The baboon: a model for the study of primate growth hormone receptor gene expression during development

G Zogopoulos^{1,2}, P Nathanielsz⁵, G N Hendy^{2,3,4} and C G Goodyer^{1,2}

¹Department of Pediatrics, McGill University, Montreal, Québec, Canada

²Department of Medicine, McGill University, Montreal, Québec, Canada

³Department of Physiology, McGill University, Montreal, Québec, Canada

⁴Department of Human Genetics, McGill University, Montreal, Québec, Canada

⁵Department of Physiology and the Laboratory for Pregnancy and Newborn Research, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

(Requests for offprints should be addressed to C G Goodyer, Endocrine Research Laboratory, Room C-1238, McGill University - Montreal Children's Hospital, Research Institute, 2300 Tupper St, Montreal, Québec, Canada H3H 1P3)

ABSTRACT

In subprimates, significant onset of growth hormone receptor (GHR) expression occurs only after birth whereas, in the human, GHR mRNA and protein are widely manifest from the first trimester of fetal life. Thus, it is likely that subprimates are not the best models for studying regulation of human GHR gene transcription, especially during early stages in development. Here we have explored the potential of the baboon as a more appropriate model. Baboon GHR cDNAs were cloned from postnatal liver by reverse transcription (RT)-PCR, using human GHR-specific primers. The encoded baboon GHR precursor protein has an identical signal peptide sequence to that of human and rhesus monkey GHRs, and the mature baboon GHR is also 620 amino acids long, with 95% and 98.5% amino acid identity to the human and rhesus monkey receptors respectively. Previous studies in the human have identified eight 5' untranslated region (5' UTR) variants of the GHR mRNA (V1 to V8, numbered according to their relative abundance). We cloned the baboon V1, V3 and V4 homologues by RT-PCR: these variants have a high degree (>92%) of sequence identity with their human counterparts

and also diverge at an identical point, 12 nucleotides upstream of the start of translation. The expression pattern of these three GHR mRNA isoforms in baboon liver during development was characterized. Strong expression of baboon V1 and V4 was evident by 49 days of postnatal life ($n=5$, 49 days and adult (18.6-19.6 kg)); very low levels of V1, but not V4, were observed in younger animals ($n=2$, 6 and 30 days). In contrast, V3 5' UTR variant mRNA was present in all fetal ($n=4$, 141-155 days gestation) and postnatal ($n=7$, 6-19.6 days and adult (18.6 kg)) hepatic specimens examined. Analysis of postnatal kidney and lung ($n=2$, 19 and 19.6 kg) revealed that V3 transcripts are present in these tissues, but not V1 and V4. Together, these data demonstrate that, as in the human, baboon V1 and V4 expression is developmentally regulated and tissue specific, while the V3 isoform is more widely expressed. Therefore, it is likely that the regulatory regions of the baboon and human GHR genes are well conserved. Our findings suggest that the baboon is an appropriate animal model in which to define the mechanisms regulating GHR gene transcription during primate development.

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INTRODUCTION

The growth hormone (GH) receptor (GHR) mediates multiple metabolic as well as growth-promoting effects in skeletal and soft tissues: expression of functional GHRs is essential for normal postnatal mammalian growth and metabolic

homeostasis (Rosenbloom *et al.* 1997). However, the physiological significance of the GHR during fetal development is poorly understood (Gluckman & Harding 1997, Hay *et al.* 1997). Ontogenic studies in subprimates (rodent, sheep, cow, rabbit and pig) show that significant onset of GHR mRNA and protein synthesis occurs only after birth (Walker

et al. 1992, Ymer & Herington 1992, Breier *et al.* 1994, Schnoebelen-Combes *et al.* 1996). In contrast, we have demonstrated that, in the human, GHR mRNA is widely expressed from the first trimester of fetal life (Zogopoulos *et al.* 1996a). In addition, GHR immunostaining has been identified in human tissues as early as 8.5 weeks of fetal age; by midgestation, the tissue distribution of the GHR is often identical to that found in the adult (Hill *et al.* 1992, Simard *et al.* 1996). Thus, significant GHR mRNA and protein synthesis begins earlier in human development than in lower species, indicating an important role for the GHR during human fetal growth.

The diversity of the 5' untranslated region (5' UTR) of GHR mRNA in the human (Pekhlitsky *et al.* 1992, Zogopoulos *et al.* 1997), ovine (O'Mahoney *et al.* 1994, Adams 1995), bovine (Heap *et al.* 1995) and rodent (Baumbach & Bingham 1995, Menon *et al.* 1995) has suggested that, in all species, multiple promoter regions may regulate transcription of the GHR gene. In the human, eight different 5' UTR variants, numbered V1 to V8 according to their relative abundance, have been cloned from adult liver by 5' rapid amplification of cDNA ends (5' RACE) (Pekhlitsky *et al.* 1992). All eight variants have distinct nucleotide sequences and only converge at -11 nucleotides prior to the start site of translation in exon 2. We have recently demonstrated that expression of two of these variants is tissue specific as well as developmentally regulated: V1 and V4 were only detected in postnatal liver (Zogopoulos *et al.* 1996b, 1997). In contrast, V3 GHR mRNA transcripts were present in all human tissues examined, from as early as 10 weeks of fetal age (Zogopoulos *et al.* 1996b, 1997). Induction of V1 and V4 expression in postnatal liver appears to be functionally significant: there is, in parallel, a 4-fold increase in total hepatic GHR mRNA levels as well as GHR receptor binding activity (Zogopoulos *et al.* 1997, 1998). Our subsequent studies have mapped the V1 and V4 sequences to the same region in the human GHR gene and revealed that their transcription is regulated by a common promoter (Zogopoulos *et al.* 1998). The V3 sequence has been localized to a distinct genomic region and preliminary studies suggest that it is transcribed from a unique start site (G Zogopoulos, C G Goodyer and G N Hendy, unpublished data). Together, these data suggest an important developmental switch in GHR gene expression due to differential promoter usage.

The finding of earlier GHR expression in the human than in subprimates (Hill *et al.* 1992, Walker *et al.* 1992, Ymer & Herington 1992, Breier *et al.* 1994, Schnoebelen-Combes *et al.* 1996, Simard

et al. 1996, Zogopoulos *et al.* 1996a) suggests that these lower species are not the best models for studying regulation of the human GHR gene during fetal and early postnatal life, and that another primate may be more appropriate. In the present study, human GHR-specific primers were used to clone baboon GHR cDNAs by reverse transcription (RT)-PCR from postnatal baboon liver total RNA. Nucleotide sequencing of multiple clones revealed that the baboon GHR has a high level of sequence identity with the human (95%) and rhesus monkey (98.5%) GHRs (present data, Leung *et al.* 1987, Martini *et al.* 1997). V1, V3 and V4 homologues were also cloned by RT-PCR from postnatal baboon liver. Subsequent semi-quantitative RT-PCR and Southern blotting analysis showed that, as in the human, baboon V1 and V4 GHR mRNA expression is developmentally regulated and tissue specific, whereas V3 is widely expressed in fetal and postnatal tissues. Therefore, it is likely that the regulatory regions in the baboon and human GHR genes are well conserved, resulting in similar GHR mRNA regulation in the two species. These data suggest that the baboon is an appropriate animal model in which to study GHR mRNA expression during primate development.

MATERIALS AND METHODS

Tissues

Fetal and postnatal baboon tissues were obtained immediately after the animals were killed ($n=8$, 141 days gestation to adult; term= ~ 184 days). Those animals that were wild caught have been identified by weight at the time of study since their ages were unknown. The human postnatal liver specimen (transplant donor age=43 years) was collected 5 h following death. Specimens were flash-frozen in dry-ice acetone and stored at -70°C for RNA extraction. Total RNA was isolated using the guanidine thiocyanate/CsCl gradient method, and treated with deoxyribonuclease I (DNase I; Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) to eliminate genomic DNA contamination. Animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee; animal facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

RT-PCR cloning of baboon GHR cDNAs

Five micrograms postnatal (49 days) baboon liver total RNA were reverse transcribed for 1 h at 48°C with $0.6\text{ }\mu\text{M}$ of the appropriate antisense human

TABLE 1. Sequence of the oligonucleotide RT-PCR primers and the internal hybridization probe (*)

Oligonucleotide	Sequence
1S	5' AGA TTG AGA ATG ACT GAT TTG GGA G 3'
2S	5' GGA GAC CTT GGA AGG GAC AGA G 3'
3S	5' GAG TAG CAA AGA TGG ATT AAG TGA G 3'
4S	5' TCA AGA ATG GAA AGA ATG CCC TG 3'
1A*	5' GAA CCT CAT CTG TCC AGT GGC AT 3'
2A	5' AGG TAT CCA GAT GGA GGT AAA CG 3'
3A	5' TAG AAT CCA TAC CCC ATC CTG TC 3'

GHR primer (Table 1 and Fig. 1), as previously described (Zogopoulos *et al.* 1996b). Parallel RT reactions were run in the absence of AMV-RT to ascertain that gene transcripts and not genomic DNA were being amplified. Six microlitres RT product were amplified for 30 cycles in 0.3 µM of the appropriate sense and antisense human GHR primers (Table 1 and Fig. 1), 0.5 mM deoxyribonucleotides (dNTPs; Amersham Pharmacia Biotech), 22.5 mM MgCl₂, 2.5 U Taq/PWO polymerase mix (Roche Diagnostics, Laval, Quebec, Canada), and 1 × Expand Long Template PCR System Buffer #3 (Roche Diagnostics). The

reaction was heated at 92 °C for 2 min, cycled 30 times for 10 s at 92 °C, 30 s at 61 °C and 2 min at 68 °C, and terminated with a final elongation of 5 min at 68 °C. The amplified cDNAs were inserted into the PGEM-T TA cloning vector (Promega, Madison, WI, USA) and three independent clones were sequenced using a Li-Cor automated system (Li-Cor Biotech, Lincoln, NE, USA).

Semi-quantitative RT-PCR

RT reactions were carried out as described above and 6 µl RT product were amplified for 25 cycles

FIGURE 1. Schematic representation of homologous baboon and human cDNA isoforms. Coding exons are indicated by numbered boxes and 5' UTRs are represented by empty boxes. The relative positions of the sense (S) and antisense (A) primers as well as the internal hybridization probe (1A*) are indicated (oligonucleotide sequences are provided in Table 1). Total RNA was reverse transcribed with primer 2A, and primer sets 1S/2A, 2S/2A and 3S/2A were used specifically to amplify the V1, V3 and V4 cDNAs respectively. The remaining baboon cDNA coding region was cloned using primer 3A in the RT step, and the 4S/3A PCR primer combination. Broken lower lines indicate the resulting PCR products after amplification with these sets of primers. The V1 and V4 internal standards, used as positive controls in the semi-quantitative RT-PCR assays, both generate PCR products of 382 bp.

with 0.25 µM human GHR sense and antisense primers (Table 1 and Fig. 1), as previously described (Zogopoulos *et al.* 1996b). Internal V1 and V4 standards were generated using the PCR method of Jin *et al.* (1994); a V3 standard could not be used since multiple (V3a, V3b, V3c) variant transcripts have been described for the human GHR (Pekhletsy *et al.* 1992). Under the specified conditions, each amplification reaction was still in the exponential phase.

Southern blotting analysis of PCR products

PCR products were resolved on 2% agarose gels and transferred to 0.45 µm positively charged nylon membranes (Schleicher & Schuell, Keene, NH, USA). Blots were hybridized, using 0.4 nCi/ml end-labelled probe (Table 1 and Fig. 1) per cm² nylon membrane, as previously described (Zogopoulos *et al.* 1996b). Bands were visualized by autoradiography following 3–14 h exposure to Kodak XAR-5 film (Eastman Kodak) using two intensifying screens.

RESULTS

Cloning of GHR cDNAs from baboon liver

To determine if, in the baboon, V1, V3 and V4 GHR mRNAs showed the same developmental patterns of expression as in the human, we used human-specific primers and the RT-PCR strategy described in Fig. 1 to clone the V1, V3 and V4 baboon cDNA homologues from postnatal liver total RNA. Nucleotide sequencing revealed that, like their human counterparts, the three baboon 5' UTR variants diverge at –12 nucleotides from the start site of translation in exon 2 (Fig. 2). The human (Pekhletsy *et al.* 1992) and baboon 5' UTR homologues are highly conserved: 96% for V1 and 92% for V4, while the shorter nucleotide stretch of the baboon V3 5' UTR matches its human counterpart precisely. It should be noted that an additional alternatively spliced V3 isoform of longer length and lower abundance has been identified in the human (Pekhletsy *et al.* 1992, Zogopoulos *et al.* 1996b). Evidence for the presence of this V3 subvariant in the baboon was obtained following longer exposure of the Southern blots to X-ray film (data not shown). The baboon V1 sequence was also a perfect match for the 5' UTR sequence of the rhesus monkey GHR cDNA reported by Martini *et al.* (1997) except, surprisingly, for the terminal 3' 12 nucleotides representing the splice site in exon 2:

these were missing in the rhesus monkey GHR mRNA sequence.

To obtain the complete baboon GHR mRNA coding sequence, a second RT-PCR, using human-specific primers within exon 5 and the 3' UTR, was carried out. The complete nucleotide sequence of the baboon GHR cDNA predicts a 620 amino acid mature protein, with 95% and 98.5% amino acid sequence similarity with the human (Leung *et al.* 1987) and rhesus monkey receptors (Martini *et al.* 1997) respectively (Fig. 3). The characteristic features of the GHR are all well conserved in the three primates: the 18 amino acid signal peptide, the seven cysteine residues, five potential N-glycosylation sites and YGEFS motif of the extracellular domain, and the proline-rich Box 1 region of the cytoplasmic tail (Fig. 3) (Argetsinger & Carter-Su 1996).

Characterization of V1, V3 and V4 mRNA expression during development

The RT-PCR/Southern blot strategy used to characterize V1, V3 and V4 mRNA expression is summarized in Fig. 1. In each analysis, aliquots from the same RT reaction were tested for expression of each variant. V1 and V4 transcripts were not detectable in the fetal livers examined ($n=4$, 141–155 days gestation), and were only readily identified in hepatic samples at and following 49 days of life ($n=4$, 49 days and adult (18.6–19.6 kg)) (Fig. 4). Low levels of V1 were also present in younger postnatal animals ($n=2$, 6 and 30 days); V4 mRNAs were not detectable at this early postnatal stage in development. There was no evidence for expression of V1 and V4 transcripts in adult baboon kidney and lung specimens ($n=2$, 19 and 19.6 kg) (Fig. 4). In contrast to V1 and V4, V3 was observed in all fetal and postnatal baboon livers examined, as well as the postnatal kidney and lung tissues (Fig. 4). Thus, similar to the human (Zogopoulos *et al.* 1996b, 1997), baboon expression of V1 and V4 GHR mRNA transcripts is developmentally regulated and tissue specific, while the V3 isoform is more widely expressed.

DISCUSSION

Using human-specific primers, we cloned by RT-PCR the coding exons as well as portions of three 5' UTRs (V1, V3 and V4) of the baboon GHR mRNA. Analysis of the cDNA clones showed that the mature GHR proteins of the baboon and rhesus monkey are extremely well conserved (98.5%), and have a similar degree of variation from the human

V1

Baboon	*****	*****	*****gaatt	ttgtgaaggt	ctatatatca	aagcagaaag
Human	agattgagaa	tgactgattt	gggag-g---	-----	t-----	-----
Baboon	tccaagaagt	tagagattaa	tacatgccaa	gttgtgacca	acaaacttct	gtgggatccc
Human	a-----t-	-----	-----g--a----	-g-----	-g-----	-----
Baboon	acctccaacc	cctccctgtc	tgccagattt	taaccaaata	agtgtgatgt	gatctgcttg
Human	-----	-----c--	-t-----	-----	-----	-----
Baboon	aatatatgag	tgaagaaaa	aggaaattta	aaaagttctt	gatataaagc	ctggaggaaa
Human	c-----	-----	-----	-----	-----	-----
Baboon	caatacgaaa	atccagcctc	tatttcagca	atatctgccg	<i>gactattggt cctacaggtA</i>	
Human	-----	-----	-----	-----	-----	-----
Baboon	TG					
Human	--					

V3

Baboon	*****	*****	***aagggtc	<i>ctacaggtAT</i>	G
Human	ggagaccttg	gaagggacag	ag-----	-----	-

V4

Baboon	*****	*****	*****aagag	gataggcggc	agcggggcca	gagggtgtgaa
Human	gagtagcaaa	gatggattaa	gtgag-----	t-----a---	---a-----	-----
Baboon	tagcctggaa	ccttctgaga	agatcccttt	gtagacagaa	gaaaggggag	atttgtgga
Human	-----ca---	-----	-----t-----	--g---g---	-----	g-----
Baboon	aatgctggaa	<i>gataaggtcc</i>	<i>tacaggtATG</i>			
Human	-----g-----	-----	-----			

FIGURE 2. Alignment of the baboon V1, V3 and V4 cDNA variants with their human counterparts. Stars indicate baboon nucleotides corresponding to the human-specific GHR primers used to clone the V1, V3 and V4 cDNAs from postnatal baboon liver. Dashes denote common nucleotides. Italicized bold letters identify the 11 nucleotide stretch prior to the ATG start site of translation that is common to the three baboon and human 5' UTR variants.

(baboon 95%, rhesus monkey 94%), with the few amino acid differences dispersed throughout the receptor sequence (present data, Leung *et al.* 1987, Martini *et al.* 1997). This is consistent with the idea that these primates are equi-distant evolutionary ancestors of the human (Pausova *et al.* 1995).

The conserved structural features of the baboon GHR include five potential N-glycosylation sites, seven cysteine residues and the YGEFS motif of the extracellular region, as well as Box 1 of the cytoplasmic domain. Since both the human and rhesus receptors are glycosylated at several sites (Leung *et al.* 1987, Martini *et al.* 1997), it is likely

that carbohydrate groups are added to the baboon GHR at the conserved asparagine residues. The formation of two extracellular domain disulphide bridges occurs in the GHRs of all species studied to date, and is also a feature of the class I division of the cytokine/GH/prolactin receptor superfamily (Argetsinger & Carter-Su 1996). Another characteristic of class I receptors is the presence of a WSXWS (where X is any amino acid) motif in the proximal region of the extracellular domain (Baumgartner *et al.* 1994, Argetsinger & Carter-Su 1996). Although this motif is replaced by a related YGEFS consensus in GHRs, the domain is still

FIGURE 3. Amino acid sequence comparison of the baboon, rhesus monkey and human GHRs. Solid vertical lines mark the boundaries of the signal peptide (18 amino acids) as well as the extracellular (246 amino acids), transmembrane (24 amino acids) and intracellular (350 amino acids) domains. Closed circles and stars identify conserved cysteine residues and N-glycosylation sites respectively. Shaded boxes show the YGEFS and Box 1 motifs. Transparent boxes indicate differences in amino acids among the three species. The absence of an alignment dot between the sequences refers to a residue difference.

FIGURE 4. Ontogenic and tissue-specific expression of the baboon V1, V4 and V3 5' UTR GHR mRNA variants. Representative Southern blots showing the expression patterns of the GHR mRNA variants in baboon fetal liver (F-li; in days gestation) as well as postnatal liver (P-li; in days or kg), lung (P-lu; in kg) and kidney (P-ki; in kg) tissues. A human liver specimen from a 43-year-old adult (HAL, 43 yr) was run in parallel. Internal V1 and V4 standards (382 bp) served as a positive amplification control. H₂O served as a negative RT-PCR control. The sizes of the V1, V4 and V3 PCR products that were cloned and sequenced are indicated (in bp).

required for maximal biological activity of the receptor, probably by directing certain conformational features (Baumgartner *et al.* 1994, Argetsinger & Carter-Su 1996). The proline-rich Box 1 domain consists of the same ILPPVPVP consensus sequence in the baboon as in all mammalian GHRs cloned to date. Box 1 is crucial for GHR cellular responses, being responsible for the binding and activation of JAK2 tyrosine kinase, an early event in the intracellular signalling cascades (Wang & Wood 1995, Argetsinger & Carter-Su 1996).

Although structural characteristics of the GHR are generally conserved among species, the amino acid sequence identities can be as low as 70% (Argetsinger & Carter-Su 1996). The present cloning of the baboon GHR, as well as the recent characterization of the rhesus monkey GHR (Martini *et al.* 1997), will permit comparative functional studies of primate versus subprimate GHRs. Martini *et al.* (1997) have already demonstrated an intriguing difference between the monkey and rat GHRs; while the rat GHR binds and activates JAK2 only after GH-induced receptor dimerization, the rhesus monkey receptor is consti-

tutively associated with JAK2 even though it only stimulates JAK2 tyrosine kinase activity after ligand binding.

The presence of 5' UTRs highly conserved between the baboon (present data) and human (Pekhletsy *et al.* 1992) GHR mRNAs suggests that the GHR gene regulatory regions will be similar in the two primates. The 5' end of the human GHR gene has recently been characterized: the V1 and V4 5' UTR variants are localized to the same region in the human gene and can be generated (along with V7 and V8) by transcription from a common promoter (Zogopoulos *et al.* 1998), whereas V3 maps to a distant genomic region and is thought to be regulated by a different promoter (G Zogopoulos, C G Goodyer and G N Hendy, unpublished data). Investigations in subprimates have identified an ovine homologue (exon 1A) to V1, as well as mouse (L1) and rat (GHR1) transcripts with nucleotide sequences similar to the human V7 variant (Pekhletsy *et al.* 1992, O'Mahoney *et al.* 1994, Baumbach & Bingham 1995, Menon *et al.* 1995). Ontogenic studies have demonstrated that, like the human or baboon V1

and V4 isoforms, these subprimate transcripts are present only in postnatal liver (present data, Pekhletsky *et al.* 1992, O'Mahoney *et al.* 1994, Baumbach & Bingham 1995, Menon *et al.* 1995, Zogopoulos *et al.* 1996b, 1997). Thus, the 5' end of the GHR gene regulating postnatal liver-specific GHR mRNA expression in the human appears to be a composite of the homologous ovine and rodent DNA structures. It will be of interest to determine whether the comparable baboon GHR regulatory region is also a composite of the subprimate genomic DNA.

The 5' UTRs of many eukaryotic mRNAs are involved in modulating translation initiation (Jansen *et al.* 1995). Given that V1 and V4 mRNA expression seems to be restricted to postnatal liver in primates (present data, Zogopoulos *et al.* 1996b, 1997), it will be important to determine how these two 5' UTR variants influence translation relative to the widely expressed V3 variant. It is also possible that differential control of fetal versus postnatal tissue expression of the receptor results in distinct GH biological responses that are critical for normal growth and development. Since these questions will be difficult, if not impossible, to answer directly in the human, especially during fetal and early neonatal stages, the baboon should prove to be a highly useful primate model.

In summary, baboon GHR cDNA isoforms have been cloned by RT-PCR from postnatal liver. The 620 amino acid mature baboon GHR protein is very similar to the human and rhesus monkey receptors. As in the human, expression of the baboon V1 and V4 5' UTR GHR mRNA homologues was found to be developmentally regulated and tissue specific, whereas the V3 isoform was detected in all fetal and postnatal tissues examined. Together, these data suggest that the baboon is an appropriate animal model in which to study GHR gene expression during primate development.

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