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Glucocorticoid effect on the level of corticotropin messenger RNA activity in rat pituitary

(adrenalectomy/wheat germ extract/cell-free protein synthesis/immunoprecipitation/sodium dodecyl sulfate–polyacrylamide gel electrophoresis)

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ABSTRACT **In an attempt to understand the molecular mechanism underlying the depressive effect of glucocorticoids on corticotropin production, the level of corticotropin messenger RNA activity in rat pituitaries was measured with the use of the cell-free protein-synthesizing system derived from wheat germ. The large translation product of corticotropin messenger RNA was identified and quantitated by indirect immunoprecipitation with antibody against corticotropin. The level of corticotropin messenger RNA activity was increased 3- to 6-fold by adrenalectomy. Dexamethasone administration to adrenalectomized rats resulted in a marked suppression of corticotropin messenger RNA activity. Cortisol and corticosterone also exhibited a suppressive effect but were less effective than dexamethasone. In contrast, nonglucocorticoids such as progesterone and aldosterone had no suppressive effect. These results indicate that at least part of the glucocorticoid effect on corticotropin production in the pituitary is exerted at the pretranslational level.**

It is well known that glucocorticoids markedly depress the level of corticotropin (adrenocorticotropic hormone, ACTH) in blood plasma (1). Accumulating evidence indicates that at least part of this depressive effect is due to a direct action of glucocorticoids on the pituitary (1). However, the molecular mechanism underlying this glucocorticoid action has not been elucidated yet. Using a mouse pituitary tumor cell line, Watanabe *et al.* (2) observed a decrease in both the intracellular and the extracellular level of ACTH after treatment with glucocorticoids, thus suggesting that the primary effect of glucocorticoids is exerted on the production of ACTH rather than on its release. On the other hand, studies by Fleischer and Rawls (3) with cultured rat pituitary cells suggested a depressive effect of glucocorticoids on the release of ACTH, although at high glucocorticoid concentrations the production of ACTH appeared to be reduced as well. If glucocorticoids indeed diminish ACTH production, their effect may involve an action at the level of transcription, translation, or post-translational processes such as cleavage of a precursor.

Our previous work has demonstrated that poly(A)-containing RNA prepared from bovine anterior pituitaries directs the synthesis of a large molecular form of ACTH in the cell-free system derived from wheat germ (4). We have also shown that this assay system serves to quantitate the mRNA coding for ACTH. In the present investigation, we have assessed the level of ACTH mRNA activity in rat pituitaries with the use of this technique in order to explore the molecular mechanism responsible for the depressive effect of glucocorticoids on ACTH production. The results obtained have demonstrated that the level of ACTH mRNA activity in pituitaries is increased markedly by adrenalectomy and is reduced specifically by glucocorticoids. This finding indicates that at least part of the glucocorticoid effect is exerted at the pretranslational level.

MATERIALS AND METHODS

Animals. Male Wistar-strain rats were used in all experiments. All rats were maintained under conditions of controlled lighting (lights on 6 a.m., off 6 p.m.) and temperature (24°) and were given a balanced diet (Funahashi, Tokyo, Japan) and water ad libitum. Rats weighing 130–160 g were adrenalectomized by the dorsal approach after being anesthetized by an intraperitoneal injection of sodium pentobarbital (7.5 mg/100 g of body weight). Adrenalectomized rats were subsequently maintained on drinking water containing 0.9% NaCl. When indicated, steroid hormones were given in the drinking water containing 0.9% NaCl. Groups of experimental rats and normal controls of the same age (at least five rats per group) were sacrificed by decapitation between 9:30 and 10 a.m. on the indicated postoperative days, and whole pituitaries were removed. Plasma was separated from the blood collected by cardiac puncture and was frozen for subsequent determination of 11-hydroxycorticosteroids.

Isolation of RNA. Pituitaries from each group of rats were pooled and homogenized at 0° with a Teflon–glass homogenizer in 5 ml of 0.25 M sucrose containing 25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, and 0.1% diethylpyrocarbonate. The homogenate was centrifuged at 1000 × *g* for 10 min. To the resulting supernatant were added sodium deoxycholate and Triton X-100 at a final concentration of 1% each. After homogenization with a Teflon–glass homogenizer, the mixture was centrifuged at 10,000 × *g* for 10 min. From the supernatant collected, RNA was extracted by sodium dodecyl sulfate ($NaDodSO_4$)/phenol/chloroform/isoamyl alcohol treatment and precipitated with cold ethanol as described previously (4). The RNA pellet was washed with 2 M LiCl and then dissolved in distilled water. Finally, the RNA was collected by cold ethanol precipitation. The yield of RNA was 55–75 μg per five pituitaries for all groups of rats.

Cell-Free Protein Synthesis and Immunoprecipitation. Assay of cell-free protein synthesis in the wheat germ system and indirect immunoprecipitation of the translation product related to ACTH with antibody against ACTH were performed as described previously (4). The synthesis of total protein was measured by determining L-[4,5-^{3}H]leucine incorporation into trichloroacetic acid-insoluble material in the postribosomal supernatant of the assay mixture as described previously (4).

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Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); ACTH-(1–24), synthetic peptide with residues 1–24 of the amino acid sequence of ACTH; ACTH-(1–18), synthetic peptide with residues 2–18 of the amino acid sequence of ACTH and glycine at the amino terminus; $NaDodSO_4$, sodium dodecyl sulfate.

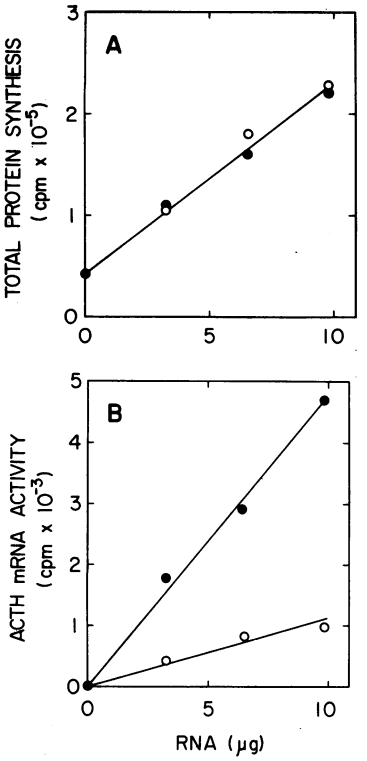

FIG. 1. Effects of various amounts of RNA on [^{3}H]leucine incorporation into total protein (*A*) and the translation product related to ACTH (*B*). The indicated amounts of pituitary RNA derived from normal rats (○) and from rats at 7 days after adrenalectomy (●) were translated. For further details, see *Materials and Methods.*

The synthesis of the translation product related to ACTH was measured by subtracting the radioactivity present in the immunoprecipitate formed with control IgG from that present in the immunoprecipitate formed with anti-ACTH. All radioactivity values given for the synthesis of total protein as well as of the translation product related to ACTH represent those per 50 μl of the assay mixture. The preparation and characterization of anti-ACTH were described previously (4). Unless otherwise stated, antibody directed to amino acid residues 19–24 of the ACTH sequence was used.

Reagents and Analytical Methods. Dexamethasone 21-phosphate, progesterone, aldosterone, and corticosterone were obtained from Merck (Darmstadt, Germany), and cortisol 21-phosphate from Sigma (St. Louis, MO). Plasma levels of 11-hydroxycorticosteroids were determined according to the method of De Moor *et al.* (5) with minor modifications. RNA concentrations were determined by assuming an $A_{254}^{1\%}$ of 250. The cell-free product was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis as described previously (4), except that 2-mm gel slices were incubated with 0.55 ml of NCS solubilizer/water (10:1, vol/vol) at 45° overnight prior to the determination of radioactivity. After electrophoresis, 70–80% of the radioactivity applied to the gel was recovered in all experiments. Other reagents and determinations were as described previously (4).

RESULTS

Effect of Adrenalectomy on the Level of ACTH mRNA Activity. It is well known that adrenalectomy results in a marked increase in the ACTH level in pituitaries (6). In order to determine whether this rise in the ACTH level is due to an increased level of ACTH mRNA activity, we assayed pituitary RNA derived from normal and adrenalectomized rats for the ability to direct the cell-free synthesis of the translation product related to ACTH in the wheat germ protein-synthesizing system. The translation product related to ACTH was quantitated by indirect immunoprecipitation with the use of antibody against ACTH. As shown in Fig. 1 *A* and *B*, a linear response in the synthesis of the translation product related to ACTH as well as of total protein was obtained with up to 10 μg of added RNA, thus enabling the assay to be used for quantitative purposes. Moreover, it was clearly demonstrated that the ACTH mRNA activity was markedly increased after adrenalectomy; the extent of the increase ranged from 3- to 6-fold in different experiments. In contrast, the synthesis of total protein was essentially unaffected. Because pituitaries from sham-operated rats exhibited a level of ACTH mRNA activity virtually identical with that of pituitaries from normal rats, it is concluded that the enhancement of ACTH mRNA activity is caused by the removal of adrenals.

Identification of the Translation Product. In order to confirm that the radioactive material isolated by indirect immunoprecipitation actually represented the translation product related to ACTH, we analyzed the immunoprecipitate by electrophoresis on NaDodSO$_4$/polyacrylamide gels. The results of this analysis are presented in Fig. 2. When the labeled immunoprecipitate that was formed with antibody directed to the amino acid 19–24 sequence of ACTH was electrophoresed, a prominent radioactivity band and a second minor one migrating more rapidly were observed (Fig. 2*A*). By comparison with marker polypeptides, the molecular weights of the two components were estimated to be approximately 33,000 and 22,000. In contrast, the immunoprecipitate formed by incubation of the same translation product with control IgG exhibited no such bands except a very small radioactivity peak in the region where the minor component of 22,000 daltons was observed. Furthermore, both radioactivity bands were almost completely abolished by the addition of an excess of ACTH-(1–24) to the translation product prior to the addition of anti-ACTH [ACTH-(1–24) refers to synthetic peptide with residues 1–24 of the amino acid sequence of ACTH]. No such competition occurred upon prior addition of excess ACTH-(1–18) [ACTH-(1–18) refers to synthetic peptide with residues 2–18 of the amino acid sequence of ACTH and glycine at the amino terminus]. In the experiment shown in Fig. 2*B*, another preparation of antibody, which is directed to ACTH-(1–18), was used for indirect immunoprecipitation. Electrophoretic analysis of the resulting immunoprecipitate likewise revealed a conspicuous radioactivity band and a second minor one; the bands migrated with mobilities corresponding to molecular weights of approximately 33,000 and 22,000, respectively. Both bands were almost fully abolished by prior addition of excess ACTH-(1–18). These results indicate that the radioactive material isolated with the use of anti-ACTH actually represents the translation product of ACTH mRNA.

In the experiments just described, the translation product analyzed was synthesized with pituitary RNA derived from adrenalectomized rats. In the experiment shown in Fig. 2*C*, pituitary RNA derived from normal rats was translated, and the product was analyzed electrophoretically after indirect immunoprecipitation with antibody directed to the amino acid residues 19–24 of the ACTH sequence. The labeled immunoprecipitate exhibited two radioactivity bands with mobilities identical with those of the bands that were observed for the translation product synthesized with pituitary RNA from adrenalectomized animals. These bands were not observed, except a small radioactivity peak with the faster mobility, when the immunoprecipitate formed with control IgG was electrophoresed. Furthermore, the amounts of radioactivity present in both bands were approximately 3-fold smaller than those that

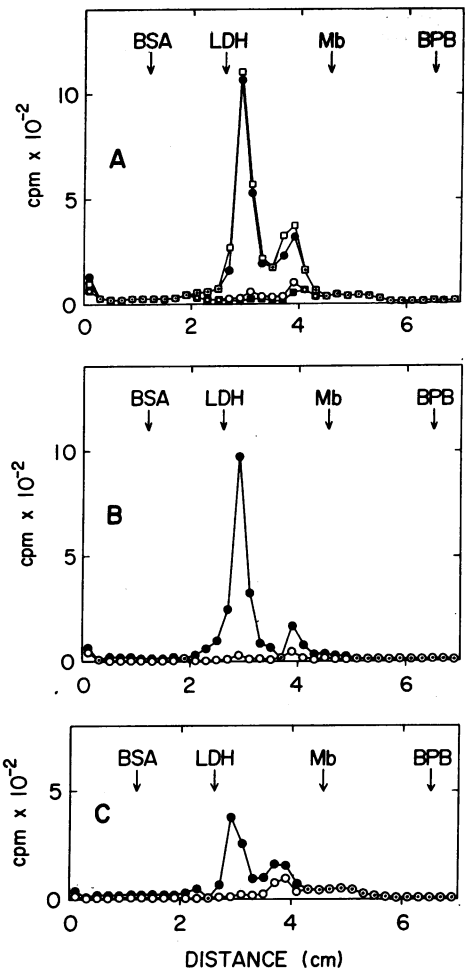

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of the immunoprecipitates. (*A*) Pituitary RNA derived from rats at 14 days after adrenalectomy was used to direct protein synthesis. Immunoprecipitation was carried out with antibody directed to the amino acid 19–24 sequence of ACTH in the presence of 5 μg of either ACTH-(1–24) (■) or ACTH-(1–18) (□) or in their absence (●). Immunoprecipitation was performed also with control IgG (○). (*B*) Pituitary RNA derived from rats at 7 days after adrenalectomy was used to direct protein synthesis. Immunoprecipitation was conducted with antibody directed to ACTH-(1–18) in the presence (○) or absence (●) of ACTH-(1–18). (*C*) Pituitary RNA derived from normal rats was used to direct protein synthesis. Immunoprecipitation was performed with antibody directed to residues 19–24 in the amino acid sequence of ACTH (●) or with control IgG (○). Other details were as described in *Materials and Methods*. The marker polypeptides used were bovine serum albumin (BSA; 67,500 daltons), rabbit muscle lactate dehydrogenase (LDH; 36,000 daltons), and equine muscle myoglobin (Mb; 17,200 daltons). Bromphenol blue (BPB) served as the tracking dye.

were found in the corresponding bands seen in Fig. 2*A*; the radioactivities were compared after subtraction of the radioactivity present in the respective band derived from the immunoprecipitates formed with control IgG. In the following experiments, therefore, we measured the radioactivity present in the whole immunoprecipitate formed with anti-ACTH (corrected for the radioactivity present in the immunoprecipitate formed with control IgG) in order to compare the levels of ACTH mRNA activity in pituitaries from rats treated in different manners.

Effect of Glucocorticoids on the Level of ACTH mRNA Activity. The increase in the ACTH mRNA activity observed after adrenalectomy was considered to be due to the removal

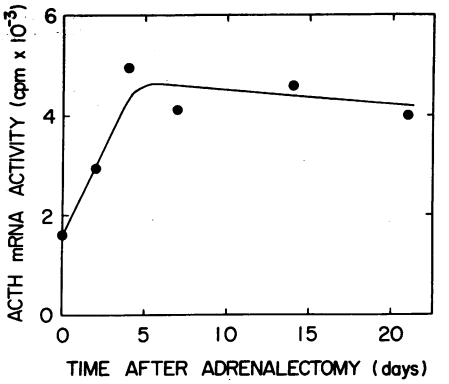

FIG. 3. Changes in the level of ACTH mRNA activity following adrenalectomy. Pituitary RNA derived from rats at various times after adrenalectomy was used to direct protein synthesis. Values for day zero represent those for normal rats. The amount of RNA added was 6.1 μg/50 μl of the reaction mixture. Other details were as described in *Materials and Methods*.

of a repressive effect of glucocorticoids on ACTH synthesis. The following experiments were designed to verify this concept.

In the experiment represented in Fig. 3, we studied the time course of changes in the level of ACTH mRNA activity in rat pituitaries following adrenalectomy. The ACTH mRNA activity increased progressively during the first 4 days, and a high level was maintained up to at least 21 days after adrenalectomy. The blood plasma from some of the animals used in this experiment was assayed for 11-hydroxycorticosteroids, the level of which was found to be actually lowered after adrenalectomy (53.7, 1.9, and 2.0 μg/100 ml for the normal rats and the rats at 4 and 7 days after adrenalectomy, respectively).

We next studied the effect of oral administration of dexamethasone on the level of ACTH mRNA activity in the pituitaries of adrenalectomized rats. As shown in Fig. 4, the level of ACTH mRNA activity decreased gradually and reached a minimal value 3 days after the initiation of dexamethasone administration. Fig. 5 exhibits the effects of various doses of dexamethasone administered for 4.5 days. The extent of suppression of ACTH mRNA activity increased with increasing doses of dexamethasone, the half-maximal concentration being approximately 0.1 μg/ml of the drinking water.

In Table 1, the effects of other steroids are compared with that of dexamethasone. Adrenalectomized rats received various steroids (10 μg/ml) in the drinking water for 4.5 days, and the levels of ACTH mRNA activity in the pituitaries of these rats were then assayed. Dexamethasone reduced the ACTH mRNA

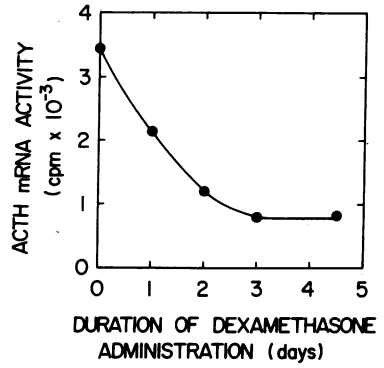

FIG. 4. Changes in the level of ACTH mRNA activity after dexamethasone administration to adrenalectomized rats. Rats at 10 days after adrenalectomy received dexamethasone (10 μg/ml) in the drinking water for the indicated lengths of time and were then sacrificed. The amount of pituitary RNA added was 6.0 μg/50 μl of the reaction mixture. Other details were as described in *Materials and Methods*.

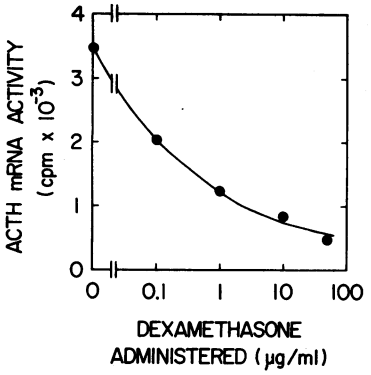

FIG. 5. Effects of various doses of dexamethasone on the level of ACTH mRNA activity. Rats at 10 days after adrenalectomy received the indicated doses of dexamethasone in the drinking water for 4.5 days and were then sacrificed. The amount of pituitary RNA added was 6.0 μg/50 μl of the reaction mixture. Other details were as described in *Materials and Methods.*

activity most markedly. Cortisol and corticosterone also exhibited a suppressive effect but were less effective than dexamethasone. In contrast, progesterone and aldosterone had no suppressive effect. These results indicate that the suppression of ACTH mRNA activity is due to a specific action of glucocorticoids.

In all the experiments shown in Figs. 3–5 and Table 1, the pituitary RNA derived from rats in various conditions was capable of directing [^{3}H]leucine incorporation into total protein to essentially the same extent. It is concluded, therefore, that the level of ACTH mRNA activity is specifically reduced by glucocorticoids.

DISCUSSION

The present investigation has demonstrated that rat pituitary RNA added to the wheat germ system directs the cell-free synthesis of polypeptides reactive with antibody against ACTH. The use of this technique has permitted the assay of the relative levels of ACTH mRNA activity in the pituitaries of rats subjected to adrenalectomy and steroid hormone treatment. Because the amount of RNA extracted from pituitaries was essentially unaffected after adrenalectomy or steroid hormone treatment, it can be concluded that the observed variations in the level of ACTH mRNA activity assayed with the cell-free protein-synthesizing system actually reflect changes in the level of functional ACTH mRNA in the pituitary.

Analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis of the cell-free translation product reactive with anti-ACTH revealed a major radioactive polypeptide of 33,000 daltons and a second one of 22,000 daltons. The results of the competition experiments indicate that both these polypeptides contain a structural component identical with the ACTH molecule. Since the amount of the minor polypeptide relative to that of the major one was somewhat variable from experiment to experiment, the minor polypeptide seems to be an artifactual product of cell-free synthesis, which may result from degradation of the major product or from premature release of nascent peptide chains from polysomes. The presence of two forms of cell-free translation product was likewise observed with the wheat germ protein-synthesizing system for placental lactogen and growth hormone (7, 8).

The present finding that rat pituitary RNA directs the cell-free synthesis of a translation product that is reactive with anti-ACTH but is much larger than ACTH is in good agreement with our previous observation of a similar large translation product synthesized with bovine pituitary RNA (4). The identity of the latter product as a large molecular form of ACTH was evidenced by the presence of common antigenic sites and a common chymotryptic peptide. Recently, Jones *et al.* (9) have also reported the cell-free synthesis of a large molecular form of ACTH with mouse pituitary tumor RNA.

Table 1. Effects of various steroids on the level of ACTH mRNA activity

Steroid	ACTH mRNA activity (cpm)
None	3390
Dexamethasone	479
Cortisol	1900
Corticosterone	2004
Progesterone	4205
Aldosterone	4170

Rats at 21 days after adrenalectomy received various steroids (10 μg/ml) in the drinking water for 4.5 days and were then sacrificed. The amount of pituitary RNA added was 7.5 μg/50 μl of the reaction mixture. Other details were as described in *Materials and Methods.*

Watanabe *et al.* (10, 11) have shown that mouse pituitary tumor cells, like other glucocorticoid-responsive tissues, contain a high-affinity glucocorticoid receptor, which undergoes translocation to the nucleus after binding glucocorticoids. Of interest is the fact that the different suppressive effects of various steroids on the level of ACTH mRNA activity, as shown in the present study, correlate generally with the binding specificity of the glucocorticoid receptor, as described by Watanabe *et al.* (10). Thus, it appears likely that the glucocorticoid receptor plays an essential role in the glucocorticoid-mediated suppression of ACTH mRNA activity in the pituitary and that a major part of the glucocorticoid action is exerted within the nucleus, where transcriptional events are regulated.

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