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Localization of growth hormone receptor messenger RNA in human tissues

H.C. Mertani[1], M.C. Delehaye-Zervas[2], J.F. Martini[2], M.C. Postel-Vinay[2] and G. Morel[1]

[1]*CNRS URA 1454, Neuroendocrinology, Lyon-Sud School of Medicine, Oullins; and* [2]*INSERM U344, Molecular Endocrinology, Hôpital Necker-Enfants Malades, Paris, France*

In order to identify GH target cells in human tissues, we investigated the cellular distribution of human GH receptor (GHR) messenger RNA. This was performed by *in situ* hybridization and Northern blot hybridization using complementary radioactive DNA probe encoding part of the extracellular domain of the GHR. Several tests were carried out to validate the detection of gene expression. Our results demonstrate that the GHR gene is expressed in all human tissues studied, including liver, muscle, kidney, heart, skin, thymus, adipose tissue, placenta, testis, ovary and mammary gland. The quantification of the *in situ* hybridization signal by densitometric analysis showed a greater expression of GHR transcript in liver, muscle, kidney, heart, and skin epidermis, and a reduced expression in thymus, mammary gland, testis, ovary, and dermis/hypodermis. These results confirm the widespread distribution of the GHR gene expression in human tissues and identify the cells that might be responsive to GH.

Keywords: growth hormone receptor; mRNA; *in situ* hybridization; human tissue; northern blot

Introduction

Growth hormone (GH) has many biological effects in various organs. The first step in its action is the binding to a membrane GH receptor (GHR). The GHR has a single transmembrane domain and it belongs to the GH/prolactin/cytokines receptor family (Kelly *et al.*, 1991). Moreover, a circulating GH binding protein (BP) corresponding to the extracellular domain of the GHR has been identified in sera of many species (Kelly *et al.*, 1991). Attempts were made to study GHR gene expression in some human tissues (Oakes *et al.*, 1992; Delehaye-Zervas *et al.*, 1994; Mercado *et al.*, 1994) and to localize the protein by immunocytochemistry (Werther *et al.*, 1990, 1993). However the presence of the GHR has still not been demonstrated in all tissues known to respond to GH. In tissues of the human gastrointestinal tract we have shown by Northern-blot analysis that the GHR is encoded by a 4.5 kb mRNA, and that no specific mRNA for the GHBP is detected (Delehaye-Zervas *et al.*, 1994).

In order to identify GH target cells in other human tissues, we investigated the cellular localization of GHR mRNA by *in situ* hybridization and relative gene expression was determined by computer-assisted densitometric analysis. In addition to the *in situ* localization, gene expression was confirmed by Northern-blot hybridization.

Correspondence: Dr Gérard Morel
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Results

In situ *hybridization (ISH)*

The ISH signal appeared in the photographs (Figures 1-10) after detection of probe-target mRNA hybrids either on film (variable grey levels) or on tissue sections (dark points under transmitted bright-field illumination). Specificity of the signal was confirmed (a) by disappearance of the signal in presence of an excess of unlabeled homologous probe, on autoradiographic film (Figures 2B and 9B) and on emulsion-coated sections (Figures 2C, 4B and 7B), (b) no modification of the signal in presence of an excess of unlabeled heterologous probe and (c) the absence of signal in other control experiments.

Muscle Homogeneous distribution of the macroautoradiographic signal was observed for the skeletal muscle (Figure 1, inset). The pattern of silver grain localization is illustrated on Figure 1, silver grains were not equally distributed over the skeletal muscle fiber section, but they were strongly localized in the cytoplasm close to the nuclei.

Kidney In the kidney, there was a homogeneous macroautoradiographic signal over the cortex, the medullary rays and the medulla (Figure 2A). Silver grains were detected with variability, all along the components of the nephron, they appeared more numerous over the proximal than the distal convoluted tubules. The glomeruli were not labeled. In the medulla, thick

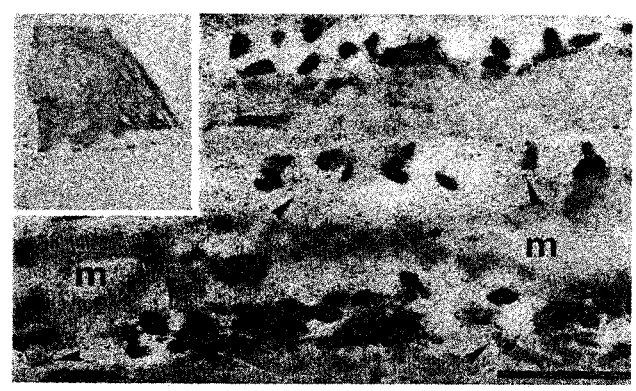

Figure 1 GHR gene expression in muscle section. Macroautoradiographic pattern obtained on film after hybridization with the GHR cDNA probe (inset, × 3.5). On emulsion-coated section, silver grains were abundantly localized in the cytoplasm next to the nuclei of scattered muscle fiber cells (arrowheads) and absent from muscular fibers (m). Magnification bar is 50 μm

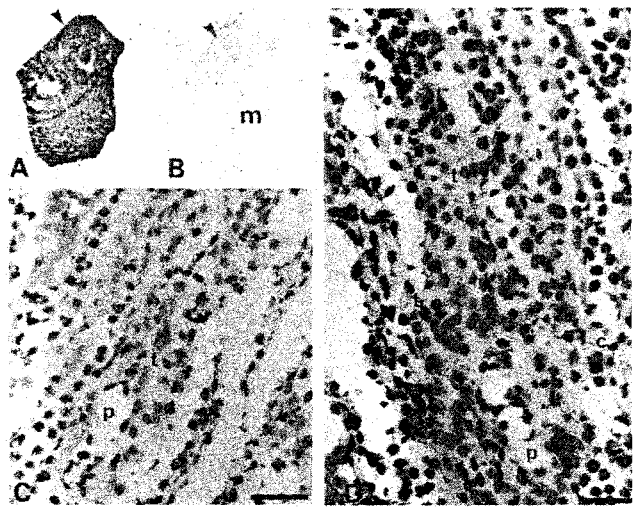

Figure 2 GHR gene expression in a part of kidney section. Homogeneous macroautoradiographic pattern over the cortex (arrowheads) and the medulla (m) (**A**, × 4). The signal on film was abolished when 100-fold excess of unlabeled probe was added to the hybridization buffer (**B**, × 4). On emulsion-coated section no silver grain was present on section hybridized with 100-fold excess of unlabeled probe (**C**). The silver grains were localized in epithelial cells of the thick ascending limbs (t), intrarenal collecting tubules (c) and proximal tubules (p) (**D**). Magnification bar is 50 μm

descending and ascending limbs and intrarenal collecting tubules were strongly labeled (Figure 2D).

Heart The macroautoradiographic signal was the same for samples of left or right ventricle, and appeared homogeneous (Figure 3, inset). In ventricular muscle, silver grains were abundantly detected in the cytoplasm of the myocytes (Figure 3). Cells of the endocardium, and some cells of the epicardium were also labeled. Smooth muscle cells and endothelial cells of blood vessels were faintly labeled.

Skin The ISH signal obtained on film showed a regionalization of the labeling in the epidermis, compared to the dermis and hypodermis (Figure 4A). In the epidermis, silver grains were preferentially localized in cells of the stratum germinativum and stratum spinosum, and reduced in the stratum granulosum (Figure 4C). In the stratum lucidum and the stratum corneum no labeling was found. Strong labeling was also seen in the cells of the sebaceous glands and in the epithelial cells layers of the follicular sheath (Figure 4C). In the dermis and hypodermis, silver grains were

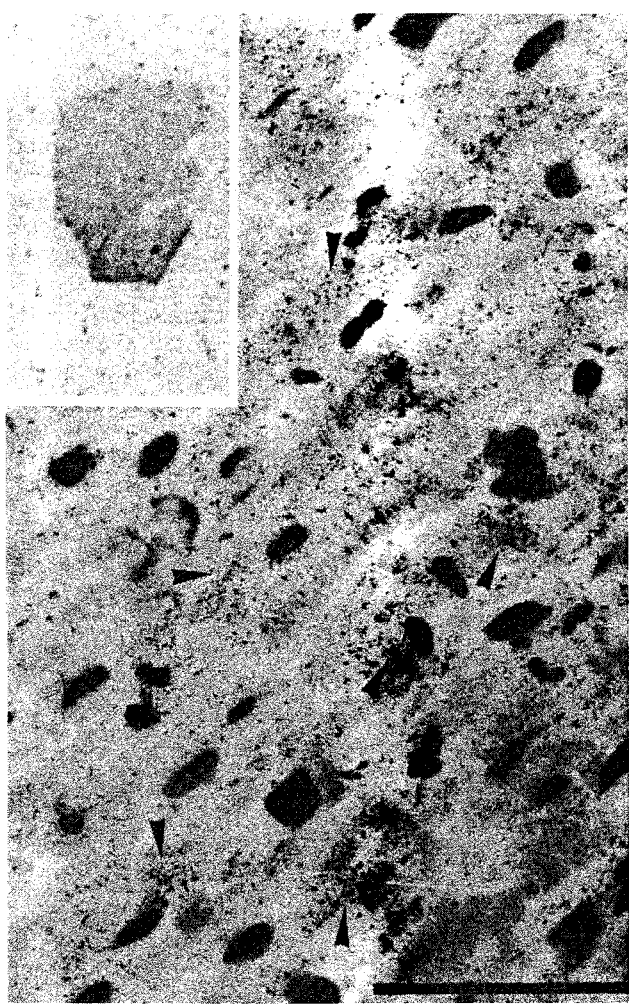

Figure 3 GHR gene expression on longitudinal section of left cardiac ventricle. Macroautoradiographic pattern (inset, × 2.5). On emulsion-coated section, silver grains were abundant in the cytoplasm of the myocardium cells (arrowheads). Magnification bar is 50 μm

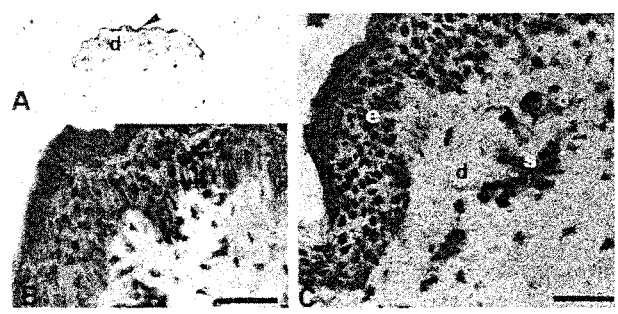

Figure 4 GHR gene expression in human skin. Macroautoradiographic pattern of ISH signal, showing the intense labeling of the epidermis (arrows) *vs* dermis (d) (**A**, × 7). On emulsion-coated section, no silver grain was present on section hybridized with 100-fold excess of unlabeled probe (**B**), whereas in absence of probe competition, silver grains were abundant in epidermis layers (e), in some cells of the dermis (d), and in sebaceous glands (s) (**C**). Magnification bar is 50 μm

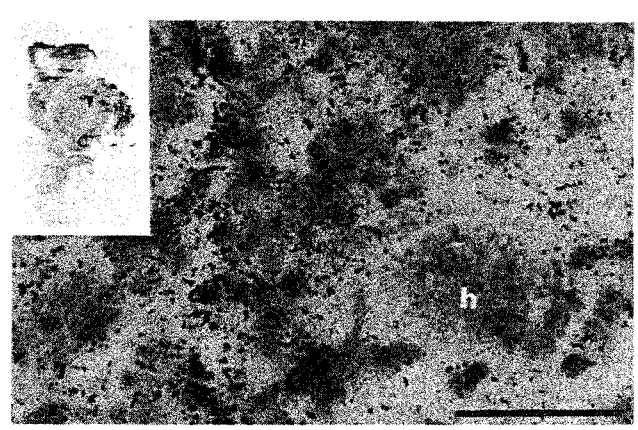

Figure 5 GHR gene expression on thymus section. Macroautoradiographic pattern showing a slight difference between the cortex (c) and the medulla (arrowhead) (inset, × 4). On emulsion-coated section, the distribution of silver grains was homogeneous in cells of the medulla, but absent from cells of a Hassall's corpuscle (h). Magnification bar is 50 μm

less abundant, but they were present in some fibroblasts, and adipocytes.

Thymus Autoradiograms of thymus sections show a preferential distribution of the ISH signal in the cortex compared to the medulla (Figure 5, inset), but this difference was slight. At the light microscopic level, silver grains were detected in all cortical cells, they were also present in epithelial cells of the medulla but absent in Hassall's corpuscles (Figure 5). Silver grains were reduced in the adipose tissue surrounding the thymic capsule and in cells of the interlobular septum, and they were absent in the fibrous capsule.

Placenta Homogeneous distribution of the macroautoradiographic signal was observed for sections of the placenta at term (Figure 6, inset). At the light microscopic level, in the placenta villi, silver grains were detected in the multinucleated syncytiotrophoblastic cells and they were reduced over the cytotrophoblastic cells (Figure 6). They were also detected in the chorionic trophoblasts but not in the decidualized cells of the endometrium.

Adipose tissue Distribution of the macroautoradiographic signal showed strong labeling in smooth muscle of arterioles and surrounding adipose tissue (Figure 7A). At the light microscopic level, silver

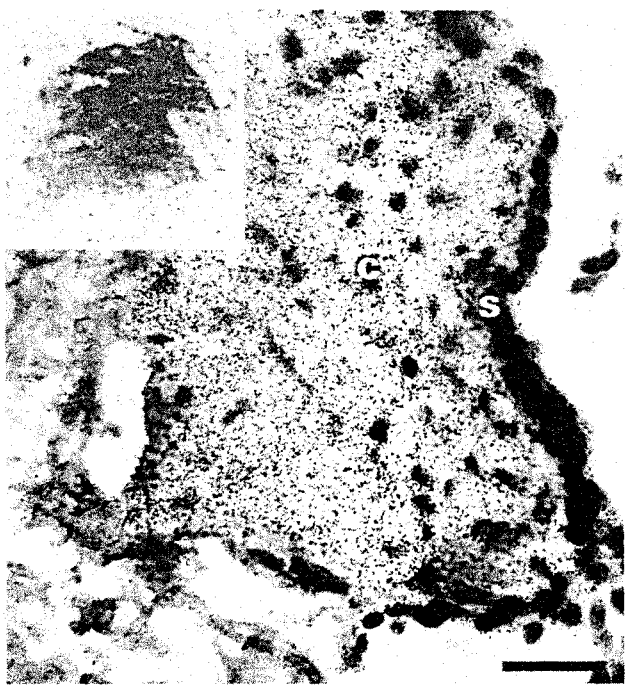

Figure 6 GHR gene expression on section of placenta at term. Macroautoradiographic pattern (inset, × 3). Silver grains were abundundly localized in the cytoplasm of the cytotrophoblastic cells (c) and reduced in the multinuclear syncytiotrophoblastic layer (s). Magnification bar is 50 μm

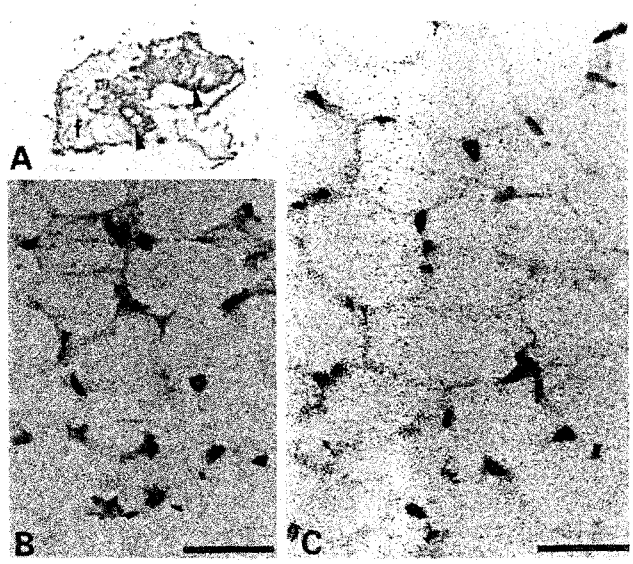

Figure 7 GHR gene expression on adipose tissue section. The macroautoradiographic signal appeared dense in arterioles (arrowheads) and in surrounding fat tissue (f) (**A**, × 5). Background level can be seen on emulsion-coated sections, hybridized with 100-fold excess of unlabeled probe (**B**), whereas in absence of probe competition silver grains were abundant in the cytoplasm of the adipocytes (**C**). Magnification bar is 50 μm

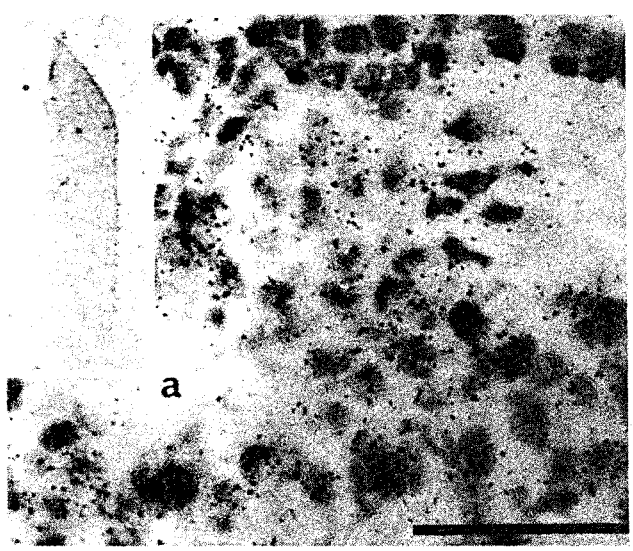

Figure 8 GHR gene expression on mammary gland section. Macroautoradiographic pattern (inset, × 4). Silver grains were localized in the cytoplasm of the epithelial cells acini. a: lumen of the acini. Magnification bar is 50 μm

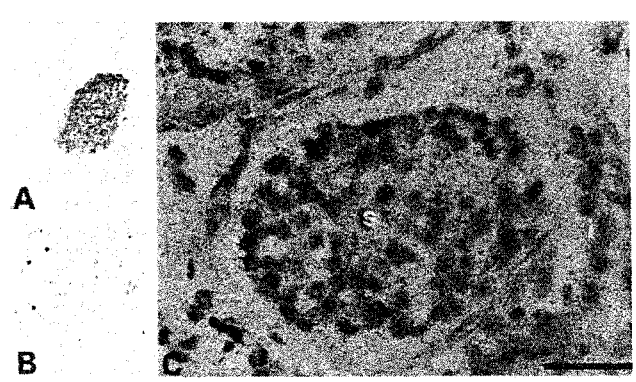

Figure 9 GHR gene expression on testis section. Macroautoradiographic pattern showing the labeling over the seminiferous tubules. (**A**, × 4.5), and its extinction when 100-fold excess of unlabeled probe was used (**B**, × 4.5). On emulsion-coated section, silver grains were distributed in all the seminiferous tubules (s) and are more abundant in centrally located cells than in interstitial cells (i) (**C**). Magnification bar is 50 μm

grains were detected in cytoplasm around the fat vesicle of the adipocytes (Figure 7C).

Mammary gland Homogeneous distribution of the macroautoradiogaphic signal was observed for sections of the mammary gland (Figure 8, inset). At the light microscopic level, silver grains were detected in the epithelial cells of the acini and they were reduced over the adipose tissue (Figure 8).

Testis Homogeneous distribution of the macroautoradiographic signal was observed over all the seminiferous tubules on sections of the human testis (Figure 9A). At the light microscopic level, silver grains were detected uniformly in Sertoli and germ cells of the seminiferous epithelium, and they were reduced in the interstitial cells of the connective tissue (Figure 9C).

Ovary Homogeneous distribution of the macroautoradiographic signal was observed over medullary and cortical parts of the ovary (Figure 10A, inset). At the light microscopic level, silver grains were detected over the surface epithelium of the ovary, and cells of the cortical stroma (Figure 10A). Silver grains were present in lutein cells of the corpus luteum. Granulosa and theca cells of developing follicles were also strongly labeled (Figure 10B).

Semi-quantitative expression of GHR

Quantification and statistical analysis of gene expression, expressed in arbitrary units, is shown in Figure 11. The highest level of GHR expression was found in liver (we used the same liver sample as described in (Delehaye-Zervas *et al.*, 1994)) followed by muscle, kidney, and epidermis. Gene expression was significantly lower ($P < 0.05$) in other tissues such as heart, thymus, ovary, testis, mammary gland and drastically reduced in the area of the dermis/hypodermis.

Characterization of GHR mRNA by Northern-blot hybridization

Hybridization of polyadenylated mRNA electrophoresed on agarose gel with a human GHR cDNA probe revealed the presence of a single mRNA form, with an estimated size of 4.5 kb in kidney, muscle, heart, placenta, adipose tissue and mammary gland (Figure 12). Discrepancies appeared between the results of the ISH semi-quantification and the spots of the Northern, principally due to the fact that measures of ISH were performed on specific areas while the Northern processed from polyA mRNAs extracted from the entire tissue, that might be contaminated or degraded.

Discussion

In this study, we localized GHR gene expression in many human tissue types using *in situ* hybridization. We also quantified differences in levels of GHR mRNA using a semi-quantitative method that we recently validated for the study of GHR gene expression in the digestive tract (Delehaye-Zervas *et al.*, 1994). The highest level of expression was observed in

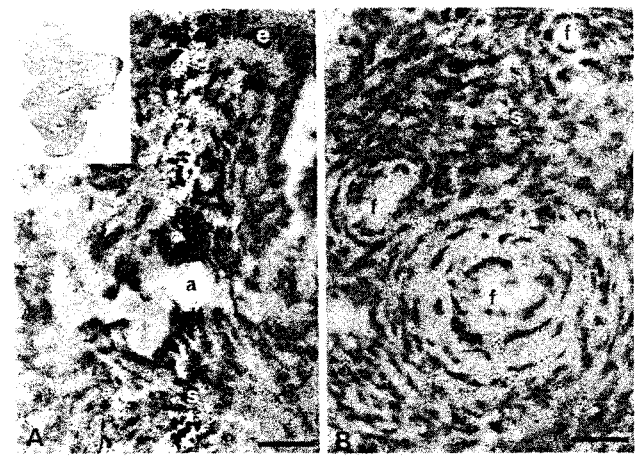

Figure 10 GHR gene expression on ovary section. Macroautoradiographic pattern (inset **A**, × 2.5). On emulsion-coated section, silver grains were distributed over the surface epithelium (e), in cells of the stroma (s), and absent within a corpus albicans (a) (**A**). In the ovary cortex, silver grains were present over granulosa and theca cells of follicles (f) at different stages (**B**). Magnification bar is 50 μm

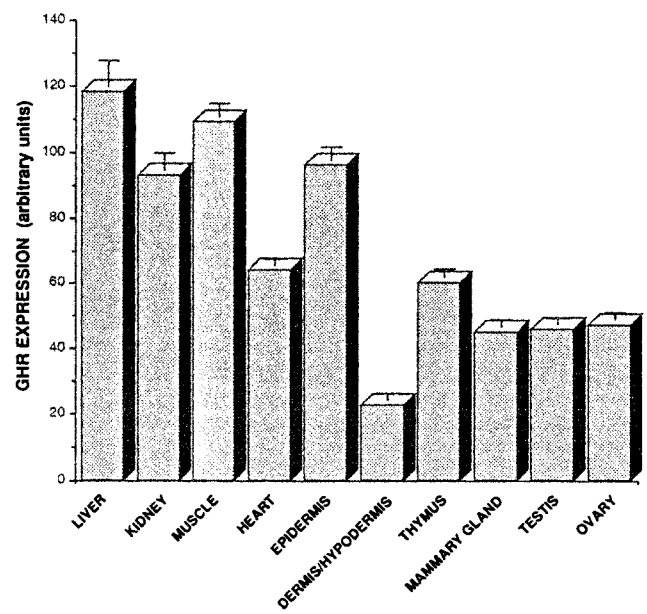

Figure 11 Level of GHR gene expression after hybridization on human tissue sections. Densitometric analysis was carried out as described in Materials and Methods. Each value is the mean ± SEM of the relative level of expression of the GHR mRNA from six autoradiograms per tissue

the liver, followed by muscle, kidney, skin epidermis, and heart. Comparable differences in GHR gene expression level have been found in rat tissues using solution hybridization assays (Mathews *et al.*, 1989). However, due to the use of human tissues from different origins, and given that the processing of mRNA in the cellular biochemistry is affected by environmental changes, it appears difficult to define a normal steady state of gene expression in human tissues, in this study. Thus, care must be taken for the interpretation of the semi-quantification results. Using a human GHR cDNA probe corresponding to the extracellular domain of GHR, we detected, as has already been shown for tissues of the gastrointestinal

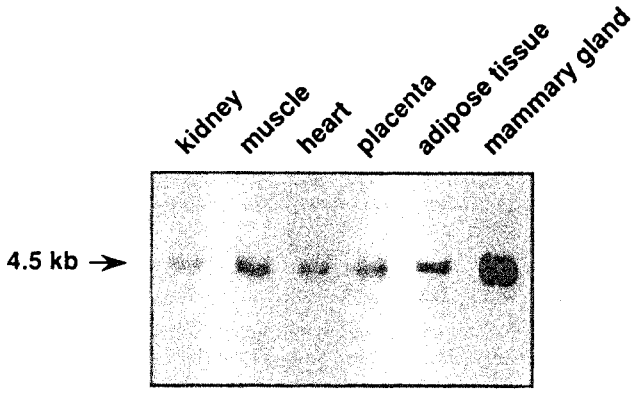

Figure 12 Northern-blot analysis of GHR mRNA in different tissues. 10 μg of polyadenylated RNA from each tissue were electrophoresed, transferred to nylon membrane and hybridized with a ^{32}P labeled cDNA probe coding for part of the extracellular domain of GHR

tract (Delehaye-Zervas *et al.*, 1994), a single 4.5 kb GHR mRNA transcript, which probably codes for the full length membrane GHR. The presence of the 4.5 kb mRNA in human tissues confirm GHR gene expression evidenced by *in situ* hybridization. The tissue processing (mRNA extraction, quantity of tissue, areas of *in situ* hybridization measures), and isotopes used for probes labeling, were different for Northern blot and *in situ* hybridization, making it difficult to compare labeling intensity of the spots to the *in situ* semi-quantification. Thus, the Northern blot results in our study are exclusively qualitative. To confirm the presence of GHR mRNA in testis and ovary tissues, which were available in small amounts, and in skin and thymus tissues, in which the signal was too low to be detected by Northern blot, we used the reverse transcription-polymerase chain reaction (RT-PCR) method, as already described (Delehaye-Zervas *et al.*, 1994). Analyses of PCR products revealed the expression of GHR mRNA in all the tissues, which confirmed the *in situ* hybridization results (data not shown).

Though skeletal muscle is considered to be a major target for GH, GH binding sites have not been identified by radioligand binding assays. Thus our results, which show a high level of expression of the GHR gene in human muscle are particularly important. In rat muscle, GHR/BP gene expression has been shown by Northern blot (Mathews *et al.*, 1989; Carlsson *et al.*, 1990; Tiong *et al.*, 1991), ISH (Jennissche *et al.*, 1991), and immunocytochemistry (Lobie *et al.*, 1992). In man, GHR mRNA has been detected in the diaphragm (Mercado *et al.*, 1994). Many GH effects on human muscle have been documented: in particular, GH stimulates myosin heavy-chain gene expression (Fong *et al.*, 1989), and enhances muscular metabolism (Yarasheski *et al.*, 1992; Fryburg *et al.*, 1993). In the rat, GH stimulates myoblast differentiation (Nixon *et al.*, 1984), and appears to be involved in the process of muscular regeneration, since it increases GHR mRNA levels after ischemic injury (Jennissche *et al.*, 1991).

The ISH signal was strong in the kidney, with a preferential localization over the medullary thick ascending and descending limbs: such a localization of GHR mRNA has been shown in the rat kidney, and has been correlated to the expression of insulin-like growth factor-I (IGF-I) (Chin *et al.*, 1992). In the rat, immunocytochemistry has been used to show that GHR/BP is present throughout the nephron (Lobie *et al.*, 1992). GHR/BP mRNA has been detected by Northern blot analysis in rat kidney (Mathews *et al.*, 1989; Carlsson *et al.*, 1990; Tiong *et al.*, 1991), and GHR gene expression has been found by RT-PCR (Urbanek *et al.*, 1992; Mercado *et al.*, 1994). These findings tend to confirm the effects of GH on renal functions; GH is able to stimulate renin secretion in GH-deficients rats (Wyse *et al.*, 1993), and also gluconeogenesis in the proximal tubules in dogs (Rogers *et al.*, 1989).

As concerns the heart, GHR/BP mRNA has been detected by Northern blot in the rat (Mathews *et al.*, 1989; Carlsson *et al.*, 1990; Tiong *et al.*, 1991) and in the rabbit (Jammes *et al.*, 1991), and the proteins have been detected by immunocytochemistry (Lobie *et al.*, 1992). GHR gene expression has been demonstrated in the human heart and aorta by RT-PCR (Mercado *et al.*, 1994). In our study, an ISH signal was found in the human heart ventricle, being essentially localized in the cytoplasm of the myocytes. Acromegaly is often associated with cardiomegaly and hyperkinetic heart syndrome (Thuesen *et al.*, 1988), heart failure and cardiovascular diseases being major causes of death in such cases.

GHR/BP mRNA are present in rat skin (Carlsson *et al.*, 1990), the receptor protein being localized predominantly in the epidermis (Lobie *et al.*, 1990b). Our finding of a high level of transcripts in epidermal cells is in accordance with immunocytological localization data (Oakes *et al.*, 1992), and confirms that GHR is synthesized in human skin. GHR transcripts have been detected in cultured fibroblasts and keratinocytes (Urbanek *et al.*, 1992; Sobrier *et al.*, 1993). *In vivo* GH treatment leads to an increase in human skin thickness (Salomon *et al.*, 1989); and this effect is not solely IGF-I-dependent, since in nude mice transplanted with human skin, GH treatment was found to result in an increase in thymidine incorporation in the transplanted human epidermal cells, as well as an increase in skin thickness. These effects are slowly reduced by the injection of an anti-IGF-I antibody concomitantly with GH (Gilhar *et al.*, 1994).

We demonstrated GHR gene expression in the human thymus. GHR/BP transcripts have not been detected in rat thymus by Northern blot analysis (Tiong *et al.*, 1991), though they have been shown by RT-PCR in fetal spleen and thymus in the cow (Scott *et al.*, 1992), which indicates that GH can affect the immune function at early developmental stages. In the GH-deficient mouse DW/J, GH administration has been shown to stimulate thymocyte proliferation and restore the deficiency of CD4/CD8 double positive thymocytes (Murphy *et al.*, 1993). In humans, GH-deficiency has been demonstrated to be associated with a diminished immune function (Fleischer *et al.*, 1980), which can be reversed by GH treatment (Rapaport *et al.*, 1986).

Binding studies and Northern blot analyses have shown the presence of GH receptors in rabbit fetal placenta (Ymer *et al.*, 1989). GHR/BP immunoreactivity is clearly detected in the decidual, trophoblastic and epithelial cells of rat placenta at embryonic day 12; it increases considerably at embryonic day 18 (Garcia-

Aragon *et al.*, 1992). The localization of GHR mRNA by ISH is quite similar to that reported for the receptor protein by immunocytochemistry in the human placenta (Werther *et al.*, 1993). A spliced GHR isoform has been identified in the human placenta and some other tissues; the transcript with the deletion of 66-base pairs encoding exon 3 does not always coexist with the normal GHR transcript (Urbanek *et al.*, 1992). The cDNA probe we used did not allow us to discriminate between the two forms of GHR mRNA. GH action in the placenta is not well known. Placental GH expressed by syncytiotrophoblasts in the placental villi may play an important role, since it binds with high affinity to GHR (Frankenne *et al.*, 1988).

GHR mRNA has been identified in human adipose tissue (Sobrier *et al.*, 1993). Many effects of GH have been shown *in vitro*: in particular GH plays a role in the differentiation of pre-adipocytes into adipose cells (Morikawa *et al.*, 1982; Nixon *et al.*, 1984).

GHR transcripts have been detected by RT-PCR in human mammary tissue (Sobrier *et al.*, 1993), and GHR mRNA has been clearly identified in rabbit mammary acini (Jammes *et al.*, 1991) and in proliferating rat mammary gland (Lincoln *et al.*, 1990), and has been localized by ISH in epithelial cells in the bovine mammary gland (Glimm *et al.*, 1990). The presence of GHR mRNA in the mammary gland is supported by the recent demonstration of *in vivo* GH effects on rat mammary development and differentiation (Feldman *et al.*, 1993).

We detected GHR mRNA, though at low levels, in the ovary and testis. GHR/BP gene expression has been identified at low levels in rat testis (Mathews *et al.*, 1989), and protein localization has been mapped in the male and female rat reproductive systems (Lobie *et al.*, 1990a). GHR gene expression has been detected by RT-PCR in the human testis and ovary (Mercado *et al.*, 1994). Little is known about the action of GH on the male reproductive function. Treatment of hypophysectomized rats with GH is known to result in an increase in testicular IGF-I gene expression (Closset *et al.*, 1989), and GH can affect steroidogenesis and cellular proliferation, probably by potentiating the action of luteinizing hormone (LH) on the production of IGF-I by Leydig cells (Spiteri-Grech *et al.*, 1992). GH is also known to increase IGF-I-immunoreactivity in the rat ovary (Davoren *et al.*, 1986), and to stimulate estradiol secretion by human granulosa cells (Barreca *et al.*, 1993). Furthermore, hGH administered concomitantly with gonadotropin is capable of inducing ovulation in partly GH-deficient menopausal women (Homburg *et al.*, 1990).

In conclusion, we demonstrated a widespread distribution of GHR mRNA in human tissues. GHR mRNA was detected in tissues, such as the muscle, which is known to respond to GH, though the receptor has not been identified by binding assay studies. Cellular localization of the GHR mRNA could give a better understanding of the physiological role of GH in some tissues.

Materials and methods

Tissues

Human tissues were obtained from patients aged 15 months to 80 years who had to undergo surgery for various pathological situations. Kidney was obtained from a 6 year old boy at the time of transplantation, muscle from a 60 year old man, skin from a woman aged 45 years, thymus from a boy and a girl aged 15 months, adipose tissue from a boy aged 3.5 years, placental tissue was collected at term (39 weeks of pregnancy), testis from a 30 year old man, ovary from a woman aged 44 years and mammary gland form a 17 year old woman. Liver obtained from a 20 year old man was used as positive control.

cDNA probe

cDNA probe was prepared from the hGHR cDNA (gift of Dr W. Wood, Genentech, San Francisco, CA, USA) as described in (Delehaye-Zervas *et al.*, 1994); a 708 bp XbaI–EcoRI fragment, which corresponds to the signal peptide and the first 218 amino-acids of the extracellular domain. DNA fragment was purfied free of vector DNA by agarose gel electrophoresis and labeled by the random priming method with [^{32}P]dCTP (3000 Ci/mmol, Amersham, Les Ulis, France) or [^{35}S]dATP (1000 Ci/mmol, Amersham) to specific activities of $1-2 \times 10^8$ c.p.m./μg (^{32}P probe) or $4-6 \times 10^7$ c.p.m./μg (^{35}S probe). Free nucleotide triphosphates were removed by chromatography on Sephadex G50 or by ethanol precipitation.

In situ hybridization

Tissues were fixed in 4% paraformaldehyde for 2 h at 4°C, cryoprotected in 0.4 M sucrose, then frozen and kept in liquid nitrogen. Cryostat sections (10 μm), mounted on 3-aminopropyl-tri-ethoxysilan coated microscope slides were rehydrated in 0.15 M NaCl, followed by 0.1 M phosphate buffer, fixed in 4% paraformaldehyde for 10 min and submitted to a 1 μg/ml proteinase K (Sigma, l'Isles d'Abeau, France) digestion in 1 × Tris-EDTA (TE) buffer (TE 10 ×: 100 mM Tris, 10 mM EDTA, pH 7.6) for 20 min at 37°C. After phosphate buffer and NaCl washing, the slides were dehydrated in ascending concentrations of ethanol and air dried. *In situ* hybridization was performed overnight at 37°C as previously described (Delehaye-Zervas *et al.*, 1994). Briefly, slides were incubated in hybridization buffer [1 ml of hybridization buffer contained 50% deionized formamide, 10% Dextran sulfate, 4 × standard saline citrate (1 × SSC: 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 1 × Denhardt's solution, (50 × = 1% bovine serum albumin, 1% Ficoll 400 and 1% polyvinylpyrrolidone), 250 μg/ml yeast tRNA, and 10^6 c.p.m. of labeled denatured cDNA probe corresponding to saturating concentration]. Sections were washed sequentially in 2 × SSC for 1 h at room temperature, 2 × SSC for 1 h at 50°C [as we reported for the liver (Delehaye-Zervas *et al.*, 1994), we found this temperature to preserve optimal conditions of hybridization and ensure elimination of non specific probe binding on muscle sections], 1 × SSC and 0.5 × SSC, for 30 min each at room temperature. After quick ethanol dehydration and air drying hybridized probes were detected by autoradiography. To prepare for macroautoradiography the slides with the tissue sections, along with slides containing calibrated tritium-labeled standards (autoradiographic [^{3}H]micro-scales, Amersham), were apposed onto autoradiographic films (Hyperfilm [^{3}H], Amersham) for 2–3 days at room temperature. The films were developed in D19 (Kodak, Paris, France). For microautoradiographical purposes the slides were dipped in NTB2 nuclear emulsion (Kodak) and exposed at 4°C for 10–30 days, then developed in D19 and stained with 1% Toluidine blue.

In situ hybridization controls

Controls on the specificity of the ISH reaction were performed on sections following those which gave a positive ISH signal. They included: (a) omission of the probe, (b) hy-

bridization with an excess of unlabeled probe in a ratio of 100:1, (c) hybridization with labeled heterologous probes specific for hGH or hPRL in the same conditions as for the labeled probe, (d) hybridization with an excess of unlabeled heterologous probe for hGH or hPRL, in a ratio of 100:1, using the same buffer conditions as for the experimental probes, (e) the restricted pattern of localization of the ISH signal in some areas and its absence in others (e.g. epidermis *vs* dermis).

Semi-quantification of in situ *hybridization*

The analysis of ISH signals on film was performed by computer-assisted microdensitometry as described (Delehaye-Zervas *et al.*, 1994) and permits a semi-quantitative estimation of gene expression in each tissue prepared under the same conditions. Optical densities of homogeneous areas were measured on six autoradiograms of each tissue, under constant parameters, and artifacts were avoided. A linear relationship (standard curve), with a slope depending on exposure time, was found by single regression between optical density values of the standards and their corresponding radioactivity. Substitution of optical density values of each tissue, into the standard curve equation permits comparison of results obtained on different autoradiographic films. The different tissue radioactivity values, expressed in arbitrary units, are mean ± SEM. Statistical analysis was performed using one-way analysis of variance, and Student's *t*-test. $P < 0.05$ was considered significant. Non-specific signal was found to represent 5% of the value of positive signal, and was subtracted from each optical density analysis.

Northern-blot analysis

Tissues samples were immediately frozen in liquid nitrogen and stored at − 80°C until used. Total RNA were extracted according to (Chomczynski *et al.*, 1987). Polyadenylated RNAs were purified on oligo (dT) cellulose affinity columns (Pharmacia Biotech). RNA concentration was quantified by absorbance at 260 nm. 10 µg of polyadenylated mRNA were electrophoresed through a 1% agarose/2.2 M formaldehyde gel and transferred to a GeneScreen$^+$ membrane (Du Pont de Nemours, Biotechnology Systems Division, les Ulis, France) by capillary blotting under ionic strength in 20 × SSC. The membrane was baked at 80°C for 2 h. Hybridization was performed at 42°C in 50% formamide buffer according to manufacturer's instructions. Washes were carried out at 55°C once in 1 × SSC, SDS 0.1% and then in 0.1 × SSC, 0.1% SDS. Autoradiography was performed at − 80°C for 5 days with intensifying screens using hyperfilm-MP (Amersham, France).

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