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Specific modulation of the transcription of cloned avian vitellogenin II gene by estradiol–receptor complex *in vitro*

(purified nuclei/cloned genes/transcription initiation/homologous system)

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ABSTRACT **Avian vitellogenin-cauliflower mosaic virus hybrid gene is effectively transcribed *in vitro* in the homologous embryonic liver nuclei system. The transcription of the hybrid gene is modulated by the addition of an estradiol–receptor preparation that has been shown to bind selectively to an upstream region of cloned vitellogenin gene. Stimulation of the transcription of cloned vitellogenin hybrid gene by estradiol receptor is α-amanitin sensitive, hormone dependent, and promoter specific. Simian virus 40 and *Escherichia coli* promoters are not stimulated by the estradiol–receptor complex. The endogenous silent vitellogenin II gene (wild type) present in the nuclei is not turned on by the addition of estradiol–receptor complex. Deletion or inversion of the DNA sequence where the estradiol–receptor complex binds results in the complete suppression of the *in vitro* stimulation of transcription by estradiol receptor. Correct initiation of the transcription was demonstrated by primer extension studies of the newly synthesized RNA.**

Recently, we have shown that a DNA sequence upstream of the chicken vitellogenin II gene binds preferentially estradiol–receptor complex (1). However, these results did not show whether binding of the receptor complex to this region had any relevant biological function—e.g., the turning on or the modulation of vitellogenin gene expression. As a possible experimental approach to this problem we thought of using an *in vitro* homologous transcription system. We have previously shown that the chicken liver nuclei were able to reinitiate RNA synthesis *in vitro* (2). RNA synthesis in isolated purified nuclei has been described for a large number of different cell types (3–15), but in all cases the endogenous DNA was used as the sole source of template. Since the cytoplasm is absent from the nuclei in the *in vitro* system one can control the nuclear environment precisely and in particular for the screening of putative cytoplasmic regulatory molecules. In addition, nuclei incubated *in vitro* have been shown to add poly(A) tails (7, 14, 15), initiate RNA synthesis (2, 3, 6, 9, 13, 14), produce discrete RNA sequences (2, 8, 10, 11–13), and process precursor mRNA (15).

We now describe experiments in which we show that the nuclei system transcribes also cloned genes added to the incubation mixture. A promoter-specific modulation of the transcription of cloned genes is also obtained by the addition of estradiol–receptor complex.

MATERIALS AND METHODS

The *in Vitro* Transcription System. The preparation and storage of liver nuclei from 18-day embryos and the *in vitro* transcription mixture were prepared as outlined by Panyim *et al.* (2) with the following modifications: endogenous ribonucleases were inhibited by the addition of 10–50 units of human placental ribonuclease inhibitor (RNasin) per 150 μl of incubation mixture. When nuclear estradiol–receptor preparation was added to the reaction mixture, care was taken to keep the KCl concentration between 0.09 and 0.1 M. One to 3 μg of cloned DNA template was added per ml of incubation mixture. Upon incubation at 25°C for 40–60 min, RNA was immediately purified from the incubation mixture as described below. For each time point and estradiol–receptor concentration, assays were run in duplicate and parallel controls were incubated in ice. The control values were subtracted from the experimental values.

Processing and Analysis of the *in Vitro* Transcription Product. At the end of the incubation, 30 Kunitz units of ribonuclease-free DNase I was added per standard incubation mixture of 150 μl and the incubation was continued for 30 min at 25°C. EDTA and sodium dodecyl sulfate were then added to final concentrations of 5 mM and 0.5%, respectively. Upon addition of 150 μg of proteinase K, the reaction mixture was incubated at 42°C for 30 min. Following three extractions with chloroform/phenol, 1:1 (vol/vol), 10 μg of tRNA was added as carrier and RNA was precipitated twice with ethanol. The sediment was dissolved into 150 μl of 10 mM Tris, pH 8/5 mM $MgCl_2$/1 mM EDTA and incubated with 5 units of RNasin and 30 Kunitz units of DNase I at 37°C for 30 min. EDTA and sodium dodecyl sulfate were then added as indicated above and the proteinase K (100 μg per incubation mixture) treatment and chloroform/phenol extractions were repeated as described above. Upon ethanol precipitation the small sediment was lyophilized, dissolved into 5 μl of H_2O, and spotted onto nitrocellulose filters as described by Thomas (16). Alternatively, the reaction mixture was mixed with 2–3 vol of 8 M guanidine·HCl/10 mM sodium acetate, pH 5/5% mercaptoethanol and RNA was centrifuged through a cushion of 5.7 M CsCl for 24 hr at 250,000 × *g* at 20°C as described by Glisin *et al.* (17). Sedimented RNA was dissolved into 0.3 M sodium acetate at pH 5, extracted with chroroform/phenol, 1:1 (vol/vol), and precipitated with ethanol. Specific sequences in RNA were determined by the dot blot hybridization assay described by Thomas (16) and the primer extension studies were carried out as described by Geiser *et al.* (18). As primer we used the 251-base-pair (bp) *Sau*3A–*Cla* I fragment prepared from the clone pVT598-1-7 (Fig. 1). The analysis of the transcription product of pVT598-1-7 was carried out with either the radioactively labeled *Cla* I-*Eco*RI (750 bp) or the *Cla* I-*Sau*3A (251 bp) DNA fragments prepared from pVT598-1-7. DNA probes were labeled by nick-translation to a specific activity of 2–8 × 10^8 cpm/μg (19).

Preparation of Nuclear Receptor. The preparation of nuclear estradiol–receptor was carried out exactly as described

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Abbreviations: bp, base pair(s); SV40, simian virus 40; XPRT, xanthine phosphoribosyltransferase; CAT, chloramphenicol acetyltransferase; RNasin, human placenta ribonuclease inhibitor.

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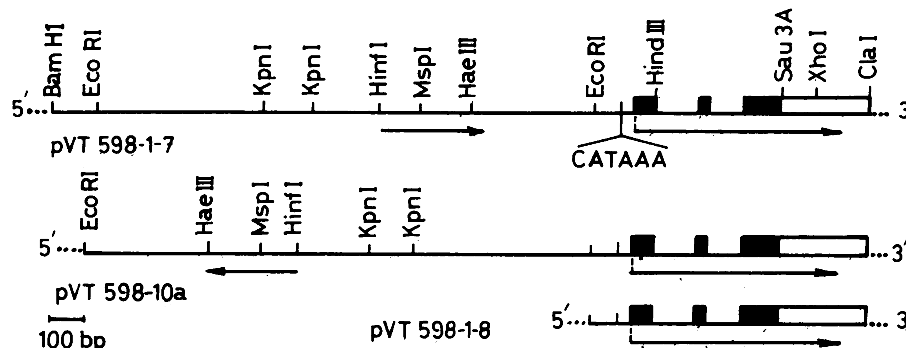

FIG. 1. Restriction map of the constructions used for the *in vitro* transcription system. pVT598-1-7 is a hybrid between vitellogenin gene and the cauliflower mosaic virus DNA (open box, *Sau*3A–*Cla* I). In addition to the structural part of the gene we show also the upstream sequence of vitellogenin gene. pVT598-10a has an inverted 1400-bp *Eco*RI DNA fragment and pVT598-1-8 has a deletion of the same DNA fragment.

by Jost *et al.* (1). There is a positive correlation between the binding capacity of the estradiol receptor to DNA and its ability to stimulate the *in vitro* transcription of cloned vitellogenin gene.

Recombinant DNA. The clone pVT598-1-7 (Fig. 1) used for the *in vitro* transcription has been obtained by the insertion of *Sau*3A–*Cla* I DNA fragment of cauliflower-mosaic virus between the *Cla* I site of pBR322 and the *Sau*3A site of the third exon of vitellogenin gene. pVT598-1-7 also contains an upstream sequence of vitellogenin II gene extending up to the *Bam*HI site at nucleotide position −1600 bp. The estradiol–receptor complex binding site is situated at the nucleotide position −597 to −620 including the *Msp* I site (1). Derivatives of pVT598-1-7 were obtained by deleting the upstream sequence *Bam*HI–*Eco*RI (pVT598-1-8, Fig. 1) or by inversion of the 1400-bp *Eco*RI DNA fragment containing the estradiol–receptor binding site plus flanking regions (pVT598-10a, Fig. 1). As *Escherichia coli* promoter we used the clone pYEJ001, which contains tandem repeats of synthetic lactose operator and *E. coli* RNA polymerase promoter linked to the chloramphenicol acetyltransferase (CAT) gene (20). The purified *Hin*dIII DNA fragment containing the CAT gene was used as labeled probe. For the simian virus 40 (SV40) promoter we used the clone pSV2 gpt (21) containing the early-region promoter linked to the bacterial xanthine phosphoribosyltransferase (XPRT) and the *Hin*dIII–*Bam*HI DNA fragment containing the XPRT gene was used as labeled probe.

Materials. Restriction enzymes were purchased either from Boehringer or from Stehelin AG Basel. The ribonuclease inhibitor RNasin was from Genofit SA Geneva. The plasmid pYEJ001 was from P-L Biochemicals and pSV2 gpt was a generous gift from C. Moroni from our institute. The radioactive nucleotide 3′-deoxyadenosine 5′-[α-^{32}P]triphosphate tetra (triethylammonium) salt (3000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Ribonuclease-free DNase I was from Worthington.

RESULTS

***In Vitro* Transcription of the Vitellogenin-Cauliflower Mosaic Virus Hybrid Gene.** We have already shown that the liver nuclei cell-free system is able to reinitiate and sustain RNA synthesis for at least 90 min (2). The aim of the following experiments was to adapt this homologous *in vitro* transcription system to study the regulation of cloned vitellogenin gene. To be able to distinguish the transcription product of the endogenous wild-type vitellogenin gene present in the liver nuclei from the added cloned DNA, we constructed a hybrid gene consisting of the first three exons of vitellogenin gene linked to a cauliflower mosaic virus DNA sequence (Fig. 1), and the *Cla* I–*Sau*3A (250 bp) DNA fragment of cauliflower mosaic virus was used as labeled hybridization probe.

Fig. 2*A* shows that under our experimental conditions both circular and linear DNA are equally well transcribed in a dose-dependent manner. For a concentration of 1.5 μg of DNA per ml of circular pVT598-1-7 DNA, there is a time-dependent transcription going up to 1 hr (Fig. 2*B*, curve a). However, if 200 fmol of estradiol receptor are added per 150 μl of incubation mixture, there is a significant increase in the

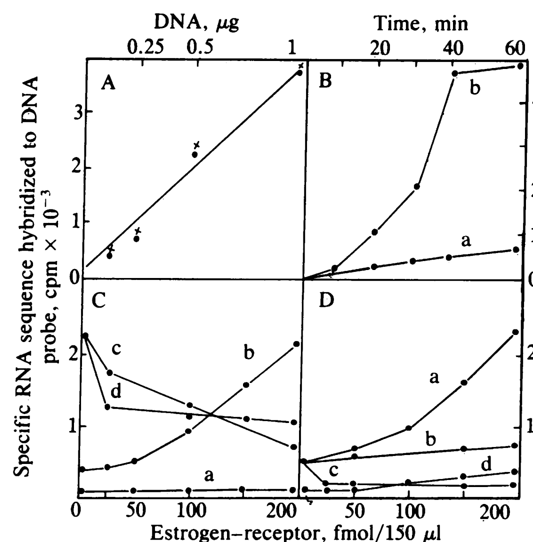

FIG. 2. Properties of the *in vitro* transcription system. (*A*) DNA concentration dependence of the transcription of pVT598-1-7. The concentration is given in μg/150-μl incubation mixture. x, Circular DNA; ●, linear DNA. (*B*) Time dependence of the *in vitro* transcription of pVT598-1-7 in the absence (curve a) and in the presence (curve b) of 200 fmol of estradiol–receptor complex. Two-hundred fifty nanograms of DNA template was incubated per 150 μl of incubation mixture. (*C*) Effect of increasing concentrations of estradiol receptor on the transcription of different promoters: curve a, wild-type endogenous vitellogenin gene; curve b, pVT598-1-7; curve c, pYEJ001 DNA containing *E. coli* promoter linked to CAT gene; curve d, pSV2 gpt has SV40 early promoter linked to bacterial XPRT. For curves b–d, the concentration of DNA template is 250 ng/150-μl incubation mixture. (*D*) Control experiments: curve a, incubation of pVT598-1-7 with increasing concentrations of estradiol–receptor complex; curve b, same experiment as in curve a except that the receptor has no estradiol (1); curve c, same incubation conditions as in curve a but in the presence of 1 μg of α-amanitin per ml (α-amanitin was added only to the incubation mixtures containing the estradiol–receptor complex); curve d, polymerase activity in the estradiol–receptor preparation. The incubation mixture is as in curve a except in the absence of nuclei. In all tests the concentration of DNA template was 250 ng/150-μl incubation mixture.

rate of transcription (Fig. 2*B*, curve b) and maximal RNA synthesis is observed after 40 min of incubation at 25°C. If we test in a parallel experiment the transcription of the endogenous wild-type vitellogenin gene present in the nuclei, there is no trace of vitellogenin mRNA synthesis even in the presence of estradiol–receptor complex (Fig. 2*C*, curve a), regardless of whether the estradiol–receptor preparation is from the liver or the oviduct nuclei. Since the estradiol–receptor preparations are not pure, many control experiments had to be done. One of the basic criteria for specific hormone–receptor interaction with the genome is to be hormone dependent. Fig. 2*D* (curve b) shows that in the absence of estradiol there is no stimulation of pVT598-1-7 transcription by the receptor preparation over the baseline value obtained without receptor. In addition, the dose-dependent stimulation of transcription by estradiol–receptor complex is α-amanitin sensitive (Fig. 2*D*, curve c) and the estradiol–receptor preparation contains too little polymerase activity to explain the increase in transcription of pVT598-1-7 (Fig. 2*D*, curve d). Additional control experiments (results not shown) indicate that estradiol–receptor preparation does not increase the association of the template with the purified nuclei and it does not increase the leakage of polymerases from the nuclei; moreover, estradiol alone is unable to stimulate the *in vitro* transcription of pVT598-1-7 DNA.

Specificity of the Promoter and Upstream Sequences. Using a crude system, Jensen's group (22) has shown that exposure of purified uterus nuclei to unpurified estradiol–receptor complex of uterus cytosol increased the total synthesis capacity of the system by 2- to 3-fold. Therefore, in the following experiments it was necessary to show the specificity of the stimulation of transcription by estradiol–receptor complex. Fig. 2*C* shows that although cloned vitellogenin hybrid gene pVT598-1-7 (curve b) is stimulated by increasing concentrations of estradiol–receptor, SV40 (curve c) and *E. coli* (curve d) promoters are inhibited by increasing concentrations of estradiol–receptor preparation. Recently, we have shown that there is a preferential binding of estradiol–receptor complex to a sequence situated 600 bp upstream from the start of the gene (1). It was therefore of interest to see what happens if we remove (pVT598-1-8, Fig. 1) or invert (pVT598-10a, Fig. 1) this sequence and its neighboring DNase I-hypersensitive sites (23). The results presented in Fig. 3 clearly show that the deletion (curve b) or inversion (curve c) of the upstream sequence completely abolishes the stimulation of transcription by the estradiol–receptor complex.

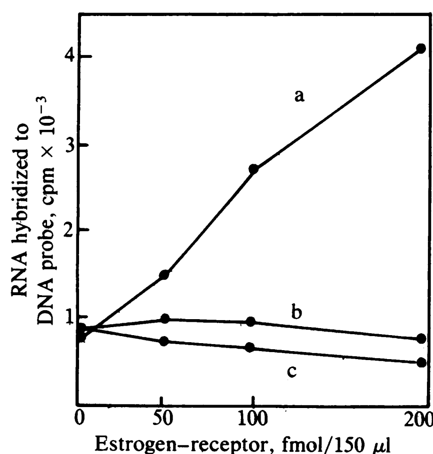

FIG. 3. Effect of inversion and deletion of the vitellogenin upstream DNA sequence of pVT598-1-7 on the *in vitro* stimulation of transcription by estradiol–receptor complex. Curve a, pVT598-1-7; curve b, pVT598-1-8, deletion of upstream sequence *Bam*HI–*Eco*RI; curve c, pVT598-10a, inversion of the upstream DNA fragment *Eco*RI. For the restriction map of these clones, see Fig. 1.

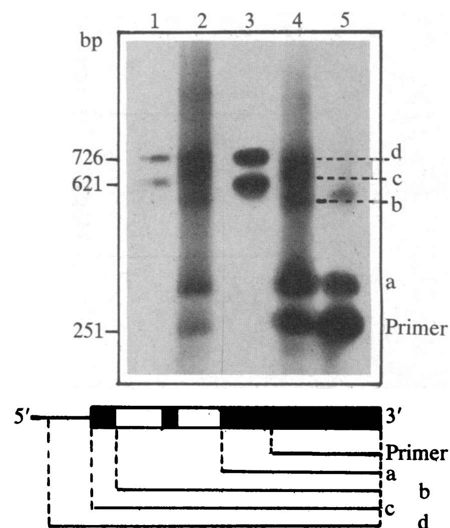

FIG. 4. Primer extension study of the *in vitro* transcription product of pVT598-1-7. Lanes 1 and 3, size markers of 726 and 621 bp. Lanes 2 and 4, primer extension of the *in vitro* transcription product of pVT-1-7 in the presence of estradiol–receptor complex; lane 5, primer extension in the absence of estradiol–receptor complex. DNA fragments were separated by electrophoresis on a 2% agarose gel. The primer is the 251-bp-long *Cla* I-*Sau*3A DNA fragment. Fragments a–d are graphically represented on the map below the autoradiogram. The black and open boxes represent the exons and introns of pVT598-1-7, respectively.

Primer Extension Studies of the *in Vitro* Transcription Product. The aim of the present experiments was to determine the starting point of the initiation of transcription of pVT598-1-7 *in vitro* in the presence and in the absence of estradiol–receptor complex. Total RNA was prepared by the guanidine·HCl procedure and primer extension was carried out as outlined in *Materials and Methods*. Results in Fig. 4 show that in the absence of estradiol receptor (lane 5) there is no apparent RNA transcribed starting at the beginning of the first exon, and fragments a and b may represent processed RNA. In sharp contrast, in the presence of estradiol–receptor complex, we find, in addition to fragments a and b (lanes 2 and 4), RNA starting at the correct initiation point (fragment c) and even higher up in a region that contains a nucleotide sequence homologous to the bacterial RNA polymerase binding site (fragment d) (18).

DISCUSSION

Under specific conditions it is possible to use chicken embryonic liver nuclei for transcription of cloned genes *in vitro*. If possible, the use of any detergent should be avoided when preparing the nuclei since detergents sharply decrease the ability of the liver nuclei to transcribe endogenous and exogenous DNA. The system presented here also requires the presence of a ribonuclease inhibitor. In reconstruction experiments (data not shown) we found that up to 20% of the input labeled DNA incubated in the cell-free system for 1 hr is associated with the nuclei and that this association is time dependent. However, we do not yet know the nature of this association. Preparations of nuclei free of cytoplasmic contaminants were also essential since we found that under our experimental conditions the embryonic liver cytoplasm had a dose-dependent inhibitory effect on the *in vitro* transcription of cloned vitellogenin gene (data not shown). This explains why we could not use a cell-free system prepared according to the protocols of Manley *et al.* (24). The experiments presented in Figs. 2*C* and 3 strongly suggest that the

estradiol–receptor complex only stimulates the transcription of genes that have the receptor-binding site, as is the case for clone pVT598-1-7. Moreover, the estradiol–receptor complex may have an effect on the correct initiation of RNA synthesis (Fig. 3).

Under the same experimental conditions, SV40 and bacterial promoters are better transcribed than pVT598-1-7; however, SV40 and bacterial promoters are inhibited by increasing concentrations of estradiol–receptor complex. In the case of SV40, it is conceivable that the homology between the core enhancer sequence and the estradiol–receptor binding site (1) favors the binding of the receptor to SV40 core enhancer, resulting in a competition for another regulatory protein. A deletion of the whole DNA fragment containing the estradiol–receptor binding site and flanking sequences also suppresses the stimulation of transcription of the vitellogenin hybrid gene by the estradiol receptor (Fig. 3). These results strongly indicate that the upstream sequence of vitellogenin gene is important for the modulation of the gene expression by estradiol–receptor complex. Furthermore, the position and orientation of this sequence cannot be changed without abolishing the stimulatory effect of the estradiol–receptor complex (Fig. 3). This means that in spite of having some homology with viral core enhancers, the estradiol–receptor binding site and flanking region do not behave like a true viral enhancer.

It is interesting to note that the silent endogenous vitellogenin gene present in embryonic liver nuclei is not turned on by the addition of nuclear estradiol–receptor complex, whereas the transcription of pVT598-1-7 is largely increased by the addition of the receptor. It is known that in 13- to 19-day embryos, vitellogenin gene can be turned on by *in ovo* injection of estradiol (ref. 25 and unpublished results). This means that the failure of the wild-type endogenous vitellogenin gene to respond to the receptor preparation *in vitro* is not due to a critical stage of differentiation of embryonic liver but is rather an indication that estradiol–receptor complex alone is not sufficient to turn on a specific silent gene, regardless of whether the receptor is prepared from liver or oviduct nuclei. With regard to the other factors possibly responsible for the turning on of the silent gene by steroid hormones, several possibilities such as covalent modifications of the DNA and specific protein modifications such as acetylation, ADP-ribosylation, and phosphorylation remain to be investigated.

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