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Cell-Free Synthesis of Rat Growth Hormone

(pituitary tumor cells/Krebs II ascites cells/membrane)

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ABSTRACT **Growth hormone has been synthesized in a cell-free system derived from Krebs II ascites cells, under the direction of RNA prepared from rat pituitary tumor (GC) cells. Growth hormone synthesized in the cell-free system was identified by precipitation with antiserum against growth hormone developed in baboon, followed by electrophoretic analysis of the dissolved precipitate on sodium dodecyl sulfate–polyacrylamide gels. RNA from both the membrane and the post-membrane fractions of the cytoplasm of GC cells stimulated protein synthesis in the cell-free system, but only RNA from the membrane fraction was found to direct the synthesis of growth hormone.**

An understanding of the processes involved in gene expression in eukaryotic cells will require the ability to detect and quantitate the messenger RNAs for particular proteins. Recently, cell-free systems capable of initiating and completing the translation of heterologous or homologous eukaryotic messenger RNAs have been developed. Messenger RNAs for embryonic chicken myosin, bovine lens α-crystallin protein, mouse hemoglobin, rabbit hemoglobin, duck hemoglobin, mouse myeloma immunoglobin light chain, hen oviduct albumin, and reovirus capid proteins have been translated in a reticulocyte cell-free system (1–11). Messenger RNAs for rabbit hemoglobin, mouse globin, human globin, mouse myeloma light chain, bovine lens α-crystallin protein, chicken oviduct ovalbumin, HeLa cell histones, encephalomyocarditis viral coat proteins, reovirus capid proteins, Qβ bacteriophage, and hepatic tryptophan oxygenase have been translated in a murine cell-free system derived from Krebs II ascites tumor cells (11–28). Messenger RNAs for reovirus, encephalomyocarditis virus, mouse Elberfeld virus, and Mengo virus have been translated in cell-free systems derived from mouse L cells, Chinese hamster ovary cells, HeLa cells, and Ehrlich ascites tumor cells (11, 29, 30). Such systems for protein synthesis provide a highly specific and sensitive means for detection and characterization of messenger RNA.

Growth hormone (GH) is a secretory pituitary protein, composed of a single polypeptide chain of molecular weight about 21,000 (34). At present, relatively little is known about the site and the control of the synthesis of growth hormone and other pituitary protein hormones. To obtain further information in this area, we have developed techniques for the partial purification of GH messenger RNA, and for its translation in a heterologous cell-free system. The source of the GH messenger RNA was a clonal strain of rat pituitary tumor cells (GC), in which GH represents about 10% of the total protein synthesis (31).

Abbreviations: HEPES, 2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; GH, growth hormone; GC cells, clonal strain of rat pituitary tumor cells.

MATERIALS AND METHODS

Cells. Krebs II ascites tumor cells were obtained from Dr. A. Burness and maintained in the peritoneal cavities of CA-1 Swiss female mice obtained from Charles River Laboratories, Wilmington, Mass. The cells were harvested 7 days after intraperitoneal injection.

The GC rat pituitary tumor cells were grown in suspension culture in Eagle's minimal essential medium (Joklik's modification, Grand Island Biological Co.) containing 15% horse serum, 2.5% fetal-calf serum, and 15 mM HEPES (2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), as described (31).

Preparation of Cell-Free Extract (S-30). The ascites fluid from twelve mice was washed three times with cold phosphate-buffered saline (pH 7.2, Ca^{++}- and Mg^{++}-free, 0.1 mM EDTA added) as described by Burness (unpublished). The cell-free extract was prepared following the procedure of Aviv, Boime, and Leder (24).

Cell-Free Protein Synthesis was carried out in a volume of 0.1 ml, containing 30 mM Tris·HCl (pH 7.5), 3.5 mM $Mg(CH_3COO)_2$, 90 mM KCl, 7 mM 2-mercaptoethanol, 0.16 mg/ml of creatine kinase, 10 mM creatine phosphate, 40 μM of the 19 L-amino acids, 3.67 μM of L-[^{14}C]leucine (0.12 μCi, 307 Ci/mol), and 30 μl of cell-free extract (S-30) from mouse Krebs II ascites tumor cells. In some experiments, the creatine kinase–creatine phosphate energy-generating system was replaced by a phosphoenolpyruvate energy-generating system. In such cases 10.3 mM sodium phosphoenolpyruvate was used and the concentrations of $Mg(CH_3COO)_2$ and KCl were changed to 4.5 and 82 mM, respectively. It was not necessary to add exogenous pyruvate kinase. The reaction was allowed to proceed for 2 hr at 29°, and was stopped by the addition of 0.4 ml of 0.1 M KOH. After a further incubation for 20 min at 36°, 2 ml of 10% trichloroacetic acid was added. The trichloroacetic acid precipitate was washed three times with 2 ml of 5% trichloroacetic acid, dissolved in 2 ml of 90% formic acid, dried under a heat lamp on a planchet, and counted in a Nuclear-Chicago gas flow counter with an efficiency of 16% for ^{14}C.

For experiments involving immune precipitations, the reaction mixture was scaled up to 1 ml, and labeling was carried out by replacing nonradioactive methionine with 0.082 μM of L-[^{35}S]methionine (1.666 μCi, 122 Ci/mM). At the end of the incubation period the reaction mixture was centrifuged at 15,000 rpm for 2 min; 0.1 ml of the supernatant was then withdrawn and processed as described above to determine the total acid-insoluble radioactivity. The remainder of the reaction mixture was subjected to immune precipitation, as described below.

Preparation of RNA. Cytoplasm was prepared from 1.2 × 10^9 GC cells, and divided into membrane and post-membrane fractions as described (32). Briefly, preparation of these fractions involved three washes of the cells with phosphate-buffered saline, swelling in hypotonic buffer, disruption in a Dounce homogenizer, removal of nuclei by centrifugation at 800 × *g* for 5 min, and separation of membrane from post-membrane fractions by centrifugation at 21,500 × *g* for 10 min.

Preparation of RNA from membrane and post-membrane fractions was as follows. The pellet containing the membrane fraction was suspended in 10 volumes of a buffer containing 0.14 M NaCl–0.01 M Tris·HCl (pH 8.4)–0.5% Na dodecyl sulfate–5 mM EDTA. This suspension was mixed with an equal volume of phenol–chloroform–isoamyl alcohol (1:1:0.01) and vigorously shaken for 5 min at 37°. The mixture was centrifuged at 10,000 × *g* for 5 min, and the resulting top aqueous phase was carefully removed. The insoluble interphase was resuspended in 10 ml of the above buffer and again extracted in the same manner. The two aqueous phases were combined and extracted with an equal volume of phenol (88% phenol, 12% H_2O) by shaking for 5 min at 37°. After centrifugation, the top aqueous phase was carefully removed and extracted with phenol in the same manner. To the final aqueous phase, 1/10 volume of solution containing 2 M sodium acetate and 10 mM EDTA (pH 5.1) was added and RNA was precipitated by the addition of 2 volumes of cold 95% ethanol. The precipitate was collected by centrifugation and washed twice with cold 95% ethanol, dissolved in water, dialyzed against 2 liters of water overnight at 4°, concentrated by lyophilization, dissolved in a small volume of water, and stored at −70°. RNA from the post-membrane fraction of the cytoplasm was prepared in a parallel fashion.

Immune Precipitation. After incubation and centrifugation as described above, reaction mixtures were analyzed for radioactivity precipitable by antibody against GH as described (31). Briefly, carrier medium containing unlabeled GH was added, followed by sufficient antiserum against rat GH prepared in baboon to yield a slight antibody excess. After incubation overnight at 4°, the immune precipitates were washed twice with phosphate-buffered saline. The precipitates were then dissolved in Na dodecyl sulfate sample buffer by boiling for 2 min, for analysis by Na dodecyl sulfate–polyacrylamide gel electrophoresis. To a small aliquot of the dissolved precipitate 0.1 ml of 10% bovine-serum albumin and 0.4 ml of 0.1 M KOH were added, and the mixture was incubated for 20 min at 36°. This mixture was then precipitated with trichloroacetic acid, and processed for determination of acid-insoluble radioactivity as described above.

TABLE 1. *Gross peptide synthesis and antibody-precipitable peptide synthesis stimulated by RNA from membrane (M) and post-membrane (PM) fractions**

Energy-generating system	Exogenous RNA: Source	Exogenous RNA: Concentration (μg/ml)	Incorporation of [^{35}S]methionine (cpm): Total trichloroacetic acid ppt	Incorporation of [^{35}S]methionine (cpm): Anti-GH serum ppt
Creatine phosphate	—	—	51,000	375
	PM	240	208,500	1,430
	M	255	192,500	3,300
Phosphoenolpyruvate	—	—	33,250	150
	PM	240	128,000	825
	M	255	122,000	3,340

* Cell-free protein synthesis was performed in 1 ml of reaction mixture for 2 hr at 29°.

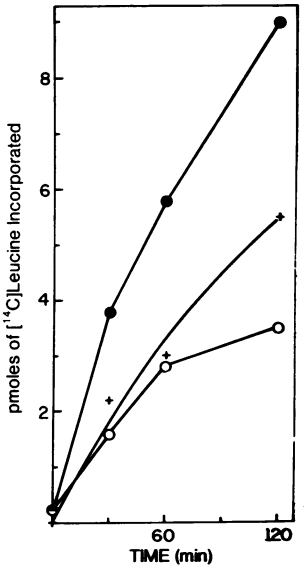

FIG. 1. Time-course of polypeptide synthesis in the cell-free system in the presence and absence of membrane-fraction RNA. Replicate samples of 0.1 ml volume, with creatine phosphate as energy source, were incubated for various time intervals. Incorporation of [^{14}C]leucine into acid-insoluble material was as described in *Methods.* (●——●) Incorporation of [^{14}C]leucine in the presence of 100 μg/ml of RNA from the membrane fraction of GC cell cytoplasm; (○——○) incorporation of [^{14}C]leucine in the absence of exogenous RNA; (+–+–+) net incorporation stimulated by membrane-fraction RNA.

^{3}H-Labeled GH was prepared by incubation of GC cells for 2 hr in the presence of [^{3}H]leucine, and precipitation of the labeling medium with antiserum against rat GH (31).

Polyacrylamide Gel Electrophoresis in the Presence of Na Dodecyl Sulfate. Na dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described (31). Gels containing only ^{35}S-labeled protein were fractionated in a Savant gel crusher (33) and counted in a Nuclear-Chicago low-background planchet counter. Gels containing both ^{35}S- and ^{3}H-labeled protein were fractionated in a Savant gel crusher into 15 ml of methoxyethanol toluene scintillant [per liter: 570 ml of toluene, 390 ml of methoxyethanol, 40 ml of "Spectrafluor-PPO-POPOP" (Amersham/Searle)], and counted in a Beckman LS-100 scintillation counter. The tritium radioactivity was corrected for ^{35}S spillover.

RESULTS AND DISCUSSION

It has been observed that the majority of newly synthesized GH in the GC cells is membrane-associated (32), suggesting that GH may be synthesized on membrane-bound polysomes. Hence, in the present experiments GC cell cytoplasm was separated into membrane and post-membrane fractions, and

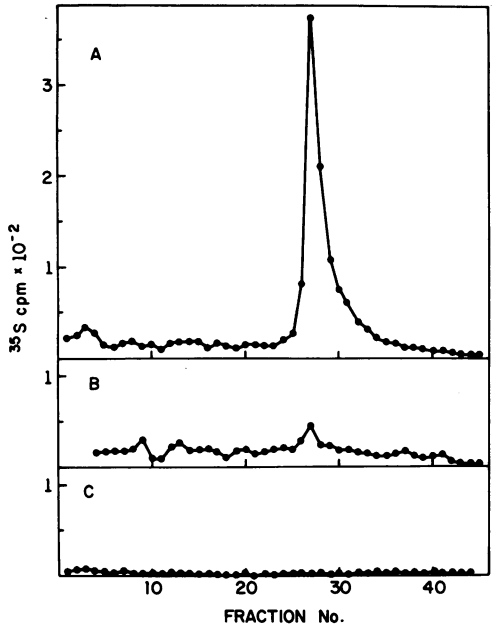

FIG. 2. Na dodecyl sulfate–acrylamide gel electrophoresis of material precipitable by antibody against GH synthesized in the cell-free system. Anti-GH antiserum precipitates of 1.0 ml of reaction mixture which contained a phosphoenolpyruvate energy-generating system were analyzed on 10-cm, 10% acrylamide Na dodecyl sulfate-containing gels. Migration was from *left* to *right*. The reaction mixtures contained RNA isolated from GC cell cytoplasm as follows: (*A*) 255 μg/ml of membrane-associated RNA; (*B*) 240 μg/ml of post-membrane RNA; (*C*) no exogenous RNA.

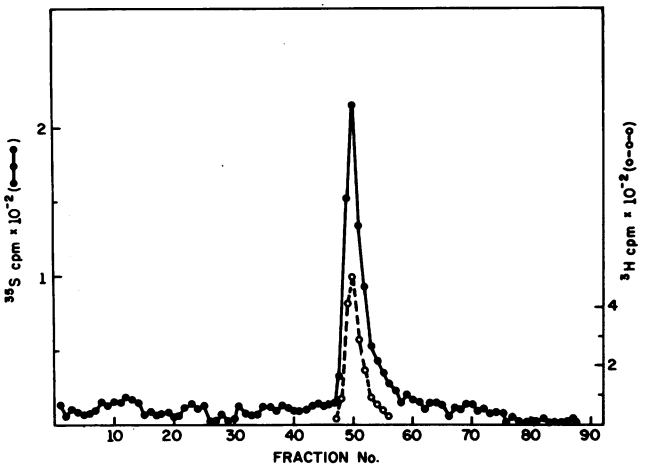

FIG. 3. Relative mobilities during Na dodecyl sulfate–acrylamide gel electrophoresis of material precipitable by antibody against GH synthesized *in vitro* and *in vivo*. The antiserum precipitate of ^{35}S-labeled material synthesized in the cell-free system, with creatinine phosphate as energy source and containing 100 μg/ml of membrane-associated RNA from GC cells, was dissolved in sample buffer. To this was added sample buffer containing ^{3}H-labeled GH, prepared by antiserum precipitation of the medium from GC cells incubated in the presence of [^{3}H]-leucine. The sample was then boiled for 2 min and subjected to electrophoresis on a 10%, 20-cm gel. ^{35}S, ●——●; ^{3}H, ○——○.

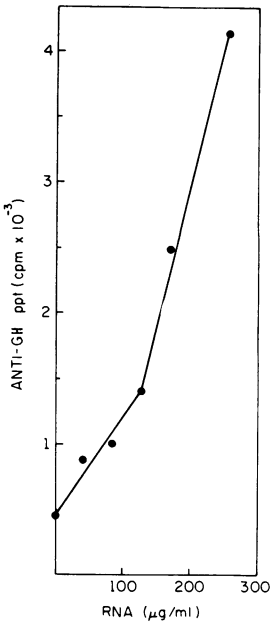

FIG. 4. Synthesis of material precipitable by antibody against GH in the presence of various concentrations of membrane-associated RNA from GC cells. Membrane-associated RNA was incubated at the indicated concentrations in the cell-free system with phosphoenolpyruvate as the energy-generating system, in the presence of [^{35}S]methionine. The volume of the reaction mixture was 0.5 ml. Radioactivity precipitable by antiserum against GH was determined as described in *Methods*.

the RNA from either fraction was incubated in the presence of ascites cell-free extract. The result of such an experiment, performed with membrane-fraction RNA, is shown in Fig. 1. Protein synthesis proceeded at a nearly linear rate for about 2 hr, attesting to the stability of the system. At the concentration of membrane-fraction RNA used, the stimulation of protein synthesis by the added RNA was only about 2-fold (Fig. 1), indicating that the preincubation procedure designed to remove endogenous messenger RNA activity was only partly effective.

GH was detected and quantitated among the peptides and proteins newly synthesized *in vitro* by a combination of immunologic and electrophoretic techniques. Antiserum against growth hormone prepared in baboon plus carrier GH were used under conditions of slight antibody excess to specifically precipitate the labeled GH. This technique has been shown to result in the specific precipitation of labeled GH from the cytoplasm of the GC cells (31). The radioactive material present in the immune precipitates (1–3% of the total trichloroacetic-acid precipitate, see Table 1) was further analyzed by electrophoresis on Na dodecyl sulfate-containing polyacrylamide gels.

RNA preparations obtained from either the membrane or the post-membrane fractions of GC cytoplasm were incubated in the cell-free system. RNA preparations from either cell fraction were found to be about equally effective in stimulating gross polypeptide synthesis, measured as acid-insoluble radioactivity (Table 1). However, the polypeptide synthesis stimulated by membrane RNA that is precipitable by antiserum against GH was about three to five times that observed with post-membrane RNA (Table 1).

The radioactively labeled, immunologically precipitated material was characterized as GH by electrophoresis of the solubilized antibody precipitate on Na dodecyl sulfate–acrylamide gels. It was observed that most of the protein precipitated by antiserum against GH which was synthesized in the presence of membrane RNA migrated as a single peak with the approximate mobility of GH (Fig. 2*A*). Only a trace of this peak was observed when postmembrane RNA was used to program protein synthesis (Fig. 2*B*), and no such peak was observed when synthesis was carried out in the absence of added RNA (Fig. 2*C*). The possibility that the lack of GH messenger RNA activity in the post-membrane RNA fraction was due to the presence in this fraction of an inhibitor of the translation of GH messenger RNA was tested by incubation in the cell-free system of equal concentrations of RNA from the membrane and post-membrane fractions. No inhibition of the peak observed with membrane-fraction RNA alone was observed (data not shown).

The identity of the material synthesized in the presence of membrane RNA as GH was further verified by the following observations. The presence of excess unlabeled rat GH during the immune reaction has been shown earlier to create a condition of antigen excess, preventing the formation of an immune precipitate, and thus preventing the precipitation of radioactive GH from the cytoplasm of the GC cells (31). In the present experiments, the presence of excess unlabeled rat GH during the immune reaction was found to abolish the peak observed under the conditions of Fig. 2*A* (data not shown). In addition, the [^{35}S]methionine-labeled product synthesized *in vitro* was found to comigrate with ^{3}H-labeled GH synthesized and secreted by the GC cells (Fig. 3).

An experiment was performed to test the usefulness of the present system for the quantitation of GH messenger RNA. Various concentrations of membrane RNA from the GC cells were incubated in the cell-free system, and immune precipitation was performed. The ratio of the radioactivity in the immune precipitate to the concentration of membrane RNA used was observed to be constant up to a membrane RNA concentration of 125 μg/ml, above which an increased ratio was observed (Fig. 4). The increased ratio at higher membrane RNA concentrations most likely arose from a nonspecific stimulatory effect of the ribosomal RNA present in this preparation on the translation of messenger RNA (35).

It is concluded that RNA isolated from GC cells may be translated in a Krebs II ascites cell-free system into polypeptide chains with the same immunological characteristics and electrophoretic mobility on Na dodecyl sulfate–acrylamide gels as GH. The observation that membrane-associated RNA directs the synthesis of a GH-like protein while postmembrane RNA does not is consistent with earlier observations that the majority of newly synthesized GH is membrane-associated (32), and implies the existence of a cellular mechanism for the specific association of GH messenger RNA with membranes.

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