Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 43–47, January 1986
Biochemistry

In vitro secondary activation (memory effect) of avian vitellogenin II gene in isolated liver nuclei

(transcription/estradiol receptor/polyamines/phosphorylation)

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Communicated by Diter von Wettstein, August 26, 1985

ABSTRACT **The vitellogenin II gene is specifically reactivated *in vitro* (secondary stimulation, memory effect) in purified liver nuclei that had ceased to express the gene *in vivo* a month after the roosters had received a single injection of estradiol (primary stimulation). The *in vitro* reactivation depends on the addition to the nuclei of nuclear and cytoplasmic extracts from estradiol-stimulated livers, polyamines (0.1–1.0 mM), and calmodulin (0.1 mM). Under identical incubation conditions the vitellogenin gene could not be reactivated in oviduct, embryonic, and immature chicken liver nuclei. Two other genes, those for ovalbumin and lysozyme, which are regulated by estradiol in the oviduct, could not be activated in the liver nuclei. The correct initiation of vitellogenin gene transcription in the liver nuclei was tested by primer extension studies. Addition of the antiestrogen tamoxifen (0.1 μM) to the system decreased vitellogenin mRNA synthesis by about 45% without affecting total RNA synthesis. Addition of quercetin (0.1 mM) and *trans*-flupenthixol (0.2 mM), inhibitors of nuclear protein kinase II and calmodulin-dependent kinase, respectively, inhibited the synthesis of vitellogenin mRNA by about 55% without affecting total RNA synthesis. The inhibitory effects of the antiestrogen and the kinase inhibitors were not additive, suggesting that both classes of inhibitor act on the same target or related targets. Depleting the estradiol receptors from the cell and nuclear extracts by means of estradiol–receptor antibodies covalently bound to Matrex beads reduced the stimulation of the vitellogenin gene by 40%. We conclude that in addition to the estradiol receptor and phosphorylation of nuclear protein(s) there are additional factors responsible for the *in vitro* secondary activation of the avian vitellogenin II gene.**

In the livers of immature chickens and *Xenopus* exposed to estradiol for the first time (primary stimulation) there is a lag period preceding the onset of vitellogenin synthesis (1–7). About 10 days after the primary stimulation, the amount of vitellogenin mRNA in the chicken liver cells returns to background level. If a second dose of estradiol is administered at this time (secondary stimulation), the vitellogenin mRNA is synthesized at the maximal rate without any time lag (3–5, 7). During the primary stimulation of the chicken liver by estradiol, the vitellogenin II gene is marked at the chromatin level by two nuclease-hypersensitive sites located at the 5′ end, which persist even after estradiol withdrawal (8, 9). Furthermore, during the primary stimulation there is hypomethylation of a *Hpa* II site (8, 10–13) where the estradiol–receptor complex binds (14). Further estradiol-dependent hypomethylation sites have been recently detected on the upstream region of the gene by means of the genomic sequencing technique (unpublished data). The persistence of the hypomethylation sites and the two DNAse I-hypersensitive sites situated between the estradiol receptor binding site and the promoter may represent important features of the committed state of vitellogenin II gene and may explain the rapidity of the secondary stimulation of vitellogenin gene *in vivo*. Furthermore, the stimulation of the gene by estradiol does not require protein synthesis *de novo* (7). This means that all factors necessary for the turning-on of the gene are already present in the cell and that estradiol probably triggers translocation, covalent modification, or both of specific *trans*-acting factors. In the present work we show that it is possible to reactivate the vitellogenin II gene *in vitro*. We have also identified some of the important parameters in this process.

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MATERIALS AND METHODS

***In Vitro* Transcription System.** Post-primary estradiol-stimulated nuclei from adult roosters were prepared 30–35 days after adult animals had received a single injection of estradiol (40 mg/kg of body weight). At this time no more vitellogenin mRNA was synthesized in the liver nuclei. The preparation and storage of purified liver nuclei were as described by Panyim *et al.* (15). The basic transcription mixture (300 μl) contained 0.1 M Hepes at pH 8, 90 mM KCl, 5 mM $Mg(CH_3COO)_2$, 2 mM dithiothreitol, 12.5% (vol/vol) glycerol, 2 mM each of ATP, UTP, CTP, and GTP, recrystallized bovine serum albumin at 2.5 mg/ml, 30–150 units of human placenta ribonuclease inhibitor (RNasin), and 40–80 $\times 10^6$ freshly thawed nuclei per ml of incubation mixture. Assay mixtures, in duplicate, were incubated at 23°C and the corresponding controls were incubated in ice with α-amanitin at 2 μg/ml. Additional controls were incubated at 23°C in the presence of heparin sulfate at 1 mg/ml. The control values were subtracted from the corresponding experimental values. Cytoplasmic and nuclear extracts were obtained by homogenizing tissues with a glass–Teflon homogenizer in 2 vol of 25% (vol/vol) glycerol, 2.6% bovine serum albumin, 5 mM $Mg(CH_3COO)_2$, 50 mM Tris·HCl at pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol, and the homogenates were centrifuged for 5 hr at 200,000 × *g* at 0°C. Nuclear extract was obtained by lysing highly purified nuclei in 0.4–0.5 M KCl containing 50 mM Tris·HCl at pH 8.3, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol, followed by centrifugation as indicated above. Cytoplasmic and nuclear extracts were stored in liquid nitrogen.

Total RNA synthesis was measured by the incorporation of [^{3}H]UTP into material precipitable by 5% trichloroacetic acid.

Processing and Analysis of the *in Vitro* Transcription Product. All samples were treated and analyzed for specific mRNA sequences as outlined by Jost *et al.* (16). As probe for vitellogenin mRNA we took the fragments A and B of pVT598 (14) covering 2 kilobases of the vitellogenin II gene. Ovalbumin and lysozyme mRNA sequences were tested with

cloned cDNA probes pHha OV (17) and pls 1 (18), respectively. Primer extension studies were carried out as described by Geiser *et al.* (11). The single-stranded DNA primer sequence 5'-AGCTTACCTACAAGGGTGAG-3', corresponding to the nucleotide positions 41 to 60 of the first exon of the vitellogenin II gene, was synthesized by the phosphoramidite approach (19, 20), using an Applied Biosystem DNA synthesizer. It was purified by ion-exchange HPLC using a Partisil SAX (Whatman) column eluted with a linear gradient of 0.001–0.3 M potassium phosphate buffer (pH 6.3) in 60% (vol/vol) formamide. Desalting was carried out on a Bio-Gel P-2 (Bio-Rad) column, eluted with a mixture of ethanol and water (2:8, vol/vol).

Immunochemical Techniques. Monoclonal antibodies directed against calf uterus estradiol receptor, linked to Matrex-102 beads (21, 22), were used to deplete the cell extracts (nuclear and cytoplasmic) of estradiol receptor. The antibodies from the clone JS-34/32 coupled to Matrex-102 beads (Amicon) cross-reacted with chicken estradiol receptor, and 10 μl of a suspension of these beads was allowed to react with 7 fmol of chicken oviduct or liver estradiol–receptor complex for 6 hr at 0°C or 4 hr at 4°C. Parallel controls were incubated with mouse IgG bound to Matrex beads (Matrex-102-mouse IgG). Estradiol receptor concentration in the cell extracts was determined according to Best-Belpomme *et al.* (23).

Inhibitors of Protein Kinases. A stock solution of 60 mM quercetin, an inhibitor of casein kinase II (24), was prepared in dimethyl sulfoxide and stored in aliquots at −20°C. A stock of 10 mM *trans*-flupenthixol, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase (25), was prepared in water and kept in aliquots at −20°C.

Materials. The tetra(triethylammonium) salt of 3'-deoxyadenosine 5'-[α-^{32}P]triphosphate (3000 Ci/mmol; 1 Ci = 3.7 GBq), the ammonium salt of [5,6-^{3}H]uridine 5'-triphosphate (40 Ci/mmol), and β-[2,4,6,7,10,17-^{3}H]estradiol (130 Ci/mmol) were purchased from Amersham. Ribonuclease-free DNAse I was from Worthington and human placenta ribonuclease inhibitor was from Genofit (Geneva). Tamoxifen and nafoxidine were from Sigma, calmodulin from bovine brain was from Fluka, and quercetin was purchased from Serva (Heidelberg). *trans*-Flupenthixol was from I. Novak-Hofer. All restriction enzymes and reverse transcriptase were from Boehringer Mannheim.

RESULTS

Factors Required for *in Vitro* Stimulation of the Vitellogenin II Gene. It has already been shown that one of the first effects of estradiol on the chicken liver is to increase the level of polyamines (26). More recently, Ca^{2+}/calmodulin has been shown to stimulate the phosphorylation of a tyrosine residue of the receptor protein (27).

Fig. 1 *Upper Left* shows that, under our initial assay conditions, nuclei isolated from roosters that had been treated with estradiol 35 days earlier do not synthesize vitellogenin mRNA (bar 1). However, when the same nuclei were incubated with nuclear extract from estradiol-treated chicken livers, a substantial increase in vitellogenin mRNA sequences was observed (bar 2). Similarly, addition of 1 mM spermine (bar 3) and 0.1 mM Ca^{2+}/calmodulin treatment (bar 4) resulted in an increase in vitellogenin mRNA synthesis. Maximal activation of the gene by spermine was observed within the concentration range of 0.1–1 mM, and higher concentrations of polyamines (up to 5 mM) were inhibitory. In addition, at the same concentrations, spermidine was only half as active as spermine (data not shown). Bar 5 shows the additive effect of nuclear extracts, spermine, and calmodulin. Under the same transcription conditions, however, nuclei prepared from immature chickens or chicken oviduct did not synthesize vitellogenin mRNA. This implies that the induction is tissue specific and occurs only in post-primary-stimulated rooster liver nuclei.

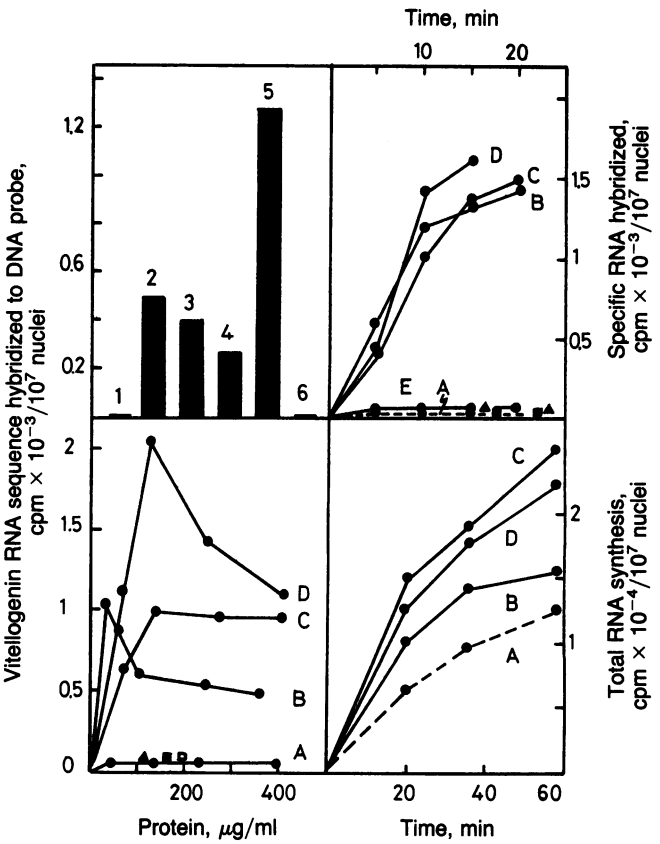

FIG. 1. Factors influencing *in vitro* secondary stimulation of the avian vitellogenin II gene. (*Upper Left*) Bar 1, post-primary-stimulated rooster liver nuclei were incubated under the basic transcription conditions. The same nuclei were also incubated under the basic transcription conditions supplemented with liver nuclear extract (50 μg/ml) from chicks treated for 48 hr with estradiol (bar 2), 1 mM spermine (bar 3), or 0.1 mM calmodulin (bar 4). The effect of combined treatments for bars 2, 3, and 4 is shown by bar 5. Bar 6 represents the same conditions as bar 5, except that the nuclei were from the oviducts of egg-laying hens, the livers of chicken embryos, or the livers of chicks. (*Lower Left*) Protein concentration dependence of nuclear and cytoplasmic extracts on *in vitro* secondary stimulation of the vitellogenin II gene. Curve A, cytoplasmic extract from immature chicken liver (●), oviduct (■), erythrocytes (□), and brain (▲). Curve B, nuclear extract from estradiol-treated chicken liver; curve C, nuclear extract from oviduct; curve D, cytoplasmic extract from liver of estradiol-treated chicks. (*Upper Right*) Time dependence of *in vitro* secondary stimulation of the vitellogenin II gene. Curve A, post-primary liver nuclei incubated under the basic transcription conditions. Curve B, same as A but with addition of 1 mM spermine, Ca^{2+}/calmodulin (0.1 mM), and cytoplasmic liver extract (50 μg/ml) from estradiol-treated (48 hr) chicks (complete system). Curve C, same as B except with extract (50 μg/ml) prepared from liver nuclei from estradiol-treated chicks. Curve D, same as B, except with liver cytoplasmic and nuclear extracts (50 μg/ml) from estradiol-treated chicks. Curve E, same as D but in the presence of heparin sulfate at 1 mg/ml. The following mRNA sequences were tested in the complete system: ●, vitellogenin; ▲, ovalbumin; and ■, lysozyme. (*Lower Right*) Effect of cytoplasmic and nuclear extracts of estradiol-treated chicks on total RNA synthesis in post-primary estradiol-treated nuclei. The conditions of incubation correspond to those described for *Upper Right*.

Fig. 1 *Lower Left* shows the effect of protein concentration in the nuclear and cytoplasmic extracts on the *in vitro* secondary stimulation of the vitellogenin gene. Among the nuclear preparations, only liver and oviduct extracts containing high concentrations of estrogen receptor gave rise to

a stimulation of the vitellogenin gene transcription, whereas for the cytoplasmic extracts only the preparation from estradiol-treated chicken showed a significant response. Other cytoplasmic preparations from embryonic liver and chick liver, erythrocytes, spleen, and brain were without effect. The possibility that the nuclear and cytoplasmic extracts of estradiol-treated chicken liver were contaminated with vitellogenin mRNA sequences has been ruled out by molecular hybridization.

Fig. 1 *Right* shows the results of a comparison between the time dependence of the *in vitro* secondary activation of the vitellogenin gene (*Upper Right*) and total RNA synthesis (*Lower Right*) in the presence of the complete transcription system supplemented with cytoplasmic or nuclear extracts or both from estradiol-treated chicken liver. Post-primary-activated nuclei incubated under the basic transcription conditions (curves A) did not show any synthesis of vitellogenin mRNA while total RNA synthesis proceeded for over 1 hr. In the complete system, however, we observed a *de novo* significant stimulation of vitellogenin mRNA synthesis while total RNA synthesis showed maximal 2-fold increase. The effects of nuclear and cytoplasmic extracts on estradiol-treated livers were not additive and the synthesis of vitellogenin mRNA proceeded for only 20 min, whereas total RNA synthesis lasted over 1 hr (Fig. 1 *Right*).

Specificity of Activation of the Vitellogenin Gene. Fig. 1 *Upper Left* (bar 6) shows that when hen oviduct nuclei, embryonic liver, or immature liver nuclei were incubated in the complete transcription system, no vitellogenin mRNA synthesis was observed. The secondary stimulation is thus probably tissue specific and occurs only in post-primary-stimulated liver nuclei. Moreover, among the cytoplasmic extracts tested, only the liver extracts from estradiol-treated chicks showed a stimulation of vitellogenin gene expression, while cytoplasmic extracts prepared from embryonic liver or chick liver, brain, spleen, or erythrocytes were without effect (Fig. 1 *Lower Left*, curve A). Two other genes that are regulated by estrogen in the oviduct, those for ovalbumin and lysozyme, were tested for their expression in the post-primary-stimulated liver nuclei. The results show (Fig. 1 *Upper Right*, curve A) that under our experimental conditions ovalbumin and lysozyme genes were not expressed in the liver nuclei incubated in the complete system.

Transcription-Initiation During *in Vitro* Secondary Stimulation of the Vitellogenin Gene. It has been shown that heparin can effectively block RNA transcription initiation *in vitro* (28, 29). The secondary stimulation of the vitellogenin gene is indeed very sensitive to heparin sulfate at 1 mg/ml (Fig. 1 *Upper Right*, curve E), suggesting that initiation of the transcription of the gene may take place. The *in vitro* initiation starting point was determined by primer extension studies. Total RNA was prepared from the incubated nuclei by the guanidine HCl-procedure, and primer extension with a synthetic primer was carried out as described in *Materials and Methods*. Fig. 2 shows the results of two independent experiments. Lanes 1 and 3 show the primer extension product obtained from RNA isolated from incubated nuclei of post-primary-stimulated chicks and immature chick liver nuclei, respectively. In neither case can apparent vitellogenin mRNA sequences be seen. However, when the post-primed nuclei were incubated in the complete transcription system as outlined for Fig. 1 (*Upper Left*, bar 5), there was substantial synthesis of vitellogenin mRNA with correct initiation of transcription. We furthermore observed a large number of smaller bands corresponding to degradation products, aborted primer extension products, or both.

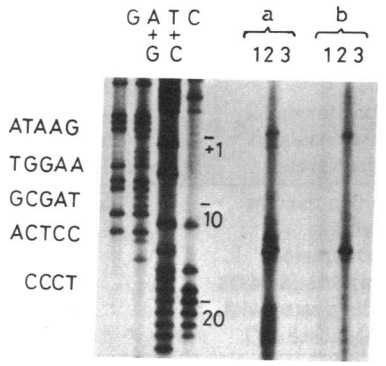

FIG. 2. Primer extension study of the *in vitro* transcription product of the vitellogenin II gene. Primer extension was carried out as previously described (11) with total RNA isolated from incubated liver nuclei prepared from post-primary-stimulated nuclei (lanes 1), *in vitro* secondary-stimulated post-primary nuclei (lanes 2), and immature chick liver nuclei (lanes 3); two independent experiments are represented by a and b. Primer extension product was separated on an 8% polyacrylamide sequencing gel in parallel with the corresponding DNA sequence (left half of gel) derived from the *Hin*dIII–*Eco*RI DNA fragment covering the first exon of the vitellogenin gene.

Effect of Antiestrogens and Protein Kinase Inhibitors on Secondary Stimulation of the Vitellogenin Gene. The aim of the

Table 1. Effect of antiestrogen and protein kinase inhibitors on the *in vitro* secondary stimulation of the vitellogenin II gene

Incubation conditions for post-primary-stimulated rooster liver nuclei	Vitellogenin mRNA sequences hybridized, cpm/1.5 × 10^7 nuclei	Inhibition, %	Incorporation of [^{3}H]UTP into total RNA, cpm/1.5 × 10^7 nuclei
Basic transcription mixture	Below detection	—	26,166
Complete system	1800	0	32,898
+ tamoxifen (0.1 μM)	990	45	32,880
+ *trans*-flupenthixol (0.1 mM)	1440	20	33,000
+ quercetin (0.2 mM)	1260	30	32,800
+ *trans*-flupenthixol + quercetin	810	55	33,090
+ *trans*-flupenthixol + quercetin + tamoxifen	815	55	32,940

Nuclei were prepared from the liver of roosters 30 days after they had received a single dose of 40 mg of estradiol per kg of body weight (post-primary nuclei). At this time the liver did not synthesize any vitellogenin mRNA. The basic transcription mixture was as described in *Materials and Methods*. The complete transcription system was the basic transcription mixture supplemented with 1 mM spermine, 0.1 mM Ca^{2+}/calmodulin, 1 μM $CaCl_2$, 10 nM estradiol, and nuclear and cytoplasmic liver extracts from estradiol-treated chicks at 50 μg of protein per ml. Incubation was carried out in duplicate for 30 min at 23°C and samples were processed. Where indicated estradiol was replaced by the antiestrogen (tamoxifen) or protein kinase inhibitors (quercetin or *trans*-flupenthixol).

Table 2. Effect of treatment of cell extracts with immobilized monoclonal antibodies against estradiol receptor on the *in vitro* secondary stimulation of the vitellogenin II gene

Incubation conditions for post-primary-stimulated rooster liver nuclei	Vitellogenin mRNA sequences hybridized, cpm/1.5 × 10^7 nuclei	Inhibition, %	Estradiol receptor, fmol			
			In 1.5 × 10^7 nuclei	In added cell extracts	Total	% of complete system
Basic transcription mixture	Below detection	—	14	—	14	15
Complete system	1404	0	14	77	91	100
+ cell extracts treated with Matrex-102-JS-34/32	879	40	14	2	16	17
+ cell extracts treated with Matrex-102-mouse IgG	1349	4	14	70	84	92

Cell extracts were nuclear and cytoplasmic extracts from the liver of estradiol-treated chicks. Aliquots were treated with the monoclonal antibody JS-34/32 in parallel with control mouse IgG, each immobilized on Matrex-102. Incubation (in duplicate) was for 30 min at 23°C.

following experiments was to use antiestrogens and protein kinase inhibitors to study the roles of the estrogen–receptor complex and protein kinases on the *in vitro* secondary stimulation of the vitellogenin gene. Nafoxidine and tamoxifen were used as specific antiestrogens (30–32) and the Ca^{2+}/calmodulin-dependent kinase and nuclear kinase II were blocked by *trans*-flupenthixol (25) and quercetin (24), respectively. The results of Table 1 show that addition of tamoxifen at 0.1 μM resulted in a 45% reduction of vitellogenin mRNA synthesis, while total RNA synthesis was not affected. Similar results were obtained by using nafoxidine (another antiestrogen) at 0.1 μM. The protein kinase inhibitors *trans*-flupenthixol and quercetin gave rise to 20% and 30% inhibition, respectively, and the effects of the two inhibitors were additive. In contrast, when the antiestrogen was combined with the two kinase inhibitors no additive effect was observed, suggesting that both antiestrogen and kinase inhibitors react with a common or related target. Doubling the concentrations of all inhibitors gave the same results. Experiments in which nuclear protein phosphatase activity was blocked with 20 mM β-glycerophosphate and in which the cyclic AMP-dependent kinases were activated were all without any effect on vitellogenin gene transcription. Moreover, increasing the protein acetylation by sodium butyrate and/or acetyl-CoA was also without any effect on the *in vitro* secondary stimulation of the vitellogenin gene transcription (data not shown).

As an alternative approach to assess the importance of estradiol–receptor complex in the secondary stimulation of vitellogenin gene, we used specific monoclonal antibodies directed against estradiol receptor (21, 22). The results in Table 2 show that depleting estradiol receptors from liver cell extracts by 83% inhibited the secondary stimulation by about 40%, whereas the same cell extract treated with control mouse IgG resulted in a reduction of only 4%. It is important to note, however, that approximately 14 fmol of estradiol receptor bound in the nuclei that could not be removed by treatment with Matrex-102-JS 34/32 were still present in these experiments. These residual estradiol receptors represented about 20% of the receptor molecules found in the full estradiol-induced livers *in vivo*.

DISCUSSION

Polyamines have been suggested as mediators for steroid hormones in mammary gland and chicken liver (26, 33). One of the roles of polyamines is to stimulate the nuclear kinases (34, 35), and it has been suggested that the phosphorylation of nuclear nonhistone proteins may play an important role on the specific regulation of gene expression (36–38). Therefore we can infer that polyamines may, in part, mediate the activation of gene expression through phosphorylation of nuclear proteins. In the present experiments we demonstrate by using specific kinase inhibitors that spermine and Ca^{2+}/calmodulin acted at least in part by activation of specific kinases (Table 1). These experiments also showed that there is no additive effect of the antiestrogen and the inhibitors of protein kinases, strongly suggesting that the kinases and antiestrogen react with the same target or related targets. Since it has been shown that the phosphorylation of steroid receptors occurs *in vivo* and *in vitro* (27, 39–43), it is tempting to conclude that the target of phosphorylation may be the estradiol–receptor complex. The combination of the antiestrogen and kinase inhibitors resulted in an inhibition of vitellogenin gene transcription never exceeding 55%. This means that in addition to the estrogen receptor and its possible phosphorylation there is another factor(s) present only in the cell extracts of estradiol-treated chicks. Preliminary experiments indicated that the other factor(s) is present only in the cytoplasm and nuclear extracts of estradiol-treated chicks, it is heat sensitive, and it has a high affinity for heparin-Sepharose. The possibility that the factor(s) is RNA polymerase II has already been ruled out.

Additional experimental evidence showing the direct involvement of estradiol–receptor complex in the *in vitro* secondary stimulation of the vitellogenin gene is proved by the results in Table 2. We show that a depletion of estradiol–receptor complex from the cellular extracts by immunochemical techniques reduced the secondary *in vitro* stimulation by 40%. The other 60% could represent the combined activity of the nuclear endogenous estradiol receptor (about 500 receptor molecules per nucleus, representing a maximum of 20% of the receptors found in a fully estradiol-induced chicken liver) and other cytoplasmic and nuclear factor(s). Further work is needed to fully characterize these factors. The *in vitro* transcription system presented here has shown to be suitable for the study of the *trans*-acting factors involved in the regulation of a specific gene in its natural environment.

We thank Mr. M. Ackerman for his expert assistance in photography. We also thank Dr. J. M. Jeltsch and Dr. A. E. Sippel for their gift of the cDNA clones pHha OV and pls 1. We are also grateful to Dr. I. Novak-Hofer for a generous gift of *trans*-flupenthixol. We thank Mrs. M. Seldran for her expert assistance.

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