Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 429–433, January 1984
Biochemistry

Preferential binding of estrogen–receptor complex to a region containing the estrogen-dependent hypomethylation site preceding the chicken vitellogenin II gene

(estrogen–receptor binding site/DNase I protection/DNA binding assay/core enhancer)

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Communicated by Diter von Wettstein, October 6, 1983

ABSTRACT **DNA-cellulose competition binding assays were used to measure the ability of cloned DNA fragments of the chicken vitellogenin II gene to displace the estrogen–receptor complex from total chicken DNA coupled to cellulose. The DNA fragment that gave the highest competition is situated in the upstream region of the gene between nucleotides −458 and −725. This DNA fragment has four small clusters of A+T-rich sequences and contains the estrogen-dependent hypomethylation site. *In vitro* methylation of the *Msp* I site does not change the capacity of the DNA fragment to compete for estrogen–receptor complex, whereas cleavage of the C-C-G-G (*Msp* I site) results in a complete loss of competition of this fragment for estrogen–receptor complex. These results, combined with deoxyribonuclease I protection experiments, suggest that the most probable binding site for estrogen–receptor complex is . . .G-C-G-T-G-A-C-C-*G*-*G*-A-*G*-C-T-*G*-*A*-*A*-*A*-*G*-A-A-C-A-C. . . . This sequence has 73% homology with the core enhancer sequence of simian virus 40, . . .*G*-*G*-T-*G*-T-G-*G*-*A*-*A*-*A*-*G*. . . (identical bases italicized).**

One mechanism in the regulation of specific gene expression by steroid hormones is the binding of the steroid receptor complex to the chromatin (1, 2). Given the large number of receptor molecules translocated to the nucleus and the relatively small number of genes that are stimulated, Yamamoto and Alberts (3, 4) proposed a two-site model of DNA binding in which the selective action of the steroid–receptor complex is achieved through interaction of a small number of high-affinity binding sites and a large number of low-affinity sites. This binding of steroid receptor to high-affinity binding sites could be, for example, responsible for the hypomethylation of a *Msp* I site situated at −611 base pairs (bp) in the upstream region of the vitellogenin II gene (5–7). Similarly, an interaction of the steroid–receptor complex with the DNA in this region of the gene may explain the presence of a deoxyribonuclease I-hypersensitive site situated approximately 700 bp upstream from the gene (8). The appearance of deoxyribonuclease I (DNase I) hypersensitivity at −700 bp and the hypomethylation of the *Msp* I site take place only in the presence of estrogen and are both observed in the estrogen-treated liver and the oviduct. The common denominator of these two phenomena in liver and oviduct is the requirement of estrogen and therefore of nuclear receptor complex by which the effect of estrogen is expressed. We therefore formulate the working hypothesis that the steroid–receptor complex binds to the 5′ upstream region of the gene where both hypomethylation and DNase I hypersensitivity are observed. We have tested this hypothesis and demonstrate that nuclear estrogen–receptor complex has a preferential binding to a DNA fragment situated upstream from the 5′ end of the gene containing the *Msp* I hypomethylation site.

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MATERIALS AND METHODS

Preparation of Nuclear Receptor. Nuclei from the oviducts of egg-laying hens were prepared according to Best-Belpomme *et al.* (9). Estrogen receptor was extracted from purified nuclei with 500 mM KCl/50 mM Tris·HCl, pH 8.7/10 mM EDTA/2 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride. Upon centrifugation of the chromatin at 150,000 × *g* for 3 hr at 0°C the supernatant fraction was chromatographed on a column of hydroxylapatite according to Gschwendt and Schneider (10). Fractions were tested for the quantity of receptor and for its binding capacity to DNA-cellulose (11). A second chromatographic step on a hydroxylapatite column was necessary to improve the DNA binding capacity of the estrogen–receptor complex. In some experiments estrogen–receptor was first "stripped" of its endogenous estrogen by a 10-min incubation at 37°C with 5% charcoal/0.5% dextran (9).

Chicken DNA-Cellulose Competition Binding Assay. The binding and competition assay was performed according to Mulvihill *et al.* (11) with few modifications. Total chicken DNA was covalently bound to *m*-aminobenzyloxymethylcellulose according to Weideli and Gehring (12). The estrogen receptor binding buffer was 100 mM KCl/50 mM Tris·HCl, pH 8.7/5 mM EDTA/2 mM 2-mercaptoethanol. Each assay was run in triplicate.

Preparation of Cloned DNA Fragments. Fragments A, B, C, D, and E of pVT598 (see Fig. 2) were inserted into the *Sma* I site of pHP34 (13). Upon transformation and selection of the appropriate clones, the inserted DNA fragments were cut out of the plasmid with *Eco*RI and DNA fragments were separated on 1% or 1.5% low-melting-point agarose gels. Upon elution of DNA by heating in 10 mM Tris·HCl, pH 8/1 mM EDTA at 70°C for 15 min, agarose was extracted with phenol and DNA was precipitated with ethanol at −20°C for 3 hr. Treatment of DNA fragments with S1 nuclease was performed according to Mulvihill *et al.* (11). *In vitro* methylation of the C_2 DNA fragment was performed with *Hpa* II methylase as described by Wigler *et al.* (14) and demethylase activity was measured according to Gjerset and Martin (15).

DNase I Protection Experiments. Experiments were essentially done according to Schmitz and Galas (16). In an incubation mixture of 2 ml we had 100 mM KCl, 50 mM Tris·HCl at pH 8.7, 2 mM EDTA, 2 mM 2-mercaptoethanol, 2 ng (11 fmol) of 5′-end-labeled C_2 DNA fragments at a specific activity of 10^8 cpm/μg of DNA (DNA fragments were labeled only on the noncoding strand), and 100 fmol of nuclear estrogen receptor with 0.1 μM estrogen or without estrogen. One part of the "stripped" receptor was reloaded with 0.1 μM estrogen and the other part was used without estrogen. The incubation mixture was first kept at 0°C for 1 hr and then 30 min at 20°C. $MgCl_2$ was added to a final concentration of 8 mM and DNase I was 0.2–10 units/ml. After incubation for 5

Abbreviation: bp, base pair(s).

min at 20°C, the reaction was stopped with 20 mM EDTA and the mixture was extracted with phenol. DNA fragments were analyzed on an 8% acrylamide sequencing gel (17) and sequence analysis was carried out according to Maxam and Gilbert (17).

Materials. Restriction enzymes *Bam*HI and *Eco*RI were from Boehringer; *Hin*fI, *Hae* III, *Msp* I, *Hpa* II methylase, and *Hpa* II were from New England BioLabs. *m*-aminobenzyloxymethylcellulose was purchased from Miles-Yeda (Rehovot, Israel) and phenylmethylsulfonyl fluoride, from Boehringer Mannheim. [2,4,6,16,17-^{3}H(N)]Estradiol (150 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear.

RESULTS

Properties of the DNA-Cellulose Binding Assay. Seasonal variations in the level of estrogen receptor, as with the chicken oviduct progesterone receptor (11), were observed. These variations were 6-fold between winter (minimum) and summer (maximum). Therefore, care was taken to use an excess of DNA-cellulose in all competition experiments and each batch of nuclear receptor was calibrated. Fig. 1*A* (curve a) shows a calibration curve obtained after equilibration of the binding reaction. Curve b of Fig. 1*A* shows that binding of the estrogen–receptor complex is abolished in the presence of DNase I at 10 μg/ml. Curve c of Fig. 1*A* shows that the binding of estrogen–receptor complex to DNA is protein dependent because the binding is abolished by proteinase K at 10 μg/ml. This result suggests that ^{3}H-labeled estrogen does not bind directly to DNA-cellulose. The binding of estrogen–receptor complex to DNA, like that of other steroid receptors, is also dependent on the salt concentration. Fig. 1*B* shows that above 0.1 M KCl there is a progressive dissociation of the estrogen–receptor complex from DNA. The temperature at which the receptor is loaded with ^{3}H-labeled estrogen (5.0 nM) has an influence on its subsequent capacity for binding to DNA. Fig. 1*C* shows that the loading of the receptor at 37°C for 30 min resulted in only 2% of the receptor molecules binding to DNA, whereas an incubation of the same receptor preparation with ^{3}H-labeled estrogen at 0°C for 5 hr resulted in 35–40% binding of the receptor to DNA. Preliminary experiments suggest that the temperature sensitivity may be due to protease activity present in the receptor preparation. Under our experimental conditions an incubation of 30–45 min at 20°C was sufficient to obtain equilibrium of the binding of estrogen–receptor to DNA-cellulose (Fig. 1*D*).

Competition Binding Assay with Purified Cloned DNA Fragments. The aim of the experiments was to determine the strongest binding site for the estrogen–receptor complex in the DNA preceding the vitellogenin gene. Fig. 2 shows the map of the different DNA fragments tested in the competition experiments. Fig. 3*A* shows the results obtained with five fragments covering 5,800 bp of the 5′ end and the flanking region of the vitellogenin II gene. For the same concentrations of competing DNA, fragment C gave the strongest competition. Fragments A and B, which cover the first 2,000 bp of the vitellogenin gene, gave the poorest competition with total chicken DNA. Fragment C was further cut with restriction enzymes into smaller pieces (Fig. 2), and these fragments were also tested in the competition assay. Fig. 3*B* shows that fragment C_2 is bound preferentially by estrogen–receptor complex in comparison with other C fragments. This fragment contains the DNase I-hypersensitive site (8) and the hypomethylation site *Msp* I (5–7). It is known that the *Msp* I site of fragment C_2 undergoes hypomethylation *in*

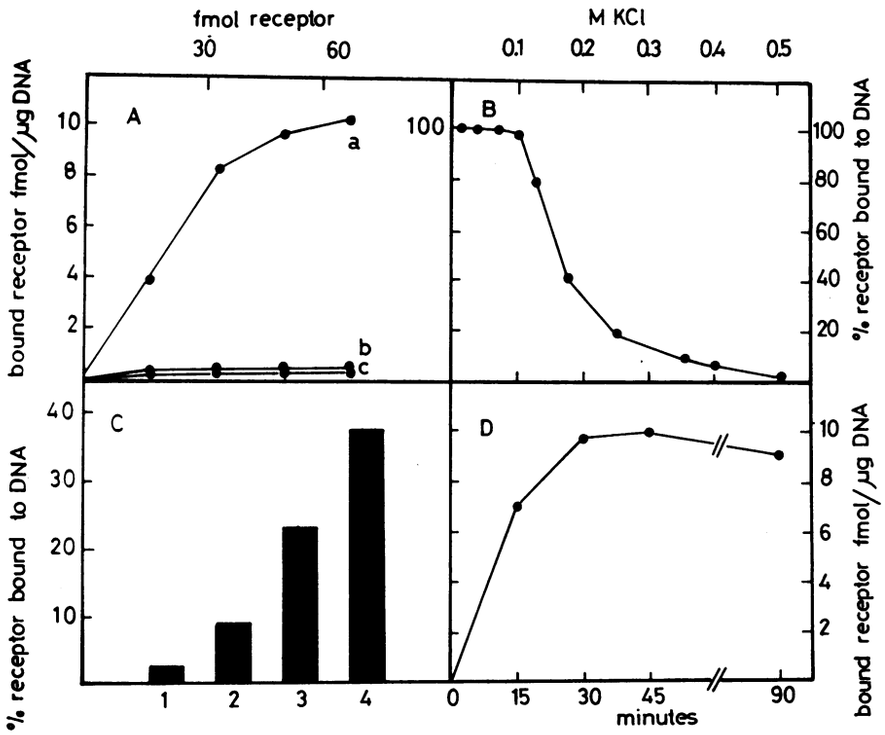

FIG. 1. Properties of the DNA-cellulose estrogen–receptor binding assay. (*A*) Binding kinetics. Curve a, increasing concentrations of nuclear estrogen–receptor complex incubated with a constant amount (4 μg) of chicken DNA bound to cellulose. Curve b, in the presence of DNase I at 10 μg/ml. Curve c, in the presence of proteinase K at 10 μg/ml. (*B*) Effect of increasing concentrations of KCl on the binding of estrogen–receptor complex to chicken DNA-cellulose. In each incubation mixture we had 30 fmol of receptor per 4 μg of chicken DNA bound to cellulose. (*C*) Effect of the temperature of the "loading" of estrogen receptor on subsequent binding to DNA. Estrogen receptor was incubated with 50 nM ^{3}H-labeled estrogen at 37°C for 30 min (bar 1), 30°C for 30 min (bar 2), 4°C for 1 hr (bar 3), or 0°C for 5 hr (bar 4) and then tested in the DNA-cellulose assay. (*D*) Time course of the binding of ^{3}H-labeled estrogen–receptor complex to DNA-cellulose at room temperature. Four micrograms of chicken DNA bound to cellulose was incubated for the time indicated with 30 fmol of estrogen–receptor complex.

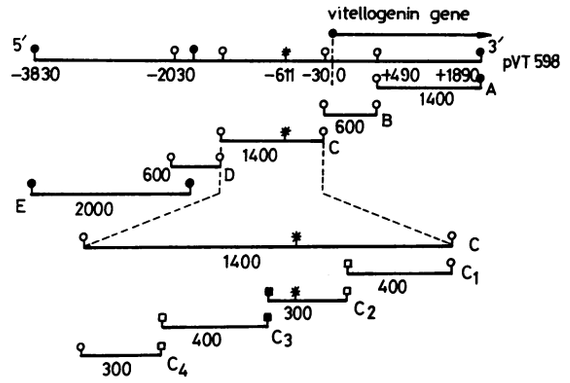

FIG. 2. Restriction map of the cloned DNA fragments used in the DNA-cellulose competition assay. ●, *Bam*HI; ○, *Eco*RI; ⁎, *Msp* I/*Hpa* II; ■, *Hin*fI; □, *Hal* III. The size of individual DNA fragments are given in bp.

vivo as an effect of estrogen (5–7), and it was of interest to see whether methylation of this site *in vitro* would affect the binding of estrogen–receptor complex to DNA. As seen in Fig. 3*C* (curves a and b) methylation of the *Msp* I site had no influence on the receptor binding. In a control experiment, no methylase activity could be detected in the receptor preparation (data not shown). Fig. 3*C* (curve c) shows that *Msp* I cleavage of fragment C_2 abolished the binding capacity of fragment C_2 for the estrogen–receptor complex. Consequently the putative binding site of estrogen–receptor must include or lie adjacent to the *Msp* I site.

DNase I Protection Experiment. Since in our experiments we used semipurified estrogen–receptor preparations, we exploited the unique feature of the steroid receptor to bind DNA only in the presence of the appropriate steroid ligand and compared the results obtained with the same preparation of receptor without the hormone. Fig. 4 shows the results obtained for the middle portion (noncoding strand) of the fragment C_2. In the presence of estrogen we observed in this area at least three stretches 20–30 nucleotides long protected by the estrogen–receptor complex. Two consist of A+T-rich regions and the other is composed of a sequence of 20–30 nucleotides containing the *Msp* I site. The two A+T-rich regions, A and C (Figs. 4 and 5; see ref. 7 for the entire DNA sequence), are situated between nucleotides −550 and −584 (34 bp, 82% A+T) and between −628 and −658 (30 bp, 83% A+T). Another region of the C_2 DNA fragment, covering nucleotides −458 to −545, did not show any DNase I protection with estrogen–receptor complex in comparison with estrogen receptor without estrogen or DNA (data not shown).

DISCUSSION

Several difficulties were encountered in the equilibrium competition binding assay for measuring the extent of displacement of estrogen–receptor complex from chicken DNA-cellulose by cloned genomic DNA fragments. Besides the seasonal variations in the level of nuclear estrogen receptor, we found that the receptor preparation could not bind to DNA unless it had been purified on a hydroxylapatite column, thereby eliminating exogenous DNA. Most receptor preparations also were temperature sensitive and an incubation at 37°C for 30 min resulted in reduced ability to bind to DNA (Fig. 1*C*). Preliminary experiments indicate that the reduction of the DNA binding ability of the estrogen–receptor complex is due to protease(s) present in the receptor preparation.

When different DNA fragments were compared in their ability to bind the estrogen–receptor complex in the competition experiment we found that the first 2,000 bp of the vitellogenin gene gave much less competition than the DNA region upstream of the gene. In the upstream region, the DNA fragment C_2 gave the highest competition. This fragment contains four clusters of A+T-rich sequences, the DNase I-hypersensitive site (8), and the *Msp* I hypomethylation site (5–7) (Fig. 5). The density of A+T-rich sequences, however, is not sufficient to explain the preferential binding of estrogen–receptor complex to this DNA fragment; C_1 (compare Fig. 3*B* and Fig. 5) has as many clusters of A+T-rich sequences without giving the same competition as fragment C_2. On the other hand, that estrogen–receptor binds preferentially to A+T-rich double-stranded DNA (18) could explain the weak binding of receptor to DNA fragment C_1. Results from DNase I protection experiments suggest that in the region of the *Msp* I site shown in Fig. 4 there are three areas protected by estrogen–receptor complex, compared

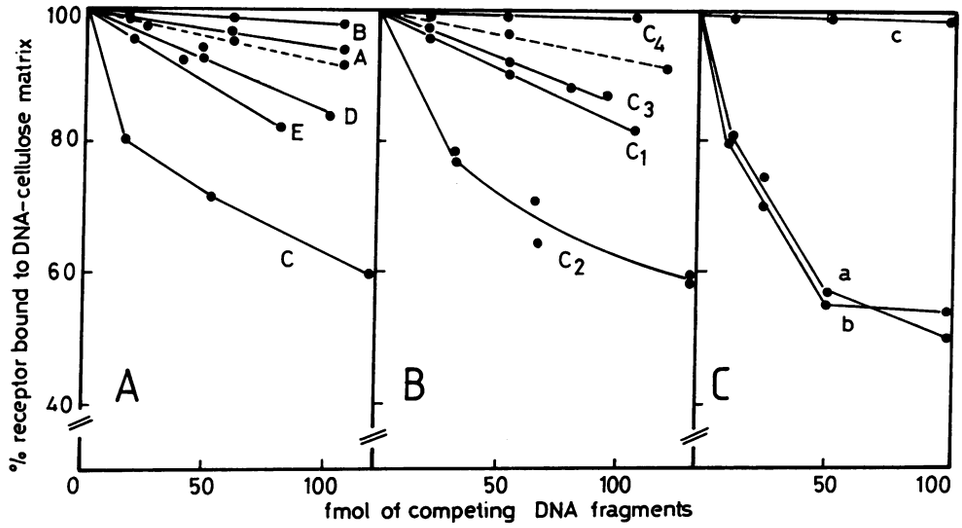

FIG. 3. Competition assay with cloned DNA fragments from the 5′ end and the upstream region of the chicken vitellogenin II gene. All experiments illustrated have been carried out with the same preparation of estrogen receptor. (*A* and *B*) Competition assay with cloned purified genomic DNA fragments A, B, C, D, and E, and subfragments of C described in Fig. 2. The broken line represents the competition with total chicken DNA. (*C*) Effect of methylation (curve a) versus no methylation (curve b) and effect of the cleavage of the *Msp* I site of C_2 DNA fragment on the competition assay (curve c).

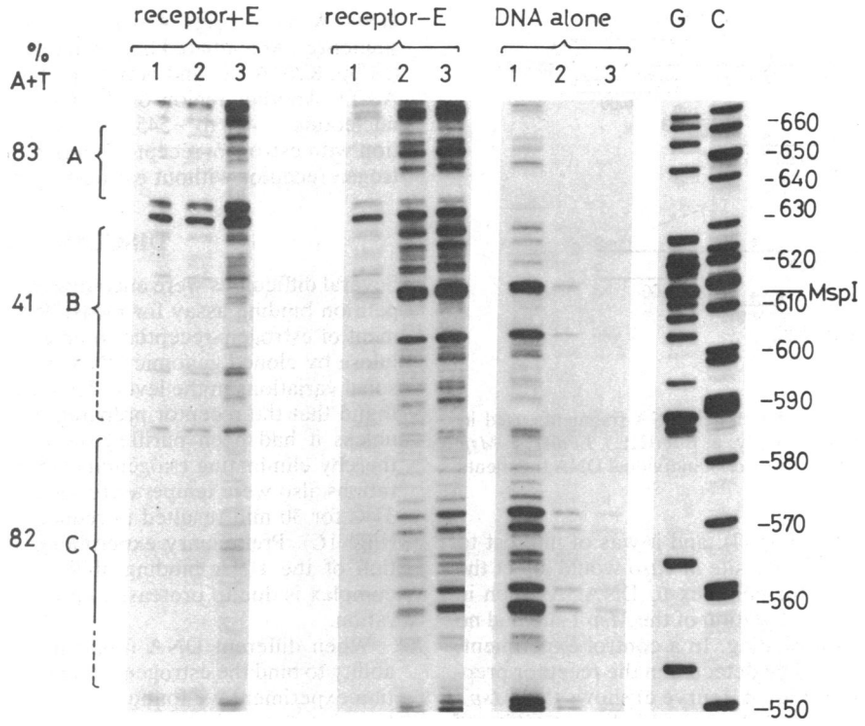

FIG. 4. DNase I protection experiment. DNAs in lanes 1, 2, and 3 were digested with 2, 4, and 8 units of DNase I per ml, respectively. The protected areas A, B, and C were determined by comparison of tests carried out in presence of receptor with and without estrogen. HS stands for DNase I-hypersensitive site and is also determined by comparison of tests made with receptor with and without estrogen. The sequence ladder of G and C nucleotides indicates the positions of nucleotides in the upstream sequence of the gene. The horizontal bars between the two sets of lanes 1–3 of receptor with or without estrogen (E) indicate where the major differences in DNase I sensitivity occur.

with controls with the receptor without estrogen. One site covers the *Msp* I and neighboring sequences and the other two are on the A+T-rich flanking sequences (Figs. 4 and 5). These protected areas are separated by DNase-hypersensitive sites. Because the integrity of C-C-G-G (*Msp* I site) is required for the binding of the estrogen–receptor complex to the C_2 DNA fragment (Fig. 3), we suggest that the sequence protected between the two DNase I-hypersensitive sites and containing the *Msp* I sequence is the most likely estrogen–receptor binding site on the upstream region of the gene (Fig. 4). The protection of A+T-rich sequences by estrogen–receptor complex may represent lower affinity binding sites since preliminary experiments show that in the presence of

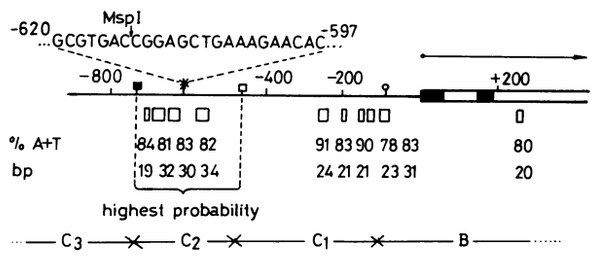

FIG. 5. Summary of the organization of the 5′ end of the chicken vitellogenin II gene and the location of a receptor binding site inferred from competition experiments and DNase I protection experiments. The open boxes below the map show the location of A+T-rich (≥78%) sequences that are 19 bp or longer (7). The length and percent A+T compositions of these sequences are shown below each box. Fragment C_2, which gave the highest competition, is designated as the most probable binding site and the sequence of the binding site is given above the map. ○, *Eco*RI; □, *Hea* III; ■, *Hin*fI; ⋆, *Msp* I/*Hpa* II. The solid bars represent the first and second exon and the adjacent open bars represent the introns of the vitellogenin gene.

0.2 M KCl these sites were not protected by the estrogen–receptor complex (data not shown). The numerous A+T-rich sequences on the upstream region of the gene (Fig. 5) may represent the low-affinity binding sites defined by Yamamoto and Alberts (3, 4) and the protected sequence between −597 and −620 could be the high-affinity binding site for the estrogen–receptor complex. The above considerations could explain the existence of two different sequences for the binding site of the chicken progesterone–receptor complex (11, 19). The putative estrogen–receptor binding site, . . .G-C-G-T-G-A-C-C-G-G-A-G-C-T-G-A-A-A-G-A-A-C-A-C. . ., has no homology with the progesterone–receptor binding sites (11, 19), but it has 73% identity with the simian virus 40 core enhancer sequence, . . .G-G-T-G-T-G-G-A-A-A-G. . ., which determines the constitutive expression of the gene (20). However, we find a major difference, the first G of the sequence G-G-A-A-A-G, which in simian virus 40 is absolutely required for gene expression (20), is replaced in the estrogen–receptor binding site by a T. What role the estrogen–receptor complex plays in the upstream region of the gene and how it does it is still not known, but it is almost certain that alone it cannot determine the organ-specific expression of vitellogenin gene. Most probably some other organ-specific nuclear nonhistone proteins may play a key role (21).

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