

# Corticotropin-releasing Hormone, Proopiomelanocortin, and Glucocorticoid Receptor Gene Expression in Adrenocorticotropin-producing Tumors In Vitro

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## Abstract

To differentiate between ectopic ACTH syndrome and Cushing's disease, gene expression of corticotropin-releasing hormone (CRH), proopiomelanocortin (POMC), and glucocorticoid receptor was examined in 10 pituitary adenomas (Cushing's disease) and in 10 ectopic ACTH-producing tumors. CRH increased plasma ACTH levels in all patients with Cushing's disease and in five patients with ectopic ACTH syndrome whose tumors contained CRH and CRH mRNA. In five CRH nonresponders, CRH was not detected in tumors that contained no CRH mRNA or that contained only long-size CRH mRNA. Dexamethasone (Dex) decreased plasma ACTH levels in all patients with Cushing's disease and in three patients with ectopic ACTH-producing bronchial carcinoid. These tumors contained glucocorticoid receptor mRNA. CRH increased and Dex decreased ACTH release and POMC mRNA levels in pituitary adenoma and bronchial carcinoid cells. PMA increased POMC mRNA levels only in carcinoid cells. These results reveal characteristics of ectopic ACTH-producing tumors: long-size CRH mRNA and PMA-induced POMC gene expression. In addition, there are two ectopic ACTH syndrome subtypes: tumors containing ACTH with CRH (CRH responder) and tumors without CRH. Dex decreases ACTH release and POMC mRNA levels in some bronchial carcinoids. Therefore, CRH and Dex tests have limited usefulness in differentiating between Cushing's disease and ectopic ACTH syndrome. (*J. Clin. Invest.* 1993. 92:2790-2795.) Key words: Cushing's disease • ectopic ACTH syndrome • pituitary • vasopressin • phorbol myristate acetate

## Introduction

ACTH-dependent Cushing's syndrome can be classified into three types; Cushing's disease due to a pituitary corticotroph adenoma, ectopic ACTH syndrome, and Cushing's syndrome due to ectopic corticotropin-releasing hormone (CRH)<sup>1</sup> pro-

duction that stimulates pituitary ACTH secretion. In pituitary corticotroph adenomas, ACTH secretion and proopiomelanocortin (POMC, the precursor of ACTH) mRNA levels were increased by CRH via the cAMP-dependent protein kinase system (1). Such a CRH-induced increase in ACTH secretion and POMC gene expression was inhibited by dexamethasone (Dex). On the other hand, the ACTH response to CRH and Dex in patients with ectopic ACTH-producing tumors differed from that in patients with Cushing's disease. In the majority of patients with ectopic ACTH syndrome, plasma ACTH did not respond to CRH (2) or Dex (3), although ACTH responded to CRH (4, 5) and Dex (6, 7, 8) in some patients. Sometimes CRH was detected in these tumors (4). In addition, the POMC mRNA in ectopic ACTH-producing tumors was heterogeneous (9-12), and short-size POMC mRNA was not translated or did not produce secretable POMC-related peptides (12). In the present study, to distinguish ectopic ACTH syndrome from Cushing's disease on a molecular basis, we examined the gene expression of CRH, POMC, and glucocorticoid receptors (GR) in pituitary corticotroph adenomas and ectopic ACTH-producing tumors, and we also compared these results with clinical data to evaluate the usefulness of the CRH test and the Dex suppression test for differentiating between the two.

## Methods

**Patients.** Pituitary adenomas were obtained during transsphenoidal surgery in 10 patients with Cushing's disease (aged 24-56-yr-old; 7 females and 3 males). Pituitary non-adenomatous tissues were obtained from 5 patients for use as controls. Of the 10 patients with ectopic ACTH-producing tumors (Table 1), 3 patients with bronchial carcinoid survived the operation. The six patients with pulmonary small-cell carcinoma and one patient with esophageal cancer died. Therefore, tumor tissues from 3 patients with bronchial carcinoid were obtained at surgery while the other tissues were obtained during autopsy.

**RIA.** Plasma ACTH and cortisol levels were determined by ACTH IRMA kit (Mitsubishiyuka Co. Ltd., Tokyo, Japan) and cortisol RIA kit (Amersham, Arlington Heights, IL) as previously described (13). Tumor tissues were extracted by 0.1 N HCl, and CRH and ACTH levels were determined by RIA as previously described (14).

**Cell culture.** The pituitary adenoma, as well as non-adenomatous tissues and bronchial carcinoid tissues were obtained at surgery. Tissues were dissected and suspended as previously described (1). Aliquots of  $2 \times 10^5$  cells were placed in multiple-well dishes with 2 ml of Hepes-buffered DME containing 10% FCS as previously described (1). After 3 d of culture, the cells were washed with serum-free Hepes-buffered DME containing 0.2% BSA, and were incubated with the test material in 1 ml of fresh medium humidified with 95% air and 5% CO<sub>2</sub> at 37°C for 15 h. After incubation, the medium was removed, and the ACTH levels in the medium were determined by RIA. Three dishes were used for each test material ( $n = 3$ ).

**Northern blot analysis.** Details of isolation of cellular RNA and Northern blot analysis were previously described (1). In brief, total

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1. Abbreviations used in this paper: AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; Dex, dexamethasone; GR, glucocorticoid receptor; IRMA, immunoradiometric assay; POMC, proopiomelanocortin.

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Table I. Plasma ACTH and Cortisol Levels in Patients with Ectopic ACTH Syndrome

Patient	Age	Sex	Tumor	Base		CRH	Metyrapone	Dex	
				ACTH*	F <sup>‡</sup>	ACTH*	ACTH*	ACTH*	F <sup>‡</sup>
1	43	M	lung ca,	242	64	251	262	238	65
2	69	M	lung ca,	141	53	218	248	153	55
3	58	M	lung ca,	148	38	229	261	151	41
4	61	M	lung ca,	860	108	921	889	912	98
5	74	M	lung ca,	755	73	778	782	761	70
6	72	M	esophagus ca	208	108	210	212	205	103
7	42	M	lung ca,	120	32	203	204	128	35
8	33	F	br. carci.	110	79	121	118	52	44
9	44	F	br. carci.	66	39	84	151	28	17
10	44	F	br. carci.	101	66	142	363	44	36

\* pg/ml. F<sup>‡</sup>, cortisol μg/dl. ca, cancer. br. carci., bronchial carcinoid. CRH, CRH test (hCRH 100 μg i.v.). Metyrapone, 1.5 g single dose Metyrapone test. Dex, 8 mg single dose Dex suppression test.

RNA samples in the tumor tissues and cultured cells (5 dishes were pooled for GR mRNA and 1 dish for POMC mRNA) were immediately isolated by the acid guanidinium thiocyanate-phenol-chloroform method (15). RNA samples from tissue extracts were applied to an oligo(dT)-cellulose column, and 0.5 μg of poly(A) RNA samples (for CRH mRNA and GR mRNA) and total RNA samples from cultured cells (1 μg for POMC mRNA and 5 μg for GR mRNA) were denatured and electrophoresed. After electrophoresis the RNA was transferred to a filter, which was then baked. The filter was prehybridized and hybridized thereafter with <sup>32</sup>P-labeled POMC cDNA probe, <sup>32</sup>P-labeled CRH cRNA probe, or <sup>32</sup>P-labeled GR cRNA probe. After washing, filters were exposed to Kodak XAR-5-x-ray film (Eastman Kodak, Tokyo, Japan). The same filter was then rehybridized with a human β-actin probe 21 d later. The results of the autoradiogram were quantified with a scanning densitometer. The levels of CRH, POMC, and GR mRNAs in each sample were corrected for differences in recovery based on the amount of β-actin mRNA detected. The pBSchCRH 1300 plasmid containing a RsaI fragment of human CRH gene and the pBSchGCR 1002 plasmid containing an EcoRI fragment of human GR gene were kindly supplied by Dr. J. A. Majzoub (Harvard Medical School, Boston, MA). The 1.1-Kb SmaI restriction fragment containing the entire third exon of the human POMC gene was supplied by Dr. J. L. Roberts (Mount Sinai School of Medicine, New York, NY). The human β-actin probe was a derivative of pMFβ A-1. The labeled POMC and β-actin cDNA and CRH cRNA were obtained as previously described (1, 15, 16). Radioactive GR cRNA copies were synthesized as previously described for the CRH cRNA probe (15, 16).

**Statistical analysis.** Relative CRH, POMC, and GR mRNA levels were expressed as a percentage of the control value (basal level). The data were statistically evaluated by analysis of variance with Duncan's multiple range test.

**Test materials.** Synthetic human CRH and vasopressin (AVP) were purchased from Peninsula Laboratories (Belmont, LA). Norepinephrine (NE), 8-bromo-cAMP (8-Br-cAMP), Ca-ionophore (A23187), and PMA were obtained from Sigma Chemical Co. (St. Louis, MO).

## Results

**Clinical data.** In all patients with Cushing's disease, plasma ACTH levels increased to more than 200% of the basal level with 100 μg of the CRH test and a 1.5-g single-dose metyrapone test, and decreased to less than 50% of the basal level with the 8-mg single-dose dexamethasone suppression test. In patients with ectopic ACTH syndrome (Table I), plasma ACTH in-

creased to more than 150% of the basal level in 3 patients (patients 2, 3, and 7) and to nearly 150% in 2 patients (patients 9 and 10). In the other 5 patients, plasma ACTH levels did not change. In 3 patients (patients 8, 9, and 10), plasma ACTH and cortisol levels decreased to about 50% of the basal level with the 8-mg single-dose dexamethasone suppression test. In 5 patients, plasma ACTH levels increased with the single-dose metyrapone test. Metyrapone responder also showed an increase in plasma ACTH level to more than 150% or nearly 150% of the basal level with the CRH test.

**Peptide content of ACTH-producing tumors.** In patients with Cushing's disease, ACTH levels in pituitary adenomas were 1.8–10.8 μg/mg wet wt. In ectopic ACTH-producing tumors, ACTH levels in the tissues obtained at surgery were remarkably higher than those in the tumors obtained at autopsies (Table II). CRH was detected in 5 tumor tissues, and the CRH levels in the tissues obtained at surgery were also remarkably higher than those obtained at the autopsies. These 5 patients showed some increase in the plasma ACTH level with the CRH and metyrapone tests. However, in the other 5 patients whose

Table II. ACTH and CRH Levels and POMC, CRH, and GR mRNAs in Ectopic ACTH-producing Tumors

Patient	Peptide		mRNA		
	ACTH	CRH	POMC	CRH	GR
	ng/g wet wt				
1	15	<1	+	–	
2	151	4	+	+	
3	150	42	+	+	
4	321	<1	+	–	
5	61	<1	+	–	
6	4	<1	+	+	
7	15	5	+	+	–
8*	80,000	<1	+	+	+
9*	22,600	166	+	+	+
10*	405,000	47	+	+	+

\* Obtained at surgery. All others were obtained at autopsy.

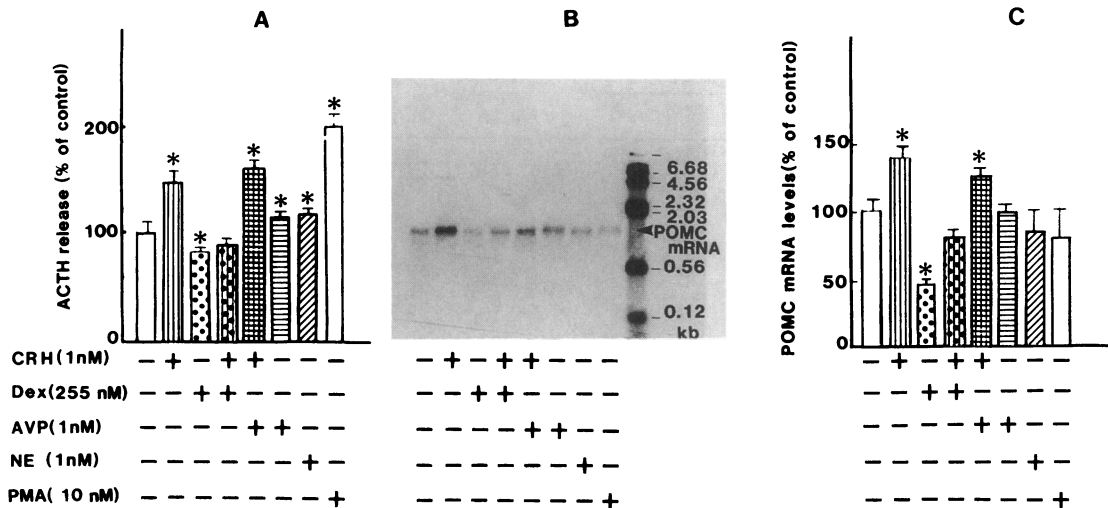


Figure 1. Effects of ACTH secretagogues on ACTH release (A) and POMC gene expression (B and C) in pituitary nonadenoma cells from five patients with Cushing's disease after 15 h of incubation. (B) An example of Northern blot of POMC mRNA. (C) Relative changes in POMC mRNA levels compared to control values. Values are the mean  $\pm$  SE.  $n = 5$  patients.

tumor tissues did not contain CRH, plasma ACTH levels did not change after CRH or metyrapone administration.

**ACTH secretion and POMC mRNA in vitro.** In pituitary nonadenoma cells, basal ACTH levels were 0.21–0.37 ng/dish per 15 h. ACTH secretion was stimulated by CRH, AVP, NE, and PMA, and was inhibited by Dex (Fig. 1). There was no POMC mRNA size heterogeneity in the pituitary tissues. In the nonadenoma cells, the POMC mRNA level was increased by CRH but not by AVP, NE, or PMA (Fig. 1). AVP failed to enhance the CRH-induced POMC mRNA level. Dex inhibited POMC gene expression.

In pituitary adenoma cells, basal ACTH levels were 12–121 ng/dish per 15 h. ACTH secretion was stimulated by CRH, AVP, NE, and PMA, but was inhibited by Dex (Fig. 2). The POMC mRNA level was increased by CRH, but not by NE, AVP, or PMA as in the nonadenoma cells (Fig. 2). Neither AVP nor PMA affected basal and CRH-induced POMC mRNA levels.

In 10 ectopic ACTH-producing tumors, the major part of POMC mRNA was authentic-size, while short-size POMC mRNA accounting for less than 10% of the total amount of POMC mRNA. In ectopic ACTH-producing tumors from patients 8, 9, and 10, basal ACTH levels were 14–92 ng/dish per 15 h. CRH, Ca-ionophore, PMA, and 8-Br-cAMP increased ACTH release and POMC mRNA levels, although AVP stimulated only ACTH release (Fig. 3). Dex decreased the POMC mRNA level. The tumors of patients 8, 9, and 10 exhibited GR mRNA and their plasma ACTH levels were decreased by Dex. In patient 7, plasma ACTH was not decreased by Dex and GR mRNA was not detected in the tumor.

**CRH mRNA.** CRH mRNA was detected in 7 ectopic ACTH-producing tumors (Table II and Fig. 4). In patient 8, only a single band of long-size CRH mRNA ( $\sim 2.0$  kb nucleotides) was detected. In patient 6, long-size and a small amount of authentic-size ( $\sim 1.5$  kb nucleotides) CRH mRNAs were detected. Significant amounts of long- and authentic-size CRH

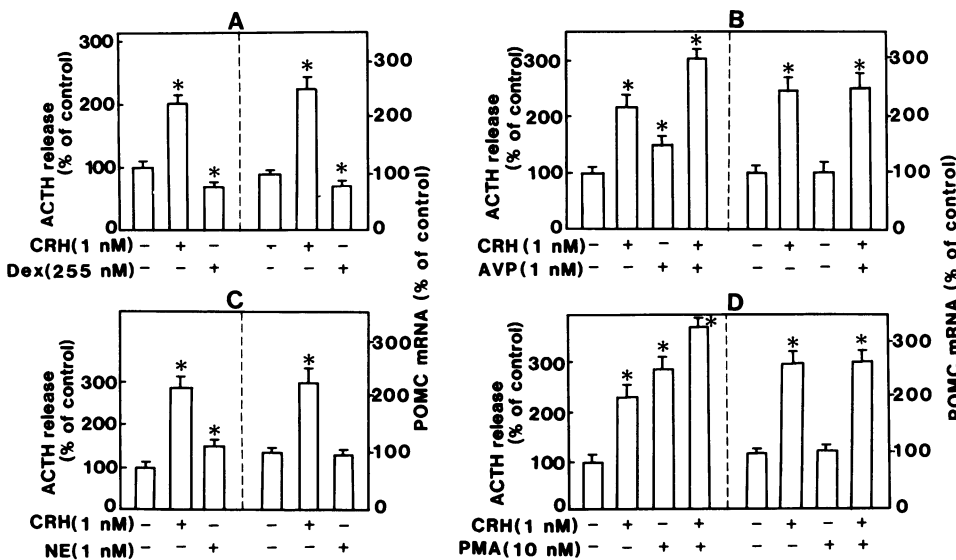


Figure 2. Effects of CRH, AVP, NE, and PMA on ACTH release and POMC gene expression in pituitary adenoma cells from 10 patients with Cushing's disease. In each figure, ACTH release is shown on the left, and the POMC mRNA level is shown on the right.  $*P < 0.05$  vs. the control value. Values are the mean  $\pm$  SEM.  $n = 10$  patients.

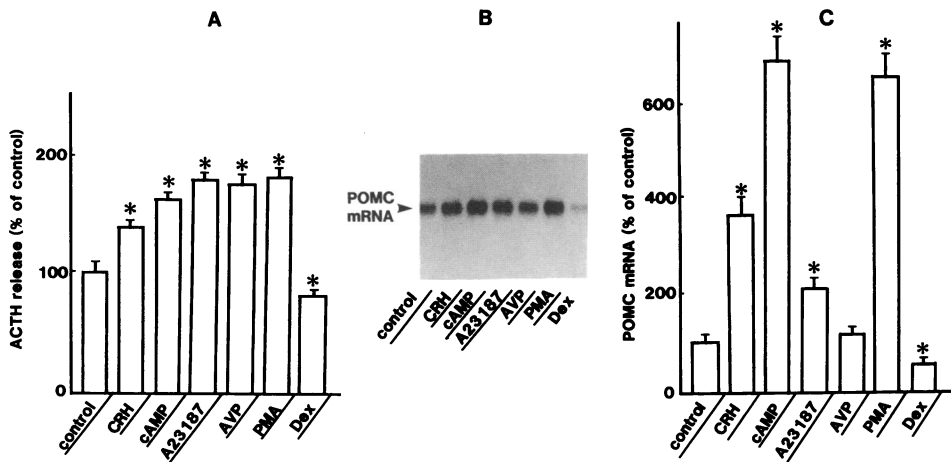


Figure 3. Effects of Dex, CRH, AVP, 8-Br-AMP (cAMP), PMA, and Ca-ionophore (A23187) on ACTH release (left) and POMC gene expression (middle and right) in bronchial carcinoid cells from patients 8, 9, and 10. An example of Northern blot analysis of POMC mRNA (middle) and relative changes in POMC mRNA levels compared to control values (right). Values are mean  $\pm$  SEM ( $n = 3$  patients).

mRNAs were detected in patients 7 and 10. Only authentic-size CRH mRNA was detected in patients 2, 3, and 9. In patients 1, 4, and 5, there was no authentic CRH mRNA or only a small amount of authentic CRH mRNA in their tumors, and plasma ACTH did not increase with the CRH test.

**GR mRNA.** Three bands (7.1 k, 6.1 k, and 5.6 k nucleotides) of GR mRNA were detected in all pituitary adenomas from patients with Cushing's disease. In cultured pituitary adenoma cells, GR mRNAs were down-regulated by CRH and Dex (Fig. 5), with the inhibitory effect of Dex being greater than that of CRH. CRH enhanced Dex-induced down-regulation of GR mRNA.

In ectopic ACTH syndrome, GR mRNAs were detected in bronchial carcinoid tumors of patients 8, 9, and 10, but were not detected in the tumor of patient 7 (Fig. 6). Plasma ACTH decreased to about 50% of the basal level with the 8-mg single-dose Dex suppression test in patients 8, 9, and 10, although it did not decrease in patient 7.

## Discussion

In clinical studies, plasma ACTH levels were increased by CRH and metyrapone in all patients with Cushing's disease and in five patients with ectopic ACTH syndrome. In these latter five patients, CRH was detected in the tumor, while tumor CRH was not detected in other patients whose plasma ACTH levels did not change after CRH administration. This result suggests that the presence of tumor CRH is necessary for

the ACTH response to CRH and metyrapone in patients with ectopic ACTH syndrome.

CRH mRNA was detected in 7 ectopic ACTH-producing tumors, and a significant amount of CRH peptide was detected in 5 of the 7 tumors. In the 2 tumors in which CRH peptide was not detected, long-size CRH mRNA was predominant and either no short-size CRH mRNA or a small amount of short-size CRH mRNA was detected. This result suggests that long-size CRH mRNA is not translated. This is one of the characteristics of ectopic ACTH-producing tumors. The difference in the size of CRH mRNA may be the result of an aberrant initiation of transcription, alternate splicing or different poly(A) tails. PMA increases the poly(A) tail length of CRH mRNA in transfected cells (17), and such long-size CRH mRNA may increase stability and translatability as shown in AVP mRNA (18). However, the present study indicates that this long-size CRH mRNA is not compatible to the CRH mRNA in the ectopic ACTH-producing tumors.

The human CRH gene was reported to have multiple transcription initiation sites (19) and two TATA boxes, authentic and proximal TATA I and distal TATA II which is a less efficient transcription site (20). There was no size heterogeneity in the CRH mRNA from different human tissues (21); however, CRH transcripts in the adrenal gland and testis were about 200 and 500 bases longer, respectively than those found in the brain of a rat (22). Therefore, there may be a tissue specificity for the transcription start site. A difference in the size of transcripts has been reported in the POMC mRNA of ectopic ACTH-producing tumors (9-12). Usually, short-size POMC transcripts have

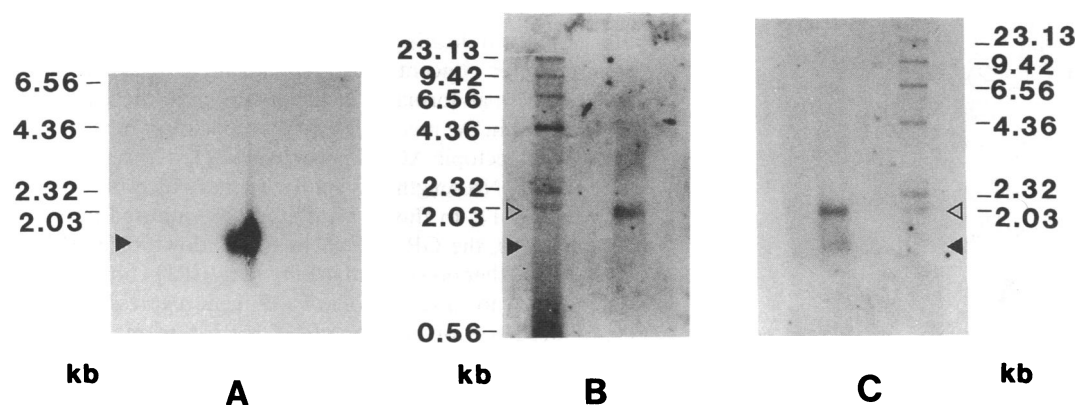


Figure 4. Heterogeneity of CRH mRNA in ectopic ACTH-producing tumors. ( $\blacktriangle$ ) authentic-size CRH mRNA (1.5 kb nucleotides). ( $\triangle$ ) long-size CRH mRNA (2.0 kb nucleotides) (A) patient 9, (B) patient 8, (C) patient 10.

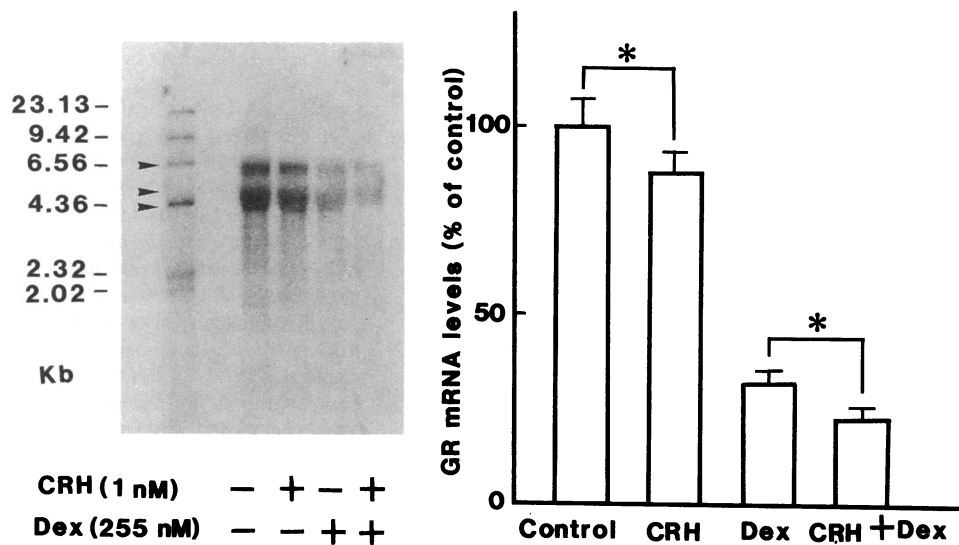


Figure 5. Effects of CRH and Dex on GR gene expression in pituitary adenoma cells from patients with Cushing's disease (15-h incubation). An example of Northern blot analysis of GR mRNA in corticotroph adenoma cells from a patient with Cushing's disease (left, 7.1, 6.1, and 5.6 k nucleotides) and relative changes in GR mRNA levels compared to the control values. Values are mean  $\pm$  SEM ( $n = 1$  in each patient, with total of 10 patients)

either no translatability or diminished translatability, although long-size transcripts are translatable (9–12). In the present study, authentic-size POMC mRNA was predominant in all ACTH-producing tumors.

In the *in vitro* study of cultured pituitary adenoma and nonadenoma cells, CRH stimulated both ACTH release and POMC gene expression, while AVP, NE, and PMA stimulated ACTH release but failed to increase the POMC mRNA level. This result is in good agreement with our previous result showing that the CRH-protein kinase A system stimulates both ACTH release and POMC gene expression, while protein kinase C-related ACTH secretagogues (AVP, NE, and PMA) only stimulate ACTH release in rat anterior pituitary cells (1).

In ectopic ACTH-producing tumor cells, CRH, 8-Br-cAMP, and Ca-ionophore increased the POMC mRNA level. This result indicates that both the accumulation of cytoplasmic cAMP and the increase in the cytoplasmic  $Ca^{++}$  level stimulate ACTH release and POMC gene expression in ectopic ACTH-producing carcinoid cells, as well as in pituitary corticotroph cells (23, 24). On the other hand, AVP stimulated ACTH release, although it did not increase the POMC mRNA level in

the carcinoid cells as in the pituitary corticotroph cells (1). However, PMA stimulated both ACTH release and POMC gene expression in the carcinoid cells. This stimulatory effect of PMA on POMC gene expression could also be observed in AtT-20 cells from a mouse anterior pituitary corticotroph tumor cell line (25). This PMA effect differs from the PMA effect on pituitary adenoma and nonadenoma cells. PMA-induced increase in the POMC mRNA level is also one of the characteristics of ectopic ACTH-producing tumors.

The action site where the CRH-protein kinase A system stimulates transcription is the cAMP-response element (CRE) of the POMC gene. The PMA-protein kinase C system generally acts on the phorbol ester response element (TRE/AP-1 site). The POMC gene has an AP-1 site-like sequence as well as CRE (26). One possible explanation for this different effect of PMA on POMC gene expression is a tissue-specific transcription factor. The PMA-stimulated transcription factor may not be present in the pituitary corticotroph cells, but rather may exist in the carcinoid or AtT-20 cells. AVP is also a protein kinase C-related ACTH secretagogue, although it failed to increase POMC mRNA levels. AVP-induced activation of protein kinase C may be less potent than PMA-induced activation.

GR mRNA was detected in all pituitary adenomas of patients with Cushing's disease, and in 3 out of 4 ectopic ACTH-producing tumors. Interestingly, these 3 tumors were bronchial carcinoid and their plasma ACTH levels were inhibited by Dex. In another patient whose tumor did not have GR mRNA, plasma ACTH was not inhibited by Dex. This result agrees with evidence indicating that there is a direct correlation between receptor concentration and hormone sensitivity (27, 28). However, abnormality of the postreceptor mechanism is also included in the mechanism of glucocorticoid resistance in patients with ectopic ACTH syndrome (29). The heterogeneity of GR mRNA length was similar to that noted in previous reports (30, 31). In this *in vitro* study on cultured pituitary adenoma cells, the GR mRNA level was down-regulated by CRH, and further down-regulated by Dex. CRH enhanced the Dex-induced down-regulation of GR gene expression. This Dex-induced down-regulation of GR mRNA seems to be one of the reasons for the resistance of ACTH release and POMC gene expression to the Dex suppression test in patients with

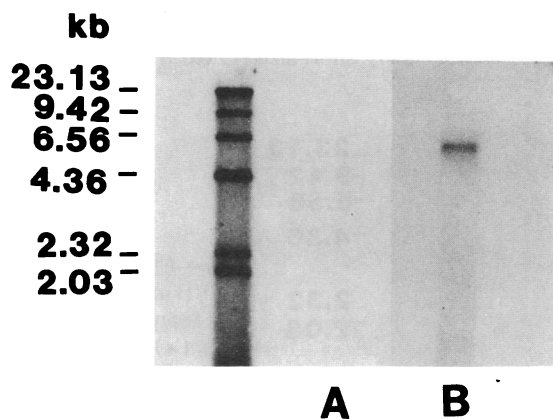


Figure 6. Northern blot analysis of GR mRNA in ectopic ACTH-producing tumors. (A) patient 7 (GR mRNA, not detected), (B) patient 9 (GR mRNA, detected)

Cushing's disease. Autoregulation of GR gene expression is quite complex and occurs at the transcriptional, posttranscriptional, or posttranslational levels (32, 33). CRH-induced down-regulation of GR gene expression is also observed in AtT/20 cells (34). The effect of CRH on GR gene expression is believed to be regulated through the adenylate cyclase system (27, 34). Enhancement of Dex-induced down-regulation of GR mRNA by CRH suggests a different mechanism of down-regulation by Dex and CRH.

From these results, it can be said that ectopic ACTH-producing tumors have the following characteristics; (a) there are at least two ectopic ACTH syndrome subtypes, tumors containing ACTH with CRH and those without CRH; (b) tumors that responded to exogenous CRH contained CRH and authentic-size CRH mRNA; (c) some tumors contained long-size CRH mRNA, which did not seem to be translated; (d) in bronchial carcinoid cells, CRH and PMA increased and Dex decreased ACTH release and POMC mRNA levels. Therefore, it can be concluded that CRH and Dex tests have limited usefulness in differentiating between Cushing's disease and ectopic ACTH syndrome, particularly due to bronchial carcinoid.

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