Selective Reduction of Proadrenocorticotropin/Endorphin Proteins and Messenger Ribonucleic Acid Activity in Mouse Pituitary Tumor Cells by Glucocorticoids†

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ABSTRACT: Mouse pituitary tumor cells (line AtT-20/D_{16v}) were used as a model system for studying the mechanism of the glucocorticoid-mediated decrease in adrenocorticotropin (ACTH) and β-endorphin production. Glucocorticoids decrease by 50–60% the levels of ACTH, β-endorphin, and the amino-terminal fragment of the common precursor of these peptides as measured by radioimmunoassay. Isoelectric focusing and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis of ACTH, endorphin, or N-terminal specific immunoprecipitated radioactive AtT-20 cell extracts showed that glucocorticoids had essentially no effect on the processing of the precursor other than to reduce by 50% the amount of label incorporated into pro-ACTH/endorphin proteins. The effect of the steroid was highly specific for the precursor and its end products as detected by two-dimensional gel analysis of pulse-labeled cellular proteins. Dexamethasone specifically reduced the total level of translatable ACTH/endorphin messenger ribonucleic acid (mRNA) with a half-maximal effective concentration at 3 nM, a 16-h half-time of deinduction, and no detectable influence on the proportion of translatable mRNA in the polysomes. The ACTH/endorphin precursor forms synthesized in the presence and absence of glucocorticoids are similar by every analytical technique used, suggesting that they are the same protein. Thus, glucocorticoids appear to reduce ACTH and β-endorphin production in AtT-20 cells by specifically decreasing ACTH/endorphin mRNA and not by altering the processing of the precursor.

It has been known for many years that glucocorticoids decrease the level of ACTH[1] in plasma (Yates & Maran, 1974). Studies with intact animals (Ganong & Hume, 1955; McCann et al., 1958; Russel et al., 1969), pituitary explants (Gonzalez-Lugue et al., 1970; Arimura et al., 1969; Berthold et al., 1970; Fleischer & Rawls, 1968), and pituitary cell culture systems (Pollack & La Bella, 1966; Fleischer & Rawls, 1970) suggest that this effect is on ACTH release in the pituitary. More recently, glucocorticoids have been observed to decrease both extracellular and intracellular levels of immunoreactive and bioactive ACTH in AtT-20 cells (Watanabe et al., 1973a; Herbert et al., 1978), suggesting that glucocorticoids may also act by inhibiting the synthesis of ACTH.

These studies were performed prior to the discovery that β-lipotropic hormone is derived from the same precursor protein as ACTH (Mains et al., 1977; Roberts & Herbert, 1977a,b; Nakanishi et al., 1977a,b). It is necessary to reconsider results of the earlier studies in the light of some of the consequences of this discovery. For one thing, when the precursor is converted to ACTH, β-lipotropic hormone, β-endorphin, and N-terminal glycopeptides (Roberts et al., 1978), processing intermediates are formed which have much lower molar reactivities in bioassays and radioimmunoassays than the ACTH end products (Eipper & Mains, 1975; Roberts et al., unpublished experiments). Therefore, measurements of ACTH levels by these assay methods cannot distinguish between glucocorticoid effects on the regulation of synthesis of the precursor or the processing of the precursor. Alteration of any step in processing would be expected to change the intracellular distribution of the forms of ACTH and endorphin. To detect such an alteration, we have studied the kinetics of labeling of the forms of ACTH and β-endorphin in AtT-20 cells with and without glucocorticoid pretreatment using radioactive amino acids and sugars.

Glucocorticoids have been shown to induce specific proteins by increasing the levels of the mRNA molecules that code for these proteins [for a review, see Johnson et al. (1979)]. However, the inhibitory effects of glucocorticoids are not as well understood. The inhibitory effect of glucocorticoids on ACTH production has been well-enough characterized in the AtT-20 cells to be useful as a model for studying the mechanism of glucocorticoid-mediated deinduction of a specific protein. By analogy with glucocorticoid induction of proteins, one might expect glucocorticoid deinduction to operate by decreasing levels of specific mRNA. Nakanishi et al. (1977b) have provided evidence for this mechanism by showing that glucocorticoids reduce the level of translatable ACTH mRNA in AtT-20 cells and in the pituitary. However, it is not clear that the observed decrease in ACTH mRNA activity completely accounts for the observed decrease in ACTH levels. Also these studies did not consider alternative modes of regulation. Recent studies have indicated that synthesis of particular proteins can be regulated at the translational level by modulating the cytoplasmic activity of mRNA molecules that code for the protein. This type of translational regulation

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[1] Abbreviations used: ACTH, adrenocorticotropin; LPH, lipotropin; $NaDodSO_4$, sodium dodecyl sulfate; DMEM, Dulbecco minimal essential medium. The forms of the ACTH/endorphin precursor, ACTH and endorphin, are designated by their apparent molecular weights determined by $NaDodSO_4$–polyacrylamide gel electrophoresis as follows: 29K, 32K, and 34K pro-ACTH/endorphin for the 29 000, 32 000, and 34 000 forms of the precursor and 13K ACTH and 4.5K ACTH for the 13 000 and 4500 forms of ACTH, respectively.

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could be accomplished by a shift in the distribution of specific mRNA molecules between an actively translating form in the polysomes and an inactive form in the nonribosomal portion of the cytosol (Civelli et al., 1976; Yap et al., 1978). mRNA activity could also be modulated at the translational level without undergoing a redistribution in the cytoplasm. For example, although casein mRNA is present in the polyribosomes of the mammary gland during pregnancy, it is not translated efficiently until hormonal stimulation at parturition (Rosen et al., 1978). To determine if synthesis of the ACTH/endorphin precursor is also regulated at the translational level by long-term treatment with glucocorticoids (48–72 h), we have isolated polysomes, polysomal RNA, and total cytoplasmic RNA from dexamethasone-treated and untreated cultures of AtT-20 cells and compared the translational activity of these fractions in a reticulocyte cell-free system.

Methods

Incubation of AtT-20 Cells with Steroids and Radioactive Precursors. Mouse pituitary tumor cells, AtT-20/D_{16v}, were grown in Dulbecco minimal essential medium (DMEM) with 10% horse serum as described by Roberts et al. (1978). For stock solutions, steroids were dissolved either in phosphate-buffered saline (50 mM $NaPO_4$, pH 7.2, 0.9% NaCl) at 10^{-5} M or in 95% ethanol at 10^{-2} M. Incubation of cultures with [^{35}S]methionine (Amersham/Searle, ~1000 Ci/mmol), [^{3}H]phenylalanine (Amersham/Searle, 40 Ci/mmol), and D-[6-^{3}H]glucosamine (Amersham/Searle, 30 Ci/mmol) was done as described previously by Roberts et al. (1978). Cell protein destined for immunoprecipitation or ACTH radioimmunoassay was extracted with 5 N acetic acid, using protease inhibitors (Roberts et al., 1978) but omitting the bovine serum albumin carrier.

Preparation of RNA and Polysomes. One confluent 10-cm culture dish of AtT-20 cells routinely yielded 0.5–1 mg of total cytoplasmic RNA. Each experimental treatment was performed with up to three dishes. After the prescribed hormonal treatment, cultures were rinsed twice with phosphate-buffered saline and the postnuclear supernatant was prepared by using a 0.5% Triton X-100 buffer as previously described by Roberts & Herbert (1977a). RNA in this supernatant was directly extracted with phenol–chloroform, or the supernatant was centrifuged through a discontinuous sucrose gradient to prepare polyribosomes free of membrane cytosol material (Roberts & Herbert, 1977a). The polyribosomes were resuspended in H_2O and used directly for translation or extracted with phenol–chloroform. Extracted RNA preparations were treated with autodigested pronase and again extracted with phenol–chloroform. RNA was precipitated with ethanol, dried, redissolved in H_2O, and stored at −20 °C. RNA concentrations were determined by measuring the optical density at 260 nm using the 1 mg/mL A_{260} value at 22.5 for RNA and 12.5 for polysomes. Reticulocyte lysate systems were treated with micrococcal nuclease prior to use in translating RNA or for completing the nascent polypeptide chains on the polyribosomes (Pelham & Jackson, 1976). Aurintricarboxylic acid (1×10^{-4} M) was used to inhibit initiation of protein synthesis in the polysome chain completion experiments (Roberts & Herbert, 1977b). RNA and polyribosomes were used in translation assays at two or three different concentrations ranging from 30 to 150 μg of RNA per mL. Incorporation of radioactivity into trichloroacetic acid precipitable material increased linearly with the amount of RNA added to the reaction mixture (Figure 1). Assay mixtures of 40 μL were incubated at 30 °C for 90 min and diluted to 100 μL with

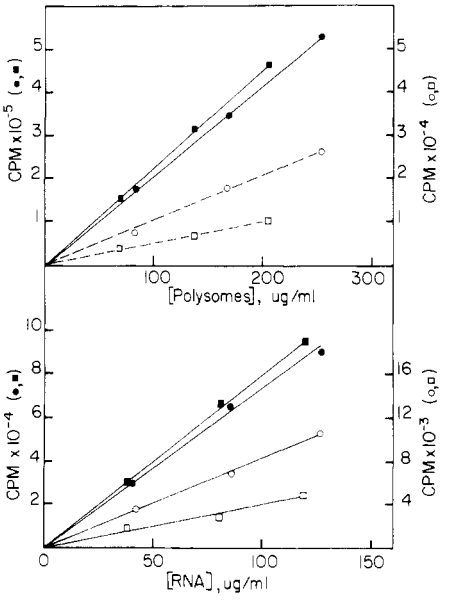

FIGURE 1: Linearity of the ACTH/endorphin mRNA translation–immunoprecipitation assay. Varying concentrations of AtT-20 polyribosomes (top panel) or phenol-extracted RNA (bottom panel) were used as templates for protein synthesis in the modified reticulocyte cell-free protein-synthesizing system. Total trichloroacetic acid precipitable radioactivity (●, ■) and ACTH specific immunoprecipitable radioactivity (○, □) were measured as described under Methods. Control cultures are represented by circles (●, ○), and dexamethasone-pretreated cultures are represented by squares (■, □).

H_2O, and two 4-μL aliquots were analyzed for trichloroacetic acid precipitable radioactivity.

Immunoprecipitations and Radioimmunoassays. Aliquots of the translation assay mixture were immunoprecipitated after fivefold dilution with the immunoprecipitation buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA). The ACTH antiserum used had previously been purified by α(1–24)-ACTH affinity chromatography (Mains & Eipper, 1976); gel electrophoretic analysis demonstrated that >95% of the radioactivity in an immunoprecipitate was in a protein of M_r 28 500. The antibody had quantitatively bound all of the pro-ACTH/endorphin precursor in the assay as evidenced by the inability of additional antibody to bind any more protein of M_R 28 500. Pro-ACTH/endorphin mRNA activity was estimated from the linear portion of a curve relating immunoprecipitable radioactivity to RNA concentration in the assay mixture (Figure 1). The mRNA activity is expressed relative to trichloroacetic acid precipitable radioactivity. This translation–immunoprecipitation assay measures the relative ability of the isolated RNAs to synthesize pro-ACTH/endorphin in the reticulocyte system and is not necessarily a measure of the amount of pro-ACTH/endorphin mRNA. ACTH and β-endorphin radioimmunoassays were performed as described by Herbert et al. (1978), and values are reported as micrograms of radioimmunoactivity per milligram of cell protein. The radioimmunoactivity for the N-terminal fragment was performed by using an antibody from a rabbit which had been injected with a partially purified concentrate of AtT-20 serum-free culture medium (Mains & Eipper, 1976). N-Terminal protein for iodinations and standards was isolated from AtT-20 culture medium by gel filtration on a G-100 Sephadex column using 10% formic acid for elution and by $NaDodSO_4$–acrylamide slab gel electrophoresis (unpublished experiments). The concentration of N-terminal protein was determined by Coomassie Blue staining, and thus the values reported in Table I for the N-terminal fragment are relative

Table I: Dexamethasone Decreases Intracellular Levels of ACTH, Endorphin, and N-Terminal Glycopeptide

	control[a]	Dex[a]	Dex/control
ACTH	2.07	0.96	0.46
β-endorphin	1.87	0.76	0.41
N-terminal glycopeptide	0.78	0.35	0.45

[a] Values are expressed in micrograms of radioimmunoactivity per milligram of protein and are the average of duplicate assays performed on two sets of cell cultures. AtT-20 cultures were treated with 10^{-6} M dexamethasone (Dex) for 72 h, acid extracted, and analyzed for protein and ACTH, β-endorphin, and N-terminal glycopeptide radioimmunoactivity as described under Methods.

to the standards used in determining the protein concentration. *Staphylococcus aureus* Cowan I bacteria were used to separate bound from unbound antigen in the N-terminal radioimmunoassay (Kessler, 1975).

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide tube gel electrophoresis was performed as described by Roberts & Herbert (1977a) with 12% Biophore gels. Slab gel electrophoretic analysis was performed as described by O'Farrell (1975), using a nonequilibrium isoelectric focusing (pH 3–10) gel for the first dimension and a 12.5% acrylamide–$NaDodSO_4$ gel for the second dimension. Peptide analysis of proteins separated by gel electrophoresis was done by digesting the material with trypsin (1:20 w/w trypsin/protein) directly in the gel, and after a second identical addition of trypsin to the solubilized protein, the resulting soluble peptides were analyzed by paper electrophoresis at pH 6.5 (Roberts & Herbert, 1977a,b).

Results

Glucocorticoid Effects on the Levels of ACTH, Endorphin, and N-Terminal Glycopeptide in AtT-20 Cells As Determined by Radioimmunoassay. AtT-20 cultures were preincubated with or without 10^{-6} M dexamethasone for 72 h. The cells were extracted, and the levels of ACTH, β-endorphin, and N-terminal glycoprotein in the extracts were determined by radioimmunoassay. The results in Table I show that dexamethasone lowers the levels of all of these components by 50–60%. It would appear from these results that dexamethasone is inhibiting overall production of these components. However, this conclusion cannot be made from these data because the antisera are known to react differently on a molar basis with different forms of ACTH and β-endorphin in the radioimmunoassay. For example, in radioimmunoassays with antiserum Hiiaka (Roberts et al., unpublished experiments), the ACTH radioimmunoactivity per mole of 4.5K ACTH is ~6 times as high as that of the 29–34K pool of ACTH/endorphin molecules. Hence, the reduction in radioimmunoactivity values following dexamethasone treatment (Table I) could be achieved by shifting the distribution of the forms of ACTH in favor of the ones with less radioimmunoactivity per mole (29–34K ACTH/endorphin) rather than by lowering overall production of the forms. This could be accomplished by a block in processing of the precursor forms. Thus, before one can conclude from results in Table I that dexamethasone inhibits the overall production of hormones, it is necessary to know whether dexamethasone alters the molecular distribution of the forms of the hormones.

Do Glucocorticoids Alter the Distribution of the Forms of ACTH in AtT-20 Cells or Inhibit the Synthesis of the ACTH/Endorphin Precursor? The first form of the precursor detected in AtT-20 cells is glycosylated (29K pro-ACTH/endorphin) (Roberts et al., 1978). This protein, which is

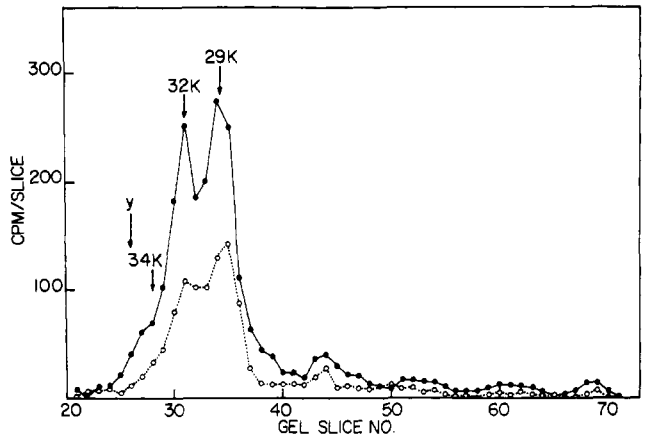

FIGURE 2: Effect of dexamethasone pretreatment on short-term labeling of ACTH proteins. Cultures of AtT-20 cells in Falcon microtest wells were preincubated 48 h in DMEM with and without 10^{-6} M dexamethasone. The medium was changed once at 24 h. To label, fresh medium (50 μL) was added containing 50 μCi of [^{35}S]methionine (~1000 Ci/mmol) with and without 10^{-6} M dexamethasone. After 20 min, the cells were extracted and the radioactive ACTH was immunoprecipitated and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using 12% acrylamide Biophore gels. Open circles refer to dexamethasone-pretreated cultures. Dansyl yeast alcohol dehydrogenase (y, arrow) was included as an internal standard. A background of 18 cpm has been subtracted. Numbers above peaks (34K, 32K, and 29K) refer to apparent molecular weights calculated as previously described (Roberts et al., 1978).

believed to have a single carbohydrate side chain, can be either processed to lower molecular weight forms of ACTH and endorphin by proteolytic cleavage or converted to higher molecular weight forms of the precursor (32K and 34K pro-ACTH/endorphin) by addition of more carbohydrate side chains (Roberts et al., 1978). To test whether glucocorticoids inhibit ACTH production by altering the processing of the precursor or by inhibiting the overall synthesis of the precursor, we first studied the kinetics of incorporation of radioactive amino acids into the various forms of ACTH and β-endorphin. AtT-20 cultures were incubated with or without glucocorticoids for 48–72 h. They were then labeled with [^{35}S]methionine for 20 min, and the forms of ACTH were analyzed by immunoprecipitation and one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis. At 20 min only the precursor forms (29–34K) are labeled and no labeled endorphin or ACTH is present in the culture medium (Roberts et al., 1978). Hence, a 20-min labeling period can be used to measure the synthetic rate of the precursor in the cells as well as the distribution of label in the precursor forms. Figure 2 shows that dexamethasone has very little effect upon the distribution of radioactivity in the pro-ACTH/endorphin precursor forms after 20 min of labeling. The major effect is to lower the amount of label incorporated into pro-ACTH/endorphin ~50% (2.7% of the total acid-insoluble radioactivity was in pro-ACTH/endorphin in the control culture and 1.3% in the dexamethasone-treated culture). Similar results were obtained with cultures that had been labeled for 60 min, 120 min, 4 h, and 8 h. Hence, dexamethasone appears to inhibit synthesis of the pro-ACTH/endorphin precursor proteins without affecting the conversion of one precursor form to another.

The inhibitory effect of glucocorticoids on the synthesis of the precursor is quite specific as shown in the data presented in Figure 3. AtT-20 cultures treated 72 h with dexamethasone or cortisol along with an untreated culture were labeled for 10 min with [^{35}S]methionine, and the total protein extracts were analyzed by a two-dimensional (isoelectric focusing and $NaDodSO_4$–polyacrylamide gel electrophoresis) gel system.

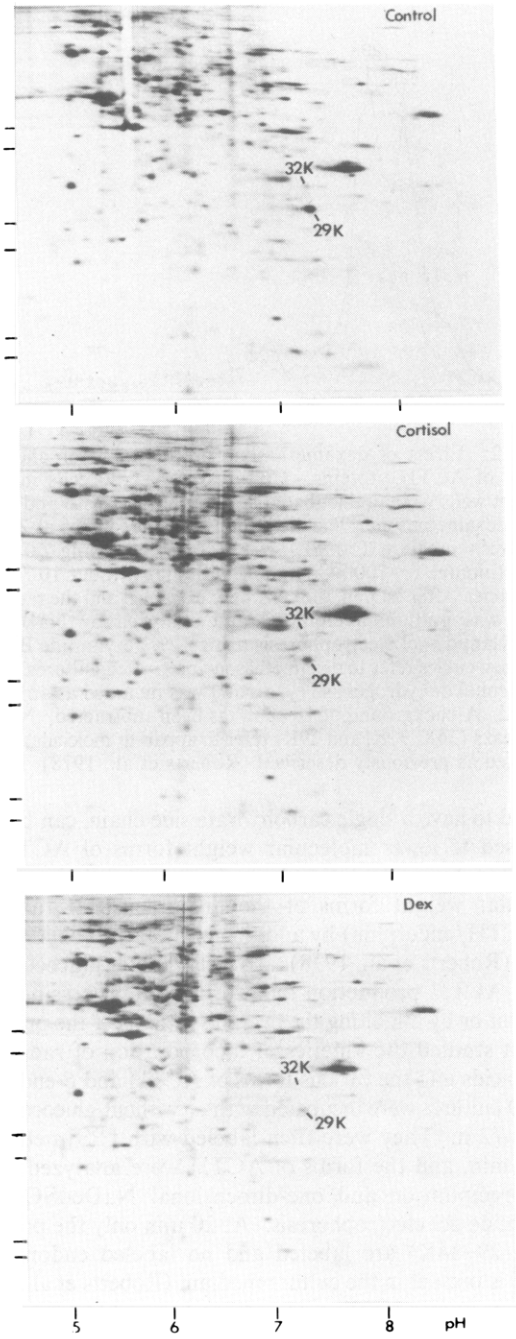

FIGURE 3: Two-dimensional gel electrophoretic analysis of pulse-labeled cells. Identical microwell cultures were treated with either no steroid, 10^{-6} M dexamethasone, or 10^{-6} M cortisol for 72 h, rinsed once in minus methionine DMEM, and then incubated in 50 μL of minus methionine DMEM containing 100 μCi of [^{35}S]methionine for 10 min with and without 10^{-6} M dexamethasone. The cells were then rinsed twice with phosphate-buffered saline and extracted with isoelectric focusing lysis buffer containing pH 3–10 ampholytes (Bio-Rad) as described by O'Farrell (1975). Approximately equal amounts of radioactive protein were applied to each gel. Isoelectric focusing was performed for 1800 Vh and the pH gradient (abscissa) measured in a parallel gel (O'Farrell, 1975). The second dimension was a $NaDodSO_4$–polyacrylamide gel. Internal protein standards for molecular weight determination were ovalbumin (43 000), yeast alcohol dehydrogenase (37 000), chymotrypsinogen A (25 700), trypsin (22 500), RNase A (13 700), and cytochrome *c* (11 600) and are indicated on the ordinate of each panel from top to bottom in the order given here. Exposure of the dried gel was for 48 h, using Kodak X-Omat film. The top panel represents the control culture, the middle panel was from cells treated with cortisol, and the bottom panel was from dexamethasone-pretreated cells. Two forms of the pro-ACTH/endorphin precursor are indicated by the symbols 29K and 32K.

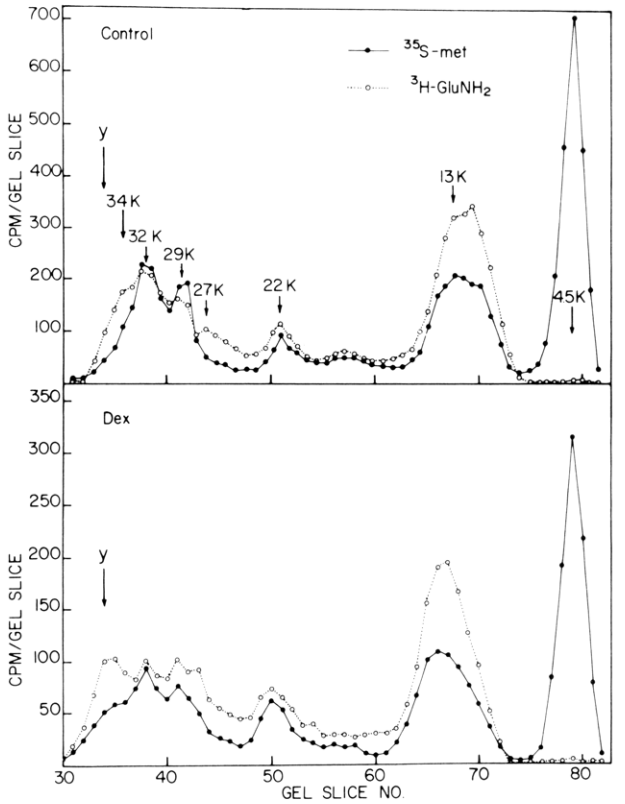

FIGURE 4: Glucocorticoid effect on the double-labeling pattern of ACTH proteins. Cultures of AtT-20 cells were treated as described in Figure 2 for 48 or 72 h. 50 μL of fresh low glucose modified medium with and without 10^{-6} M dexamethasone (see Methods) containing 125 μCi of [^{3}H]glucosamine and 80 μCi of [^{35}S]methionine was added to the cultures. After 2 h of incubation, the cells were extracted and immunoprecipitated with an ACTH specific antiserum. Immunoprecipitates were analyzed by 12% $NaDodSO_4$–polyacrylamide gel electrophoresis as in Figure 2. [^{3}H]Glucosamine label is represented with open circles and [^{35}S]methionine label with closed circles. The ^{3}H channel has been corrected for ^{35}S spillover (5%), and background has been subtracted. Upper panel, control cells; lower panel, dexamethasone-treated cells (48 h).

The levels of both the 29K and 32K forms of the precursor (whose positions in the gel are identified by specific immunoprecipitation as in Figure 5) are reduced by treatment with glucocorticoids. However, the overall labeling pattern of the cell protein is not affected.

The 29K, 32K, and 34K forms of the ACTH/endorphin precursor are known to be glycosylated (Eipper et al., 1976; Roberts et al., 1978). In order to test whether glucocorticoids have any effect on the addition of carbohydrate to these proteins, we incubated dexamethasone-treated and untreated cultures with both [^{35}S]methionine and [^{3}H]glucosamine. The forms of ACTH were isolated by immunoprecipitation and separated by $NaDodSO_4$ gel electrophoresis. Since the number of methionine residues in each form of ACTH is known (Roberts et al., 1978), it is relatively easy to estimate the amount of glucosamine relative to the amount of protein in each form of ACTH from the data in Figure 4. This is possible because the precursor forms and the ACTH intermediates are labeled to a steady-state level in this experiment (Roberts et al., 1978). The results show that the distribution of the forms is the same in both treated and untreated cultures with the exception that a little more of the 34K form of pro-ACTH/endorphin precursor is present in the glucocorticoid-pretreated cultures. The carbohydrate content of the various forms appears to be similar in the two kinds of culture, although there is more carbohydrate in the 29K form in the

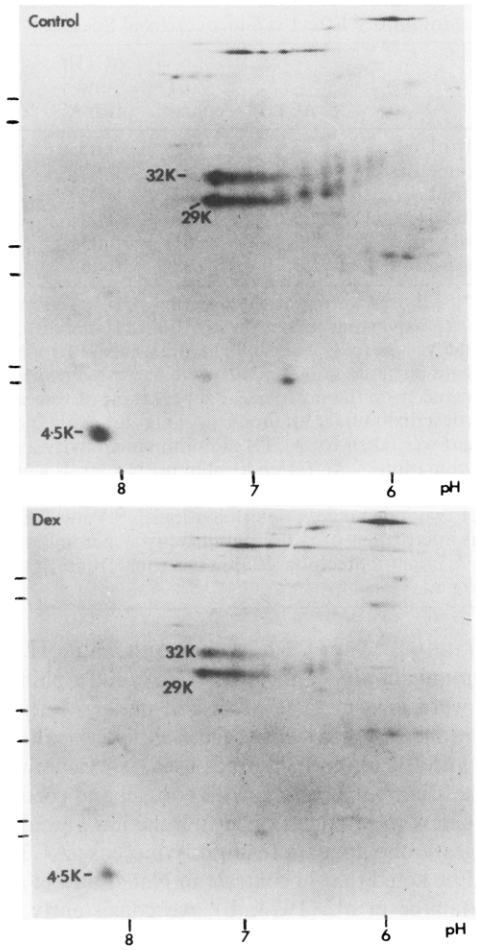

FIGURE 5: Two-dimensional gel electrophoretic analysis of ACTH immunoprecipitates of steroid-treated and control cultures. Cultures were treated as described in Figure 3 except that they were labeled for 3 h and the ACTH specific proteins were isolated from the cell extracts as described under Methods. Gel electrophoresis, the pH gradient, and molecular weight markers are as described in Figure 3. The top panel is of a control culture and the bottom panel of a 72-h 10^{-6} M dexamethasone-pretreated culture. The different forms of ACTH are indicated by their apparent molecular weight (29K, 32K, 4.5K, etc.).

dexamethasone-treated culture than in the control culture.

Immunoprecipitates of AtT-20 cultures pretreated with and without dexamethasone and labeled with [^{35}S]methionine for 2 h were analyzed by the two-dimensional electrophoretic system as shown in Figure 5. Dexamethasone does not appear to alter either the isoelectric point or the molecular weight of any of the immunoprecipitated species of ACTH, although it does decrease the intensity of all of the ACTH specific spots in the figure, in agreement with the results in Figures 2–4.

To test if there is any effect of dexamethasone on β-LPH, β-endorphin, or N-terminal fragment production in these cells, we performed similar labeling experiments with the use of a β-endorphin specific antiserum and an antiserum against the N-terminal region of the precursor. These experiments, using both the one- and two-dimensional gel electrophoretic analyses, indicated that dexamethasone does not alter the pattern of processing of the different forms of β-endorphin or of the N-terminal fragment (data not shown).

Effect of Glucocorticoids on Cell-Free Synthesis of Pro-ACTH/Endorphin. To determine directly if the decrease in pro-ACTH/endorphin protein is due to a decrease in the translation capacity of the system, we isolated polysomes from cultures pretreated with 10^{-6} M dexamethasone as described under Methods and allowed them to complete their nascent

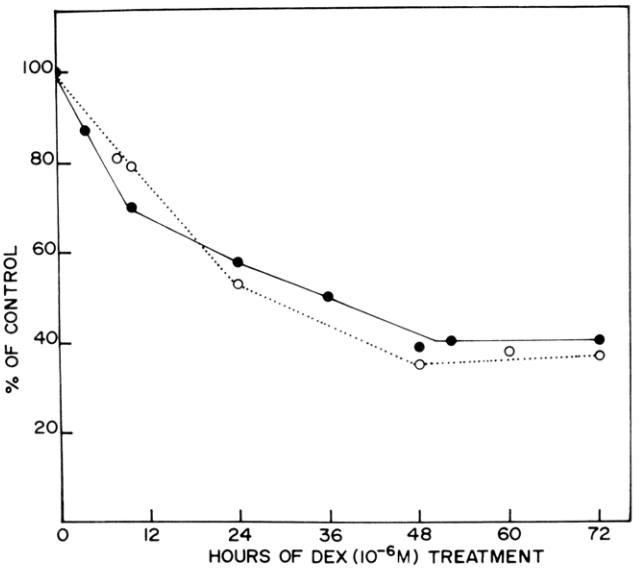

FIGURE 6: Time course of glucocorticoid inhibitory effect. Identical cultures of AtT-20 cells in 10-cm dishes were grown to ~30% confluency. The medium was changed, and after appropriate intervals, dexamethasone was added. Medium was changed again at 24 and 48 h, including steroid if already present. Cells in all cultures were harvested at the same time, and RNA was prepared from the postnuclear supernatant as described under Methods. An aliquot of the postnuclear supernatant was taken for ACTH radioimmunoactivity and protein determinations. Values were expressed as a percentage of the zero-time value, where intracellular ACTH radioimmunoactivity (○) was determined as micrograms of ACTH radioimmunoactivity per milligram of cell protein and pro-ACTH/endorphin mRNA levels (●) were measured as ACTH immunoprecipitable cpm per trichloroacetic acid precipitable cpm.

chains in the presence of aurintricarboxylic acid (a specific inhibitor of initiation of protein synthesis) in a reticulocyte cell-free system. Labeled precursor was isolated by immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis. Again, dexamethasone pretreatment caused a 2–2.5-fold decrease in label incorporated into the pro-ACTH/endorphin precursor, in agreement with radioimmunoassay results shown in Table I and with results obtained by pulse labeling the cells (Figures 2 and 3). Furthermore, total cytoplasmic RNA from 10^{-6} M dexamethasone-pretreated cultures is also approximately half as active (per milligram of RNA) in directing synthesis of the precursor protein in a reticulocyte cell-free system as cytoplasmic RNA from untreated cells. Thus, it appears that the 50–75% reduction of the ACTH level in the AtT-20 tumor cells after 48–72 h of dexamethasone treatment can be accounted for by a similar reduction in the level of translatable mRNA that codes for ACTH/endorphin proteins.

Time Course of Reduction of Pro-ACTH/Endorphin mRNA Activity. Previous studies have shown that dexamethasone decreases the secretion of ACTH in AtT-20/D_{16v} cultures with a maximal effect obtained after ~48 h (Herbert et al., 1978). To determine if dexamethasone reduces the levels of ACTH radioimmunoactivity and pro-ACTH/endorphin mRNA activity with a similar time course, cultures of AtT-20 cells were incubated for varying periods of time with 10^{-6} M dexamethasone and their ACTH radioimmunoactivity contents and pro-ACTH/endorphin mRNA activities were assayed as described under Methods. Figure 6 shows the results of these experiments. Both the intracellular ACTH radioimmunoactivities and the pro-ACTH/endorphin mRNA activities exhibited similar time courses of inhibition, and the effect appeared to be maximal after ~48 h of glucocorticoid treatment. Thus, it appears that the pro-ACTH/endorphin

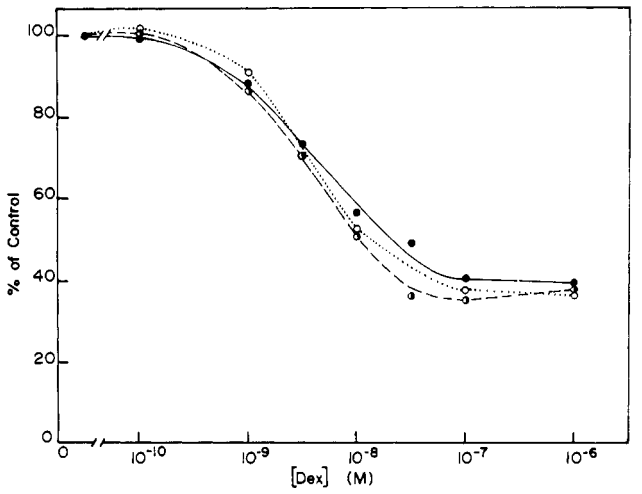

FIGURE 7: Dexamethasone dose response. 10-cm dishes of AtT-20 cells at ~30% confluency were treated with varying concentrations of dexamethasone for 60–72 h. Total cytoplasmic RNA and polysomes were prepared from these cultures, and their ability to direct the synthesis of the pro-ACTH/endorphin precursor was measured as described under Methods. Intracellular ACTH and total cytoplasmic protein were also measured. Values are expressed as a percentage of the minus dexamethasone control by averaging two independent determinations as described in Figure 6. Symbols used are as follows: ACTH radioimmunoactivity (●); pro-ACTH/endorphin mRNA level (○); and pro-ACTH/endorphin polysome level (◑).

mRNA activity directly reflects the ACTH protein level during the deinduction period. The half-time of dexamethasone inhibition is ~14 h with 10^{-6} M dexamethasone.

Effect of Dexamethasone Concentration on Pro-ACTH/Endorphin mRNA Levels. As is shown in Figure 7, dexamethasone lowers the intracellular ACTH radioimmunoactivity levels in the AtT-20 cells in a dose-dependent fashion. The half-maximal effect is ~4 nM dexamethasone, in agreement with results of previous studies (Herbert et al., 1978). Experiments were then performed to determine if dexamethasone reduces pro-ACTH/endorphin mRNA activity in polysomes, polysomal RNA, and total cytoplasmic RNA in the same dosage range. Cultures were treated with different dexamethasone concentrations for 60–72 h to reach a new steady-state level of pro-ACTH/endorphin mRNA activity (Figure 6). Total cytoplasmic RNA, polysomal RNA, and polysomes were isolated. The pro-ACTH/endorphin mRNA activity of each fraction was assayed in the reticulocyte cell-free system as described under Methods. As can be seen in Figure 7, the activity of pro-ACTH/endorphin mRNA present in total cytoplasmic RNA and in polysomes closely parallels the level of the pro-ACTH/endorphin proteins present in cell extracts at different doses of dexamethasone. Similar results were obtained by using isolated polysomal RNA (data not shown). This again suggests that the level of pro-ACTH/endorphin proteins present in AtT-20 cells directly reflects the level of the pro-ACTH/endorphin mRNA in the cells. It can also be seen in Figure 7 that there is no differential effect of glucocorticoids on pro-ACTH/endorphin mRNA activity either in the total cytoplasmic RNA fraction or in total polysomes. They both give curves that are parallel to the ACTH radioimmunoactivity curve.

Inhibition of Synthesis of Pro-ACTH/Endorphin Is Glucocorticoid Specific. It has previously been shown that the inhibitory effect of steroids on ACTH secretion in AtT-20/D_{16v} cultures is glucocorticoid specific (Herbert et al., 1978). To determine if the reduction of pro-ACTH/endorphin mRNA activity by dexamethasone is specific for glucocorticoids, we incubated AtT-20 cultures with several different steroids at 10^{-7} M for 72 h as shown in Table II. ACTH radioimmunoactivity and pro-ACTH/endorphin mRNA activity were measured as described under Methods. The ACTH radioimmunoactivity levels closely parallel mRNA activity with the different steroids used. Dexamethasone has the greatest inhibitory effect, with cortisol and corticosterone being the next most potent. Aldosterone has a weaker effect, and progesterone appears to slightly increase ACTH levels. It should be noted that in contrast to Nakamura et al. (1978) and Watanabe et al. (1973a,b), we consistently find that cortisol is a more potent glucocorticoid than corticosterone (Herbert et al., 1978). One possibility for this difference is that we are working with a different subclone of the AtT-20 cell line.

Table II: Inhibitory Effect Is Glucocorticoid Specific[a]

	ACTH[b]	% of control	ACTH/end mRNA[c]	% of control
control	3.51	100	0.061	100
dexamethasone	1.45	41	0.029	48
cortisol	1.67	48	0.031	51
corticosterone	2.09	60	0.036	59
aldosterone	2.85	81	0.051	84
progesterone	3.79	108	0.062	102

[a] AtT-20 cultures were pretreated with 1×10^{-7} M steroid for 3 days prior to harvesting cells. Media, 10 mL/10-cm dish, were changed daily. Steroids were added from a sterile 10^{-5} M stock in phosphate-buffered saline. Cells were harvested and polysomes were prepared from the postnuclear supernatant of the cell homogenate as described under Methods. An aliquot of the postnuclear supernatant was taken for ACTH radioimmunoactivity and protein determinations. ACTH/endorphin mRNA levels were measured as described under Methods. Values presented here are the average of two independent determinations. [b] Values are expressed as micrograms of radioimmunoactivity per milligram of protein. [c] Immunoprecipitable cpm per trichloroacetic acid precipitable cpm.

Complexity of the Glucocorticoids Response in the AtT-20 Cells. Several kinds of measurements show that glucocorticoids do not inhibit the synthesis of the bulk of the protein or RNA in AtT-20 cells. First, dexamethasone does not alter the incorporation of radioactive amino acids into trichloroacetic acid insoluble material, the amount of cell protein in a culture, or the growth rate of the cells (Herbert et al., 1978). Second, the quantity of RNA isolated from the glucocorticoid-treated cells is approximately the same as the amount of RNA isolated from the untreated cells (per milligram of cell protein). Third, sucrose density gradients of polysomes from glucocorticoid-treated and untreated cultures show that glucocorticoids have essentially no effect on the size distribution of polysomes. Fourth, the RNA isolated from dexamethasone-treated cultures is as efficient in directing the synthesis of total protein as RNA isolated from untreated cultures in the reticulocyte cell-free system (Figure 1). Finally, the overall distribution of proteins synthesized in AtT-20 cultures preincubated with or without synthetic or natural glucocorticoids is very similar, as shown in Figure 3. These observations suggest that glucocorticoids are specifically inhibiting pro-ACTH/endorphin synthesis rather than causing a general inhibition of protein synthesis or a decrease in the amino acid pools.

The results in Figures 2–5 also demonstrate that dexamethasone treatment of AtT-20 cells does not alter the apparent molecular weights of the pro-ACTH/endorphin proteins or their derivatives. There was also no apparent change in the isoelectric points of any of the pro-ACTH/endorphin proteins in response to glucocorticoid treatment (Figures 3 and 5). Studies with antisera to the ACTH, β-endorphin, and N-

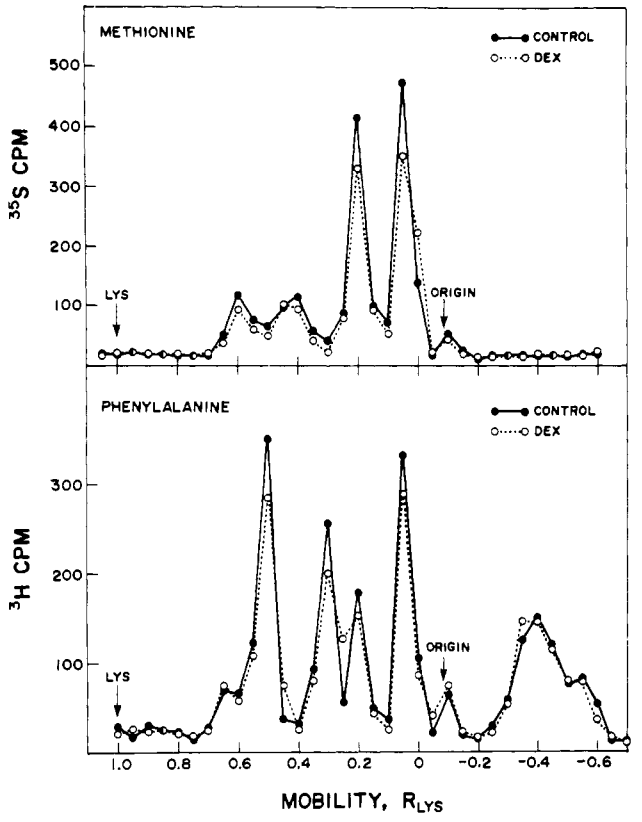

FIGURE 8: Tryptic peptides of pro-ACTH/endorphin cell-free product. RNA isolated from AtT-20 cells treated with or without 10^{-6} M dexamethasone was translated in the reticulocyte system by using either [^{35}S]methionine or [^{3}H]phenylalanine as the radioactive amino acid (Methods). The pro-ACTH/endorphin precursor was isolated, equal amounts of radioactivity were trypsin digested, and the resulting peptides were analyzed by paper electrophoresis as previously described by Roberts et al. (1978). The top panel shows the methionine peptides and the bottom panel the phenylalanine tryptic peptides. Control samples are indicated by solid circles and dexamethasone-treated samples by open circles.

terminal regions of the precursor show that the immunological properties of the precursor proteins are also the same whether they are synthesized in the presence or absence of dexamethasone. Finally, the precursor proteins synthesized in the reticulocyte cell-free system under the direction of mRNA from dexamethasone-treated and untreated cells have identical [^{35}S]methionine- and [^{3}H]phenylalanine-labeled tryptic peptides (Figure 8). Similar results were obtained when leucine or lysine was used to label the precursor (data not shown). Thus, it appears that the same pro-ACTH/endorphin protein is being synthesized in the presence or absence of dexamethasone.

Discussion

In this study we have used the AtT-20/D_{16v} cell line to study the mechanism of glucocorticoid-mediated inhibition of ACTH and endorphin production. Watanabe et al. (1973a,b) have shown that glucocorticoids depress the levels of bioreactive and immunoreactive ACTH in AtT-20 cells and that this effect is correlated with the ability of these steroids to bind to a cytoplasmic factor that has the properties of a glucocorticoid receptor. Previous studies have shown that the AtT-20 cell line is a good model system for studying biosynthesis and processing of ACTH and endorphin. The AtT-20 cells closely resemble anterior pituitary corticotrophs in hormonal content (Roberts et al., 1978), response to steroids, hypothalamic releasing factors (Allen et al., 1978), and processing of the precursor (Herbert et al., 1979).

A model of the structure of the common precursor has been formulated in which β-endorphin is located at the C-terminal portion of the molecule and ACTH is adjacent to β-LPH near the middle of the molecule, leaving an unidentified sequence of ~100 amino acids at the N terminus (Roberts & Herbert, 1977b; Mains & Eipper, 1978). Therefore, there are three functionally distinct domains in the precursor. The results in Table I show that the levels of the peptides derived from these domains (as measured by radioimmunoassay) are reduced to almost the same extent by dexamethasone treatment of AtT-20 cells. This result could be explained by postulating that glucocorticoids (1) depress synthesis of the precursor, (2) enhance degradation of the precursor and/or low molecular weight forms of the hormones, or (3) block a step in conversion of the precursor to lower molecular weight forms of ACTH and endorphin. Since the precursor forms are less immunoreactive (on a molar basis) than the lower molecular weight forms of ACTH, a block of this kind could lower radioimmunoassay values simply by redistributing the forms of the hormone.

To determine if a processing step is inhibited by glucocorticoids, we measured the effect of dexamethasone on the kinetics of labeling of the forms of ACTH and endorphin with radioactive amino acids and sugars. The results in Figures 2–5 show clearly that the reduction of intracellular ACTH immunoreactivity in cells after 48–72 h of treatment with dexamethasone (Table I) cannot be accounted for by a major shift in the distribution of the forms of ACTH. It appears that glucocorticoids induced a decrease in the level of all of the forms of ACTH. This decrease cannot be accounted for by a glucocorticoid inhibition of the uptake of the radioactive amino acids or the total protein synthesis since there is no effect on the other cell proteins (Figure 3). In addition, there appears to be no change in the molecular weights or isoelectric points of the different ACTH proteins after hormonal treatment. Similar conclusions can be drawn for the distribution of the forms of β-endorphin and the N-terminal fragment (unpublished experiments). However, treatment for 72 h with dexamethasone does cause a slightly greater accumulation of highly glycosylated forms of pro-ACTH/endorphin (Figure 2). This shift is being investigated in more detail.

Experiments were then performed to determine if glucocorticoids inhibit the synthesis of precursor proteins. To accomplish this, we used short pulses of radioactive amino acids which labeled the precursor proteins without labeling the intermediates or end products of processing. In this way we could measure synthesis of the precursor independently of processing, turnover, or secretion. The results in Figure 2 show that dexamethasone treatment for 48 h reduces the incorporation of radioactive amino acids into the precursor proteins to about the same extent as it reduces the levels of immunoreactive ACTH and endorphin in AtT-20 cells (Table I). These results suggest that glucocorticoids decrease the hormone levels in AtT-20 cells by suppressing synthesis of the precursor and not by altering processing or degradation of the precursor. It should also be noted that all of these labeling studies have been performed on cultures treated for long periods of time (48–72 h) with steroids. Glucocorticoids may elicit some short-term, transient effects on the processing of the precursor, a possibility which is being investigated in more detail.

Dexamethasone could inhibit the rate of synthesis of the precursor either by reducing the total number of copies of precursor mRNA through an effect on transcription, processing, or turnover of the mRNA or by a translational effect. Translational regulation of protein synthesis has been observed

in several systems. In the case of albumin synthesis in rat liver, translational regulation is associated with shuttling of albumin mRNA selectively between an actively translating form (in the polysomes) and an inactive form (a nonribosomal–ribonucleoprotein complex) depending upon the nutritional state of the animal (Yap et al., 1978). Other examples of this type of regulation have also been reported (Civelli et al., 1976; Kurtz et al., 1978).

We have compared the translating activity of polysomes in a chain completion assay with that of phenol-extracted total cytoplasmic RNA and phenol-extracted polysomal RNA from dexamethasone-treated and untreated cells. If dexamethasone reduces the level of translatable precursor mRNA without causing a redistribution of the mRNA between an active form (in polysomes) and an inactive form, one would expect the steroid to reduce the translational activity of all three fractions to the same extent. This is the result that we found. Furthermore, dexamethasone reduction of translating activity of precursor mRNA in all three preparations exhibited the same dexamethasone dose response. Finally, it should be noted that dexamethasone reduced mRNA translating activity to the same extent and with the same dose response as intracellular levels of ACTH and endorphin (Table I). These results suggest that glucocorticoids decrease the synthesis of the precursor protein in AtT-20 cells by reducing the level of precursor mRNA in the cytoplasm and not by causing a redistribution of precursor mRNA between active and inactive forms. The availability of a cloned cDNA probe to pro-ACTH/endorphin mRNA (Roberts et al., 1979) will make it possible to determine mRNA levels directly in glucocorticoid-treated and untreated cells by RNA–DNA hybridization.

The effect of glucocorticoids on pro-ACTH/endorphin mRNA was found to be specific in terms of steroid structure, in agreement with the results of Nakamura et al. (1978), and selective in terms of the number of gene products affected. Glucocorticoids did not affect total protein or RNA synthesis by the cells, cell growth, or viability. Within the limits of resolution of the gel electrophoresis method used, we can say that the synthesis of other cellular proteins was not grossly affected (Figure 3). These studies suggest, therefore, that the effect of glucocorticoids in these cells is specific and directed at a subset of the expressed genes of the cell.

It is of interest to note that glucocorticoids are unable to reduce the level of pro-ACTH/endorphin mRNA to less than 20% of the control value both in vivo and in vitro [these studies and those of Nakanishi et al. (1977b) and Nakamura et al., (1978)]. One possible explanation is that two or more genes for pro-ACTH/endorphin are being expressed and only one can be regulated by glucocorticoids, resulting in maintenance of a constant low level of the mRNA by the unregulated genes and a variable amount of mRNA by the glucocorticoid-regulated genes. (Such a possibility could only be tested at the present time if there were structural differences in the pro-ACTH/endorphin protein derived from the two different genes.) Haralson et al. (1979) have recently reported that AtT-20/D_{16v} mRNA directs the synthesis of at least two different pro-ACTH/endorphin proteins in a wheat germ cell-free system. However, we have never observed this heterogeneity (Roberts & Herbert, 1977a,b) and other investigators have also reported only one pro-ACTH/endorphin cell-free translation product using AtT-20 mRNA (Nakamura et al., 1978). Structural studies will be necessary to determine the relationship between the multiple pro-ACTH proteins observed by Haralson and colleagues. The tryptic peptide and two-dimensional gel electrophoretic data reported here strongly suggest that the pro-ACTH/endorphin protein being synthesized in the presence and absence of glucocorticoids is the same. Subtle differences, however, such as a change of a single amino acid residue (from alanine to glycine, for example), could only be detected by amino acid sequence determination of the pro-ACTH/endorphin proteins isolated from hormone-treated and untreated cells. Recently, Nakanishi and colleagues (1978, 1979) have reported the complete amino acid sequence of pro-ACTH/endorphin protein from the intermediate lobe of bovine pituitary, and Roberts et al. (1979) have reported a partial sequence of pro-ACTH/endorphin from AtT-20 cells by DNA sequence analysis of a cloned cDNA copy of pro-ACTH/endorphin mRNA from these sources. Such techniques will be extremely valuable in analyzing the pro-ACTH/endorphin mRNA sequence expressed in different tissues before and after hormone treatment.

In summary, long-term glucocorticoid treatment of AtT-20 cells appears to have no major effect on the pathway of processing of the pro-ACTH/endorphin precursor or on the intracellular distribution of the different molecular forms of ACTH and endorphin. However, glucocorticoids do decrease the synthetic rate of the precursor, and this decrease directly reflects an overall decrease in the level of translatable pro-ACTH/endorphin mRNA present in the cytoplasm of the cell. The steroid does not appear to be "shuttling" the ACTH/endorphin mRNA between actively translating polysomes and inactive mRNP particles and also does not appear to be altering the translational efficiency of the pro-ACTH/endorphin mRNA. The glucocorticoid specificity of this effect along with the known presence of specific glucocorticoid receptors in the AtT-20 cells suggests that the mRNA decrease may be mediated by a direct nuclear event of the glucocorticoid–receptor complex.

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Fluorescent Chemoaffinity Labeling. Potential Application of a New Affinity Labeling Technique to Glucocorticoid Receptors†

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ABSTRACT: A new, general methodology for affinity labeling is proposed. We call this method chemo-, or chemically activated, affinity labeling (CAL) since the addition of a specific chemical is required to initiate the affinity labeling process. Chemoaffinity labeling thus combines control over the timing of covalent bond formation, a distinguishing feature of photoaffinity labeling (PAL), with the advantages of the relatively stable and specific "reactive" functional groups found in conventional, or electrophilic, affinity labeling (EAL). Our chemical initiator for CAL is *o*-phthalaldehyde (OPTA), which rapidly reacts with primary amines and thiols to give 1-(alkylthio)-2-alkylisoindoles in high yield [Simons, S. S., Jr., & Johnson, D. F. (1978) *J. Org. Chem. 43*, 2886–2891]. Since these isoindoles are intensely fluorescent [Simons, S. S., Jr., & Johnson, D. F. (1978) *Anal. Biochem. 90*, 705–725], this CAL reaction will usually result in fluorescent chemoaffinity (FCA) labeling, or FCAL. In order to obtain a covalent receptor–steroid complex, we have prepared three hydrolytically stable glucocorticoid derivatives which should participate in this CAL reaction. All three steroids exhibit high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induce the enzyme tyrosine aminotransferase (TAT). In model studies with OPTA and an added amine or thiol, each steroid gives a good yield of the desired isoindole, two of which are strongly fluorescent and display good stability and response to changes in solvent polarity. Thus, these synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors. OPTA is also a new cross-linking reagent, and cross-linked protein–isoindole derivatives readily display energy transfer from tryptophan to isoindole.

Affinity labeling has become a powerful method for studying the binding sites of ligand–macromolecule complexes (Baker et al., 1961; Singh et al., 1962; Jakoby & Wilchek, 1977). Presently, there are two general methods of affinity labeling (Wold, 1977). Conventional, or electrophilic, affinity labeling (EAL) (Baker et al., 1961) relies on the presence of a substituent (HNuc; usually a nucleophile) in the binding cavity of the macromolecule (B) which can attack a chemically reactive functional group (X; e.g., an α-halo ester) attached to the ligand (A). While these reactions are reasonably specific for a given X–HNuc combination, no control over the reaction is possible since covalent bond formation occurs upon approximation of the two groups. The frequency of approxi-

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