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Identification of Adrenocorticotropin Receptor Messenger Ribonucleic Acid in the Human Pituitary and Its Loss of Expression in Pituitary Adenomas

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The ACTH receptor (ACTH-R) is the second member of the melanocortin (MC-2) receptor family that includes five seven-transmembrane G protein-coupled receptors and has been shown to be predominantly expressed in the adrenal cortex. It has been postulated that ACTH may regulate its own secretion through ultra-short-loop feedback within the pituitary. ACTH-secreting adenomas are characterized by resistance to glucocorticoid feedback, and they may have dysregulated ACTH feedback. We therefore investigated the ACTH-R in normal and adenomatous human pituitary tissue. We report here the identification of ACTH-R mRNA in the human pituitary gland, which was confirmed by direct sequencing. We studied the expression of the ACTH-R in 23 normal pituitary specimens and 53 pituitary adenomas (22 ACTH-secreting, nine GH-secreting, eight prolactin-secreting, one TSH-secreting, one FSH-secreting, 10 nonfunctioning, and two silent corticotroph adenomas), using the sensitive technique of real-time quantitative PCR. Contamination of ACTH-secreting adenomas and nonfunctioning pituitary adenomas with nonadenomatous tissue was excluded by lack of Pit-1 expression. ACTH-R mRNA was detected in all normal pituitary specimens, and *in situ* hybridization colocalized expression to ACTH staining cells only. However, ACTH-R mRNA levels were undetectable in 16 of 22 ACTH-secreting tumors and in both silent corticotroph tumors. Diagnostic preoperative plasma ACTH levels were significantly lower in the ACTH-R positive ACTH-secreting tumors, compared with those who were ACTH-R negative ($P = 0.0006$). Direct sequencing of the coding region of the ACTH-R in cDNA from three ACTH-secreting tumors positively expressing the receptor showed no mutations, as did sequencing of genomic DNA in three receptor negative ACTH-secreting tumors and the two silent corticotrophs. These results provide further evidence compatible with an ACTH feedback loop in the pituitary and suggest that loss of expression of the ACTH-R in corticotroph adenomas of patients with Cushing's disease may play a role in the resistance to feedback of the pituitary-adrenal axis seen in these patients. (*J Clin Endocrinol Metab* 88: 6080–6087, 2003)

CUSHING'S DISEASE, OR pituitary-dependent Cushing's syndrome, usually is due to a corticotroph adenoma and typically is characterized by a derangement in corticosteroid feedback. The tumors, which have generally been considered to be monoclonal in origin, produce excessive levels of ACTH that remain unsuppressed by levels of circulating cortisol that would otherwise inhibit ACTH release (1). At a molecular level, although abnormalities of the structure or expression of the glucocorticoid receptor have been repeatedly sought, such changes appear to be a rare cause of the abnormal feedback (2, 3). Changes in cortisol feedback could also theoretically be due to altered expression of the 11β-hydroxysteroid dehydrogenase enzymes, which modulate cortisol access to the glucocorticoid receptor: These have been recorded in pituitary tumors but are probably not specific for the corticotroph phenotype (4). The cause of the deranged feedback, and the shift of the cortisol-ACTH feedback curve, thus remains unclear.

Early studies in animals suggested that an elevated plasma ACTH concentration could in itself inhibit the secretion of ACTH, independent of steroid feedback (5, 6), and this was subsequently confirmed in murine corticotroph tumor cells (7), although not in normal rat pituitary tissue (8). ACTH implants into the rat median eminence have also been shown to reduce circulating corticosteroid levels (9). In the human, there is also evidence that ACTH may inhibit its own secretion via an ultra-short-loop feedback on the pituitary. Infusions of a shorter (1–24 amino acid) biologically active ACTH were performed in patients with Addison's disease treated with conventional replacement therapy (10): endogenous

Abbreviations: ACTH-R, ACTH receptor; DIG, digoxigenin; dNTP, deoxynucleotide triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gDNA, genomic DNA; ISH, *in situ* hybridization; NFPA, nonfunctioning pituitary adenoma; POMC, proopiomelanocortin; PRL, prolactin; RQ-PCR, real-time quantitative PCR; RT, reverse transcription; SSC, saline sodium citrate; UTR, untranslated region.

ACTH secretion was measured using a highly specific two-site immunometric assay that did not cross-react with ACTH (1–24). Endogenous ACTH was shown to be significantly reduced 15 min into the infusion, compared with placebo; in addition, the endogenous ACTH response to ovine-sequence CRH was obliterated by ACTH (1–24) infusion, suggesting that the effect was likely to be at the level of the pituitary rather than the hypothalamus. These results were contrary to an earlier article in patients with Addison's that had demonstrated no evidence of inhibition of endogenous ACTH by the synthetic ACTH analog alsactide (β-Ala1, Lys17-ACTH1–17-4-amino-N-butylamide) (11). The discrepancy in findings between these two studies is not clear, although it has been postulated to be due to a possible difference in specificity between ACTH (1–17) and ACTH (1–24) at the receptor level (10).

Feedback at the hypothalamic level by ACTH has also been demonstrated in the rat (12) and human subjects (13). Clinically, we observed that patients with familial glucocorticoid deficiency, caused by germline inactivating mutations of the ACTH receptor (ACTH-R) (14, 15), were often persistently pigmented while receiving normal or even excessive glucocorticoid replacement, and had persistently detectable ACTH levels. We therefore speculated that these patients demonstrate deranged feedback as a consequence of the mutated ACTH-R. The ACTH-R is the second member of the melanocortin (MC-2) receptor family that includes five seven-transmembrane G protein-coupled receptors, signal transduction occurring largely through stimulation of adenylate cyclase and cAMP production (16). Previous studies have shown predominant expression of the ACTH-R in the adrenal cortex, as expected, but it has also been described in human extraadrenal tissues; these include testis, heart, kidney, ovary (17), skin (18), and vascular endothelial cells (19) as well as murine adipose tissue (20, 21), but it has not been described in the pituitary, although the melanocortin receptors subtypes MC1-R, MC4-R, and MC5-R are known to be expressed in this tissue (17). We therefore further speculated that the ACTH-R might be expressed in the normal human pituitary and that changes in its sequence or relative expression may be part of the molecular defect seen in Cushing's disease.

The aim of this study was therefore to investigate the possible presence of the ACTH-R in human pituitary and to quantify and compare the level of expression of mRNA in corticotroph tumors, compared with normal pituitary tissue and other pituitary tumors. Protein expression could not be studied because there are currently no specific antisera available, but *in situ* hybridization together with immunocytochemistry was used to colocalize expression. We also wished to examine the expression of proopiomelanocortin (POMC) and T-pit, a recently described specific marker of corticotroph cells (22, 23), in these normal and tumor samples in relation to ACTH-R status.

Materials and Methods

Tumor specimens

A total of 56 human pituitary adenomas were examined: 25 ACTH-secreting tumors, two silent corticotroph tumors, nine GH-secreting tumors, 10 nonfunctioning pituitary adenomas (NFPAs), eight prolactin (PRL)-secreting tumors, one FSH-secreting, and one TSH-secreting tumor were obtained at the time of transsphenoidal surgery. Tumors were collected in liquid nitrogen and then stored at −80 C. The tumor type was determined on the basis of clinical and biochemical findings before surgery and by morphological and immunocytochemical analysis of the removed tissue sample. The size of the tumor was based on the preoperative magnetic resonance imaging findings. Clinical details, including baseline 0900 h serum cortisol and plasma ACTH levels, are given in Table 1. Normal human pituitary autopsy specimens (23 samples collected in liquid nitrogen and then stored at −80 C, and four samples formalin fixed), obtained within 24 h of death, were obtained from patients with no evidence of any endocrine abnormality. Informed consent was obtained from all patients, and the study was approved by the local Ethics Committee.

RNA extraction and reverse transcription

Total RNA was homogenized and extracted from frozen tissue using an SV isolation kit (Promega, Southampton, UK), which includes a DNase step; quantification and purity of the total RNA was determined by spectrophotometry.

Total RNA (1 μg) was reverse transcribed into cDNA using TaqMan reverse transcription (RT) reagents (PE Biosytems, Warrington, UK). Each 50-μl reaction mix contained 5.5 mM $MgCl_2$, 2 mM deoxynucleotide triphosphate (dNTP) mix, 2.5 μM random hexamers, 20 U RNase inhibitor, 62.5 U Multiscribe reverse transcriptase, and 1 μg RNA. Thermal cycling conditions were as follows: 25 C for 10 min, 48 C for 30 min, and 95 C for 5 min.

PCR

The integrity of the mRNA and the exclusion of genomic DNA contamination from each specimen was verified by PCR for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), on cDNA and RT-control reactions, with omission of reverse transcriptase. Contamination of ACTH-secreting, silent corticotroph, FSH-secreting tumors, and NFPAs by nonadenomatous pituitary tissue was excluded by PCR for the gene Pit-1, as previously published (24) (Fig. 1).

PCR for the corticotroph cell-expressed genes POMC and T-pit were also performed. Confirmatory PCR of the ACTH-R was performed in corticotroph tumors after real-time quantitative PCR analysis. Intron-spanning primers sequences were as follows: GAPDH (196-bp product; GenBank accession no. M33197, forward 5′ CCATGGAGAAGGCTGGGG 3′; reverse 5′ CAAAGTTGTCATGGATGACC 3′), Pit-1 (560-bp product; GenBank accession no. D10216, forward 5′ AGTGCTGCCGAGTGTCTACCA 3′; reverse 5′ TTTCTTTTCCTTTCATTTGCT 3′), Tpit (400-bp product; GenBank accession no. NM005149, forward 5′ CGTCTACATTCACCCGGACT 3′; reverse 5′ CACTCCATCTGGATTGGAAAA 3′), POMC (320-bp product; GenBank accession no. V01510, forward 5′ AGGACCTCACCACGGAAAG 3′; reverse 5′ CATGGAGTAGGAGCGCTTG 3′), and ACTH-R (248-bp product; GenBank accession no. Y10100 and S77555, forward 5′ CCCAGAAAGTTCCTGCTT 3′; reverse 5′ ATATCTCCTCCGGCAAAA 3′). PCR using 2.5 μl cDNA was performed in 25-μl reaction volumes on the GeneAmp 9700 thermal cycler (PE Biosytems), with reagent concentrations for the different genes as follows: GAPDH and Pit-1, 0.2 μM primers, 1.5 mM MgCl, 200 μM dNTPs, and 0.625 U Taq DNA polymerase in storage buffer A (Promega); T-pit, POMC, and ACTH-R 0.5 μM primers, 1.5 mM MgCl, 400 μM dNTPs, 5 μl Q-Solution, and 1.25 U HotStarTaq (Qiagen, Sussex, UK). An initial denaturing/activating step of 95 C for 5 and 15 min, respectively, for the Promega and Qiagen Taq polymerases was required, followed by a repeated cycles of 95 C for 30 sec, a variable annealing temperature for 30 sec, and 72 C for 1 min. Annealing temperatures and cycle numbers were as follows: GAPDH (55 C, 26 cycles); Pit-1 (55 C, 30 cycles); T-pit (55 C, 35 cycles); POMC (58 C, 30 cycles); and ACTH-R (55 C, 40 cycles). A final extension step of 72 C for 10 min was used. PCR products were run on ethidium bromide-stained 2% agarose gels.

Real-time quantitative PCR (RQ-PCR) of the ACTH-R

The ACTH-R contains two exons, the first encoding the 5′ untranslated region (UTR), whereas the second encodes part of the 5′-UTR, the

TABLE 1. The preoperative tumor size and biochemical characteristics of the corticotroph adenomas

Tumor no.	Clinical diagnosis	ICC	ACTH-R expression	Sex	Age (yr)	Size of tumor	0900 h Cortisol (nmol/liter)	0900 h ACTH (ng/liter)	Suppression on LDDST
1	Cushing's disease	ACTH, scattered GH and PRL	+	F	49	Micro	347	16	No
2	Cushing's disease	ACTH	+	F	40	Micro	578	17	No
3	Cushing's disease	ACTH	+	M	24	Macro	725	50	No
4	Cushing's disease	ACTH	+	F	20	Macro	741	65	No
5	Cushing's disease	ACTH	+	M	59	Macro	603	28	No
6	Cushing's disease	ACTH	+	F	56	Micro	557	17	No
7	Cushing's disease	ACTH	−	F	25	Macro	822	117	No
8	Cushing's disease	ACTH	−	F	17	Micro	873	96	No
9	Cushing's disease	ACTH	−	M	19	Micro	788	68	No
10	Cushing's disease	ACTH	−	F	48	Micro	925	128	No
11	Cushing's disease	ACTH	−	F	41	Macro + SSE	780	133	No
12	Cushing's disease	ACTH	−	F	39	Macro + SSE	800	103	No
13	Cushing's disease	ACTH	−	F	6	Micro	623	98	No
14	Cushing's disease	ACTH	−	F	37	Micro	707	44	NA
15	Cushing's disease	ACTH	−	F	58	Micro	520	110	NA
16	Cushing's disease	ACTH	−	F	55	Micro	369	26	No
17	Cushing's disease	ACTH	−	F	69	Macro	662	71	No
18	Cushing's disease	ACTH	−	F	50	Micro	737	70	No
19	Cushing's disease	ACTH	−	F	35	Micro	844	71	No
20	Cushing's disease	ACTH	−	M	70	Macro	383	155	No
21	Cushing's disease	ACTH, scattered TSH and FSH	−	F	35	Micro	1150	181	No
22	Nelson's syndrome	ACTH	−	F	50	Macro	0	>1500	ND
23	Nonfunctioning	ACTH	−	M	33	Macro + SSE	414	35	ND
24	Nonfunctioning	ACTH	−	F	34	Macro	83	17	Yes

The three corticotroph tumors excluded from further analysis by positive Pit-1 expression are not shown in this table. Tumors 1–22 were clinically ACTH-secreting. Tumors 23 and 24 were silent corticotrophs on clinical and immunocytochemical grounds. Tumors 1–6 showed ACTH-R expression. Tumors 7–22 were ACTH-R negative. ICC, Immunocytochemistry; Micro, microadenoma (<10 mm); Macro, macroadenoma (≥10 mm); SSE, suprasellar extension; LDDST, low-dose dexamethasone suppression test; NA, data not available; ND, test not performed; F, female; M, male.

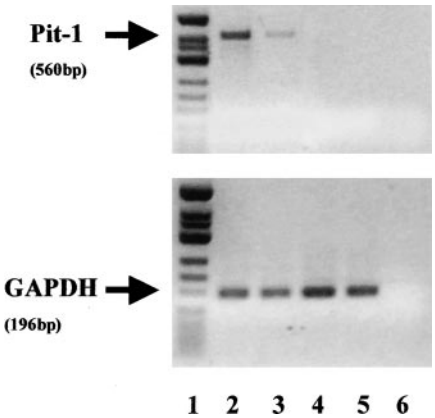

FIG. 1. Representative 2% agarose gels showing Pit-1 and GAPDH expression. Lane 1 shows size marker ϕX 174 DNA/*Hinf*-1 digest. Lane 2 shows a normal postmortem pituitary as a positive control for Pit-1 expression. Lane 3 shows an ACTH-secreting adenoma positive for Pit-1 and therefore excluded from further analysis. Lanes 4 and 5 show an ACTH-secreting adenoma and NFPA, respectively, negative for Pit-1, and therefore free of contamination from nonadenomatous tissue. All samples show detectable expression of the internal control gene GAPDH (*lower gel*). Lane 6 shows the water control.

entire coding region, and the 3′-UTR. Recently an alternative exon 1 has been described, which leads to an alternative transcript (25), although this does not predict a change in the translated protein. We wished to quantify both transcripts, and therefore intron-spanning probe and primer sets were designed for the normal (ACTH-R) and alternative (ACTH-R1A) transcripts. The RQ-PCR reactions and analysis for the ACTH-R were carried out using the ABI PRISM 7900 sequence detection system (PE Biosytems), which allows for detection of PCR products as they accumulate during the PCR process, rather than assaying the final product after a fixed number of cycles. For each experiment, RQ-PCR for the endogenous control gene 18s-rRNA was also performed for each sample, allowing for data to be expressed relative to 18s-rRNA, therefore compensating for any differences in RT efficacy. We previously determined 18s-rRNA to be the most consistent internal control gene in pituitary tissue, both adenomatous and normal, using the TaqMan human endogenous control plate (PE Biosytems) (26). To allow for comparison between RQ-PCR experiments, standard curves were constructed for ACTH-R/ACTH-R1A and 18s-rRNA using human adrenal total RNA, which was shown to express both genes. Serial 5-fold dilutions (20 ng/μl to 32 pg/μl) in TE (Tris 10 mM EDTA 1 mM, pH 8.0) buffer were performed followed by RT, and these stocks of cDNAs were used for all experiments. Data from standard curves with a correlation coefficient of less than 0.97 were discounted. RQ-PCR was carried out in triplicate using 10-μl reaction volumes containing 5 μl TaqMan Universal PCR master mix, 200 nM probe, 50 nM (ACTH-R), 300 nM (ACTH-R1A) or 300 nM (18s-rRNA) forward primer, 900 nM (ACTH-R), 300 nM (ACTH-R1A), or 300 nM (18s-rRNA) reverse primer at 50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min.

Primer and probes were synthesized by MWG Biotech (Ebersberg, Germany), with sequences as follows: ACTH-R (GenBank accession no. Y10100 and S77555), forward 5′ CTCATTCATTTTGCCCAGAAAGTT 3′, reverse 5′ GGATCTTTTCTTCCTTGTAGCACTTG 3′, probe 5′ TCTCCCAATCACCTTCAGCTCTGAAGCA 3′; ACTH-R1A (GenBank accession no. AB013459), forward 5′ GTGAAAGCTTGGTGATTAATATAAAGACTT 3′, reverse 5′ CCTTGTAGCACTTGCTGGAGATC 3′, probe 5′ AGTTAAAATCTCCCAATCACCCTCCACATGAA 3′; and 18s-rRNA (GenBank accession no. X03205), forward 5′ TCCCCATGAACGAGGAATTC 3′, reverse 5′ GTGTACAAAGGGCAGGGACTTAA 3′, probe 5′ CAACGCAAGCTTATGACCCGCACTTACT 3′. The probes were labeled with the reporter dye (6-carboxy-fluorescein) at the

5′ end and the quencher dye (6-carboxy-tetramethylrodamine) at the 3′ end. Experiments were performed on at least two occasions for each sample.

ACTH-R sequencing

Direct sequencing of the entire coding region of the ACTH-R (894 bp) was performed on cDNA from three ACTH-R-positive ACTH-secreting tumors and one each of a positive NFPA; GH-, PRL-, and TSH-secreting tumor; and two normal pituitary samples. In addition, for three of the ACTH-negative ACTH-secreting adenomas and the two silent corticotrophs, sufficient tissue was available to allow tumor genomic DNA (gDNA) preparation using the QIAamp DNA minikit (Qiagen). Quantification and purity of the gDNA was determined by spectrophotometry and sequencing subsequently performed. PCR products were prepared using the following two overlapping primer sets: 48 forward 5′ GAAAAGATCCTGAAGAATCAATCAA 3′, 656 reverse 5′ GAACAGCGACGTGAAGGTG 3′; 506 forward 5′ GGACCGCTACATCACCATCT 3′, 1073 reverse 5′ GGCACTTGGCAACGTTATTC 3′. PCR using 5 μl cDNA or 2 μl gDNA was performed in 25-μl reaction volumes containing 1 μM primers, 2.5 mM MgCl, 400 μM dNTPs, 5 μl Q-Solution, and 2.5 U HotStarTaq (Qiagen). PCR parameters were 95 C for 15 min followed by for the cDNA and the gDNA, respectively, 45 or 35 cycles of 95 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min, and then 72 C for 10 min. The PCR product size was checked by ethidium bromide-stained 2% agarose gel electrophoresis before purification using the ExoSAP-IT kit protocol (USB Corp., Cleveland, OH). Each PCR product was sequenced in both the forward and reverse direction using a 10-μl sequencing reaction, consisting of 4.5–5.5 μl purified PCR product, 4 μl BigDye terminator ready reaction mix (PE Biosytems), and 1 μM primer. The primers were the same as those used for the PCR amplification. The extension products obtained were purified using Sephadex column precipitation plates (Amersham Life Science Ltd., Buckinghamshire, UK) to remove the excess of dye terminators, which can interfere with base calling. Electrophoresis of the samples was performed on the ABI Prism 3700 DNA analyser (PE Biosytems). The obtained sequences were analyzed using the Phred/Phrap/Consed software package (www.phrap.org) and compared with the published ACTH-R sequence.

In situ hybridization (ISH)

ISH was performed using probes produced from an 894-bp cDNA for the ACTH-R incorporated into plasmid pcDNA3 (Invitrogen, Paisley, UK). After linearalization with *Hin*dIII, the antisense riboprobe was transcribed with SP6 RNA polymerase in the presence of digoxigenin (DIG) 11 uridine 5-triphosphate. The sense probe was generated using *Xba*I and T7 RNA polymerase (all reagents Roche Diagnostics, Penzberg, Germany). Sections (4 μm thick) were cut from four formalin-fixed paraffin-embedded normal pituitary glands. Following rehydration, sections were washed in 0.1 M HCl solution, 0.3% Triton X 100, and digested with proteinase K (100 mg/ml) in PBS at 37 C (all reagents Sigma-Aldrich, Gillingham, UK). Slides were incubated with DIG-labeled probe overnight at 42 C in a hybridization buffer [containing Denhardts, 2 × saline sodium citrate (SSC), 50% formamide, SDS and salmon sperm DNA]. Stringency washes started with 2 × SSC for 30 min, 0.1 × SSC for 15 min, and then 0.1 × SSC at 50 C for 30 min. Slides were then incubated for 1 h in alkaline phosphatase-conjugated anti-DIG antiserum, diluted 1:2000 in Tris buffer pH 7.4. Sections were washed in buffer and finally incubated in nitroblue tetrazolium/bromo-chloro-indolyl phosphate, containing levamisole until a dark blue reaction product developed. Sections were counterstained with hematoxylin, mounted in Glycergel, and examined under a light microscope (all reagents Sigma-Aldrich). Controls included incubation with DIG-labeled sense probe and DIG-labeled probe to albumen mRNA.

Immunocytochemistry

Serial sections of pituitary were immunostained for ACTH, GH, PRL, TSH, LH, FSH, and S100 protein to identify folliculostellate cells, using the EnVision technique (Dako, Ely, UK). The distribution of positive ISH signal was compared with the hormone staining patterns.

Statistical analysis

ACTH-R data were expressed as the ratio of ACTH-R quantity to 18s-rRNA quantity. Data are shown as median with minimum and maximum values. The nonparametric Kruskal-Wallis ANOVA was used to calculate differences in expression between groups of tissues. The Mann-Whitney *U* test was used to calculate differences in preoperative characteristics. Significance was taken as $P < 0.05$.

Results

A total of three ACTH-secreting tumors were shown to be Pit-1 mRNA positive and therefore probably contaminated with nonadenomatous pituitary tissue and thus were excluded from further analysis (Fig. 1). None of the NFPAs or the single FSH-secreting tumor studied showed Pit-1 positivity.

RQ-PCR for the ACTH-R and ACTH-R1A was performed on 22 ACTH-secreting tumors, two silent corticotroph tumors, nine GH-secreting tumors, 10 NFPAs, eight PRL-secreting tumors, one FSH-secreting tumor, and one TSH-secreting tumor as well as 23 normal pituitary specimens. ACTH-R mRNA was identified in all normal autopsy pituitary samples, although at variable expression levels (Fig. 2). In the ACTH-secreting tumors as a group, ACTH-R mRNA was significantly underexpressed (median 0, range 0–1.16), compared with normal pituitary samples (median 4.40×10^{-2}, range 1.51×10^{-3} to 4.52×10^{-1}) ($P = 0.008$; Fig. 2).

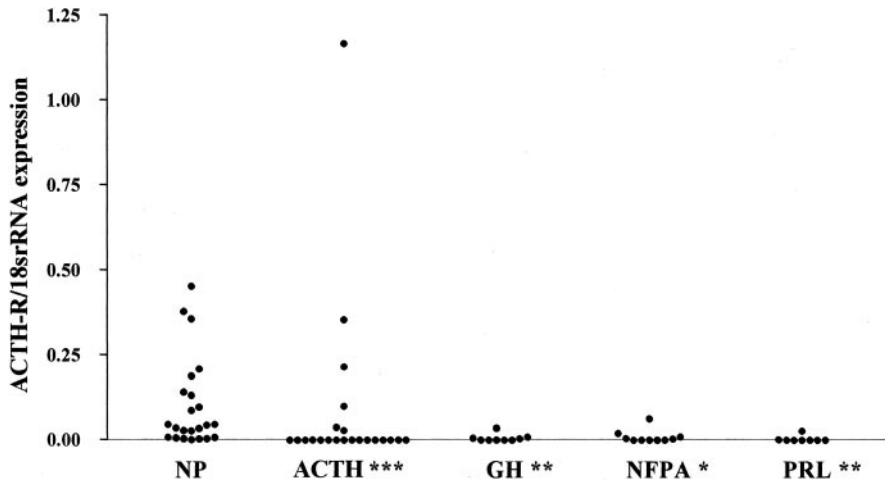

FIG. 2. Scatterplot of ACTH-R mRNA expression in pituitary tumors and normal pituitary: NP, Normal pituitary, n = 23; ACTH, ACTH-secreting adenomas, n = 22; GH, GH-secreting adenomas, n = 9; NFPA, n = 10; PRL, PRL-secreting adenomas, n = 8. Actual values of ACTH-R/18srRNA expression are shown. ***, $P = 0.008$ *vs.* normal pituitary; **, $P \leq 0.01$; *, $P = 0.03$ *vs.* normal pituitary.

Only six of the 22 ACTH-secreting tumors had detectable expression of the ACTH-R and in these expression levels were comparable with the normals in five, but it was higher in one tumor (Fig. 2). Undetectable ACTH-R expression was confirmed by conventional PCR (Fig. 3). There was also significantly reduced ACTH-R expression in the GH-secreting tumors (median 0, range 0–3.47×10^{-2}, $P = 0.01$), PRL-secreting tumors (median 0, range 0–6.26×10^{-2}, $P = 0.004$), and NFPAs (median 1.67×10^{-3}, range 0–6.26×10^{-2}, $P = 0.03$; Fig. 2). In addition, the single TSH-secreting tumor showed very weak expression of the ACTH-R (7.91×10^{-3}), whereas the single FSH-secreting tumor showed no expression (data not shown).

ACTH-R1A expression was detected in five normal pituitary samples, one ACTH-secreting tumor, and one GH-secreting tumor, all of which showed expression of ACTH-R. Expression levels of the alternative transcript were 30- to 70-fold less than that of the normal transcript in these specimens (data not shown).

Plasma 0900 h ACTH levels at diagnosis were significantly lower ($P = 0.0006$) in the ACTH-secreting tumors positive for ACTH-R expression, compared with those not expressing the ACTH-R (Fig. 4). The patient with Nelson's syndrome and ACTH levels greater than 1500 ng/liter was excluded from this analysis. There was no statistical difference in any of the other diagnostic baseline characteristics between ACTH-R-positive and ACTH-R-negative ACTH-secreting tumors (Table 1).

ISH was used to localize the ACTH-R within the normal pituitary gland. The antisense probe gave a positive signal in cells that corresponded to a subpopulation of corticotrophs, as defined by immunocytochemical staining (Fig. 5, A and B). Neither the sense probe nor the albumen probe gave any signal (Fig. 5C). The pattern did not correspond to cells immunopositive for any of the other hormones or to folliculostellate cells.

T-pit and POMC mRNA expression was positive in all the corticotroph tumors, although only weak expression for T-pit was present in two of these tumors. There was no discernible difference in T-pit or POMC expression between ACTH-R-positive and ACTH-R-negative adenomas (Fig. 6A). POMC expression was present in all the normal pituitary samples, whereas T-pit expression was present in all but one normal sample (Fig. 6B). In nine of the 12 ACTH-R-positive noncorticotroph tumors, there were faint bands for T-pit. However, POMC expression was seen in only four of these samples. In the ACTH-R-negative noncorticotroph tumors, eight of 17 showed very weak expression in either T-pit or POMC (data not shown).

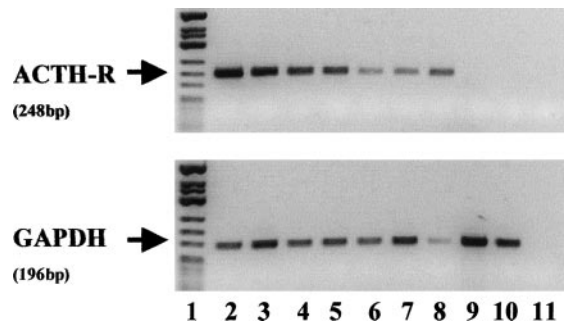

FIG. 3. The 2% agarose gels showing ACTH-R and GAPDH expression in ACTH-secreting adenomas. Lane 1 shows size marker ϕX 174 DNA/*Hinf*-1 digest. Lane 2 shows an adrenal sample as a positive control. Lanes 3–8 show the ACTH-secreting adenomas with detectable ACTH-R expression by RQ-PCR. Lanes 9 and 10 show two representative ACTH-secreting adenomas showing no detectable ACTH-R expression by RQ-PCR. Lane 11 shows the water control.

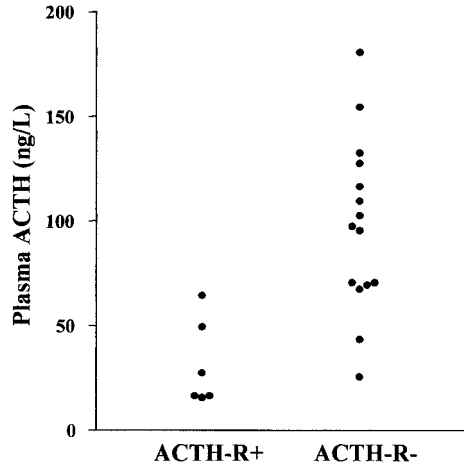

FIG. 4. Scatterplot showing lower plasma ACTH levels at diagnosis in the ACTH-R positive (ACTH-R+), compared with the ACTH-R negative (ACTH-R−) tumors ($P = 0.0006$ between groups).

Direct sequencing of the coding region of the ACTH-R was performed on the cDNA of two normal pituitary samples, three ACTH-secreting adenomas shown to express the ACTH-R, and one of each ACTH-R-positive GH-secreting, PRL-secreting, and TSH-secreting adenomas. In addition, direct sequencing of the coding region was performed on the gDNA of three ACTH-R-negative ACTH-secreting adenomas and both silent corticotrophs. All sequences were identical to the published ACTH-R sequence, with no base alterations.

Discussion

We have demonstrated the presence of ACTH-R mRNA in the normal human pituitary gland and have localized its expression to corticotroph cells. Because it has been suggested that ACTH may itself be involved in the regulatory feedback of ACTH secretion (5, 9, 10, 13) and because dysregulation of the usual negative feedback pathways on ACTH secretion is characteristic of Cushing's disease, it is possible to speculate that qualitative or quantitative changes in the ACTH-R are present in ACTH-secreting pituitary adenomas.

We found that the ACTH-R was absent in around 70% of the ACTH-secreting tumors studied but was present in all the normal human pituitary specimens, with the normal transcript the predominant species. It was also present in some of the noncorticotroph tumors. Because it appears the ACTH-R is expressed in the normal pituitary on corticotrophs, with no evidence of expression in noncorticotroph cells, then any homogeneous population of corticotrophs would be expected to have a much higher expression of the ACTH-R than nontumorous pituitary; even in those tumors that expressed the ACTH-R, in five of these six ACTH-R-

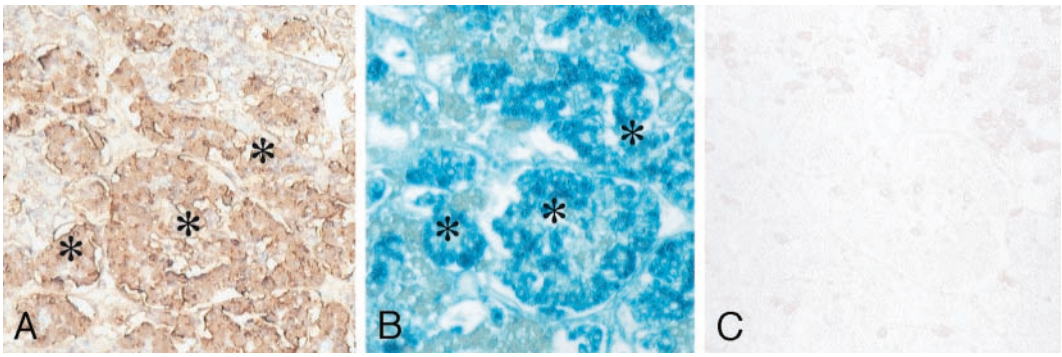

FIG. 5. Colocalization of the ACTH-R in normal human pituitary gland. A, Immunohistochemistry for adrenocorticotrophin. B, ISH. Antisense probe for mRNA for ACTH-R. C, ISH. Sense probe for mRNA for ACTH-R. The pattern of signal for ACTH-R mRNA corresponds to corticotrophs. There is no binding of the sense probe. Because the sections are not immediately adjacent (there were intervening control slides), the *asterisks* serve to identify corresponding groups of cells in the immunohistochemical and ISH slides.

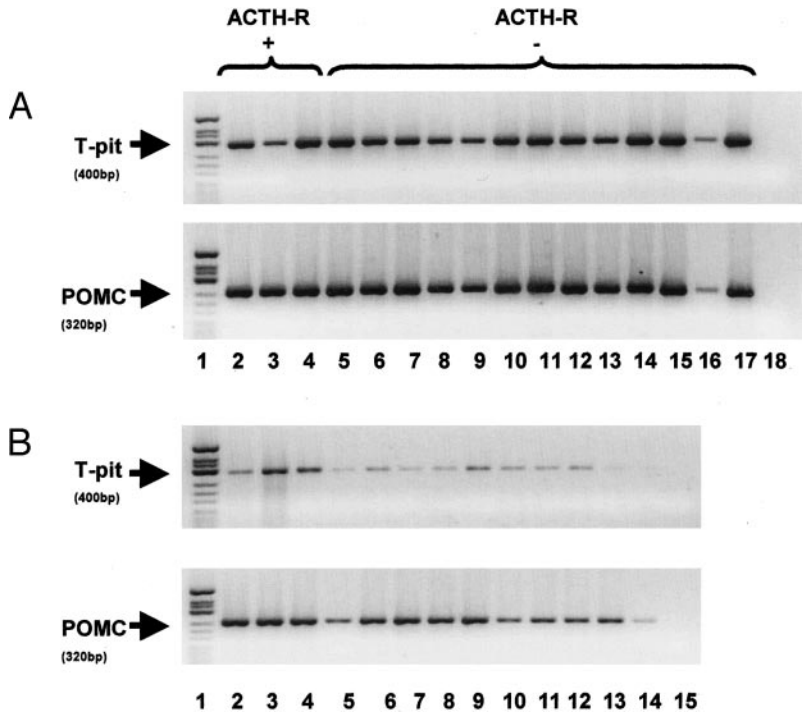

FIG. 6. A, Representative 2% agarose gels of T-pit (*upper gel*) and POMC (*lower gel*) expression in ACTH-secreting (lanes 2–15) and silent corticotroph adenomas (lanes 16 and 17). Lane 1 shows size marker ϕX 174 DNA/*Hinf*-1 digest. Lanes 2–4 shows ACTH-R-positive tumors. Lanes 5–15 show ACTH-R-negative tumors. Lane 18 shows the water control. B, Representative 2% agarose gels of T-pit (*upper gel*) and POMC (*lower gel*) expression in normal postmortem pituitary specimens all positive for the ACTH-R. Lane 1 shows size marker ϕX 174 DNA/*Hinf*-1 digest. Lanes 2–14 show normal pituitary samples. Lane 15 shows the water control.

positive ACTH-secreting tumors, absolute expression levels of the ACTH-R were comparable with those seen in the normal pituitary specimens (Fig. 2). Therefore, even in these five tumors, it seems likely that the expression level of the ACTH-R per adenomatous corticotroph cell is reduced relative to a normal corticotroph cell. What is perhaps more interesting is the observation that the ACTH-secreting tumor with the high level of ACTH-R expression (tumor no. 6 in Table 1) was the smallest tumor studied, a microadenoma approximately 3 mm in diameter. From this it is tempting to hypothesize that loss of ACTH-R expression occurs as the tumor develops. However, this is not borne out in the other ACTH-secreting tumors, in which there appeared to be no correlation between tumor size and ACTH-R positivity or negativity.

The mechanism for the loss of ACTH-R expression in corticotroph tumors remains unclear. One possibility is that sequence mutations lead to abnormal transcripts of the ACTH-R in ACTH-secreting tumors that might undergo nonsense-mediated decay of abnormal mRNA, as previously described in Carney complex GH-secreting tumors (27); however, in three ACTH-secreting tumors and the two silent corticotrophs negative for the ACTH-R, no coding region mutations could be identified in the tumor gDNA. This renders an explanation based on mutated sequences leading to nonsense-mediated decay for the loss of message improbable. In three ACTH-secreting adenomas that showed ACTH-R expression, no mutations were found in the mRNA coding region that might predict inactivation of the receptor in these tumors. Similarly, no mutations were detected in the small group of noncorticotroph tumors studied. One possibility is that loss of the ACTH-R could be causal in the pathogenesis of ACTH-secreting adenomas: if ACTH is an inhibitor of tumoral ACTH secretion and/or growth, then gradual loss of the ACTH-R by a clone or clones could in theory lead to selective accumulation of that clone. However,

an argument against this speculation is that these tumors do not routinely develop in patients with familial glucocorticoid deficiency who have germline-inactivating mutations of the ACTH-R. It is therefore more likely that this phenomenon is a consequence of the tumoral process. In the adrenal gland, ACTH-R expression is up-regulated rather than down-regulated by ACTH (28, 29), but the influence of high levels of ACTH and corticosteroids on the pituitary ACTH-R is unknown.

The finding that morning plasma ACTH levels were significantly lower in those ACTH-secreting tumors positive for the ACTH-R fits with the hypothesis that ACTH exerts negative feedback on its own secretion at the level of the pituitary. It is unclear at the current time how ACTH-R signaling might influence ACTH levels. There was no discernible difference in POMC expression between ACTH-R positive and negative ACTH-secreting tumors, although we did not perform RQ-PCR. Therefore, it may be that the mechanism is via inhibition of POMC processing or secretion of ACTH from the corticotroph cell, rather than an influence on POMC mRNA expression. It is also possible that the higher the level of ACTH, the higher the cortisol burden, and it is possible that cortisol itself down-regulates the ACTH-R. We did not find any correlation between the single basal cortisol level, but this may not adequately reflect the integrated burden of glucocorticoid excess over time.

T-pit is a novel T box factor identified in the mouse that activates POMC gene transcription and has been shown to be present only in murine and human pituitary POMC-expressing lineages, *e.g.* corticotrophs and melanotrophs (22, 23). There are currently no published data on T-pit mRNA expression in normal human pituitary tissue or adenomas using RT-PCR. As might be anticipated, we have shown that in both the normal pituitary specimens and ACTH-secreting adenomas, POMC was expressed in all samples, whereas T-pit was expressed in all but one of the normal pituitary specimens. In the corticotroph tumors, T-pit and POMC were expressed in all samples, and there was no obvious difference in the strength of bands between ACTH-R-positive and ACTH-R-negative tumors. We were intrigued by the weak expression of the ACTH-R in some noncorticotroph tumors. In nine of the 12 ACTH-R positive noncorticotroph tumors, there were faint bands for T-pit and in some positive POMC expression. We assume this indicates some degree of contamination with trapped normal corticotroph cells, a well-recognized phenomenon (30) (Asa, S., and K. Kovacs, personal communications). However, there was weak expression of POMC and/or T-pit in some of the other ACTH-R-negative noncorticotroph tumors, and it may be that in these the levels of ACTH-R if present were too small to detect.

In summary, we have identified mRNA for the ACTH-R in the normal human pituitary gland. ACTH-R mRNA expression appears to be lost in the majority of ACTH-secreting tumors, compared with the normal pituitary, but in those tumors in which ACTH-R expression is still detectable, plasma ACTH levels are significantly lower. We suggest that this provides further support for the concept of ACTH autoregulation at the pituitary level, and this may help to explain the resistance to feedback seen in Cushing's disease. ACTH-R levels were also significantly reduced in GH-secreting adenomas, PRL-secreting adenomas, and NFPAs as might be expected, considering the ACTH-R colocalizes to corticotroph cells on ISH in normal pituitaries. However, as noted above, there is currently no antibody available to the ACTH-R to investigate its protein expression by either Western blotting or immunostaining. Furthermore, functional studies on human corticotroph tumors are extremely difficult because of the small size of such tumors and a lack of normative data on the normal human pituitary. Nevertheless, it is clearly important to consider the translation and functionality of the ACTH-R before concluding as to the functional relevance of these findings.

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