The growth hormone receptor (GHR) cDNA was cloned from the liver of Rhesus macaque using polymerase chain reaction. As deduced from the nucleotide sequence, the mature GHR is a protein of 620 amino acids which presents 94.1% identity with the human receptor. The monkey GHR (mkGHR) expressed in 293 cells presented the expected specificity for a primate GHR and was able to transduce a transcriptional effect of GH. Human GH was able to activate tyrosine phosphorylation of both the tyrosine kinase JAK2 and the receptor in 293 cells co-transfected with mkGHR and JAK2 cDNAs. The GH binding protein (GHBP), the soluble short form of the GHR, was also present in monkey serum. Expression of the GHR cDNA in eucaryotic cells indicated that the GHBP can be produced by proteolytic cleavage of the membrane receptor. Northern blot analysis of GHR gene expression in different tissues allowed us to identify three different transcripts of 5.0 and 2.8 kilobase pairs and a smaller one of 1.7 kilobase pairs which could encode a GHBP. Rapid amplification of cDNA extremities (3'-RACE-polymerase chain reaction) was used to identify a cDNA encoding a protein in which the transmembrane and cytoplasmic domains of the receptor are substituted by a short sequence of 9 amino acids. This transcript was present in various tissues and could encode a GHBP as well, suggesting for the first time that two different mechanisms can coexist for the generation of the GHBP: proteolytic cleavage of the membrane receptor and a specific mRNA produced by alternative splicing.

Growth hormone (GH) has a variety of biological effects in a large number of target tissues, particularly metabolic and growth effects on skeleton and soft tissues (1). GH actions at the cellular level, such as mitogenic effects (2, 3), insulin-like and anti-insulinic metabolic effects (4), as well as gene regulating actions (5–7), are mediated by receptors which belong to the same family as the prolactin (PRL) and many cytokine/ hematopoietin receptors (8).

In many species including man, only the liver GH receptor (GHR) has been well characterized (9, 10). The rabbit liver GHR was purified to homogeneity and sequenced, and the cDNA encoding the GHR was cloned first in rabbit and subsequently in man (11), which permitted study of the expression of the GHR gene in many tissues (12, 13).

Some molecular events following the binding of GH to its receptor have been identified: dimerization of the GHR is induced (14) and initiates the association with a tyrosine kinase of the JAK family, followed by a cascade of protein phosphorylations, including the JAK2 kinase and the receptor itself (15). Moreover, transcriptional activation by the GHR has been determined by co-transfecting the promoter of a GH-responsive gene coupled to a reporter gene along with the cDNA of rat GHR (16) or of rabbit GHR (17). These transcriptional effects of GH involved signal transducers and activators of transcription (STATs) factors, and it has been shown that GH is able to activate the tyrosine phosphorylation of STAT1, STAT3, and STAT5 (18). Furthermore, several alternative signaling pathways have been identified to be induced by the activated GHR, such as stimulation and tyrosine phosphorylation of mitogen-activated protein kinase (19), and activation of voltage-dependent Ca2+ channels (20).

In addition to the membrane GHR, a soluble GH-binding protein (GHBP) has been identified in serum (21, 22). In rabbit, the amino acid sequences of the GHBP and of the extracellular domain of the membrane receptor are identical (11, 23). Two separate mechanisms have been proposed for the genesis of the GHBP. In mice and rats, two distinct mRNAs of 4.5 and 1.5 kb encode the full-length membrane receptor and the GHBP, respectively. In these species, the transmembrane region of the receptor has been replaced by a short hydrophilic tail (24, 25). In man, rabbit and several other species, the major mRNA transcript is ~4.5 kb as detected by Northern blot analysis; it has been proposed that GHBP results from proteolytic cleavage of the membrane-bound receptor. The release of GHBP was first demonstrated in the medium of IM-9 cells (26). More recently, direct evidence of the production of GHBP in cultured medium of cells transfected with the cDNA encoding the rabbit GHR has been presented (27).
In monkeys, GH has been purified (28), its primary structure identified (29) and its cDNA was cloned many years later (30). This hormone is 96% identical to human GH. The tissue distribution of the monkey GHR has been studied by ligand binding (31) but was not well characterized because of low expression of the GHR in most tissues. The only effect of GH studied in monkeys is the preservation of bone mass in hypogonadal females treated with recombinant human GH (32).

Although the human GHR cDNA was cloned several years ago, experiments using the full-length human cDNA have been impeded by difficulties such as growth of bacterial clones and altered specificity of expressed receptors, as reported by several authors (3, 11). We sought to clone another primate (Rhesus macaque) GHR cDNA for the development of tools to study signal transduction pathways. Evidence that two different mechanisms could coexist to generate the GHBP has been obtained in monkey.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human GH was kindly provided by Serono-Ares Laboratories (Geneva, Switzerland), recombinant bovine GH by Dr. J. Thibault (Hubrecht Laboratory, Utrecht, The Netherlands), and recombinant human PRL by Dr. J. Martial (University of Liege, Belgium).

**Animals**—All tissues were obtained immediately after death from two adult male Rhesus macaque (8 and 9 years old) killed according to procedures recommended by the Panel on Euthanasia of the French Health Ministry. Tissues were frozen in liquid nitrogen and stored at −80 °C until used. Serum samples were of young and old adult male Rhesus macaque from the breeding of the Centre d'Etude et de Recherche en Médecine Aéropatiale, Brétigny-sur-Orge, France.

**DNA Isolation and Plasmid Constructions**—Total liver RNA was recovered by spectrophotometer (Beckman DU-64, Beckman) at 260 nm. Some aliquots were loaded on a 1% agarose gel to compare the relative intensity of 28 S and 18 S ribosomal RNAs. Similar intensities were observed in all samples (data not shown). Polyadenylated RNAs were purified on oligo(deoxythymidine)-cellulose affinity columns (Pharmacia Biotech Inc.).

**cDNA Isolation**—cDNA was transcribed into cDNA in a 20-μl reaction using 200 units of Moloney murine leukemia virus reverse transcriptase (RT) primed with 1 μg of oligo(deoxythymidine)12–18 primer (Boehringer Mannheim) and conditions described by the manufacturer (Life Technologies, Inc.). Half of each product was used as template for polymerase chain reaction (PCR) using primer pairs hGRG-hGR2 or hGR15-hGR3 specific for each part of the GH receptor (see Table I and Fig. 1A). The reaction was carried out in PCR buffer (50 mM KCl, 2 mM MgCl2, and 2 mM Tris-HCl, pH 8.3), 200 μM deoxy-NTPs, 25 pmol of forward and reverse primers, and 1 units of Taq polymerase (Perkin-Elmer) in a total volume of 50 μl. The experiments proceeded for 20 cycles, where a cycle consisted of 45 s at 94 °C, 1 min at 53 °C, and 1.5 min at 72 °C. After a 10-min final extension at 72 °C, the products were isolated on a 2% agarose gel. After a second PCR, the products were isolated on a 2% agarose gel and transferred to a nylon membrane (Amersham) by capillary blotting (10). Double stranded DNA was sequenced by the dideoxy chain termination method (34) using modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.) and by the Sequenase technique (35). This construction contained the cDNA encoding the full-length human GH receptor (see Table I and Fig. 1A) but was not well characterized because of low expression of the GHR. Cells were deprived of serum for an overnight period and hGH binding was studied. The conditioned medium was recovered for GHBP measurement.

For GH dependent induction of reporter construct, 293 cells were plated in 6-well plates before being transiently cotransfected as described before with 0.5 μg of pCH110 (β-galactosidase expression vector from Pharmacia), 100 ng of plasmid pCDNA3/mkGHR, and 75 ng of the fusion gene construct containing the luciferase-coding sequence linked to either the sequence −175/59 of Spi 2.1 promoter (plasmid Spi-luc) or to 2.3 kb of the β-casein promoter (plasmid β-Cas-luc). One day after transfection, cells were incubated with serum-free medium containing 50 μM rhGH and 250 μM dexamethasone (Sigma) or 250 μM dexamethasone and 48 h. Cells were then lysed in lysis buffer (Promega), and whole cell extracts were used for detection of luciferase and β-galactosidase activities. To correct for differences in transfection efficiencies between plates within an experiment, luciferase activity was normalized to β-galactosidase activity.

For purification of GHR complexes and Western blot analysis, 293 cells (5 × 105, in 100-mm dishes) were transfected with 4 μg of plasmid pCDNA3/mkGHR and 2 μg of cDNA encoding JAK2 (kindly provided by Dr. J. Ihle), according to the same protocol.

**Immunoprecipitation and Western Blot Analysis**—Transfected 293 cells extracts in 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.06% Triton X-100) were incubated with the designated antibody and with protein-A-Sepharose (50%, v/v) under non-denaturing conditions, overnight at 4 °C. Immunoprecipitated complexes were washed three times in cold lysis buffer, boiled in SDS sample buffer, and subjected to 7% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose filter and immunodetected with either an anti-phosphotyrosine antibody or a mouse monoclonal antibody (clone 4G10; Upstate Biotechnology, 0.1 mg/ml) or an anti-JAK2 antibody (Upstate Biotechnology, dilution 1:4000) overnight at 4 °C. Membranes were incubated with an anti-mouse or anti-rabbit antibody coupled to peroxidase for 1 h at room temperature before being washed four times for 30 min in washing buffer (50 mM Tris–HCl, pH 7.6, 200 mM NaCl, 0.05% Tween 20) and revealed by chemiluminescence (ECL kit from Amersham). Membranes were stripped overnight at 4 °C in a 0.1% SDS sample buffer (10 mM Tris pH 6.8, 100 mM NaCl, 0.1% Nonidet P-40, 0.05% SDS, and 0.005% β-mercaptoethanol) and reprobed.

**Binding Studies**—[125I]hGH was prepared using chloramine-T, as described previously (9), with a specific activity ranging between 80 and 180 μCi/μg.

Liver and kidney were cut into pieces and homogenized with an equal volume of 250 mM sucrose, buffered at pH 7.4 with 3 mM imidazole–HCl, using a Teflon-glass homogenizer. Microsomal membrane fractions were prepared by differential centrifugation (9). Incubation of [125I]hGH (1 × 10−10 M) with liver membrane fraction in a volume of 450 μl (25 mM Tris–HCl and 20 mM MgCl2, pH 7.4, with 0.1% bovine serum albumin) was performed for 18 h at 4 °C. Incubations were stopped by addition of 3 ml of chilled binding buffer. Bound and free hormones were separated by centrifugation at 3000 × g for 30 min. Transfected COS-7 or 293 cells (1 × 105 cells/well of 6-well plates) were incubated with [125I]hGH in phosphate-buffered saline containing 0.5% bovine serum albumin. Incubations were carried out at room temperature for 4 h. Cells were washed twice with ice-cold phosphate-buffered saline; then cells were lysed by 1 ml of 1 N NaOH. Pellets and cell suspensions were counted using a LKB Phosphorimager gamma-spectrometer.

Binding of [125I]hGH to serum GHBP was measured by gel filtration and SDS-polyacrylamide gel electrophoresis after isoelectric focusing (37). For cells in culture, media were concentrated (20 times) using Centriprep 30 column (Amicon). Scatchard analyses were performed using the Ligand program (38).

**Northern Blot Analysis**—Ten to twenty μg of polyadenylated RNA were electrophoresed through a 1.1% agarose, 2.2 μM formaldehyde gel and transferred to Hybond-N+ membrane (Amersham) by capillary
RESULTS

Binding Characteristics of the Monkey GHR—The specific binding of \(^{125}\text{I}\)-hGH to microsomal membrane fractions from two male Rhesus macaques was similar: 3.5 and 4.9% of total radioactivity/300 \(\mu\)g of membrane protein. A microsomal fraction prepared from kidney of the same two animals had a very low level of binding, less than 1%. Binding affinities to liver GHR were 1.95 \(\pm\) 0.08 \(\times\) 10\(^9\) \(\text{M}^{-1}\) and 3.31 \(\pm\) 0.16 \(\times\) 10\(^9\) \(\text{M}^{-1}\), with a comparable binding capacity of 26.37 \(\pm\) 0.58 and 22.27 \(\pm\) 0.51 fmol/mg protein, respectively.

Cloning, Sequencing, and Characterization of Monkey GHR-CDNA—The cDNA of the monkey GHR was cloned in two parts, hGR5-hGR23 called 5’ and hGR15-hGR3 called 3’, with the common hGR15-hGR23 region (Fig. 1). These two parts had a size of 1087 and 1389 bp, respectively. The hGR5 and hGR3 primers were chosen with regard to the sequence alignment in the 5’- and 3’-untranslated regions that are conserved over species (Table I). To confirm the sequence obtained by PCR amplification of two long fragments, amplification of given portions of the sequence was performed using specific pairs of primers mGR0-mGR56 and mGR46-mGR104 (Table I). The sequences obtained were identical using the two methods. The overall amino acid identity is 94.1% with the human GHR receptor, and all the characteristic domains of GHR family are conserved.

COS-7 cells were transiently transfected with the cDNA of the full-length receptor inserted in the pcDNA3 eukaryotic expression vector. Specific binding of \(^{125}\text{I}\)-hGH to whole cells demonstrated that the cloned receptor was expressed by the transfected cells. The association constant calculated by Scatchard analysis from competition experiments shown in Fig. 2 was 5.75 \(\pm\) 0.16 \(\times\) 10\(^9\) \(\text{M}^{-1}\) and the binding capacity was of 22,000 sites/cell. Scatchard analysis from competition binding was also performed with 293 cells transiently transfected with the mGHR cDNA; the affinity constant was the same, 4.35 \(\pm\) 0.11 \(\times\) 10\(^9\) \(\text{M}^{-1}\).

As expected for a primate GHR receptor, recombinant bovine GH failed to compete with \(^{125}\text{I}\)-hGH; a small effect could be observed only at high concentrations of unlabeled hormone demonstrating the specificity of this primate receptor for primate GH (Fig. 2). Somatogenic specificity was also attested by absence of competition by recombinant human PRL with \(^{125}\text{I}\)-hGH binding (Fig. 2).

**Table I.** Sequences of Primers for Amplification and Sequence Orientation

| Primer Pair | Sequence |
|-------------|----------|
| mGR0-mGR56 | GACAGCTACGTGACCTGCTG |
| mGR46-mGR104 | GACAGCTACGTGACCTGCTG |

**Fig. 1.** Cloning, sequencing, Northern, and Southern strategies used to identify the monkey GH receptor. A, schematic representation of mkGHR mRNA derived from the nucleotide sequence of the two cDNAs inserts shown in B. 5’- and 3’-untranslated regions are represented by solid lines; coding region, enclosed box; signal peptide, black box; transmembrane domain, stippled box. The dotted lines at the ends represent as yet non-identified regions of mRNA. Positions of PCR primers and size of amplicons 5’ and 3’ (in base pairs) are indicated. B, the two cDNAs and the location of restriction fragments (E, extracellular; I, intracellular) and oligonucleotide (TMD) specific of the transmembrane domain, used as Northern hybridization probes. C, position of PCR primers: mGR46, mGR104; and nucleotide probe, mGR73, used for Southern blot.
Functional Studies of the Monkey GHR—We have used the constructs β-casein/luciferase (β-Cas-luc) or Spi 2.1/luciferase (Spi-luc) to test the ability of GH to activate β-casein or serine protease inhibitor gene transcription. Human 293 fibroblast cells were transiently cotransfected with a plasmid containing the mkGHR (in pcDNA3 vector) and reporter gene constructs (β-Cas-luc or Spi-luc). These cotransfections resulted in a clearly inducible luciferase activity by GH. The maximal effect could be achieved with 50 nM hGH, as described previously (16). Maximal fold induction is 6.2 ± 0.6 for the β-Cas-luc construct and 4.1 ± 0.5 for the Spi-luc.

The association between monkey GHR and JAK2 kinase was studied in 293 cells transiently cotransfected with mkGHR and JAK2 cDNA. The phosphorylation of both JAK2 kinase and GHR is stimulated by hGH. As shown in Fig. 3A, the stimulation of the phosphorylation is about 8-fold for GHR and 2.2-fold for JAK2. Fig. 3B confirms that the same quantity of protein was loaded on lanes corresponding to stimulated and unstimulated cells following immunoprecipitation by anti-JAK2 and anti-GHR. A 2.5-fold induction of JAK2 is also seen after immunoprecipitation by anti-phosphotyrosine following GH stimulation (Fig. 3B). Basal phosphorylation of GHR and especially of JAK2 is detectable as shown in Fig. 3A, in lanes (GH), using anti-JAK2 and anti-GHR antibodies, suggesting a constitutive association of JAK2 with GHR. Also, in the absence of GH stimulation, a complex containing the GHR and JAK2 is immunoprecipitated by the anti-receptor mAb 263 (Fig. 3B). Interestingly, this association does not appear to be modified by GH stimulation. In fact, stimulation by GH results in induction of tyrosine phosphorylation of JAK2 and the GHR but there is no further recruitment of JAK2 by the activated receptor.

2. H. Buteau, A. Pezet, P. A. Kelly, and M. Edery, manuscript in preparation.
Detection of GHBP in Monkey Serum and Culture Medium of Cells Expressing the Monkey GHR—The elution profile of \([^{125}\text{I}]\text{hGH}\) incubated with serum obtained from an adult Macaque is shown in Fig. 4A. A single peak is observed at the elution time expected for GHBP (37). An excess of unlabeled hGH inhibited this peak demonstrating the specificity of the hGH binding. In 12 different sera, the specific binding ranged from 2.1 to 11.0% of total radioactivity (for 100 \(\mu\)l of serum). Recombinant bovine GH and human PRL had no effect on the binding of \([^{125}\text{I}]\text{hGH}\) to the GHBP, demonstrating that it is a primate GH-binding protein without lactogenic specificity (data not shown).

In the culture medium of 293 cells transiently expressing monkey GHR, the presence of the soluble receptor was also detected (Fig. 4B), with a comparable elution profile to that obtained with serum. The level of GH binding was 23% using concentrated culture medium (\(\times 20\)). Scatchard analysis from competition binding showed an affinity constant of \(2.0 \pm 0.6 \times 10^{8}\) M\(^{-1}\) for the GHBP produced by proteolytic cleavage. No soluble GHR can be detected in culture medium of non-transfected 293 cells.

Gene Expression of the Monkey GHR—Northern blot analysis was performed to determine the size and the distribution of the GHR transcripts in various monkey tissues. A major transcript with an estimated size of 5.0 kb hybridized to probe E (Fig. 5A), in liver, heart, kidney, pancreas, intestine, stomach, muscle, and fat. Two other mRNAs were detected: one of 2.8 kb in liver, kidney, and adipose tissue; another one of 1.7 kb was detected in heart, kidney, and stomach. In the stomach, the intensity of the signal of the 1.7-kb transcript was comparable to that of the 5.0-kb mRNA (Fig. 5A).

To look for differences in the coding region, the same membranes were hybridized with probe I (Fig. 5B) which is specific for the cytoplasmic domain of the receptor. This probe hybridized with the two large mRNAs of 5.0 and 2.8 kb, but not with the 1.7-kb transcript (Fig. 5B). Using an oligonucleotide probe specific for the transmembrane domain, TMD primer (Table I), the hybridization profile is similar (data not shown). Hybridization of all membranes with a cyclophilin cDNA probe was also performed to normalize the results (Fig. 5C). As observed in all other species the liver showed the highest expression level of the long form; the expression of GHR mRNA was also...
Two Mechanisms for the Generation of the Monkey GHBP

Fig. 6. RT-PCR amplification of GHR mRNA in different monkey tissues. A, ethidium bromide staining of PCR products using mGR46 and mGR104 primers. B, hybridization of the gels shown in A after transfer using an internal probe (mGR73). C, ethidium bromide staining of PCR products using Y1 and Y2 primers of ubiquitous cyclophilin. The size of amplicons are indicated on the right in base pairs.

We used RT-PCR with forward primer mGR46 and reverse primer mGR104, which spans a part of the extracellular coding region, the transmembrane domain, and a part of the intracellular region of the monkey GHR cDNA (Table I, Fig. 1B), to investigate GHR gene expression in various tissues. Ethidium bromide staining of amplified products separated on agarose gel showed in all tissue samples a band of the predicted length, 600 bp (Fig. 6A). The specificity of the amplification was confirmed by Southern hybridization using an internal probe, oligonucleotide mGR73 (Fig. 6B). The efficiency of the RT-PCR reaction was confirmed by a control amplification of the ubiquitous protein cyclophilin, using human primers (Fig. 6C).

During the analysis of the expression of GHR gene in monkey tissues, at least two additional bands were detected using RT-PCR with primer mGR0-mGR104. These products visualized by ethidium bromide staining showed one band with the expected size and two other smaller bands (data not shown). We subsequently purified each band, subcloned, and identified the sequence of these additional PCR products. The first clone we characterized resulted from an alternative splicing of exon 3 and corresponded to the human GHRd3 isoform (41, 42). It was observed in the different monkey tissues studied. The second clone was also a deletion mutant lacking exons 4, 5, and 6. This deletion resulted in a frameshift and introduced a stop codon in exon 7, before the region encoding the transmembrane domain. The putative protein product would be a secreted form of mkGHR of 44 amino acids.

Molecular Cloning of a cDNA Encoding a Putative GHBP—To further establish the existence of a specific transcript for the GHBP in monkey we used 3′-RACE-PCR to identify a specific sequence for a GHBP. Two subsequent amplifications performed on heart and kidney cDNA using upstream primers mGR0 and mGR31, respectively, gave a 250-bp cDNA fragment that was subcloned in the pcDNA3/mkGHR plasmid by substituting the transmembrane and cytoplasmic domains. The analysis of this sequence relative to the genomic sequence of the human GHR gene shows that this cDNA could be generated by a lack of splicing between exon 7 and intron 7. Thus, 236 bp of intron 7 are integrated in the coding sequence of the GHBP resulting in the addition of 8 new amino acids and a stop codon (Fig. 7). The 3′ noncoding sequence includes an additional poly(A) signal followed by a poly(A) tract. This alternatively spliced mRNA would permit the production of a secreted extracellular domain of the GHR.

We have used RT-PCR with primers prepared from highly conserved regions of mammalian GH receptors to clone the cDNA of the Macaque rhesus GH receptor. Using several tests we demonstrate that this cDNA encodes a receptor protein which presents the expected specificity for a primate GHR and is fully functional. The monkey GHR gene is expressed not only in the liver, but in all the tissues analyzed. A 5.0- and 2.8-kb transcripts were detected with different probes and likely encode the full-length membrane receptor. In some tissues, another mRNA of 1.7 kb is seen that could result from alternative splicing and encode a GHBP. Using 3′-RACE-PCR we identified a cDNA encoding a GHBP that has been characterized.

The cDNA of the monkey GHR was constructed using two fragments of PCR of 1.0–1.4 kb to limit the amplification errors observed for long size fragments. The sequence obtained was also confirmed by amplification and sequencing of smaller fragments. Assembling, subcloning into eukaryotic expression vectors, and propagating the cDNA encoding the full-length monkey GHR in E. coli was successfully achieved. We were also able to express these constructs in eukaryotic cells to produce the expected fully active receptor. It is important to note that propagation in bacteria and also transfection of full-length human GHR cDNA with all the expected characteristics has proven difficult (3, 11), although recently, modifications in nucleotide sequences in and around the transmembrane region have permitted some amelioration (43).

The analysis of the mkGHR cDNA shows that it encodes a precursor protein of 638 amino acids which has a very high degree of identity with the human GHR (94.1%). Comparing the sequence of the hormones, human and monkey GH share 96.0% identity (29), a value similar to that for the GH receptor: this supports the hypothesis of a co-evolution of hormone and receptor in different species. Thus, the structure of human and monkey GHRs are probably very similar.

The functional activity of the receptor encoded by this cloned cDNA has been established. Expression of the monkey cDNA in COS cells results in a receptor protein with all the characteristics of a primate GHR. This receptor is able to bind hGH with an affinity similar to that found for the receptor present in monkey liver membranes. Moreover, this receptor is specific for hGH and is unable to bind nonprimate GH; it has somatogenic specificity and is devoid of lactogenic specificity. We also performed functional studies in 293 cells using the promoters of genes encoding β-casein and Spi 2.1 in bioassays based on specific activation by GH as described previously (16, 17). Our findings demonstrate that β-casein, specific for a lactogenic effect, and Spi 2.1 promoters are inducible by hGH in 293 cells transfected with the mkGHR cDNA. The fold induction obtained with the monkey GHR is similar to or greater than that obtained with the rat (16) or rabbit GHR cDNA (17).

Using forward primer mGR46 and reverse primer mGbp (Table I), we investigated the expression of this putative GHBP mRNA in 6 different tissues. Ethidium bromide staining of amplified products separated on agarose gel showed a band of the predicted length, 468 bp (Fig. 8A). The specificity of the amplification was confirmed by Southern hybridization using an internal probe, oligonucleotide mGR56 (Fig. 8B). The amplicon is present in kidney, heart, and is very abundant in stomach as expected from Northern blot analysis, but it is also detectable in the liver; in intestine and pancreas no signal could be observed.

**DISCUSSION**

The secreted mkGHBP had an affinity constant of $2.94 \times 10^{-9}$, a value similar to that of the GHBP produced by proteolytic cleavage of the membrane bound receptor.

Using forward primer mGR46 and reverse primer mGbp the sequence of the hormones, human and monkey GH share 96.0% identity (29), a value similar to that for the GH receptor: this supports the hypothesis of a co-evolution of hormone and receptor in different species. Thus, the structure of human and monkey GHRs are probably very similar.
in the generation of mkGHR and mkGHBP transcripts. Exon and exon sequences are denoted by shaded lowercase letters and boxed uppercase letters, respectively. Resulting amino acid sequences are shown above and below. Boldface lowercase letters indicate the intrinsic nucleic acids involved in the sequence of the GHBPs. The extracellular domain-transmembrane junction is marked by an asterisk in the amino acid sequence.

**FIG. 7.** Alternative splicing mechanism generating mkGHR and mkGHBP. Diagrammatic representation of the genomic DNA fragment involved in the generation of mkGHR and mkGHBP transcripts. Introns and exons sequences are denoted by shaded lowercase letters and boxed uppercase letters, respectively. Resulting amino acid sequences are shown above and below. Boldface lowercase letters indicate the intrinsic nucleic acids involved in the sequence of the GHBPs. The extracellular domain-transmembrane junction is marked by an asterisk in the amino acid sequence.

**Fig. 8.** RT/PCR amplification of GHBs mRNA in different monkey tissues. A, ethidium bromide staining of PCR products using mGR46 and mGbp primers. B, hybridization of the gel shown in A after transfer using an internal probe, mGR56 primer. The size of amplicons are indicated on the right in base pairs.

To further establish the functional activity of this receptor, we investigated its signal transduction pathways. Binding of GH to cell surface receptors is the initial step of hormone action. An early event that occurs following hormone binding and homodimerization of the GHR (14, 44) is the rapid induction of tyrosine phosphorylation of several proteins, one of which has been identified as the tyrosine kinase JAK2 (15, 45). We have examined the association and the activation of tyrosine kinase JAK2 in the monkey GHR, using the transient cotransfection system of both JAK2 and mkGHR cDNAs in 293 cell line. The fact that JAK2 and the receptor are phosphorylated even in the absence of GH stimulation could be due to autoactivation because of overexpression; and/or reveal a state of basal phosphorylation, amplified by the high copy number. Our studies demonstrate that the monkey GHR is able to associate with JAK2 and to activate its phosphorylation; we also observed GHR tyrosine phosphorylation following GH stimulation. Both the internal deleted form of PRL receptor found in the Nb2 cell line and the long form of the rat PRL receptor were shown to be constitutively associated with the tyrosine kinase JAK2 (46–48), through the conserved proline-rich region, Box 1 (47, 49). In contrast to other models (17), the monkey GHR appears to be constitutively associated with JAK2 and stimulation by GH increases the level of tyrosine phosphorylation of both JAK2 and the GHR, but does not appear to promote the recruitment of JAK2 molecules.

Even if the biological functions of the GHBPs remain to be clarified, it is of major interest to understand the mechanisms by which this protein is generated. GHBP is present in the serum of many species (50), and also in monkey serum. The binding affinity of the monkey GHBP is lower than the membrane R, as previously reported in human serum GHBP (9, 10). In our study, GHBP is detected in culture medium of cells transiently transfected with the monkey GHR full-length cDNA, suggesting that the GHBP measured in the serum of animals could be generated by proteolytic cleavage of the membrane receptor as demonstrated for rabbit OHR (27).

The expression pattern of the monkey GHR gene was studied. In man, GH receptor gene expression has been detected in many different tissues (12, 13), although the level of mRNA in nonhepatic tissues tends to be low and difficult to detect. In Northern blot analyses, large amounts of polyadenylated mRNA were required to detect the presence of transcripts, supporting the fact that GHRs are expressed in low abundance. Analysis of RT-PCR products revealed the widespread distribution of monkey GHR gene expression; as expected, mRNA were detected in all tissues tested, in gastrointestinal tract, muscle, spleen, kidney, and adipose tissue but with variations in the level of expression.

In the course of this study, we isolated and characterized two isoforms of the monkey GHR that have a deletion in the extracellular domain. These isoforms are thought to be generated by alternative splicing, since they specifically lack one or three exons. Such isoforms generated by alternative splicing have also been reported for the human PRLR (51). The GHR isoforms were detected in many different tissues and may have a general biological importance. The first one corresponds to the human GHRd3 isoform; the protein product of this GHR has been shown to be expressed on the cell surface and is capable of binding GH with the same affinity as the wild type (41). The other clone lacking exons 4, 5, and 6, would encode a secreted protein lacking GH binding activity.

Interestingly, different forms of monkey GHR mRNAs have been characterized by Northern blot analyses. The 5.0- and 2.8-kb transcripts are of sufficient size to encode the full-length membrane receptor, and they hybridize to both extra and intracellular probes and to an oligonucleotide specific to the transmembrane region. The different sizes could arise from multiple transcription starts or polyadenylation sites or from differential processing of a single transcript. The latter could affect either the coding region, the noncoding regions, or both. Furthermore, these sizes are consistent with the 4.5- and 2.8-kb transcripts reported in man (12). Also, a smaller mRNA of 1.7 kb, only detected with the extracellular probe, is expressed in stomach, heart, and kidney. In these three tissues of the two monkeys described, there was great variation in the level of expression of this mRNA, as well as in the relative expression of the 1.7-kb mRNA to the long form. The size of this transcript is not sufficient to encode the full-length receptor but is similar to the small mRNAs (1.2–1.5 kb) found in mouse and rat tissues and that had been demonstrated to encode the GHBPs (24, 25). In man and rabbit, short forms of mRNA with an estimated size of 2.8 and 1.3 kb, respectively, were sometimes detected; but they have never been well characterized (12, 52).

3'-RACE-PCR allowed us to identify a specific monkey sequence for the GHBPs. In fact, RT-PCR revealed that the expression of this transcript is specific to some tissues and its
abundance is variable, highly expressed in stomach, heart, and kidney, less so in liver, and absent in some other tissues like intestine or pancreas. Thus, a local production of GHB could occur in monkey and the nature of its tail is not directly related to that identified for the mouse or rat GHB (24, 25). Recently, the presence of a splice variant of the GHR has been demonstrated in human tissues (53, 54). This mRNA results from a deletion in exon 9 leading to a stop codon at position 280. This short form of the membrane GHR could generate large amounts of GHB. We were not able to find such a transcript capable of generating a GHB in monkey tissues.

Taken together, these results demonstrate that the cloned cDNA expresses a fully functional GHR. The GHB found in monkey serum could be generated through two separate mechanisms: proteolytic cleavage of the membrane receptor and a specific mRNA expressed in several monkey tissues. This is the first demonstration of a possible co-existence of the two mechanisms of GHB generation within the same subject.

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REFERENCES

1. Kelly, P. A., Djiane, J., Postel-Vinay, M. C., and Edery, M. (1991) Endocrinology 129, 883–888
2. Colosi, P., Wong, K., Leong, S. R., and Wood, W. I. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1148–1152
3. Mathews, L. S., Norstedt, G., and Palmitter, R. D. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9343–9347
4. Lebrun, J.-J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. (1994) EMBO J. 13, 3573–3579
5. Delehaye-Zervas, M. C., Mertani, H., Martini, J. F., Nihoul-Fekete, C., Morel, G., and Postel-Vinay, M. C. (1994) J. Clin. Endocrinol. Metab. 78, 141–148
6. Mertani, H. C., Delehaye-Zervas, M. C., Martini, J. F., Postel-Vinay, M. C., and Morel, G. (1995) Endocrinology 136, 135–142
7. Cunningham, B. C., Ueltsch, M., De Vos, A. M., Mullerin, M. G., Clauser, K. R., and Wells, J. A. (1991) Science 254, 821–825
8. Argetsinger, L. S., Campbell, G. S., Wang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter-Su, C. (1993) Cell 74, 237–244
9. Goujon, L., Allevato, G., Simonin, G., Piquerez, L., Le Cam, A., Clark, J., Nielsen, J. H., Djiane, J., Postel-Vinay, M. C., Edery, M., and Kelly, P. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 567–961
10. Edery, M., Levi-Meyrueis, C., Paly, J., Kelly, P. A., and Djiane, J. (1994) Mol. Cell. Endocrinol. 102, 39–44
11. Baumann, G., Chastre, E., Choquet, A., Bara, J., Gaspach, C., and Kelly, P. A. (1995) Am. J. Physiol. 268, G431–G442
12. Ross, R. J. M., Esposito, N., Shen, X. Y., Ven Laue, S., Chew, S. L., Postel-Vinay, M. C., and Finidori, J. (1997) Mol. Endocrinol. 11, 265–273
13. Dastot, F., Sobier, M. L., Duquesnoy, P., Duriez, B., Gossens, M., and Ameisen, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10723–10728