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In vitro RNA synthesis and expression of vitellogenin gene in isolated chicken liver nuclei

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ABSTRACT

Optimal conditions for prolonged in vitro synthesis of RNA in isolated chicken liver nuclei have been described. It is shown by incorporation of γ^{32}P-GTP into RNA, analysis of the product on sucrose density gradient, and digestion with alkaline phosphatase and ribonuclease A that there is reinitiation of RNA synthesis. Polynucleotide kinase activity has been ruled out as an explanation for the incorporation of γ^{32}P-GTP. α-Amanitin inhibits RNA synthesis by about 50%. Nuclei prepared from estradiol-treated chicks have twice the RNA synthesis activity as the controls. RNA is synthesized in the presence of Hg-UTP and the mercurated product separated by affinity chromatography on sulfhydryl-Sepharose column under stringent conditions. Vitellogenin mRNA sequences are measured by hybridization with DNA complementary to vitellogenin mRNA. Estradiol treatment leads to a 10-fold increase in vitellogenin mRNA sequences.

INTRODUCTION

Vitellogenin is the precursor of egg yolk proteins which is synthesized in the liver of all egg-laying animals[1-3]. In chicken and Xenopus, vitellogenin has a molecular weight of about 200,000-240,000 daltons and is synthesized in large quantity in response to estradiol[2-3]. The stimulation is almost immediate, reaching maximum in 3-4 days and requires no new DNA synthesis[4,5]. Many studies have shown that induction of vitellogenin by estradiol is preceded by a large increase in total liver RNA[5,6], vitellogenin mRNA[7-11] and polysomes[12-15]. The exact mechanism for this increase is not understood. Current evidence suggests that an estradiol-receptor complex may activate specific genes[16]. Furthermore, the pleiotropic effect

of estradiol on overall RNA synthesis may also involve a direct activation of RNA polymerase I and II by the steroid-receptor complex[17,18]. Evidence for direct estradiol action on chicken liver chromatin are limited and at the present stage there is no compelling evidence that estradiol-receptor complex changes the template specificity of chromatin as it has been shown in the case of ovalbumin[19]. The main difficulties in employing chromatin in *in vitro* studies are the loss of loosely bound proteins during the isolation procedure and the random shearing of DNA. In addition, because of its very low endogenous polymerase activity, addition of exogenous homologous polymerases is often required. In order to circumvent these difficulties we have studied the optimal conditions for prolonged RNA synthesis in isolated chicken liver nuclei.

MATERIALS AND METHODS

Animals and hormone treatment

White Leghorn immature chicks weighing 80-100 g and 200-300 g were used for primary[5] and secondary stimulation[5], respectively. They were injected intramuscularly with 50 mg per kg body weight of 17β-estradiol dissolved in propylene glycol, livers were perfused *in situ* with 50 ml 0.9% NaCl and used immediately for nuclei preparation.

Preparation of nuclei

Nuclei were prepared at 4°C by homogenising the minced liver in 10 volumes of a 1.5 M sucrose - 3 mM $MgCl_2$ solution (pH not adjusted), using a glass teflon homogenizer at 500 rpm and with 4-6 up and down strokes. After filtration through 7 layers of sterile gauze, the homogenate was layered on top of 7 ml of 1.5 and 2.2 M sucrose containing 1 mM $MgCl_2$. Centrifugation was performed in a Beckman SW 27 rotor at 21,000 rpm and 4°C for 1 hour. The nuclear pellets were suspended in 50 mM Tris HCl buffer pH 8 containing 2.6% purified bovine serum albumin, 25% glycerol and 5 mM $Mg(CH_3COO)_2$ and kept in small aliquots frozen in liquid nitrogen. After one month of storage

no detectable loss of RNA synthesizing capacity was observed. Nuclei prepared from liver of estradiol-treated chicks did not pellet to the bottom of the centrifuge tube unless the concentration of the bottom layer of sucrose was reduced to 2.0 M.

In vitro RNA synthesis

Unless otherwise indicated in vitro RNA synthesis was performed by modifying the procedure described by Marzluff et al[20] and by Ernest et al[21]. The incubation mixture (325 µl) contained 50 mM Tris-HCl, pH 8, 90 mM KCl, 5 mM $Mg(CH_3COO)_2$, 0.05 mM EDTA, 2.5 mM dithiothreitol or 10 mM distilled 2-mercaptoethanol, 12.5% glycerol, 1.6 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.1 mM labeled UTP (final specific activity 1 Ci/mmole), 2.5 mg/ml purified bovine serum albumin and $20\text{-}80 \times 10^6$ freshly thawed nuclei/ml. When Hg-UTP was used in the incubation mixture (^{3}H)-UTP was replaced by (^{3}H)-CTP. The reaction mixture was incubated at 25°C and at regular intervals 10 µl aliquots were pipetted into 1 ml of a cold 5% TCA solution containing 50 mM $Na_4P_2O_7$. Samples were collected on Whatman GF/C filters, washed extensively with the 5% TCA solution containing 50 mM $Na_4P_2O_7$ and absolute ethanol. The dried filters were counted in 7 ml of toluene-based scintillation liquid.

RNA extraction and purification

Nuclear RNA was extracted from the nuclei by the procedure described by Penman[22]. The incubation was stopped by lysing the nuclei with an equal volume of 0.02 M Tris HCl, pH 7, buffer containing 1 M NaCl, 0.1 M $MgCl_2$, 1 mg/ml yeast RNA and 1 mg of iodoacetate-treated deoxyribonuclease 1. After 3 minutes of incubation at 37°C sodium dodecyl sulfate and EDTA were added to a final concentration of 0.5% and 0.1 M, respectively. RNA was extracted at 60°C by phenol and phenol-chloroform as described by Penman[22]. After precipitation with ethanol, RNA was further purified by two precipitations with cetyltrimethylammonium bromide[23].

Synthesis of mercurated nucleotides

Mercurated UTP was prepared according to Dale and Ward[24]. The mercurated UTP was identified by the shift of the peak of absorbance at 267 nm and by thin layer chromatography on polyethylenimin-cellulose[25].

Affinity chromatography of mercurated RNA

Sulfhydryl-Sepharose (Affi-Gel 401, BioRad) was packed into a 5 ml sterile plastic syringe in the presence of 0.25 M distilled 2-mercaptoethanol, 0.1% sodium dodecyl sulfate and 2 mM EDTA dissolved in a 10 mM Tris HCl, pH 7.4, buffer. The column was extensively washed with the same buffer but without 2-mercaptoethanol. The mercurated RNA was dissolved in 0.5 ml sterile distilled water, heated at 100°C for 90 sec in a sterile, silicone-treated glass tube, quickly cooled in ice, loaded onto the column and incubated in the column at room temperature for 30 minutes. The column was first washed with 15 ml (flow rate: 5 ml per hour) of 10 mM Tris HCl, pH 7.4, buffer containing 2 mM EDTA 0.1% sodium dodecyl sulfate, then with 15 ml of the same buffer containing 85% deionized formamide, and finally with 15 ml of buffer without formamide. Mercurated RNA was eluted with the same buffer containing 0.25 M distilled 2-mercaptoethanol. The fractions containing mercurated RNA were pooled, adjusted to 3 M 2-mercaptoethanol and left at room temperature overnight. Such treatment was sufficient to remove the Hg from the RNA[24,26]. The demercurated RNA was precipitated with ethanol after addition of NaCl (0.2 M final concentration) and carrier RNA (100 µg).

Isolation of vitellogenin mRNA and synthesis of complementary DNA

Vitellogenin mRNA was prepared from estradiol-treated chicken liver as previously described[27]. Complementary DNA to vitellogenin mRNA was synthesized in a mixture containing 0.1 M Tris HCl, pH 8.3, 0.01 M dithiothreitol, 0.01 M $MgCl_2$, 5 µg/ml oligo-dT, 50 µg/ml vitellogenin mRNA and 0.5 mM each

of dATP, dGTP, (^{3}H)-dTTP, (^{3}H)-dCTP. The reaction was done in 20 µl batches with 90 units of AMV-reverse transcriptase. After 30 minutes of incubation at 42°C the reaction mixture was chilled and 0.3 M NaOH and 60 µg of carrier DNA (Micrococcus luteus) were added. After overnight incubation at 37°C, the cDNA was separated from the free nucleotides by chromatography on Sephadex G-50 (1x35 cm) column equilibrated with 0.01 M Tris HCl, pH 7.4, buffer containing 0.005 M EDTA and 0.1 M NaCl. After ethanol precipitation and solubilization in H_2O, aliquots were analyzed by electrophoresis on 5% polyacrylamide gels containing 98% formamide[12]. Restriction fragments from ΘX 174 DNA digested with Hae III were used as standard for size determination. The cDNA was found to be heterogeneous in size with a distribution between 100 and 1,500 nucleotides. The specific activity of the cDNA was 5.9×10^7 dpm/µg.

RNA-cDNA hybridization

Samples of RNA ranging from 6.5 to 350 ng were hybridized in silicone-treated sterile glass capillaries with 70 pg of vitellogenin cDNA in 5 µl of 0.02 M Tris-HCl, pH 7, buffer containing 0.6 M NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate and 10 µg carrier yeast RNA. The capillaries were sealed, placed for 2 minutes in boiling water and incubated at 65°C for 63 hours.[10] Hybridization was stopped by chilling and hybrid formation tested by S1 nuclease digestion (2,000 units/ml) as described by Leong *et al*[28]. Each point on Fig. 6 represents average of duplicate determinations.

Analysis of RNA on sucrose density gradients

Samples of nuclear RNA were dissolved in H_2O, heated for 90 seconds at 95°C and, after quick cooling, loaded on a linear gradient of 5-20% sucrose containing 0.02 M LiCl, 0.005 M EDTA and 10 mM Tris HCl, pH 7.4[27]. Centrifugation was performed for 135 minutes at 60,000 rpm and 6°C in a Beckman SW 60 rotor. Alternatively, we used a 5-20% sucrose gradient containing 85% deionized formamide, 10 mM Tris HCl, pH 7.6, 1 mM EDTA and centrifuged it for 15 hours at 40,000 rpm in a SW 40 rotor, at 25°C.

Materials

2-Mercaptoethanol from Fluka AG was distilled under vacuum and kept in small aliquots at -20°C. Bovine serum albumin from Sigma (Fraction V) was freed of ribonucleases by chromatography on carboxymethyl- and DEAE-cellulose[29]. (^{3}H)-UTP (35 Ci/mmole), γ^{32}P-GTP (28 Ci/mmole), (^{3}H)-dTTP (17.3 Ci/mmole), (^{3}H)-dCTP (15 Ci/mmole) were purchased from New England Nuclear. (^{3}H)-CTP (21 Ci/mmole) was from the Radiochemical Center, Amersham. α-Amanitin was obtained from Boehringer. S1 Nuclease from *Neurospora crassa* was obtained from Seikagaku Kogyo Ltd, Tokyo, Japan. Alkaline phosphatase from *E. coli*, pancreatic ribonuclease A and deoxyribonuclease 1 (RNAse-free) were purchased from Worthington. The deoxyribonuclease 1 was treated with iodoacetate as described by Zimmermann and Sandeen[30]. Dr. J. Beard (Life Sciences Inc.) kindly provided the AMV-reverse transcriptase.

RESULTS

RNA synthesis in the isolated nuclei

To study *in vitro* transcription in isolated nuclei it is necessary that they retain their *in vivo* components. Thus, they were isolated in a hypertonic solution which has been shown to retain maximal RNA polymerase activity[31]. In addition, we omitted the widely used treatment with detergents to avoid alteration in chromatin appearance[32] and loss of outer nuclear membranes. As judged by light and electron microscopy the preparation showed no detectable contamination with cells or cellular debris and yielded about 500 μg DNA per gram of liver. These purified nuclei, under our conditions, support RNA synthesis for over 90 minutes (Fig. 1A). This prolonged synthesis resulted from an adjustment of the concentration of nuclei and of mono- and divalent cations. Using the conditions described by Marzluff *et al*[20] or by Ernest *et al*[21] we failed to maintain RNA synthesis for more than 30 minutes. As seen in Figure 2, employing their con-

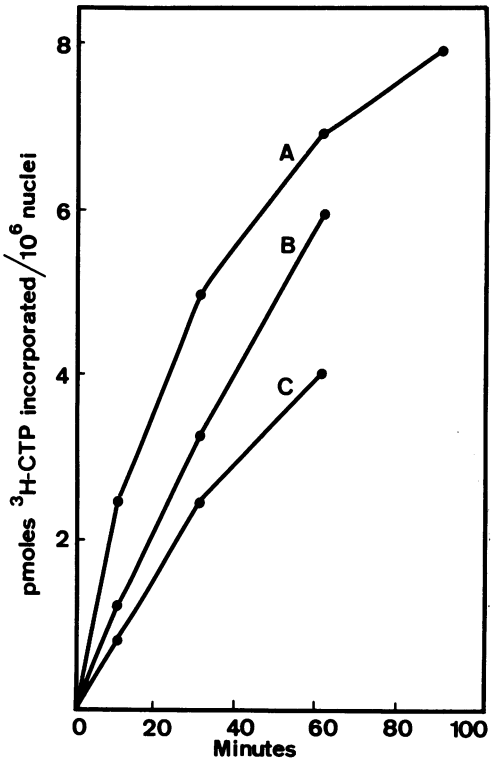

Fig. 1

In vitro RNA synthesis and effect of mercurated nucleotides. Nuclei ($20x10^{6}$/ml) were incubated in the absence of mercurated UTP (A) or with 33% of the UTP replaced by Hg-UTP (B). In C, UTP was totally replaced by Hg-UTP.

centrations of 150 mM KCl and 1 mM $MnCl_2$ RNA synthesis was inhibited. Inhibition by $MnCl_2$ (Fig. 2B) was unexpected, since $MnCl_2$ seems to be essential for the activity of eukaryotic RNA polymerase II[33]. However, Schwartz et al[33] have shown that concentrations of $MnCl_2$ higher than 2 mM were inhibitory to RNA polymerase II. As regards other constituants, Mg^{++} can be used over a broad optimal range (Fig. 2B), the four nucleoside triphosphates were required in excess and, under our experimental conditions, the Km for UTP was 33 µM. As reported by Ernest et al[21], bovine serum albumin was required for keeping the integrity of nuclei during incubation. However, we found that one

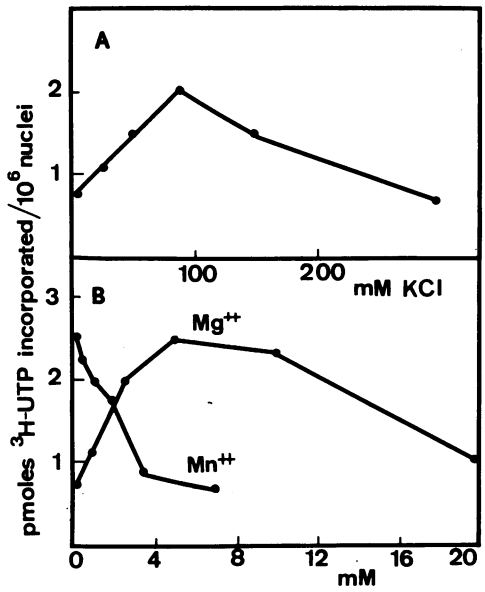

Fig. 2
Effect of KCl, $Mg(CH_3COO)_2$ and $MnCl_2$ on in vitro RNA synthesis in isolated nuclei. Panel A: Effect of KCl. RNA synthesis was performed in 100 µl aliquots as described in Methods, except that varying amounts of KCl were used. Samples were incubated for 10 minutes at 25°C and processed as described in Methods. Panel B: Effect of $Mg(CH_3COO)_2$ and $MnCl_2$. RNA synthesis was performed as described in Panel A, except that KCl concentration was adjusted to 90 mM.

fourth (2.5 mg/ml) of the concentration they used was sufficient for our system.

RNA synthesis in the presence of mercurated nucleotides

As already observed by others[34,35], a reduction in the rate of RNA synthesis was observed in the presence of Hg-UTP (Fig. 1B,C). The inhibitory effect was more pronounced as the ratio of Hg-UTP to UTP increased. In subsequent in vitro experiments we successfully employed a mixture of UTP containing 50% Hg-UTP. Under these conditions mercurated RNA could be fractionated on sulfhydryl-Sepharose columns. As seen in figure 3, approximately 20% of the radioactivity was found in the "run off" peak. This probably represented RNA chains with relatively small content of mercurated nucleotides at the 5' end.

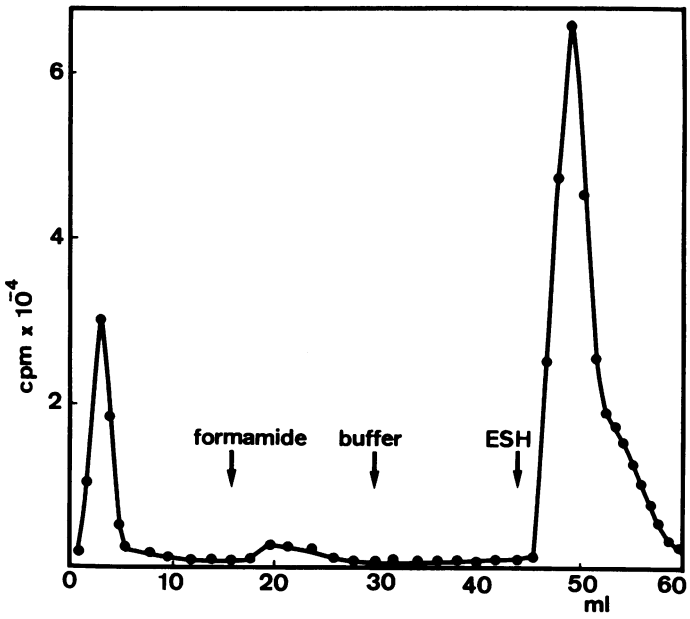

Fig. 3
Chromatography of mercurated RNA on sulfhydryl-Sepharose column (Affi-Gel 401, BioRad). RNA was synthesized as described in Fig. 1, except that the incubation mixture was scaled up 20 times, mercurated UTP was 50% of total UTP and α^{32}P-UTP was added to a final specific activity of 0.1 Ci/mmole. After 30 minutes of incubation RNA was exttracted and chromatographed on Affi-Gel 401 as described in Methods. Aliquots were directly measured for radioactivity.

Effect of estradiol treatment on in vitro RNA synthesis

Nuclei prepared from estradiol-treated chicks were twice as active in RNA synthesis as nuclei from control animals (Fig. 4). The results indicate that our conditions for in vitro RNA synthesis retain in part the in vivo properties conferred by estradiol treatment. RNA synthesis was inhibited by 50% in the presence of α-Amanitin in both control and treated chick nuclei (Fig. 4). Since low concentrations of α-Amanitin selectively inhibit mRNA synthesis, this indicates that mRNA synthesis was contributing to the synthesis of total RNA (Fig. 4). Moreover, there was no apparent selective increase of polymerase II activity due to estradiol treatment.

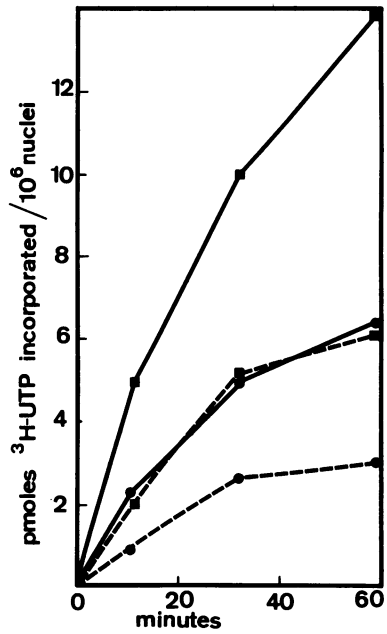

Fig. 4
Effect of in vivo estradiol and of α-Amanitin on the in vitro RNA synthesis in isolated nuclei. Nuclei from control and from chicken which had been treated for 18 hours with estradiol were incubated in the presence of mercurated UTP as described in Methods.
●——●, ■——■ : RNA synthesis in nuclei from control chicks and estradiol-treated chicks, respectively.
●----●, ■----■ : same as above, except that 2 μg/ml α-Amanitin were added to the incubation mixture.

Initiation of RNA synthesis

In order to determine whether our system was capable of initiating RNA synthesis, nuclei were incubated with γ^{32}P-GTP in the presence or absence of 1 mg/ml heparin, an inhibitor of RNA initiation[36]. Under these conditions we were able to detect a linear (up to 60 minutes) but low level of γ^{32}P-GTP incorporation into RNA which was abolished by 85% in the presence of heparin. Indeed, that RNA initiation was taking place was also supported by the analyses of the double-labeled RNA on sucrose density gradients (Fig. 5) and by digestion of the labeled RNA with alkaline phosphatase or pancreatic ribonuclease A (Table I).

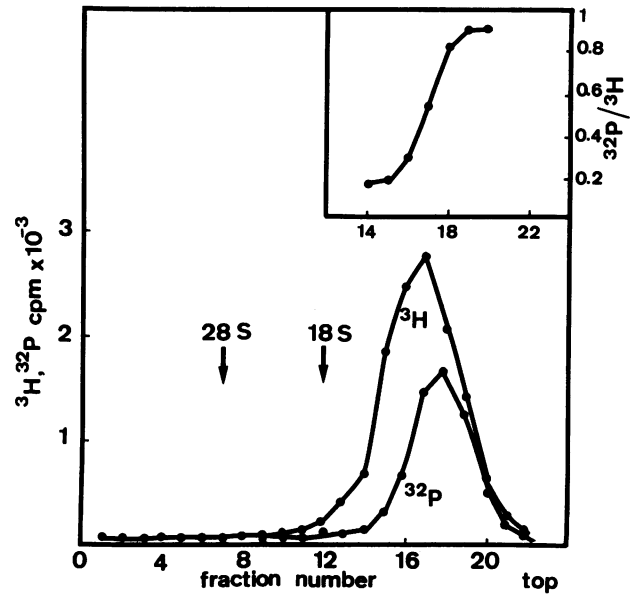

Fig. 5
Sucrose density gradient centrifugation of ^{3}H-CTP and. γ^{32}P-GTP double-labeled nuclear RNA. RNA was synthesized as in Fig. 1, except that GTP concentration was reduced to 0.05 mM and mercurated UTP represented 50% of total UTP. γ^{32}P-GTP and ^{3}H-CTP had a final specific activity of 5 and 1 Ci/mmole, respectively. After 30 minutes of incubation, RNA was extracted and analyzed on a 5-20% sucrose gradient as described in Methods. Each fraction (0.2 ml) was directly counted for radioactivity. The inset represents the ratio of $^{32}P/^{3}H$.

In figure 5 the sucrose gradient pattern showed a shift to the right (small molecular weight RNA) of ^{32}P-labeled product when compared with the (^{3}H)-RNA. This is consistent with the expectation that under conditions where there is reinitiation, the ratio of $^{32}P/^{3}H$ should increase as the size of RNA decreases. Furthermore, as shown in Table I, both ^{32}P and ^{3}H counts in the double-labeled RNA were sensitive to ribonuclease A, while only ^{32}P incorporation was sensitive to alkaline phosphatase treatment. This strongly argues that γ^{32}P-GTP was incorporated at the 5' end of the RNA[37]. To rule out the activity of polynucleotide kinase as an explanation for the incor-

Enzyme treatment	Incorporation of label into RNA	
	(^{3}H)-CTP cpm	(γ^{32}P)-GTP cpm
Control	1,075	307
Alkaline phosphatase	998	110
Ribonuclease A	112	20

Table I
Sensitivity of double-labeled RNA (γ^{32}P-GTP, ^{3}H-CTP) to alkaline phosphatase and ribonuclease A. *In vitro*-synthesized RNA was separated on Affi-Gel 401 as shown in Fig. 3. The RNA was dissolved in water and 10 µl aliquots were mixed with either 500 µl of 50 mM Tris HCl, pH 8.2, buffer containing 1 unit of alkaline phosphatase or 500 µl of 0.03 M sodium citrate, pH 7.5, buffer containing 5 µg ribonuclease A and 0.3 M NaCl. A control containing only 50 mM Tris HCl, pH 8.2, was run in parallel. The incubation was carried out at 37°C for 30 minutes. Incubation was stopped by adding 5% TCA (final concentration) together with 50 µg of carrier yeast RNA. Precipitates were collected and processed as described in Methods. Each value represents the average of two measurements.

poration of γ^{32}P-GTP into RNA[38] we separated the *in vitro* γ^{32}P-GTP-labeled RNA from non-labeled *in vivo* synthesized RNA by chromatography on sulfhydryl-Sepharose column (as in Fig. 3) and determined the specific radioactivity. The specific radioactivity of the *in vitro* synthesized RNA was about 2,000 times higher than that of the *in vivo* synthesized RNA. If the incor-

Nuclear RNA synthesized	^{32}P-GTP cpm	RNA µg	specific activity cpm/µg
in vivo	517	50	10.3
in vitro	2,931	0.15	19,520

Table II
Distribution of γ^{32}P-GTP label between *in vivo* and *in vitro* synthesized nuclear RNA. Nuclei were incubated in the presence of mercurated UTP for 10 minutes, the RNA was then extracted in the absence of carrier RNA and chromatographed on Affi-Gel as described in Methods. RNA in the "run off" peak represents the RNA which had been synthesized *in vivo* while the RNA eluting with 2-mercaptoethanol was synthesized *in vitro*. Each value represents the average of two measurements.

poration of γ^{32}P-GTP into RNA was solely due to polynucleotide kinase then the specific radioactivity of the in vivo and in vitro synthesized RNA should be equal (Table II).

Detection of vitellogenin gene product among nuclear RNA

Having shown that there is an increase in RNA synthesis in nuclei derived from estradiol-treated chicks we next wanted to determine if there was also a selective increase in vitellogenin mRNA synthesis. As seen in figure 6, the content of vitellogenin mRNA sequences synthesized in vitro by nuclei from estrogen-treated chicks was 5 times as high as in the control nuclei. Similarly, the RNA from the "flow through" of the Affi-Gel 401 (not shown), which represented the majority of the in

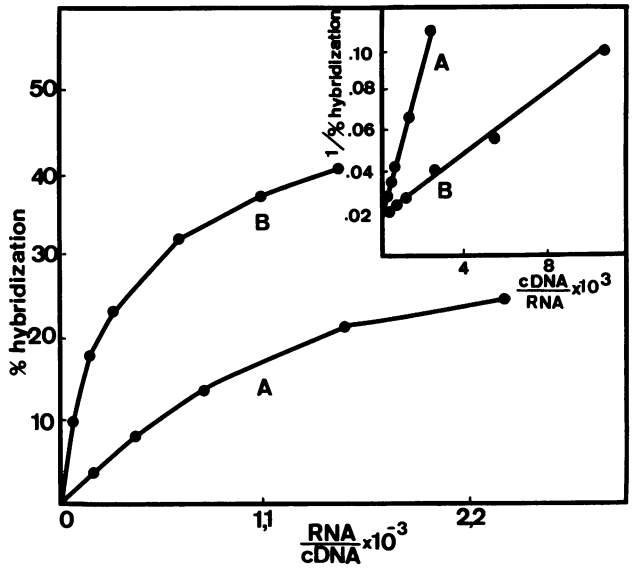

Fig. 6
Hybridization of newly synthesized RNA with vitellogenin cDNA. Newly, in vitro synthesized RNA was separated on Affi-Gel and hybridized, in 5 µl mixtures, as described in Methods. A and B are the S1-resistant hybrids of the cDNA with the newly synthesized RNA from control and from estradiol-treated chick nuclei, respectively. The insert represents the double-reciprocal plot of the same results.

vivo synthesized RNA, also contained a similar amount of vitellogenin mRNA sequences as compared with controls. From a double-reciprocal plot the maximal hybridization and sequence enrichment can be stimated. The results show that about 48% maximal hybridization and vitellogenin mRNA sequences, expressed in weight ratio of cDNA/RNA at half maximal hybridization, are 0.5×10^{-3} and 2.7×10^{-3} for control and estradiol-treated chicken liver, respectively.

DISCUSSION

The experiments described here were designed in order to obtain conditions which should make nuclei isolated from chicken liver suitable for studying in vitro expression of the vitellogenin gene. Prolonged RNA synthesis is necessary in this case, since vitellogenin mRNA is over 7,000 nucleotides long. If we assume a similar rate of vitellogenin mRNA synthesis as that of RNA of HeLa cells nuclei, which was estimated to be 6-7 nucleotides per second[39], it will require a minimum of 50-20 minutes of linear synthesis to complete one chain of vitellogenin mRNA. This estimate does not take into consideration the fact that a larger precursor of vitellogenin mRNA may exist. The synthesis of vitellogenin mRNA is most probably catalyzed by RNA polymerase II which is known to be responsible for mRNA production. The results that α-Amanitin, which specifically inhibits RNA polymerase II[40,41], decreased RNA synthesis by about 50%, indicates active participation of this enzyme. Therefore, the system should be suitable for the study of in vitro vitellogenin mRNA synthesis. In addition, we presented evidence that under our incubation conditions RNA reinitiation is taking place. Thus, we should also be able to use this system to study the control of initiation of RNA synthesis. In this regard it has been shown for chick oviduct chromatin that an increase in RNA initiation was responsible for the large increase in RNA synthesis triggered by estradiol[42,43]. In our in vitro system, twice as much RNA is synthesized in nuclei prepared from estradiol-treated chicks as in the control. This difference is probably real and unlikely due to an increase in ribonuclease activi-

ty in the control. Using either 3'end labeled ^{3}H-tRNA or uniformely labeled total polysomal RNA as a substrate, we could not detect any significant difference in ribonuclease activity between the two preparations of nuclei (data not shown). Similarly, using the same system Weckler and Gschwendt[44] found no difference in the rate of RNA degradation in crude extract prepared from nuclei from control and estradiol-treated chicks.

In our system the relatively small size of the in vitro synthesized RNA may be ascribed to the Hg-UTP. It has been suggested that high concentrations of Hg-UTP give rise to premature termination of RNA synthesis[45]. The quantitation of vitellogenin mRNA sequences among de novo synthesized RNA in isolated nuclei from estradiol-treated chicks shows a 5-fold enrichment in vitellogenin sequences as compared with the controls. Taking into consideration that total synthesis of estradiol-treated chicks is twice that of the controls (Fig. 4), this corresponds to an overall 10-fold increase in vitellogenin mRNA sequences. Therefore, it can be concluded that the isolated nuclei incubated under our conditions selectively express the vitellogenin gene. Knowing that 18 hours after secondary stimulation there is about a 1,000-fold increase in vitellogenin mRNA sequences[10], it is difficult to explain why, in isolated nuclei, there is only a 10-fold increase in vitellogenin mRNA sequences over the control. A similar phenomenon has been observed in the case of the ovalbumin gene transcribed from chicken oviduct chromatin[43,46]. Several possibilities could account for this observation: First, isolated nuclei may suffer a loss of regulatory factors which confer selective synthesis of vitellogenin mRNA. Second, the incubation conditions optimizing RNA synthesis may not be optimal for vitellogenin gene transcription. For example, Yamamoto and Seifart[47] have demonstrated that conditions for maximal total RNA synthesis were different from those for 5S RNA synthesis. Third, the cDNA used could contain a complementary sequence to a "constitutive" RNA, arising, for instance, from a gross contamination by such an RNA during the mRNA preparation. As a consequence, the control chick will exhibit excessively high

levels of "vitellogenin mRNA" sequences making the increment triggered by estradiol abnormally low. This possibility is unlikely, since we employed for cDNA synthesis an RNA preparation which contained 90-95% vitellogenin mRNA (as judged by the translation of mRNA in a cell-free system). Fourth, our cDNA represents about 100-1,500 nucleotides from the 3'end, while full length vitellogenin mRNA is 7,000 nucleotides long. Therefore, all vitellogenin mRNA sequences which are prematurely terminated will not be detected by our probe, leading to an underestimation of our mRNA concentration. In addition, very short RNA chains as well as short cDNA sequences may give unstable hybrids which would not be detected under our stringent conditions. To study this possibility it would require the synthesis of a full length cDNA, which is at the present not available. Finally, the vitellogenin gene in chick liver may be "leaky" or the mechanism giving rise to the very large increase in vitellogenin mRNA sequences in response to estradiol treatment may operate at several levels including processing, transport and stabilisation of the mRNA. At least in one case there is evidence that estradiol affects the turnover of ovalbumin mRNA[48].

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