An Estradiol-dependent Protein from Chicken Liver Binds Single-stranded DNA and RNA*

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We have identified two estradiol-dependent single-stranded DNA binding proteins in the nucleus and cytoplasm of chicken hepatocytes that bind the sequence 5’TACACCTCGATATG3’ in the first exon of the chicken vitellogenin gene. As judged by chromatography on heparin-Sepharose and by proteolytic clipping experiments performed with bromouridine-substituted single-stranded RNA, reveal that an estradiol-dependent hepatocyte cytoplasmic protein with a Mₐ of 71,000 binds to the mRNA-like single-stranded RNA.

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It is generally accepted that the rate of transcription is of fundamental importance in regulating the level of mRNAs in the cell (1, 2). Individual mRNA species have widely differing half-lives, and the stability of many mRNAs varies dramatically in response to hormone stimuli and stages of the cell cycle (1, 3, 4, 5-10). One of the most striking examples is the 30-fold increase in vitellogenin mRNA stability following estradiol treatment of Xenopus laevis hepatocytes (1).

Using the ssDNA 5’TACACCTCGATATG3’ from the first exon of the vitellogenin gene we show in this paper that ssDNA binding activities found in both the nuclei and cytoplasm of chicken cells are estradiol-induced, that they are distinct from previously described dsDNA binding activities (11, 12), and that the cytoplasmic protein recognizes specific features of the nucleotide sequence. Since the ability of a protein to bind ssDNA may reflect its capacity to bind RNA (13), we have also examined the possibility that the cytoplasmic protein in fact binds to the mRNA.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 ligase, T4 polynucleotide kinase, and T7 RNA polymerase were from Boehringer Mannheim and Biofine (Praroman, Switzerland). [γ-³²P]ATP (3000 Ci/mmol) and [α-³²P]CTP (3000 Ci/mmol) were from Amersham Corp. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 380A synthesizer and purified as described (12).

Hormone Treatment of Animals—Mature and immature white Leghorn roosters were treated with a single intramuscular injection of 17β-estradiol (40 mg/ml propylene glycol, 40 mg/kg of body weight) into the leg.

Preparation of Cell Extracts—Twenty-four hours after administration of estradiol, livers from hormone-treated or control animals were perfused with cold NaCl (150 mM) and then homogenized in 4 volumes (v/w) of ice-cold buffer (50 mM Tris·HCl, pH 8, 5 mM MgCl₂, 2 mM dithiotreitol, 20 mM β-glycerophosphate, 0.5 mMphenylmethylsulfonyl fluoride, 25% glycerol) with 4–8 strokes of a loose-fitting glass homogenizer at 1500 rpm. Following centrifugation (120,000 × g for 4 h) the supernatant was stored in small aliquots in liquid nitrogen. Liver nuclei were prepared and then extracted with 400 mM (NH₄)₂SO₄, according to published procedures (14, 15). Heparin-Sepharose chromatography was carried out as described (12).

Protein-Nucleic Acid Interactions—The gel retardation and competition assays were performed as described (12, 16, 17). As a template for the synthesis of the RNA competitor used in RNA competition assays, the 130-base pair DNA fragment extending from the EcoRI site at position +73 to the HindIII site at position +63 at the start of the chicken vitellogenin gene (18, 19) was cloned into the vector pSP715 (Pharmacia LKB Biotechnology Inc.). Full-length RNA was purified on a denaturing 8% polyacrylamide gel.

Missing Contact Probing the Protein-DNA Interaction—Specific contacts between protein and DNA were tested essentially as described by Brunelle and Schleif (20). The bound and free DNA were separated on a 1% low melting agarose gel, extracted, and subjected to piperidine cleavage. The resulting fragments were analyzed on a 20% sequencing gel.

Proteolytic Clipping Bandshift Assay—This was carried out using trypsin, as described by Schreiber et al. (22).

Protein-RNA Cross-linking—For cross-linking experiments 250 μM bromo-UTP and 250 μM UTP replaced 500 μM UTP in the RNA synthesis reaction. Protein-RNA cross-linking with UV irradiation was performed essentially as described by Moore et al. (21).

RESULTS AND DISCUSSION

To determine whether the ssDNA and dsDNA binding activities observed between nucleotide positions +3 and +16 of the chicken vitellogenin gene (11) were mediated by the same protein, heparin-Sepharose fractions of a liver nuclear lysate from mature hens were screened by gel retardation assay for their ability to bind the single- and double-stranded oligodeoxyribonucleotide (Fig. 1A). The single-stranded DNA binding protein (ssDBP) was found in the flow-through (0.1 M KCl) from the heparin-Sepharose column, whereas the major double-stranded binding protein designated as NHP4 (11), was eluted with 0.4–0.5 M KCl. This shows that the ssDBP and NHP4 are distinct from one another. Moreover, this experiment confirms that the ssDBP binds specifically to the upper strand which has the nucleotide sequence 5’TACACCTCGATATG3’. Either the synthesis or the nucleic acid binding activity of the ssDBP is hormone-induced, as it was observed in the nuclear extracts of hen liver and oviduct but not rooster liver (Fig. 1B).

Fig. 2 shows that the same single-stranded oligonucleotide
**FIG. 1.** An estradiol-dependent nuclear protein binds ssDNA from the start of the chicken vitellogenin gene. Gel retardation assays were carried out (A) with 5 μg of protein from heparin-Sepharose fractions of hen hepatocyte nuclear extract (lanes 1–6 represent 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M KCl, respectively), 1 μg of E. coli DNA, and 0.5 ng (~100,000 cpm) of single-stranded oligodeoxyribonucleotide, upper strand (US, 5′TCACCTTCGCTATG3′), lower strand (LS, 5′CATAGCGAAGGTGAB′) and double-stranded (DS) DNA, respectively, and (B) with 10 μg of protein from the same heparin-Sepharose fractions as in A of hen liver (HL), oviduct (HO), and rooster liver (RL) nuclear extracts and the upper strand oligodeoxyribonucleotide. The protein-bound (b) and free (f) DNA are indicated.

was also bound by an estradiol-inducible cytoplasmic factor found in both the oviduct and hepatocytes of estradiol-treated immature chicks. Like the nuclear protein, it was specific for the upper strand and did not bind the duplex form of the oligodeoxyribonucleotide. No DNA binding activity was detected in the cytoplasm of livers from untreated immature chicks or roosters. In contrast to the nuclear protein, the cytoplasmic protein was eluted from heparin-Sepharose by 0.3 M KCl, suggesting that the two proteins may be different. This was confirmed using the proteolytic clipping bandshift assay described by Schreiber et al. (22), where trypsin digestion of the nuclear and cytoplasmic proteins resulted in different banding patterns (Fig. 3). The more abundant cytoplasmic protein was used in subsequent experiments.

The fact that the ssDBPs have a distinct preference for oligonucleotide 5′TCACCTTCGCTATG3′ rather than its complementary strand shows that their DNA binding is to some extent sequence-specific.

To examine the contact points of the cytoplasmic ssDBP with DNA, we tested the effect of the partially depurinated and depyriminated single-stranded oligonucleotide on protein binding (Fig. 4). The removal of any one of the five bases, CCTT-G, at the center of the oligodeoxyribonucleotide severely interfered with the protein-DNA interaction. Comparison of this sequence with the sequences of other oligodeoxyribonucleotides that were strong competitors does not reveal any striking similarities in the sequence (data not shown), supporting the view that this protein does not have a strict recognition sequence. However, all the strong competitors

**FIG. 2.** An estradiol-dependent cytoplasmic protein binds ssDNA from the start of the chicken vitellogenin gene. A, heparin-Sepharose fractions of hen oviduct cytoplasmic extract (8 μg of protein/assay, lanes 1–5 represent 0.1, 0.2, 0.3, 0.4, and 0.5 M KCl, respectively) were screened by gel retardation assay with upper strand (US), lower strand (LS), and double-stranded (DS) oligodeoxyribonucleotide. B, 0.5 ng of labeled upper strand oligodeoxyribonucleotide (5′TCACCTTCGCTATG3′) was tested as above with the heparin-Sepharose fractions (5 μg of protein/assay) of the liver cytoplasmic extracts from estradiol-induced (+E2, secondary stimulation) and uninduced (−E2) immature chickens.

**FIG. 3.** Proteolytic clipping bandshift assay. Protein-DNA complexes formed between nuclear or cytoplasmic extracts and the upper strand oligonucleotide were subjected to limited proteolysis with trypsin (lanes 1–8 represent no enzyme, 0.02, 0.04, 0.08, 0.2, 0.4, 2, and 10 units of enzyme, respectively. Undigested complex (D) and proteolytic products (→) are indicated.
contain the sequence YTNG, where Y is one or more pyrimidine residues and N is any nucleotide. Statistically this sequence should occur often in a given stretch of DNA, so our results are not at variance with the accepted view that, while displaying a preference for certain nucleotides (23), ssDBPs do not usually recognize highly specific sequences (13).

Proteins binding to ssDNA often have a significant affinity for ssRNA (24, 25). We have demonstrated the ability of the ssDBPs to binding RNA in two ways. In the first, RNA corresponding to the upper DNA strand from -73 to +53 of the chicken vitellogenin gene was used to compete for the binding of the ssDBP to the labeled oligonucleotide. Fig. 5A shows that this ssRNA could compete for the binding of the cytoplasmic protein more efficiently than either Escherichia coli tRNA or yeast rRNA. In the second, the protein was cross-linked to the ssRNA. Fig. 5B shows a protein, M, 71,000, from the hepatocyte cytoplasm of estradiol-treated immature chickens and a hormone-independent protein, M, 126,000, bind to this ssRNA in vitro. Dreyfuss and co-workers (26) have shown that the two major bands of heterogeneous RNA ribonucleoproteins detected by Coomassie Blue staining have a similar size (68,000 and 120,000, respectively). The band at M, 68,000 represented a cluster of proteins of which two have been shown to bind preferentially to pyrimidine-rich RNA (27), and it may be that the 71-kDa protein described here is a member of that family of proteins. Two less abundant proteins (M, 74,000 and 85,000) whose activities are reduced by estradiol also appear to bind this RNA, but their significance remains to be determined.

In view of this estradiol dependence and ability to bind ssRNA, it is tempting to think that the cytoplasmic protein plays a role in the hormone-stimulated stabilization of mRNA. Similarly Brock and Shapiro (1) have proposed that a trans-acting protein could be responsible for the massive increase in vitellogenin mRNA stability upon estradiol treatment of X. laevis hepatocytes. There is a substantial body of evidence suggesting that certain structural features or cis-acting sequences at both the 5' and 3' ends of mRNA mediate their characteristic rates of degradation (9, 28–35). Recent experiments with chicken very low density apolipoprotein II and vitellogenin mRNAs suggest that estradiol regulates a specific degradative activity that only functions once estradiol is withdrawn (36). So an alternative possibility is that during estradiol treatment the RNA binding protein marks the RNA as a target for subsequent degradation upon withdrawal of the hormone. Experiments are currently in progress to test these hypotheses.
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