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Estradiol-Dependent Trans-Acting Factor Binds Preferentially to a Dyad-Symmetry Structure Within the Third Intron of the Avian Vitellogenin Gene

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The secondary activation of the avian vitellogenin II gene in isolated liver nuclei by cytoplasmatic liver extracts of estradiol-treated chicks is accompanied by the binding of a protein from the extract to the structural part of the cloned gene. Both the DNA-binding and gene-stimulatory activities, which cochromatograph on heparin-Sepharose, are apparently present only in the cytoplasmatic liver extracts of estradiol-treated roosters and in the oviduct extracts of egg-laying hens. DNA-binding competition assays combined with exonuclease III footprinting showed that the factor binds to the imperfect dyad-symmetry structure $^{5'}$GTCTTGTTCCAAAC$^{3'}$ within the third intron of the gene. The factor is sequence specific and binds equally well to both single- and double-stranded DNA with an estimated dissociation constant of 3.5×10^{-10} M.

Key words: estradiol-dependent trans-acting factor, binds preferentially, dyad-symmetry structure, third intron, avian vitellogenin gene

The elucidation of the regulation mechanisms of eucaryotic gene expression represents one of the major challenges of modern-day biology. Several models of gene regulation have been proposed to date, and in all of these, protein-DNA complexes have been assigned essential roles. A number of proteins associated with the regulation of gene activity have been recently described in the literature. Several of these were shown to bind to DNA structures possessing dyad symmetry [1–7], while others exhibited sequence-specific binding requirements [8–13]. These protein/DNA complexes could either represent an entry point for RNA polymerase from which it could migrate to the RNA start site, or a gene activator site, from which a structural change could be transmitted along the DNA. Alternatively, they could facilitate a protein–protein interaction with RNA polymerase or other transcription factors [14].

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The avian vitellogenin II gene represents a highly suitable model system for studying the molecular mechanisms of gene regulation. The in vivo primary activation of this gene with estradiol was shown to be accompanied at the chromatin level by the appearance of clusters of hormone- and tissue-specific nuclease hypersensitive sites [15,16] and at the DNA level by a strand-specific demethylation of several CpG dinucleotides, located within the upstream region of the gene [7,17]. In addition, the active gene was shown to be selectively and reversibly associated with the nuclear matrix [18]. These observations are indicative of a complex interaction of this gene with a number of hormone- and tissue-specific factors.

In an attempt to study these trans-acting factors and their recognition sites on the vitellogenin gene, a functional in vitro transcription assay was recently independently developed in two laboratories [19,20]. Using nuclei isolated from the livers of estradiol-treated roosters Jost et al [20] demonstrated that the gene could be reactivated by the addition of spermine, calmodulin, nuclear, or cytoplasmic extract from the livers of hormone-treated roosters. We now report that this estradiol-dependent in vitro secondary activation of the gene coincides with the binding of a factor to a region of dyad symmetry within its third intron.

MATERIALS AND METHODS

Preparation of DNA Fragments and Oligonucleotide Synthesis

DNA fragments A, B, C, and D (Fig. 1) were all derived from the plasmid pVT598 [21] by *Eco*RI/*Bam*HI digestion and separated by electrophoresis on low-melting agarose gel (1–1.8%). The DNA was recovered by heating the gel slices in 300 mM sodium acetate, pH 5, 1 mM EDTA at 70°C for 15 min, followed by phenol extraction and ethanol precipitation. Further desalting was carried out by using NENsorb 20 cartridges (NEN). Fragments shorter than 200 bp were purified by electrophoresis on 8% polyacrylamide gels.

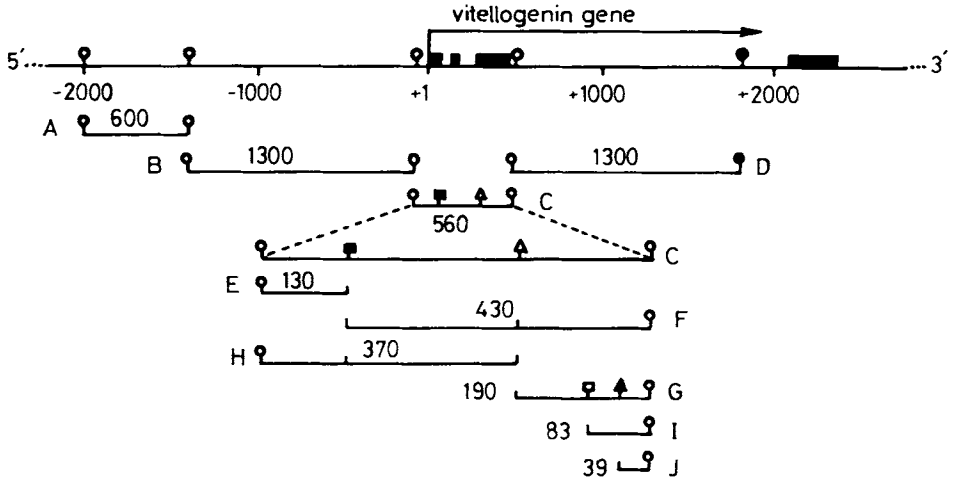

Fig. 1. Restriction map of the cloned DNA fragments used in the DNA-binding studies *Eco*RI (○), *Bam*HI (●), *Hin*dIII (■), *Hin*fI (△), *Sau*3AI (□), *Hae*III (▲). The fragment sizes (in base pairs) are indicated.

Oligodeoxyribonucleotides were synthesized by using an Applied Biosystems model 380A synthesizer and purified by electrophoresis on 8% polyacrylamide gels. The bands corresponding to the desired products were cut out and the DNA was recovered by elution into 500 mM ammonium acetate, 5 mM EDTA. Desalting was performed on Sep-Pak C_{18} (Millipore) or NENsorb 20 (NEN) cartridges. The oligonucleotides were labeled to a specific activity of 2–5 $\times$ 10^8 cpm/μg of DNA; 5′-ends were labeled with T4 polynucleotide kinase/[gamma-^{32}P]ATP, while 3′-end labeling was achieved by filling in the sticky ends of the restriction fragments with DNA polymerase I (Klenow fragment)/[alpha-^{32}P]dATP, following published procedures [22].

Hormone Treatment of Animals

Adult mature and immature white Leghorn roosters were treated with a single intramuscular injection of estradiol (40 mg/ml of propyleneglycol, 40 mg/kg of body weight) into the leg.

Preparation of Cytoplasmic and Nuclear Liver Cell Extracts

Livers from estradiol-treated (24 hr post-treatment) or control animals were perfused with cold NaCl (150 mM) and then homogenized in four volumes (v/w) of ice-cold extraction buffer [500 mM Tris·HCl pH 8, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride (PMSF), 25% glycerol] with four strokes of a loose-fitted glass/Teflon homogenizer at 1,500 rpm. Following centrifugation (120,000g, 4 hr), the supernatant was stored in small aliquots in liquid nitrogen. Liver nuclei were prepared as described by Panyim et al [23], extracted with 400 mM KCl, 50 mM Tris·HCl pH 8, 5 mM $MgCL_2$, 2 mM 2-mercaptoethanol, 1 mM PMSF, and centrifuged at 150,000g for 4 hr. The supernatant was stored in small aliquots in liquid nitrogen.

Heparin-Sepharose Chromatography

Cytoplasmic proteins (160 mg) were applied onto a column of heparin-Sepharose (8-ml bed volume, Pharmacia) equilibrated at 0°C with the extraction buffer. Fractionation was achieved by elution with a stepped KCl gradient. Ammonium sulfate was added to the collected fractions (to 60% saturation) and the precipitate was resuspended in extraction buffer. Following extensive dialysis against the same buffer, the protein fractions were stored in small aliquots at −70°C. They were assayed for the specific vitellogenin transcription and DNA binding in vitro (see below). The fraction eluted with .15–.35 M KCl contained 4.8 mg of protein.

In Vitro Transcription

This experiment was described previously [20]. RNA was extracted and analyzed for specific mRNA sequences following a published procedure [24]. Fragments A and B of the plasmid pVT598 [21] containing 2 kb of the vitellogenin coding sequence were used as the radioactive probes.

DNA-Protein Binding Assays

The polyacrylamide gel retention assay was carried out essentially as described [12,25–27]. The binding buffer (100 mM NaCl, 10 mM Tris·HCl pH 8, 1 mM EDTA, 12.5% glycerol) was supplemented with $MgCl_2$ (to 5 mM) for the exonuclease

III footprinting experiments; 2.5–3fmol (.5–.6 ng) of labeled DNA were incubated in 20 μl of binding buffer with increasing concentrations of protein (1–5 μg). The nonspecific competing DNA was from *E coli*.

Gel retardation studies were carried out using 4% polyacrylamide gels. The acrylamide/bis-acrylamide ratio for DNA fragments in excess of 600 bp was 80:1; for shorter fragments it was 30:1. The electrophoresis buffer system was as described by Singh et al [12]. Following autoradiography, the bands representing the specific DNA-protein complex were cut out and assayed for radioactivity. A gel slice of equivalent size was cut out from the control lane containing the labeled DNA alone, and the activity contained in this slice was substracted from the experimental values. In some experiments the DNA-protein complex was separated from the unbound DNA by filtration on nitrocellulose membranes (Schleicher & Schuell, BA85, 9-mm diameter, 0.45-μm pore size) presoaked for several hours at room temperature in the binding buffer containing 30 μg/ml of sonicated *E coli* DNA. The reaction mixture was slowly passed through the filter, which was then washed with 50–100 sample volumes of binding buffer. The filters were assayed for radioactivity and the background values (typically 0.03% of input) were subtracted. When necessary, the DNA could be recovered from the membranes by incubation in 250 μl of 500 mM ammonium acetate, pH 7.5, 0.1 mM EDTA, 0.1% SDS, and 100 μg of proteinase K. Following incubation at 37°C for 30 min, the samples were extracted with phenol/chloroform and the DNA was precipitated with 2.5 volumes of ethanol at −20°C.

Exonuclease III Footprinting

The experiments were carried out as described by Wu [2,3,28]; 2.5fmol (0.5 ng) of fragment J, labeled either on the upper or on the lower strand, was incubated at 23°C in 20 μl of binding buffer containing 3 μg of the protein (the fraction eluted from the heparin-Sepharose column with 0.35 M KCl) and a 1,000-fold excess (500 ng) of sonicated *E coli* DNA. After 15 min exonuclease III was added to a final concentration of 100–400 units/ml and the incubation was continued for a further 15 min. The reaction was stopped by the addition of EDTA (5 mM total concentration) and the protein-DNA complex was separated from the free DNA by nitrocellulose membrane filtration.

Immunochemical Techniques

Monoclonal antibodies, coupled to Matrex-102 beads, were used to deplete the cell extracts of estradiol receptor. Although raised against calf uterine estradiol receptor (clone JS-34/32, [29,30]), the antibodies cross-reacted with the chicken liver estradiol receptor; a 10-μl suspension of the functionalized Matrex beads was allowed to react with 5 fmol of the chicken liver estradiol receptor complex for 6 hr at 0°C. Under these conditions the antibody removed over 98% of the estradiol-receptor activity. Estradiol receptor concentration in the cell extracts was determined before and after treatment with antibodies by the method of Best-Belpomme et al [31].

RESULTS

DNA-Protein Binding Assay

In an attempt to characterize the mode of action of potential trans-acting factors responsible for the in vitro secondary activation of the vitellogenin gene, we tested

the cytoplasmic liver extracts for specific DNA-binding activity. DNA fragments covering approximately 2 kb of the 5′-end of the gene, and 2 kb of its upstream sequence were used as substrates (Fig. 1). Fragment C, a 560-bp-long *Eco*RI fragment, which carries the first three introns of the vitellogenin gene, was shown to possess a specific binding activity for a protein(s) present in the cytoplasmic extract from the livers of estradiol-treated roosters. This specific DNA-protein complex (Fig. 2, lanes 3 and 4) was only observable in the presence of a 1,000–2,000-fold excess of carrier DNA, which was required to compete out the nonspecific binding (Fig. 2, lane 2). The presence of unlabeled fragment C in the assay (50–100-fold excess) led to a quantitative displacement of the labeled fragment from the specific DNA-protein complex (Fig. 2, lanes 5 and 6).

Correlation Between the Binding of the Factor and the In Vitro Secondary Activation of the Vitellogenin Gene

Figure 3 shows the dependence of the in vitro secondary activation of the vitellogenin gene on the concentration of various protein fractions. Serum albumin and the cytoplasmic fraction from the liver extracts of control roosters failed to activate vitellogenin mRNA synthesis (curves A and B, respectively). The DNA binding activity of the control extract to fragment C was also very low (Fig. 4, curve B). By contrast, estradiol treatment of roosters resulted in a significant increase of both gene stimulatory activity (Fig. 3, curve C) and DNA binding to fragment C (Fig. 4, curve C). Following partial purification of this latter extract on heparin-Sepharose, the fraction eluted with 0.35 M KCl showed an approximately tenfold increase in both activities (Fig. 3, curve D, and Fig. 4, curve D). Neither activity was present in the fractions eluted with 0.5 and 1.0 M KCl. Interestingly, nuclear

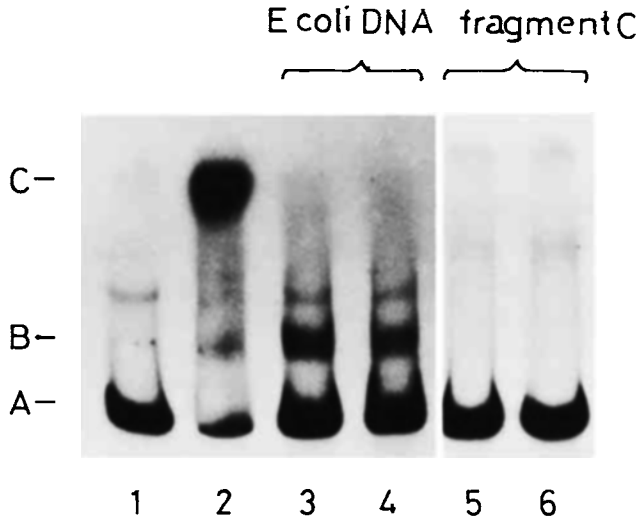

Fig. 2. Gel retardation of fragment C by the protein factor from liver extracts of estradiol-treated roosters. **Lane 1:** Labeled fragment C. **Lane 2:** Fragment C + 2 μg of protein fraction. **Lane 3:** Fragment C + 2 μg of protein fraction + 1,000-fold weight excess of *E coli* DNA. **Lane 4:** Same as 3, but with 2,000-fold excess of *E coli* DNA. **Lane 5:** Same as 3 + 50-fold excess of unlabeled fragment C. **Lane 6:** Same as 5, but with a 100-fold excess of unlabeled fragment C. **Band A:** Unbound DNA fragment C. **Band B:** Specific DNA-protein complex. **Band C:** Nonspecific DNA-protein complex.

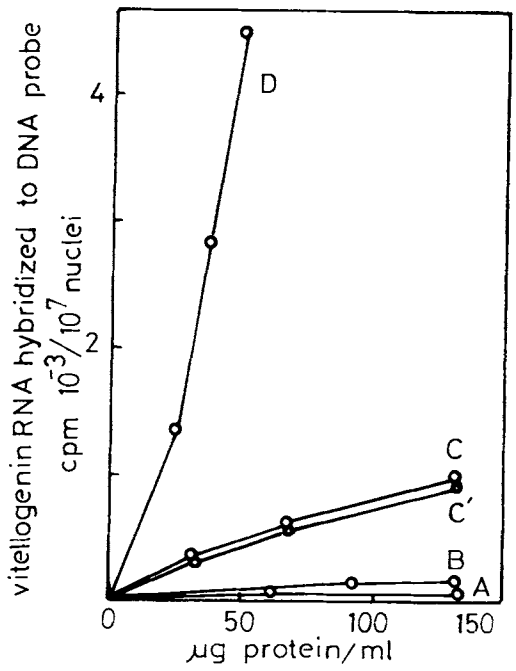

Fig. 3. The dependence of the in vitro secondary stimulation of vitellogenin mRNA synthesis on protein factor concentration. **Curve A:** Bovine serum albumin. **Curve B:** Cytoplasmic liver extract from control (nontreated) roosters. **Curve C:** Cytoplasmic liver extract from treated roosters. **Curve C′:** Same as C, but after removal of estradiol receptors with immobilized antibodies. **D:** Heparin-sepharose-purified fraction eluted with 0.35 M KCl.

extracts from mature hen oviduct and the liver of estradiol-treated roosters that have been shown to activate in vitro vitellogenin gene [20] presented also DNA binding activity to fragment C (Fig. 4, curves E and F).

Estradiol Dependence of the DNA Binding Activity

The in vitro secondary activation of the vitellogenin gene in isoalted liver nuclei is restricted to extracts from tissues possessing estradiol receptors [20]. As our experiments show, the above-described DNA-binding activity exhibited a similar specificity: cytoplasmic extracts from the liver of estradiol-treated roosters were found to bind very efficiently to fragment C, whereas a cytoplasmic extract from erythrocytes of the same rooster failed to show any significant binding (Fig. 4, curves C and G, respectively).

In order to demonstrate that the factor present in the active cytoplasmic extracts was not estradiol-receptor complex, a portion of the extract was treated with antiestradiol receptor monoclonal antibodies linked to Matrex beads. This treatment removed over 98% of the estradiol-receptor complex from the preparation. An extract thus treated was still able to activate the gene in vitro (Fig. 3, curve C′) and bind to the DNA fragment C (Fig. 4, curve C′). Additional evidence that the protein binding to fragment C is not estradiol-receptor complex is given by the DNA competition experiment. As seen in Figure 5, fragment B, which contains the estradiol-receptor

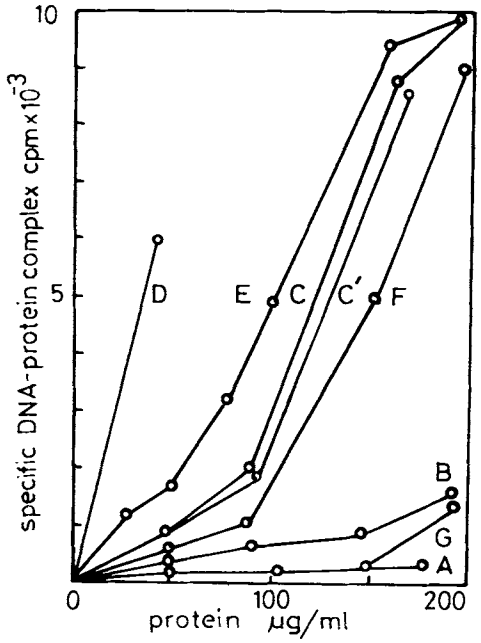

Fig. 4. Estradiol dependence of binding of the protein fraction to vitellogenin DNA (fragment C). **A:** Bovine serum albumin. **B:** Cytoplasmic extract from livers of control roosters. **C:** Cytoplasmic extract from livers of estradiol-treated roosters. **C′:** Same as C but after removal of estradiol receptors with immobilized antibodies. **D:** Fraction of cytoplasmic extract from livers of estradiol-treated roosters, eluted from heparin-Sepharose column with 0.35 M KCl. **E:** Nuclear extract from livers of estradiol-treated roosters. **F:** Nuclear extract from oviduct of egg-laying hens. **G:** Cytoplasmic extract from mature erythrocytes. The DNA binding activity was determined by gel retardation assay.

binding site [21,33–35], is a poor competitor for the protein-DNA fragment C complex.

The Factor Binds Preferentially to a Dyad-Symmetry Structure Within the Third Intron of the Vitellogenin Gene

The precise location of the factor binding site was established by a series of competition binding assays, followed by exonuclease III footprinting. The results of preliminary experiments provided a saturation curve for the specific binding of the factor to fragment C (Fig. 4, curve C). The assays, in which unlabeled purified DNA fragments (Fig. 1, fragments E–J) were used to displace the protein-bound fragment C from the protein/DNA complex, were then carried out at equilibrium with subsaturation concentrations of the factor. The strong competitive binding exhibited by the fragments F and G (Fig. 5) indicated that the factor must bind preferentially to the 3′-proximal region of fragment C. Further experiments with fragments G, I, and J provided strong evidence for the binding site being located on the 39-bp *Hae*III-*Eco*RI fragment J (Fig. 6). The exact position and size of the binding site was then ascertained from exonuclease III footprinting experiments. The synthetic 39-mer duplex, 5′ labeled in either the upper- or lower- strand, was incubated with the cell extracts until equilibrium conditions were reached. Exonuclease III digestion of the resulting DNA-protein complex, followed by separation of the oligonucleotide frag-

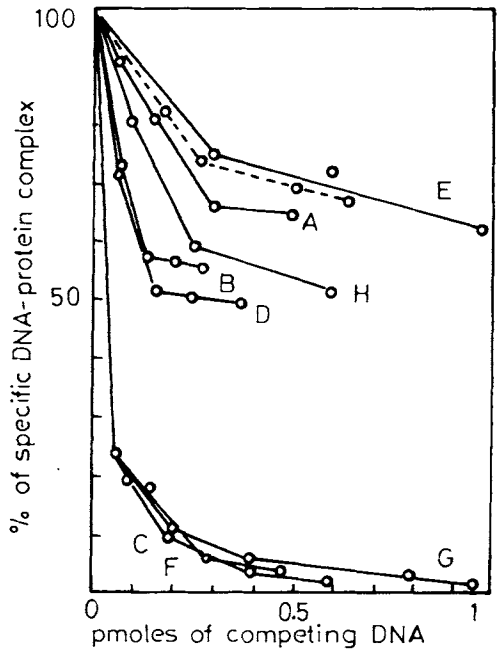

Fig. 5. Competitive binding assay between the fragment C/protein factor complex and restriction fragments A–H (see Figure 1). The 20 μl incubation mixture contained 3 μg protein, 800 ng *E coli* DNA, 0.1 ng of P^{32}-labeled DNA substrate, and increasing concentrations of competing nonlabeled purified DNA fragments. The value of 100% represents the amount of radioactivity present in the polyacrylamide gel band containing the protein-DNA complex prior to the addition of the specific competing DNA.

ments by denaturing polyacrylamide gel electrophoresis, indicated (Fig. 7) that the protected DNA region lies between the nucleotide positions 454 $\pm$ 1 and 466 $\pm$ 1, the protected sequence being $^{5'}$GTCTTGTTCCAAAC$^{3'}$. This imperfect dyad-symmetry structure lies within the third intron of the gene and is flanked at the 3'-end by a methylatible CpG site [32]. The shorter minor bands appearing below the main DNA bands at nucleotide positions 454 and 466 (Fig. 7) probably reflect the "breathing" of the protein factor on the DNA duplex.

The Estradiol-Dependent Factor Binds to Both Single- and Double-Stranded DNA in a Sequence-Specific Fashion

The DNA binding properties of the factor were further investigated by comparing its affinity for duplex DNA with that for the individual single strands of the same fragment. Under our standard assay conditions, the protein factor was shown to bind equally well to double-stranded (Fig. 8, curve A) and single-stranded DNA (Fig. 8, curves B,C). The protein possessed no affinity for the other synthetic oligonucleotides tested (Fig. 8, curves D–F), which suggests that the binding of the factor to DNA is sequence-specific.

Kinetic and Equilibrium Parameters of the Binding Reaction

Kinetics of the binding reaction were studied in order to establish the equilibrium conditions. Figure 9 (panel A) shows that at 23°C, the reaction between the

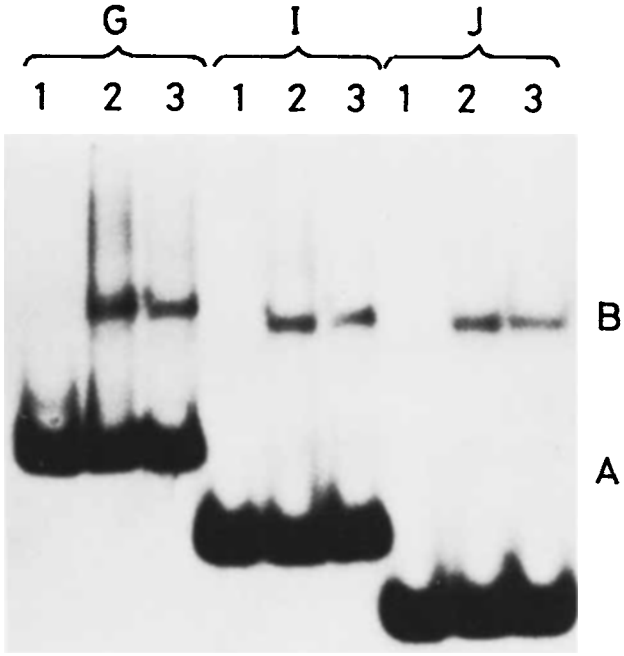

Fig. 6. Gel retardation assay of protein complexes with DNA fragments **G, I,** and **J** (see Figure 1). The fragments were labeled by filling in the sticky ends and purified by polyacrylamide gel electrophoresis. **Lanes 1:** DNA fragment alone (0.1 ng). **Lanes 2:** DNA fragment (0.1 ng) + cell extract fraction (3 μg) + 1,000-fold excess of *E coli* DNA (100 ng). **Lanes 3:** Same as 2 but with 2,000-fold excess of *E coli* DNA. **Band A** represents the unbound DNA fragments. **Band B** represents the protein-DNA complex.

heparin-Sepharose-purified factor and the synthetic 39-bp fragment J reached equilibrium after 20 min. The relative stability of the specific DNA-protein complex, formed in the presence of a 1,000-fold excess of carrier DNA, was determined by adding a 100-fold excess of the unlabeled fragment J as the specific competitor. An apparent half-life of the complex under these conditions was approximately 30 min (inset of Fig. 9, curve a), whereas in the absence of the specific competitor the protein-DNA complex was stable throughout the duration of the experiment (inset of Fig. 9, curve b). The equilibrium constant was estimated by titrating the binding protein fraction with the labeled fragment J and estimating the amount of protein-DNA complex by a nitrocellulose filter binding assay as described in Methods. Scatchard analysis yielded a straight line, indicative of a single bimolecular reaction. Assuming that each DNA fragment carries only a single binding site, an average dissociation constant $K_d = 3.5 \times 10^{-10}$ M was obtained. The intercept of the line with the abscissa allowed the estimation of the concentration of the binding molecules in our assay. Based on the estimate that a diploid liver cell contains approximately 60 pg of protein, each cell would be expected to have an average of 10,000–20,000 molecules of the binding factor.

DISCUSSION

We have recently shown that cell extracts from estradiol-responsive tissues (eg, liver or oviduct) were able to activate the avian vitellogenin II gene in a gene-specific

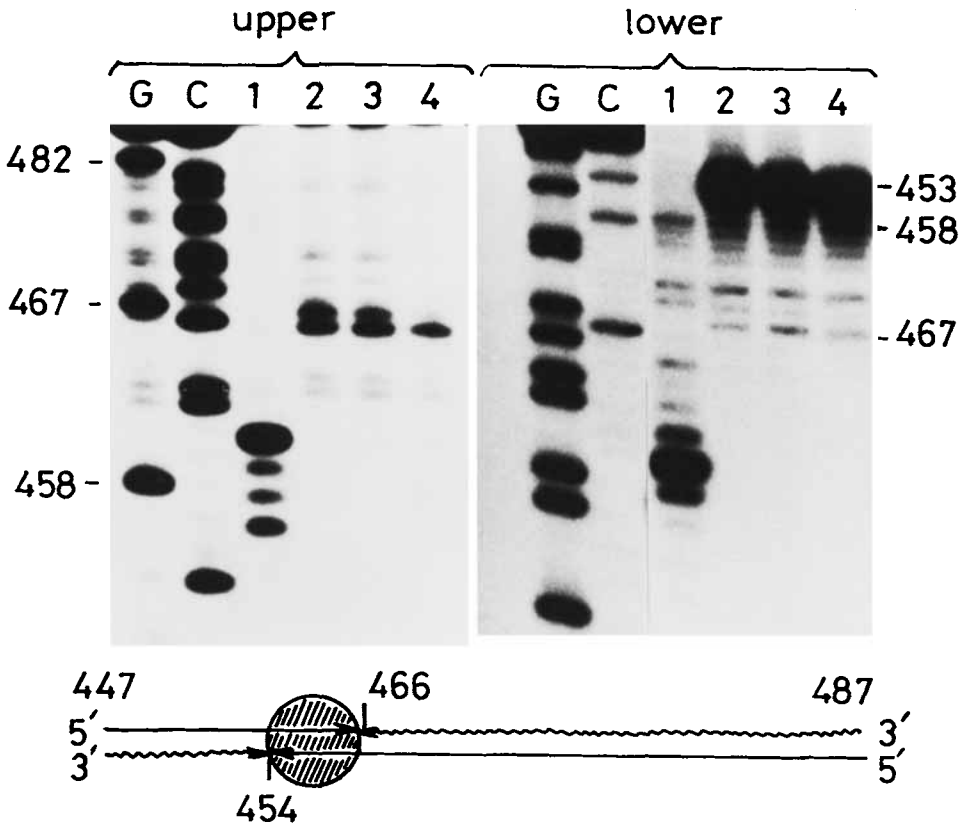

Fig. 7. Exonuclease III footprinting experiments. The strategy of the binding site mapping is diagrammatically represented under the autoradiogram. The DNA duplexes, selectively 5′-labeled either in the upper or in the lower strand, were incubated with the protein factor, followed by exonuclease III. The duplex labeled in the upper strand was digested up to nucleotide 466 ± 1 (wavy line), while the duplex labeled in the lower strand was digested up to nucleotide 454 ± 1. The binding conditions are described in the text. **Lanes 1** represent exonuclease III digests in the absence of added protein. Exonuclease III concentrations are as follows: 100 U/ml (**lanes 2**), 200 U/ml (**lanes 1 and 3**), 400 U/ml (**lanes 4**). **G** and **C lanes** represent the guanine- and cytidine-specific sequencing tracks [41], respectively. The sequence of the upper strand of the synthetic duplex J is $^{5'}$GGCCATGTCTTGTTCCAAACGCAC CAACCAACACTGAATTC$^{3'}$.

and hormone-specific manner, while extracts from other tissues had no effect on vitellogenin gene stimulation in vitro [20]. Our current results show a positive correlation between this in vitro stimulation and a DNA binding activity present in the active cell extracts. This correlation persists following fractionation of the extracts by heparin-Sepharose chromatography (cf. Figs. 3 and 4).

DNA binding competition studies and exonuclease III footprinting traced the DNA binding activity to a 14-bp motif, $^{5'}$GTCTTGTTCCAAAC$^{3'}$, situated within the third intron of the gene. The binding sites of this and one other estradiol-dependent trans-acting protein from chicken liver identified to date show certain similarities. The consensus sequence $^{5'}$GGTCANNNTGACC$^{3'}$ of the estradiol response element [7,21,33–35] and the sequence $^{5'}$GTCTTGTTCCAAAC$^{3'}$ of the above-described factor are both capable of forming secondary structures with 5 bp in the stem and 3

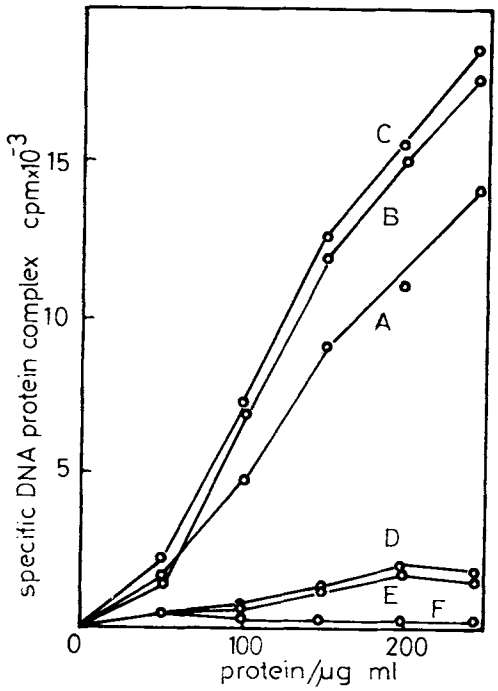

Fig. 8. The binding of the estradiol-dependent factor to DNA. **Curve A:** Duplex DNA fragment J. **Curve B:** Fragment J, lower strand. **Curve C:** Fragment J, upper strand. **Curve D:** Single-stranded sequence $^{5'}$GAATCTCTGCAGGTTTTAATT$^{3'}$. **Curve E:** Single-stranded sequence $^{5'}$TGATACA TTGAAACTTCTGGTC$^{3'}$. **Curve F:** Single-stranded sequence $^{5'}$GAAGGGCGGTCGGTGC$^{3'}$. The binding conditions were as described in the text. The product of reaction was analyzed by the gel retardation assay.

bases in the loop. Furthermore, both these sequences are flanked by a methylatible CpG dinucleotide. The demethylation of the CpG within the estradiol receptor binding site was shown to follow similar kinetics to those of the vitellogenin mRNA synthesis [7]. Interestingly, measurements made during estradiol induction of this mRNA in vivo indicated that the newly identified DNA binding activity present in the liver extracts could also be closely correlated with the kinetics of the mRNA synthesis. Upon cessation of transcription of the gene, the DNA binding activity returned to base level (data not shown). The influence of DNA methylation on the function of the latter motif should therefore also be studied. While testing 4 kb (Fig. 1) of vitellogenin DNA for the possible binding of trans-acting factors we found in the cytoplasmic liver extract only one protein binding to a specific sequence with high affinity. In contrast, for the same 4-kb DNA we found in the high salt nuclear extracts at least eight other proteins binding to the estradiol response element and to the promoter region. However, none of these nuclear proteins was hormone or organ specific (unpublished results).

Despite the apparent similarities between these two factors, a DNA fragment containing the estradiol-receptor binding site failed to displace the protein from its complex with fragment C in DNA binding competition experiments (data not shown), suggesting that these two activities exhibit sequence specificities for their respective binding sites and are thus quite distinct from one another. Surprisingly, a gene bank

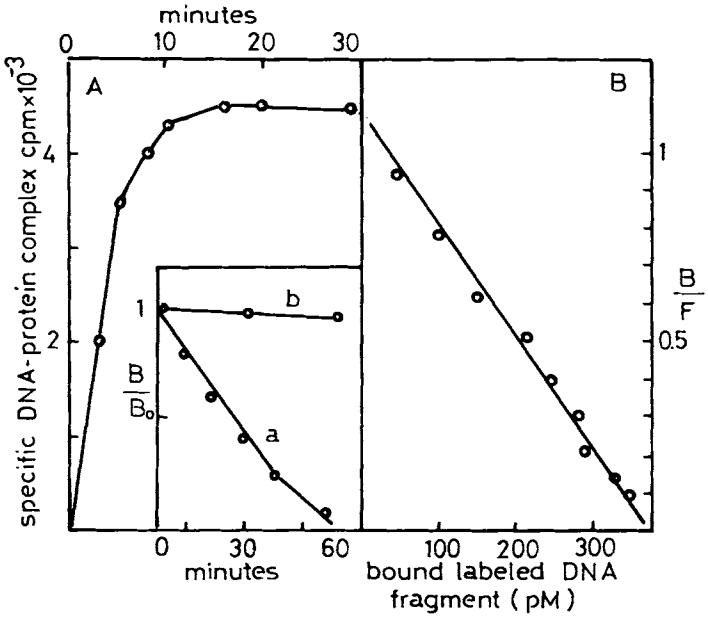

Fig. 9. Association and dissociation rates of the DNA-protein complex and estimation of the equilibrium dissociation constant. **Panel A:** 100 fmol of labeled fragment J was incubated at 23°C in 200 μl of binding buffer in the presence of a 1,000-fold-weight excess of sonicated *E coli* DNA and 30 μg of the partially purified binding protein. Aliquots were withdrawn at the time intervals indicated and rapidly filtered through nitrocellulose membranes. The rate of formation of the DNA-protein complex was estimated from the amount of filter-bound radioactivity. The inset of panel A shows the dissociation kinetics of the specific protein-DNA complex. The protein was incubated with the DNA fragment J as described above, except that after 20 min (t=0) a 100-fold-mol excess of unlabeled fragment J was added (curve a). The time course of the reaction was followed by withdrawing small aliquots from the reaction mixture as described above. The DNA-protein complex was relatively stable in the absence of unlabeled competitive DNA (curve b). B/B_0 represents the ratio of filter-bound DNA at time=t and time=0, respectively. **Panel B:** 4 μg of the partially purified protein fraction was titrated with the labeled DNA duplex J under standard assay conditions in the presence of a 1,000-fold excess of sonicated *E coli* DNA. The amount of filter-bound DNA was estimated as described. Assuming that each DNA fragment has only a single binding site, an apparent dissociation constant $K_d = 3.5 \times 10^{-10}$ was calculated. Nonspecific binding to the nitrocellulose membrane in the presence of bovine serum albumin was typically not more than 0.03% of the input labeled DNA. B and F represent the bound and free DNA, respectively.

computer search revealed only three DNA sequences with better than 85% homology to the above-described factor binding site. These are located within the v-fms oncogene of the feline sarcoma virus, the Epstein-Barr virus, and the yeast enolase (Table I). The apparent lack of homologous sequences within other estradiol-regulated genes may, however, be purely due to the lack of available sequence data.

Although the precise nature of the factors involved in the activation pathway is as yet unknown, RNA polymerase II and estradiol-receptor complex have been ruled out as potential candidates. Furthermore, as the induction of vitellogenin mRNA synthesis does not require protein synthesis [36], it seems likely that the factors required for the specific gene activation are already present in the cell, albeit in their inactive form. Estradiol treatment presumably results in the direct or, more likely, indirect activation of these trans-acting factors. Evidence for the latter activation

TABLE I. Sequence Homology of the Dyad-Symmetry Structure of the Binding Site of the Estradiol-Dependent Protein Factor With Other Genes*

Sequence	Nucleotide position	Gene	Reference
G T C T T G T T C C A A A C	453–466	Avian vitellogenin	
G T C T T G C G C C A A A C	2,152–2,165	Feline sarcoma virus	[42]
G T T T T G A T C C A A A C	253–266; 336–349 6,791–6804	Epstein-barr virus	[43]
G T G T T G C T C C A A A C	993–1,006; 820–833	Yeast enolase	[44]

*The computer search was made from the data bank of the European Molecular Biology Laboratory, Heidelberg.

pathway comes from a series of experiments [37,38], which implicated polyamines as mediators for the steroid hormone in the mouse mammary gland and in chicken liver, respectively. Polyamines can act as stimulators of nuclear protein kinases and, as our recent work shows [20], the in vitro secondary activation of the chicken vitellogenin II gene is both polyamine- and protein kinase-dependent. It will be interesting to establish whether the above-described estradiol-dependent factor requires phosphorylation for activation.

It is tempting to speculate about the possible role of this factor in the regulation of vitellogenin gene expression. As shown above (Fig. 8), the estradiol-dependent factor exhibits sequence specificity on both double- and single-stranded DNA. It is, however, unlikely to be related to any of the known single-strand binding proteins, such as the product of the *ssb* gene of *E coli*, the T4 gp32 phage protein, adenovirus DBP, or the thymus UP1 protein [for review, see 39]. All these proteins lack sequence specificity as well as having a far greater affinity for single-stranded as compared to duplex DNA. In our case the binding of the factor to a single DNA strand could result in the destabilization of the duplex, facilitating thus the progress of RNA polymerase II along the gene. It is also conceivable that this estradiol-dependent protein could influence the metabolism of the vitellogenin hnRNP. Regulation at this level would not be surprising as estradiol was already shown to increase the half-life of vitellogenin mRNA in vivo, and this at the exclusion of other mRNAs [40]. A further possibility is that the dyad-symmetry structure and the protein factor which binds to it constitute a part of a hormone-dependent enhancer [4]. Experiments aimed at elucidating the role of this estradiol-dependent DNA binding protein in the regulation of vitellogenin gene expression are currently in progress.

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