Regulation of Growth Hormone Receptor and Binding Protein Expression in Domestic Species (43741)

BRENDAN BINGHAM, ELIZABETH R. OLDHAM,[1] AND WILLIAM R. BAUMBACH[2]
Molecular and Cellular Biology Group, Agricultural Research Division, American Cyanamid Company, Princeton, New Jersey 08543

Abstract. **Growth hormone receptor (GHR) expression has been analyzed at the RNA level. In the rat, relative expression of the RNA species encoding the GHR and the GH-binding protein (GHBP) appears to be sensitive to endocrine status. Full-length GHR cDNA clones from ovine, porcine, and chicken were used as probes to investigate the existence of unique RNAs for GHBPs in these species. In the sheep and pig, only a single, ~4.5-kb RNA is apparent. Although quite high levels of GH binding activity are found in pig serum, a variety of methods failed to isolate a separate GHBP message, suggesting that porcine GHBP is produced via a mechanism different from that which is known for rat. One class of chicken GHR cDNA, resulting from alternative use of a splice acceptor 17 bases upstream of the Intron 6/Exon 7 junction, is also presented.**

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The effects of growth hormone (GH) are mediated by high-affinity binding to the cell surface GH receptor (GHR), which is produced in largest quantity in the liver and to a lesser extent in peripheral tissues. In addition, a high-affinity, soluble GH binding protein (GHBP) has been identified in several species (1–3). In rat and mouse, a unique, alternatively spliced form of the GHR transcript encodes the GHBP. In rats, the smaller (GHBP) message lacks the transmembrane domain of the GHR, but contains the entire sequence of the extracellular domain, as well as sequence encoding a hydrophilic carboxy-terminal domain of 17 amino acids (4) (24 amino acids in mice [5]). In rat serum, GHBP deriving from the 1.2 kb GHBP transcript is sufficient to account for all circulating GHBP (6). In humans and rabbits, however, there is both circumstantial (7) and *in vitro* (8) evidence that the soluble GHBP arises by the proteolytic cleavage of the extracellular domain from the membrane bound receptor. In the chicken, four distinct GHR transcripts exist (9), but none has been shown to be a specific GHBP message.

Herein, we present a study of differential GHR and GHBP mRNA expression in rats, including some evidence bearing on the endocrine regulation of that expression. In addition, we present GHR mRNA expression data from livestock species (pigs, sheep, and chickens). Although pigs and sheep lack a separate GHBP transcript, we present evidence for GHBPs in these animals as well. Finally, a putative GHBP mRNA in the chicken is presented.

Materials and Methods

Animals and Tissues. Animal experiments were conducted with approval of the Institutional Animal Care and Use Committee. Rats, including operated (Taconic Labs, NY), were housed under 12:12-hr light:dark conditions; hypophysectomized (hypox) animals were in addition maintained at 28°C. Castrated, ovariectomized, and hypox rats were operated upon at 5 weeks and used for studies 1–2 weeks later. Tissues were frozen in liquid nitrogen immediately upon dissection and stored at −80°C.

RNA Preparation, Northern Blotting, Quantification, and Statistical Analysis. Total cellular RNA preparation, poly(A) selection of RNA, Northern blot

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[1] Current address: DNX, 303B College Road East, Princeton Forrestal Center, Princeton, NJ 08540.

[2] To whom requests for reprints should be addressed at American Cyanamid Co., Clarksville Road, P.O. Box 400, Princeton, NJ 08543.

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preparation and analysis were as described (9). Radiolabeled probes are identified in the text.

For quantification, filters were exposed to storage phosphor screens, digitized on a Phosphorimager and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and analyzed by single classification ANOVA (10) for the experiments displayed in Table I. Probabilities (*P*) are based on tabled values (11).

GH Binding Activity in Serum. Serum samples (200 ml) were incubated with 32,000 cpm ^{125}I bovine GH (bGH) (50–60 µCi/µg) in 25 m*M* Tris pH 7.4, 0.1% BSA and 10 m*M* $MgCl_2$ overnight at 4°C, with or without 1.0 µg unlabeled bGH. Bound and free fractions were separated by chromatography on a Sephacryl S-200 column as described (1, 3). Peaks for free GH and GH/GHBP complexes were integrated and expressed as a percentage of recovered counts.

GHR and GHBP Cloning. Low stringency screening (filter wash in 2× SSC, 0.2% SDS, 55°C) of a porcine liver cDNA library (Clontech, Palo Alto, CA) was performed (12) using a radiolabeled probe corresponding to the extracellular domain of the rat GHR. Positive λ phage clones were subcloned and sequenced (Sequenase; USB, Cleveland, OH).

The porcine liver cDNA (λgt11) library was screened against monoclonal antibody 4.3 (6) as described (13). Positive phage clones were subcloned and sequenced. The porcine cDNA library was also screened at low stringency (filter wash in 1× SSC, 0.2% SDS, 37°C) with a radiolabeled 51 base oligonucleotide, RatBP, which is complementary to the sequence of the hydrophilic C-terminal domain of the rat GHBP.

Reverse transcription of porcine liver mRNA (20-week-old female) was performed using each of the following primers: RatBP, random hexamers, and a porcine GHR (pGHR) intracellular primer (PS8, complementary to pGHR Position 1197 to 1178), followed by PCR amplification using RatBP and a pGHR extracellular primer (PS3, corresponding to pGHR Position 247 to 266). RatBP and PS3 were also used in a PCR amplification from the porcine cDNA library, as were PS8 and PS3.

Our chicken GHR (cGHR) cDNA cloning is described elsewhere (9). Exon 6- and Exon 7-specific cGHR fragments were used as probes to a chicken genomic library (Stratagene, CA). One positive λ clone was subcloned, and the intronic regions surrounding Exon 6 and 7 were sequenced.

Table I. Statistical Analysis of the GHBP to GHR mRNA Ratio in Rats of Varying Endocrine Status

Study	Groups[a]	*n*	Mean[b]	Analysis[c]
1	F, mock ovex	4	11.5	ns
	F, ovex	4	13.1	
	M, mock cast	4	12.6	
	M, cast	4	11.6	
2	F, mock ovex	4	6.76	$P > 0.10$
	F, ovex	4	7.17	
	M, mock cast	4	10.3	
	M, cast	4	6.65	
3	F, intact	3	4.39	ns
	F, mock ovex	4	4.24	
	F, ovex	4	4.52	
	M, intact	3	4.51	$P > 0.25$
	M, mock cast	4	4.90	
	M, cast	4	3.90	
4	F, hypox	10	2.85	$P < 0.005$
	F, hypox, GH-injected	4	5.76	
5	F, hypox	4	2.13	$P < 0.005$
	F, hypox, GH-infused	4	3.13	
	F, hypox, GH-infused + Est	4	3.25	

[a] Treatments: estrogen, 50 µg/day for 3 days by single ip injection in sesame oil; GH-injected, 500 µg ip in PBS, analyzed 4 hr later; GH-infused, 150 µg/day for 3 days by osmotic pump. F, female; M, male; ovex, ovariectomized; cast, castrated; Est, estrogen.

[b] Squared arithmetic mean of the square-root transformed variates.

[c] For analysis, data were square-root transformed. Probability (*P*) values (Model I ANOVA) are reported.

Results

Expression of Rat GHR and GHBP RNA. A radiolabeled cDNA fragment that includes the entire coding region which is common to the GHR and GHBP messages was used in the Northern analysis of poly(A) liver RNA from rats ranging in age from 1 day to 50 weeks (Fig. 1). Linear regression of this ratio reveals no significant age-related trend. A tissue distributional study of a 10-week-old virgin female rat,

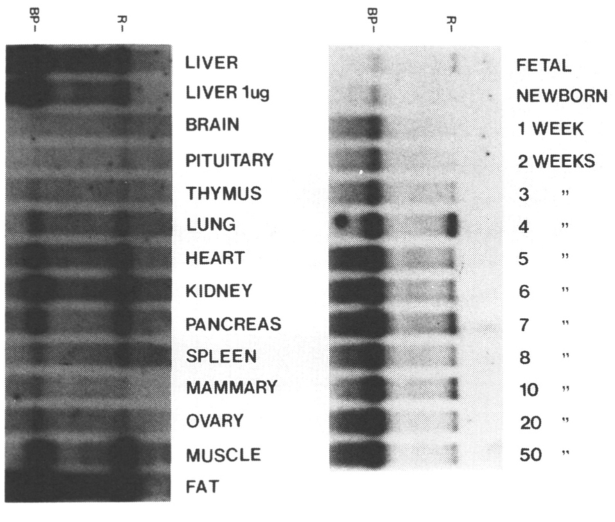

Figure 1. Northern analysis of rat growth hormone receptor and binding protein mRNA during ontogeny of female liver (right) and among female tissues (left). The 4.75-kb receptor band and the 1.2-kb binding protein band are indicated (R and BP, respectively).

however, reveals clear differences in the GHBP to GHR ratio (Fig. 1): liver, 7.1; kidney, 0.6; ovarian fat pad, 1.7; skeletal muscle, 0.4; brain, 1.2; thymus, 1.0; lung, 0.5; heart, 0.5; pancreas, 1.4; spleen, 0.2; ovary, 3.7; mammary, 1.5; and pituitary, 0.3 (autoradiogram is overexposed to reveal low level expression).

Several studies were performed to investigate differential GHR and GHBP mRNA production in rats, in which Northern blots of rat liver mRNA were probed and analyzed as above (Table I). Three separate experiments were performed to determine the effect of sexual status on the GHBP to GHR ratio. In Study 1, 2, and 3, there were no significant differences among gonadectomized or intact males and females. The effects of GH and GH + estrogen treatment on the GHBP to GHR ratio were investigated in Study 4 and 5, in which a significant increase in the GHBP to GHR ratio was seen in hypox females treated with GH. Estrogen treatment had no effect above and beyond the effects of GH.

Expression of Ovine and Porcine GHR and GHBP. Results of our ovine GHR cDNA cloning (14) are similar to earlier work (15). We also cloned the pGHR cDNA, and discovered five nucleotide differences between our clone and the published sequence (16). We find T (vs G) at Position 1143, T (vs G) at 1225, G (vs C) at 1666, G (vs C) at 1739, and C (vs G) at Position 1827 of the coding sequence. These nucleotide differences are predictive of amino acid differences, Asp_{381}, Ser_{409}, Val_{556}, and Gly_{580}, compared with the reported Glu_{381}, Thr_{409}, Leu_{556}, and Ala_{580}.

The results of GHBP determinations in the serum of sheep, pigs, and cows is given in Table II, where the relative increase in labeled GH (in the absence or presence of unlabeled GH) found in a high molecular weight complex is indicative of serum GH-binding activity. GHBP activity in rat serum is so high that even 5 μg/ml added unlabeled bGH appears not to be saturating. For the pig, a substantial amount of GHBP is demonstrated, whereas the sheep appears to have lower but detectable levels, and the bovine serum displays no detectable GHBP activity.

Upon Northern analysis, one specific GHR message is seen in all tissues examined from pigs and sheep (Fig. 2, with quantified GHR RNA levels from these and similar blots being shown in Table III). A search for a separate porcine GHBP transcript (similar to the rat GHBP transcript) was nonetheless undertaken, since a specific GHBP transcript might be indistinguishable in size from the GHR message or otherwise undetectable upon Northern blotting. However, two methods of cDNA cloning (screening with a specific monoclonal antibody and probing with the oligonucleotide RatBP) each failed to produce phage showing any similarity in sequence to the pGHR. In addition, RatBP was used in attempts to amplify a porcine GHBP fragment either from the porcine liver cDNA library or reverse transcribed porcine liver mRNA. In each case, however, no specific porcine GHBP fragments were amplified. Finally, if the porcine GHBP derives from an alternatively spliced GHR transcript that retains the intracellular sequence, then PCR using GHR-specific primers would be expected to amplify two products, corresponding to the GHR and GHBP transcripts. However, such a PCR generated only one amplified band, whose restriction pattern matched the GHR (not shown).

Table II. Detection of GHBP in Animal Serum. Percent [^{125}I]labeled Bovine GH Found as Part of a High Molecular Weight Complex

Species	w/o GH[a]	w/ GH
Rat	82.8%	16.1%
Pig	62.9%	7.2%
Cow	8.5%	9.2%
Sheep	17.9%	5.2%

[a] Samples incubated in the absence (w/o) or presence (w/) of unlabeled bovine GH.

Cloning of a cDNA Variant of the cGHR. Chicken GHR cDNA cloning (9) produced a product (called CH17) which includes a 17-base insertion (TGC-AAA-GAC-AGA-AAT-GC) at Position 531 of the GHR coding sequence. The insertion and the resultant frame-shift generates a predicted protein structure which would include six novel amino acids (His_{178}, Phe_{179}, Cys_{180}, Leu_{181}, Cys_{182}, and Ser_{183}) followed by a termination codon. The original CH17 isolate includes a GHR sequence ranging from nucleotide

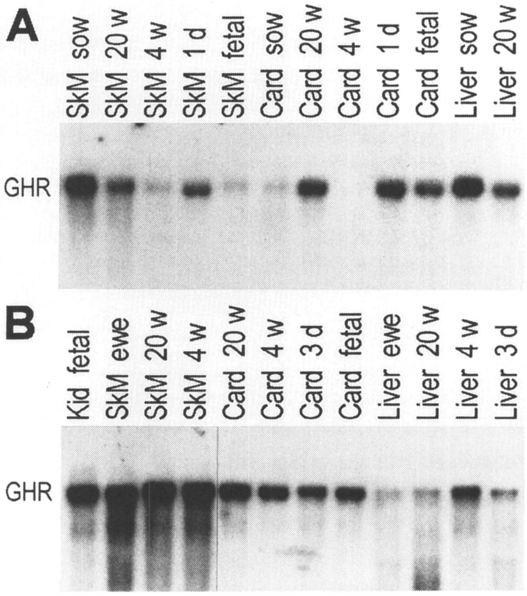

Figure 2. Northern analysis of porcine (A) and ovine (B) growth hormone receptor mRNA. The 4.5-kb GH receptor band is indicated (GHR). SkM, skeletal muscle; Card, cardiac muscle; Kid, kidney; w, week-old; d, day-old.

Table III. Tissue Distribution of GHR mRNA in Pigs and Sheep. Levels of mRNA Derived from Northern Blots in Arbitrary Units

	Fetus[a]	3 days	4 weeks	20 weeks	Pregnant
Porcine					
Liver	23.4	18	26	175	180
Heart	135	192	10	174	33
Musc	34	117	35	175	279
Stomach	79	194	161	11	28
Lung	193	125	3.4	39	14
Brain	8.5	4.1	1.0	1.7	0.5
Fat	NA	NA	NA	150	125
Kidney	176	133	10	96	87
Ovine					
Liver	6	12.4	24	22	23
Heart	50.4	37.2	54	75	46.7
Musc	6.5	11.6	206	205	131
Abo	46	41	42	61	148
Lung	99	49	4.7	59	86.3
Brain	9.7	8	1.3	10.3	12.4
Fat	NA	NA	NA	124	175

[a] Fetus, <1 week prior to birth; NA, not available for analysis; musc, skeletal muscle; abo, abomasum.

Position 422 to 1360, but the CH17 transcript includes all upstream extracellular sequences, as judged by the sequence of an RT-PCR fragment primed by an oligonucleotide complementary to the 17 base insertion and amplified by that primer, and a primer corresponding to Position 2–21 of the cGHR. That the CH17 cDNA includes at least 600 base pairs of the intracellular sequence indicates that it cannot correspond to the 2.1-kb GHR message seen on Northern blots, since that message does not hybridize to a GHR intracellular probe (16). Genomic sequence analysis confirms that the 17 inserted bases are derived from the 3′ end of Intron 6 (predicted from the known human GHR genomic structure [17]), suggesting that the origin of the CH17 is splicing to an alternative acceptor site 17 bases upstream of the normal Exon 7 sequence.

Discussion

An understanding of the endocrine control of GHR and GHBP synthesis might lend some insight into the functional role of the GHBP in growth and physiology. Both RNA species appear to be coregulated (e.g., throughout development in liver) (Fig. 1). Among tissues, however, there are clear differences in GHBP:GHR ratios (Fig. 1) (18). Although the relative expression of the rat GHR and GHBP transcripts does not appear to be affected by sexual status, Study 4 and 5 demonstrate the clear effect of GH on the GHBP to GHR ratio (Table I). This may be similar to previous observations, where high serum GH levels were seen to correlate with higher ratios of GHBP to GHR during pregnancy in mice (19) and in rats (18).

In many animals, including pigs and sheep (Fig. 2), only one GHR message is visible on Northern blots, and the GHBP is best believed to be a product of proteolytic cleavage of the GHR. In light of the disparity between serum GHBP concentrations in sheep and pigs (Table II), it would be interesting to test whether these receptors are similarly processed *in vitro* into GHBPs as was shown for the rabbit (8). The data in Table III are suggestive of some interesting patterns of expression (e.g., expression in adult ovine liver is rather modest in comparison to that of skeletal muscle). Also, in both pigs and sheep, the tissue distribution is somewhat unusual at 4 weeks of age, which coincides with the time of weaning.

Chicken GHR mRNA expression has been described (9, 20–22). Of four GHR mRNA transcripts, only the 4.7- and 4.0-kb species encode full length receptors, while the 2.1- and 0.7-kb species potentially encode GHBPs. Evidence exists (using ligand blotting) in chickens for soluble serum GHBPs (23). Although the 0.7-kb message is unlikely to encode a GHBP, the 2.1-kb species does not encode a full-length receptor, so it may encode a GHBP (9). The CH17 transcript that we describe herein must also be viewed as a potential GHBP message, even though it does not appear to arise from an abundant GHR transcript. Evidence also exists for at least one other candidate chicken GHBP cDNA arising from alternative splicing (24).

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