# Glucocorticoid Regulation of Proopiomelanocortin Messenger Ribonucleic Acid Content of Rat Hypothalamus

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**We have verified the possibility that the POMC gene of the rat hypothalamus might be subject to regulation by glucocorticoids. Adrenalectomy increased the concentration of POMC mRNA in anterior pituitary and in hypothalamus, but not in the neurointermediate lobe of the pituitary gland. Dexamethasone and, to a slightly lesser extent, corticosterone treatments reversed the adrenalectomy-induced increase in POMC mRNA concentrations in both anterior pituitary and hypothalamus. Dexamethasone caused a slight decrease of POMC mRNA levels in the neurointermediate lobe of the pituitary gland, while corticosterone had no effect. These results indicate that the POMC gene of the rat brain hypothalamus is also under negative control by glucocorticoids. (Molecular Endocrinology 2: 727–731, 1988)**

## INTRODUCTION

ACTH, $\beta$-endorphin ($\beta$-end), and $\alpha$-MSH are derived from the same precursor, POMC. The nucleotide sequence of the POMC gene was first described in the bovine pituitary gland (1) and later in the rat and porcine gland (2–4). Although metallothionein-IIa (5), $\alpha_1$-acid glycoprotein (6), and the GH gene (7–9) are all under positive control by glucocorticoids, the POMC gene in anterior pituitary gland is under negative control by these steroids (10, 11) and under positive control by CRF (12).

The POMC gene is expressed in the intermediate lobe of the pituitary gland but it is not regulated by glucocorticoids (10). The reason for this is perhaps a lack of glucocorticoid receptor in this tissue (10). The presence of POMC-related peptides in the hypothalamus (13) raises the question as to whether or not the POMC gene in this tissue is also regulated by glucocorticoids. This question is of importance in view of the well known innervation of the paraventricular nucleus of the hypothalamus by ACTH and $\beta$-end-containing terminals (14) and of the stimulatory role of opioid peptides in the control of the secretion of CRF (15). Recent immunohistochemical studies have also demonstrated the presence of glucocorticoid receptor immunoreactivity in numerous monoaminergic neurons including those of the arcuate nucleus (16). Finally, it is of primordial importance to gain a better understanding of the limbic-hypothalamic mechanisms regulating ACTH secretion, particularly in view of the ACTH and CRF hypersecretion observed in patients suffering from endogenous depressive illness (17–19) and of the abnormal hypothalamic-pituitary-adrenal function in anorexia nervosa (20).

Our results demonstrate a negative regulation of hypothalamic POMC gene expression by glucocorticoids.

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## RESULTS

As expected, plasma ACTH levels were greatly increased by adrenalectomy (1564 ± 290 pg/ml, n = 22, for ADX *vs.* 110 ± 30 pg/ml, n = 30, for intact animals) and brought back to normal levels by treatment with corticosterone (CORT) (85 ± 10 pg/ml, n = 28) or dexamethasone (DEX) (78 ± 28 pg/mL, n = 25). It is worthwhile to note that neither CORT nor DEX decreased plasma ACTH below normal values (calculated Q = 0.59 and 0.70, respectively, while theoric Q for $P < 0.05 = 2.77$ and 2.92, respectively). This clearly indicates that the replacement doses of CORT or DEX used resulted in the equivalent effects of physiological mean values of circulating levels of glucocorticoids.

In agreement with the increased plasma ACTH concentrations observed after adrenalectomy, the POMC mRNA content of the anterior pituitary was also increased (Fig. 1A) while DEX reversed this action. In contrast to its effect on plasma ACTH values, CORT failed to completely reverse the effects of ADX on POMC mRNA levels, indicating a differential sensitivity of POMC mRNA synthesis and/or degradation and of



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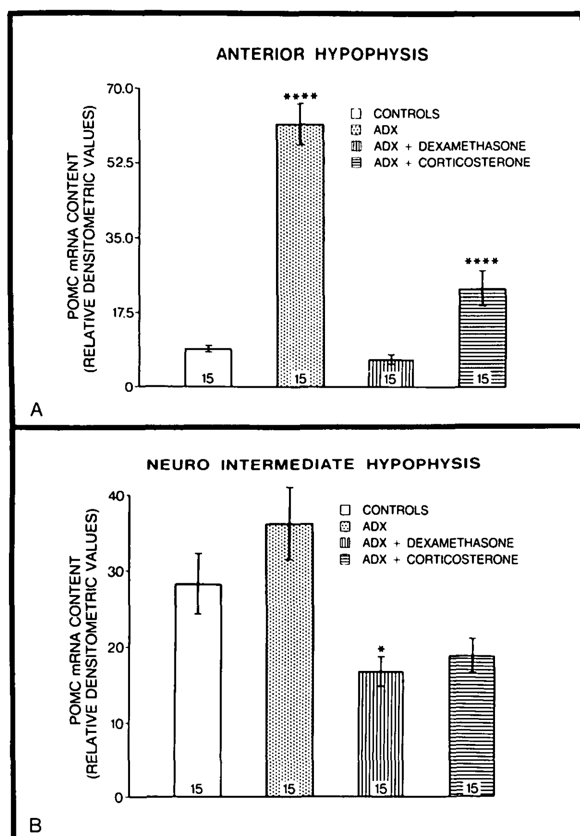


**Fig. 1.** Relative POMC mRNA Content of the Anterior Pituitary (A) or Neurointermediate Lobe (B) in the Four Experimental Groups of Animals Described in *Materials and Methods*

$P < 0.001$ when compared to sham-operated control; *, $P < 0.05$ when compared to sham-operated control. *Arrow bars* represent SEM. The number of separate experiments is indicated at the *bottom of each column.*

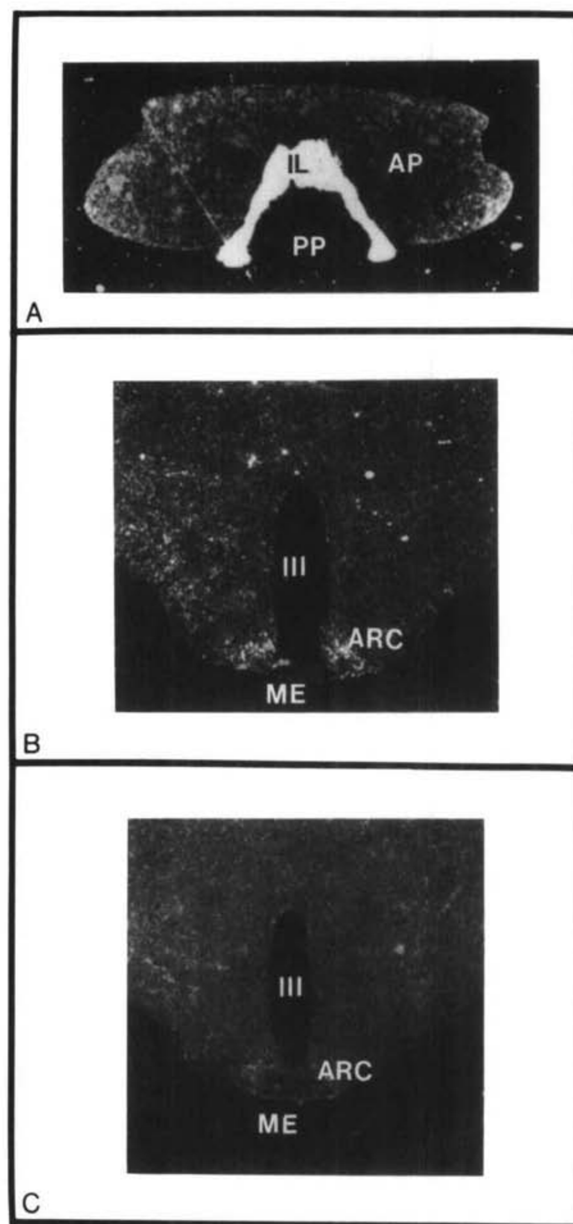


**Fig. 2.** *In situ* Hybridization Using $\alpha$-[$^{32}$P]UTP-Labeled cRNA (Anti-Sense) Probe (A, B) or $\alpha$-[$^{32}$P]UTP-Labeled mRNA (Sense) Strand (C) in the Pituitary (A) and in the Hypothalamus (B, C)

Specific hybridization signal was observed in the intermediate lobe of the pituitary (A), the anterior lobe of the pituitary (A), and the arcuate nucleus of the hypothalamus (B). No hybridization signal was obtained with the $\alpha$-[$^{32}$P]UTP-labeled mRNA (sense) strand (C). AP, Anterior Pituitary; ARC, arcuate nucleus of the hypothalamus; III, third ventricle; IL, intermediate lobe of the pituitary gland; ME, median eminence; PP, posterior lobe of the pituitary gland.

ACTH secretion to this steroid. Adrenalectomy did not influence the concentration of POMC mRNA in the neurointermediate lobe of the pituitary (Fig. 1B). DEX, however, caused a slight decrease in POMC mRNA levels ($P < 0.05$) while CORT was devoid of any significant effect in this structure. These control experiments performed on anterior and neurointermediate lobes of pituitary gland agree with those of others (10) and indicate the specificity of our hybridization probe used. Anatomical specificity was established by *in situ* hybridization. Figure 2 illustrates that the probe hybridizes very strongly in the intermediate lobe of the pituitary gland. In addition, radioautographic grains were observed in the anterior pituitary (Fig. 2A) and the arcuate nucleus of the hypothalamus (Fig. 2B). Moreover, hybridization using $^{32}$P-labeled POMC sense (*i.e.* mRNA sequence) strands generated from pGEM 1 vector POMC cDNA insert by SP6 RNA polymerase run-off transcription, failed to generate hybridization signal (Fig. 2C).

Northern blot analysis indicates that the POMC mRNA content of the hypothalamus was strikingly increased 6 days after adrenalectomy (Fig. 3). Both DEX and CORT treatments caused a reversal of this increase in adrenalectomized (ADX) animals, although CORT appeared to be less potent in this respect than was DEX. In order to obtain statistical confirmation of these effects, hypothalamic POMC mRNA from each of 21 experiments in the four treatment groups was individ-

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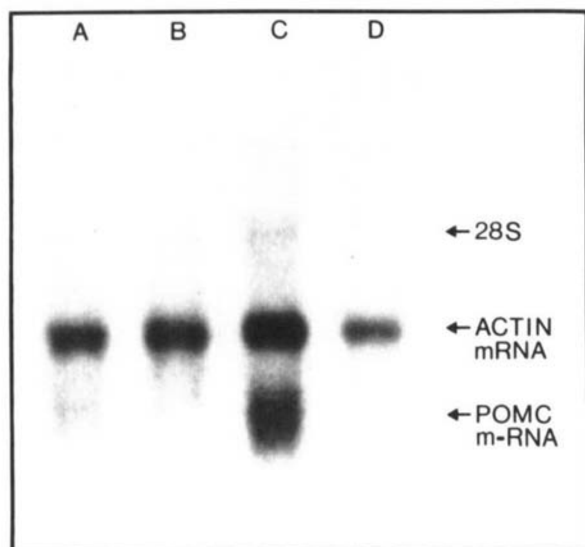


**Fig. 3.** Northern Blot Analysis of POMC mRNA Relative to β-Actin mRNA Levels in the Hypothalamus

RNA from animals with the following treatments is shown: lane A, ADX + CORT; lane B, ADX + DEX; lane C, ADX; lane D, sham operated.

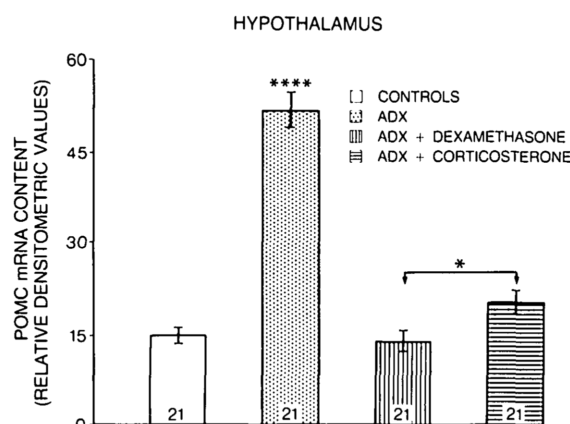


**Fig. 4.** Relative POMC mRNA Content of the Hypothalamus of the Four Experimental Groups of Animals

CORT was less effective than DEX treatment ($P < 0.05$) in restoring POMC mRNA content to normal values in ADX animals. ****, $P < 0.001$ when compared to sham-operated control; *, $P < 0.05$ when compared to ADX-DEX-treated group. *Arrow bars* represent SEM. The number of separate experiments is indicated at the bottom of each column.

ually analyzed by dot blot hybridization techniques. The results shown in Fig. 4 clearly demonstrate the significance of the ADX-induced increase in POMC mRNA content of the hypothalamus and its complete reversal by DEX. CORT replacement therapy also reversed the effect of ADX on POMC mRNA, although the concentration remained slightly, but significantly, greater than that of ADX-DEX-treated animals.

## DISCUSSION

Our results suggest the presence of a glucocorticoid-regulated POMC gene in rat brain hypothalamus. The presence of POMC mRNA in rat hypothalamus has been shown by solution hybridization (21), Northern blots, and *in situ* hybridization (22) and immunocytochemical evidence have demonstrated the presence of POMC-derived peptides (13, 23–25). In some studies, this latter methodological approach failed to disclose any glucocorticoid-sensitive mechanism of regulation of POMC-derived peptides in the hypothalamus (23, 26, 27) while in others (28) an increased concentration of β-end in rat hypothalamus, midbrain, and hindbrain after adrenalectomy was reported. This latter conclusion is supported by our results which indicate an increased POMC mRNA concentration in the hypothalamus 6 days after adrenalectomy. This increase is reversed by glucocorticoid treatment and the relevance of this effect is emphasized by our use of doses of CORT or DEX reported to restore physiological concentrations of circulating glucocorticoids without any metabolic side effects, particularly on protein catabolism (29, 30).

Birnberg *et al.* (31) reported an absence of effects of adrenalectomy or glucocorticoid treatment on the level of POMC mRNA content in the hypothalamus 8 days and 16 days after adrenalectomy. The reason for this discrepancy remains obscure, but the great variability of the values of POMC mRNA content that they report and the use of small number of tissue samples (5) in comparison to us (21) may have contributed to their negative findings since the hypothalamus contains but little POMC mRNA compared to anterior or intermediate lobes of the pituitary gland (21). Our techniques permitted confirmation of the effects of ADX and glucocorticoids in these latter two tissues (10–12, 22, 31) and thus demonstrated the specificity of our probe and hybridization stringency conditions. This is further demonstrated by *in situ* hybridization experiments which demonstrate that our probe only hybridizes with material in the POMC-containing arcuate nucleus region of the hypothalamus. The presence of the necessary mechanisms for glucocorticoid-mediated responses of arcuate nucleus neurons is provided by immunocytochemical studies which have shown that 50% of the neurons in the parvocellular arcuate nucleus and 20% of the magnocellular part of this nucleus are glucocorticoid receptor-like immunopositive (32). Moreover, glucocorticoid receptor-like immunoreactivity has been colocalized in the dopaminergic neurons of the hypothalamic arcuate nucleus (16).

Further studies are required to fully understand the role of POMC gene products in the arcuate nucleus and the reason for their negative regulation by glucocorticoids. One possibility could be the implication of the β-end terminals, which innervate the paraventricular nucleus of the hypothalamus (33), in CRF and/or vasopressin secretion.

## MATERIALS AND METHODS

### Animals

Sprague-Dawley rats (Charles River Canada, St. Constant, Quebec, Canada) weighing between 225–250 and 300–325

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g, were divided into four groups: sham-ADX, ADX + CORT treatment, and ADX + DEX treatment. All ADX animals (treated or not) were provided with saline (0.8% NaCl + 0.1% KCl) *ad libitum*. Sham-ADX received water. All animals were kept two per cage with food *ad libitum* and under 10-h dark, 14-h light cycle.

### Treatments

Surgical procedures were performed on day 0. All animals were weighed on day 1 and were injected with vehicle or hormone between 0900 and 1100 hr each day thereafter for a period of 7 days. Sham, and one group of ADX animals, received the vehicle (1% ethanol in corn oil; 0.1 ml/100 g, BW). CORT and DEX were administered to the other ADX groups in the same vehicle and at the respective doses of 2.5 mg/100 g, BW (2.5 mg/0.2 ml vehicle) and of 0.1 mg/100 g, BW (0.1 mg/0.2 ml vehicle). Twenty-one different experiments were performed according to this protocol with 15 animals per group.

### Isolation of Total Cellular RNA

Rats were decapitated on the seventh day of treatment, 4 h after the last injection. Brains were rapidly removed and three areas were dissected: the anterior and neurointermediate lobes of the pituitary gland and the hypothalamus. Tissues were pooled according to their group of origin and were homogenized with a Polytron apparatus in 4 M guanidinium isothiocyanate, 5 mM sodium citrate (pH 7.0), 0.1 M $\beta$-mercaptoethanol, 0.5% (wt/vol) sarkosyl and 0.33% (vol/vol) antifoam-A (Sigma, St. Louis, MO). Homogenates were prepared for ultracentrifugation on cesium chloride gradients as described by Maniatis *et al.* (34). After centrifugation at 36,000 rpm (Beckman SW-60 or SW-40 rotor) for 20 h, the total RNA pellet was redissolved in 1 × TE (34) containing 0.25% (wt/vol) sodium dodecyl sulfate (SDS) and extracted with phenol-chloroform before precipitation with ethanol from 0.3 M Na acetate.

### Blotting Procedures

Samples of RNA (in ethanol) were centrifuged at 12,000 × *g*, 15 min at 4 C. The pellets were dried and resuspended in 100 $\mu$l 1× TE. Aliquots of 5 $\mu$l were diluted in 400 $\mu$l (final) of 1× TE. Absorbance was measured with a DU-6 Beckman spectrophotometer at 260 and 280 nm in order to determine the quantity and the purity of the RNA preparation.

Eight serial 1:1 dilutions of each sample were prepared in order to deposit on nitrocellulose filters a maximal amount of 5 $\mu$g RNA for the anterior and neurointermediate pituitary lobes and of 30 $\mu$g for the hypothalamus. All dilutions were made in 10× SSC. Blotting and nitrocellulose filters were presoaked in water (10 min) and then in 10× SSC (10 min). Nitrocellulose filters were baked at 80 C under vacuum for 2 h to fix the RNA.

### Northern Blots

Agarose gel electrophoresis of formaldehyde-denatured RNA was done according to standard procedures (34) and transferred to nitrocellulose filters.

### Preparation of POMC cDNA and cRNA Probes

The rat cDNA probe used was a 750 base pair fragment corresponding to most of the exon 3 of rat POMC gene and was a generous gift from Dr. J. Drouin (Institut de Recherche Clinique de Montréal, Montreal, Quebec, Canada). The cDNA probe was labeled with $\alpha$-$^{32}$P-dCTP (2000–3000 Ci/mmol, Amersham, Arlington Heights, IL) by standard nick translation techniques. The labeled probe was purified by elutions on Sephadex G-50 in 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA. The 750 bp fragment was also subcloned into the plasmid pGEM 1 and $\alpha$-$^{32}$P-UTP-labeled cRNA probes were prepared from this plasmid by T7 RNA polymerase run-off transcription of the cDNA insert. Complementary RNA probes for $\beta$-actin mRNA were generated by SP6 RNA polymerase from a 1.5 kilobase pair $\beta$-actin *PstI* fragment inserted in pGEM-2.

### Filter Hybridization Procedures

Filters were prehybridized for 2 h at 42 C in the following buffer: 50% (vol/vol) formamide, 5× SSC, 5× Denhardt, 0.1% SDS, 50 mM phosphate, and 0.025% (wt/vol) denatured salmon sperm DNA. Hybridization buffer was composed of four parts of prehybridization buffer, one part of 50% dextran sulfate and 18–20 × $10^6$ cpm (for pituitary gland) or 30 × $10^6$ cpm (for hypothalamus) of denatured labeled cDNA probe in a final volume of 15 ml. Hybridization took place at 42 C for 20 h. Filters were washed two times (10 min each) in 2× SSC, 0.1% SDS at room temperature and once (15 min) in 0.1× SSC, 0.1% SDS at 55 C. The filters were then wrapped in Saran wrap and put in cassettes with Kodak XOmat R films either with or without intensifying screens.

### *In Situ* Hybridization

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and paraffin-embedded according to standard procedures. Seven micrometer thick slices were mounted on slides and hybridized during 24 h at 60 C according to the procedure of Watson *et al.* (35). $^3$H-Labeled cRNA probes were used in these experiments. The slides were covered with Kodak NTB-2 emulsion and exposed during 40–50 days in the dark at 4 C before developing in Kodak D-19 developer. Slides were stained with cresyl violet before viewing under dark field illumination.

### Data Analysis

Films were analyzed with Joyce and Loebl microdensitometer. The range of total RNA quantities deposited on filters allowed us to obtain a linear relationship between these quantities and the relative densitometric values of hybridization signal. All densitometric analyses were performed in the range of RNA concentrations responding to this criteria of linearity. Each film was scanned over three to four series of data, each series corresponding to an equal amount of RNA deposited in each experimental group and the slope calculated by linear regression. Statistical analysis was performed with the Duncan-Kramer (36) test after analysis of variance.

### ACTH RIA

Blood samples were centrifuged at 1500 × *g*, the plasma was removed and assayed for its ACTH content by specific RIA (37). The intra- and interassay coefficients of variance were less than 7% and 12%, respectively.

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