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Messenger RNA for Hepatic Tryptophan Oxygenase: Its Partial Purification, Its Translation in a Heterologous Cell-free System, and Its Control by Glucocorticoid Hormones

(enzyme synthesis/steroid)

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ABSTRACT **Messenger RNA from rat liver was partially purified by chromatography on cellulose on the basis of its poly(A) content. Microgram amounts of this RNA stimulate protein synthesis manyfold in a heterologous cell-free system, derived from Krebs ascites cells supplemented with reticulocyte initiation factors. The messenger RNA directs the initiation, synthesis, and release of a product that was identified as complete subunits of hepatic tryptophan oxygenase (EC 1.13.1.12) by immunoprecipitation with monovalent antibodies prepared against homogeneous tryptophan oxygenase and subsequent sodium dodecyl sulfate–polyacrylamide electrophoresis of the solubilized immunoprecipitate. This may represent the first complete translation in a heterologous system of a mammalian messenger RNA coding for an enzyme protein. Analysis of the messenger RNA content of the liver after glucocorticoid administration demonstrates that the hormonally enhanced rate of synthesis of tryptophan oxygenase is accompanied by an increased quantity of its corresponding messenger RNA.**

Insight into the intimate biochemical processes controlling gene expression during cell differentiation and hormone action in eukaryotes will require the quantitation of changes in the cellular content and rate of synthesis of specific mRNAs. It has only recently become possible to unequivocally identify certain cellular mRNAs by their coding function for specific proteins in homologous and heterologous cell-free protein-synthesizing systems. This identification has, however, been limited to mRNA preparations coding for proteins that represent major components of highly specialized cells, e.g., globin, myosin, lens crystalline, immunoglobulin, and ovalbumin (1–8). The recent discovery that eukaryotic mRNA contains a sequence of poly(A) residues has permitted the isolation of mRNAs in high yield and partially purified form (8–10). This development, in conjunction with the availability of sensitive heterologous cell-free protein-synthesizing systems, encouraged us to investigate the hepatic quantity of the mRNA for the enzyme tryptophan oxygenase even though this enzyme represents only a minimal fraction of the rat-liver proteins. Furtherfore, the mRNA for tryptophan oxygenase was selected because this enzyme is under delicate regulatory control (11).

The increased activity of tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12) after glucocorticoid administration was the first demonstration of an inducible enzyme in a mammalian system (12). Feigelson and Greengard subsequently showed that this increased enzyme activity resulted from an increased amount of enzyme protein present (13), which was preceded and accompanied by enhanced rates of incorporation of precursors into hepatic RNA, reflecting hormonal regulation of DNA transcription (14–18). By studying the incorporation of labeled amino acids into the immunologically isolated enzyme during hormonal induction, Schimke *et al.* concluded that the changes in the amount of enzyme were due to an increased rate of synthesis of the enzyme *in vivo* (19). To study whether the increased rate of tryptophan oxygenase synthesis is accompanied by a higher quantity of its mRNA, we developed a heterologous cell-free protein-synthesizing system that allowed the faithful translation of the partially purified mRNA for tryptophan oxygenase. These studies demonstrate that hydrocortisone administration causes a rise in the cellular amount of mRNA for tryptophan oxygenase concurrent with the hormonally enhanced rate of synthesis of this enzyme protein.

MATERIALS AND METHODS

Extraction and Purification of RNA. RNA was extracted from livers of untreated rats or those that had been injected 3 hr previously with 5 mg/100 g body weight of hydrocortisone acetate. The livers were homogenized in a Waring Blendor at maximal speed for 1 min in 15 volumes of 0.05 M sodium acetate (pH 5.0)–10 mM EDTA–0.5% sodium dodecyl sulfate to which an equal volume of buffer-saturated phenol–chloroform 1:1 had been added. After it was vigorously shaken for 5 min at room temperature (25°), the suspension was rapidly cooled and centrifuged at 6000 $\times g$ for 15 min. The aqueous phase was again extracted five times with the buffer-saturated phenol–chloroform, made 0.3 M in LiCl; the RNA was precipitated with two volumes of ethanol at −20°. The precipitated RNA was washed three times with 75% ethanol, three times with absolute ethanol, and finally dissolved in water. The RNA was then purified by cellulose chromatography as described, yielding an enriched poly(A)-containing mRNA fraction that consisted of between 2.5 and 3.5% of the applied RNA (8). The poly(A)-containing RNAs from chicken oviducts and rabbit retriculocytes were similarly isolated (8).

In Vitro Protein Synthesis. The purified RNA, in the amounts indicated in the figure legends, was tested for mRNA activity in the Krebs ascites cell-free system supplemented with tRNA and rabbit reticulocyte initiation factors as described (8). The amount of L-[^{3}H]leucine (30–50 Ci/mmol) incorporated into the total protein and into the released chains of newly synthesized, immunologically isolated tryptophan oxygenase was determined (8).

Abbreviations: S-30 preparation, supernatant after centrifugation at 30,000 $\times g$ of Krebs ascites tumor cells.

Preparation of Anti-Tryptophan Oxygenase. Rat hepatic tryptophan oxygenase was purified to homogeneity, and antibodies were prepared in rabbits. The antibodies were purified and analyzed by double-diffusion techniques (20).

Immunoprecipitation of Tryptophan Oxygenase. The equivalence point of the precipitation of the enzyme with anti-tryptophan oxygenase was determined by incubation of 8 μg of homogeneous tryptophan oxygenase in 10 μl of 0.1 M sodium phosphate (pH 7.0) containing 0.01 M L-tryptophan, with increasing amounts of anti-tryptophan oxygenase gamma globulins purified by ammonium sulfate precipitation (33.5 mg of protein per ml) in 0.35 ml of 0.01 M sodium phosphate (pH 7.5)–0.14 M NaCl–2% Triton X-100 for 30 min at room temperature and 30 min at 4°. The immunoprecipitates were washed five times with 0.35 ml of 0.01 M sodium phosphate (pH 7.5)–0.14 M NaCl–2% Triton X-100; the precipitated protein was quantitated according to Lowry *et al.* (21). Enzyme activity was measured, in the absence of Triton X-100, as described (22).

In the *in vitro* studies of aminoacid incorporation the immunologic and electrophoretic analysis of the newly synthesized released proteins was performed after removal of the ribosomes from the protein-synthesizing mixtures by centrifugation at 180,000 × *g* for 60 min. To each 100 μl of reaction supernatant, 3.2 μg of purified tryptophan oxygenase as carrier and 40 μl of anti-tryptophan oxygenase were added; the immunoprecipitate was allowed to form as described above. The immunoprecipitates were washed five times in 0.01 M sodium phosphate–0.14 M NaCl–2% Trition X-100, containing 0.01 M unlabeled L-leucine. The immunoprecipitate was then dissolved in 75 μl of 10 mM sodium phosphate pH 7.0–2% sodium dodecyl sulfate–2% 2-mercaptoethanol, electrophoresed on 10-cm long 10% polyacrylamide gels, and analyzed for radioactivity as described (8). Each gel was standardized with at least three internal protein markers, one of them always being homogeneous hepatic tryptophan oxygenase.

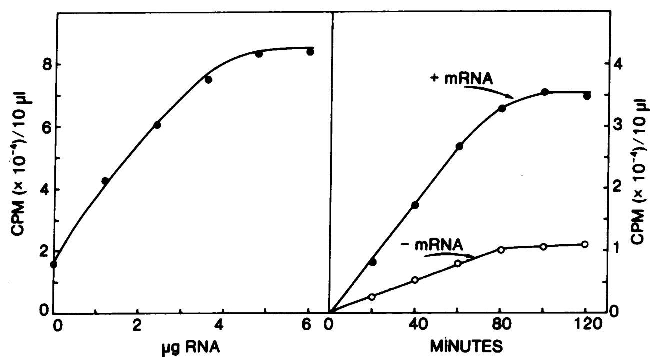

FIG. 1. Stimulation of protein synthesis by poly(A)-rich mRNA from rat liver. *Left panel:* Incorporation of L-[^{3}H]leucine into protein was measured after 60-min incubation of replicate 50-μl reaction mixtures containing the indicated amounts of cellulose-purified RNA (8), extracted from livers of rats 3 hr after treatment with hydrocortisone acetate. *Right panel:* At the indicated incubation times, 10 μl of reaction mixtures in the absence of (○——○) and containing 20 μg/ml of cellulose-purified RNA (●——●) were removed for determination of the incorporated radioactivity. Different Krebs ascites tumor S-30 preparations were used in these two experiments.

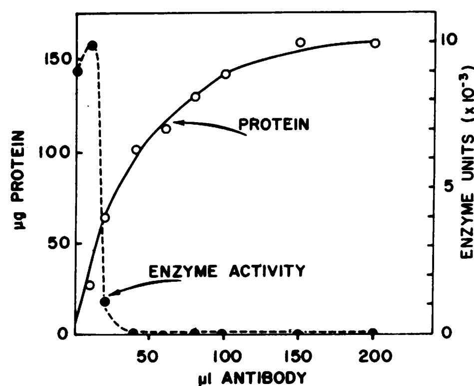

FIG. 2. Determination of the equivalence point for immunoprecipitation of tryptophan oxygenase by anti-tryptophanoxygenase. 8 μg of tryptophan oxygenase were incubated with the indicated amounts of anti-tryptophan oxygenase as described in *Methods*. The washed immunoprecipitates were analyzed for protein content, and the residual tryptophan oxygenase activity was determined in the supernatant after removal of the immunoprecipitate.

RESULTS

Fig. 1 (*left*) shows the stimulation in the incorporation of L-[^{3}H]leucine into total protein as a function of the amount of rat-liver poly(A)-containing RNA purified on cellulose (8). 6 μg of this mRNA fraction per 50-μl reaction volume led to more than a 5-fold stimulation of protein synthesis with this particular 30,000 × g supernatant (S-30 preparation) from Krebs ascites cells, which displayed a rather high basal-incorporation rate. As we have observed that S-30 preparations with a high background incorporation led to increased synthesis of specific products, these were usually used for the experiments to be described, even though other S-30 preparations with lower basal incorporation rates were stimulated up to 15-fold by optimal amounts of the poly(A)-rich RNA fraction. As shown in Fig. 1, (*right*), the endogenous and stimulated protein synthesis are linear for 1 hr.

To unequivocally identify tryptophan oxygenase among the newly synthesized and released protein chains, we applied immunological and electrophoretic techniques to the supernatants obtained after removal of the ribosomes from the incubated assay mixtures. In view of our final goal—to quantitate the mRNA for tryptophan oxygenase in the course of glucocorticoid induction—we took great care to obtain monospecific antibodies by using homogeneous preparations of tryptophan oxygenase (20). As has been previously shown (20), and confirmed with the present enzyme and antibody preparations, antibodies to homogeneous tryptophan oxygenase interact with both the purified enzyme and high-speed hepatic cytosol, yielding a single precipitin line on double-diffission Ouchterlony plates. Furthermore, incorporation of the enzyme into the agar eliminates the precipitin line with the cytosol, confirming the exclusive presence of monospecific anti- tryptophan oxygenase antibodies.

The determination of the equivalence ratio of homogeneous tryptophan oxygenase and anti-tryptophan oxygenase is depicted in Fig. 2. Soluble enzyme–antibody complexes form that are catalytically inactive and complete precipitation of the enzyme protein takes place at the equivalence point.

For isolation of the radioactive newly synthesized and released chains of tryptophan oxygenase, authentic carrier tryptophan oxygenase and anti-tryptophan oxygenase were

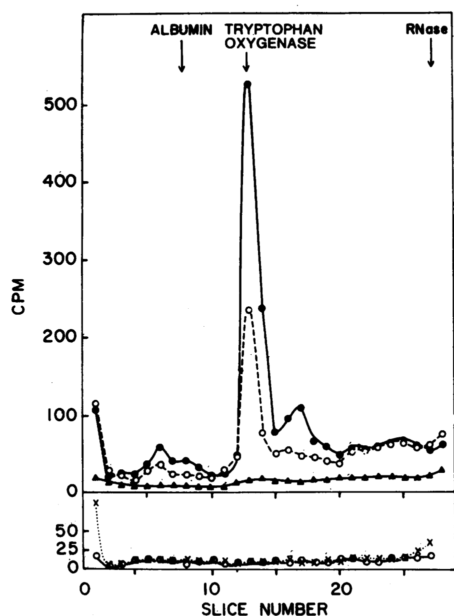

FIG. 3. Sodium dodecyl sulfate-polyacrylamide electrophoresis of proteins synthesized *in vitro* after immunologic isolation. The released proteins after 60 min incubation of 0.5-ml reaction mixtures containing cellulose-purified mRNA fractions were immunoprecipitated as described below and subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis. *Upper panel*: Proteins directed by 60 μg of liver mRNA from uninduced animals (O- - -O) and by 60 μg of liver mRNA from animals that had received hydrocortisone (●——●) were precipitated with carrier tryptophan oxygenase and anti-tryptophan oxygenase. Another sample stimulated by 60 μg of liver mRNA from induced animals was precipitated with chicken ovalbumin and anti-ovalbumin (▲——▲). *Lower panel*: Proteins directed by 7 μg of cellulose-purified mRNA from rabbit reticulocytes (X- - -X) and 25 μg of RNA from chicken oviducts (O——O) were precipitated with carrier tryptophan oxygenase and anti-tryptophan oxygenase. *Arrows* indicate the position of proteins used as internal markers in the sodium dodedyl sulfate-acrylamide gel electrophoresis.

added to the released chains in the optimal proportions established in immunotitration experiments. In control experiments, chicken ovalbumin and rabbit anti-ovalbumin (8) were used in optimal combining proportions similarly determined. The radioactive immunoprecipitates obtained with these techniques were extensively washed with buffer containing 2% Triton X-100 and 0.01 M nonradioactive L-leucine to minimize unspecific precipitation and binding. Since we felt the necessity to unambiguously identify the nascent polypeptide, we combined the immunological isolation with electrophoresis of the thus obtained products on sodium dodecyl sulfate-polyacrylamide gels. From our previous work on the purification and physicochemical characterization of rat-liver tryptophan oxygenase, we knew that the enzyme is composed of two pairs of subunits of identical molecular weight of 43,000 ($\alpha_2\beta_2$) (20, 23). Thus, we required the same electrophetic behavior for these enzyme subunits synthesized *in vitro*.

From Fig. 3 we can conclude that rat-liver RNA, purified on cellulose, directs the synthesis of a product that is precipitated by anti-tryptophan oxygenase and comigrates with authentic tryptophan oxygenase subunits upon sodium dodecyl sulfate-acrylamide gels. Four replicate experiments with three different hepatic mRNA preparations and two different Krebs ascites S-30 preparations have yielded such a definitive demonstration for the synthesis and release of hepatic tryptophan oxygenase in this heterologous system. To ensure the specificity of the analysis and to rigorously exclude the possibility of nonspecific trapping of immunologically unrelated products, samples directed by rabbit reticulocyte mRNA and chicken oviduct mRNA, purified on cellulose, were processed in an identical fashion with carrier tryptophan oxygenase and anti-tryptophan oxygenase. As is evident (Fig. 3, *lower panel*), no radioactivity peak was obtained in the position corresponding to the tryptophan oxygenase subunits. Likewise, as a further control, when products of the synthesis directed by rat-liver mRNA were treated with chicken ovalbumin and anti-ovalbumin (rather than with hepatic tryptophan oxygenase and anti-tryptophan oxygenase), no precipitation of the newly synthesized tryptophan oxygenase moieties was detectable (Fig. 3).

Fig. 3 (*upper panel*) also compares the hepatic contents of mRNA for tryptophan oxygenase in livers derived from control and hormonally induced animals. RNA extracted from equal amounts of liver derived from control animals and from those that had received a glucocorticoid hormone 3 hr before they were killed, were identically processed as described in *Methods*. Both these RNA preparations showed the same absorbancy at 260 nm and overall stimulatory capacity in the *in vitro* protein synthesis. From the gel patterns of the products, isolated as described above, it is evident that the mRNA from hormone-treated animals lead to a 3-fold higher incorporation of [^{3}H]leucine into tryptophan oxygenase components, thus indicating the presence in hormone-induced liver of elevated quantities of mRNA for tryptophan oxygenase.

It is interesting to note in Fig. 3 that a small radioactive peak is apparent at a molecular weight of about 85,000. As we have described earlier (20), a minor fraction of homogeneous tryptophan oxygenase, when analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, migrated in this zone, which we interpreted as representing dimeric structures. Since the newly synthesized enzyme displays similar characteristics, this may indicate that the enzyme synthesized *in vitro* has attained certain quaternary characteristics of the native enzyme. Furthermore, we have repeatedly observed the presence of low molecular weight, labeled polypeptide components that were immunoprecipitated only by anti-tryptophan oxygenase and that may represent released incomplete fragments of this enzyme protein.

DISCUSSION

Theoretically, several alternative mechanisms can be envisaged to explain the induction of specific enzyme proteins by steroid hormones. They possibly might enhance the rate of translation by a more efficient "read-out" of the corresponding mRNA, the amount of which does not change, i.e., translational control. On the other hand, hormonal regulation of the amount of mRNA itself may be the determinative parameter. To experimentally distinguish between these alternatives, we developed a cell-free system that allows the faithful translation and assay of the mRNA of tryptophan oxygenase. Great care was taken to ensure the specificity of our identification procedure, since we estimated from the turnover rate and

steady-state activity of tryptophan oxygenase and its turnover number, that tryptophan oxygenase synthesis represents only a minor fraction of the total hepatic protein synthesis (in the order of 0.1%). Thus, enzyme homogeneous by several criteria (20) was used for preparation of the immunesera. To give further support to the identification of the tryptophan oxygenase synthesized *in vitro*, we analyzed the immunoprecipitated products by sodium dodecyl sulfate–mercaptoethanol electrophoresis, allowing comparison of the molecular weights of the products synthesized *in vitro* with the subunits of authentic tryptophan oxygenase. Since unspecific coprecipitation represents a potentially serious problem in such radio-immunologic analysis, we introduced controls by (*i*) processing samples that had been directed by mRNA preparations derived from rabbit reticulocytes and chicken oviduct with carrier tryptophan oxygenase and anti-tryptophan oxygenase and (*ii*) by analyzing products instructed by rat-liver mRNA with the carrier chicken ovalbumin–anti-ovalbumin system. On the basis of these negative control experiments and the definitive demonstration of the presence of released radioactive polypeptides that are precipitated by anti-tryptophan oxygenase and comigrate with subunits of authentic tryptophan oxygenase, we believe that the described experiments demonstrate the synthesis of tryptophan oxygenase in the heterologous Krebs cell-free system. This represents, to our knowledge, the first demonstration for the complete translation in a heterologous system of a mammalian mRNA that codes for a protein with enzymatic function.

Tomkins and coworkers have recently demonstrated an augmentation in the number of nascent chains of tyrosine aminotransferase in steroid-induced hepatoma cells (24) as well as an increased rate of synthesis of this enzyme in extracts from induced cells (25). They concluded that "induction control probably operates on either the amount of messenger RNA or the rate of specific polypeptide-chain initiation" (24). As shown in Fig. 3, the poly(A)-containing mRNA isolated from livers of animals that had received glucocorticoid 3 hr before they were killed contain about three times the control amount of mRNA coding for tryptophan oxygenase. Thus, our investigations directly measuring the total amount of mRNA for tryptophan oxygenase in liver support the hypothesis that the hormonally increased rate of synthesis of tryptophan oxygenase (13, 19) is a consequence of an enhanced amount of the mRNA coding for this enzyme protein. It remains unknown whether the elevated amount of the mRNA for tryptophan oxygenase in the tissue is due to an increased rate of its synthesis or to a decreased rate of its degradation. Furthermore, the rapidity of the hormonal augmentation of the amount of hepatic mRNA for tryptophan oxygenase suggests that these steroids modulate gene expression within existing cells and do not cause the cell differentiation that characterizes estrogen-mediated regulation of ovalbumin synthesis in chicken oviducts (6, 7).

Since we have extracted the mRNA from the total tissue, we cannot infer the location of the mRNA for tryptophan oxygenase within the cell. mRNA exists in the form of ribonucleoprotein particles, some of which are not functionally associated with polysomes (26, 27). To avoid possible nucleolytic degradation during cell fractionation, we isolated the mRNA directly from the entire tissue. Thus, our estimation of amounts of mRNA for tryptophan oxygenase may include species of mRNA that, by virtue of compartmentation or other subtle regulation, may not be functionally available for translation. Nevertheless, the weight of the existing evidence indicates that hormonally enhanced amounts of poly(A)-rich mRNA coding for tryptophan oxygenase are responsible for the hormonally increased rate of synthesis of this enzyme protein.

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