size and number of the fragments obtained, it is possible that more than one type of particle exists with differing arrangements of protein as suggested by Weintraub and Van Lente (1974). Alternatively each particle may contain an identical arrangement of groups of histones which may exist in a number of conformational states, thus generating the observed series of discrete lower molecular weight DNA fragments.

Acknowledgments

The author wishes to thank Dr. Sol Spiegelman for helpful discussions and his generous support, Dr. Tom Maniatis for providing sized fragments of λ DNA, and Elizabeth Lacy for her excellent technical assistance.

References

Axel, R., Cedar, H., and Felsenfeld, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2029.
Axel, R., Melchior, W., Sollner-Webb, B., and Felsenfeld, G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4101.
Burgoyne, L. A., Hewish, D. R., and Mobbs, J. (1974), *Biochem. J. 143,* 67.
Clark, R. J., and Felsenfeld, G. (1971), *Nature (London), New Biol. 229,* 101.
Clark, R. J., and Felsenfeld, G. (1974), *Biochemistry 13,* 3622.
Hewish, D. R., and Burgoyne, L. A. (1973), *Biochem. Biophys. Res. Commun. 52,* 504.
Kornberg, R. (1974), *Science 184,* 868.
Kornberg, R., and Thomas, J. O. (1974), *Science 184,* 865.
Maurer, R., Maniatis, T., and Ptashne, M. (1974), *Nature (London) 249,* 221.
Noll, M. (1974), *Nature (London) 251,* 249.
Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330.
Peacock, A., and Dingman, C. (1967), *Biochemistry 6,* 1818.
Rill, R., and Van Holde, K. E. (1973), *J. Biol. Chem. 248,* 1080.
Sahasrabuddhe, C. G., and Van Holde, K. E. (1974), *J. Biol. Chem. 249,* 152.
Sollner-Webb, B., and Felsenfeld, G. (1975), *Biochemistry,* preceding paper in this issue.
Van Holde, K. E., Sahasrabuddhe, C. G., Shaw, B. R., Van Bruggen, E. F., and Arnberg, A. (1974), *Biochem. Biophys. Res. Commun. 60,* 1365.
Weintraub, H., and Van Lente, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4249.

Adrenocorticotropic Hormone Stimulation of Adrenal RNA Polymerase I and III Activities. Nucleotide Incorporation into Internal Positions and 3′ Chain Termini†

Shella A. Fuhrman* and Gordon N. Gill

ABSTRACT: In the presence of 50 m*M* $(NH_4)_2SO_4$ and low concentrations of α-amanitin (7.7 μg/ml), adrenal nuclei synthesize predominately rRNA as characterized by size and base composition. Approximately 10% of the RNA synthesized under these conditions sediments at 4–5 S; this RNA synthesizing activity is inhibited by high concentrations of α-amanitin (231 μg/ml) indicating the presence of RNA polymerase III activity. ACTH administration to guinea pigs results in a twofold increase in adrenal nuclear RNA polymerase I and III activities at 14 hr of hormone treatment. Analysis of the amount of radiolabeled nucleoside triphosphate incorporated in vitro into 3′ chain termini and into internal nucleotide positions has been utilized to measure the number of RNA chains and the average chain length synthesized in vitro. Incorporation into 3′ chain termini is not changed by ACTH; incorporation into internal nucleotides is doubled in parallel with the increase in RNA polymerase I activity. These results are not due to an altered K_m of RNA polymerase I for the four nucleoside triphosphates, nor to differential RNase or phosphatase activity. These studies suggest that the regulation of RNA polymerase I by ACTH is accomplished in part through an increase in the rate of RNA chain elongation.

Increased rRNA synthesis occurs in target tissues in response to a variety of hormonal and growth stimuli. An induction of rRNA[1] synthesis and/or increased nuclear RNA polymerase I activity occurs with estrogen (Notebloom and Gorski, 1963; Barry and Gorski, 1971; Hamilton et al., 1968), glucocorticoids (Garren et al., 1964; Yu and Feigelson, 1971), growth hormone (Oravec and Korner, 1971), chorionic gonadotropin (Jungman and Schweppe, 1972), adrenocorticotropic hormone (ACTH) (Farese and

† From the Department of Biology and the Department of Medicine, Division of Endocrinology, University of California—San Diego, La Jolla, California 92037. *Received October 3, 1974.* This investigation was supported by National Institutes of Health Research Grant No. AM13149 and National Institutes of Health Training Grant No. AM05643 both from the National Institute of Arthritis, Metabolism and Digestive Diseases. S.A.F. is a National Institutes of Health Predoctoral Trainee and G.N.G. is a recipient of Public Health Service Research Career Development Award No. AM70215 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; rRNA, ribosomal RNA; TGMED, 50 m*M* Tris (pH 7.9), 25% glycerol, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 5 m*M* dithiothreitol; PEI, polyethyleneimine.

Schnure, 1967; Fuhrman and Gill, 1974), thyrotropin (Adiga et al., 1971), and thyroxin (Tata and Widnell, 1966; Griswold and Cohen, 1972). An increase also occurs with growth stimuli such as serum or amino acid addition to fibroblasts (Emerson, 1971; Rovera et al., 1971; Franze-Fernandez and Pogo, 1971), phytohemagglutinin addition to lymphocytes (Pogo, 1972), and during hepatic regeneration stimulated by partial hepatectomy (Blatti et al., 1970).

We have been studying the response of the adrenal cortex to ACTH. After 24 hr of ACTH administration, adrenal cortical RNA content is increased approximately 50% with the primary increase being rRNA (Fiala et al., 1956). Adrenal nuclear RNA polymerase I activity has increased by 4 hr and reaches a maximum at 14 hr, while total RNA polymerase II activity remains constant over this time period (Fuhrman and Gill, 1974).

Recently, RNA polymerase III, the enzyme involved in pre 4 S and 5 S RNA synthesis, has been characterized as resistant to low but sensitive to high concentrations of the cyclic octapeptide α-amanitin (Weinmann and Roeder, 1974). Since RNA polymerase III contributes to activity observed under standard RNA polymerase I assay conditions, it became important to assess the effects of ACTH on adrenal RNA polymerase III activity. The present studies indicate that approximately 10% of the RNA synthesizing activity of both control and ACTH stimulated adrenal cortical nuclei assayed under low ionic strength conditions is resistant to low but sensitive to high concentrations of α-amanitin. The present experiments indicate that ACTH administration in vivo results in a twofold increase in RNA polymerase III activity in addition to confirming the increase in RNA polymerase I activity.

Identification of increased RNA polymerase activity in nuclei isolated from stimulated cells does not per se allow a distinction between an increased number of RNA polymerase enzyme moieties and increased activity of RNA polymerase which could be due to alterations in the enzyme, template, or both. The present studies were designed to explore further the increased nuclear RNA polymerase activity induced by ACTH. Analysis of the amount of radiolabeled nucleoside triphosphate incorporated into the 3′ chain ends and into the internal nucleotide positions has been utilized to measure the number of RNA chains being synthesized, the average chain length synthesized in vitro, and the elongation rate (Barry and Gorski, 1971; Cox et al., 1973). This allows for quantitation of the effects of ACTH administration on the number of growing RNA chains and on the average chain size synthesized in vitro. Since one RNA polymerase molecule is engaged in the transcription of each RNA chain (Richardson, 1966) determination of the number of RNA chains provides an estimate of the number of RNA polymerase molecules actively engaged in transcription. Under the assay conditions employed, most of the activity measured is derived from RNA polymerase I. The data presented suggest that the regulation of adrenal cortical nuclear RNA polymerase I activity by ACTH is accomplished through an increase in the average chain length synthesized and in the rate of RNA chain elongation.

Methods

Treatment of Animals and Preparation of Adrenal Nuclei. Female guinea pigs weighing 300–350 g were injected intraperitoneally with 40 U of long-acting depot ACTH (Acthar gel, Armour Pharmaceutical Company) every 12 hr beginning at time zero and sacrificed at 14 hr after the first injection. Variation of the timing of the injections did not alter the results. Control animals received no treatment. All animals were sacrificed between 10 a.m. and 12 noon. Adrenals from 10 animals were pooled; nuclei were prepared and suspended in TGMED (50 m*M* Tris (pH 7.9), 25% glycerol, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 5 m*M* dithiothreitol) as previously described (Fuhrman and Gill, 1974). Control nuclei and ACTH nuclei refer to adrenal nuclei isolated from untreated animals and from animals treated for 14 hr with ACTH, respectively.

RNA Polymerase I Assay. RNA polymerase I reaction mixtures contained 50 m*M* Tris (pH 7.9), 1.23 m*M* $MnCl_2$, 1.54 m*M* $MgCl_2$, 4.6 m*M* NaF, 2 m*M* 2-mercaptoethanol, 7.7% glycerol, 0.03 m*M* EDTA, 1.54 m*M* dithiothreitol, 50 m*M* $(NH_4)_2SO_4$, each unlabeled nucleoside triphosphate at 0.75 m*M*, and 7.7 μg/ml of α-amanitin. The concentrations of nuclear DNA and of labeled nucleoside triphosphate are indicated in the appropriate figure legends. These reaction conditions are optimal for RNA polymerase I activity in adrenal cell nuclei (Fuhrman and Gill, 1974). Unless otherwise stated, reactions were terminated by spotting an aliquot onto DE81 filter paper discs (Whatman, 2.4 cm); the discs were processed as described by Blatti et al. (1970) and counted in 1 ml of toluene–Liquifluor (160 ml Liquifluor to 8 pints of toluene).

RNA Polymerase III Assay. Since eukaryotic RNA polymerase III is inhibited by high concentrations of α-amanitin (Weinmann and Roeder, 1974), RNA polymerase III was assayed using the same conditions as described above for RNA polymerase I except that two α-amanitin concentrations (7.7 and 231 μg/ml) were used. RNA polymerase III activity is derived from the difference in incorporation observed at the two concentrations of α-amanitin. α-Amanitin at 7.7 μg/ml inhibits RNA polymerase III about 5%; 231 μg/ml inhibits it greater than 95%. The monovalent and divalent cation concentrations used are optimal for pre 4S and 5S RNA synthesis (McReynolds and Penman, 1974a).

Extraction and Gradient Analysis of RNA. RNA polymerase I reaction mixtures (0.325 ml) were incubated at 37° for varying time periods. *Dictyostelium discoidum* ribosomal and tRNAs labeled with ^{32}P were added at the beginning of the extraction to measure recovery of RNA and to serve as markers for the gradients. RNA was extracted by the proteinase K method of Firtel and Lodish (1973). The RNA pellet was dissolved in 0.5 ml of H_2O, heated for 1 min at 90°, and layered onto 17 ml of 15–30% linear sucrose gradients containing 0.1 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.5), and 0.5% sodium dodecyl sulfate. After centrifugation for 22 hr at 22,000 rpm in an SW27 rotor, 0.5-ml fractions were collected and 0.5 ml of 10% trichloroacetic acid containing 0.1 *M* sodium pyrophosphate was added to each fraction. Precipitates were collected on GF/C filters, washed with 5% trichloroacetic acid containing 0.1 *M* sodium pyrophosphate, and dried. Radioactivity was determined by scintillation counting in 2 ml of toluene–Liquifluor.

Measurement of RNA Chains. A modification of the procedure described by Barry and Gorski (1971) was used. RNA polymerase I reactions in a final volume of 0.325 ml were incubated at 37° for the indicated times. Incubations were stopped by the addition of 50 μg of yeast RNA and 1.5 ml of 10% trichloroacetic acid containing 0.1 *M* sodium pyrophosphate. The precipitate was collected on GF/C filters and washed first with 5% trichloroacetic acid and then with

2 ml of ethanol at −20°. The filters were dried, placed in test tubes containing 2 ml of 0.3 *M* KOH, and incubated for 16 hr at 37°. Carrier nucleoside (200 nmol) was added 30 min after the start of the incubation. Hydrolysis was stopped with the addition of 0.4 ml of 10% perchloric acid followed by 0.5 ml of 5% perchloric acid. The precipitated $KClO_4$, DNA, protein, and GF/C filter were removed by filtration through a Millipore filter (0.45 μm). The filters were then washed with 0.01 *M* HCl until no radioactivity remained on either filter. Charcoal (25 mg) was added to the combined filtrates to adsorb nucleosides and nucleotides. After incubation at 0° for 1 hr, the mixture was centrifuged and the supernatant was discarded. The charcoal pellet was washed twice with 4 ml of cold H_2O, resuspended in 1 ml of 50% ethanol and 1 *M* NH_4OH, and incubated for 1 hr at 37° to elute the adsorbed nucleotides and nucleosides. Charcoal was removed by filtration through Millipore filters. The filters were then washed twice with 0.5 ml of the elution mixture. The combined filtrates were lyophilized and the residue was dissolved in 200 μl of 50% ethanol. Recovery of both nucleoside and nucleoside monophosphate was 90%.

Separation of nucleoside from nucleoside monophosphate was accomplished by ascending chromatography on polyethyleneimine (PEI)-cellulose thin layer plates (J. T. Baker Chemical Company). The PEI-cellulose plates were prewashed twice with H_2O, then 75 μl of the sample was spotted 2 cm from one end of the plate. The chromatograms were developed first in methanol to 13–14 cm from the origin (Cox et al., 1973), then in H_2O to the methanol solvent front, then in 2.0 *M* sodium formate (pH 3.4) to 3–4 cm from the origin. Nucleosides and nucleoside monophosphates were located by their uv absorption. All nucleosides were found within 3 cm of the H_2O solvent front and all nucleoside monophosphates were found within 1 cm of the sodium formate solvent front; 1-cm portions of each plate were placed in scintillation vials containing 100 μl of 1 *M* HCl; 0.5 ml of NCS (Amersham-Searle Corporation) and 4 ml of toluene–Liquifluor were added; ^3H was counted with 50% efficiency.

Ribonuclease Assay. RNA polymerase I reactions were prepared as described above, except that 10 μ*M* cold UTP was used instead of [^3H]UTP. ^3H-labeled yeast RNA (270,000 cpm/ml of reaction) was added to each reaction mixture. Equal amounts of control and ACTH adrenal nuclei (25 μg of DNA) were added to reactions which were incubated at 37°. Aliquots of 40 μl were removed at various times after the beginning of the reaction and spotted on DE81 filter paper discs which were processed as described by Blatti et al. (1970). The rate of degradation of the [^3H]RNA was determined by fitting the experimental data to a straight line using a computer program for least-squares regression analysis.

Phosphatase Assay. Phosphatase activity was measured by addition of 25 μl of control or ACTH nuclei in TGMED to a reaction mixture (0.5 ml) containing 1 *M* Tris(pH 8.0) and 1 m*M* *p*-nitrophenyl phosphate. After incubation at 37° for 20 min, reactions were stopped by addition of 1 ml of 1 *M* NaOH. Activity was measured as the change in optical density at 410 nm, assuming an E_{410} of 1.62×10^4 for *p*-nitrophenol (Garen and Levinthal, 1960; Wykes and Smellie, 1966).

DNA and Protein Determinations. DNA concentrations were determined by the ethidium bromide method of LePecq and Paoletti (1966) using calf thymus DNA as a standard. Proteins were removed from the samples by adding NaCl to a concentration of 1 *M*, followed by extraction with chloroform–isoamyl alcohol (24:1, v/v) prior to DNA measurement. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

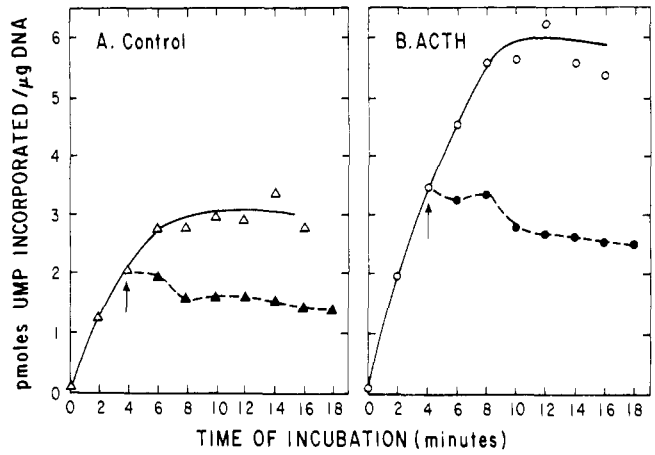

FIGURE 1: Effect of in vivo ACTH administration on in vitro guinea pig adrenal nuclear RNA polymerase activity. Nuclei were isolated from the adrenal glands of untreated guinea pigs and from the adrenal glands of guinea pigs treated in vivo with ACTH for 14 hr. As described under Methods, RNA polymerase activity was assayed at 37° in the presence of 7.7 μg/ml of α-amanitin at an initial [^3H]UTP concentration of 0.015 m*M* (2.02 Ci/mmol). At the time indicated by the arrow, unlabeled UTP was added to one set of incubations to a final concentration of 0.53 m*M* and incubations were continued for the times indicated. Aliquots (20 μl) were spotted onto Whatman DE81 filter paper discs and processed as described under Methods. The closed symbols indicate activity in the presence of excess unlabeled UTP.

Results

Effect of ACTH Administration on Adrenal Nuclear RNA Polymerase I and III Activities. Nuclei isolated from the adrenals of ACTH-treated guinea pigs have increased RNA polymerase activity in low concentrations (7.7 μg/ml) of α-amanitin when compared to nuclei from the adrenals of untreated animals (Figure 1). Since the peak increase in nuclear RNA polymerase activity resistant to 7.7 μl/ml of α-amanitin occurs 14 hr following initiation of ACTH treatment (Fuhrman and Gill, 1974), animals treated for this time period have been utilized in the present experiments. Total nuclear RNA polymerase II activity is not changed by ACTH treatment during this time period (Fuhrman and Gill, 1974). The increased rate of RNA synthesis is observed using each of the four nucleoside triphosphates as radiolabel. To assess the stability of the RNA synthesized in vitro, an excess of unlabeled UTP sufficient to prevent further [^3H]UTP incorporation was added to the reactions at the times indicated and incubation continued. Though some degradation of RNA occurs in both sets of nuclei (Figure 1), the stability of the RNA is not changed by ACTH treatment.

In order to assess the contribution of RNA polymerase III to the RNA polymerase activity observed at low concentrations of α-amanitin, nuclei were incubated at α-amanitin concentrations of 7.7 μg/ml to inhibit RNA polymerase II activity and at 231 μg/ml to inhibit RNA polymerase III activity. This concentration of α-amanitin has been shown to inhibit RNA polymerase III activity at least 95% (Weinmann and Roeder, 1974; S. A. Fuhrman and G. N. Gill, un-

Table I: RNA Polymerase I and III Activities in Adrenal Nuclei.[a]

	RNA Polymerase I (pmol of UMP/μg of DNA)	RNA Polymerase III (pmol of UMP/μg of DNA)
Control	0.94 ± 0.12	0.11 ± 0.01
ACTH	1.47 ± 0.02 ($p < 0.05$)	0.18 ± 0.01 ($p < 0.001$)

[a] Adrenal nuclei isolated from control and ACTH-treated guinea pigs were incubated as described under Methods in reactions containing 0.05 M $(NH_4)_2SO_4$ and α-amanitin at either 7.7 or 231 μg/ml. After incubation for 5 min at 37°, incorporation of radiolabeled UTP was determined as described under Methods. RNA polymerase III activity is calculated as the difference between incorporation at 7.7 μg/ml of α-amanitin and incorporation at 231 μg/ml of α-amanitin. RNA polymerase I activity is the activity observed at 231 μg/ml of α-amanitin. Values shown are the means ± the standard error of the mean. p values were calculated using Student's t test.

Table II: Base Composition of RNA Synthesized in Vitro.[a]

	Control	ACTH Treated
% A	22	19
% G	37	39
% U	17	15
% C	24	27

[a] Adrenal nuclei isolated from control and ACTH-treated guinea pigs were incubated as described under Methods in reactions containing 0.05 M $(NH_4)_2SO_4$ and α-amanitin (7.7 μg/ml). Four separate reaction mixtures were prepared, each with one radiolabeled nucleoside triphosphate. The final concentration of each nucleoside triphosphate in the reaction was 7.7 μM. After incubation for 3 min at 37°, incorporation of each nucleoside triphosphate into RNA was measured as described under Methods. Radiochemical purity of the nucleoside triphosphates was determined immediately prior to use by chromatography on PEI-cellulose (Randerath and Randerath, 1967) and the corrected specific activities used in the calculation of incorporation into RNA. The radiochemical purity of each nucleoside triphosphate was >90%.

published). The results shown in Table I indicate that RNA polymerase III accounts for approximately 10% of the RNA polymerase activity in the presence of 7.7 μg/ml of α-amanitin, and that RNA polymerase III activity is increased approximately twofold after 14 hr of ACTH treatment. The major portion of the RNA polymerase activity which is resistant to 7.7 μg/ml of α-amanitin is also resistant to 231 μg/ml of α-amanitin. This activity, representing RNA polymerase I, is also increased by ACTH treatment.

Partially purified RNA polymerase I has been shown to direct the synthesis of rRNA using *Xenopus laevis* DNA enriched with ribosomal cistrons (Beebee and Butterworth, 1974). Under low ionic strength conditions and in the presence of α-amanitin, nuclei isolated from several sources synthesize RNA which is characterized by base composition, size, and competition hybridization as predominantly rRNA (Zylber and Penman, 1971; Reeder and Roeder, 1972). The RNA synthesized by guinea pig adrenal nuclei in the presence of α-amanitin and 0.05 M $(NH_4)_2SO_4$ has been characterized as rRNA-like by base composition and by size. The RNA is G-C rich (Table II); the base composition resembles that of HeLa cell 45S rRNA precursor and clearly differs from that of HeLa cell heterogeneous nuclear RNA (Willems et al., 1968).

Analysis by sodium dodecyl sulfate sucrose gradient sedimentation of the RNA synthesized in control and ACTH nuclei reveals the presence of large molecular weight

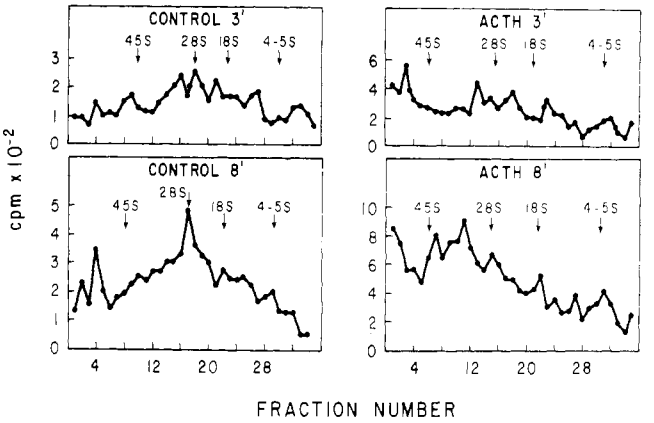

FIGURE 2: Sodium dodecyl sulfate sucrose gradient sedimentation of the RNA synthesized in vitro. Adrenal nuclei isolated from control and ACTH-treated guinea pigs were incubated as described under Methods in reaction mixtures containing 7.7 μg/ml of α-amanitin and 6 μM [^{3}H]UTP (5.08 Ci/mmol) at 37° for the times indicated. Incubations contained 17.6 μg of nuclear DNA from control animals and 18.5 μg of nuclear DNA from ACTH-treated animals. RNA was extracted and analyzed by sedimentation on 15–30% sucrose gradients as described under Methods. The ^{32}P marker recovery from each gradient equaled 75–80%.

species. At an early time point (3 min of incubation), the distribution of radioactivity incorporated into RNA is fairly evenly distributed across the gradient (Figure 2). This is the result expected if 100–200 nucleotides are added to growing RNA chains distributed across the rRNA cistron. At a later time point (8 min of incubation), radioactivity is incorporated primarily into higher molecular weight species of RNA (Figure 2). The 4–5S peak accounts for approximately 10% of the radioactivity on the gradients in agreement with the results using high concentrations of α-amanitin (Table I). The 4–5S peak is abolished by high concentrations of α-amanitin in the incubation assay (data not shown).

Effect of ACTH Administration on Nucleotide Incorporation into 3' Chain Termini. The mechanism of ACTH stimulation of adrenal RNA polymerase I activity was studied by measuring the incorporation of radiolabel into nucleoside and nucleotide residues. RNA synthesized in vitro in assay mixtures containing [^{3}H]CTP or [^{3}H]UTP was extracted and hydrolyzed with alkali. The products of alkaline hydrolysis are nucleoside tetraphosphate from the 5′ end of the RNA chain, 3′-nucleoside monophosphate from the interior of the RNA chain, and nucleoside from the 3′-hydroxyl end of the RNA chain. The products of alkaline hydrolysis were quantitated after chromatographic separation on PEI-cellulose. Nucleoside tetraphosphate was not measured in the present experiments because RNA synthesis in isolated nuclei under low ionic strength conditions consists almost entirely of elongation of already initiated chains (Jacobson et al., 1974; McReynolds and Penman, 1974a). At early time points, incorporation of radiolabel into 3′-hydroxyl chain terminal nucleoside provides a measure of the number of RNA chains and, therefore, of the number of active RNA polymerase I enzymes present. The incorporation of radiolabel into internal nucleoside monophosphate per chain provides a measurement of average chain lengths synthesized in vitro; during active elongation the incorporation into internal nucleoside monophosphate per unit time provides a measure of the rate of nucleotide polymerization.

Results using [^{3}H]CTP and [^{3}H]UTP are shown in Fig-

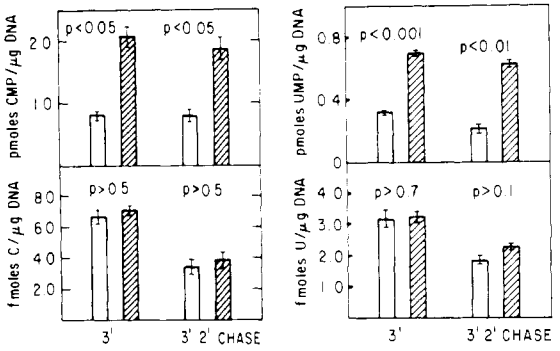

FIGURE 3: Effect of ACTH on nucleoside triphosphate incorporation into nucleoside monophosphate and nucleoside. Reactions containing 30–50 μg of nuclear DNA, 7.7 μg/ml of α-amanitin, and 6.8 μM of [^{3}H]CTP (specific activity 1.8 or 3.65 Ci/mmol) or 6 μM [^{3}H]UTP (specific activity 1.94 or 5.16 Ci/mmol) were incubated at 37° for the times shown. For the chase experiments, the specific activity of the isotope was diluted 100-fold and incubation continued for 2 min. Incorporation into nucleoside and into nucleoside monophosphate was determined as described under Methods. Values shown are the means ± the standard error of the mean. p values were calculated using Student's t test. Nuclei from control adrenals (open bar); nuclei from 14-hr ACTH adrenals (shaded bar).

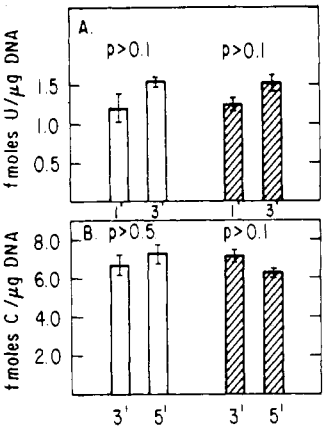

FIGURE 4: Effect of increasing incubation time on incorporation of nucleoside triphosphate into nucleoside. Incubations were performed as described in the legend of Figure 3 for the times indicated, and incorporation into nucleoside quantitated as described under Methods. Values shown are the means ± the standard error of the mean. p values were calculated using Student's t test. The p values shown on the figure compare nucleoside incorporation at two time points for one set of nuclei. The p values calculated for comparing control and ACTH nuclei at any one time point are: U/μg, 1 min, $p > 0.7$; U/μg, 3 min, $p > 0.7$; Ci/μg, 3 min, $p > 0.5$; Ci/μg, 5 min, $p > 0.1$. Nuclei from control adrenals (open bar); nuclei from 14-hr ACTH adrenals (shaded bar).

ure 3. Incorporation into nucleoside monophosphate from the internal positions of the RNA chains is increased by ACTH treatment (upper portion, Figure 3). This increase, which is observed when either [^{3}H]CTP or [^{3}H]UTP is used in the RNA polymerase I reaction, parallels the observed increase in total RNA polymerase I activity. In contrast, the amount of nucleoside from the 3′-hydroxyl ends of the RNA chains is statistically unaltered (lower portion, Figure 3) indicating that the number of RNA chains is equal in nuclei from control and ACTH treated animals. The larger values obtained using [^{3}H]CTP reflect the higher cytidine content of the RNA synthesized.

If radiolabeled nucleoside represents the 3′-hydroxyl end of the growing RNA chains, addition of excess unlabeled nucleoside triphosphate to incubation mixtures should result in replacement of the labeled nucleoside by an unlabeled one. Such cold chase experiments resulted in a decrease in labeled nucleoside incorporation. After incubation with [^{3}H]CTP for 3 min, the specific activity of the isotope was diluted 100-fold and the reaction continued for an additional 2 min (Figure 3, left). The 2-min cold chase reduced the incorporation of label into cytidine by 50% but had no effect on the incorporation into internal nucleoside monophosphate. A similar cold chase experiment using [^{3}H]UTP resulted in a 42% decrease in labeled uridine (Figure 3, right). When a 100-fold excess of unlabeled UTP was added after 1 min of incubation with [^{3}H]UTP, the incorporation into uridine was reduced 80%. It would appear that after 1 min of incubation 80% of the RNA chains measured are actively growing; after 3 min of incubation 40–50% of the measured RNA chains are growing.

Since RNA chain initiation is not occurring, nucleoside incorporation which reflects chain termini should not change with incubation time. UTP incorporation into uridine was statistically unchanged from 1 to 3 min of incubation (Figure 4A), as was CTP incorporation into cytidine from 3 to 5 min of incubation (Figure 4B). The amount of nucleoside measured could be influenced by sequential activity of RNase generating strand scissions followed by production of nucleoside with phosphatase. The stability of the synthesized RNA shown in Figure 1 is similar in the two sets of nuclei indicating similar levels of ribonuclease. The gradient analysis shown in Figure 2 indicates that extensive cleavage to small molecular weight species does not occur. Since only a small portion of an average rRNA chain is labeled in vitro (about 3% at early time points), random endonucleolytic cleavages in this region at a specific nucleotide should be uncommon. In addition, when [^{3}H]RNA is added to control and ACTH nuclei as described under Methods, identical rates of degradation are found (1.1% degradation of input [^{3}H]RNA/min). Phosphatase activity, measured as the conversion of p-nitrophenyl phosphate to p-nitrophenol as described under Methods was statistically unaltered by ACTH treatment. The activity in control nuclei was 115 ± 7 pmol of p-nitrophenol formed per μg of protein per 20 min; in ACTH nuclei, 129 ± 7 pmol per μg of protein per 20 min ($p > 0.1$). These data argue that while some error in the quantitation of nucleoside may be introduced by the activity of RNase and phosphatase, it is small and constant in both the control and ACTH nuclei. Although a small percentage of measured nucleoside may derive from internal scission points or from 3′-OH termini created by processing of the RNA, it is important to note that under all conditions of incubation and labeling, the nucleoside incorporation in control and ACTH nuclei was equal.

These data, in addition to stability of the chain number at different incubation times (Figure 4) and the ability of unlabeled nucleoside triphosphate to decrease the incorporation of labeled nucleoside (Figure 3), indicate that the nucleoside quantitation presented largely reflects the termini of RNA chains being synthesized.

Effect of ACTH Administration on the Average Chain Length Synthesized in Vitro. The ratio of internal nucleoside monophosphate to 3′ chain terminal nucleoside provides an estimate of the average length of RNA synthesized in vitro. As shown in Table III, the average length RNA synthesized in vitro in ACTH nuclei in the presence of 7.7 μg/ml of α-amanitin is approximately twice that synthesized in control nuclei. The rate of nucleotide polymeriza-

Table III: Nucleotide/Nucleoside Ratio of RNA Synthesized in Vitro by Adrenal Nuclei from Control and 14 hr ACTH-Treated Guinea Pigs.

Time of Incubation (min)	Source of Nuclei	3′-UMP/U	3′-UMP/U (× min^{-1})
1	Control	53.7	53.7
	ACTH	91.3	91.3
3	Control	127.8	42.6
	ACTH	249.2	83.1
3	Control	101.6	33.9
	ACTH	213.8	71.3
		3′-CMP/C	3′CMP/C (× min^{-1})
3	Control	116.8	38.9
	ACTH	292.2	97.4
5	Control	147.2	29.4
	ACTH	317.9	63.6

Table IV: Average Chain Length Added in Vitro to RNA Sedimenting above 10 S.[a]

	3′-CMP/C	
Control	180 ± 22	$p < 0.05$
ACTH	302 ± 9	

[a] Adrenal nuclei isolated from control and ACTH-treated guinea pigs were incubated as described under Methods in reaction mixtures containing 4.8 μM [^{3}H]CTP (5.52 Ci/mmol) and 30–50 μg of nuclear DNA at 37° for 3 min. RNA was extracted and sized by sedimentation on 15–30% sucrose gradients as described under Methods. ^{32}P-labeled marker rRNA was displayed on a parallel gradient. RNA sedimenting above 10 S was pooled and collected by ethanol precipitation after the addition of 100 μg of yeast RNA. RNA was hydrolyzed, and nucleoside and nucleotide were separated and quantitated as described under Methods. Values shown are the means ± the standard error of the mean. p values were calculated using Students' t test.

tion is calculated from the ratio of incorporation of internal nucleoside monophosphate to nucleoside per unit time. ACTH treatment results in an approximate doubling of the elongation rate (Table III). The apparent decrease in elongation rate with increasing incubation time reflects the termination which is occurring even at early time points. The 1 min time point where 80% of the ^{3}H-labeled nucleoside can be chased provides the most accurate reflection of the in vitro elongation rate. Although the apparent rate of elongation slows with time of in vitro incubation, the twofold increase in nucleotide incorporation per chain seen with ACTH nuclei was maintained at all times of incubation.

Since RNA polymerase III is active under the low α-amanitin concentrations used in these experiments, synthesis of pre-4S and 5S RNA contributes to the nucleoside and nucleotide quantitation observed, and thus to the calculated average chain length synthesized in vitro. Since RNA polymerase III has been reported to reinitiate in vitro in HeLa cell nuclei (McReynolds and Penman, 1974a), the contribution to nucleoside quantitation may be greater than the observed 10% contribution to total incorporation. Since the present experiments were done at early time points (1-, 3-, and 5-min incubation), the extent of reinitiation should be minimal. McReynolds and Penman (1974b) have reported that the pre-4S RNA synthesized by HeLa and CHO cell nuclei terminates exclusively in U. In the present experiments, the nucleoside quantitated using either [^{3}H]UTP or [^{3}H]CTP is equal in both control and ACTH nuclei suggesting that most of the observed incorporation arises from rRNA precursors.

In order to more directly evaluate the effects of ACTH on rRNA synthesis, RNA, isolated from adrenal nuclei incubated in vitro for 3 min at 37° in the presence of 7.7 μg/ml of α-amanitin, was subjected to sodium dodecyl sulfate sucrose gradient sedimentation, and the material sedimenting above 10 S was analyzed for incorporation of [^{3}H]CTP into nucleoside and nucleotide. The results, which exclude any contribution of pre-4S and 5S RNA, are shown in Table IV. The average chain length added in a 3-min incubation to RNA larger than 10 S is increased in the ACTH nuclei, and compares favorably with the values shown in Table III. These data indicate that any errors in the quantitation of nucleoside introduced by the activity RNA polymerase III in adrenal nuclei are not excessive in short incubations and do not alter the basic observation of an increase in the average chain length synthesized in vitro.

An altered K_m of the nuclear RNA polymerase I for UTP or CTP could explain an apparent increase in the average chain length synthesized in vitro. However, the apparent K_m for each of the four nucleoside triphosphates was similar when control and ACTH nuclei were compared excluding an altered K_m of the enzyme for nucleoside triphosphate as the explanation for the observed increase in rate of nucleotide polymerization (Table V). Double reciprocal plots of RNA polymerase I activity as a function of the UTP and CTP concentrations are illustrated in Figure 5. The plots are linear at low nucleotide concentrations which argues against a significant pool of unlabeled nucleoside triphosphate in either set of nuclei. When nuclei are assayed using [^{3}H]CTP or [^{3}H]UTP as label in the absence of the other three nucleoside triphosphates, no incorporation is found, again indicating that significant pools of nucleoside triphosphate are not present. Similar linear plots were obtained when the concentrations of GTP and ATP were varied. Although phosphatase activity in the nuclei could introduce a systematic error in the K_m determinations, the fact that the K_m's are unaltered after ACTH treatment argues against differential phosphatase activity. It can be calculated from the phosphatase data given above that less than 0.03% of the total nucleoside triphosphate present would be converted to nucleoside diphosphate in a 5-min reaction. These experiments also indicate that there is no differential transport of labeled nucleoside triphosphate into the control and ACTH nuclei.

Since one RNA polymerase I enzyme is involved in the synthesis of each RNA chain, the constant chain number in control and ACTH nuclei implies that the number of active RNA polymerase I moieties remains constant. This also implies increased activity of each RNA polymerase I enzyme transcribing on the endogenous template.

Discussion

In mammalian cells the amounts of each of the major classes of RNA (mRNA, rRNA, and tRNA) increase during the transition from a resting to a growing state (Johnson et al., 1974). Increased rRNA and tRNA contents are associated with increased rates of synthesis, increased nuclear RNA polymerase I and III activities (Johnson et al., 1974; Mauck and Green, 1974), and increased rRNA and tRNA stability (Abelson et al., 1974); increased mRNA content

Table V: Effect of ACTH on the K_m of RNA Polymerase I for the Nucleoside Triphosphates.[a]

		$K_m(\mu M)$	V_{max} (pmol of NMP per μg of DNA per 5 min)
UTP	Control	3.1	1.40
	ACTH	3.5	3.05
CTP	Control	13.0	3.13
	ACTH	11.1	5.44
GTP	Control	18.3	3.81
	ACTH	20.1	8.40
ATP	Control	11.2	1.20
	ACTH	10.0	1.94

[a] K_m and V_{max} values were determined as described in the legend of Figure 5.

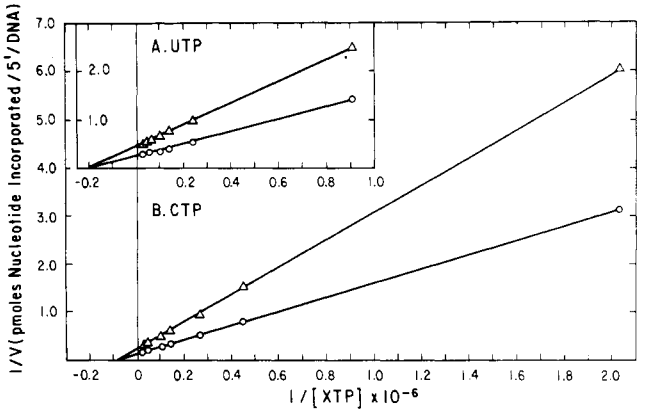

FIGURE 5: Double reciprocal plots of the rate of RNA synthesis as a function of UTP and CTP concentration. RNA polymerase I reaction mixtures (65 μl) containing the final concentrations of [^{3}H]UTP or [^{3}H]CTP indicated and 5–10 μg of nuclear DNA were incubated at 37° for 5 min and incorporation determined as described under Methods. Lines were fitted to the experimental points by a computer program for least-squares regression analysis and K_m and V_{max} values calculated from that analysis. The K_m and V_{max} values determined for each nucleoside triphosphate are listed in Table V. Nuclei from control guinea pig adrenal glands (Δ). Nuclei from guinea pig adrenal glands obtained from animals treated with ACTH for 14 hr (O).

may be associated with increased precursor processing since nuclear RNA polymerase II activity and mRNA stability are not altered (Abelson et al., 1974; Mauck and Green, 1974; Rudland, 1974). Where studied the growth of the adrenal cortex in response to in vivo ACTH treatment resembles the growth of mammalian cells in culture. Increased nuclear RNA polymerase I activity precedes the increase in DNA synthesis beginning 16 hr following initiation of ACTH administration while nuclear RNA polymerase II activity remains constant (Fuhrman and Gill, 1974; Masui and Garren, 1970). The present studies indicate that ACTH treatment also results in increased nuclear RNA polymerase III activity during the prereplicative period.

The rate of RNA synthesis depends on the number of RNA chains being synthesized and the rate at which ribonucleotide residues are added to each chain. In the present experiments RNA polymerase I activity accounts for the largest part of the in vitro incorporation observed. The number of RNA chains undergoing elongation in vitro has been quantitated from the 3′-OH nucleoside ends. Though increased nuclear RNA polymerase I activity is observed in adrenals from ACTH treated guinea pigs, the nucleoside content is unchanged implying an equal number of rRNA chains undergoing in vitro elongation. The average chain length synthesized in vitro, calculated using data that includes all RNA synthesized in the presence of 7.7 μg/ml of α-amanitin (Table III) and data that include only RNA sedimenting faster than 10 S (Table IV), is increased approximately twofold in agreement with the increased nucleotide incorporation. At 3 min of incubation added radioactivity is distributed in all size classes of RNA; at later time points the larger RNA precursors are more heavily labeled (Figure 2). Since the calculated elongation rates do not provide for enough nucleotide addition to result in the size shift observed on gradient sedimentation, a preferential in vitro addition to larger RNA precursor molecules may be occurring at later incubation times. Studies of McReynolds and Penman (1974a) in fact indicate that larger RNA chains elongate faster than shorter ones in vitro. The increased labeling of larger RNA precursors in nuclei from ACTH-treated animals may thus reflect especial labeling of RNA chains nearing completion. Alternatively, since termination is occurring at early time points, the shift of radiolabel to higher molecular weight regions may reflect elongation of a small population of rRNA precursors at a more rapid rate.

The calculated elongation rate is increased approximately twofold in ACTH-treated nuclei. Because termination occurs rapidly in nuclei assayed in vitro, the calculated elongation rates are necessarily inexact. Because 80% of the labeled nucleoside can be replaced by unlabeled nucleoside after 1 min of incubation, assay at this time allows the most accurate estimation of in vitro elongation rates.

If the primary effect of ACTH is to increase the elongation rate of rRNA precursors without altering the number of active RNA polymerase I moieties, the final level of labeled nucleotide incorporation in control nuclei should, after prolonged incubations, equal that in ACTH nuclei. This did not occur during the in vitro incubation times studied (Figure 1) suggesting that the increase in RNA synthesis observed may also result from an increased number of growing chains. However, nucleoside residues were equal under all conditions studied indicating that the number of growing RNA chains were equal. Barry and Gorski (1971) noted a similar discrepancy between the differing plateau values of RNA synthesis in control and estrogen-treated nuclei and identical amounts of 3′-OH chain termini; Guilfoyle and Hanson (1974) also noted this discrepancy in studies of auxin stimulated soybean hypocotyls. The failure of control nuclei to reach an equivalent extent of incorporation may have resulted from the premature termination occurring in vitro. The calculated in vitro elongation rates of 50–100 nucleotides/min (Table III) are comparable to the rates of 25–50 nucleotides/min observed in uterine nuclei in vitro (Barry and Gorski, 1971) and in HeLa cell nuclei in vitro (McReynolds and Penman, 1974a), but are significantly slower than those of 650–2600 nucleotides/min calculated by Emerson (1971) in studies of chick embryo fibroblasts in vivo. Under the assay conditions used here, it would require 130–260 min to complete an entire 45S rRNA precursor. Though some RNA chains may be completed (Figure 2), it is evident that accumulation of label has ceased by 10 min of incubation (Figure 1). The half-life of the RNA polymerase I reaction was measured by incubation of nuclei at 37° for varying times in complete reaction mixtures minus radiolabeled nucleoside triphosphate, followed by addition of radiolabeled nucleoside triphosphate for 3 min of further incubation. The observed half-life of

the reaction is approximately 8 min in both control and ACTH nuclei (data not shown), suggesting that the active nuclear RNA polymerase I–template complex is not stable enough in vitro to allow RNA polymerase I moieties located near the 5′ end of a 45S rRNA gene to actually reach proper 3′ termini.

Premature in vitro termination of RNA chains is thus a significant limitation to determination of accurate elongation rates and assay at early time points is necessary for accurate reflection of chain number and average chain length synthesized in vitro.

An increased elongation rate has been observed in other systems which demonstrate increased rRNA synthesis. In their study of increased uterine nuclear RNA polymerase I activity in estrogen-stimulated rats, Barry and Gorski (1971) observed an increased rate of RNA chain elongation and concluded that this was the major alteration responsible for the increased rate of rRNA synthesis. Emerson's study (1971) of the in vivo rates of rRNA precursor synthesis in chick embryo fibroblasts also indicated an increased elongation rate of the 45S rRNA precursor after refeeding confluent or serum-starved cells.

Using the data present in Figures 3 and 4, one can calculate that there are approximately 100 RNA chains, and, therefore, 100 RNA polymerase I enzymes active in transcription on each rRNA cistron in both control and ACTH nuclei.[2] Since RNA polymerase III activity contributes to nucleoside quantitation at low α-amanitin concentrations, the true ribosomal polymerase number is at least 10% less than this estimate. This value compares favorably with the value of 42 RNA polymerase I molecules per ribosomal gene calculated by Barry and Gorski (1971) for rat uterine nuclei and with the theoretical value of about 240 *Escherichia coli* RNA polymerases that could be packed onto one 45S rRNA cistron (Manor et al., 1969). It is also within the range calculated by Schwartz and Roeder (1974) of 0.6×10^4–15×10^4 RNA polymerase I molecules/cell.

The present studies do not distinguish between primary alterations in the RNA polymerase I enzyme and alterations in the chromatin template. The finding of an equal RNA chain number in control and ACTH nuclei implies that the number of active template sites as well as the polymerase number remains constant. Since the ribosomal genes have not been well separated from the bulk of mammalian chromatin, reported studies have dealt primarily with the levels of solubilized RNA polymerase I under conditions of increased rRNA synthesis. Cox et al. (1973) found a 400% increase in extractible RNA polymerase I activity in uterine nuclei from rats treated with estrogen for 25 hr. Griswold and Cohen (1973) reported a primary increase in solubilized RNA polymerase I activity from thyroxine-treated tadpole liver. Yu and Feigelson (1971) have observed that liver nuclei from cortisone-treated rats demonstrate increased transcription of poly(dC) and poly[d(A-T)] when endogenous transcription is blocked with actinomycin D and α-amanitin. Sajdel and Jacob (1971) have observed altered forms and increased activity of rat liver RNA polymerase I on DEAE-Sephadex chromatography after cortisone administration. Guilfoyle et al. (1975) have reported that auxin stimulation of rRNA synthesis in soybean seedlings results in increased extractible RNA polymerase I, though the number of RNA polymerase I molecules actively transcribing chromatin in vitro is equal (Guilfoyle and Hanson, 1974). These data have all been interpreted as indicating that the concentration and/or activity of RNA polymerase I is increased.

In contrast, Benecke et al. (1973) reported that cortisol treatment did not alter the amount of activity nor chromatographic properties of extractible RNA polymerase I from rat liver. In yeast, readdition of amino acids or glucose results in increased rRNA synthesis and increased nuclear RNA polymerase I activity (Gross and Pogo, 1974). The amount of extractible RNA polymerase I activity was unchanged under the conditions of increased or decreased nuclear RNA polymerase I activity indicating that it is the interaction of the enzyme with the nuclear DNA which is regulated.

The ultimate goal of distinguishing between alterations of RNA polymerase I itself or its chromatin template which result in an increased rate of RNA synthesis will require methods of purification of RNA polymerase I which will favor maintenance of an altered function, if such exists; as well as methods for purification of chromatin containing ribosomal genes.

Acknowledgments

The authors thank Dr. Richard Firtel for his suggestions concerning the extraction and gradient analysis of the in vitro synthesized RNA, and for the gift of the [^{32}P]*Dictyosteleum* RNA.

References

Abelson, H. T., Johnson, L. F., Penman, S., and Green, H. (1974), *Cell 1,* 161.

Adiga, P. R., Murthy, P. U. N., and McKenzie, J. M. (1971), *Biochemistry 10,* 702.

Barry, J., and Gorski, J. (1971), *Biochemistry 10,* 2384.

Beebee, T. J. C., and Butterworth, P. H. W. (1974), *Eur. J. Biochem. 45,* 395.

Benecke, B. J., Fevencj, A., and Seifart, K. H. (1973), *FEBS Lett. 31,* 53.

Blatti, S. P., Ingles, C. J., Lindell, T. J., Morrie, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 649.

Cox, R. F., Haines, M. E., Carey, N. H., and Catlin, G. H. (1973), *Eur. J. Biochem. 32,* 513.

Emerson, C. P., Jr. (1971), *Nature (London), New Biol. 232,* 101.

Farese, R. V., and Schnure, J. J. (1967), *Endocrinology 80,* 872.

Fiala, S., Sproul, E. E., and Fiala, A. (1956), *J. Biophys. Biochem. Cytol. 2,* 115.

Firtel, R. A., and Lodish, H. F. (1973), *J. Mol. Biol. 79,* 295.

Franze-Fernandez, M. T., and Pogo, A. O. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3040.

Fuhrman, S. A., and Gill, G. N. (1974), *Endocrinology 94,* 699.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38,* 470.

[2] The assumptions made in the calculation of the packing of RNA polymerase I enzymes per ribosomal RNA cistron are that each cell has 5.9 pg of DNA (Vendrely and Vendrely, 1949), that there are about 1000 copies of the rRNA genes per cell (Steele, 1968; Mohan et al., 1969), and that the base composition of the RNA is that shown in Table I. For example:

$$\left(\frac{7 \times 10^{-15} \text{ mol cytidine}}{\mu\text{g DNA}}\right)\left(\frac{4 \text{ mol nucleoside}}{1 \text{ mol cytidine}}\right)\left(\frac{5.9 \times 10^{-6} \mu\text{g DNA}}{\text{cell}}\right) \times \left(\frac{6.02 \times 10^{23} \text{ molecules}}{\text{mol}}\right)\left(\frac{1 \text{ cell}}{1000 \text{ rRNA cistrons}}\right) = \frac{99 \text{ RNA polymerase I}}{\text{rRNA cistron}}$$

Garren, L. D., Howell, R. R., and Tomkins, G. M. (1964), *J. Mol. Biol. 9,* 100.
Griswold, M. D., and Cohen, P. P. (1972), *J. Biol. Chem. 247,* 353.
Griswold, M. D., and Cohen, P. P. (1973), *J. Biol. Chem. 248,* 5354.
Gross, K. J., and Pogo, A. O. (1974), *J. Biol. Chem. 249,* 568.
Guilfoyle, T. J., and Hanson, J. B. (1974), *Plant Physiol. 53,* 110.
Guilfoyle, T. J., Lin, C. Y., Chen, Y. M., Nagao, R. T., and Key, J. L. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 69.
Hamilton, T. H., Widnell, C. C., and Tata, J. R. (1968), *J. Biol. Chem. 243,* 408.
Jacobson, A., Firtel, R. A., and Lodish, H. F. (1974), *J. Mol. Biol. 82,* 213.
Johnson, L. F., Abelson, H. T., Green, H., and Penman, S. (1974), *Cell 1,* 96.
Jungman, R. A., and Schweppe, J. S. (1972), *J. Biol. Chem. 247,* 5542.
LePecq, J. G., and Paoletti, C. (1966), *Anal. Biochem. 17,* 100.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Manor, H., Goodman, D., and Stent, G. S. (1969), *J. Mol. Biol. 39,* 1.
Masui, H., and Garren, L. D. (1970), *J. Biol. Chem. 245,* 2627.
Mauck, J. C., and Green, H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2819.
Mauck, J. C., and Green, H. (1974), *Cell 3,* 171.
McReynolds, L., and Penman, S. (1974a), *Cell 1,* 139.
McReynolds, L., and Penman, S. (1974b), *Cell 3,* 185.
Mohan, J., Dunn, A., and Casola, L. (1969), *Nature (London) 223,* 295.
Notebloom, W. D., and Gorski, J. (1963), *Proc. Natl. Acad. Sci. U.S.A. 50,* 250.
Oravec, M., and Korner, A. (1971), *J. Mol. Biol. 58,* 489.
Pogo, G. B. T. (1972), *J. Cell Biol. 53,* 635.
Randerath, K., and Randerath, E. (1967), *Methods Enzymol. 12A,* 323.
Reeder, R. H., and Roeder, R. G. (1972), *J. Mol. Biol. 67,* 433.
Richardson, J. P. (1966), *J. Mol. Biol. 21,* 115.
Rovera, G., Farber, J., and Baserga, R. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1725.
Rudland, P. S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 750.
Sajdel, E. M., and Jacob, S. T. (1971), *Biochem. Biophys. Res. Commun. 45,* 707.
Schwartz, L. B., and Roeder, R. G. (1974), *J. Biol. Chem. 249,* 5898.
Steele, W. J. (1968), *J. Biol. Chem. 243,* 3333.
Tata, J. R., and Widnell, C. C. (1966), *Biochem. J. 98,* 604.
Vendrely, R., and Vendrely, C. (1949), *Experientia 5,* 327.
Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.
Willems, M., Wagner, E., Laing, R., and Penman, S. (1968), *J. Mol. Biol. 32,* 211.
Wykes, J. R., and Smellie, R. M. S. (1966), *Biochem. J. 99,* 347.
Yu, F.-L., and Feigelson, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2177.
Zylber, E. A., and Penman, S. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2861.

Synthetic Position 5 Analogs of Adrenocorticotropin Fragments and Their in Vitro Lipolytic Activity†

Michael W. Draper,* Martin A. Rizack, and R. B. Merrifield

With the Assistance of Barbara H. Guernsey

ABSTRACT: Twenty-three analogs of the ACTH-(4–10)-heptapeptide sequence, which forms the "active core" of adrenocorticotropin (ACTH) and related hormones, have been synthesized by the solid-phase method. These analogs all contain structural modifications at or near the 5-glutamic acid residue of ACTH. The peptides were purified to electrophoretic and chromatographic homogeneity. The peptides were assayed for lipolytic activity in an isolated cell system derived from rabbit adipose tissue. In this system, it was determined that residue 5 plays a very important "spacer" role in the peptide, but that this spacer function is not very dependent on the nature of the side chain of the position 5 amino acid. It was found, however, that a number of analogs containing *basic* residues (arginine or lysine) in position 3 and/or position 5 of ACTH-(3–10) and ACTH-(4–10) fragments have 5 to 10 times the activity of the respective parent peptides. The presence of a latent anionic locus in the rabbit fat-cell receptor for ACTH is suggested by this study.

The ACTH-(4–10)-heptapeptide sequence (Met-Glu-His-Phe-Arg-Trp-Gly) has been found to be absolutely invariant in the adrenocorticotropins (ACTH), melanocyte-stimulating hormones (MSH), and lipotropins (LPH)[1] of all species sequenced to date (Riniker et al., 1972; Li, 1972; Hechter and Braun, 1972; Lee et al., 1963; Lowry and

† From the Rockefeller University, New York, New York 10021. *Received January 13, 1975.* This work was supported in part by National Science Foundation Grant No. BMS 571-01352.

* Present address: Department of Medicine, Harbor General Hospital, Torrance, Calif. 90509

[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; LPH, lipotropin; Boc, *tert*-butoxycarbonyl.