased GHR1A mRNA expression in the liver is mediated by the JAK2-STAT5 pathway.

Although the JAK2-STAT5 signaling pathway seems to mediate GH regulation of many genes in the liver, STAT5 binding sites have been identified in only a small number of genes, including serine protease inhibitor (Spi) 2.1 (45), IGF-I (35, 46, 47), acid-labile subunit (48), suppressor of cytokine signaling 2 (SOCS2) (49), cytokine-inducible SH2-containing protein (50), cytochrome P450 subfamily IIC polypeptide 11 (CYP2C11), CYP2A2 and CYP4A2 (51), CYP3A10 (52), CYP2C12 (53), hepatocyte nuclear factor 6 (HNF-6) (54), and glutamine synthetase (55). In reporter gene assays, these STAT5 binding sites mediate only modest GH responses (*i.e.* changes of only a few-fold) (45–55). In contrast, the STAT5 binding site in the GHR1A promoter identified in this study mediated a greater than 10-fold GH response in reporter gene expression, suggesting that it is a transcriptionally strong STAT5 binding site. The sequence of this STAT5 binding site is not identical to any of those in IGF-I, Spi2.1, and other GH-target genes (45–55). It therefore remains to be determined whether it is this sequence difference or the difference in promoter context that makes this STAT5 binding site in the GHR1A promoter transcriptionally more active than other known STAT5 binding sites.

As mentioned earlier, GH also stimulates hepatic expression of GHR1 mRNA, the rat ortholog of the bovine GHR1A mRNA (20). Aligning the promoter sequence of the bovine GHR1A with that of the rat GHR1 as well as those of the human V1 and mouse L1 indicated that the STAT5 binding site in the bovine GHR1A promoter is not conserved in any of those other species (data not shown), suggesting that if GH stimulation of rat GHR1 or human V1 or mouse L1 mRNA expression is mediated by the JAK2-STAT5 pathway, the STAT5 binding site mediating this regulation is likely located outside their proximal promoters. In addition to STAT5, many other transcription factors have been shown to be activated by GH in the liver, including STAT1, STAT3, HNF-1, HNF-3, HNF-4, HNF-6, CAAT/enhancer-binding protein (C/EBP), c-jun, c-fos, and forkhead box proteins m1b (56, 57). Therefore, GH stimulation of rat GHR1 or GHR mRNA from any other species might be mediated by one or more of these transcription factors. Similarly, although the results of this study indicate that binding of STAT5 to the bovine GHR1A promoter is more than sufficient to induce

significant GHR1A mRNA expression (10-fold change in reporter gene expression *vs.* 2-fold change in GHR1A mRNA expression), they do not exclude the possible involvement of other transcription factors in this regulation. On top of the list of these transcription factors is HNF-4, because this transcription factor has been shown to cooperate with STAT5 in GH regulation of several genes in the liver (42) and because the bovine GHR1A promoter contains a functional HNF-4 binding site (34).

In summary, the results of this study suggest that continuous action of GH stimulates the expression of GHR1A mRNA through the JAK2-STAT5 signaling pathway, thereby increasing GHR protein expression in the bovine liver. GH action is known to be down-regulated by multiple negative feedback mechanisms (2, 58). GH stimulation of GHR1A mRNA and hence GHR protein expression may be a positive feedback mechanism that counteracts these negative feedback mechanisms and/or amplifies overall GH action in the bovine liver.

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