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HGH, PRL and βHCG/βLH gene expression in clinically inactive pituitary adenomas detected by in situ hybridization*

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Summary. Within our surgical collection clinically inactive pituitary adenomas represent 30.7% of all pituitary tumours. To characterize their endocrine activity we studied 40 clinically inactive pituitary adenomas with in situ hybridization (ISH) using cRNA probes labelled with ^{35}S encoding growth hormone (GH), prolactin (PRL) and chorionic gonadotrophin (βHCG). No tumour was associated with clinical evidence of elevated hormone secretion. A mild hyperprolactinaemia not correlated with hormone or the mRNA content of the cells was interpreted to be incidental in 11 patients. By histological analysis, immunohistochemistry (IH) and electron microscopy the adenomas were diagnosed as small cell chromophobic ($n=16$) and large cell chromophobic ($n=8$) adenomas, and oncocytomas ($n=16$). Gene expression of one or more hormones was identified by ISH in 18 of 40 adenomas in few cells. GH and PRL gene expression was rare (GH mRNA in 3 of 40 tumours and PRL mRNA in 8 of 40 tumours) whereas in 14 of 40 adenomas βHCG/βLH gene expression was identified in scattered cells. Five of 40 adenomas lacking hybridization signals revealed hormones by IH. The detection of mRNA was accompanied by positive immunostaining for the respective hormones in 72%. The combination of ISH and IH reveals good evidence that the hormones are synthesized in the tumours and not taken up from the serum and stored in the cells. The two methods used together permit a more precise analysis of tumour biology than each alone.

Key words: Pituitary adenoma – In situ hybridization – Immunohistochemistry

Introduction

Progress in the characterization of pituitary adenomas depends on the development of techniques for defining cell types and hormone production in tumour cells. Besides immunohistochemical methods (IH) demonstrating hormone contents of cells, in situ hybridization (ISH) has now become a very valuable technique for examining gene expression. Positive hybridization signals in addition to detection of the hormone by IH reveal strong evidence that the hormone is synthesized in the cell and not taken up from circulation and stored. Positive hybridization signals without detection of the respective hormone give information on cellular activity at the level of gene transcription.

Clinically inactive pituitary adenomas are tumours unassociated with clinical symptoms due to hypersecretion of adenohypophyseal hormones. In our surgical collection (1983–1987) of 616 pituitary adenomas 30.7% appear to be clinically inactive.

Apart from the pituitary hormones GH (growth hormone) and PRL (prolactin) our main interest concerned the synthesis of glycoprotein hormones, since their overproduction generally produces no clinical signs (Kwekkeboom et al. 1989). The glycoprotein hormones include follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and the placental hormone chorionic gonadotrophin (HCG). They consist of two subunits: the α-subunit, which all gonadotrophins have in common, and the β-subunit (βFSH, βLH, βTSH and βHCG) with biological and immunological specificity (Jameson et al. 1987). Synthesis of the glycoprotein hormones LH, FSH and TSH and the α-subunit in clinically inactive pituitary adenomas has been increasingly reported (Ridgway et al. 1981; Klibanski et al. 1983; Black et al. 1987; Saeger et al. 1991).

We studied the gene expression of GH, PRL and βHCG/βLH in 40 clinically inactive pituitary adenomas by ISH and IH in order to obtain more information about their endocrine activity. We chose βHCG as one of the glycoprotein hormones as its synthesis has not been studied previously in a representative collection of pituitary adenomas. Because of the great nucleic acid sequence homology of βHCG mRNA and βLH mRNA we added immunohistochemical analyses of βHCG and βLH, performed with antibodies of proven specificity.

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Materials and methods

From a collection of pituitary adenomas surgically removed between 1983 and 1987 at the Department of Neurosurgery of the University of Hamburg (FRG) we selected 40 adenomas from patients without symptoms of endocrine hyperfunction. Preoperative measurements of hormone serum levels included PRL and in many cases GH, adrenocorticotropic hormone (ACTH), TSH and the gonadotrophins LH, FSH and TSH.

After surgical removal, parts of the adenoma tissue were fixed in neutral buffered formalin for 3 h, dehydrated, and embedded in paraffin. Sections (4–6 μm) were cut, mounted on 3-aminopropyl-triethoxysilane-coated slides and deparaffinized. They were fixed in paraformaldehyde, rinsed in phosphate buffered saline (PBS), dehydrated and stored at 4° C from 1 day up to 2 months until used for ISH or IH.

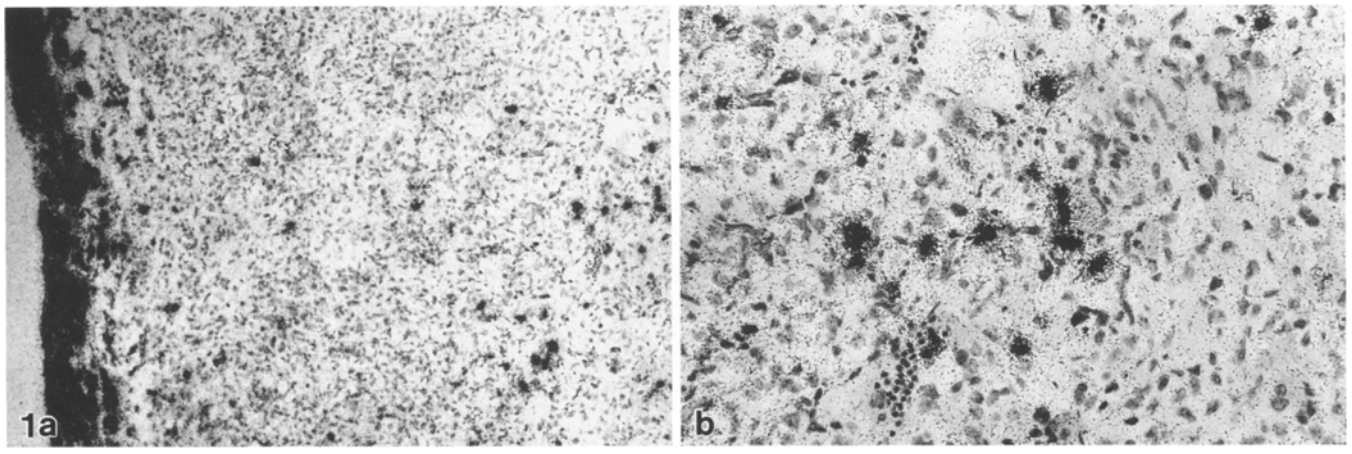
cDNAs coding for GH (316 bp), PRL (248 bp) and βHCG (267 bp) were obtained from ATCC (American Type Culture Collection, Rockville, Maryland, USA), subcloned into pGEM vectors (Uhlig 1991) and labelled with ^{35}S CTP by in vitro transcription in the Sp6 system (Amersham-Buchler, Braunschweig, FRG).

Hybridization was carried out as described (Fehr et al. 1987). Briefly, slides were prehybridized for 3 h at 50° C with 1 ml/slide of the following solution: 5× Denhardts (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5× hybridization salt [0.6 M NaCl, 0.025 M piperazinediethane-sulphonic acid (PIPES), 0.025 M ethylenediaminetetra-acetate (EDTA)], 50% deionized formamide, 0.2% sodiumdodecyl sulphate (SDS), 0.01 M dithiothreitol (DTT), 250 μg/ml yeast tRNA and 250 μg/ml sonicated salmon sperm DNA.

Slides were then hybridized each with 70 μl of the same solution except that 4 ng of probe (ca. $1–2 \times 10^6$ cpm) and 10% dextran sulphate (Sigma, Munich, FRG) were added. The slides were covered with coverslips. After incubation for 16–20 h at 50° C coverslips were removed and the slides were washed twice for 5 min each in 4× SSC (standard sodium citrate) +20 mM β-mercaptoethanol. Another two washes for 5 min each in 4× SSC were followed by incubation with 40 mg/ml RNase A (Boehringer, Ingelheim, FRG) in 0.5 M NaCl, 10 mM Tris pH 7.5, and 1 mM EDTA for 30 min at 37° C. After washing for 30 min at 37° C in the same buffer without RNase A slides were placed twice for 15 min each in 2× SSC at 50° C. After dehydrating through graded alcohols, slides were allowed to air dry and dipped into Kodak NTB-3 Nuclear track emulsion diluted 1:1 with 0.3 M ammonium acetate. After exposure for 5–9 days slides were developed in Kodak D-19, counterstained with Mayer's haemalaun, and mounted with Entellan (Merck, Darmstadt, FRG).

Positive controls were carried out with tissues expressing the respective mRNA (pituitary tissue for GH mRNA and PRL mRNA, human placenta for HCG mRNA). Negative controls included (a) substitution of pituitary tissue by rat tongue sections; (b) hybridization of tumour sections with cRNA transcribed from a clone isolated from von Ebner's glands (pta-2, for further details see Schmale et al. 1990) to check for non-specific affinity of the pituitary adenoma tissue for RNA; and (c) incubation of tissue with RNase A. In none of these controls were signals above background level found.

IH was performed on paraffin sections by the avidin biotin complex technique of Hsu et al. (1981) using IgG fractions of the following polyclonal rabbit antibodies to GH (1:500), PRL (1:300), βHCG [1:200, show slight cross-reaction (about 3%) with luteinizing hormone], and βLH (1:800, with cross-reaction to βHCG in 6.5%), all obtained from Dakopatts (Hamburg, FRG) and α-subunit of the gonadotrophins (Gräßlin/Saeger, Hamburg, FRG; 1:600). Normal pituitaries served as positive controls for the pituitary hormones, human placenta tissue as positive control for βHCG.



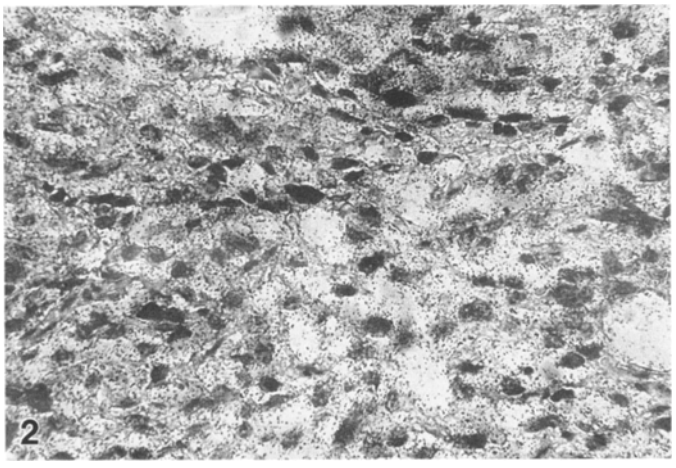


Fig. 1. a Large cell chromophobic adenoma with scattered cells positive for prolactin (PRL) mRNA. On the left adjacent pituitary with many positive cells. Paraffin section, in-situ hybridization (ISH) with ^{35}S-labelled PRL cRNA, autoradiography for 5 days. ×90. **b** Part of same section: some adenoma cells with high intensity of signals and many with low intensity of signals. ×230

Fig. 2. Large cell chromophobic adenoma with some cells with low hybridization signals for βHCG mRNA in single cells and high background. Paraffin section, ISH with ^{35}S-labelled βHCG cRNA, autoradiography for 6 days. ×360

Results

Morphological classification revealed that 40% ($n=16$) of the adenomas were small cell chromophobic adenomas, 20% ($n=8$) were large cell chromophobic adenomas, and 40% ($n=16$) were oncocytic adenomas.

Evaluation of PRL serum hormone levels of the patients showed a mild hyperprolactinaemia (range 15.2–64.0 µg/l, normal <15.0 µg/l) in 11 patients (4 male, 7 female) suffering from one of the three morphological categories of tumour. Despite this finding we regarded these adenomas as clinically inactive; they did not cause clinical symptoms and may have originated from paraadenomatous pituitary. No correlation could be found between PRL serum levels and the detection of PRL gene expression and hormone content in the adenoma cells. Six adenomas of the patients with mild hyperprolactinaemia showed no positive reaction to ISH and IH; 5 adenomas showed hybridization signals and hormone content of PRL in scattered cells (Fig. 1a, b). Within the group of patients without elevated serum hormone levels PRL gene expression (mRNA and/or hormone) was identified in 5 adenomas from all the three classified groups.

No elevation of GH serum levels was detected. GH gene expression was demonstrated in 3 tumours (7.5%) in scattered cells. Positive ISH signals and hormone detection by IH correlated in 1 large cell chromophobic adenoma and in 1 oncocytic adenoma (Table 1).

Beta-HCG/βLH gene expression was found in 14 (35.0%) of the clinically inactive pituitary adenomas: 7 small cell chromophobic adenomas, 3 large cell chromophobic adenomas, and 4 oncocytomas showed signals for βHCG/βLH hybridization. All signals were found in single cells and proved to be less intense than those in placental tissue, which served as positive control (Fig. 2).

IH showed βHCG in 6 of the 14 adenomas with positive ISH for βHCG/βLH (Table 1), which was accompanied by positive immunostaining for βLH and the α-subunit of the gonadotrophins in 2 adenomas in single cells and for βLH in 1 adenoma. Another 3 adenomas with signals for βHCG/βLH mRNA showed βLH content. Four tumours with negative ISH for βHCG/βLH mRNA contained βLH and the α-subunit, and 2 adenomas only showed signals for βLH.

In toto the α-subunit was detected by IH in 11 adenomas. There was no obvious correlation between the signals for the α-subunit and those of βHCG, βLH and the other hormones.

In 17 of the 40 adenomas no signs of endocrine activity could be revealed by ISH or IH.

Signals for all the three hormones under study were detected in 3 adenomas. Five of the adenomas expressed two hormones simultaneously, 2 of them GH and PRL, and the remaining 3 PRL and βHCG/βLH. Fifteen adenomas showed expression of one hormone (in 13 adenomas signals for βHCG/βLH were detected and in 2 adenomas PRL activity by either hormone or hormone and mRNA was revealed) (Table 2). The results of the pituitary adenoma mRNA hybridization and the IH for all 40 patients are summarized in Tables 1–3.

Table 1. Correlation of in situ hybridization (ISH) and immunohistochemistry (IH) in 40 clinically inactive adenomas

Gene expression of	mRNA and hormone detected	Only mRNA signals	Only hormone detection
	(Number of cases)		
GH	2/40	1/40	1/40
PRL	7/40	1/40	2/40
βHCG/βLH	9/40	5/40	6/40

Discussion

Following initial reports of identification of secretory granules in clinically silent and/or chromophobic adenomas by electron microscopy (Saeger et al. 1976), pituitary hormones and uncombined α- and β-subunits of the gonadotrophins except βHCG have been recognized immunohistochemically with increasing frequency in clinically inactive pituitary adenomas (Snyder et al. 1984; Black et al. 1987; Kovacs et al. 1989; Saeger et al. 1990).

We found that 45% ($n=18$) of our 40 clinically inactive adenomas showed signs of hormone synthesis in scattered cells shown by ISH. This was accompanied in part by immunostaining for the respective hormone.

Table 2. Endocrine activity in 40 clinically inactive pituitary adenomas as revealed by ISH and IH for GH, PRL and βHCG/βLH[a]

Adenoma type	No activity	Activity for 1 hormone	Activity for 2 hormones	Activity for 3 hormones
Small cell chromophobic ($n=16$)	3 1 (PRL)	9 (βHCG/βLH) –	2 (PRL+βHCG/βLH)	–
Large cell chromophobic ($n=8$)	4	1 (PRL)	1 (PRL+βHCG/βLH)	2
Oncocytic ($n=16$)	8	6 (βHCG/βLH) 1 (PRL)	1 (GH+PRL)	–

[a] Immunohistochemical findings of βHCG and βLH are subsumed

Table 3. HGH, PRL and βHCG/βLH gene expression, PRL serum level, and immunohistochemistry in 40 clinically inactive pituitary adenomas

Number of cases	Adenoma type	HGH		PRL			βHCG/βLH			
		ISH	IH	ISH	IH	Serum level	ISH	IH βHCG	IH βLH	IH α-subunit
1	Small cell chromophobic	0	0	0	0	n	+	++	0	0
1	Small cell chromophobic	0	0	0	0	n	+	+	0	0
1	Small cell chromophobic	0	0	0	0	n	+	0	++	0
1	Small cell chromophobic	0	0	++	0	n	+	0	0	0
1	Small cell chromophobic	0	0	0	0	n	0	0	0	+
1	Small cell chromophobic	0	0	0	0	n	0	0	0	+++
1	Small cell chromophobic	0	0	0	++	n	0	0	0	+
1	Small cell chromophobic	0	0	++	+++	16.7(f)	+	0	0	0
1	Small cell chromophobic	0	++	++	+	45.4(f)	0	0	0	+
1	Small cell chromophobic	0	0	0	0	30.0(f)	+	0	0	0
1	Small cell chromophobic	0	0	0	0	28.0(f)	+	0	++	0
1	Small cell chromophobic	0	0	0	0	17.4(m)	0	0	++	0
1	Small cell chromophobic	0	0	0	0	n	0	0	+	0
3	Small cell chromophobic	0	0	0	0	n	0	0	0	0
1	Large cell chromophobic	0	0	(+)	++	n	0	0	++	+
1	Large cell chromophobic	0	0	0	0	n	0	0	0	+
1	Large cell chromophobic	0	0	++	++	51.1(f)	+	0	0	+
1	Large cell chromophobic	+	++	++	++	23.3(m)	+	0	0	0
1	Large cell chromophobic	++	0	++	++	21.2(f)	++	+	+	++
3	Large cell chromophobic	0	0	0	0	n	0	0	0	0
1	Oncocytic	+	++	0	+	n	0	0	0	0
1	Oncocytic	0	0	+	++	n	0	0	0	0
1	Oncocytic	0	0	0	0	n	+	+	++	+
1	Oncocytic	0	0	0	0	n	+	+	0	0
1	Oncocytic	0	0	0	0	n	+	0	++	0
1	Oncocytic	0	0	0	0	16.8(m)	0	0	++	+
1	Oncocytic	0	0	0	0	64.0(m)	0	0	++	+
1	Oncocytic	0	0	0	0	40.0(f)	+	++	++	0
7	Oncocytic	0	0	0	0	n	0	0	0	0
Total number of positive cases		3	3	8	9		14	6	12	11

For measurement of HGH and PRL gene expression in situ hybridization (ISH) signal intensity was compared with that obtained in normal pituitary: 0, no signals detected; +, signal intensity less than in normal pituitary and only in scattered cells; ++, signals comparable to that in normal pituitary in scattered cells

βHCG/βLH mRNA hybridization signals were compared to those in placental tissue: 0, no signals detected; +, signals of less intensity than in placental tissue and in scattered cells; ++, signals comparable to those detected in placental tissue in scattered cells

For immunohistochemistry (IH): 0, no immunostaining; +, positive immunostaining of less intensity than in control tissue sections in scattered cells; ++, immunostaining comparable to control sections but in scattered cells; +++, immunostaining comparable to control sections in numerous positive cells

PRL serum level is expressed in μg/l (normal <15 μg/l); n, normal serum level; m, male; f, female

In 5 adenomas (12.5%) IH revealed hormone content in scattered cells whereas the respective mRNA could not be detected. Whether the cell products identified reflect imbalanced biosynthesis resulting in hormones with structural or functional abnormalities is not answered by this study. Positive cases were found in all of the three adenoma types: small and large cell chromophobic and oncocytic adenomas.

The cRNAs used for hybridization to pituitary GH mRNA and PRL mRNA are highly specific (Uhlig 1991). Because βLH mRNA and βHCG mRNA possess extensive structural homology with about 90% nucleic acid homology, one cannot differentiate between hybridization of the labelled βHCG probe with βHCG mRNA and βLH mRNA. Therefore, we subsumed the ISH of βHCG/βLHm RNA. Immunohistochemistry was performed with antibodies showing only slight cross-reaction to other gonadotrophins.

The in situ hybridization technique was found to exhibit lower hybridization signals on formalin-fixed and paraffin-embedded tissue than on frozen, cryostat-cut sections (Bäz 1990). However, hybridization signals were high enough to allow an evaluation, and paraffin sections offer the advantage of detailed histological examination (Lloyd 1988; Saeger et al. 1991).

In our study 3 adenomas showed hybridization with GHm RNA, which was accompanied by hormone immunostaining in 2 cases. We detected PRL mRNA and the hormone prolactin in 7 adenomas and only PRL mRNA in 1 tumour. Simultaneous gene expression of GH and PRL was found in 2 adenomas.

Mild hyperprolactinaemia was observed in 11 of 40

patients ranging up to 64.0 µg/l. In 5 of these cases the ISH and IH was positive in scattered cells indicating PRL hormone synthesis in these cells. As observed by others (Black et al. 1987) slightly elevated PRL serum levels are found in varying numbers of patients without clinical symptoms. It has been suggested that this PRL elevation does not result so much from PRL secretion of the adenoma as from disturbances in the hypothalamus-pituitary axis (for details and review, see Riedel et al. 1986). Horvath et al. (1988) propose that some as yet uncharacterized hormonal product of tumour cells may be capable of releasing PRL from the non-adenomatous pituitary, either directly or through dopaminergic pathways. We found no correlation between the serum PRL levels and the extent of immunoreactivity and mRNA content, and regarded these adenomas as clinically inactive.

Jameson et al. (1987) detected neither PRL mRNA nor GH mRNA by Northern blot analysis in 12 clinically non-functioning pituitary adenomas. But signals of α- and β-subunit mRNA of the glycoprotein hormones were observed in some adenomas. Levy and Lightman (1988) examined the anterior pituitary hormone mRNA species in 16 chromophobic adenomas; they detected GH mRNA in 1 tumour, GH and PRL gene expression in 1 adenoma, and signals of the α-subunit and the LH mRNA in 2 adenomas. Our data concerning GH and PRL gene expression in clinically inactive pituitary adenomas correlate with these reports.

In 14 of 40 clinically inactive adenomas we found βHCG/βLH mRNA in scattered cells accompanied by the finding of βHCG in 6 adenomas (accompanied by βLH in 3 adenomas) and βLH in 3 adenomas. The antibody used to identify βHCG shows slight cross-reaction (3%) to βLH; the βLH antibody cross-reacts with βHCG in about 6%. No immunostaining of βHCG was observed in normal pituitary tissue. The human genome contains seven βHCG genes or pseudogenes in addition to a single copy of βLH gene. Three of the seven HCG genes seem to be expressed in placenta (Talmadge et al. 1984). We do not know whether the βHCG/βLH gene expression detected in the clinically inactive pituitary adenomas is identical with that in placental tissue or is caused by activation of one of the remaining four HCG genes. Jameson et al. (1986) detected βHCG-like mRNA in an α-subunit-secreting pituitary adenoma by northern blot analysis. Despite the close relationship between βLH mRNA and βHCG mRNA they found strong evidence for the identification of βHCG mRNA. A wide variety of tumours are associated with ectopic secretion of HCG (Braunstein et al. 1973). Do our findings suggest a new example of ectopic βHCG production in pituitary adenomas or do they imply the activation of one of the pituitary HCG genes whose expression in normal pituitary lies below level of detection? To answer this question more details of βHCG biosynthesis are required.

Miura et al. (1985) stressed the absence of clinical symptoms caused by gonadotrophin hypersecretion in patients suffering from pituitary adenomas, which leads to the diagnosis of clinically inactive pituitary adenoma, despite endocrine activity. The production of gonadotrophins in clinically inactive pituitary adenomas has been reported by several investigators (Asa et al. 1986; Demura et al. 1986). The significance of α-subunit of the gonadotrophins as a tumour marker for gonadotrophin-producing adenomas has also been discussed (Demura et al. 1986). We believe that in the absence of hypersecretion of other anterior pituitary hormones elevated α- and β-subunits of gonadotrophins including βHCG can probably be used as a further diagnostic approach to clinically inactive adenomas. Further characterization of the glycoprotein hormones, their gene expression and storage patterns should allow new steps in characterizing pituitary adenomas and defining their cytogenesis in order to improve diagnostic and therapeutic approaches.

Hormone synthesis in clinically inactive pituitary adenomas shown with ISH and IH is even higher than that revealed by IH alone. In our study ISH detected gene transcripts in 17.5% of all tissue sections lacking positive immunostaining for the corresponding hormone. One reason for the discrepancy between mRNA and hormone content in tumours could be loss of mRNA during steps of fixation. Absence of detectable hormones may be due to either hormone production below the level of detection (Tramu et al. 1978; Mashiter et al. 1981) or may result in defects in hormone stability (Beck-Peccoz et al. 1989). Changes in hormone storage and secretion patterns in the tumour cells can explain negative immunostaining (Horvath and Kovacs 1974) as does the thesis of post-translational catabolism of peptide hormones (Kovacs et al. 1978). Tumours also may produce uncombined subunits that are clinically inactive (Snyder et al. 1984). To stress the failure of histological, immunohistochemical and ultrastructural markers to give definitive data on cell origin, Kovacs et al. (1980) applied the term "null cell" adenomas. They discussed the thesis of differentiation of null cells into hormone-producing cells and the possibility that null cells are not yet dedifferentiated and still have the ability to synthesize immunoreactive markers. Alternatively, because of the high incidence of gonadotrophin immunostaining in clinically inactive pituitary adenomas, Saeger et al. (1990) considered that most of the clinically inactive pituitary adenomas may derive from gonadotroph cells.

We conclude that ISH and IH can be combined to localize both the mRNA and the hormone in the same cell. Together the methods offer the possibility of detailed study of the endocrine activity of clinically inactive pituitary adenoma cells and may help to resolve these issues.

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