Endocrine, *vol. 12, no. 3, 265–271, June 2000* 0969–711X/00/12:265–271/$11.75

Increased Expression of Growth Hormone and Prolactin Receptors in Hepatocellular Carcinomas

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The liver is an essential target tissue for growth hormone (GH) and prolactin (PRL). The aim of this study was to determine the *in situ* expression of growth hormone receptor (GHR) and prolactin receptor (PRLR) in hepatocellular carcinomas and to compare the results with normal liver. For this purpose, *in situ* hybridization (ISH) and immunohistochemical techniques were performed and several tests were conducted to validate the results. By radioactive ISH, all the hepatocellular carcinomas studied showed labeling for GHR and PRLR mRNAs. Relative expression levels, determined by computer-assisted microdensity, were higher in hepatocellular carcinomas than in normal liver. Immunohistochemistry led us to confirm the constant expression of both receptor proteins in hepatocellular carcinomas and normal liver and to demonstrate their localization not only in the cytoplasm but also in the nucleus. These results confirm that the liver is a major GH and PRL target tissue and suggest that in hepatocellular carcinomas the proliferative effects of these hormones may be increased by a higher expression of their receptors.

Key Words: *In situ* hybridization; immunohistochemistry; mRNA expression; hepatocyte; liver.

Introduction

Growth hormone receptor (GHR) and prolactin receptor (PRLR) were first identified and characterized in the liver *(1,2)*. Both receptors show significant homology, share a common ancestral gene, and belong to the class 1 of the GH/PRL/cytokine receptor family or hematopoietin receptor superfamily *(3–5)*. GHR and PRLR were shown in many tissue types *(4,6–10)*, and in recent years multiple factors have been described that regulate their expression *(11,12)*. Hepatic actions of GH constitute the greatest number of any tissue (>120). In the same way, PRL actions are described in liver *(13)*. These two hormones have a growth factor role, by acting directly or through growth factor synthesis. GHR and PRLR, after ligand-induced dimerization, trigger a cascade of intracellular events leading to cell proliferation *(14,15)*. It has been reported that hepatoma cells express PRLR *(16)*. However, controversial results exist about the expression of GHR in hepatocellular carcinomas *(17–20)*. The aim of the present study was to determine whether these receptors show a differential expression in hepatocellular carcinomas vs normal liver. For this purpose we used two complementary methods: *in situ* hybridization (ISH) and immunohistochemistry. The basal expression of these receptors in the normal tissue led us to use these morphological methods to distinguish between normal and carcinoma cell expressions of mRNA and protein.

Received December 1, 1999; Revised January 18, 2000; Accepted January 19, 2000.

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Results

In Situ Hybridization

In normal human liver, a homogeneous expression of hGHR and hPRLR mRNAs was found on macroautoradiograms (Fig. 1A,B). No signal was observed with sense probes (Fig. 1C,D). The microscopic study showed abundant silver grains in the cytoplasm of hepatocytes (Fig. 1 E). No labeling was detected when the sense probes were employed (Fig. 1F). In hepatocellular carcinomas, every sample tested was positive for the presence of hGHR and hPRLR mRNA. The signal obtained on macroautoradiograms was heterogeneous

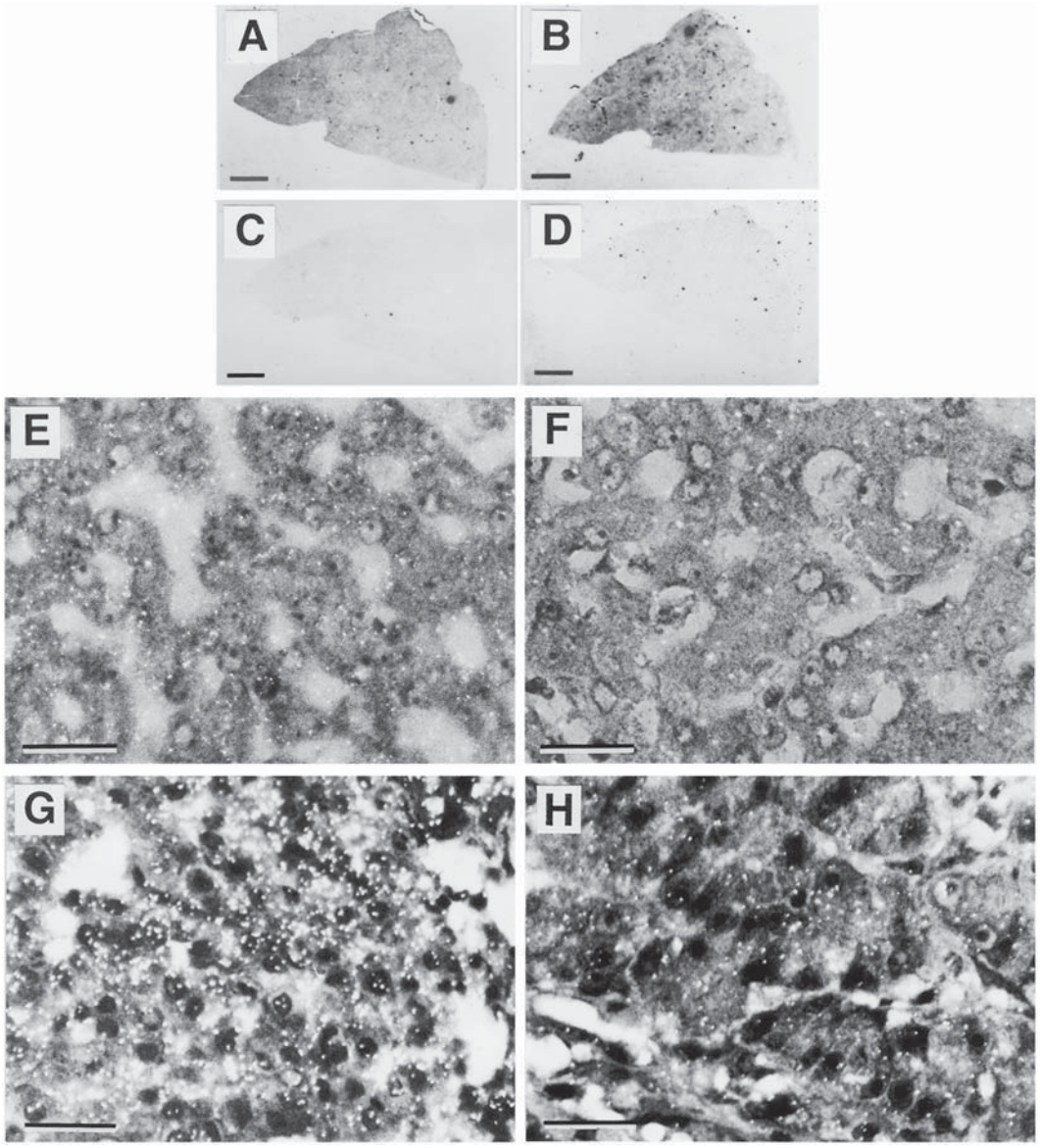

Fig. 1. Expression of **(A–F)** hGHR and hPRLR mRNA in normal liver tissue and **(G, H)** hepatocellular carcinomas. Macroautoradiograms from adjacent liver sections showed specific signals for hGHR mRNA in (A) and hPRLR mRNA in (B), whereas liver sections hybridized under the same experimental conditions with hGHR sense probe and an excess of unlabeled hPRLR probe showed no significant labeling in (C) and (D), respectively. Microscopic analysis showed epipolarized silver grains for hGHR mRNA (E) and hPRLR mRNA (F) over all normal hepatocytes. In (G) and (H), microscopic analysis showed a high density of signal over hepatocellular carcinoma cells for hGHR mRNA (G) and hPRLR mRNA (H) (A–D: ×2, bars: 0.3 cm; E–H ×300, bars: 50 μm).

(Fig. 1G, H, insets), and a diffuse cytoplasmic labeling was demonstrated in the microscopic observation over the liver carcinoma cells (Fig. 1G,H).

Semiquantitative Analysis of mRNAs

The measurement of optical density (OD) on macroautoradiograms allowed us to estimate the relative expression of the level of hGHR and hPRLR genes. The hGHR and hPRLR mRNA were significantly increased in hepatocellular carcinomas in comparison to normal liver. The results obtained for hGHR mRNA were 30.26 ± 2.06 (arbitrary units) in hepatocellular carcinomas and 17.85 ± 1.01 in normal liver ($p < 0.000263$), and for hPRLR mRNA, 31.01 ± 1.41 and 17.93 ± 1.09, respectively ($p < 0.000006$) (Fig. 2A,B).

Immunohistochemistry

The results obtained by immunohistochemical techniques were in good agreement with those obtained by ISH. Normal liver showed a homogeneous positivity for GHR and PRLR throughout the hepatic lobule. Both the cytoplasm and nucleus of hepatocytes were immunostained (Fig. 3A,C). No immunoreactivity was found when the primary antibodies were substituted by either unrelated monoclonal antibodies (MAbs) or mouse IgG_1 directed towards substances not present in mammalian tissues (Fig. 3B,D). All the hepatocellular car-

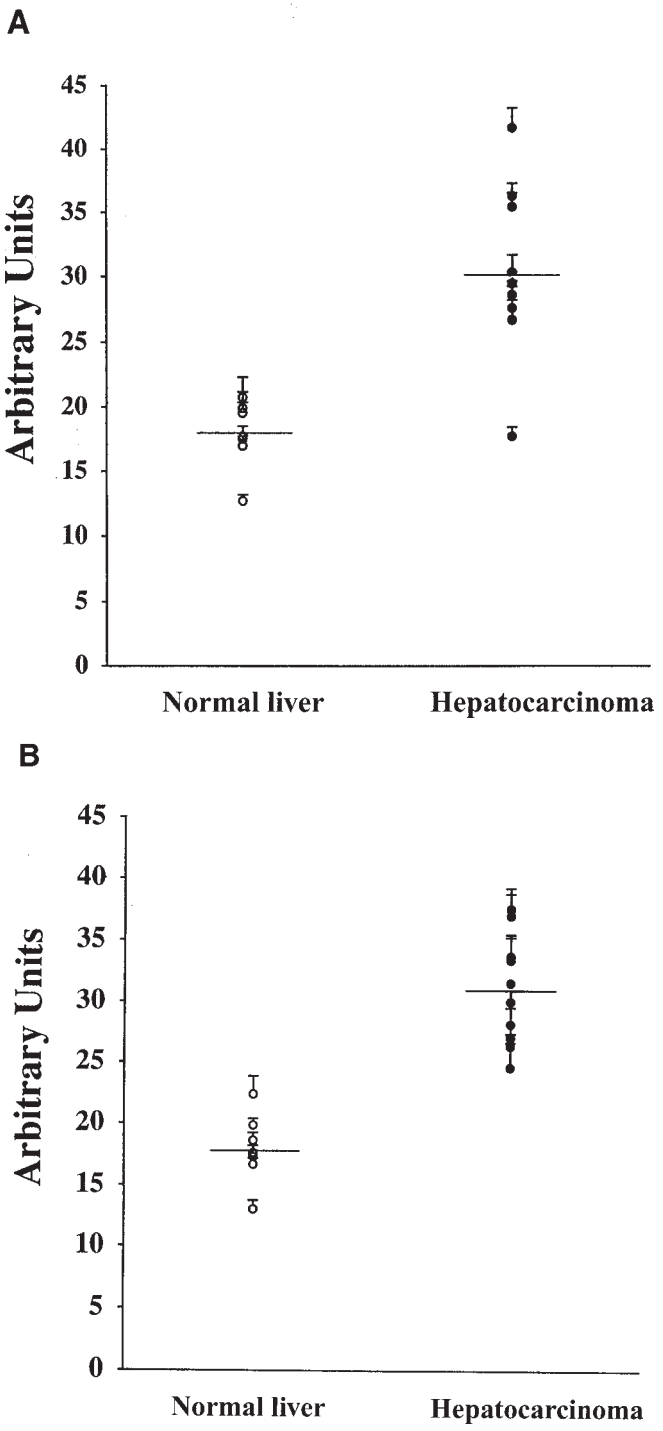

Fig. 2. Evaluation of **(A)** hGHR and **(B)** hPRLR mRNA in normal liver and hepatocellular carcinomas. Signal density was quantified on autoradiographic film and expressed in arbitrary units. The means of the density of signal are about twofold higher in hepatocellular carcinomas than in normal liver.

cinomas studied exhibited a diffuse positivity for GH and PRL receptors. In general, the intensity of immunostaining was higher than in normal cells. The pattern of cellular immunoreactivity was similar to that obtained in normal liver; that is, both the cytoplasm and nucleus were also positive in malignant cells (Fig. 3E,F).

Discussion

This study has demonstrated by two complementary morphological techniques—ISH and immunohistochemistry—the expression of the GHR and PRLR gene and their translation in normal human liver and hepatocellular carcinomas. Controls carried out in our study proved the specificity of the ISH technique for the detection of GHR and PRLR mRNA transcripts and of the immunohistochemical technique for the detection of the proteins themselves such as previously described for GHR and PRLR visualization in normal *(9,21,22)* and pathological human tissues *(10,23)*.

Previous reports have shown the expression of GHR and PRLR in normal liver of rodents *(1,2,7)* and humans *(9,20,22,24)*. In agreement with our results, a similar homogeneous pattern of immunostaining among hepatocytes of different zones of the hepatic lobules (except for some around the central veins) was reported for PRLR in rat liver *(25)*.

Hepatocellular carcinomas are hormone-dependent tumors whose proliferation can be stimulated by GH *(26)* and PRL *(27)*. Moreover, in transgenic mice with the GH gene put under transcriptional control of the mouse metallothionein promoter, hepatocellular neoplasms including both adenoma and carcinoma were frequently found *(28)*. In the same way, rats treated with GH during early phases of promotion of liver regeneration after partial hepatectomy showed overexpression of c-*myc* and c-*fos* and promotion of liver carcinogenesis *(29)*. The expression of PRLR in hepatoma cells was previously demonstrated *(16)*. However, controversial findings on the expression of GHR in hepatocellular carcinomas have been reported with decreasing concentration *(17)* and negative *(18)* or positive results *(19,20)*. In our study, every tumor sample analyzed showed expression of both receptors, indicating that this is a general phenomenon. Semiquantitative analysis of the expression of mRNAs on macroautoradiograms showed that the levels, although variable from case to case, were higher in hepatocellular carcinomas than in normal liver for both GHR and PRLR.

The intracellular localization of GHR and PRLR in both normal liver and hepatocellular carcinomas is not surprising given that the members of the GH/PRL/cytokine receptor family are known to cycle rapidly between the plasma membrane and a large pool of intracellular receptors *(2,30)*. It has been proposed that the GHR and PRLR complexes are rapidly internalized by endocytosis *(31,32)*. In the current study, the receptors for GH and PRL were found not only in the cytoplasm but also in the nuclei. This subcellular distribution is to be expected because previous studies have demonstrated the presence of GHR *(33)* and PRLR *(34,35)* at the nuclear level. Furthermore, the JAK 2 kinase, which mediates the proliferative effects of GH and PRL, is localized in the nucleus *(36)*. Once in the nucleus, GH and PRL can generate signals for gene transcription and cell proliferation *(14,37–39)*.

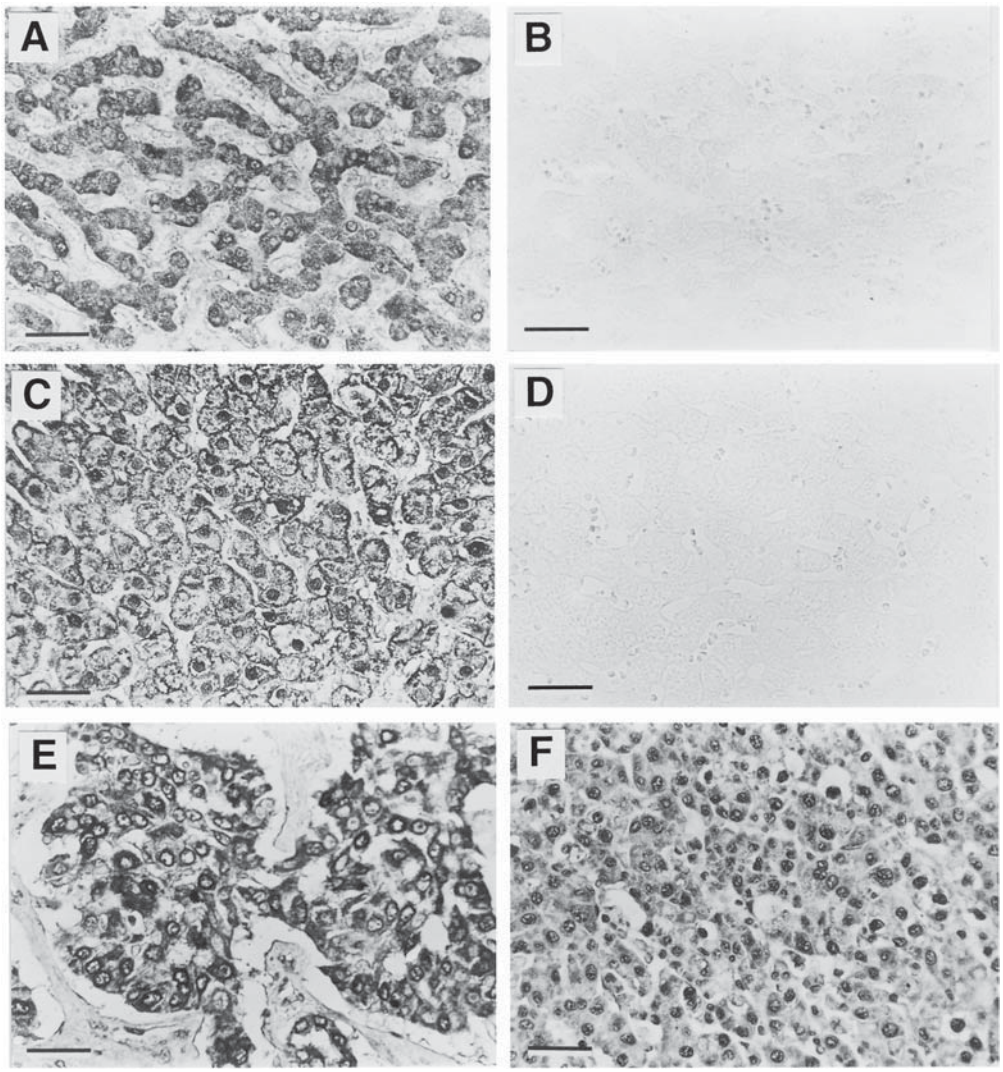

Fig. 3. Immunoreactivity for GHR and PRLR in **(A–D)** normal liver and **(E–F)** hepatocellular carcinomas. Immunoreactivity for GHR in normal liver was demonstrated in all hepatocytes in both cytoplasm and nucleus (A). In the control performed by substitution of the GHR antibody with an unrelated antibody of the same class (ER-PR8), no immunostaining was shown (B). Immunoreactivity for PRLR in normal liver was found in the cytoplasms and nuclei of hepatocytes (C). In the control performed by substitution of the PRLR antibody with a mouse IgG_1 that recognizes an enzyme not present in mammalian tissues, no immunoreactivity was found (D). Immunoreactivity for GHR (E) and PRLR (F) in hepatocellular carcinomas showed a diffuse pattern, with the positivity observed in the cytoplasms and nuclei of tumor cells. No nuclear counterstain was used. (×275, bars: 40 μm).

The demonstration of an increased expression of GHR and PRLR mRNA in hepatocellular carcinomas confirms the role of the liver as the major GH and PRL target tissue, as well as the proliferative effects of these hormones. GH induces insulin-like growth factor-1 (IGF-1), transforming growth factor-β, and DNA synthesis *(40,41)*, and PRL induces IGF-1 and prostaglandin synthesis *(13,42)*. The increased expression of IGF-1 receptors in hepatocellular carcinoma cell lines has been demonstrated *(43)*.

In conclusion, we have shown by immunohistochemistry the presence of GHR and PRLR in normal liver and hepatocellular carcinomas, and by ISH the higher level of their specific mRNAs in hepatocellular carcinomas compared to normal hepatocytes. These findings confirm the direct effect of GH and PRL in normal human liver and suggest that in hepatocellular carcinomas, their actions may be increased by higher expression of their receptors, and lend evidence to the proliferative effect of GH and PRL in tumors. Moreover, the demonstration of an increased expression of GHR and PRLR in hepatocellular carcinomas could raise the possibility of new therapeutic strategies (by use of specific antibodies or antagonists) in this agressive type of tumor.

Materials and Methods

Tissues

Surgical samples were selected from the pathology department files of the Marcel Merieux Institut of Lyon

(France) and the University Hospital of Santiago de Compostela (Spain). The samples included human normal liver from transplantation or autopsies (n=6; 3 men; 3 women; ages 26–87 yr) and hepatocellular carcinomas (n = 20; 13 men; 7 women; ages 34–82 yr). Tissues were immersion fixed in 10% buffered formalin for 24 h, dehydrated, and embedded in paraffin by a standard procedure. Sections 5 μm thick were mounted on sterilized 3-aminopropyl-triethoxysilane-coated slides and deparaffinized before processing for ISH or immunohistochemistry.

Probes and Antibodies

Two 30-mer oligodeoxyribonucleotide probes were synthesized (Molecular Genetic Center, Lyon, France) to detect hGHR mRNAs. The probes were complementary to nucleotides 6–35 localized in the exon 3, and 46–75 in the exon 4, according to the reported hGHR complementary DNA sequence *(44)*. hPRLR mRNA was detected with a oligodeoxyribonucleotide probe complementary to nucleotides 797–827 localized in the intracellular domain of the hPRLR sequence *(16)*. The probes were 3' end labeled with [^{35}S]dATP (Amersham, Les Ulis, France) and purified as previously described *(24)*. The specific activity was 1000 ± 108 Ci/m*M*.

Murine MAb 263 (IgG_1) was raised against the extracellular domain of the GHR *(45)*. It recognizes a cross-species determinant with high affinity and does not crossreact with insulin or PRL receptors. B6.2 anti-PRLR mouse MAb (IgG_1) was prepared against a membrane-enriched fraction of human metastatic breast cancer *(46)*. Both antibodies have been extensively validated for immunohistochemistry in human tissues *(1,21–23,46,47)*.

In Situ *Hybridization*

Deparaffinized sections were digested with 2 μg/mL of proteinase K (Boehringer, Mannheim, Germany) in a Tris (20 mmol/L)-$CaCl_2$ (2 mmol/L) buffer for 30 min at 37°C. The slides were dehydrated in ethanol series and air-dried. Sections were then covered with hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 4X standard saline sodium citrate (SSC) (1X SSC = 0.15 mol/L of NaCl, 0.03 mol/L of sodium citrate, pH 7.0), 1X Denhardt's solution (50X Denhardt's solution = 1% bovine serum albumin (BSA), 1% Ficoll 400, 1% polyvinylpyrolidone), 250 μg/mL of yeast transfer RNA, 10 mmol/L of dithiothreitol, and labeled probe (2.5 pmol/mL of hybridization buffer). ISH was performed overnight at 40°C. Sections were washed sequentially in 2X SSC for 1 h at room temperature, 2X SSC for 1 h at 50°C, and 1X and 0.5X SSC for 30 min each at room temperature. To prepare for macroautoradiography, dehydrated sections were apposed onto autoradiographic films (Hyperfilm [^{3}H]; Amersham) for 2 to 3 d at room temperature. For microautoradiographic purposes, the slides were dipped in NTB2 nuclear emulsion (Kodak, Paris, France), exposed at 4°C for 5–15 d, and then developed in D19 (Kodak) and stained with 1% toluidine blue. Slides were observed under a fluorescent light microscope by epipolarization. Controls on the specificity of the ISH included the following:

1. Omission of the probe.
2. Hybridization with an excess of unlabeled probe at a ratio of 200:1.
3. Hybridization with a labeled oligonucleotidic-sense probe.
4. Hybridization with labeled heterologous 30-mer probes specific for hGH or hPRL and containing the same percentage of G-C bases, in the same conditions as for the homologous probes.
5. Hybridization with an excess of unlabeled heterologous probes for hGH or hPRL, at a ratio of 200:1.

Semiquantification of ISH

A semiquantitative analysis of gene expression was performed as previously described *(7,9,24)* on more than 30 macroautoradiograms from 6 samples of normal liver and 10 hepatocellular carcinomas obtained in similar conditions. Detection of hGHR and hPRLR mRNAs was run through the same hybridization, washing, and detection assays in order to render the signal levels comparable. The levels of mRNA in each sample were determined in at least four separate experiments. Autoradiograms were analyzed under constant parameters, using a densitometric computer imaging system (Cambridge Q570 system analysis, Centre de Quantimétrie, Université Claude Bernard-Lyon I, France). ODs for each sample were measured on six autoradiograms, on homogeneous areas, excluding artifacts, and then averaged. Nonspecific signal in each sample was measured using the sense probe and subtracted from each measure. A linear relationship (standard curve) with a slope depending on exposure time was found by single regression between OD values of the standards and their corresponding radioactivity. It was then possible to compare the different radioactivity values, which were expressed in arbitrary units, by inserting the OD value of each sample into the standard curve equation. The levels of the signals obtained after hybridization were expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance, followed by student's t-test. Differences were considered significant at $p < 0.05$.

Immunohistochemistry

The streptavidin-biotin-peroxidase complex procedure was employed for immunohistochemical techniques. The sections were consecutively incubated as follows:

1. In 0.1% streptavidin (Sigma, St. Louis, MO) and 0.01% d-biotin (Sigma) for 30 min each, to block endogenous biotin.
2. In MAb 263 (at a concentration of 10 μg/mL) or B6.2 (at a dilution of 1:250) for 1 h.
3. In 3% hydrogen peroxide for 10 min (Merck, Darmstadt, Germany) in order to block endogenous peroxidase.

4. In biotinylated goat antibodies to mouse/rabbit Igs (Duet kit; Dakopatts) at a dilution of 1:100 for 30 min.
5. In streptavidin-biotin-peroxidase complex (Duet kit; Dakopatts) prepared according to the manufacturer's protocol provided, for 30 min.
6. In DAB solution prepared by dissolving 1 DAB-buffer tablet (Merck) in 10 mL of distilled water for 10 min.

Between steps, the sections were washed twice for 5 min with phosphate buffered saline (PBS) (0.01 mol/L of phosphate-buffer, pH 7.4, containing 0.15 mol/L of NaCl), and after step 6, with distilled water. All dilutions were made in PBS. This buffer was added with 0.1% BSA (Sigma) for dilution of the primary antibodies (step 2) and with 2% normal goat serum (Dakopatts) for biotinylated antibodies (step 4). No counterstaining was done. Controls for specificity included the following:

1. Substitution of the MAb 263 and B6.2 with culture supernatants containing mouse IgG_1 (Dakopatts) directed toward *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues.
2. Substitution of MAb 263 and B6.2 with unrelated MAbs of the same class (IgG_1): MAb 4.3 (gift from Genentech, South San Francisco, CA) raised against a synthetic hydrophilic carboxy tail of the recombinant rat GHBP; IT-KS 20.3 (1:50) (Progen, Heidelberg, Germany) that recognizes cytokeratin 20; and ER-PR8 (1:5) (Dakopatts) that recognizes prostatic specific antigen.
3. Alternate use of PBS in place of one of the other incubation steps.

Acknowledgments

We wish to thank Dr. M. J. Waters (Department of Physiology and Pharmacology, University of Queensland, Brisbane, Australia) for the generous gift of MAb263 antibody. We also gratefully acknowledge the expert technical assistance of Dolores Fernández-Roel and Angel Vázquez-Boquete and the photographic assistance of Manuel Ríos. This work was supported by the Xunta de Galicia (grants Xuga 20811B97 and PGIDT99Px120806B) and the Association de Recherche contre le Cancer (grant 1209).

References

1. Husman, B., Andersson, G., Norstedt, G., and Gustafsson, J. A. (1985). *Endocrinology* **116,** 2605–2611.
2. Boutin, J. M., Jolicoeur, C., Okamura, H., Gagnon, J., Edery, M., Shirota, M., Banville, D., Dusanter–Fourt, I., Djiane, J., and Kelly, P. A. (1988). *Cell* **53,** 69–77.
3. Bazan, J. F. (1989). *Biochem. Biophys. Res. Commun.* **164,** 788–795.
4. Kelly, P. A., Ali, S., Rozakis, M., Goujon, L., Nagano, M., Pellegrini, I., Gould, D., Djiane, J., Edery, M., Finidori, J., and Postel–Vinay, M. C. (1993). *Recent. Prog. Horm. Res.* **48,** 123–164.
5. Cosman, D. (1993). *Cytokine* **5,** 95–106.
6. Waters, M. J., Barnard, R., Lobie, P. E., Lim, L., Hamlin, G., Spencer, S. A., Hammonds, R. G., Leung, D. W., and Wood, W. I. (1990). *Acta Paediatr. Scand.* **366,** 60–72.
7. Ouhtit, A., Morel, G., and Kelly, P. A. (1993). *Endocrinology* **133,** 135–144.
8. Nagano, M. and Kelly, P.A. (1994). *J. Biol. Chem.* **269,** 13,337–13,345.
9. Mertani, H. C., Delehaye–Zervas, M. C., Martini, J. F., Postel–Vinay, M. C., and Morel, G. (1995). *Endocrine* **3,** 135–142.
10. Mertani, H. C., García–Caballero, T., Lambert, A., Gerard, F., Palayer, C., Boutin, J. M., Vonderhaar, B. K., Waters, M. J., Lobie, P. E., and Morel, G. (1998). *Int. J. Cancer* **79,** 202–211.
11. Hu, Z. Z., Zhuang, L., and Dufau, M. L. (1996). *J. Biol. Chem.* **271,** 10,242–10,246.
12. Schwartzbauer, G. and Menon, R. K. (1998). *Mol. Genet. Metab.* **63,** 243–253.
13. Dave, J. R., Brown, N. V., and Knazek, R. A. (1982). *Biochem. Biophys. Res. Commun.* **108,** 193–199.
14. Lebrun, J. J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. (1994). *J. Biol. Chem.* **269,** 14,021–14,026.
15. Carter–Su, C., Schwartz, J., and Smit, L. S. (1996). *Annu. Rev. Physiol.* **58,** 187–207.
16. Boutin, J. M., Edery, M., Shirota, M., Jolicoeur, C., Lesueur, L., Ali, S., Gould, D., Djiane, J., and Kelly, P. A. (1989). *Mol. Endocrinol.* **3,** 1455–1461.
17. Roitman, A., Lev–Ran, A., Carr, B. I., Hwang, D. L., and Barseghian, G. (1986). *Endocrinology* **118,** 1869–1871.
18. Chang, T.–C., Lin, J.–J., Yu, S.–C., and Chang, T.–J. (1990). *Hepatology* **11,** 123–126.
19. Esposito, N., Paterlini, P., Kelly, P. A., Postel–Vinay, M.–C., and Finidori, J. (1994). *Mol. Cell. Endocrinol.* **103,** 13–20.
20. Zogopoulos, G., Albrecht, S., Pietsch, T., Alpert, L., Vonschweinitz, D., Lefebvre, Y., and Goodyer, C. G. (1996). *Cancer Res.* **56,** 2949–2953.
21. Mertani, H. C., Pechoux, C., García–Caballero, T., Waters, M. J., and Morel, G. (1995). *J. Clin. Endocrinol. Metab.* **80,** 3361–3367.
22. García–Caballero, T., Morel, G., Gallego, R., Fraga, M., Pintos, E., Gago, D., Vonderhaar, B. K., and Beiras, A. (1996). *J. Clin. Endocrinol. Metab.* **81,** 1861–1866.
23. García–Caballero, T., Gallego, R., Fraga, M., and Beiras, A. (1997). In: *Visualization of receptors: methods in light and electron microscopy.* Morel, G. (ed.). CRC Press: Boca Raton, FL.
24. Delehaye–Zervas, M. C., Mertani, H. C., Martini, J. F., Nihoul–Fekete, C., Morel, G., and Postel–Vinay, M. C. (1994). *J. Clin. Endocrinol. Metab.* **78,** 1473–1480.
25. Smirnova, O. V., Petraschuk, O. M., and Kelly, P. A. (1994). *Mol. Cell. Endocrinol.* **105,** 77–81.
26. Gatmaitan, Z., Jefferson, D. M., Ruiz–Opazo, N., Biempica, L., Arias, I. M., Dudas, G., and Leinwand, L. A. (1983). *J. Cell. Biol.* **97,** 1179–1190.
27. Pollack, R., Mishkin, S. Y., Morris, H. P., and Mishkin, S. (1982). *Hepatology* **2,** 836–842.
28. Wolf, E., Kahnt, E., Ehrlein, J., Hermanns, W., Brem, G., and Wanke, R. (1993). *Mech. Ageing Dev.* **68,** 71–87.
29. Hallstrom, I. P., Gustafsson, J. A., and Blanck, A. (1989). *Carcinogenesis* **10,** 2339–2343.
30. Roupas, P. and Herington, A. C. (1989). *Mol. Cell. Endocrinol.* **61,** 1–12.
31. Eshet, R., Peleg, S., and Laron, Z. (1984). *Acta Endocrinol.* **107,** 9–15.
32. Genty, N., Paly, J., Edery, M., Kelly, P. A., Djiane, J., and Salesse, R. (1994). *Mol. Cell. Endocrinol.* **99,** 221–228.
33. Lobie, P. E., Wood, T. J. J., Chen, C. M., Waters, M. J., and Norstedt, G. (1994). *J. Biol. Chem.* **269,** 31,735–31,746.

34. Buckley, A. R., Montgomery, D. W., Hendrix, M. J., Zukoski, C. F., and Putnam, C. W. (1992). *Arch. Biochem. Biophys.* **296,** 198–206.
35. Morel, G., Ouhtit, A., and Kelly, P. A. (1994). *Neuroendocrinology* **59,** 78–84.
36. Lobie, P. E., Ronsin, B., Silvennoinen, O., Haldosen, L.–A., Norstedt, G., and Morel, G. (1996). *Endocrinology* **137,** 4037–4045.
37. Buckley, A. R., Crowe, P. D., and Russell, D. H. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 8649–8653.
38. Yu–Lee, L.–Y. (1990). *Mol. Cell. Endocrinol.* **68,** 21–28.
39. Gronowski, A. M. and Rotwein, P. (1994). *J. Biol. Chem.* **269,** 7874–7878.
40. Strain, A. J., Hill, D. J., Swenne, I., and Milner, R. D. (1987). *J. Cell. Physiol.* **132,** 33–40.
41. Zvibel, I., Halay, E., and Reid, L. M. (1991). *Mol. Cell. Biol.* **11,** 108–116.
42. Francis, M. J. O. and Hill, D. J. (1975). *Nature* **255,** 167–168.
43. Kim, S. O., Park, J. G., and Lee, Y. (1996). *Cancer Res.* **56,** 3831–3836.
44. Godowski, P. J., Leung, D. W., Meacham, L. R., Galgani, J. P., Hellmiss, R., Keret, R., Rotwein, P. S., Parks, J. S., Laron, Z., and Wood, W. I. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 8083–8087.
45. Barnard, R., Bundesen, P. G., Rylatt, D. B., and Waters, M. J. (1985). *Biochem. J.* **231,** 459–468.
46. Banerjee, R., Ginsburg, E., and Vonderhaar, B. K. (1993). *Int. J. Cancer* **55,** 712–721.
47. Werther, G. A., Haynes, K. M., Barnard, R., and Waters, M. J. (1990). *J. Clin. Endocrinol. Metab.* **70,** 1725–1731.