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IDENTIFICATION AND TISSUE DISTRIBUTION OF MESSENGER RNA FOR THE GROWTH HORMONE RECEPTOR IN THE RABBIT

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Rabbit liver, a rich source of specific growth hormone (GH) receptors, contains three mRNA transcripts (4.2-4.5 kb, 3.1-3.2 kb, and 1.8-2.0 kb) which hybridize strongly to oligonucleotide probes complementary to nucleotide sequences in the extracellular and cytoplasmic regions of the rabbit liver GH receptor. The ~4.5 kb transcript was the most abundant and showed some sex difference (male > female) and a significant, ~2 fold increase in late pregnancy - observations consistent with changes seen in the specific ^{125}I-hGH binding capacity of rabbit liver membranes prepared from the same tissue samples. The ~4.5 kb mRNA species, but not the smaller transcripts, was also detected, at lower abundance, in rabbit kidney, heart and lung but not in mammary gland, which is known to lack ^{125}I-GH binding activity. These studies have identified the nature of the mRNA transcripts coding for the GH receptor in recognized/potential GH target tissues in the rabbit. The regulation of the major GH receptor mRNA in rabbit liver appears to broadly reflect known changes in expressed receptor protein.

The liver is a well-known target tissue for growth hormone (GH). Rabbit liver has long been regarded as a prime model for studies on GH receptor characterization, structure and physiological regulation because of the very high abundance of this receptor in this tissue (1,2). Interpretation of several such studies, particularly those on the regulation of the GH receptor, has been ambiguous; partly because of apparent interaction of GH and the structurally-related hormone, prolactin (PRL), with each others receptors, and partly because of the wide use of the ligand ^{125}I-human GH (hGH) which binds equally well to either somatotrophic (GH) or lactogenic (PRL) receptors (1,2). These practical difficulties in distinguishing between GH and PRL receptors have been given a rational basis recently with the elucidation, via

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cDNA cloning techniques, of the full nucleotide, and therefore amino acid, sequences of the human/rabbit GH receptor (3) and the rat PRL receptor (4). These two receptors have quite marked (~30%) homologies in the extracellular hormone-binding domains. The cloning of the rabbit GH receptor provides an opportunity to study directly and unambiguously the physiological/hormonal/pharmacological regulation of this receptor by examining the regulation of expression of the GH-receptor gene in the liver and other target tissues of the rabbit.

In the present study, Northern blot hybridization has been used to identify the mRNA species coding for the rabbit GH receptor and their abundance and distribution in various rabbit tissues.

MATERIALS AND METHODS

Probes and hormones

Two 30-mer oligonucleotide probes homologous to regions of rabbit liver GH receptor cDNA were synthesized. One probe (coded #302) was complementary to the cDNA nucleotide sequence corresponding to amino acids 71-80 in the extracellular domain of the receptor. The second probe (#301) corresponded to a cytoplasmic domain (amino acids 289-298). A 30-mer oligonucleotide probe complementary to a part-sequence of 18S ribosomal RNA was used as a control to standardize densitometric measurements. The probes were end-labeled with (γ^{32}P)ATP (~2000 Ci/mmol; BRESA Pty. Ltd. Adelaide, Australia) to a specific activity of 0.5-1x10^8 cpm/µg. hGH (NIAMDD-hGH-I-1) used for iodination was a gift of the National Hormone and Pituitary Program (NIADDK, Bethesda, MD, USA) and was iodinated by the Iodogen method (5) before being purified by gel chromatography on Ultrogel AcA 54 as described previously (6). ^{125}I-hGH specific activity (30-50 µCi/µg) was determined by either trichloroacetic acid precipitation of the iodination mixture or by a tracer dilution method (7).

Collection of tissues

Tissues (liver, heart, kidney, lung and/or mammary gland) from adult male (7 month old), adult female and pregnant female (30 day gestation) New Zealand white rabbits were collected both for RNA extraction and ^{125}I-hGH binding studies. The tissues were removed rapidly and immediately frozen in liquid nitrogen and stored at -80°C for RNA preparation; for binding studies, tissues were collected into 0.3 M sucrose containing 1 mM PMSF and 1000 Kallikrein inactivator units (KIU) of Trasylol/ml and stored at -20°C until processing.

Preparation of RNA

Total RNA was isolated according to the method of Chirgwin et al. (8). In brief, the frozen tissues were homogenised in 10 volumes of 5 M guanidine thiocyanate (Fluka, Buchs, Switzerland) in the presence of 5% mercaptoethanol and the homogenates layered over a cushion of 4 volumes of 5.7 M cesium chloride (Boehringer, Mannheim, W. Germany) in Beckman quick-seal tubes. The RNA was sedimented by ultracentrifugation at 85000 g for 12-16 h at 20°C and then resuspended, ethanol-precipitated, and quantitated [based on

spectrophotometric determination at 260 nm (1.0 OD $_{260}$ = 40 µg/ml)]. The RNA preparation was adjusted to a final concentration of 2 µg/µl.

Before hybridization analysis, the quality, integrity, and the level of DNA contamination was assessed by size separation on 1% non-denaturing horizontal agarose gels followed by ethidium bromide (0.5 µg/ml) staining.

Northern blot hybridization

Northern blotting was performed as previously described (9). Briefly, 10-20 µg of total RNA was denatured in 1 M glyoxal-50% dimethylsulfoxide and then electrophoresed on a 1% agarose gel in 10 mM phosphate buffer, pH 7.0. RNA was then transferred to Hybond-N membrane (Amersham International, Amersham, UK) by capillary blotting. The membranes were baked at 80°C for 2 h, UV cross-linked for 20 min and prehybridized at 50°C overnight in hybridization solution consisting of 5xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.4), 50 mM sodium phosphate pH 7.0, 5x Denhardt's solution (1x Denhardt's solution is 0.02% bovine serum albumin, Ficoll 400, polyvinyl pyrolidone), 1% sodium dodecyl suplhate (SDS), 0.1% sodium pyrophosphate and 3 mg/ml herring sperm DNA. Labelled probe (0.5-1 x 10^6 cpm/ml) was added to the pre-hybridization buffer and hybridization continued at 42°C for 24 h. The nylon filter was washed initially with 2 x SSC/0.5% SDS at room temperature (20 min), followed by washes of increasing stringency up to 0.1 x SSC at 50°C. Autoradiographs were obtained by exposure to Kodak XAR-5 film using Dupont Lightning Plus intensifying screens at -70°C. Prior to rehybridization (eg. with ^{32}P-labelled 18S probe), the blots were washed with 0.1% SDS, 0.1xSSC in the presence of 50% formamide at 37°C for 20 min; the complete removal of previously hybridized probe was determined by autoradiography at -70°C overnight. Densitometry (ISCO gel scanner 1312, Lincoln, Nebraska, USA) was performed to quantitate the changes between tissues in hybridization of the GH receptor probes on the Northern blots relative to the level of the 18S control probe.

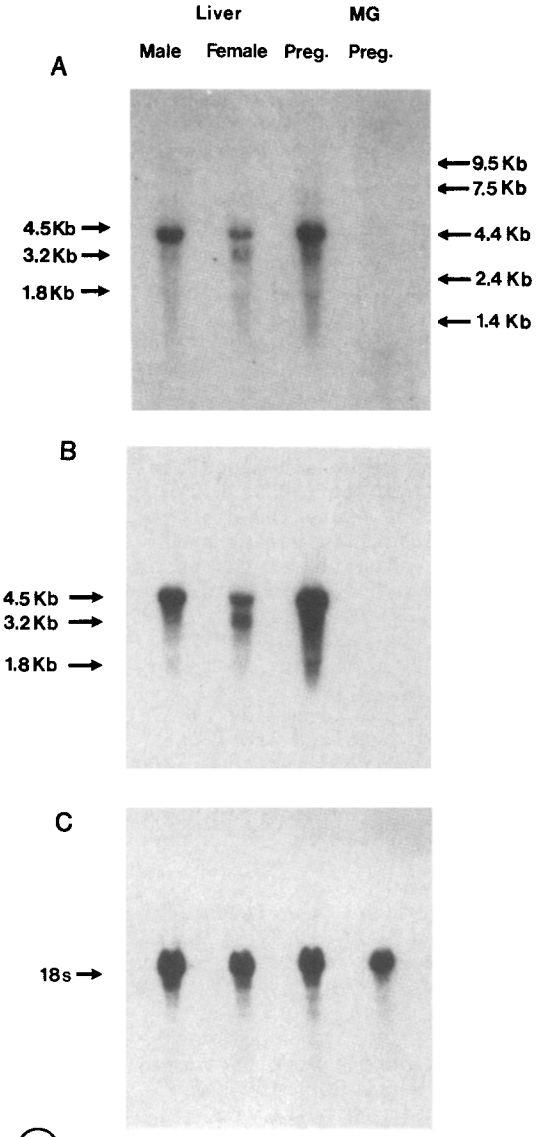
^{125}I-hGH binding studies

Microsomal membranes (100,000 g) were prepared as described previously (10) in the presence of 1 mM PMSF and 1000 KIU Trasylol/ml. The pellet was resuspended in 25 mM Hepes-HCl buffer pH 7.5 containing 10 mM $MgCl_2$, 0.02% NaN_3 and 1000 KIU Trasylol/ml. Protein concentrations were determined by the Lowry method (11).

Rabbit liver membranes (50 µl, 2 mg of protein/ml) were incubated with ^{125}I-hGH (25,000 cpm, 0.2-0.4 ng in 25 mM Hepes-HCl, 10 mM $MgCl_2$, 0.1% BSA, 0.02% NaN_3) in the presence (non-specific binding) or absence (total binding) of unlabeled hGH, in a final volume of 250 µl at 23°C for 2 h. The reaction was terminated by diluting with 1 ml ice-cold Hepes buffer and centrifuging at 1800 g for 30 min at 4°C. The supernatant was aspirated and the membrane-bound ^{125}I-hGH in the precipitate was determined in a gamma counter.

RESULTS AND DISCUSSION

In order to determine the size and number of mRNA transcripts for the growth hormone receptor gene in the rabbit, total RNA was extracted from liver of adult male, female and pregnant rabbits and analysed by Northern blotting. As shown in Fig. 1A and B, three major transcripts (4.2-4.5 kb, 3.0-3.2 and 1.8-2.0 kb) were shown to hybridize strongly to both GH receptor oligonucleotide probes. The



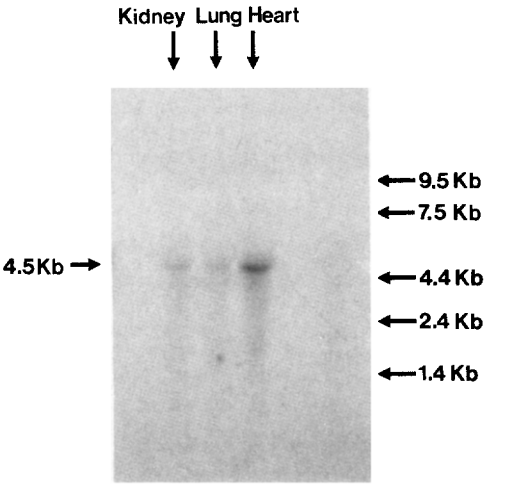


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Fig. 1. Northern blot analysis of total RNA (25 µg/lane) from rabbit liver (male, female and pregnant) and mammary gland hybridized with ^{32}P-labeled oligonucleotide probes, complementary to nucleotide sequences in the extracellular (1A) and cytoplasmic regions (B) of the GH receptor protein and in 18S ribosomal RNA (C). Autoradiography was carried out for 4 days (A,B) or 1 day (C) at -70°C with Kodak X-AR film and intensifying screens. The size (kb) of each specific GH receptor mRNA transcript is indicated on the left and was estimated from the relative mobilities of an RNA standard ladder (kb shown on the right).

Fig. 2. Northern blot analysis of total RNA (25 µg/lane) from adult rabbit kidney, lung and heart, hybridized to ^{32}P-labeled oligonucleotide probe (#301, corresponding to a cytoplasmic region). The size (~4.5 kb) of the only mRNA species detected was estimated from the relative mobilities of a commercial RNA ladder (kb shown on the right). Autoradiography was carried out as described for Fig. 1.

~4.5 kb transcript was the most abundant in each preparation and corresponds to the size of the full-length, cloned GH receptor cDNA (3) and to the ~4.7 kb band detected by those authors on a Northern blot of male rabbit liver poly (A^+) RNA. The ~4.5 kb species, but not the smaller transcripts, was also seen, at lower abundance in rabbit kidney, lung and heart preparations (Fig. 2). No specific hybridizing bands were observed in mammary gland, which is known to lack ^{125}I-hGH binding activity.

Three mRNA species were detected by both cytoplasmic (#301) and extracellular (#302) oligonucleotide probes in liver, especially in pregnant and non-pregnant females. Multiple RNAs could be explained at least in part by alternative processing/splicing of a primary transcription product from a single gene or by products of different genes. The number of GH receptor gene(s) present in the rabbit has not been reported to date. The physiological significance of the three mRNA species and whether more than one is translated is not known. Interestingly, however, more than one class of GH-binding protein has been identified in rabbit liver based on immunological data (12) and binding parameters for the two major variant forms (22K and 20K) of GH (13). Furthermore, a serum GH-binding protein has been identified (14,15), which would appear to be a truncated (i.e. extracellular) form of the membrane receptor (3). Whether these receptor forms result from translation of different mRNAs or from post-translational modifications is not yet known.

It is well known that rabbit liver GH receptors exhibit a sex and pregnancy dependence (1,2,16). This was also evident at the mRNA level with the ~4.5 kb mRNA appearing to be greater in abundance in male than female rabit liver and being further increased in late pregnancy (Fig. 1A and B). Quantitation of these changes was made by densitometry by measuring areas under densitometric peaks. The very same blots of the male, female and pregnant rabbit liver RNAs were

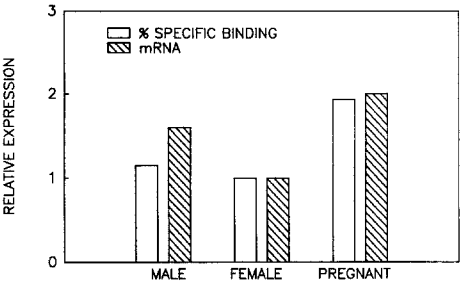

Fig. 3. Relationship between levels of ^{125}I-hGH binding capacity (□) and the relative abundance of GH receptor mRNA (▨). The binding capacity (fmol/mg protein, calculated by Scatchard analysis) and mRNA abundance (quantitated by densitometry) were each expressed relative to the values for female liver (defined as 1.0 unit).

stripped of GH-receptor probe and reprobed with an 18S ribosomal RNA oligonucleotide (Fig.1C) to determine the relative amounts of each total RNA preparation used for the Northern blot. The changes in GH receptor probe densitometric readings were then standardized and expressed relative to the reading obtained for female rabbit liver (defined as 1.0). As shown in Fig. 3 (filled histograms), there was a 1.5-fold increase in GH receptor mRNA abundance between male and female liver and an ~2-fold increase occurred in late pregnancy. These changes were consistent with changes seen in the ^{125}I-hGH binding capacity of rabbit liver membranes prepared from the same tissue samples (open histograms, Fig. 3). Scatchard analysis of displacement curves of rabbit liver membrane incubated with ^{125}I-hGH showed, as expected, that changes in specific binding were due to changes in binding capacity (male, 8949; female, 8521; pregnancy, 14380 fmol GH bound/mg protein) rather than binding affinity ($2.1\sim2.6\times10^{9}$ M^{-1}). Scatchard plots were linear in each case and were analysed by the LIGAND computer program of Munson and Rodbard (17).

The binding specificity of the ^{125}I-hGH binding proteins present in the rabbit liver membrane preparation was also determined by incubating in the presence and/or absence of a highly species-specific GH receptor monoclonal antibody (MAb) [derived, as described previously (8) from hybridoma D_6 of Simpson et al. (19)] and a

prolactin receptor MAb [M110 as reported by Katoh et al. (20)]. The data showed that the GH receptor MAb inhibited over 80% of the binding of ^{125}I-hGH to the liver receptors in each preparation whereas the PRL receptor MAb was essentially ineffective (5-10% inhibition). The reverse was true for ^{125}I-hGH binding to rabbit mammary gland receptors (data not shown). These data indicate that the livers of young adult male, female and pregnant rabbits contain primarily GH-specific receptors whilst mammary gland contains only PRL receptors. This is quite consistent with previous observations (e.g. 16,21,22) and is an important issue with regard to the GH- and/or PRL-receptor specificity of the major mRNA species (~4.5 kb) observed in rabbit liver. Since there are significant homologies between the rabbit GH and PRL receptor structures (4) it is important to distinguish between their mRNAs in a tissue-specific sense to allay concerns that the GH-receptor oligonucleotide probes might also detect PRL receptor mRNA. This appears not to be the case since no mRNAs were detected in rabbit mammary gland with either probes 301 or 302, despite long (96 h) exposure to autoradiography and high (25 µg) RNA loadings on Northern blots.

In conclusion, we have demonstrated, essentially for the first time, the nature of the mRNA transcripts coding for the GH receptor in well known GH target tissues in the rabbit. The relative abundance of the major mRNA species (~4.5 kb) in the liver correlates well with specific binding of ^{125}I-hGH in this tissue. Emphasis on the regulation of mRNA levels for the GH receptor in future should extend, and provide further insight into, our understanding of the role of the GH receptor in the regulation of GH action.

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