Transcription of the α_{2u}-Globulin Gene in Male Rat Liver Nuclei in Vitro†

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ABSTRACT: α_{2u}-Globulin is a male rat liver protein under multihormonal control which represents approximately 1% of hepatic protein synthesis. We have measured the rate of transcription of the α_{2u}-globulin gene using a nuclear cell-free transcriptional system with mercurated CTP as substrate for the endogenous RNA polymerases. The newly synthesized, mercurated RNA was purified free of endogenous RNA by chromatography on sulfhydryl agarose and hybridized to ^{3}H-labeled α_{2u}-globulin cDNA. It was found that, in male rat liver nuclei, α_{2u}-globulin RNA sequences represent 0.005% of the total newly synthesized RNA. Actinomycin D or α-amanitin completely blocks the synthesis of α_{2u}-globulin RNA in these nuclei. No α_{2u}-globulin RNA synthesis was detectable in female rat liver nuclei. Thus, within the limits of our detection the absence of hepatic α_{2u}-globulin mRNA in female rats appears to be due to a lack of transcription of the α_{2u}-globulin gene in these animals.

Recently this laboratory has focused on elucidating the biochemical mechanisms underlying the multihormonal control of the synthesis of a male rat liver protein, α_{2u}-globulin. This protein represents approximately 1% of hepatic protein synthesis in a mature male rat, and no detectable α_{2u}-globulin synthesis occurs in females (Sippel et al., 1976). The hepatic biosynthesis of this protein is under complex hormonal control: androgens, glucocorticoids, thyroid hormone, and pituitary growth hormone induce α_{2u}-globulin synthesis, and estrogens repress the synthesis of this protein (Sippel et al., 1975; Kurtz et al., 1976a,b; Roy & Dowbenko, 1977). We have shown, using a cell-free translational system (Kurtz et al., 1976a,b), and, more recently, using a pure α_{2u}-globulin cDNA[1] (Kurtz & Feigelson, 1977) that androgens, glucocorticoids, and thyroid hormones induce α_{2u}-globulin biosynthesis via induction of the cytoplasmic level of α_{2u}-globulin mRNA, whereas growth hormone regulates α_{2u}-globulin biosynthesis via a translational control mechanism.[2]

The modulation of hepatic α_{2u}-globulin mRNA level by steroids and thyroid hormone may be the result of control at the level of α_{2u}-globulin gene transcription, processing of the primary gene transcript, or stabilization of the α_{2u}-globulin mRNA. In order to distinguish between these alternatives, we have used a nuclear cell-free transcriptional system, with Hg-CTP as substrate for endogenous RNA polymerase II, to study the rate of transcription of the α_{2u}-globulin gene in nuclei derived from male and female animals.

Problems with the use of mercurated triphosphates in cell-free transcriptional systems have been well documented (Zasloff & Felsenfeld, 1977a,b; Schafer, 1977; Giesecke et al., 1977). In chromatin systems using exogenous *Escherichia coli* RNA polymerase, mercurated nucleotides can be incorporated into RNA which is complementary to endogenous RNA (Giesecke et al., 1977) or can be added to the 3′ ends of endogenous RNA species (Giesecke et al., 1977). Also, during the extraction and purification of the mercurated RNA, endogenous RNA sequences may be chemically mercurated or may aggregate with the mercurated RNA (Zasloff & Felsenfeld, 1977a,b; Schafer, 1977; Giesecke et al., 1977). Any of these effects would result in endogenous RNA sequences being copurified with the newly synthesized mercurated RNA. However, the RNA-dependent RNA polymerase activity and 3′-end addition do not seem to occur in nuclear transcriptional systems using endogenous polymerase (Nguyen-Huu et al., 1978). Furthermore, sufficiently denaturing conditions can be employed during the purification of mercurated RNA to prevent aggregation of endogenous RNA sequences. Nguyen-Huu et al. (1978) have found, using a nuclear transcription system, that the rate of ovalbumin RNA synthesis in oviduct nuclei is identical as measured using two different methods, one which involved mercurated triphosphates and one which did not.

This indicates that, if sufficient care is exercised, Hg-CTP can be used as substrate for the endogenous RNA polymerase to obtain accurate values for the rates of transcription of specific genes in nuclear transcription systems. We have used a similar system to study the rate of transcription of the α_{2u}-globulin gene in rat liver nuclei. It was found that, in nuclei prepared from male rat liver, α_{2u}-globulin mRNA sequences represent approximately 0.005% of the newly synthesized RNA, while nuclei prepared from female rat liver synthesize no detectable α_{2u}-globulin RNA sequences.

Materials and Methods

Sprague-Dawley male and female rats, weighing 250–300 g, were used throughout all experiments.

[^{3}H]UTP was purchased from New England Nuclear Inc.; α-amanitin, ribonucleoside triphosphates, *N*-acetyl-DL-homocysteine thiolactone, and Ellman's reagent were from Sigma Co.; S_1 nuclease was purchased from Miles, Inc.

Isolation of Nuclei. Nuclei were prepared as described by Marshall & Burgoyne (1976) except that the homogenate was filtered through 110-mesh nylon monofilament cloth prior to

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[1] Abbreviations used: cDNA, complementary DNA; $NaDodSO_4$, sodium dodecyl sulfate; mRNA α_{2u}, α_{2u}-globulin messenger RNA; Hg-CTP, 5-mercuricytidine triphosphate; Hg-RNA, RNA containing Hg-CMP; SH-agarose, sulfhydryl agarose; PEI-cellulose, polyethyleniminecellulose.

[2] Kurtz, D. T., Chan, K.-M., & Feigelson, P., manuscript in preparation.

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centrifugation. Nuclear pellets were washed and resuspended in 50 mM Tris-HCl, pH 8.0, containing 25% glycerol. Resuspended nuclei were quick-frozen by dropwise addition into liquid nitrogen and were stored at −80 °C. The DNA content was assayed by the diphenylamine method of Burton (1956).

Preparation of Mercurated CTP and Sulfhydrylagarose. Mercurated CTP was prepared by mercuration of CTP with mercuric acetate as described by Dale et al. (1975). The mercurated CTP was characterized by a 5 nm red shift of the CTP 261 nm absorption maximum, and by a decrease in R_f upon thin-layer chromatography on PEI-cellulose in 1:1 ethanol:1 M ammomium acetate (Crouse et al., 1976). Sulfhydryl-Sepharose 6B was prepared according to the procedure of Cuatrecasas (1970). The SH content of the resin, as determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959), was 1.5–3 μmol/mL packed resin.

In Vitro Synthesis of RNA. The conditions for RNA synthesis were optimized and are a modification to those described by Ernest et al. (1976). Unless otherwise specified, standard assays were performed at 25 °C in 50 μL reaction mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 14 mM β-mercaptoethanol, 150 mM KCl, 10% glycerol, 1 mM each of ATP, CTP and GTP, 0.4 mM of [^{3}H]UTP (0.5 Ci/mmol), and nuclei containing 5 μg of DNA. Hg-CTP, when present, was at a concentration of 0.5 mM. The incorporation of the [^{3}H]UTP into Cl_3CCOOH-precipitable material was determined as previously described Ernest et al. (1976).

Preparation and Isolation of in Vitro Synthesized Hg-RNA. Nuclei (0.2 mg of DNA/mL) were incubated at 25 °C for 1 h in a 10-mL reaction mixture described above containing 0.5 mM Hg-CTP and in the presence or absence of [^{3}H]UTP (0.5 Ci/mmol). At the end of incubation, $NaDodSO_4$ was added to 0.5%, EDTA to 10 mM, and NaCl to 0.4 M. The mixture was digested with proteinase K (100 μg/mL) at 37 °C for 30 min. The reaction was then diluted with an equal volume of buffer containing 30 mM sodium acetate, pH 5.0, 10 mM EDTA, and 0.5% $NaDodSO_4$, and the RNA was extracted three times with buffer saturated phenol–chloroform (1:1) as described previously (Schutz et al., 1973). RNA was then digested with iodoacetate-treated DNase (Worthington Inc.) and separated from low molecular weight material by chromatography on Sephadex G-50 (1.5 × 30 cm). The Hg-RNA was isolated on an SH-agarose column as described by Konkel & Ingram (1977).

The Hg-RNA which was eluted from SH-agarose column with β-mercaptoethanol was demercurated by treatment with 1.5 M β-mercaptoethanol for at least 3 h at room temperature. NaCl was added to 0.4 M and the RNA was precipitated with ethanol and collected by centrifugation. In some experiments as indicated, yeast RNA was added as a carrier prior to ethanol precipitation.

Preparation of α_{2u}-Globulin cDNA and Hybridization to RNA Synthesized in Vitro. α_{2u}-Globulin mRNA was partially purified by immunoadsorption of α_{2u}-globulin-synthesizing polysomes as described earlier (Kurtz & Feigelson, 1977). An α_{2u}-globulin-enriched cDNA was prepared using this mRNA as template for AMV reverse transcriptase using the reaction conditions described by Kacian & Myers (1976). Non-α_{2u}-globulin cDNA species were removed from this cDNA preparation by hybridization to female rat liver mRNA to a R_0t of 400, and the cDNA remaining unhybridized was collected using hydroxylapatite chromatography. This single-stranded cDNA, containing the α_{2u}-globulin cDNA, was hybridized to male liver mRNA to a R_0t of 10, and the unhybridizable cDNA species were digested using S_1 nuclease. cDNA prepared in this manner was found to be specific for α_{2u}-globulin mRNA sequences (Kurtz & Feigelson, 1977).

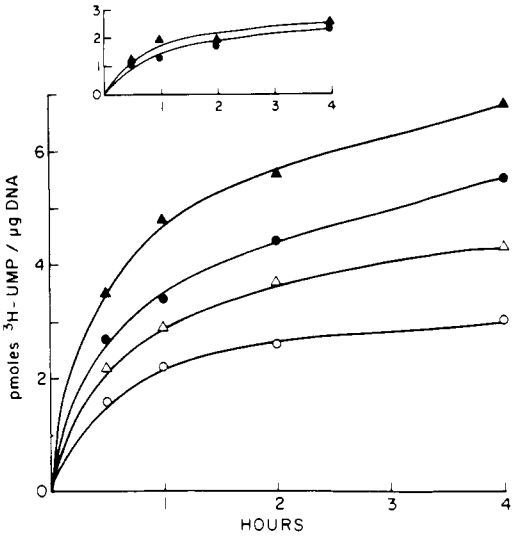

FIGURE 1: RNA synthesis in rat liver nuclei in vitro. RNA was synthesized in isolated rat liver nuclei with [^{3}H]UTP and CTP (▲ and △) or [^{3}H]UTP and Hg-CTP (● and ○), as substrates, in the presence (open symbols) or absence (closed symbols) of 2.5 μg/mL α-amanitin. Aliquots were removed at the indicated times and acid-precipitable radioactivity was determined and converted to picomoles of [^{3}H]UMP incorporated per microgram of DNA. Insert show incorporation of [^{3}H]UTP into RNA directed by α-amanitin sensitive RNA polymerase (polymerase II) in the presence of CTP (▲) and Hg-CTP (●), respectively.

Rat globin mRNA was prepared from reticulocytes of rats rendered anemic by daily injections of phenylhydrazine hydrochloride. Blood was collected and polysomes prepared as described by Kryostek et al. (1975). The polysomes were brought to 0.5% $NaDodSO_4$ and applied directly to oligo(dT)-cellulose equilibrated with a buffer containing 0.5% $NaDodSO_4$. The poly(A)-containing RNA was eluted from the column and sedimented on a 5–20% sucrose gradient as described by Merkel et al. (1975) and the poly(A) content of the gradient fractions was estimated by hybridization to [^{3}H]poly(U) (Sippel et al., 1974). The peak in the 9–10S region of the gradient was pooled and precipitated with ethanol. [^{3}H]cDNA was prepared from this mRNA as described above. This globin cDNA annealed to its template RNA with a $R_0t_{1/2}$ of approximately 8×10^{-4} and showed no hybridization with rat liver mRNA by a R_0t of 400.

Hybridization of these cDNAs to the in vitro synthesized RNAs was performed using cDNA excess hybridization as described by Hynes et al. (1977).

Results

In Vitro RNA Synthesis Using Hg-CTP. Male rat liver nuclei were prepared as described in Materials and Methods. As described previously (Ernest et al., 1976), incorporation of [^{3}H]UTP into RNA in this system continues for over 4 h of incubation at 25 °C (Figure 1). Approximately 30–35% of this RNA synthesis represents transcription by RNA polymerase II, since the addition of α-amanitin decreases overall RNA synthesis by this value (Figure 1). Polymerase II dependent transcriptive synthesis is essentially completed by 60 min of incubation (Figure 1). Addition of Hg-CTP to the nuclear system reduced the overall rate of [^{3}H]UTP incorporation by approximately 20% (Figure 1), while the polymerase II mediated polymerase activity remained essentially unchanged (Figure 1).

TABLE I: α_{2u}-Globulin Synthesis in Vitro.

incubation conditions	per cent of total RNA: α_{2u}-globulin mRNA, newly synthesized	α_{2u}-globulin mRNA, endogenous	globin mRNA, newly synthesized	globin mRNA, endogenous
male nuclei + HgCTP	0.005	0.002	undetectable	
male nuclei + HgCTP + α-amanitin or actinomycin D	undetectable	0.002	undetectable	
male nuclei + HgCTP + 10 μg globin mRNA	0.005	0.002	undetectable	0.02
female nuclei + HgCTP	undetectable		undetectable	

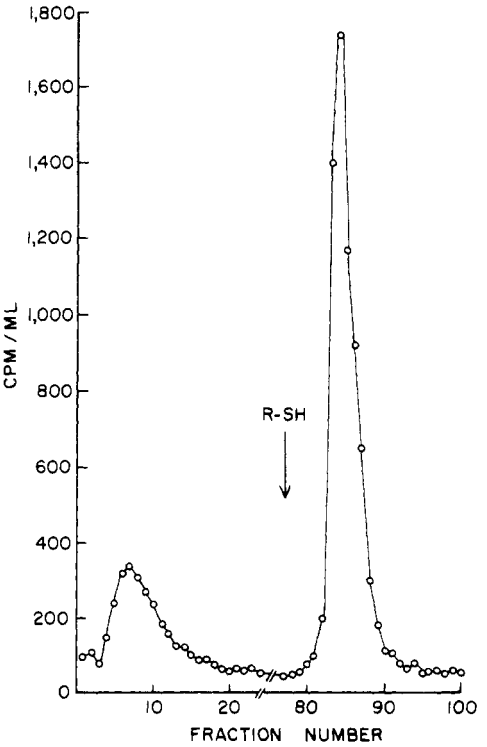

FIGURE 2: Sulfhydryl-agarose chromatography of Hg-RNA synthesized in vitro. Nuclei were transcribed in vitro with [^{3}H]UTP and Hg-CTP, and the RNA was extracted as described in Materials and Methods. The RNA was treated with DNase and separated from low molecular weight material by chromatography on Sephadex G-50. The RNA collected in the void volume was applied to a sulfhydryl-agarose column as described. Vertical arrow marks start of elution with 0.25 M β-mercaptoethanol. Two milliliter fractions were collected and the acid-precipitable radioactivity was determined.

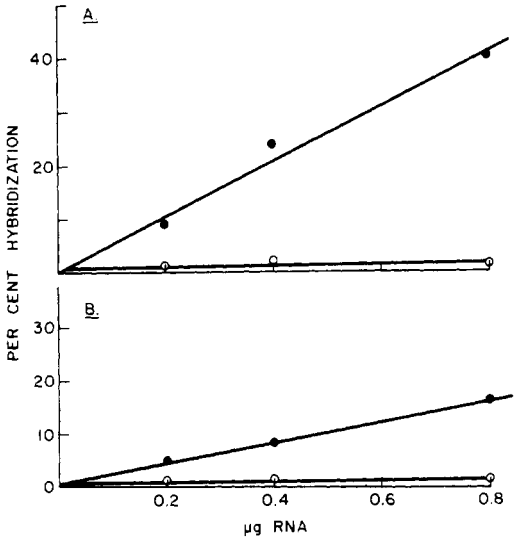

FIGURE 3: α_{2u}-Globulin mRNA synthesis in vitro in male rat liver nuclei. Male rat liver nuclei were transcribed in the presence of 0.5 mM Hg-CTP for 1 h at 25 °C. RNA was prepared and chromatographed on SH-agarose as described in Materials and Methods. The RNAs in both (A) the β-mercaptoethanol eluate and (B) the flowthrough were ethanol precipitated and aliquots were hybridized to 0.1 ng (~1500 cpm) of either α_{2u}-globulin cDNA (●) or rat globin cDNA (○). Hybridization was carried out under paraffin oil in 5-μL reaction mixtures containing 1 mM Tris (pH 7.9), 0.4 M NaCl, 2 mM EDTA and 0.1% $NaDodSO_4$ for 48 h. Hybridization was monitored using S_1 nuclease as described in Materials and Methods.

The mercurated RNA synthesized in vitro was separated from endogenous RNA by chromatography through sulfhydryl agarose as described above. Approximately 70% of the labeled RNA synthesized in the presence of Hg-CTP bound to the column and could subsequently be eluted with β-mercaptoethanol (Figure 2).

Transcription of the α_{2u}-Globulin Gene in Vitro. The level of mRNA α_{2u} sequences in the isolated newly synthesized mercurated RNA was titrated by cDNA excess hybridization to α_{2u}-globulin cDNA. It was found that male rat liver nuclei synthesized α_{2u}-globulin mRNA at a level which represents 0.005% of the total newly synthesized RNA (Figure 3 and Table I). This is a reasonable value since α_{2u}-globulin sequences represent approximately 0.002% of endogenous nuclear RNA obtained from the flow-through of the SH-agarose column. These nuclei synthesized no detectable globin mRNA, as measured by hybridization to globin cDNA (Figure 3 and Table I). Thus, transcription in these nuclei employing their endogenous RNA polymerase II remains tissue specific. If α-amanitin or actinomycin D is included in the reaction mixture, no newly synthesized mRNA α_{2u} sequences are detectable (Figure 4 and Table I), while the level of endogenous mRNA α_{2u} sequences remains unchanged (Figure 4 and Table I). This demonstrates that the newly synthesized mRNA α_{2u} sequences detected in the absence of these inhibitors is indeed the result of transcriptive synthesis of α_{2u}-globulin mRNA, and not due to contamination by endogenous mRNA α_{2u} sequences.

Exogenous Globin mRNA Sequences Are Not Copurified with Mercurated RNA. The absence of newly synthesized mRNA α_{2u} sequences in the presence of RNA synthesis inhibitors suggests that endogenous RNA sequences are not being copurified with the mercurated RNA. To explore this question further, nuclei were transcribed with Hg-CTP, and 10 μg of globin mRNA was added to the reaction mixture prior to RNA extraction and SH-agarose chromatography. No globin mRNA sequences were detectable in the β-mercaptoethanol eluate of the SH-agarose column (Figure 5 and Table I), while the flow-through contained all the globin mRNA which had been added (Figure 5 and Table I). This indicates that no chemical mercuration or aggregation of endogenous RNA is occurring during the RNA extraction and chromatography.

α_{2u}-Globulin Synthesis in Female Liver Nuclei. Female rats synthesize no detectable α_{2u}-globulin in vivo nor do their livers contain a measurable level of functional α_{2u}-globulin mRNA

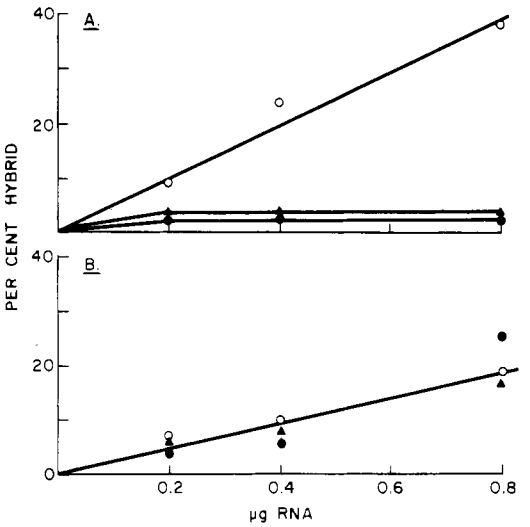

FIGURE 4: The effect of transcriptional inhibitors on the in vitro synthesis of α_{2u}-globulin mRNA in male rat liver nuclei. In vitro transcription was performed as described in Materials and Methods at 25 °C for 1 h in a 10-mL reaction mixture containing 0.5 mM Hg-CTP in the absence of inhibitors (○) or in the presence of actinomycin D (10 μg/mL) (▲), or α-amanitin (2.5 μg/mL) (●). RNAs were prepared and separated in SH-agarose column as described in Materials and Methods and hybridized to α_{2u}-globulin cDNA. (A) Hybridization of RNA in the β-mercaptoethanol eluate of the SH-agarose column. (B) Hybridization of RNA in the flow-through of the SH-agarose column.

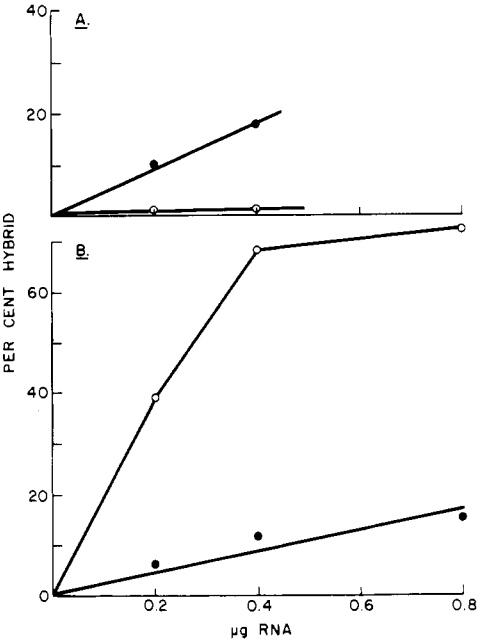

FIGURE 5: Exogenously added globin mRNA is not copurified with newly synthesized Hg-RNA. Nuclei were transcribed as described in Materials and Methods in the presence of Hg-CTP. Globin mRNA was added to the reaction mixture prior to RNA extraction and SH-agarose chromatography. The RNAs in both (A) the β-mercaptoethanol eluate and (B) flow-through were ethanol precipitated and hybridized to either α_{2u}-globulin cDNA (●) or globin cDNA (○).

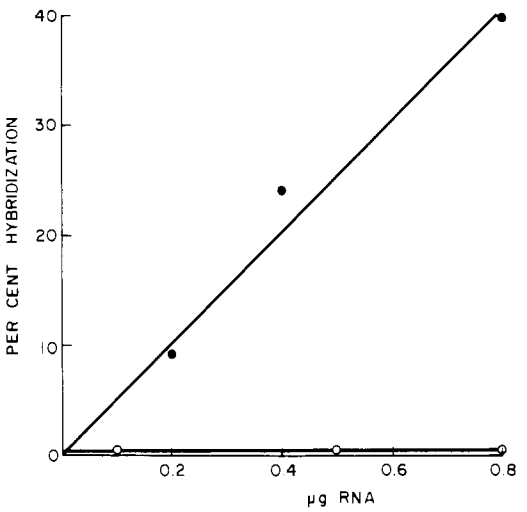

FIGURE 6: α_{2u}-Globulin RNA synthesis in vitro in male and female rat liver nuclei. Nuclei were prepared from both male and female rat liver and transcribed in the presence of Hg-CTP. Hg-RNAs were purified by SH-agarose chromatography, demercurated, and hybridized to α_{2u}-globulin cDNA as described. Hybridization of α_{2u}-globulin cDNA to RNA synthesized by male liver nuclei (●) and female liver nuclei (○).

(Sippel et al., 1975). Furthermore, titration with α_{2u}-globulin cDNA indicates that there is less than one copy of α_{2u}-globulin mRNA per cell in female rat liver (Kurtz & Feigelson, 1977). To determine if this is due to the lack of transcription of the α_{2u}-globulin gene in females, nuclei were prepared from female rat liver and transcribed in vitro. The newly synthesized RNA was collected and hybridized to the α_{2u}-globulin cDNA. No mRNA α_{2u} sequences were detectable in the newly synthesized RNA from female rat liver (Figure 6 and Table I). This indicates that, within the limits of our detection, the α_{2u}-globulin gene is not being transcribed in female liver nuclei.

Discussion

The study of the hormonal regulation of the level of specific cytoplasmic mRNAs would be greatly facilitated by a cell-free transcriptional system which would allow the measurements of the rates of transcription of specific genes. As described herein, we have prepared a nuclear transcriptional system, using Hg-CTP as substrate for endogenous polymerases, to measure the rate of synthesis of α_{2u}-globulin mRNA in male and female rat liver. It was found that, in male liver nuclei, α_{2u}-globulin mRNA sequences represent 0.005% of newly synthesized RNA. This is a reasonable value since α_{2u}-globulin mRNA represents approximately 0.002% of the steady-state nuclear RNA in male rat liver. This figure represents approximately 1/500 of the relative level of α_{2u}-globulin mRNA sequences in the cytoplasm (1%). A very similar ratio of cytoplasmic to nuclear RNA sequences has been reported for ovalbumin mRNA (Nguyen-Huu et al., 1978).

α-Amanitin completely blocks the synthesis of α_{2u}-globulin mRNA in male liver nuclei, indicating that the transcription of this gene is carried out by RNA polymerase II, as expected. No detectable synthesis of rat globin RNA sequences occurs in these nuclei, indicating that the transcription remains tissue specific.

That the mercurated α_{2u}-globulin mRNA sequences are indeed newly synthesized and do not represent contamination of endogenous sequences is shown by two sets of controls.

1. α-Amanitin and actinomycin D completely block the appearance of α_{2u}-globulin mRNA sequences in newly synthesized mercurated RNA, while the level of α_{2u} mRNA sequences in the endogenous RNA remains constant.

2. Exogenous rat globin mRNA added to the nuclei prior to RNA extraction does not copurify with the mercurated RNA, but was found in RNA which is unbound to SH-agarose.

Female rats synthesize no α_{2u} globulin in vivo and contain no detectable hepatic α_{2u}-globulin mRNA. Female rat liver nuclei are now shown to synthesize no detectable $\alpha_{2u\text{-globulin}}$ RNA sequences in vitro. Thus, within the limits of our detec-

tion, the α_{2u}-globulin gene is transcriptionally silent in female rat liver nuclei.

The availability of this nuclear transcriptional system will allow us to measure the rate of transcription of the α_{2u}-globulin gene in rats in various endocrine states. It may thus be possible to determine whether the multihormonal control of α_{2u}-globulin mRNA levels is in all instances the result of control at the level of transcription, or whether it involves modulation of RNA processing, transport, or stability.

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Immunoglobulin Genes in DNA Restriction Fragments†

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ABSTRACT: We have investigated the organization of immunoglobulin genes in mice. High molecular weight DNA from myelomas and Krebs ascites cells was cleaved with *Eco*RI restriction endonuclease and fractionated using preparative agarose gel electrophoresis. Each fraction was then hybridized to an immunoglobulin mRNA or a cDNA transcribed from the mRNA. In two series of experiments, one with a κ chain probe (MOPC 41 mRNA), the other with a λ chain probe (SAPC 178 mRNA), we analyzed a variety of myeloma DNAs and Krebs DNA. In contrast to previously reported findings (Tonegawa, S., et al. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41,* 877), we did not observe any unique restriction map pattern in the DNA from cells which express a given immunoglobulin gene. We also found that restriction fragments containing c region genes do not appear to transpose, while DNA sequences corresponding to other portions of the κ and λ mRNAs do in some cases.

Immunoglobulins are proteins which appear to be coded for by separate genes, genes for the variable (v) and constant (c)[1] regions. Evidence from genetic (Nisonoff et al., 1975) and nucleic acid hybridization experiments (Stavnezer et al., 1974; Leder et al., 1973; Faust et al., 1974) indicates that there exist one, or very few, c genes. Although the hybridization evidence may be an underestimate, as pointed out by Smith (1976), results with cloned immunoglobulin λ chain genes are compatible with the hypothesis that there may be only one c gene

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[1] Abbreviations used: cDNA, complementary DNA; c(v) region, constant (variable) region of an immunoglobulin polypeptide chain; L chain, immunoglobulin light chain.

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