Hormonal Regulation of the Hepatic Messenger RNA Levels for α_{2u} Globulin†

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ABSTRACT: The messenger RNA for rat α_{2u} globulin has been identified and quantitated in a cell-free translational system derived from Krebs II ascites cells. Hepatic tissue of the mature male rats which normally produce α_{2u} globulin was also found to contain a high level of α_{2u} mRNA. Approximately 1.6% of all poly(A) containing RNA of the adult male rat liver could be accounted for α_{2u} messenger activity. Female rats do not produce α_{2u} globulin and no α_{2u} mRNA activity could be detected in the poly(A) containing RNA fraction obtained from the livers of these animals. However, androgen treatment to spayed female rats was found to induce the parallel appearance of both α_{2u} globulin and its corresponding mRNA. Both hypophysectomy and adrenalectomy which are known to reduce the level of α_{2u} globulin in the urine of male rats were found also to reduce the hepatic level of α_{2u} mRNA. The results indicate that hormonal control of α_{2u} globulin synthesis in rat liver is achieved primarily through regulation of its translatable mRNA level and that more than one hormone may participate in this regulation.

An androgen-dependent urinary protein named α_{2u} globulin has been described by Roy and Neuhaus (1966a). α_{2u} globulin has been purified and partially characterized (Roy *et al.*, 1966). Both liver perfusion as well as immunofluorescent studies have shown the hepatic origin of α_{2u} globulin (Roy and Neuhaus, 1966b; Roy and Raber, 1972). The hepatic synthesis of α_{2u} globulin is known to be regulated by various hormones. Androgens act as specific inducers, whereas estrogens serve as specific anti-inducers. Other general growth regulatory hormones such as pituitary growth hormone, thyroid hormones, insulin, and glucocorticoids seem to act synergistically with the androgens to stimulate α_{2u} synthesis (Roy and Neuhaus, 1967; Kumar *et al.*, 1969; Irwin *et al.*, 1971; Roy, 1973; Roy and Leonard, 1973). The above observations have led us to utilize α_{2u} globulin as a probe to investigate the hormonal regulation of a specific messenger RNA (mRNA) and its translation in rat liver.

Reports from various laboratories have shown that regulation of specific protein synthesis by steroid hormones is associated with changes in the level of the corresponding mRNA for these proteins (Rhoads *et al.*, 1973; Schutz *et al.*, 1973; Chan *et al.*, 1973; Palmiter and Smith, 1973). Regulatory effects of these hormones appear to be mediated by specific receptor proteins (Jensen and DeSombre, 1973; O'Malley and Means, 1974). Whereas, the obligatory role of a cytoplasmic androgen receptor in the androgen dependent synthesis of α_{2u} globulin in rat liver has been suggested (Milin and Roy, 1973; Roy *et al.*, 1974), none of the studies on α_{2u} globulin reported thus far provides evidence for hormone dependent regulation of the amount of translatable mRNA coding for this protein in the hepatic tissue.

Recent investigations with chick oviduct proteins, ovalbumin, and avidin (Rhoads *et al.*, 1971; Means *et al.*, 1972; O'Malley *et al.*, 1972) as well as with tryptophan oxygenase from the rat liver (Schutz *et al.*, 1973) have shown that messenger dependent heterologous cell-free systems can be used as a powerful tool for studying the mechanism of hormonal regulation of protein synthesis. In this article we report the successful use of a Krebs II ascites cell-free system for the identification of α_{2u} mRNA and quantitation of its hepatic level under various endocrine manipulations. These results establish that the control of functional mRNA levels plays a key role in the hormonal regulation of α_{2u} synthesis in rat liver.

Experimental Procedure

1. Animals and Surgical Operations. Experiments were carried out with 300–350 g of albino rats of Sprague-Dawley strain. Unless otherwise specified, animals were maintained with Purina rat chow and tap water *ad libitum* and were housed in an air-conditioned animal room with 12 hr of light and 12 hr of darkness. For the collection of 24-hr urine samples, the animals were placed in stainless steel metabolism cages, and uncontaminated urine samples were collected at room temperature with thymol, penicillin, and streptomycin as preservatives. The samples were frozen immediately after collection and were stored in the freezer until assayed for α_{2u} globulin. Bilateral adrenalectomy was performed under ether anesthesia and the operated animals were maintained on 1% NaCl solution instead of drinking water. Hypophysectomy was performed under sodium pentobarbital (Nembutal) anesthesia through parapharyngeal approach (Zarrow *et al.*, 1964). The hypophysectomized rats were provided with 6% glucose instead of drinking water. All operated animals were allowed 7 days of postoperative rest before any further treatment. Before the sacrifice of the animals for the removal of livers, 24-hr urine samples were collected in the metabolism cages. For the extraction of messenger RNA, samples of hepatic tissue were removed from animals under ether anesthesia and the tissues were quickly frozen in liquid nitrogen. The liver samples were stored at −85° until further processing.

2. Isolation of α_{2u} Globulin, Preparation of Antiserum, and Immunoassay of Urinary α_{2u} Globulin. α_{2u} globulin was isolated from mature male rat urine. Antiserum against purified α_{2u} globulin was prepared by repeated injections of

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an emulsion of the antigen and Freund's complete adjuvant into rabbits. Urinary α_{2u} concentrations were assayed by means of a quantitative single diffusion technique. Detailed descriptions of these procedures have appeared elsewhere (Roy, 1973).

3. Determination of Antigen–Antibody Equivalence. Anti-α_{2u} globulin rabbit immune serum was reconstituted by dissolving 70 mg of lyophylized protein in 1.0 ml of phosphate-buffered saline (0.01 M sodium phosphate (pH 7.0)–0.14 M NaCl). 10 μg of purified α_{2u} globulin were incubated at 0° for 4 hr with increasing amounts of antibody solution in 0.5 ml of phosphate-buffered saline containing 2% Triton X-100. The immunoprecipitate was collected by centrifugation at 2000*g* and was washed four times with 4 ml of the same buffer. The precipitated protein was quantitated by the procedure of Lowry *et al.*, (1951). For 10 μg of α_{2u} globulin, maximum immunoprecipitation was obtained with 100 μl of antibody solution.

4. Isolation of Liver mRNA Fractions. RNA was prepared from 5 g of frozen liver tissue derived from three animals as described previously (Schutz *et al.*, 1973). RNA extracted with the phenol–chloroform mixture was chromatographed on cellulose to obtain the poly(A)-containing mRNA fraction (Schutz *et al.*, 1972). The total yield of RNA after cellulose chromatography varied from 0.5 to 0.6 mg/5 g of frozen liver. Hemoglobin mRNA was isolated from rabbit reticulocytes by following the procedure of Schutz *et al.* (1972) with further purification of the globin mRNA on a sucrose gradient.

5. In Vitro Synthesis of Liver Proteins. For the *in vitro* protein synthesis a Krebs II ascites cell-free system supplemented with tRNA and rabbit reticulocyte initiation factors was prepared (Mathews and Korner, 1970; Metafora *et al.*, 1972; with modifications described by Schutz *et al.*, 1974). The complete reaction mixture in 100-μl volume contained: 30 mM Tris-HCl (pH 7.5), 95 mM KCl, 3.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 5 mM creatine phosphate, 0.016 mg of creatine kinase, 0.125 A_{260} of tRNA, 0.04 mM of each of the other 19 natural amino acids, 30 μCi of [^{3}H]leucine (59 Ci/mmol), 0.45 A_{260} of the preincubated ascites cell S-30, 0.6 A_{280} of the reticulocyte initiation factor fraction, and 7 μg of rat liver mRNA.

After incubation at 37° for 90 min, the amount of [^{3}H]leucine incorporated into total protein was determined in a 10-μl aliquot according to the method described by Bollum (1968). Released polypeptide chains were separated from the polysomes by centrifugation for 1 hr at 150,000*g* at 2°. The amount of [^{3}H]leucine incorporated into the released chains was determined in a 10-μl aliquot of the supernatant.

6. Determination of α_{2u} Globulin Synthesized in Vitro. The released polypeptide fraction obtained by ultracentrifugation was brought to 2% Triton X-100, 0.01 M unlabeled leucine, 0.01M sodium phosphate (pH 7.0), and 0.14 M NaCl. After addition of 10 μg of unlabeled carrier α_{2u} globulin, immunoprecipitation was allowed to develop at 0° for 4 hr following the addition of 100 μl of anti-α_{2u} globulin solution (70 mg/ml). The antigen–antibody precipitate was collected according to a slight alteration of the method described by Rhoads *et al.* (1973) by centrifugation at 2000*g* in a conical glass tube through 1 ml of 1 M sucrose, in phosphate-buffered saline containing 2% Triton X-100. The top layer was then removed by aspiration and the surface of the sucrose layer was washed twice with 2 ml of phosphate-buffered saline and then the sucrose layer was removed. The immunoprecipitate was washed twice with 2 ml of phosphate-buffered saline and suspended in 150 μl of solution containing 2% SDS, 2% β-mercaptoethanol, 10 mM sodium phosphate (pH 7.2), 10% glycerol, and 1 μl of 1% Bromophenol Blue. The suspension was solubilized by heating for 2 min in a boiling water bath and submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The electrophoresis was performed at 8 mA/gel for 5.5 hr. After electrophoresis the 10% polyacrylamide gels were frozen at −20° and cut into 1-mm slices. The radioactivity was determined as described earlier (Sippel, 1973), except that Yorktown Hydromix was used instead of Aquasol. Protein markers including purified α_{2u} globulin were run on parallel gels and stained with Coomassie Brilliant Blue.

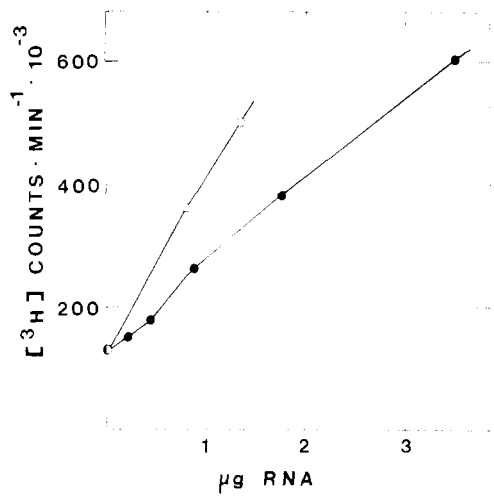

FIGURE 1: Messenger dependency of the translational system. Incorporation of [^{3}H]leucine into total protein by the Krebs II ascites cell-free system was measured after 60-min incubation at 37° in a 50-μl routine assay as described under Materials and Methods. Indicated amounts of poly(A)—containing RNA from livers of male rats (●) and rabbit reticulocyte globin mRNA (○) were added.

Results

1. α_{2u} Globulin mRNA Levels of Male and Female Rat Livers. Since earlier investigations have shown that α_{2u} globulin is synthesized in the liver (Roy and Neuhaus, 1966b; Roy and Raber, 1972), it was of interest to show that mRNA fractions from male livers contain translatable messenger for α_{2u} globulin. Total poly(A) containing mRNA from rat liver, isolated by chromatography on unmodified cellulose stimulates the synthesis of proteins in a cell-free system from Krebs II ascites cells only slightly less than equivalent amounts of purified rabbit reticulocyte globin mRNA (Figure 1). A preincubated ascites cell 30,000*g* supernatant with a relative high background incorporation was chosen in order to achieve maximal activity for the incorporation of exogenous mRNA dependent protein synthesis. Other cell-free ascites systems with lower background incorporations were found to be usually less active.

Hepatic mRNA from adult male rats (Figure 2a) directs the synthesis of a product which can be precipitated with monospecific antibodies against purified α_{2u} globulin and whose electrophoretic mobility in sodium dodecyl sulfate acrylamide gel is also identical with that of authentic α_{2u} globulin. Female rat liver mRNA, although able to direct the synthesis of roughly the same amount of total protein,

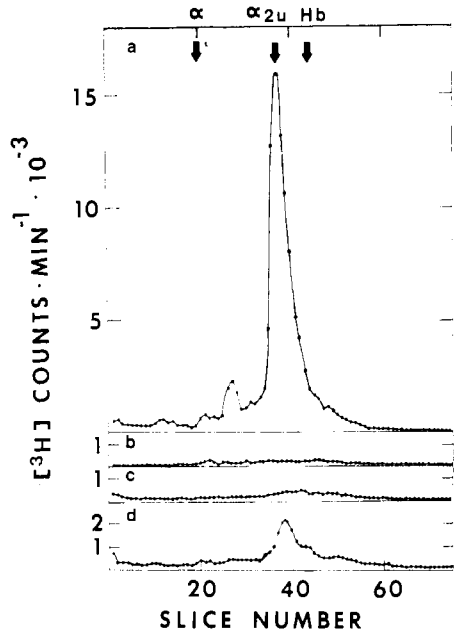

FIGURE 2: Sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of immunologically precipitated α_{2u} globulin synthesized *in vitro* by liver mRNA from normal male and ovariectomized female rats. Samples were prepared as described under Materials and Methods from the released chain fraction of a 250-μl reaction mixture incubated 90 min at 37°. The arrows indicate the positions of marker proteins (α subunit of *E. coli* RNA polymerase, 40,000 daltons; α_{2u} globulin, 19,500 daltons; Hb, rabbit globin, 16,500 daltons). (a) Synthesis directed by 17.5 μg of male liver mRNA; (b) synthesis directed the cell-free system without addition of exogenous mRNA; (c) synthesis directed by 17.5 μg liver mRNA from ovariectomized females; (d) synthesis directed by 17.5 μg of liver mRNA from ovariectomized females, treated for 10 days with dihydrotestosterone (50 μg/100 g of body weight).

Table I: *In Vitro* Synthesis of α_{2u} Globulin with Male Rat Liver mRNA.

Expt[a]	[3H]Leucine Incorporation	Cpm[b]	pmol[c]	%[d]
1	Total protein	6,530,000	768	275
	Released chains	2,370,000	279	100
	α_{2u} Globulin	68,000	4.71	1.68
2	Total protein	13,466,000	1584	284
	Released chains	4,791,000	564	100
	α_{2u} Globulin	122,000	8.43	1.49
3	Total protein	2,072,000	244	188
	Released chains	1,104,000	130	100
	α_{2u} Globulin	0	0	0

[a] The incubation volume for experiment 1 was 250 μl and contained 17.6 μg of male rat liver mRNA. Experiments 2 and 3 had incubation volumes of 500 μl containing 35 μg of male rat liver mRNA and no additional mRNA, respectively. [b] [3H]Leucine incorporation for total proteins, released chains, and α_{2u} globulin was determined after 90 min of incubation at 37° as described under Materials and Methods. [c] The radioactivity was converted to molar concentration by reference to appropriate standards which were established by counting known amounts (pmoles) of [3H]leucine in appropriate reaction mixtures. For the filter paper analysis (total proteins and released chains), a standard of 8500 cpm was equivalent to 1 pmol (counting efficiency 6.5%) and for the gel slices (α_{2u} globulin) a standard of 14,500 cpm was found to be equivalent to 1 pmol of [3H]leucine (counting efficiency 11.2%). [d] Relative amount of [3H]leucine incorporation into various fractions was computed as percentage of picomoles of [3H]leucine found in the released chains.

does not contain any specific product identifiable by the same criteria; neither does the endogenous background product of the heterologous cell-free protein synthesis system (Figure 2b,c). The fact that no radioactivity is found in the latter two cases excludes the possibility that the radioactive peak in Figure 2a is due to nonspecific precipitation or nonspecific binding of radioactivity. From these results we conclude that RNA from adult male rat livers contains the mRNA for α_{2u} globulin, whereas RNA from female livers does not. The above results extend the earlier findings concerning the hepatic synthesis of this androgen-dependent urinary protein (Roy and Neuhaus, 1966b; Roy and Raber, 1972).

The experimental approach used allows a quantitation of the amount of this specific protein among the total liver protein synthesized *in vitro*. Table I gives the results of two independent experiments to determine the percentage of [3H]leucine incorporation in α_{2u} globulin within the total radioactivity of the released chains. Assuming the 7.9% leucine content of α_{2u} globulin (Roy *et al.*, 1966) is comparable to that of other typical hepatic proteins, one can estimate that 1.5–1.7% of the total protein synthesized *in vitro* is α_{2u} globulin. Unpublished results from our laboratories showed α_{2u} globulin with 19,500 molecular weight (estimated on the basis sodium dodecyl sulfate polyacrylamide gel electrophoresis) to be an average sized protein from liver RNA synthesized *in vitro*. It may therefore be inferred that the level of α_{2u} globulin messenger within the total mRNA fraction of adult male rat liver is in the same range. However, since the mRNA fraction used for *in vitro* synthesis was obtained from whole liver tissue and the processing as well as compartmentalization may play a significant role in functional mRNA activity *in vivo*, only tentative conclusions can be drawn as to the absolute amount of active messenger for α_{2u} globulin in hepatocytes.

2. DHT Mediated Induction of α_{2u} Globulin mRNA in the Spayed Female Rats. Treatment of spayed female rats with androgenic hormones induces the synthesis of α_{2u} globulin with its subsequent appearance in the urine (Roy and Neuhaus, 1967). At a dose of 50 μg/100 g of body weight, 5α-dihydrotestosterone acts as the most effective inducer of α_{2u} globulin (A. K. Roy, unpublished observations). Results presented in Figure 2c,d and Table IIA show a good correlation between the 24-hr urinary level of α_{2u} globulin and the α_{2u} messenger RNA content of livers of 5α-dihydrotestosterone treated and untreated ovariectomized animals. Ovariectomized females which were treated for 10 days with a daily dose of 50 μg of 5α-dihydrotestosterone/100 g of body weight (subcutaneously) excreted 28.5% α_{2u} globulin in the 24-hr urine as compared to adult male rats. The hepatic α_{2u} messenger RNA activity in these animals were found to be 22.6% of the normal males. The untreated ovariectomized females did not produce any urinary α_{2u} globulin nor did their hepatic poly(A) containing RNA fraction show any detectable α_{2u} messenger activity. These results clearly show that the androgenic induction of α_{2u} globulin output in the urine of spayed female rats is associated with the appearance of α_{2u} messenger RNA in their livers.

3. Effects of Hypophysectomy and Adrenalectomy on

Table II: Hormonal Regulation of α_{2u} mRNA Levels in the Liver and Corresponding Variations in the Urinary Output of α_{2u} Globulin.

	Sex	Endocrine[a] Lesion	Hormone[a] Treatment	Hepatic[b] α_{2u} mRNA (%)	24-hr Urinary[c] α_{2u} Globulin (%)
	Male	None	None	100	100
A	Female	Ovariectomized	None	<0.4	0
	Female	Ovariectomized	10 days of DHT[d]	22.6	28.5
B	Male	Hypophysectomized	None	0	0
	Male	Hypophysectomized	10 days of DHT	0	0
C	Male	Adrenalectomized	None	20.7	5.0
	Male	Adrenalectomized	10 days of corticosterone	32.3	25.5

[a] Animals, their endocrine manipulations, and their hormone treatments are as described under Materials and Methods. [b] Poly(A)-containing RNA fractions isolated from the livers pooled from three animals were assayed for their ability to code for α_{2u} globulin synthesis *in vitro* according to the procedure described under Materials and Methods. The relative proportion of [^{3}H]leucine in immunologically and electrophoretically purified α_{2u} globulin synthesized within the released chain fraction was determined for each case as described in the legend of Table I. The values given above for hepatic α_{2u} mRNA represent the percentage of that found in normal adult male rat liver. [c] Average values from the same three animals, determined as described under Materials and Methods; 31 mg of α_{2u} globulin per day was the average urinary output of adult male rats and was set as 100%. [d] DHT, 5α-dihydrotestosterone.

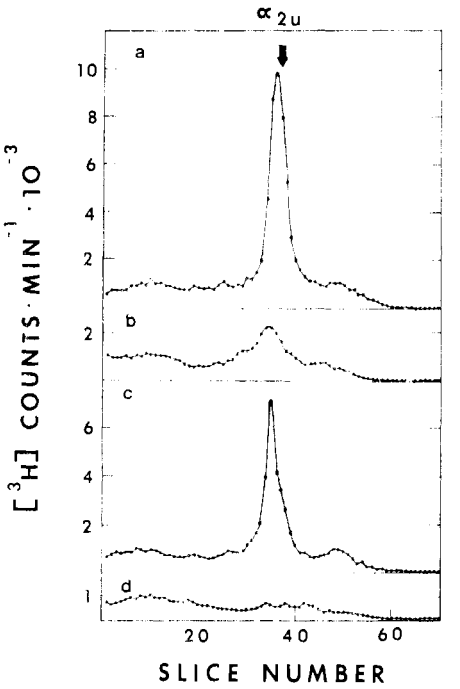

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of immunologically precipitated α_{2u} globulin synthesized *in vitro* by the liver mRNA from normal and adrenalectomized male rats. Samples were prepared as described under Materials and Methods from the released chain fraction of a 500μl reaction mixture incubated for 90 min at 37°. The arrow indicates the position of authentic α_{2u} globulin in the gel. Protein synthesis directed by 17.5 μg of liver mRNA from: (a) normal adult males; (b) adrenalectomized males; (c) adrenalectomized males treated for 10 days with corticosterone (3.0 mg/100 g of body weight); and (d) synthesis directed by the cell-free system without addition of exogenous mRNA.

α_{2u} mRNA Activity. The presence of both the pituitary gland as well as the adrenal cortex have been shown to be required for the androgen dependent synthesis of α_{2u} globulin (Kumar *et al.*, 1969; Irwin *et al.*, 1971; Roy, 1973). Results presented in Figure 3 and Table II depict the correlation between urinary α_{2u} level and the hepatic mRNA activities in the hypophysectomized and adrenalectomized male rats. Within 3 days after hypophysectomy, a complete loss of urinary α_{2u} globulin has been observed (Roy, 1973). Cell-free translation of the poly(A)-containing RNA fraction obtained from the livers of hypophysectomized male rats showed no detectable mRNA for α_{2u} globulin. Androgen treatment (50 μg of DHT/100 g of body weight, for 10 days) of these hypophysectomized rats did not cause any detectable output of α_{2u} nor cause the appearance of any detectable hepatic α_{2u} messenger activity (Table IIB). These results indicate that along with androgens, hypophysial hormones are also required directly or indirectly, for the induction of functional mRNA for α_{2u} globulin.

Unlike hypophysectomy, adrenalectomy does not completely stop α_{2u} synthesis. An examination of the urinary level of α_{2u} globulin (Table IIC) and hepatic level of functional mRNA for α_{2u} globulin (Table IIC) and Figure 3) in the adrenalectomized animals with and without corticosterone supplementation indicates that glucocorticoids may also participate in the hormonal control of the hepatic α_{2u} messenger RNA level. However, with respect to glucocorticoidal control a strict concordance between urinary output of α_{2u} globulin and the hepatic level of the corresponding mRNA has not been observed.

Discussion

Both the immunological as well as electrophoretic identification of the α_{2u} globulin synthesized with a heterologous protein synthesizing system *in vitro* under the direction of poly(A)-containing RNA fractions from rat liver provides a reasonably firm assay procedure for the functional level of the specific messenger for α_{2u} globulin within the total hepatic mRNA. The activity of the translational system and the sensitivity of the immunoprecipitation technique together with the relatively high amount of α_{2u} mRNA in the fully induced state makes it possible to detect levels of α_{2u} globulin mRNA down to 0.5% of that found in the mature male rat livers. The absence of α_{2u} globulin in the urine of female rats is closely correlated to the absence of the specific α_{2u} globulin mRNA within the hepatic mRNA fraction. The parallel appearance of α_{2u} globulin in the urine as well as its corresponding mRNA in the liver after androgen treatment of spayed famale rats indicates that the induction of this protein is mediated by the rise in the level of its specific translatable mRNA. We conclude from the parallelity of these results that the androgen dependent synthesis of α_{2u} globulin is not controlled by translational mechanism of

regulation. The results are consistent with an entirely pretranslational regulation mechanism of hormone action. The steady-state level of an active mRNA in the cytoplasm under any endocrine condition is the result of a number of events, including the rate of nuclear synthesis of mRNA sequences, the rate of processing to translatable mRNA, the rate of transport from nucleus to the polysomal mRNA fraction, and the rate of cytoplasmic mRNA inactivation. The results presented in this article do not identify which of the above described processes are hormonally modulated leading to altered tissue levels of the specific mRNA coding for the induced protein. Earlier studies of the glucocorticoid induction of the hepatic tryptophan oxygenase level have demonstrated coordinacy between hormonal saturation of the cytoplasmic steroid receptor (Beato *et al.*, 1972), its migration into the nucleus (Kalimi *et al.*, 1973; Beato *et al.*, 1974), elevation in hepatic level of both specific mRNA coding for tryptophan oxygenase, and induced levels of this enzyme (Schutz *et al.*, 1973). These findings and other recent reports (reviewed by O'Malley and Means, 1974), along with results of the present investigation, are compatible with and support the hypothesis that the hormonal regulation of specific protein synthesis may be mediated by selective gene transcription.

In an earlier publication dealing with the role of the pituitary gland on α_{2u} synthesis, it was speculated that both pituitary as well as adrenocortical secretions may regulate α_{2u} synthesis by modulating various post-transcriptional processes in the hepatic cells (Roy, 1973). The above speculation was based on the stimulatory effect of pituitary growth hormone and glucocorticoids on hepatic ribosome synthesis and translational processes (Jefferson and Korner, 1967; Greenman *et al.*, 1965; Yu and Feigelson, 1969). The results presented in this article show that both the adrenal and the pituitary gland are required for the maintenance of the high level of α_{2u} mRNA in the hepatic tissue. The observed lack of strict correlation between the urinary levels of α_{2u} globulin and the hepatic levels of the corresponding mRNA in the adrenalectomized male rats suggests that additional factors contribute to the control of the urinary output of α_{2u} globulin in these animals. In the case of hypophysectomized males, however, the level of both α_{2u} globulin secretion and its corresponding hepatic mRNA was reduced to zero and neither could be restored with androgen alone. These results suggest that the androgens require the supportive influence of other synergistically acting hormones for the maintenance of the high level of specific mRNA for α_{2u} globulin. A similar synergistic influence between estrogen and progesterone in the induction of conalbumin and ovomucoid mRNA in the chick oviduct has also been reported (Palmiter and Smith, 1973).

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