Superinduction of α_{2u} globulin by actinomycin D: Evidence for drug-mediated increase in α_{2u} mRNA

(androgenic induction/post-transcriptional repression/cell-free translation/hormone action/urinary protein)

BANDANA CHATTERJEE, JAMES HOPKINS, DENISE DUTCHAK, AND ARUN K. ROY

Department of Biological Sciences, Oakland University, Rochester, Michigan 48063

ABSTRACT   Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, increased the hepatic concentration of α_{2u} globulin, an androgen-inducible protein in the rat. Spayed female rats with a marginally induced state of α_{2u} synthesis showed an approximately 5-fold increase in hepatic α_{2u} globulin within 3–6 hr after treatment with actinomycin D. Initial treatment of these animals with 5α-dihydrotestosterone, followed by actinomycin D, resulted within 2–3 hr in a more than 2-fold increase in hepatic α_{2u} globulin compared to animals treated with the androgen alone. In spite of inhibition of hepatic synthesis of poly(A)-containing RNA to less than 25% of control, superinduction with actinomycin D resulted in a parallel increase in the translatable mRNA for α_{2u} globulin. These results showing increase in both α_{2u} globulin and its translatable mRNA after superinduction with actinomycin D support the concept of post-transcriptional repression of α_{2u} synthesis.

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, increases the level of several inducible proteins, a phenomenon termed "superinduction" (1, 2). Because actinomycin D inhibits mRNA synthesis, superinduction by this drug indicates an apparent paradox, the resolution of which may provide important clues concerning the mechanism of gene regulation in the eukaryotic cell. In fact, the post-transcriptional model for the steroidal regulation of gene activity, proposed by Tomkins and coworkers, is based primarily on their interpretation of the superinduction of glucocorticoid-mediated synthesis of tyrosine aminotransferase (TAT) by actinomycin D in hepatoma cells and in rat liver (3). A similar model of translational repression was also suggested by McAuslan (4). The above investigators have proposed that the mRNAs for the inducible proteins are continually being synthesized within the permissive phase of the cell cycle. However, the simultaneous presence of rapidly turning over post-transcriptional repressors prevents the translation of these mRNAs and may lead to their degradation. It is also proposed that, in the presence of inducing steroids, the repressor is inactivated and, because of its short half-life, is preferentially eliminated by actinomycin D, both of which phenomena would favor an accumulation of the inducible mRNA leading to increased synthesis of the inducible protein (3). Due to technical limitations, experimental verification of the McAuslan–Tomkins model has been difficult, and various other interpretations for the phenomenon of superinduction by actinomycin D have been proposed (5–7).

In our laboratory we have developed a model system for the study of hormone action in rat liver (8, 9). The system involves androgen-dependent synthesis of a low molecular weight (23,000) protein called α_{2u} globulin (10, 11). We have also identified the mRNA for α_{2u} globulin in rat liver and have established a strong correlation between the androgen-dependent increase in α_{2u} synthesis and the hepatic concentration of the

mRNA for this protein (9, 12, 13). Recently, with a sensitive radioimmunoassay, we have observed that actinomycin D causes superinduction of α_{2u} globulin in the hepatic tissue. In this article we describe the effect of actinomycin D on hepatic α_{2u} globulin and show that the drug-mediated increase of this protein is associated with a corresponding rise in its mRNA concentration.

MATERIALS AND METHODS

Experiments were performed on albino rats of the Yale strain. Actinomycin D was a gift from Walter Gall of Merck, Sharp & Dohme. α-Amanitin was kindly provided by Th. Wieland of the Max-Planck Institute (Heidelberg, West Germany). Cordycepin, cycloheximide, and 5α-dihydrotestosterone (H$_2$testosterone) were obtained from Sigma. Actinomycin D and H$_2$testosterone were administered in 0.2 ml of solvent containing equal volumes of ethanol and dimethyl sulfoxide. Cycloheximide, cordycepin, and α-amanitin were administered in 0.14 M NaCl. All of these chemicals were administered by intraperitoneal injections.

Preparation of the hepatic cytosol and radioimmunoassay of α_{2u} globulin were performed according to the procedure described (14). Total protein was determined by the method of Lowry et al. (15). For radioimmunoassay, electrophoretically pure α_{2u} globulin was iodinated with ^{125}I by chloramine-T activation (16). Antibody against α_{2u} globulin was produced in rabbits, and the anti-rabbit gamma globulin was made in goats. The radioimmunoassays were performed in duplicate along with known concentrations of α_{2u} globulin. All α_{2u} concentrations as presented in this article are the mean values from at least three experimental animals.

For extraction of hepatic RNA, the animals were stunned by cervical dislocation and the livers were quickly (within 30 sec) removed and blended in sodium dodecyl sulfate (NaDodSO$_4$)/phenol according to Rosenfeld et al. (17). After ethanol precipitation, the poly(A)-containing hepatic RNA was isolated by affinity chromatography on oligo(dT)-cellulose (18). Poly(A)-enriched hepatic mRNA was translated in the wheat germ cell-free system (19). The cation optima (K$^+$ and Mg^{2+}) and RNA saturation for maximum amino acid incorporation were tested for every batch of wheat germ S-30 preparation. Translations were performed at less than 75% saturation of amino acid incorporation (12 μg/100 μl) and optimal concentrations of KCl (80 mM) and Mg acetate (3 mM). [^{3}H]Leucine was used as the tracer amino acid. Total protein synthesis was monitored with 5-μl aliquots of the reaction mixture on filter paper discs (20). Specific α_{2u} synthesis was monitored by immunoprecipitation of α_{2u} globulin from the released peptide chains, followed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The antigen–antibody precipitate was washed through

Abbreviations: H$_2$testosterone, 5α-dihydrotestosterone; NaDodSO$_4$, sodium dodecyl sulfate; TAT, tyrosine aminotransferase.

sucrose (21) and was dissolved by incubating it for 3 min at 100°C in 100 μl of 0.0625 M Tris·HCl (pH 6.8)/3% Na-DodSO$_4$/10% (vol/vol) glycerol/5% (vol/vol) 2-mercapto-ethanol/0.001% bromphenol blue. The dissolved immunoprecipitate was subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis (14% gel) according to Laemmli (22). After electrophoresis the gels were divided up with a Gilson gel fractionator (Gilson Medical Electronics, Middleton, WI), and minced fractions from 2-mm portions of the gel were collected in scintillation vials containing a counting medium composed of 0.5 g of Omnifluor in 100 ml of liquid base consisting of toluene, Protosol, and Triton X-100 (67:3:30, vol/vol). The mixture was incubated for 16 hr at 37°C and the radioactivity was measured in a Packard liquid scintillation spectrometer after the vials had cooled in the counter for 1 hr.

Analytical disc gel electrophoresis of RNA was performed in 2% agarose gel containing 6 M urea according to the procedure described by Rosen *et al.* (23). Prior to electrophoresis, RNA samples were disaggregated by heating at 70°C for 1 min, and electrophoresis was carried out in 0.025 M citric acid at 2 mA/gel at 4°C. Gels were stained in methylene blue and photographed in 50% glycerol on Kodak Panatomic-X film through a red filter. Size distribution of the labeled RNA on agarose gel was analyzed by automatic fractionation of the gel (2-mm fractions) through a Gilson gel fractionator. The minced gel fractions were assayed for radioactivity in a manner similar to that for the polyacrylamide gel, as described above.

RESULTS

Effect of Actinomycin D on the Hepatic Concentration of α$_{2u}$ Globulin. Hepatic tissue of normal female rats does not synthesize α$_{2u}$ globulin and does not respond to H$_2$testosterone treatment (14, 24). Treatment of normal female rats with actinomycin D also did not cause any induction of α$_{2u}$ globulin. Approximately 2 weeks after ovariectomy the animals became responsive to androgens and, possibly because of androgenic hormones from the adrenal cortex, a small but detectable level
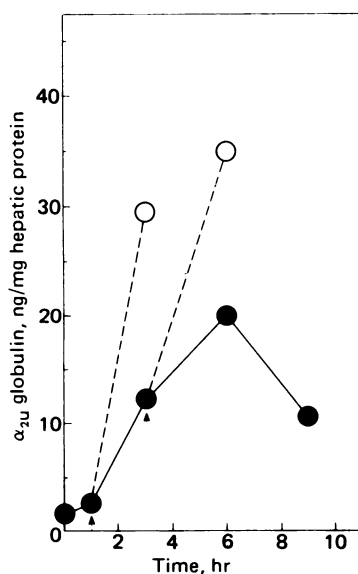
(<5 ng/mg of hepatic protein) of α$_{2u}$ globulin could be detected in the liver of these animals. The extent of the androgenic induction and actinomycin D superinduction was found to depend on this basal level of hepatic α$_{2u}$ globulin, which was found to rise gradually after ovariectomy. In order to minimize variations in androgen sensitivity and actinomycin D response between batches, we have therefore conducted each experiment so that all the animals of that experiment were from the same batch 21 days after ovariectomy (unless otherwise mentioned). The relationship between androgen sensitivity and actinomycin D response is shown by the fact that prepubertal male rats (30–35 days of age), which fail to synthesize α$_{2u}$ globulin due to lack of hepatic androgen receptor, also failed to respond to actinomycin D treatment. On the other hand, pubertal male rats (40 days of age) with a low basal level of α$_{2u}$ globulin responded to actinomycin D with enhancement of hepatic α$_{2u}$ globulin (data not presented). Fig. 1 shows the effect of treatment of spayed female rats with H$_2$testosterone followed by actinomycin D on the hepatic concentrations of α$_{2u}$ globulin. Administration of actinomycin D at 1 and 3 hr after H$_2$testosterone treatment resulted in approximately 2-fold enhancement of the H$_2$testosterone response. Once the liver developed androgen sensitivity after ovariectomy and a basal level of α$_{2u}$ globulin was detectable, actinomycin D alone, without any H$_2$testosterone treatment, was able to increase hepatic α$_{2u}$ globulin. Fig. 2 shows that a single injection of actinomycin D into spayed female rats (3 weeks after ovariectomy) resulted

FIG. 1. Superinduction of α$_{2u}$ globulin by actinomycin D after initial induction by H$_2$testosterone. All animals (spayed female rats) were treated at 0 hr with H$_2$testosterone (33 μg/100 g), and two groups of animals were subsequently treated with actinomycin D (80 μg/100 g) at 1 hr and 3 hr after H$_2$testosterone treatment (arrows). ●——●, Animals treated with H$_2$testosterone alone; ●- - -○, animals treated with H$_2$testosterone followed by actinomycin D.
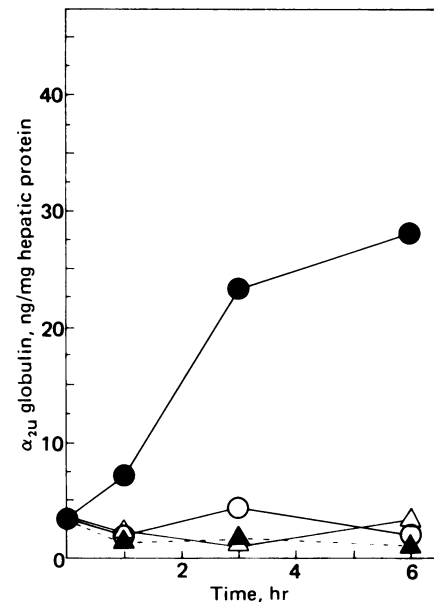
FIG. 2. Effect of actinomycin D, cycloheximide plus actinomycin D, cordycepin, and α-amanitin on the hepatic concentration of α$_{2u}$ globulin. Spayed female rats (3 weeks after ovariectomy) were treated with various drugs at 0 hr and were sacrificed at different times. ●——●, Actinomycin D (80 μg/100 g); ▲- - -▲, α-amanitin (50 μg/100 g); △——△, cordycepin (3 mg/100 g). ○——○, A group of three animals received cycloheximide (600 μg/100 g) 15 min prior to actinomycin D treatment at 0 hr. The extents of inhibition of RNA synthesis by actinomycin D, α-amanitin, and cordycepin and of protein synthesis by cycloheximide were measured by 1-hr pulses (from 1 to 2 hr after the drug administration) with [^{14}C]orotic acid and [^{3}H]leucine, respectively. The above doses of the inhibitors of RNA synthesis caused inhibition of poly(A)$^+$ hepatic RNA to the following control levels: actinomycin D, 24%; α-amanitin 30%; cordycepin 18%. Cycloheximide at the above dose reduced [^{3}H]leucine incorporation into the hepatic proteins to 8% of control. The animals treated with vehicle alone showed no change in hepatic α$_{2u}$ globulin within the experimental period (data not shown in the figure).
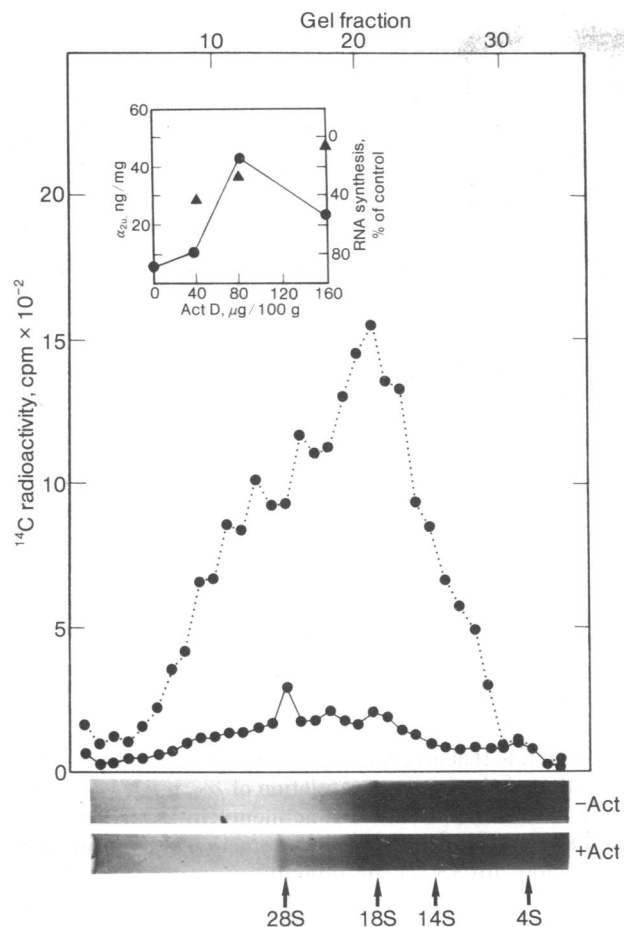
FIG. 3.   Effect of actinomycin D on the incorporation of labeled orotic acid into poly(A)-enriched hepatic RNA. Spayed female rats were treated with either actinomycin D (Act D) or vehicle (control) at time 0 and were given a pulse of [^{14}C]orotic acid (100 μCi per rat; 1 Ci = 3.7×10^{10} becquerels) 1 hr after treatment. The animals were sacrificed 1 hr later and 0.7 A_{260} unit of the poly(A)-enriched hepatic RNA was subjected to urea/agarose gel electrophoresis. Distribution of radioactivity within the gel fractions containing RNA from actinomycin D-(80 μg/100 g) treated ($\bullet$——$\bullet$) and control ($\bullet$·····$\bullet$) animals are shown in the large frame. Stained gel patterns of the same RNA samples are shown at the bottom of the figure. (*Inset*) Dose-dependent increase in hepatic α_{2u} globulin ($\bullet$——$\bullet$) and decrease in incorporation of [^{14}C]orotic acid into poly(A)-enriched RNA ($\blacktriangle$).

in a 5- to 6-fold increase in the hepatic concentration of α_{2u} globulin 3–6 hr after administration of the drug. Pretreatment of these animals with cycloheximide completely abolished the actinomycin D-mediated increase of hepatic α_{2u} globulin. Two other inhibitors of RNA synthesis, α-amanitin and cordycepin, at doses that resulted in inhibitions of hepatic poly(A)$^+$ RNA synthesis (to 30% and 18% of control, respectively) nearly equivalent to the inhibition by actinomycin D (24%), failed to cause any increase in hepatic α_{2u} globulin.

Dose Response to Actinomycin D on the Superinduction of α_{2u} Globulin and Inhibition of Poly(A)$^+$ RNA Synthesis in the Liver. The effect of various doses of actinomycin D on the hepatic concentrations of α_{2u} globulin in the spayed female rats showed that a dose of 80 μg/100 g of body weight was most effective in causing superinduction of this hepatic protein (Fig. 3). The same dose of the drug reduced incorporation of labeled orotic acid into total poly(A)$^+$ RNA and the 14S species of poly(A)$^+$ RNA to 25% and 15%, respectively. In urea-agarose gel electrophoresis the mRNA for α_{2u} globulin is known to
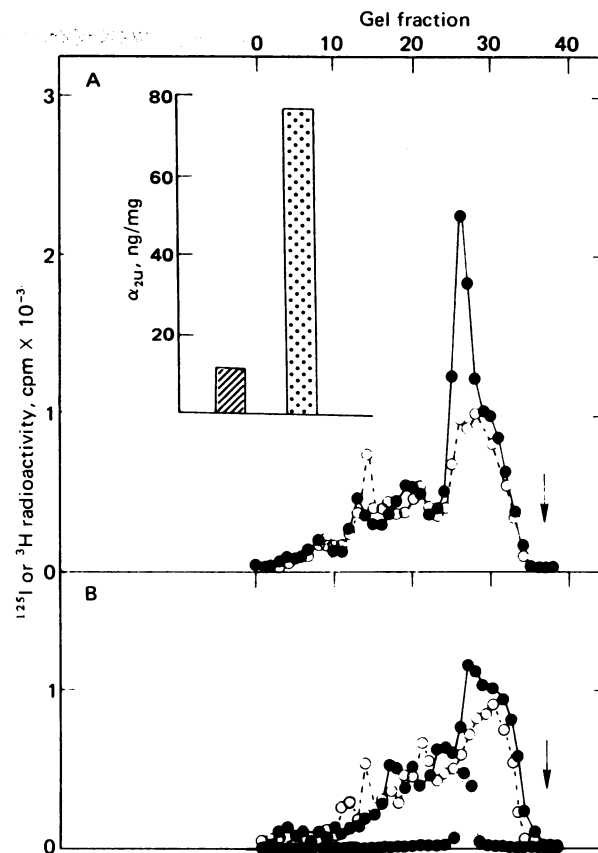

FIG. 4.   Translatable mRNA for α_{2u} globulin within the total hepatic mRNA in spayed female rats with and without actinomycin D treatment. Poly(A)-enriched hepatic RNA obtained from animals with and without actinomycin D treatment was found to be almost equally effective in promoting amino acid incorporation into trichloroacetic acid-insoluble proteins. (*A*) Distribution of radioactivity in the NaDodSO$_4$/polyacrylamide gel after electrophoresis of the immunoprecipitated α_{2u} globulin from *in vitro* translation product primed with hepatic mRNA obtained from animals treated with actinomycin D ($\bullet$——$\bullet$) or vehicle (O---O) for 3 hr. Equal amounts of labeled released peptide chains (5.95×10^6 cpm) were used for immunoprecipitation in the actinomycin D-treated and control samples. (*Inset*) Hepatic concentration of α_{2u} globulin (ng/mg of hepatic protein) in the same group of animals. Hatched bar, vehicle-treated control; stippled bar, actinomycin D-treated rats. (*B*) ($\bullet$——$\bullet$), NaDodSO$_4$/polyacrylamide gel electrophoretic profile of the radioactivity of the second α_{2u}-anti-α_{2u} immunoprecipitate obtained after removal of the labeled α_{2u} globulin from the released peptide chains synthesized in the presence of hepatic mRNA from the actinomycin D-treated animals by first immunoprecipitation (solid circles in *A*). The immunoprecipitation was 85% complete as judged by removal of ^{125}I-labeled α_{2u} globulin. O---O, NaDodSO$_4$ gel electrophoretic profile of the α_{2u}-anti-α_{2u} immunoprecipitate obtained from released peptide chains (5.96×10^6 cpm) of the wheat germ cell-free translational product without any addition of RNA. $\bullet$·····$\bullet$, ^{125}I-labeled α_{2u} globulin in the NaDodSO$_4$/polyacrylamide gel.

migrate as a 14S band (9). These results, therefore, indicate that actinomycin D-mediated superinduction of α_{2u} globulin may not involve transcriptional stimulation of α_{2u} mRNA synthesis. The stained agarose gel showed no major difference between actinomycin D- and vehicle-treated hepatic RNA, except a slightly higher level of 28S RNA in the actinomycin D-treated animals.

Correlation between Hepatic Concentrations of α_{2u} Globulin and Its Translatable mRNA with and without Treatment with Actinomycin D. Several groups of investigators have examined the levels of specific mRNA for super-

inducible proteins under conditions of superinduction (6, 25, 26). Due to differences in experimental conditions, and possibly also to differences in tissue sensitivity to actinomycin D toxicity, these studies have not resulted in clear and consistent patterns of change in mRNA concentration after superinduction. Therefore we have examined the hepatic concentration of mRNA for α_{2u} globulin in animals with and without actinomycin D treatment (Fig. 4). In order to circumvent the technical difficulties associated with the detection of very low concentrations of α_{2u} mRNA (<0.01%) in spayed female rats, we have used 36-day postoperative animals (rather than the usual 21-day), which contain higher basal levels of α_{2u} globulin. These animals show similar sensitivity to actinomycin D superinduction as the 21-day postoperative animals. In addition we have used larger than usual volume (1.0 ml instead of 0.2 ml) of the reaction mixture for the *in vitro* mRNA translation. The use of a larger volume of reaction mixture resulted in a corresponding rise of background radioactivity in the NaDodSO$_4$/polyacrylamide gel. However, the authenticity of the *in vitro*-synthesized α_{2u} globulin programmed with the hepatic mRNA was shown by: (*i*) immunoprecipitation of the *in vitro*-synthesized α_{2u} globulin by monospecific antibody against this protein; (*ii*) its coelectrophoresis with authentic α_{2u} globulin; (*iii*) removal of the labeled α_{2u} globulin (synthesized *in vitro*) after first immunoprecipitation with anti-α_{2u} globulin (Fig. 4B). A similar pattern of background radioactivity was present in the blank gel containing the α_{2u}–anti-α_{2u} immunoprecipitate that had been obtained in the presence of the same amount of labeled peptide chains from control translation (wheat germ S-30 without any hepatic RNA). Analysis of the levels of hepatic α_{2u} globulin and its translatable mRNA in the actinomycin D- and vehicle-treated rats (Fig. 4A) showed a definite correlation between the degree of actinomycin D-mediated superinduction of α_{2u} globulin and the increase in the hepatic concentration of the mRNA for this protein.

DISCUSSION

A low basal induced level of α_{2u} synthesis is a prerequisite for the actinomycin D-mediated increase of hepatic α_{2u}. In the case of both spayed female rats and maturing male rats 40 days of age, a very low but detectable level of α_{2u} globulin has consistently been observed. This low level of α_{2u} synthesis may be due to the presence of trace amounts of androgenic hormones in these animals. Prepubertal male rats (<35 days of age) do not contain hepatic androgen receptor and show a total lack of α_{2u} synthesis and complete insensitivity to androgens (27, 28). These animals also show insensitivity toward actinomycin D-mediated increase of the hepatic α_{2u} globulin. Therefore, the drug-mediated increase in the concentration of this protein represents "superinduction" rather than "induction."

The molecular mechanism of actinomycin D-mediated superinduction of the inducible proteins has been the subject of controversy for more than a decade, and various theories and hypotheses have been proposed to explain the phenomenon (3–7). Considerable attention has been focused on the findings of Palmiter and Schimke (6) implicating an enhanced rate of translation of the inducible mRNA as the mechanism of superinduction. These investigators have shown that the super-induction of ovalbumin by actinomycin D is associated with an increase in the rate of both initiation and elongation of the ovalbumin polypeptide chain. On the basis of these observations, they have proposed that, under normal conditions, limiting concentrations of the translational factors regulate the rate of translation of various mRNA species. However, after inhibition of RNA synthesis by actinomycin D, short-lived mRNA

species are quickly depleted and the long-lived mRNA species that remain could be translated at a more favorable rate. The Palmiter–Schimke model would suggest that any inhibitor of mRNA synthesis will be able to cause superinduction. However, for reasons yet unexplained, in the cases of both hepatic TAT (29, 30) and α_{2u} globulin, neither α-amanitin nor cordycepin is able to cause superinduction. Moreover, the above explanation for superinduction cannot be applicable for proteins such as hepatic TAT or tryptophan oxygenase whose mRNAs have relatively short half-lives, in the range of only a few hours (25, 31). In addition, Thompson *et al.* (32) have argued that hepatoma cells possess a large pool of monomeric ribosomes (33), and thus translational factors, especially ribosomes, cannot be rate limiting under normal conditions of mRNA translation. Furthermore, no evidence for the limitation of translational factors in the normal liver is available in the literature. Therefore, the argument of increased availability of translational factors may not be universally acceptable as the basic mechanism of superinduction.

Recently, Leinwand and Ruddle (7) have observed that actinomycin D at certain concentrations causes 2- to 3-fold stimulation of the *in vitro* translation of myeloma mRNA and tobacco mosaic virus mRNA in the wheat germ cell-free system. They have suggested that superinduction of actinomycin D may involve drug-mediated changes in the secondary structure of the mRNA, resulting in enhancement of the mRNA translation. We have found that actinomycin D does not cause any selective stimulation of the *in vitro* translation of α_{2u} mRNA (unpublished). Therefore, we feel that actinomycin D-mediated superinduction is not due to a direct interaction of the drug with the inducible mRNA for this protein.

The role of the nucleus in the phenomenon of superinduction has been indicated by the results of Ivarie *et al.* (34). These authors have shown that enucleation of hepatoma cells after induction of TAT with dexamethasone produces a condition in which a high level of TAT is maintained in the cytoplasm even after withdrawal of the inducing steroid. The above results have been interpreted to mean that, analogously to enucleation, actinomycin D may cause superinduction by canceling the influence of the nucleus on the cytoplasmic protein synthesis through a short-lived mRNA repressor of nuclear origin. These and other data have been used to support the hypothesis of hormonal regulation of protein synthesis through post-transcriptional repression (for review, see ref. 35). However, authentication of the post-transcriptional model would require evidence for an increased level of translatable mRNA for the inducible protein under the conditions of superinduction and identification of the post-transcriptional repressor. Recently the convincing results of Sehgal *et al.* (26) showing strong correlation between the levels of biologically active interferon and its translatable mRNA in human fibroblasts during induction, shut-off, and superinduction of interferon [by poly(I)·poly(C) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole] have provided considerable support for the above theory of post-transcriptional repression. The result, showing a direct correlation between the hepatic concentrations of α_{2u} globulin and its mRNA after superinduction with actinomycin D at a dose that reduces the synthesis of 14S poly(A)$^+$ RNA (the size for α_{2u} mRNA) to less than 15% of control, indicates that the drug-mediated increase in α_{2u} mRNA activity may be due to the stabilization, activation, or both of the already-synthesized RNA sequences. A parallel increase in α_{2u} mRNA and α_{2u} globulin in the liver of actinomycin D-treated rats also indicates that the increase of hepatic α_{2u} globulin is not due to changes in the rate of hepatic protein secretion. Moreover, studies from this laboratory have shown that actinomycin D does not cause any in-

hibition of α_{2u} secretion in the perfused rat liver (36). In addition, electrophoretic analysis and densitometric quantitation of the intracellular hepatic albumin in the spayed rats 3 hr after treatment with actinomycin D (80 μg/100 g) and the vehicle failed to show any detectable difference in intracellular albumin concentration in these two groups of animals (unpublished).

The correlation between the biologically active interferon and its mRNA after superinduction with dichlororibosylbenzimidazole (26) and hepatic α_{2u} globulin and its mRNA after superinduction with actinomycin D tends to support the concept of the McAuslan–Tomkins model for post-transcriptional repression of protein synthesis. However, it should be pointed out that an overwhelming body of data indicates that both steroid and nonsteroid hormones are involved in the regulation of gene transcription in their target cells (12, 13, 21, 24, 37–42). These two apparently contradictory conclusions can be reconciled if one considers that the post-transcriptional regulation serves only as an additional control over the transcriptional regulation and could play an important physiological role in the suppression of "leaky" genes.

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