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IN VIVO AND *IN VITRO* PROTEIN–DNA INTERACTIONS AT THE DISTAL OESTROGEN RESPONSE ELEMENT OF THE CHICKEN VITELLOGENIN GENE: EVIDENCE FOR THE SAME PROTEIN BINDING TO THIS SEQUENCE IN HEN AND ROOSTER LIVER

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Summary—The major egg white protein, vitellogenin, is synthesized in a tissue specific and oestradiol dependent manner in the liver of egg-laying hens. In this paper, we describe a detailed study of the protein–DNA interactions at the distal oestrogen response element (ERE_D) located 600 bp upstream of the start of transcription. *In vivo* footprinting of hepatocytes from adult hens and roosters with 0.5–0.0005% dimethylsulphate (DMS) revealed, at critical concentrations of DMS, protection of distinct guanosine residues within the ERE_D and adjacent downstream sequence in both cases. From this, it was concluded that there were proteins present in both tissues binding to this region *in vivo*. *In vitro* studies using missing base contact probing and proteolytic clipping band shift assays with hen and rooster liver nuclear extracts identified the ERE binding protein to be the same or very closely related in both tissues. Furthermore, the protein from rooster nuclear extracts bound to the ERE sequence even when the DNA was methylated at CpG dinucleotides. u.v. cross-linking experiments performed with bromodeoxyuridine substituted ERE, revealed that a nuclear protein with M_r of about 75,000–80,000 bound specifically to this sequence. These studies demonstrate that apart from the oestrogen receptor, at least one other protein can interact specifically with the chicken vitellogenin ERE, independently of hormonal expression of the gene.

INTRODUCTION

Steroid hormones regulate the transcription of specific genes via intracellular receptor proteins that interact with *cis*-acting DNA elements associated with target genes [for reviews see Refs 1–3]. Steroid receptors therefore represent an important class of *trans*-acting factors that are activated on binding their cognate ligand.

The 13 bp motif 5′ **GGTCANNNTGACC** 3′ has been identified upstream of a number of oestrogen responsive genes [4–8], and has been functionally and structurally defined as an ERE [9–12]. Two EREs have been delineated upstream of the chicken vitellogenin gene, at nucleotide positions −613 and −335 [13]. Upon oestradiol activation of the gene a number of changes occur in the upstream flanking sequence. These include the appearance of DNAse I hypersensitive sites [14] and changes in the methylation state of specific CpG dinucleotides, that occurred in a strand specific manner and in parallel to the onset of transcription [15]. Recently, protein–DNA complexes have been visualized at the ERE of the Xenopus vitellogenin B2 gene [16]. The size of the complexes observed suggested that more than one protein may be binding to this sequence.

Previous work from our laboratory has shown, that in DNA competition studies the oestrogen receptor preferentially bound to fragments of the upstream region containing the distal ERE [17]. More recently, we have shown that a ubiquitous non-histone protein bound with high affinity to a synthetic DNA fragment containing the ERE [18, 19]. This protein did not bind [^{3}H]oestradiol and did not crossreact with monoclonal antibodies directed against the oestrogen receptor [18 and J. P. Jost, unpubl. observations].

In order to understand the mechanism whereby upstream elements influence the

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activity of target promoter sequences, it is important to have a comprehensive picture of the protein–DNA interactions at such elements. In the present study we have examined the protein–DNA interactions at the distal ERE of the chicken vitellogenin gene *in vivo*, using the technique of genomic footprinting with DMS [see Ref. 20 and references therein]. The binding of a protein(s) from hen and rooster liver nuclear extracts was then compared *in vitro* by proteolytic clipping bandshift assay, u.v. cross-linking and footprinting studies with partially depurinated and depyrimidinated DNA.

EXPERIMENTAL

Animals

Adult White Leghorn egg-laying hens and roosters were used throughout these experiments. Where indicated adult roosters received a single subcutaneous injection of 40 mg 17β-oestradiol/kg body wt, 24 h prior to sacrificing.

In vivo footprinting

Genomic footprinting was carried out as described previously [21]. Briefly, cell suspensions were prepared by digestion of 10 g of minced liver with collagenase (0.5 mg/ml) and hyaluronidase (1.0 mg/ml). Preparation of cell suspensions from liver tissue with trypsin gave inconsistent results (I. J. McEwan and J.-P. Jost, unpublished observations). Cells were resuspended in Dulbecco's modified Eagle's medium ($\simeq 1$–2×10^8 cells/ml) and treated with different concentrations of DMS (0.5–0.0005%) for 5 min at 20°C. After quenching the reaction with cold saline buffer containing 50 mM-Hepes pH 7.5, 100 mM β-mercaptoethanol and 1% bovine serum albumin, the crude nuclei were isolated using 1% NP-40 and digested overnight with Proteinase K (300 μg/ml, Boehringer Mannheim GmbH). The genomic DNA was subsequently recovered, and treated as described for genomic sequencing [22, 23].

Protein extracts

Nuclear extracts from hen and rooster liver were prepared as described [24]. The dialyzed crude nuclear fractions were chromatographed on heparin Sepharose (Pharmacia) and the protein eluted from the column by a stepped KCl gradient. For these studies the 0.3–0.4 M KCl fraction was routinely used; proteins were precipitated with 70% ammonium sulphate, and dialyzed against 25 mM Hepes (pH 8.0), 1 mM EDTA, 10% glycerol, 2 mM β-mercaptoethanol buffer. Protein fractions were stored as aliquots at −80°C.

Labelled DNA

Oligonucleotides were prepared as described before [24], and end-labelled with T4 polynucleotide kinase (Biofinex, Switzerland) using adenosine-5′-[γ-^{32}P]triphosphate (3000 Ci/mmol; Amersham, U.K.) [25].

Biochemical studies

The oestradiol binding assay in the nuclear fractions was carried out according to Best-Belpomme *et al.* [26] using 17β-[2,4,6,7,10-^{3}H]oestradiol (130 Ci/mmol; Amersham, U.K.). The relative binding activities with double-stranded oligonucleotide (ERE) were calculated by quantitative gel shift analysis [24]. The relative concentrations of vitellogenin mRNA in the hen, rooster and induced rooster liver were determined by molecular hybridization as previously described [27].

Proteolytic clipping bandshift assay

Labelled double stranded DNA (0.1 ng) was incubated with 5 μg of protein in the presence of 2.5 μg non-specific DNA competitor for 10 min at 23°C. Protein–DNA complexes were then partially digested with either trypsin or V8 protease, essentially as described [28]. A range of concentrations for each enzyme was used: 0–20 μg of trypsin or 0–8 μg of V8 protease per reaction mixture. The protein–DNA complexes were incubated with each enzyme for 10 min at 23°C before loading directly onto a 4% native polyacrylamide gel.

Missing base contact probing

Missing base contact probing was carried out essentially as described by Brunelle and Schleif [29], except that the labelled DNA was partially depurinated (G + A reaction) by formic acid treatment at 20°C for 5 min. After the protein–DNA binding reaction (see above), the bound and free DNA were separated on a 1% low gelling agarose gel, eluted, cleaved with piperidine (0.1 M at 90°C for 30 min) and subsequently analyzed on a 20% sequencing gel.

u.v. cross-linking studies

Labelled DNA containing 5-bromodeoxyuridine was synthesized by "fill in" reaction using Klenow polymerase. 0.15 ng of

labelled double stranded DNA was incubated with 5 μg of protein extract and 2.5 μg of non-specific competitor DNA for 15 min at 23°C. Samples were u.v. light irradiated for 5 min at room temperature. $MgCl_2$ and $CaCl_2$ were each added to a final concentration of 10 mM, and the DNA digested with DNaseI (2.5 μg/sample) and micrococcal nuclease (1 U/sample) for 5 min at 37°C. The cross-linked products were then resolved by SDS–polyacrylamide gel electrophoresis.

RESULTS

In vivo DMS footprinting of the expressed and silent chicken hepatic vitellogenin II gene

Hepatocytes in suspension were treated with varying concentrations of DMS and the genomic DNA after isolation, digestion with Hinf I and piperidine treatment was examined on an 8% sequencing gel. The resolved target DNA fragments were visualized by indirect end-labelling with radiolabeled probes for the upper and lower strands respectively [22, 23]. Results obtained were compared for at least two to three independent experiments for each strand.

Figure 1 shows the genomic footprints obtained for the upper and lower DNA strands of the vitellogenin gene upstream region containing the ERE_D and GRE sequences, in hen and rooster livers. High concentrations of DMS (0.5 and 0.05%) resulted in a strong reaction with almost all the guanosine residues (Fig. 1, lane 1). Decreasing the concentration of DMS to as low 0.0005% produced a more selective reaction. The results show that the ERE was protected (Fig. 1, ■), and that only minor differences in the protein–DNA interaction pattern between adult egg laying hens and roosters were visible. Similarly, a region of protection was seen in both hen and rooster liver for the adjacent downstream sequence (nucleotide positions −609 to −585; Fig. 1, GRE). This region has been shown to act synergistically with the ERE in transfection studies [30, 31]. Furthermore, this sequence binds the glucocorticoid [30, 32] and progesterone [31] receptors *in vitro*.

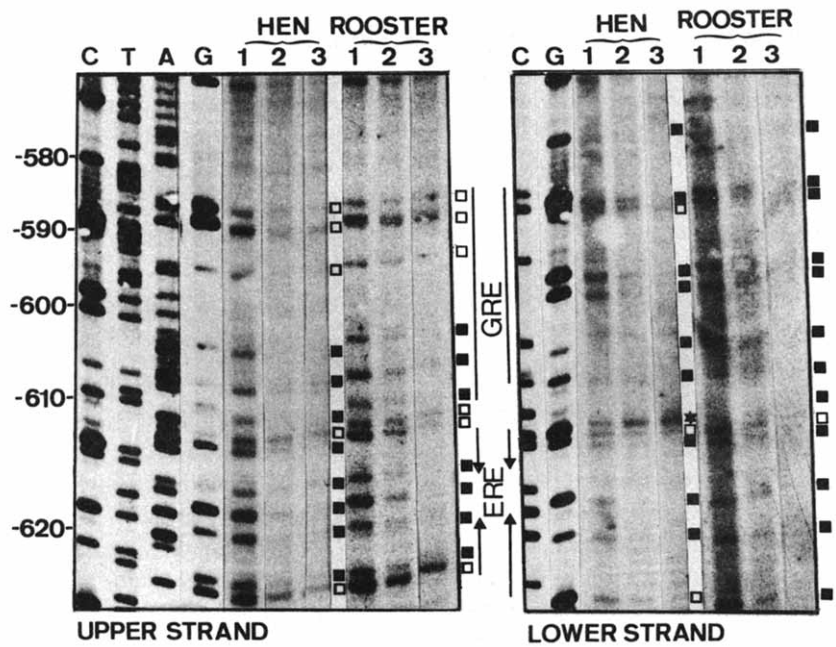


Fig. 1. *In vivo* DMS footprinting of the vitellogenin ERE_D from adult egg-laying hen and rooster livers. Genomic DNA was digested with Hinf I and 50 μg DNA/sample treated with piperidine and resolved on an 8% sequencing gel, electroblotted and hybridized with a labelled single stranded DNA probe (Upper Strand). After autoradiography, the Genescreen filter was stripped of probe and rehybridized with a labelled probe specific for the complementary strand (Lower Strand). Lanes 1–3 represent decreasing concentrations of DMS: 0.5, 0.05 and 0.0005% for hen and 0.05, 0.005 and 0.0005% for rooster respectively. The position of the ERE is indicated (arrows), and the boundaries of the DNase I footprint of the glucocorticoid receptor [32] are shown (GRE). Protected guanosine residues in both hen and rooster liver are indicated by closed boxes (■). The open boxes (□) represent signals that become weaker with decreasing DMS, but which remain clearly visible even at the lowest DMS concentrations. The star (*) indicates the appearance of a strong signal on the lower strand in hen hepatocytes at low DMS concentrations.

Protection of the ERE_D in both hen and rooster liver is surprising, since the oestrogen receptor, which is essential for activation of the vitellogenin gene, cannot be functional at the levels present in rooster liver nuclei (see below). To resolve this apparent paradox, the proteins from hen and rooster liver nuclear extracts, binding to the ERE sequence were characterized further *in vitro*.

Correlation between the levels of vitellogenin mRNA, oestradiol binding activity and DNA binding activity at the ERE

The levels of vitellogenin mRNA expression, oestradiol binding activity and the amount of protein–DNA complex formed with a synthetic DNA fragment (ERE) were determined in whole nuclei or liver nuclear extracts from egg-laying hens, roosters and oestradiol treated roosters (Table 1). As expected, the highest level of vitellogenin transcription was seen in the hen liver, which correlated well with the high level of [^{3}H]oestradiol binding activity (133 fmol/mg protein). There was no detectable vitellogenin mRNA in the rooster liver extract, although there were measurable levels of oestrogen binding activity (32 fmol/mg protein). After treatment of the rooster with oestradiol, the levels of oestrogen binding activity and vitellogenin mRNA increased in parallel. In addition, there was about a 2-fold increase in the relative *in vitro* protein–DNA binding activity at the ERE. The amount of protein binding to the ERE was 4- to 5-fold higher than the oestradiol binding activity, under all conditions tested (Table 1, Ratio b/a).

Comparison of a protein(s) from hen and rooster liver that binds to the ERE: proteolytic clipping bandshift assay

The relatedness of the ERE-binding proteins from hen and rooster liver was assessed by comparing the peptides that could bind to the ERE sequence after limited proteolysis of the protein–DNA complexes. Digestion of protein–DNA complexes with trypsin (Fig. 2A) or V8 protease (Fig. 2B), gave a limited number of peptides retaining DNA binding activity, and were the same for both hen and rooster nuclear extracts. Moreover, the same pattern of tryptic peptides was obtained by digestion of the protein–DNA complex formed between the rooster protein fraction and the DNA fragment containing ^{5m}CpG (compare Fig. 2C with 2A).

These data provide strong evidence, that the same or a closely related protein, present in both hen and rooster liver, can bind specifically to the unmethylated and methylated forms of the ERE.

u.v. cross-linking experiments

The protein binding from hen and rooster liver was further characterized by u.v. cross-linking to a bromodeoxyuridine substituted DNA fragment, and subsequent analysis by SDS–PAGE. For both tissues a strong double band with a M_r of about 80 k and a number of smaller molecular weight bands of 50 k–55 kDa were seen (Fig. 3). Cross-linking could be abolished by competing with a 10- to 20-fold mole excess of cold ERE sequence, but not with a mutated sequence (data not shown). The 50 k–55 kDa proteins, which were more prominent in rooster liver extracts, were thought to arise as breakdown products of the 80 kDa protein (I. J. McEwan, unpublished observations).

The molecular weight (80 kDa) of the specific protein is similar to that of non-histone protein 1 (NHP-1), which has recently been purified and characterized from HeLa cells [19].

In vitro footprinting with partially depurinated and depyrimidinated ERE

We have used the missing base contacts probing method, described by Brunelle and Schleif [29], to examine the base contacts important for the binding of proteins from hen and rooster nuclear extracts to the ERE *in vitro*.

Partially depurinated or depyrimidinated end-labelled DNA was incubated with a protein fraction from hen or rooster liver, and the bound and free DNA separated. After recovery

Table 1. Summary of biochemical studies on nuclear extracts used

Nuclear extract from:	Vitellogenin mRNA (molecules/ haploid genome)	a [^{3}H]E_2 binding activity (fmol/mg protein)[1]	b Protein–DNA complex ERE (fmol/mg protein)[1]	Ratio b/a
Hen liver	14,000	133 ± 29	644 ± 45	4.8
Rooster liver	ND	32 ± 14	144 ± 7	4.5
Rooster liver (oestrogen treated)	7000	71 ± 2	266 ± 9	3.7

E_2 = 17β-oestradiol; ND = not detectable.
[1]Mean ± SD, n = 4.

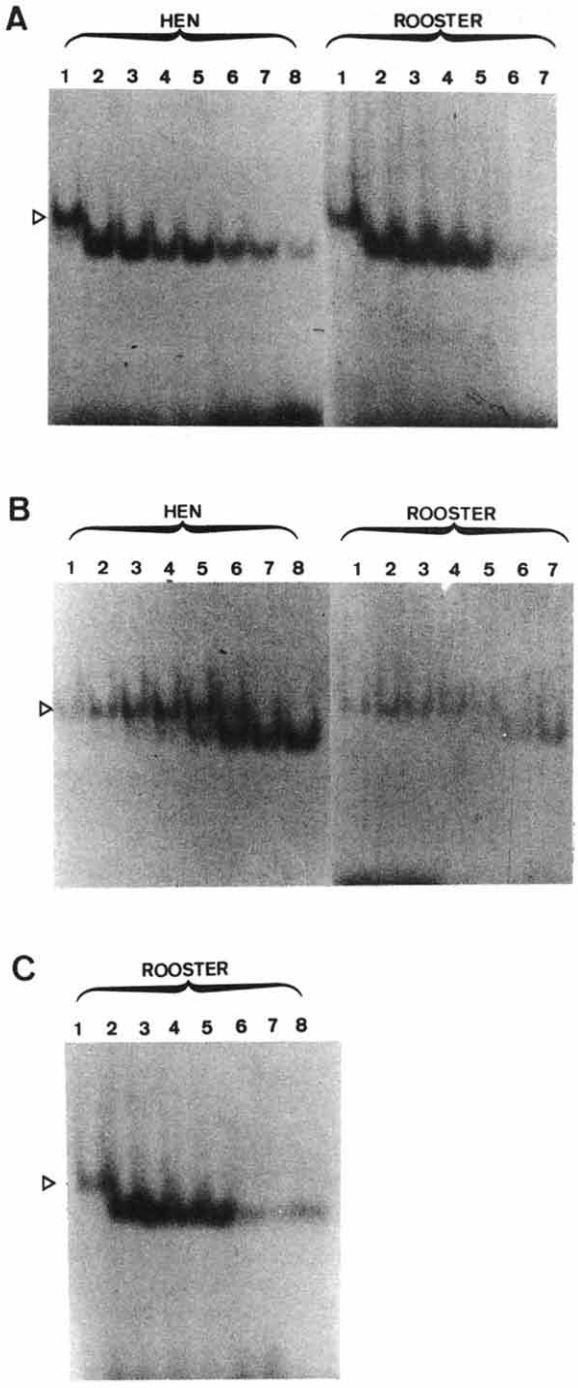


Fig. 2. Proteolytic clipping band shift assay of the binding activity present in hen and rooster liver nuclear extracts with double stranded oligonucleotides

5′ TCCTGGTCAGXGTGACXGGA 3′.
AGGACCAGTCGXACTGGXCT

Where X = C or ^{5m}C. Protein–DNA complexes digested with either 0 (lane 1), 20 ng (lane 2), 100 ng (lane 3), 200 ng (lane 4), 1 μg (lane 5), 5 μg (lane 6), 10 μg (lane 7) and 20 μg (lane 8) of trypsin (A) or 0 (lane 1), 8 ng (lane 2), 40 ng (lane 3), 80 ng (lane 4), 400 ng (lane 5), 2 μg (lane 6), 4 μg (lane 7) and 8 μg (lane 8) of V8 protease (B), respectively. (C) Trypsin treatment of the protein–DNA complex formed between the rooster protein and the ERE containing ^{5m}C. The open arrow head (▷) indicates the position of the undigested protein–DNA complex, the free DNA is not shown.

of the DNA and chemical cleavage with piperidine the fragments were resolved on a 20% sequencing gel. The principle of the technique is that the removal of a base that is critical for the binding of a protein will result in that fragment being under-represented in the bound DNA fraction and so appear as a missing or reduced signal on the sequencing gel.

Figure 4A shows the binding of a protein(s) from hen and rooster fractionated nuclear extracts to the unmethylated (X = C) or methylated (X = ^{5m}C) double stranded DNA fragment

5′ TCCTGGTCAGXGTGACXGGA 3′.
AGGACCAGTCGXACTGGXCT

For both the hen and rooster proteins removal of specific bases (Fig. 4A highlighted) reduced the binding of the protein(s), while the removal of others had little or no effect on binding.

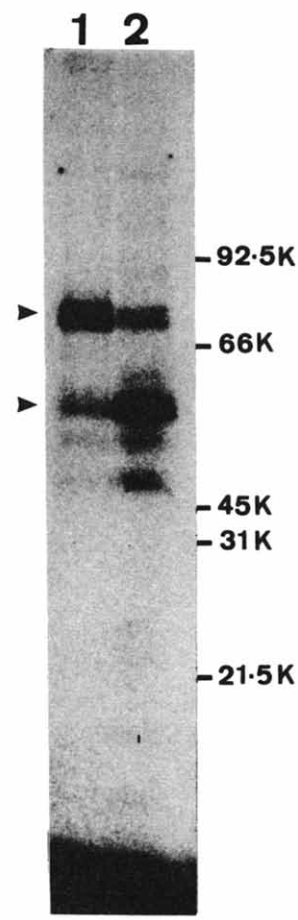
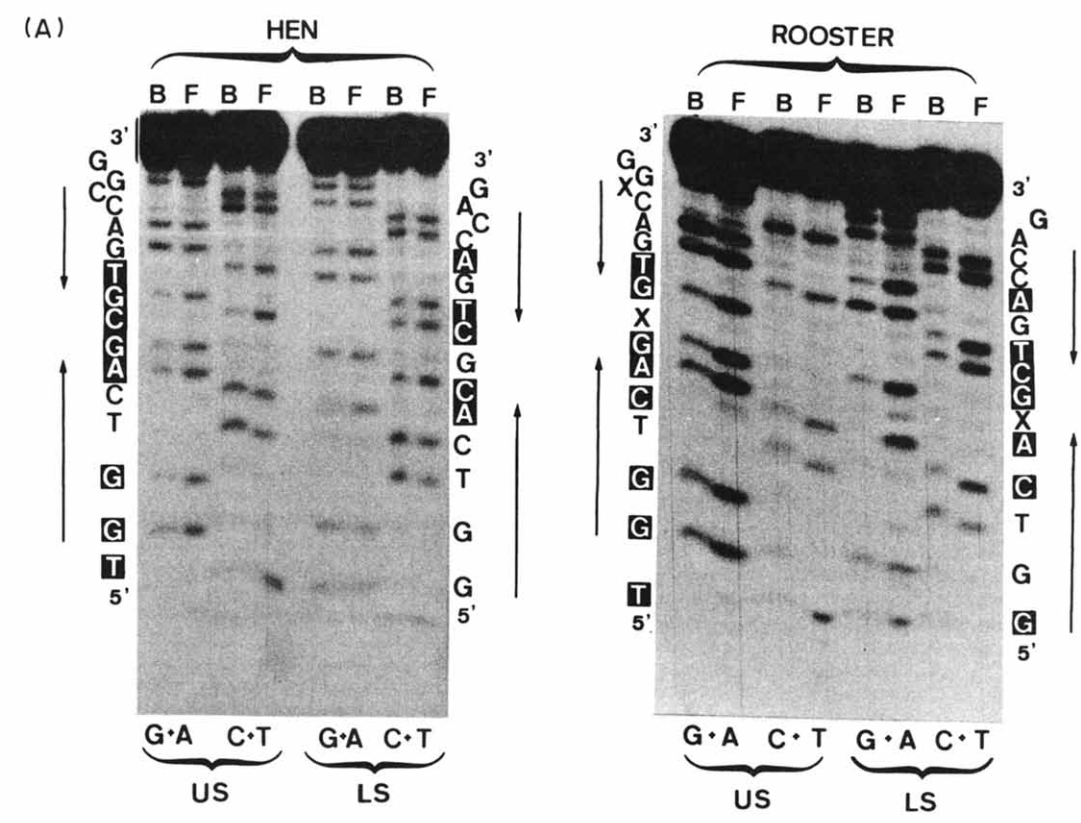


Fig. 3. u.v. cross-linking of a protein(s) from hen and rooster liver that binds to the ERE. The u.v. cross-linked products for hen (lane 1) and rooster (lane 2) nuclear extracts were resolved on a 10% polyacrylamide–SDS gel. The major cross-linked products are indicated by arrowheads. The position of M_r markers are indicated to the right.



(B)

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      ***  *****                    Hen (see above)
      *** *** **                    Rooster (see above)
      * *  ** **                    Rooster (data not shown)
5' TCCTGGTCAGXGTGACXGGA 3'
   AGGACCAGTCGXACTGGXCT
      **********                    Rooster (data not shown)
      * *** **  *                   Rooster (see above)
      * ** **                       Hen (see above)
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Fig. 4. Missing base contact probing of a protein(s) from hen and rooster liver binding to the ERE. (A) Comparison of the binding of a protein(s) from hen nuclear extract to the unmethylated (X = C) and rooster nuclear extract to the methylated (X = ^{5m}C) double stranded DNA fragment

5′ TCCTGGTCAGXGTGACXGGA 3′.

AGGACCAGTCGXACTGGXCT

B and F represent bound and free DNA, and the purine (G + A) and pyrimidine (C + T) lanes are indicated. The sequences of the upper and lower strands (US and LS) are shown at the side, with the bases important for binding highlighted. The arrows indicate the arms of the ERE palindromic sequence. (B) Summary of the data from (A), together with results for the rooster nuclear extract and unemthylated ERE sequence (data not shown).

These results and those obtained for the rooster extract with the unmethylated DNA are summarized in Fig. 4B. Although some differences exist between the hen and rooster extracts, the base contacts clustered around the central GCG were found to be important for protein binding in all cases. These results are also in good agreement with those obtained for the NHP-1 protein purified from HeLa cells [19].

DISCUSSION

We have previously described the specific binding of a non-receptor protein from HeLa cells to the ERE [19, 40]. In the present study, we show the presence of the same or a related protein in hen and rooster liver, that binds specifically to the ERE_D of the vitellogenin II gene. The technique of genomic footprinting

allowed the detection of proteins binding to the ERE_D and adjacent sequence of both the expressed (hen) and non-expressed (rooster) hepatic vitellogenin gene.

In a recent study, Philipsen *et al.* [33] using a single concentration of DMS (0.5%), also noted changes in the *in vivo* reactivity of the two guanosine residues at the centre of the ERE_D on the upper strand. However, unlike the present study, these changes were dependent on the oestrogen activation of the gene. The difference between this study and our results may be explained by the different experimental conditions used. Using a range of DMS concentrations (i.e. 0.5–0.0005%) has revealed a more detailed picture of the protein–DNA interactions at the ERE, including the binding of protein(s) from rooster liver.

The lack of apparent differences between the *in vivo* footprints from hen and rooster liver is surprising, as an effect of the activated oestrogen receptor complex in the hen liver would be expected. Although *in vivo* footprinting of the glucocorticoid receptor has been successfully described by Becker *et al.* [34] for the tyrosine amino transferase gene, Cordingley *et al.* [35] using Exonuclease III footprinting of nuclei were unable to show a footprint (ExoIII stop) for the glucocorticoid receptor on mouse mammary tumour virus DNA. This suggests that receptor dependent footprints may be difficult to demonstrate *in vivo*. However, we cannot exclude the possibility that the footprint seen in hen hepatocytes also involves the oestrogen receptor complex. In view of this, it is interesting to note the appearance of an enhanced signal in hen hepatocytes with decreasing DMS concentrations (Fig. 1*).

A comparison of fractionated nuclear extracts from hen and rooster liver by proteolytic clipping band shift assay and u.v. cross-linking of protein to DNA, revealed the same or a closely related protein of about 80 kDa, binding specifically to the ERE (Figs 2 and 3). As judged by quantitative band shift assays, this 80 kDa protein was at least 4–5 times more abundant than the oestrogen receptor and, in addition, the mole ratio of the two proteins remained approximately constant whether the gene was active or not (Table 1).

Previously, we have identified a ubiquitous non-histone protein (NHP-1) in different organs of the chicken and in different mammalian cell lines [18]. DNA competition studies showed that this protein had a binding preference for the GCG sequence in the middle of the palindrome of the ERE [18]. These results are in good agreement with the above *in vivo* and *in vitro* footprinting and cross-linking studies. Recently, NHP-1 has been isolated and purified to near homogeneity from HeLa cells [19], and its binding characteristics and molecular weight (under denaturing conditions) found to be similar to those of the protein from hen and rooster liver described here.

In attempting to ascribe a function to this protein in the oestrogen regulation of vitellogenin expression, two possibilities can be considered. Firstly, that this protein plays a direct role in the interaction of the oestrogen receptor with the ERE. Although the recent studies of Kumar and Chambon [36] and Klein-Hitpaß *et al.* [37] showed that no other proteins were required for the binding of the oestrogen receptor dimer to DNA *in vitro*, it is possible that this is not the case *in vivo*. The non-histone protein, NHP-1 could act by stabilizing receptor dimer-DNA contacts *in vivo* or by "tagging" the ERE for subsequent binding of the activated oestrogen receptor. Interestingly, recent evidence suggests that the thyroid hormone receptor requires additional accessory protein(s) for DNA binding [38, 39]. The second possibility is that this protein has a more general function, involved with the active demethylation of ^{5m}CpG. It is clear from previous studies [15, 21] that changes in the methylation state of certain CpG dinucleotides at the ERE and promoter region correlate strongly with expression of the gene. The ubiquitous nature of this factor [18, 19, 40] is in keeping with this proposal of a more general function. Experiments are currently in progress with the purified protein to address these hypotheses directly.

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