Control of the mRNA for Hepatic Tryptophan Oxygenase During Hormonal and Substrate Induction

(cell-free protein synthesis/steroid/cellulose chromatography/hydrocortisone/enzyme synthesis)

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ABSTRACT Glucocorticoid hormones increase the level of hepatic tryptophan oxygenase [EC 1.13.11.11; L-tryptophan:oxygen 2,3-oxidoreductase (deacylizing)] by increasing its rate of synthesis. Studies were performed to determine whether this induction is mediated by controlling the level of the mRNA for tryptophan oxygenase or by changing the translational efficiency of a fixed level of mRNA. Activity of tryptophan oxygenase mRNA was quantitated in a Krebs ascites cell-free, protein-synthesizing system, supplemented with tRNA and rabbit reticulocyte initiation factors. *De novo* synthesis of the protomeric unit(s) of the enzyme was a linear function of the amount of mRNA added. Time course and dose-response studies in which the enzyme level and mRNA activity in livers from rats injected with inducing doses of hydrocortisone were compared indicate that the induction of this enzyme is accompanied by a proportional increase in the level of its mRNA. This was true for mRNA isolated from total liver as well as from cytoplasmic polysomes. Induction of the enzyme by its substrate, tryptophan, however, was not accompanied by a parallel increase in mRNA activity.

Since steroid hormones lead to specific changes in macromolecular synthesis, their mechanism of action represents a suitable model system for the study of gene regulation in higher organisms. A classical example of such a change is the increased activity of the enzyme, tryptophan oxygenase [EC 1.13.11.11; L-tryptophan:oxygen 2,3-oxidoreductase (deacylizing)], in rat liver after administration of glucocorticoid hormones (1), which is the result of an increased rate of synthesis of the enzyme (2, 3). Since the increased rate of specific protein synthesis evoked by glucocorticoids is preceded by marked changes in the synthesis of RNA (4-8), we asked whether this enzyme induction is linked to changes in the concentration of the corresponding mRNA within the target tissue. To study this problem we developed a heterologous cell-free, protein-synthesizing system, which allows the faithful translation of eukaryotic mRNAs (9-11), including the mRNA for tryptophan oxygenase (12). We then determined the intracellular concentration of the mRNA for tryptophan oxygenase during induction and deinduction of the enzyme and as a function of the amount of hormone administered. Our results indicate that the rise in tryptophan oxygenase activity is accompanied by a parallel increase in the functional level of the mRNA for this enzyme. This mRNA is functionally associated with polysomes.

MATERIALS AND METHODS

Poly(A)-containing RNA was isolated from rat liver as described (12), and assayed for mRNA activity in the Krebs ascites cell-free system (10, 12). The rate of synthesis of

tryptophan oxygenase was determined by isolating the newly synthesized tryptophan oxygenase from the total released chains with monospecific anti-tryptophan oxygenase γ -globulin, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the solubilized immunoprecipitate. The location on the gel of the protomeric units of tryptophan oxygenase and marker proteins was determined with Coomassie blue. After the gel was sliced and the radioactivity of the slices was determined, tryptophan oxygenase synthesis was quantitated either planimetrically or by summing the radioactivity in three peak fractions after subtraction from each fraction of a background determined by averaging the radioactivity in three fractions on either side of the delineated peak area. The enzyme activity of tryptophan oxygenase was determined as described (13).

For the isolation of polysomes the procedure in ref. 14 was slightly modified as follows: The postmitochondrial supernatant of the 25% homogenate containing 1% Triton X-100 and 1% deoxycholate was layered over a discontinuous sucrose gradient of 2 ml, 12 ml, and 8 ml, respectively, of 0.5 M, 1.0 M, and 2.5 M sucrose, all containing 100 μ g/ml of sodium heparin. This was centrifuged for 3 hr at 25,000 rpm in a SW 27 rotor at 2°. The polysomes, which banded at the interface between the 1.0 M and 2.5 M sucrose, were collected by puncture of the tube wall, and the RNA was extracted as described (15). To obtain the poly(A)-containing mRNA fraction, the polysomal RNA was chromatographed on cellulose (9, 10).

RESULTS

Fig. 1 shows that tryptophan oxygenase synthesis is a linear function of the amount of rat liver mRNA added. Maximal incorporation of [3 H]leucine into tryptophan oxygenase is achieved at the same concentration of mRNA as maximal incorporation into the total or released proteins. Since no change occurs in either of these three parameters at the plateau level, we conclude that the mRNA for tryptophan oxygenase saturates available ribosomal subunits at the same concentration as the other liver mRNAs, suggesting non-preferential translation of the various liver mRNAs contained in our preparation.

Fig. 2 illustrates how the analysis of the cell-free synthesis of tryptophan oxygenase was performed. Equivalent amounts of mRNA from livers of control rats or those that received hydrocortisone 2 or 4 hr before they were killed (5 mg/100 g of body weight) were added to the *in vitro* protein-synthesizing system. Newly synthesized tryptophan oxygenase was precipitated from the released chains by the addition of carrier

TABLE 1. *Effect of hydrocortisone acetate on the intracellular concentration of the functional mRNA for tryptophan oxygenase*

Animal status	Amount of mRNA assayed ($\mu\text{g}/0.5 \text{ ml}$)	cpm incorporated into total proteins	cpm incorporated into released proteins	cpm in tryptophan oxygenase*	Tryptophan oxygenase as % of released chains
Control	60	4.40×10^6	1.25×10^6	290	0.023
Hydrocortisone acetate-induced	60	4.20×10^6	1.18×10^6	940	0.079

* Represented by the area of tryptophan oxygenase subunits on sodium dodecyl sulfate-polyacrylamide gels. Poly(A)-containing RNA (60 μg) of control animals and animals that had received 5.0 mg of hydrocortisone/100 g of body weight 3 hr before they were killed was used in 0.5 ml of the complete translational reaction mixture. Incorporation of [^3H]leucine into total and released proteins was determined and the amount of tryptophan oxygenase synthesis estimated as described.

tryptophan oxygenase and antibody against tryptophan oxygenase. The resultant immunoprecipitates were washed, dissolved, and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The major peak of radioactivity corresponds to the position of authentic tryptophan oxygenase subunits (molecular weight 43,000) (16). As can be seen, the mRNA level for tryptophan oxygenase increases during the induction of this enzyme by hydrocortisone (Fig. 2).

Table 1 illustrates the technical problems that had to be overcome in the identification and quantitation of tryptophan oxygenase synthesis in a cell-free system. These arise because its synthesis represents only a minute portion of total liver protein synthesis, approximately 0.025 and 0.10% in uninduced and induced animals, respectively. When equal amounts of mRNA from control and hormone-treated animals are added to the system, they stimulate the incorporation of [^3H]leucine into total protein to the same extent. However, only 30% of the total incorporation is into released chains. Of the released chains, only about two parts per ten thousand represent tryptophan oxygenase synthesis. Because of this low relative rate of synthesis, and to assure its purity, we always subjected the immunoprecipitate to electrophoretic fractionation.

Fig. 3 shows that tryptophan oxygenase mRNA activity rises and falls in parallel with the enzyme level during induction by hydrocortisone. In this, as well as in several other experiments, the decline in enzyme activity was preceded by a fall in the level of mRNA for tryptophan oxygenase.

Fig. 4 demonstrates that there is a corresponding rise in tryptophan oxygenase activity and the activity of its mRNA

with increasing doses of hormone. The data in this figure are expressed as the percent of the fully induced values.

Since the mRNA fractions used in the preceding experiments were always isolated by phenolization of the total liver, it was uncertain whether the elevated mRNA for tryptophan oxygenase after induction of the enzyme was localized at the polysomes or compartmentalized elsewhere in the cell. To rule out any storage of the mRNA, rendering it unavailable for translation, we prepared mRNA fractions from isolated polysomes. Identical amounts of mRNA derived from polysomes of control animals and from animals that had received 2 mg of hydrocortisone/100 g of body weight 4 hr before they were killed led to incorporation of 1470 cpm and 4800 cpm of [^3H]leucine, respectively, into tryptophan oxygenase, an increase comparable to that observed with mRNA extracted from the total liver. Thus, the increased messenger activity is associated with polysomes.

It had previously been demonstrated that the substrate, L-tryptophan, leads to enzyme induction not by increasing the rate of synthesis of the enzyme, but rather, by its stabilization

FIG. 1. Tryptophan oxygenase synthesis as a function of the amount of mRNA added to the cell-free translational system. The indicated amounts of poly(A)-containing mRNA were incubated in 0.5 ml of the complete reaction mixture. After incubation for 60 min at 37°, the amount of [^3H]leucine incorporated into the newly synthesized released subunits of tryptophan oxygenase was measured as described in *Materials and Methods*.

FIG. 2. Sodium dodecyl sulfate-polyacrylamide electrophoresis of tryptophan oxygenase synthesized *in vitro*. The released chains, synthesized in response to 60 $\mu\text{g}/0.5 \text{ ml}$ of poly(A)-containing RNA extracted from the livers of animals that had received no hydrocortisone and 5 mg of hydrocortisone/100 g of body weight 2 and 4 hr before they were killed, were immunoprecipitated and subjected to sodium dodecyl sulfate gel electrophoresis.

TABLE 2. Effect of L-tryptophan on hepatic tryptophan oxygenase activity and mRNA

Animal status	Catalytic activity	mRNA translational activity			
	Tryptophan oxygenase (μ mol of kynurenine/hr per g of liver)	cpm incorporated into total protein	cpm incorporated into total released proteins	cpm in tryptophan oxygenase	Tryptophan oxygenase as % of total protein synthesis
Control	1.5	6.70×10^6	2.34×10^6	606	0.026
L-Tryptophan induced	12.0	5.70×10^6	1.96×10^6	572	0.029

One-half milliliter of the complete Krebs II translational reaction mixture contained 40 μ g of poly(A)-containing RNA derived from the livers of control and L-tryptophan-treated animals (100 mg of L-tryptophan/100 g of body weight administered 4 hr before the animals were killed). Incorporation of [3 H]leucine into released proteins was determined and the amount of tryptophan oxygenase synthesis estimated as described.

(2, 3). As shown in Table 2, L-tryptophan gives rise to an 8-fold increase in enzyme activity, while as anticipated, the functional levels of the mRNA for tryptophan oxygenase in the livers of induced and control animals are indistinguishable.

DISCUSSION

Since quantitation of the mRNA for tryptophan oxygenase is entirely dependent on positive identification of the newly synthesized enzyme in the cell-free assay system, we always combined immunological isolation with electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, thus requiring for identification immunological relatedness as well as the expected molecular weight of the newly synthesized tryptophan oxygenase components. In view of the small proportion of the total protein synthesis that is tryptophan oxygenase *per se* (see Table 1), we felt that these criteria should always be fulfilled. In our previous communication (12), we detailed the precautions for the immunological detection of tryptophan oxygenase synthesized *in vitro*, as well as control experiments to ensure the specificity of our identification procedure. In the present series of experiments, "minus mRNA," reactions were routinely included in order to confirm the identity of the nascent enzyme.

Fig. 1 demonstrates that tryptophan oxygenase synthesis

FIG. 3. Comparison of tryptophan oxygenase activity with mRNA levels for tryptophan oxygenase in the livers of rats as a function of time after intraperitoneal administration of 2 mg of hydrocortisone/100 g of body weight. mRNA (60 μ g) was incubated for 60 min in the complete reaction mixture of 0.5 ml.

is a function of the amount of poly(A)-containing RNA added to the reaction mixture. Using this assay for the determination of the level of the mRNA for tryptophan oxygenase in time-course (Figs. 2 and 3) and dose response experiments (Fig. 4), we conclude that the concentration of functional mRNA changes essentially in parallel with the enzyme activity. It has been previously demonstrated (3) that the rise in enzyme level is due to an increase in the rate of synthesis of the enzyme; thus we can directly correlate this increase with an increased activity of the tryptophan oxygenase mRNA. The increase in mRNA concentration and enzyme activity shown in Fig. 4 correlates with the degree of saturation *in vivo* of the cytoplasmic glucocorticoid receptor (17).

Furthermore, since concomitant increases in mRNA activity are observed when mRNA is extracted from total tissue or polysomes, we infer that induction of this enzyme does not occur through subcellular liberation of a large reservoir of functionally inactive mRNA. Mechanisms of hormonal elevation of the mRNA for tryptophan oxygenase involving cytoplasmic factors, which had been postulated based on the phenomenon of the "superinduction" by actinomycin D (18), seem improbable as we do not observe a rise in the mRNA concentration for tryptophan oxygenase when this enzyme is superinduced by actinomycin D (L. Killewich, G. Schutz, and P. Feigelson, submitted for publication).

Since poly(A) sequences are not necessary for the translation of eukaryotic mRNAs *in vitro* (19), the increase in messenger activity for tryptophan oxygenase after hormone

FIG. 4. Hepatic tryptophan oxygenase (T.O.) activity and mRNA concentration as a function of inducing dose of hydrocortisone. Enzyme activity and mRNA concentration for tryptophan oxygenase were determined in livers of animals that had received intraperitoneally 0.2, 1.0, and 5.0 mg of hydrocortisone/100 g of body weight 4 hr before they were killed. Protein synthesis was carried out as in Fig. 3.

administration cannot be the result of a modification of this nucleotide sequence. However, accumulation by hormonal stabilization of the mRNA via poly(A) modification cannot be excluded.

Changes in messenger RNA concentrations after hormonal induction of the sex-steroid-dependent proteins of the chick oviduct (ovalbumin, conalbumin, ovomucoid, and avidin) have been reported (20-23). The present study demonstrates that the rate of synthesis of a glucocorticoid-inducible enzyme is a consequence of the hormonal control of the tissue level of the corresponding mRNA.

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