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Functional level of rat liver tryptophan 2,3-dioxygenase messenger RNA during superinduction of enzyme with actinomycin D

(cell-free protein synthesis/hydrocortisone/cellulose chromatography/translational control)

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ABSTRACT **Tryptophan 2,3-dioxygenase [EC 1.13.11.11; L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing)] activity is induced by glucocorticoid hormones and superinduced by actinomycin D. Previous experiments had shown that hormonal induction of the enzyme activity is accompanied by parallel increases in tryptophan 2,3-dioxygenase mRNA level. In this study, we measured the tryptophan 2,3-dioxygenase mRNA levels during superinduction as well as hormonal induction, to determine whether superinduction of the enzyme activity is also mediated through changes in mRNA concentration. Tryptophan 2,3-dioxygenase mRNA was measured in a Krebs ascites cell-free protein synthesizing system supplemented with rabbit reticulocyte initiation factors. We found that during superinduction of the enzyme activity by actinomycin D, the mRNA level is identical to that of the actinomycin D-free controls. Our results do not, therefore, support the hypothesis that hormonal induction and/or superinduction of tryptophan 2,3-dioxygenase mRNA are regulated by a rapidly turning over repressor.**

Actinomycin D, an inhibitor of RNA synthesis, is known to increase the activity and/or the concentration of numerous proteins in eukaryotic cells. This phenomenon, termed "superinduction", is paradoxical because one might expect the drug to inhibit protein synthesis through its inhibition of RNA synthesis. It was first discovered with the rat liver enzyme, tryptophan 2,3-dioxygenase [L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] (1), and has since been studied most intensively with tyrosine aminotransferase in cultured hepatoma cells. Tomkins' group (2–5) has found that if growth inhibited cells, preinduced with steroid, are treated with actinomycin D, the tyrosine aminotransferase activity is increased above the hormonally induced level during the period of induction. Furthermore, the fall in the rate of tyrosine aminotransferase synthesis normally seen during the deinduction period, is slowed by the addition of actinomycin D. Tomkins *et al.*, suggest that this is due to a stabilization of tyrosine aminotransferase mRNA which, under normal conditions, has a short half-life of 1–1.5 hr (6).

The superinduction phenomenon, as well as the requirement for ongoing RNA synthesis during hormonal induction and deinduction, led Tomkins *et al.*, to suggest a mechanism of gene regulation (4) based on the existence of a short-lived, post-transcriptional repressor. The postulated repressor is thought to be transcribed from a regulatory gene and interacts with the mRNA coding for the inducible protein, decreasing its activity by either promoting its degradation or preventing its translation. According to this hypothesis, induction of enzyme synthesis by steroid hormones is the result of inhibition of either the activity or the synthesis of the repressor by the hormone.

The experiments of Tomkins' laboratory with actinomycin D are compatible with his theory if one assumes that the repressor and its mRNA have a rapid turnover rate. Actinomycin D would then inhibit the synthesis of the repressor, which would lead to an increase in either the level or activity of the mRNA coding for the inducible protein. During superinduction, the effect of actinomycin D would be synergistic to that of the steroid inducer, i.e., an even greater number of mRNA molecules would be available for translation. The rate of synthesis of the inducible enzyme, and ultimately its catalytic activity, would be increased above the hormonally induced levels.

Certain of the results of Tomkins' laboratory have been questioned by Kenney *et al.* (7–9), who have done similar experiments with contradictory results. Their data indicate that the rate of tyrosine aminotransferase synthesis, measured as the incorporation of radioactive amino acids into the enzyme, is consistently inhibited in cells treated with actinomycin D during the period of hormonal induction, and that the rate of degradation of the enzyme is also diminished. They maintain that superinduction of the enzyme activity is a consequence of stabilization of preexisting enzyme molecules rather than increased enzyme synthesis.

Superinduction has also been described for the secretory proteins of the chick oviduct (10, 11), as well as for glutamine synthetase in cultures of chick embryonic neural retina (12–14), and for interferon production in diploid human fibroblasts (15). Palmiter and Schimke (10) have found increases in both the relative and absolute rates of synthesis of egg white proteins after actinomycin D treatment and have reported that this is the result of increased rates of translation of the mRNAs coding for these proteins. They suggest that this is due to the longer half-lives of the mRNAs of the inducible proteins, which, after actinomycin D treatment, cause them to become a larger proportion of the total mRNA population and thus allow them to compete more favorably for rate-limiting factors in protein synthesis. However, it is unlikely that this interpretation explains superinduction in cases such as tryptophan 2,3-dioxygenase and tyrosine aminotransferase, since the mRNAs coding for these proteins are known to have relatively short half-lives.

The post-transcriptional repressor hypothesis has been controversial because of the absence of direct evidence for the existence of the repressor and the uncertainty regarding the mechanism of superinduction of tyrosine aminotransferase and tryptophan 2,3-dioxygenase. Since our previous experiments (16, 17) had demonstrated that induction as well as deinduction of rat liver tryptophan 2,3-dioxygenase is accompanied by corresponding changes in the mRNA concentration, we addressed ourselves to the question of whether superinduction was also accompanied by changes in mRNA

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concentration. We reasoned that direct support for the repressor-mRNA stabilization model would be the demonstration of an increase in the level of the mRNA coding for the inducible protein above the hormonally induced level during superinduction by actinomycin D. If, however, superinduction was not mediated by changes in the mRNA concentration, the mRNA level during superinduction should be similar to the level during deinduction.

We used a cell-free protein synthesizing system to quantitate rat liver tryptophan 2,3-dioxygenase mRNA in the basal state, 4 hr after hormonal administration, at which point maximal induction of enzyme activity as well as maximal accumulation of the mRNA had occurred, and 4 hr later in the presence and absence of actinomycin D. Enzyme activity measurements had indicated that maximal superinduction by actinomycin D was observed under these conditions. Tryptophan 2,3-dioxygenase mRNA is increased in parallel with the enzyme activity during induction by hydrocortisone and falls more quickly than the enzyme activity during deinduction. We now report that during superinduction of the enzyme activity by actinomycin D the mRNA level is indistinguishable from that of the animals treated only with hormone (18).

MATERIALS AND METHODS

Tryptophan 2,3-dioxygenase activity was determined as described (19). RNA was extracted from 4 g of rat liver by homogenization 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 vol/vol) had been added. After shaking vigorously for 5 min at room temperature (25°), the suspension was centrifuged at 6000 × *g* for 15 min. The aqueous phase was re-extracted three times with phenol–chloroform, made 0.3 M in LiCl, and precipitated with 2 volumes of ethanol at −20°. After washing three times with absolute ethanol, the RNA was dried, redissolved in water, and chromatographed on cellulose (Sigma 38). The poly(A)-containing mRNA fraction bound to the column in high salt buffer (10 mM Tris·HCl at pH 7.6–500 mM KCl–0.2 mM $MgCl_2$), whereas ribosomal RNA species did not (17, 18). The mRNA was then eluted from the column with neutralized water, adjusted to 0.3 M LiCl, and precipitated with 2 volumes ethanol at −20°. The RNA was washed twice with 75% ethanol and twice with absolute ethanol, dried, and dissolved in water to a final concentration of 1 mg/ml (20).

Tryptophan 2,3-dioxygenase mRNA was quantified by its ability to code for the synthesis of tryptophan 2,3-dioxygenase in an *in vitro* protein synthesizing system composed of a Krebs II ascites S-30 fraction supplemented with rabbit reticulocyte initiation factors. Newly synthesized tryptophan 2,3-dioxygenase was identified by immunoprecipitation from the total released proteins with monospecific antibodies to tryptophan 2,3-dioxygenase and subsequent electrophoresis of the immunoprecipitate on sodium dodecyl sulfate polyacrylamide gels. The details of the experimental procedures have been described elsewhere (16, 20–22).

RESULTS

Fig. 1 depicts the results of an experiment in which rat liver tryptophan 2,3-dioxygenase mRNA activity was measured during induction by hydrocortisone and superinduction by actinomycin D. Animals received hydrocortisone acetate (1 mg/100 g of body weight) for 4 or 8 hr before sacrifice. One group of these animals received an additional injection of actinomycin D (0.4 mg/100 g of body weight) 4 hr after administration of the hormone and was sacrificed at 8 hr. The livers of each group of four animals were combined. Poly(A)-containing RNA was extracted and translated in the *in vitro* protein synthesizing system. Total mRNA activity, measured

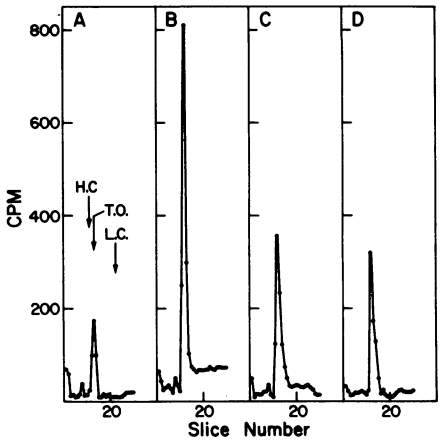

FIG. 1. The effect of actinomycin D and glucocorticoids upon the hepatic level of tryptophan 2,3-dioxygenase mRNA. Total rat liver mRNA (80 μg) was added to 500 μl of a cell-free protein synthesizing system composed of a Krebs II ascites S-30 fraction supplemented with rabbit reticulocyte initiation factors. After incubation at 37° for 1 hr, the polysomes were removed by centrifugation. The released chains were treated with anti-tryptophan 2,3-dioxygenase and carrier tryptophan 2,3-dioxygenase, and the resulting immunoprecipitates were washed, dissolved in 0.1% sodium dodecyl sulfate–2% mercaptoethanol–10 mM NaP_2 pH 7.0 and electrophoresed on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The gels were stained for protein with Coomassie brilliant blue, then sliced and counted. Cpm in tryptophan 2,3-dioxygenase was determined as described (16, 17). A, control rats received no hydrocortisone; B, rats received hydrocortisone (1 mg/100 g of body weight) 4 hr before sacrifice; C, rats received hydrocortisone 8 hr before sacrifice at the same concentration as in B; D, rats received hydrocortisone 8 hr before sacrifice at the same concentration as in B and actinomycin D (0.4 mg/100 g of body weight) 4 hr before sacrifice. Markers included immunoglobulin heavy chain (H.C. 55,000 daltons), tryptophan 2,3-dioxygenase (T.O. 43,000 daltons), and immunoglobulin light chain (L.C. 24,000 daltons).

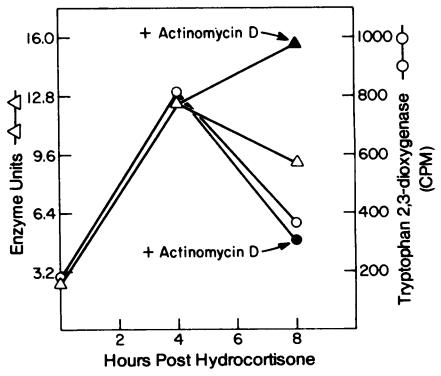

FIG. 2. The hepatic tryptophan 2,3-dioxygenase catalytic activity and mRNA level during superinduction by actinomycin D. Protein synthesis from rat liver mRNA was carried out as described for control animals, for animals receiving hydrocortisone acetate (1 mg/100 g of body weight) for the indicated times before sacrifice (unfilled symbols), and for animals also receiving an injection of actinomycin D (0.4 mg/100 g of body weight) 4 hr after administration of the hormone (solid symbols). Tryptophan 2,3-dioxygenase mRNA level is shown as cpm incorporated into tryptophan 2,3-dioxygenase. Tryptophan 2,3-dioxygenase activity was determined as described (18).

Table 1. Tryptophan 2,3-dioxygenase activity and mRNA level during superinduction by actinomycin D

Treatment (hr before sacrifice)		Enzyme activity (%)	Cpm in tryptophan 2,3-dioxygenase (%)
Hydrocortisone	Actinomycin D		
—	—	100	100
4	—	480 ± 40	470 ± 30
8	—	400 ± 30	280 ± 30
8	4	690 ± 80	270 ± 30

Rats were injected with 1–2 mg of hydrocortisone acetate per 100 g of body weight and sacrificed at the indicated times thereafter. One group (as indicated) received an additional injection of 0.4–0.6 mg/100 g of body weight of actinomycin D, 4 hr before sacrifice. Tryptophan 2,3-dioxygenase activities and mRNA level (cpm in tryptophan 2,3-dioxygenase) were determined as described earlier (16–18). Each value represents the average of the results of four experiments, in which the control value, expressed as 100%, represents tryptophan 2,3-dioxygenase activity of 2.2 μmol of kynurenine/hr per g of liver and the mRNA dependent incorporation of [^{3}H]leucine (275 cpm) into the protomeric units of tryptophan 2,3-dioxygenase. Different components for the protein synthesizing system were used in each of the four experiments; thus the absolute cpm incorporated into tryptophan 2,3-dioxygenase varied.

as the incorporation of [^{3}H]leucine into acid-precipitable counts, was the same for all samples. The level of tryptophan 2,3-dioxygenase mRNA was determined as the amount of radioactivity incorporated into the tryptophan 2,3-dioxygenase peak obtained from sodium dodecyl sulfate polyacrylamide gel electrophoresis of the immunoprecipitate. In Fig. 2, the mRNA activities from the preceding experiment are compared with the tryptophan 2,3-dioxygenase catalytic activities of the same livers. It is clear that the enzyme activity is induced 4-fold by hydrocortisone and superinduced by actinomycin D. As previously shown, the tryptophan 2,3-dioxygenase mRNA increases in parallel with the enzyme activity during induction by hydrocortisone and falls more quickly than the enzyme activity during deinduction (16, 17). The early decrease in mRNA activity is a reproducible phenomenon and may be due to a shorter half-life of the mRNA rather than of the enzyme itself. Although the enzyme activity after actinomycin D treatment is superinduced by 70%, the level of the tryptophan 2,3-dioxygenase mRNA under these conditions is identical to that of the actinomycin D-free controls.

Analysis of tryptophan 2,3-dioxygenase mRNA activity in cell-free systems is difficult since tryptophan 2,3-dioxygenase synthesis represents only a minute fraction of total liver protein synthesis, approximately 0.025% and 0.1% in uninduced and induced animals, respectively. Therefore, we have previously performed control experiments to insure the specificity of our identification procedure (16); the present set of experiments always included "minus mRNA" reactions. In four experiments, the mRNA levels in the hydrocortisone and actinomycin D-treated animals were comparable to those in the animals which received only hydrocortisone before sacrifice (Table 1).

DISCUSSION

These findings are consistent with previous experiments done in this laboratory (16, 17, 21) and demonstrate that induction of tryptophan 2,3-dioxygenase activity by glucocorticoid hormones is mediated through an increased level of the mRNA coding for the enzyme, which in turn increases its rate of synthesis. The present studies do not lend support to the belief that superinduction of the enzyme by actinomycin D is accompanied by, or due to, an increased level of its translatable mRNA and therefore do not support the concept that a rapidly turning over repressor regulates this mRNA level. We cannot, however, exclude the possibility of an actinomycin D sensitive translational repressor since this would not be reflected in our mRNA dependent cell-free translational assay.

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1. Garren, L.D., Howell, R. R., Tomkins, G. M. & Crocco, R. M. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 1121–1129.
2. Tomkins, G. M., Levinson, B. B., Baxter, J. D. & Dethlefsen, L. (1972) *Nature New Biol.* **239**, 9–14.
3. Thompson, E. B., Granner, D. K. & Tomkins, G. M. (1970) *J. Mol. Biol.* **54**, 159–175.
4. Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Samuels, H. H. & Thompson, E. B. (1969) *Science* **166**, 1474–1480.
5. Steinberg, R. A., Levinson, B. B. & Tomkins, G. M. (1975) *Cell* **5**, 29–35.
6. Steinberg, R. A., Levinson, B. B. & Tomkins, G. M. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 2007–2011.
7. Reel, J. R. & Kenney, F. T. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 200–206.
8. Kenney, F. T., Lee, K. L., Stiles, C. D. & Fritz, J. E. (1973) *Nature New Biol.* **246**, 208–210.
9. Lee, K. L. & Kenney, F. T. (1971) *Acta Endocrinol. Suppl.* **153**, 109–125.
10. Palmiter, R. D. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 1502–1512.
11. Palmiter, R. D., Oka, T. & Schimke, R. T. (1971) *J. Biol. Chem.* **246**, 724–737.
12. Moscona, A. A., Moscona, M. H. & Saenz, N. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 160–167.
13. Jones, R. E., Moscona, M. H. & Moscona, A. A. (1974) *J. Biol. Chem.* **249**, 6021–6023.
14. Schwartz, R. J. (1973) *J. Biol. Chem.* **248**, 6426–6435.
15. Vilcek, J. & Havell, E. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3909–3913.
16. Schutz, G., Beato, M. & Feigelson, P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1218–1221.
17. Schutz, G., Killewich, L., Chen, G. & Feigelson, P. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 1017–1020.
18. Killewich, L., Schutz, G. & Feigelson, P. (1974) *Fed. Proc.* **33**, 1467.
19. Feigelson, P. & Greengard, O. (1961) *J. Biol. Chem.* **236**, 153–157.
20. Schutz, G., Beato, M. & Feigelson, P. (1972) *Biochem. Biophys. Res. Commun.* **49**, 680–689.
21. Schutz, G., Beato, M. & Feigelson, P. (1974) in *Methods in Enzymology*, eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 30, pp. 701–709.
22. Sippel, A. E., Feigelson, P. & Roy, A. L. (1975) *Biochemistry* **14**, 825–829.