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Analysis of Endocrine Active and Clinically Silent Corticotropic Adenomas by *In Situ* Hybridization

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The distribution of pro-opiomelanocortin (POMC) messenger RNA (mRNA) in 7 functional and 17 clinically silent corticotropic adenomas was analyzed by* in situ *hybridization (ISH) with ^{35}S-labeled oligonucleotide probes using formalin-fixed paraffin-embedded tissue sections cut from blocks that were in storage between 1 to 14 years. All 7 functional adenomas and 4 subtype 1 tumors had detectable POMC mRNA, while 3 of 6 subtype 2 and 1 of 7 subtype 3 silent adenomas contained detectable POMC mRNA.* In situ *hybridization analysis with an ^{35}S-labeled β-actin probe showed a positive hybridization signal in 22 of 22 cases, indicating that the absence of detectable POMC mRNA in some adenomas was not due to loss of the mRNAs during processing of the tissues or because of the age of the embedded tissue blocks. Northern hybridization analysis with the oligonucleotide probes in 2 normal pituitaries and an adenoma causing Cushing's disease detected a 1.2-Kb mRNA in all three tissues, indicating that the oligonucleotide probes were very specific. These results indicate that subtype 1 silent adenomas and clinically active adenomas associated with Cushing's disease contain POMC mRNA that is readily detectable by ISH in routinely processed tissue specimens, while only a few of the subtypes 2 and 3 adenomas have POMC mRNA that can be detected in paraffin blocks with the oligonucleotide probes used in this study. (Am J Pathol 1990, 137:479–488)

Cushing's disease may be caused by pituitary corticotropic adenomas[1–3] as well as by other conditions, including idiopathic pituitary ACTH cell hyperplasia[4,5] and ectopic production of corticotropin-releasing hormones (CRH) from gangliocytic hamartomas[6] and other endocrine tumors,[7,8] leading to ACTH cell hyperplasia. In all of these conditions, ACTH can be detected by immunostaining the pituitary, which correlates with excess ACTH secretion.

Recent immunohistochemical and ultrastructural studies have shown that some pituitary adenomas that contain immunoreactive ACTH or related pro-opiomelanocortin (POMC) cleavage products are not associated with Cushing's disease.[9–12] Many of these neoplasms have ultrastructural features that are similar to functional corticotropic adenomas found in patients with Cushing's disease. In a few reported cases, Cushing's disease has developed from pituitary adenomas that were previously silent that have become endocrine-active adenomas.[13–15]

The endocrine inactive or silent corticotropic adenomas have been divided into subtypes based on morphologic, ultrastructural, and immunochemical findings.[9–12] Subtype 1 adenomas are morphologically and ultrastructurally similar to adenomas associated with Cushing's disease and Nelson's syndrome, but ACTH or cortisol excess does not occur in patients with these adenomas. Subtype 2 tumors have some features of functional corticotropic adenomas, except that ultrastructurally the size of the secretory granules are smaller and type 1 microfilaments are not present in these neoplasms. Subtype 3 silent corticotropic adenomas are a more varied group and other pituitary hormones, in addition to ACTH, may be associated with these neoplasms, but the ultrastructural features are distinct and include large nucleoli and multiple spheridia or spherical nuclear inclusions.[12]

Various hybridization studies, including Northern and *in situ* hybridization (ISH) have been used to characterize POMC messenger RNA (mRNA) in the pituitary gland and from ectopic sources.[16–20] *In situ* hybridization has been used to analyze gene-product expression in the pituitary gland and in other tissues in recent years.[21–24] This technique allows for the visualization of the mRNA in specific cell types within heterogeneous tissues. We undertook

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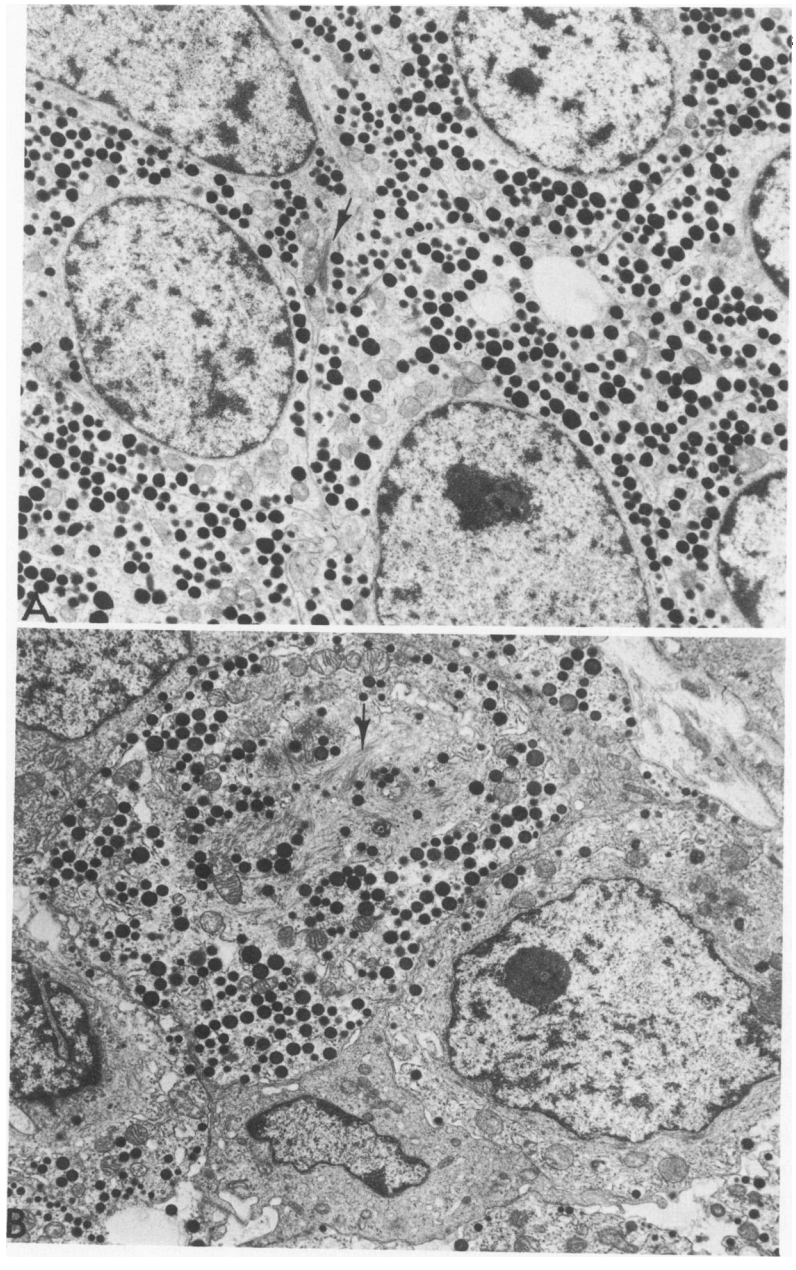

Figure 1. *Ultrastructural features of endocrine-active and silent corticotropic adenomas.* **(A)** *Adenoma from a patient with Cushing's disease showing many secretory granules ranging from 150 to 400 nm in diameter and bundles of type 1 microfilaments (arrow) ×6764.* **(B)** *Silent adenoma of subtype 1 with prominent secretory granules 150 to 400 nm in diameter and prominent type 1 microfilaments (arrow) 6,462.* **(C)** *Silent adenoma of subtype 2 with spherical and tear drop shaped granules measuring 100 to 300 nm. No microfilaments are present (×7143).* **(D)** *Silent adenoma of subtype 3 with smaller secretory granules 50 to 200 nm and large amounts of rough endoplasmic reticulum and intranuclear spheridia (arrow) (×6803).*

this study to investigate if the POMC gene was expressed in both functional and silent corticotropic adenomas. Because these silent tumors are relatively uncommon, and fresh pituitary tissues were not available, a retrospective analysis of tumors from formalin-fixed paraffin-embedded tissue sections was done using radioactively labeled oligonucleotide probes.

Materials and Methods

Tissues

Pituitary adenomas from 17 silent and 7 functional corticotropic adenomas, previously characterized by immunochemistry and electron microscopy,[9,10,12] were studied. The adenomas were fixed and embedded between 1975 and 1988, which is indicated by the last two digits in the case numbers in Table 1. In addition, frozen and paraffin sections of two normal pituitaries obtained within 6 hours postmortem and paraffin sections from a surgically excised non-neoplastic pituitary were also analyzed.

Frozen tissue sections of normal pituitaries were covered with optimal cutting temperature (OCT) embedding medium (Miles Laboratory Naperville, IL) and 8-μ-thick sections were cut in a cryostat at −20°C and then mounted on 3-aminopropyltrimethoxysilane–coated slides and then fixed in 4% paraformaldehyde, pH 7.2, for 20 minutes for ISH studies. The slides were then washed in 2× SSC (1× SSC = 0.15 mol/l [molar] NaCl and 0.015

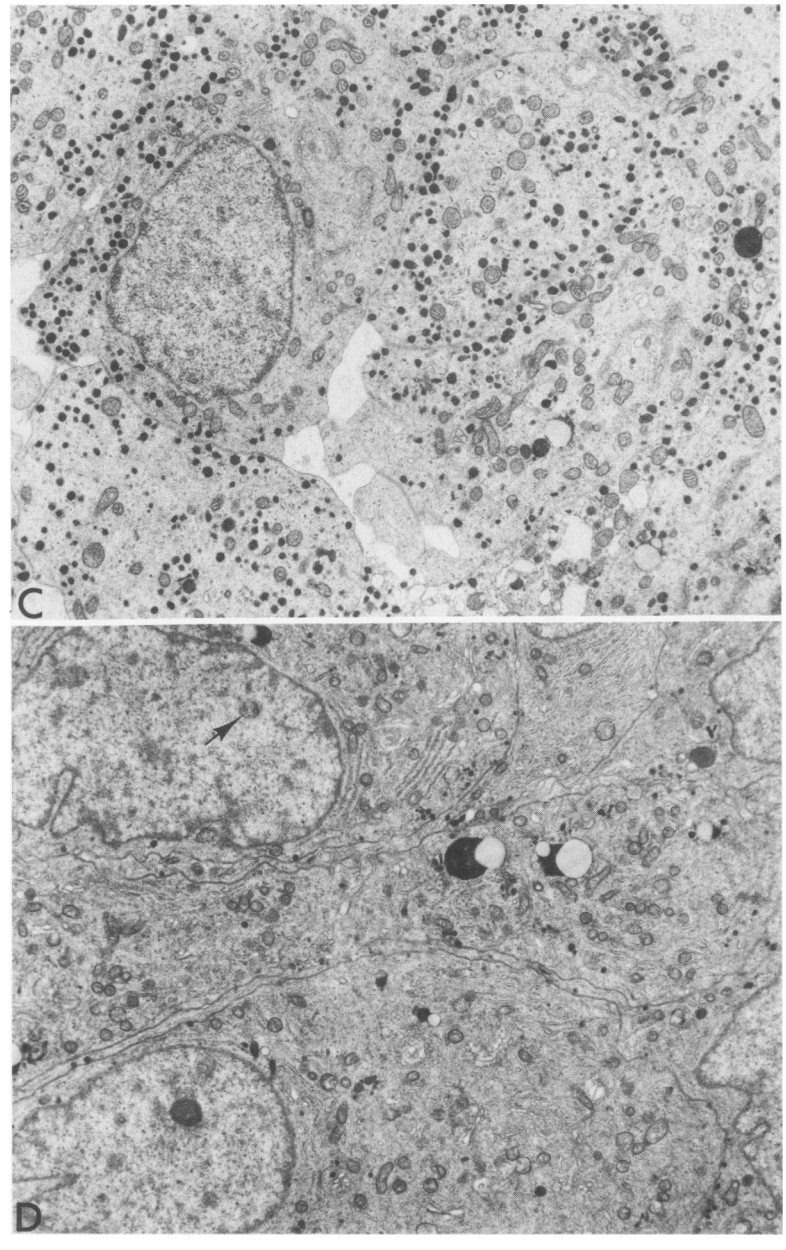

Figure 1 *(Continued)*

mol/l Na citrate), dehydrated in 95 and 100% ethanol, then stored at −70°C for up to 1 month before use. Paraffin sections were cut from specimens fixed in neutral buffered formalin, pH 7.2. Three- to four-micron sections were cut on 3-aminopropylmethoxysilane–coated slides and heated at 60°C overnight before use. Gloves were used to handle all tissue sections and RNAse-free solutions (water treated with 0.1% diethylpyrocarbonate and autoclaved) were used routinely.

Probes

The oligonucleotide POMC probes used were 5′-d [GCC CAC CGG CTT GCC CCA GCG]-3′ for probe 1 and 5′-d [CTT GCC CCA GCG GAA GTG CTC]-3′ for probe 2. Oligonucleotide probes for hPRL and hGH were used as previously described.[25] These were synthesized by the solid-phase beta cyanoethyl phosphoramidite method[26] on an automated DNA synthesizer (Gene Assembler) by Pharmacia (Piscataway, NJ). The probes were purified by gel electrophoresis on 20% polyacrylamide gels. The oligonucleotides were complementary to the region of POMC 5–11 (probe 1) and 8–14 (probe 2) coding for these residues.[27] The other probes had the following sequences: hPRL 5′ [GGC TTG CTC CTT GTC TTC GGG] - 3′ and hGH 5′-d[GGC GCG GAG CAT AGC GTT GTC]-3′ complementary to the regions of hPRL 66–72[28] or hGH 11–17[29] coding for these residues. An oligonucleotide probe for beta actin 5′-d[GGC TGG GGT GTT GAA GGT

Table 1. *Immunochemical and* In Situ *Hybridization Data in Patients with Silent and Functional Corticotropic Adenomas*

Case	Dx	Age/Sex	Immunochemistry	*In situ* hybridization for POMC	
				Probe 1	Probe 2
1 (237-85)	SA-1	48/F	ACTH	+	+
2 (156-80)	SA-1	39/F	ACTH	+	+
3 (24-87)	SA-1	35/F	ACTH	+	+
4 (1-75)	SA-1	44/M	ACTH, β-End, β-LPH	+	+
5 (266-84)	SA-2	60/F	ACTH	0	0
6 (108-84)	SA-2	20/M	ACTH	+	+
7 (51-77)	SA-2	53/M	ACTH, β-LPH	+	+
8 (15-79)	SA-2	60/M	ACTH	0	0
9 (114-80)	SA-2	46/M	ACTH, β-End, α-End	0	0
10 (205-82)	SA-2	28/M	ACTH, β-End	+	+
11 (140-77)	SA-3	28/F	ACTH, β-LPH, α-End	0	0
12 (85-80)	SA-3	17/F	ACTH, α, β-End, αSU, TSH	+	+
13 (37-85)	SA-3	34/M	GH, TSH, FSH, αSU, PRL	0	0
14 (8-88)	SA-3	60/F	TSH, GH	0	0
15 (131-79)	SA-3	27/F	α, β-End	0	0
16 (7-79)	SA-3	30/F	Negative	0	0
17 (106-86)	SA-3	58/M	ACTH	0	0
18 (21-75)	FA-N	30/F	ACTH	+	+
19 (70-84)	FA	46/F	ACTH	+	+
20 (204-88)	FA	32/F	ACTH	+	+
21 (182-88)	FA	36/F	ACTH	+	+
22 (17-88)	FA	48/M	ACTH	+	+
23 (16-88)	FA	47/F	ACTH	+	+
24 (13-88)	FA	54/M	ACTH	+	+

Dx, diagnosis.
SA, silent corticotropic adenoma, subtypes 1, 2, and 3.
FA, functional corticotropic adenoma associated with Cushing's disease.
N, Nelson's syndrome.
0, negative; +, positive hybridization signal with more than six silver grains per cell.
Other abbreviations include ACTH, adrenocorticotropin; β-End, beta endorphin; α-End, alpha endorphin; β-LPH, beta lipotropin; PRL, prolactin; GH, growth hormone; and TSH, thyroid-stimulating hormone.

CTC]-3′ complementary to beta actin 125–131 coding for these residues[30] was used as a positive-control probe.

Labeling

The oligonucleotides were labeled by the 3′ end-labeling method[17,31] using 100 ng of oligonucleotide incubated with 45 μCi of [^{35}S]dATP (1300 to 1500 Ci/mmol/l), 2 mmol/l (millimolar) $CoCl_2$, 0.5 mmol/l dithiothreitol, and 20 units of terminal deoxynucleotidyl transferase in 100 mmol/l potassium cacodylate (pH 7.2) for 45 minutes at 37°C. Probes were purified as previously described.[23]

Northern Hybridization

Total cellular RNA was glyoxylated,[32] electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters.[33] Prehybridization was done for 1.5 hours in Denhardt's solution with 0.1% SDS and 100 μg/ml denatured salmon sperm DNA. The blots were hybridized for 16 hours in the same solution containing 10% dextran sulfate and 1×10^6 cpm/ml of ^{32}P-labeled probes. Filters were subsequently washed twice for 30 minutes in 6xSSC-1% SDS at 42°C and then for 1 hour at 55°C in 6xSSC-0.1% SDS. They were air dried and exposed to a Kodak X-OMAT film (Eastman Kodak, Rochester, NY) at −70°C for 1 to 3 days.

In Situ *Hybridization*

In situ hybridization was performed, as previously described,[23–25] using 1×10^6 cpm of probe for frozen and paraffin sections for POMC probes, 5×10^5 cpm of hGH and hPRL probes and 1×10^6 cpm of the β-actin probe. Slides were incubated in 100% and 95% ethanol for 5 minutes each, washed in water at room temperature, and the paraffin sections were treated with 0.2 N HCl for 20 minutes. After this the slides were treated with 0.25% acetic anhydride in triethanolamine (vol/vol) for 10 minutes, followed by incubation in 2× SSC for 30 minutes at 70°C and washing in 2× SSC at room temperature for 5 minutes. The slides were covered with 40 μl of prehybridization buffer (2× SSC containing Denhardt's solution (0.20% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpryrrolidine) and 10% dextran sulfate, 1 mg/ml yeast tRNA, 100 μ/ml of sonicated salmon sperm DNA, and

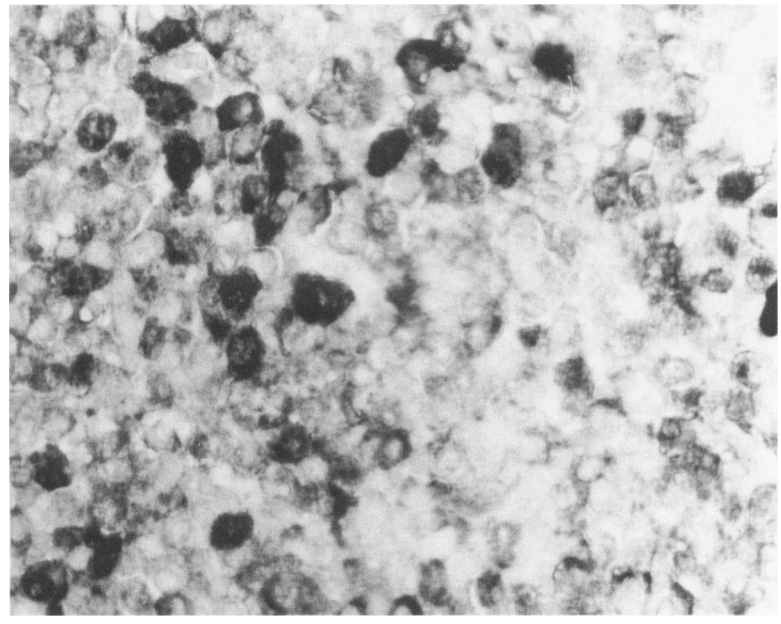

Figure 2. *Immunochemical staining of a subtype 1 adenoma showing diffuse positive immunoreactivity for ACTH (×450).*

50% formamide in 20 mmol/l sodium phosphate, pH 7.2) for 1 hour at room temperature. The labeled probes were then diluted in hybridization buffer containing 20 mmol/l dithiothreitol. They were covered with coverslips treated with Sigmacote (Sigma Chemical Co., St. Louis, MO) and incubated overnight at 44°C in moist chambers. After 18 hours, the coverslips were gently removed in 2× SSC and the slides were washed at room temperature in 2× SSC for 2 hours then in 1× SSC for 1 hour and 0.5× SSC for 1 hour. A subsequent wash in 0.5× SSC for 30 minutes at 44°C was followed by a 30-minute wash at room temperate in 0.5× SSC. The slides were then dehydrated in 95% and 100% ethanol with 0.3 mol/l ammonium acetate, and air dried. When combined ISH and immunohistochemistry procedures were done, the immunochemistry was carried out after 2× SSC washings, as described previously using ACTH antiserum at 1:1000 (Dako, Santa Barbara, CA) with the avidin biotin peroxidase system.[23] Autoradiographic detection of the hybrids was carried out by dipping in Kodak NTB2 emulsion diluted 1:1 with distilled water containing 0.3 mol/l ammonium acetate for 3 seconds. The slides were allowed to air dry for 1 hour and then placed in 4°C desiccated chamber for 1 week. They were subsequently developed for 3 minutes in Kodak D-19 developer, washed in water for 1 minute, and fixed for 5 minutes in Kodak Fixer. After washing for 1 hour, they were stained with hematoxylin and eosin (H&E) and coverslipped. Slides used for combined ISH and ICC were counterstained only with hematoxylin. Controls for ISH consisted of 1) prior digestion of pituitary tissues with 100 μg/ml of RNAse A (Sigma) at 37°C for 45 minutes before ISH, 2) competition studies with a 100-fold excess of unlabeled probes to check specificity and each probe, 3) substituting human liver for the pituitary tissue in hybridization studies, and 4) immunohistochemical localization of ACTH in the same tumors. Immunohistochemical staining for ACTH was done on adjacent sections of tissues used for ISH analysis in all cases. All cases, including the posi-

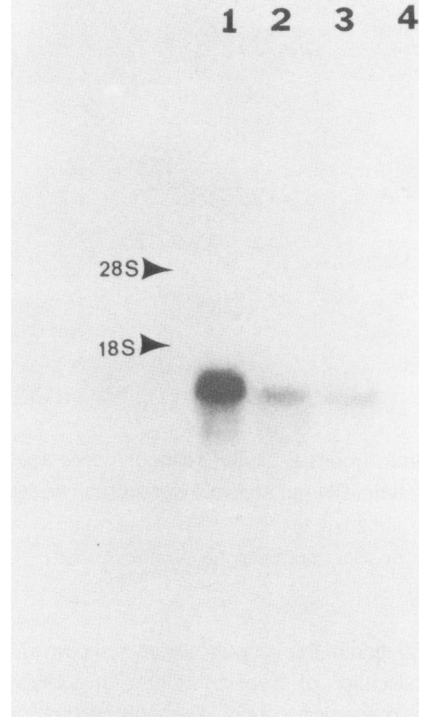

Figure 3. *Northern blot hybridization of total RNA from the pituitary tumor of a patient with Cushing's disease (lane 1), two normal pituitaries obtained at autopsy (lanes 2 and 3), and human liver RNA (lane 4) with a ^{32}P-labeled oligonucleotide POMC probe 1. A single band of mRNA at 1.2 Kb is present. After autoradiography, the blot was washed, then hybridized with POMC probe 2, and the identical distribution of mRNAs was present. Twenty micrograms of total RNA is present in each lane.*

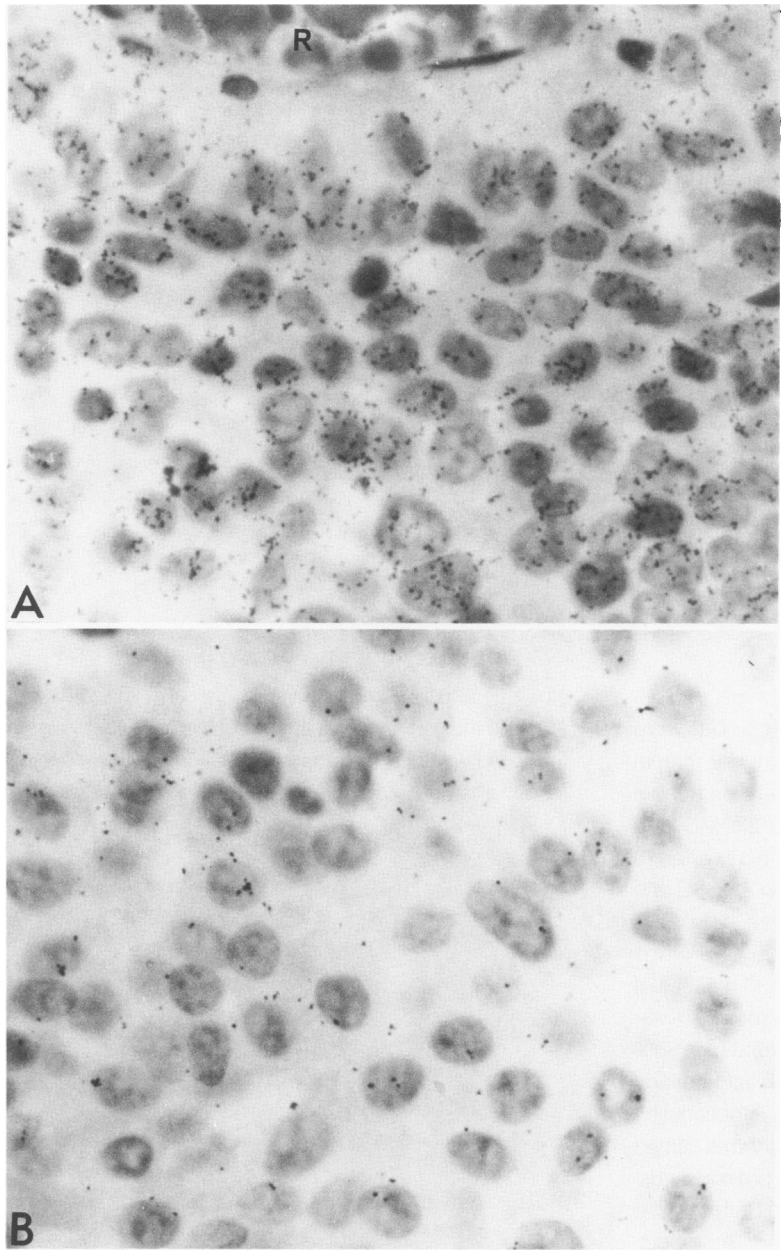

Figure 4. **A:** *In situ hybridization detecting mRNA in a silent adenoma of subtype 1 showing diffuse hybridization signal indicated by the black silver grains in formalin-fixed paraffin-embedded tissue sections using POMC 2 probe. The red blood cells (R) do not show any silver grains (×710).* **B:** *Pretreatment with ribonuclease A reduced the hybridization signal in this adenoma (×710).*

tive control of normal pituitary tissues, were analyzed at the same time. RNAse controls were run on all samples.

Results

The distribution of the various tumors is shown in Table 1. The classification of silent adenomas of subtypes 1, 2, and 3 was made using previously published immunohistochemical and ultrastructural features[10,12] (Figs. 1 and 2).

Northern hybridization studies using RNA from a functional pituitary adenoma from a patient with Cushing's disease and from two normal pituitaries obtained at autopsy showed a band of mRNA of 1.2 Kb for both probes 1 and 2 (Fig. 3).

Labeling of the adenomas with both probes provided similar signals, except that probe 1 appeared slightly more sensitive relative to the intensity of the signal as judged by the number of silver grains per cell. Positive cells had more than six silver grains per cell, while the negative controls had less than three grains per cell. These results could not be quantified because of the variable fixation and processing times as well as the different ages of the paraffin blocks. The numbers of tumors with positive hybridization signal were 4 of 4 for subtype 1, 3 of 6 for subtype 2 and 1 of 7 for subtype 3 (Fig. 4A). Pretreatment of adenomas with RNAse before hybridization diminished the hybridization signal (Fig. 4B). All seven of the functional corticotrophic adenomas had positive hybridization signals (Fig. 5A), including the tumor from a

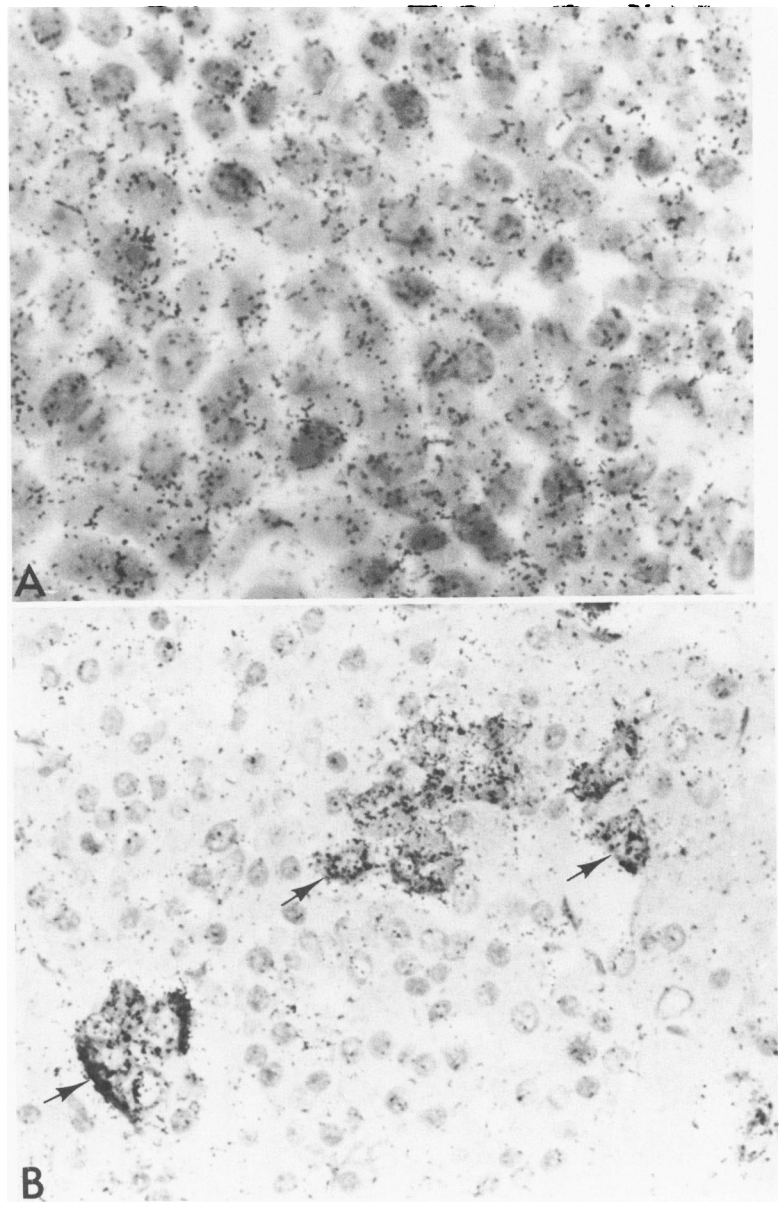

Figure 5. **A:** In situ *hybridization of an adenoma from a patient with Cushing's disease showing abundant message for POMC mRNA (×710).* **B:** *Combined* in situ *hybridization and immunohistochemistry in formalin-fixed paraffin-embedded section of normal pituitary tissue. The POMC 1 probe hybridizes with the ACTH-producing cells. (arrows) (×300).*

patient with Nelson's syndrome (case 18). In general, the intensity of silver grains in the functional tumors and the tumors from subtype 1 adenomas were strong, while a weaker hybridization signal was seen in the subtype 2 and 3 tumors.

Hybridization with the hPRL probe revealed a positive signal in 1 case of a subtype 3 tumor (case 14). A positive hybridization signal for hGH mRNA was not detected in any tumors. Hybridization with the beta actin probe revealed a positive signal in 22 of 22 cases examined (Fig. 6). Sufficient tissues from cases 1 and 20 were not available for hybridization with the beta actin probe.

In situ hybridization studies with normal pituitary tissues revealed an intense hybridization signal in 10% to 20% of the anterior pituitary cells, which is consistent with ACTH-producing cells (Fig. 5B). The hybridization signal was markedly reduced after RNAse treatment and when unlabeled POMC probes were used during the prehybridization step in competition studies before the ^{35}S-labeled probes (data not shown).

Discussion

Analysis of a group of silent and endocrine-active ACTH-producing adenomas in this study revealed that the mRNA for POMC is expressed by tumors from all groups. However the subtype 1 silent adenomas were most similar to the endocrine-active tumor with respect to detectable POMC mRNA and morphologic features at the light

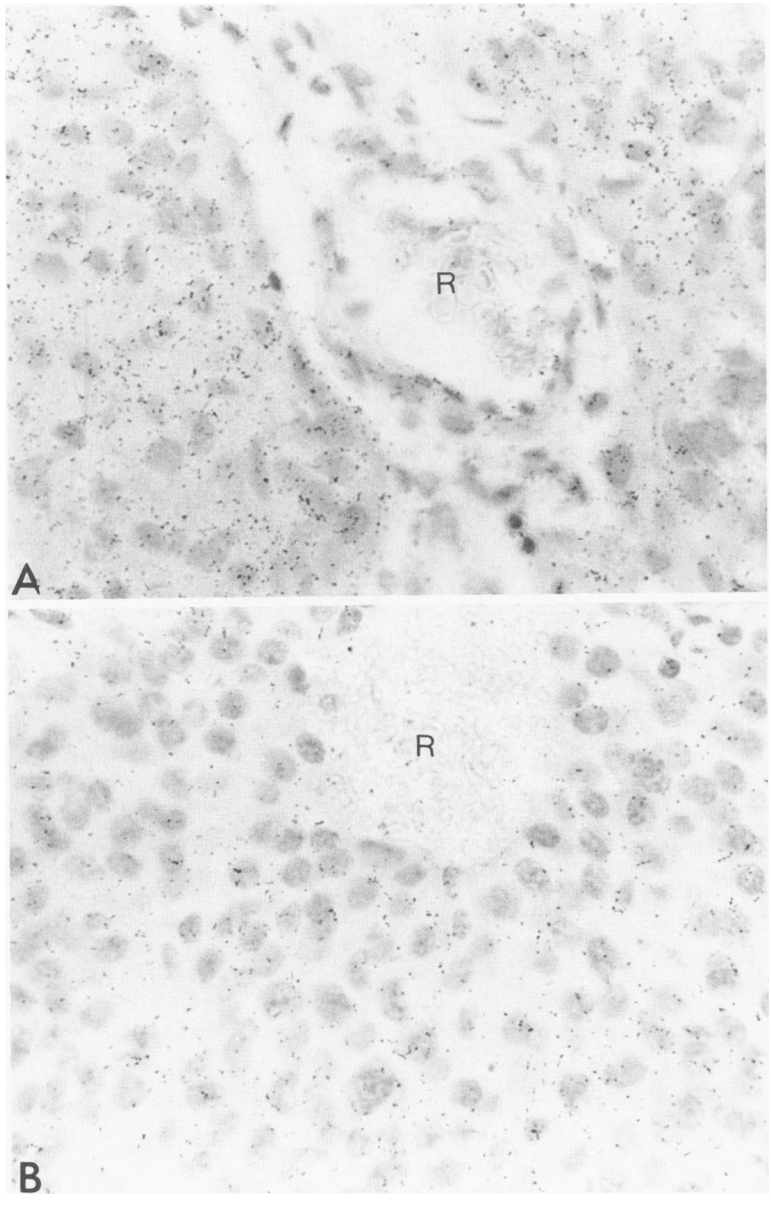

Figure 6. In situ *hybridization with a beta actin ^{35}S-labeled oligonucleotide probe in formalin-fixed paraffin-embedded tissue sections show a positive hybridization signal in subtype 3 silent adenoma tumors from case 11* **(A)** *(×400) and case 12.* **(B)** *The red blood cells (R) do not show a positive hybridization signal with this probe (×400).*

and electron microscopic levels. Only a small percentage of adenomas from subtype 2 and 3 had POMC mRNA detectable by ISH. Failure to detect POMC mRNA in these groups could be related to sensitivity of the assay in these paraffin-embedded blocks that were stored for up to 12 years. However this seems unlikely because older blocks were also present in the other groups. Sampling variation among tumors is another possible explanation for the differences because many of the tissue sections of tumors were small. Sampling differences are probably the reason for our failure to detect GH in two subtype 3 tumors, although GH was detected by immunochemistry. It is also possible that the POMC mRNA in tumors in groups 2 and 3 may be different from the other groups and were not recognized by the oligonucleotide probes. To investigate this possibility, Northern blot studies with purified RNA from the various groups of tumors would be needed and such samples are not available.

The detection of PRL mRNA in a tumor from group 3 but not in the other groups indicates that the hyperprolactinemia associated with some cases of Cushing's disease may not be related to PRL production by the neoplastic cells, but the PRL may be produced by adjacent non-neoplastic cells that may have had the dopamine regulation of PRL secretion altered by the neoplasm.

Silent corticotropic tumors are usually macroadenomas with suprasellar extensions, suggesting a more rapid growth.[10,12] In contrast, endocrine-active ACTH-producing adenomas are often microadenomas.[2] This difference may suggest differences in growth and differentiation be-

tween these two groups. Endocrine tumors that are rapidly growing are often less likely to produce hormone products that are similar to the normal gland. The mechanism responsible for endocrine inactivity of the silent adenomas is unknown but may be related to 1) defective packaging and release of POMC products or 2) production of a different POMC precursor that lacks the biologic activity of ACTH. Evidence that some endocrine active and silent ACTH-producing adenomas secrete high molecular weight ACTH-related peptides has been reported by various groups.[34,35] Abnormal processing of the POMC product has been responsible for some of these differences. Reports of pituitary tumors that were endocrine inactive and that subsequently became active[13,15] are especially intriguing and suggest that changes in cellular processing enzymes or POMC mRNA may lead to production of functional ACTH molecules capable of producing signs and symptoms associated with Cushing's disease.

Recent studies have suggested that there may be different types of ACTH tumors causing Cushing's disease. It has been proposed that some tumors may originate in the anterior or the intermediate lobe of the pituitary and may have different responses to drugs such as bromocriptine, thus implying differences in cell-surface receptors.[36] Other researchers have not corroborated these findings.[37] However differences in dopamine or other receptors, such as epidermal growth factor, have not been excluded as a possible explanation for differences in growth and differentiation of endocrine-active and silent corticotropic adenomas.

Northern hybridization analyses have revealed that many nonpituitary tissues and neoplasms express POMC gene products.[18-20] In some cases the mRNAs are heterogeneous and generally shorter than the 1200-nucleotide pituitary species by 200 to 400 bases. Failure to encode a complete POMC molecule often results in truncated POMC molecules that cannot be translated and lack signal peptides needed for membrane translocation and precursor processing. However some tumors do have the normal 1200-nucleotide mRNA species and are sometimes associated with Cushing's syndrome, such as pheochromocytomas, and pancreatic endocrine tumors produce ectopic ACTH.[18-20] Whether such mechanisms contribute to the failure of silent corticotropic adenomas to be clinically active must be shown in further studies. A recent report by Nagaya et al[38] analyzed one silent corticotropic adenoma and three adenomas from patients with Cushing's disease by Northern hybridization and found that the size of the pro-opiomelanocortin mRNA present in the silent adenoma was indistinguishable from that in the adenomas associated with Cushing's disease. While this limited study does not fully address the question about the pathophysiology of silent corticotropic adenomas, it suggests that the absence of Cushing's disease symptoms did not result from abnormalities in the coding sequence of the pro-opiomelanocortin gene or in ribonucleic acid processing in this case. Our studies revealed that all of the subtype 1 silent corticotropic adenomas and some subtype 2 and 3 tumors contain POMC mRNA that is readily detectable by ISH in routinely processed tissue specimens. Additional molecular biologic analyses of these tumors should help to elucidate the pathophysiology of these silent corticotropic adenomas.

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