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Tissue-specific and steroid-dependent interaction of transcription factors with the oestrogen-inducible apoVLDL II promoter *in vivo*

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Using *in vivo* dimethylsulphate footprinting, we have analysed protein–DNA interactions within the promoter region of the oestrogen-inducible gene encoding chicken apo very low density lipoprotein II (apoVLDL II). Most of the guanosine–protein contacts found, are located within the 230-bp DNA 5′ flanking the gene and can be grouped into separate protein-binding sites. Two of these sites resemble the oestrogen-responsive element (ERE) which is the target site for the oestrogen receptor. A third site has some features in common with the chicken ovalbumin upstream promoter element binding the COUP transcription factor. All protein contacts identified are present in the apoVLDL-II-expressing liver exclusively, and are not found in the hormone-naive liver, in erythrocytes or the oviduct tubular gland. Our results demonstrate the binding *in vivo* of a protein, presumably the oestrogen receptor, to the ERE and suggest that the hormone activates transcription by establishing a transcription complex comprising several factors at the apoVLDL II promoter.

Key words: oestrogen-responsive element/COUP box/*in vivo* footprinting/tissue specificity/oestrogen receptor

Introduction

Expression of steroid-controlled genes requires the interaction of specific transcription factors with *cis*-acting DNA sequences. Of the regulatory sequences, hormone-responsive elements (HREs) have a decisive role in steroid inducibility of target genes (for a review, see Yamamoto, 1985). Specific HREs for glucocorticoid- and oestrogen-regulated genes have been identified by gene-transfer experiments and are termed GREs (Geisse *et al.*, 1982; Robins *et al.*, 1982) and EREs (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Klock *et al.*, 1987; Martinez *et al.*, 1987) respectively. The elements act as inducible enhancers by increasing the rate of transcription from promoters in a relative position- and orientation-independent manner. Evidence that the HREs are the recognition sequences of the respective steroid receptor proteins, was provided by two types of experiments. Firstly, transcription of a HRE-linked reporter gene, transfected into receptor negative cell lines, is rendered steroid dependent by co-transfection of a receptor gene carried on an expression vector (Kumar *et al.*, 1987). Secondly, GREs and EREs are contained in the *in vitro* DNA-binding sites of their respective, purified receptors (Scheidereit *et al.*, 1986; Jantzen *et al.*, 1987; Maurer and Notides, 1987). However, the mechanism by which the receptor protein activates the enhancer is less clear. Binding of the glucocorticoid receptor protein to its target sequence *in vitro* appears to occur irrespective of the presence of the hormone (Willman and Beato, 1986). This is unexpected in view of the observation that the hormone is required for tight binding of receptor to the nucleus *in vivo*. Explanations for these conflicting data have been proposed, including artificial receptor activation *in vitro*, removal during isolation of a receptor-binding protein and the absence *in vitro* of the large excess of competing, non-specific DNA normally present in nuclei (for a review, see Green and Chambon, 1986). Therefore, it is essential to study the binding of receptor proteins to their targets *in vivo* as a function of cell type and hormone stimulation.

Several models have been proposed to explain how factors such as steroid receptors, that recognize enhancers at variable distances from the gene, can activate transcription. One model proposes that these factors interact with other proteins bound to promoter elements, with the intervening DNA looping out (Ptashne, 1986). Recently, evidence has been obtained for the involvement of additional factors in transcriptional activation of specific steroid-inducible promoters. Transcriptional activation of the glucocorticoid-inducible mouse mammary tumour virus (MMTV) promoter is accompanied by the binding of two factors, one of which was identified as a homologue of nuclear factor 1 (NF-1) (Cordingly *et al.*, 1987). Two different transcription factors cooperate in the transcription of the oestrogen-inducible, chicken ovalbumin gene (Tsai *et al.*, 1987). One of these factors binds to the so-called chicken ovalbumin upstream

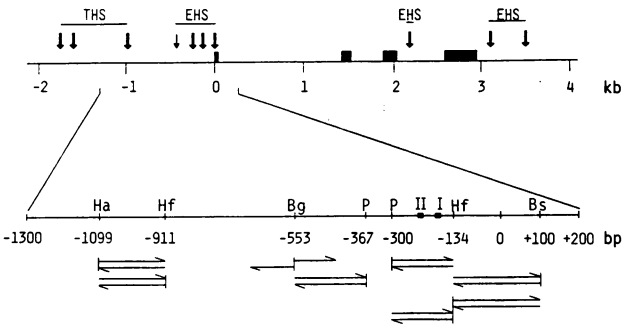

Fig. 1. Map of the apoVLDL II gene region and strategy of *in vivo* footprinting. **Upper line**: map of the chicken apoVLDL II gene, including flanking regions; black boxes, exons; arrows, oestrogen-dependent (EHS) and tissue-specific (THS) DNase I-hypersensitive site (Kok *et al.*, 1985). Numbering represents distance from the major cap site. **Lower line**: enlargement of the region analysed by *in vivo* methylation footprinting; black boxes labelled with roman numerals, sequences resembling the ERE. Positions and 5′ to 3′ direction of radiolabelled probes synthesized on single-stranded M13 templates are indicated by horizontal arrows. Restricton sites are: Ha, *Hae*III; Hf, *Hin*fI; Bg, *Bgl*II; P, *Pvu*II; Bs, *Bst*EII.

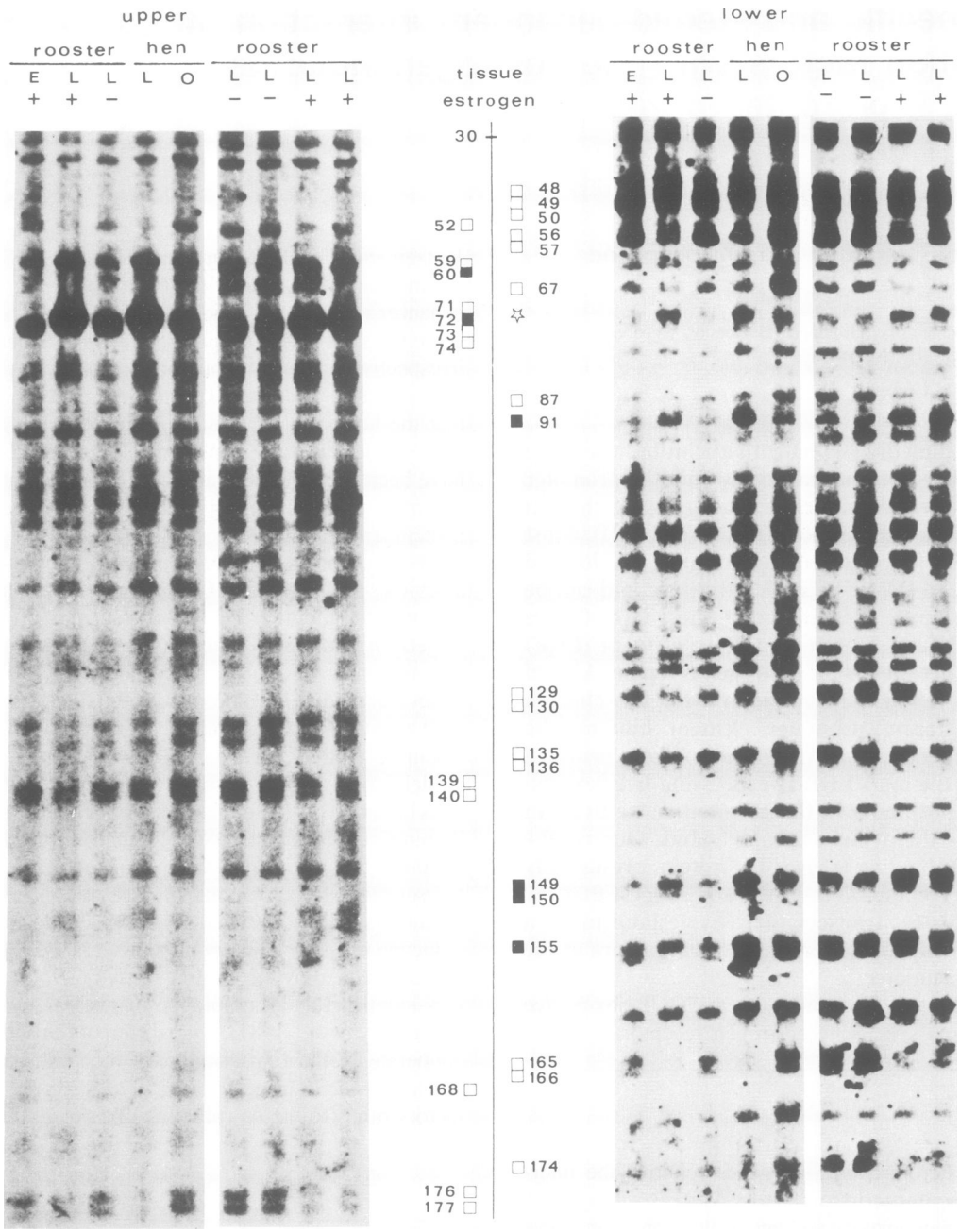

Fig. 2. Tissue-specific and oestrogen-dependent protein−DNA interaction analysed by *in vivo* DMS footprinting. Liver cells (L) and erythrocytes (E) from oestrogen-treated (+) or hormone-naive (−) roosters, and of liver cells (L) and oviduct tubular gland cells (O) from laying hen were incubated with DMS. DNA isolated from these cells was restricted with *Pvu*II and cleaved at the methylated guanosines with piperidine. The membrane carrying the electrophoretically separated fragments was hybridized with a single-stranded *Pvu*II(−300)−*Hin*fI(−134) probe and autoradiographed to visualize the guanosine ladder of the lower strand (**right panel**). The complementary probe was used in a second hybridization of the same blot to visualize the upper strand (**left panel**). Guanosines whose reactivity is enhanced (filled squares) or decreased (open squares) in apoVLDL-II-transcribing liver are indicated. Numbering indicates distance upstream from the cap site. Star indicates non-guanosine band mentioned in the text.

promoter (COUP) element at −80 bp from the cap site.

We have been studying the promoter of the oestrogen-inducible apoVLDL II gene (Van het Schip *et al.*, 1983, 1986) encoding the apo very low density lipoprotein II of chicken. The rapid activation of the gene in the expressing liver (Wiscocil *et al.*, 1980) is accompanied by the simultaneous appearance of DNase I-hypersensitive sites within the 300-bp upstream region, indicating changes in protein−DNA interactions (Kok *et al.*, 1985). To study the interaction of transcription factors with the presumed enhancer and promoter elements of the apoVLDL II gene in further detail, we have analysed a region extending from 1300 bp upstream to 200 bp within the gene for protein-binding sites, using *in vivo* dimethylsulphate (DMS) footprinting (Church *et al.*, 1985; Ephrussi *et al.*, 1985; Becker *et al.*, 1986, 1987). We find tissue-specific, hormone-dependent association of proteins to sequences resembling the ERE and the COUP element.

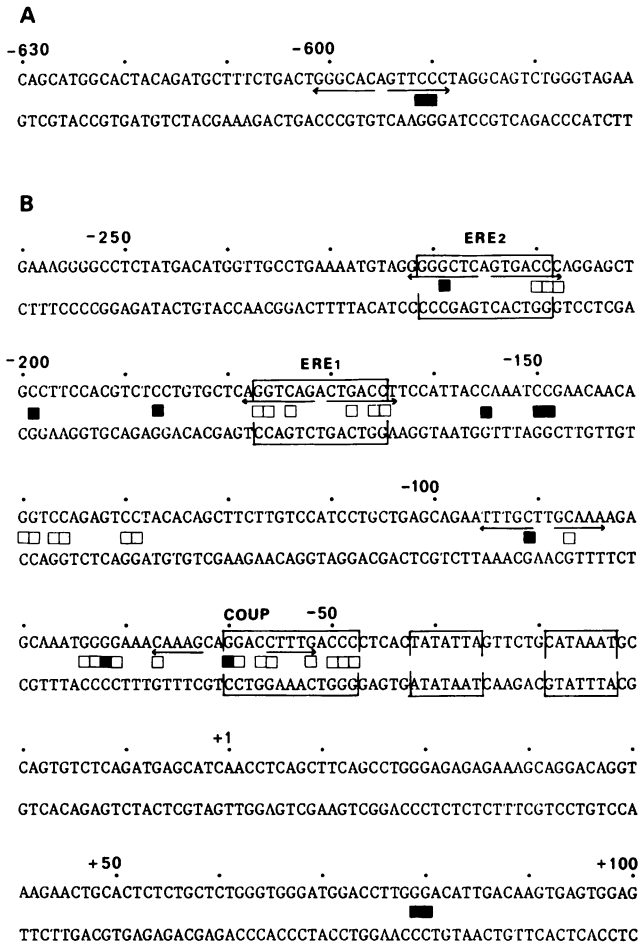

Fig. 3. Summary of protein−DNA interactions in the apoVLDL II 5′-end region *in vivo*. Sequences between positions −630 and −570 (**A**) and between −260 and +100 (**B**), containing altered guanosine reactivities, are shown. Altered reactivity against DMS in expressing liver versus non-expressing tissues is indicated by open squares (protections) and filled squares (enhancements). Sequences resembling the ERE sequences, the COUP sequence and the TATA sequences are boxed. Arrows indicate the palindromic sequences discussed in the text.

Results

Alterations of G-reactivity are liver specific and map within DNase I hypersensitive sites

Sites of protein−DNA interaction within the region 5′ flanking the chicken apoVLDL II gene were mapped by analysing the reactivity of guanosines (Gs) to DMS. The fast penetration of DMS into cells and nuclei permits its use on intact cells, thereby increasing the probability that the chromatin keeps its native conformation. Mild dispersion of fresh tissues preceded the DMS treatment. Reaction times of a few minutes at 0°C are sufficient to obtain the required level of partial methylation at the N7 of guanosines (Ogata and Gilbert, 1979). Protein contacts close to the major groove of the DNA helix can result either in protection against methylation, or in enhancement of the reactivity. The latter phenomenon is probably explained by a local increase in DMS concentration in hydrophobic pockets of the protein. The methylation pattern was analysed following the genomic sequencing protocol (Church and Gilbert, 1984; Saluz and Jost, 1986). To this end, the partially methylated genomic

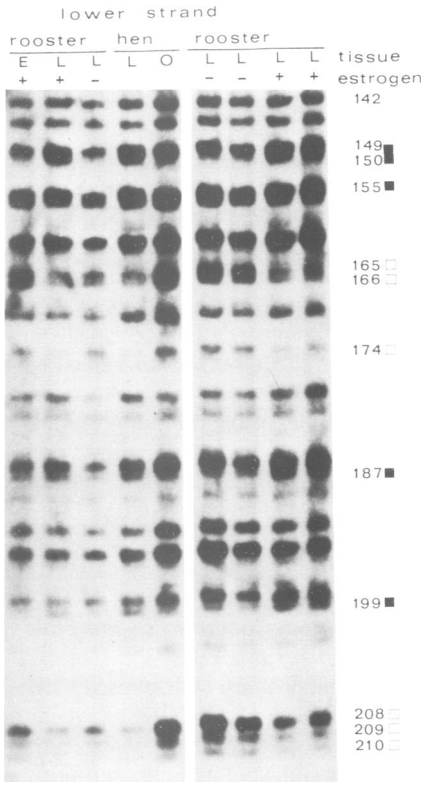

Fig. 4. DMS footprinting of protein−DNA interaction in ERE sequences *in vivo*. Restriction of methylated DNAs from DMS-treated tissues with *Pvu*II and hybridization with the *Pvu*II (−300)−*Hin*fI (−134) probe complementary to the lower DNA strand was as in Figure 2, but the gel was run for a shorter period of time to display the region immediately downstream of *Pvu*II site −300. Symbols and numbering are as described in Figure 2.

DNA was isolated, cleaved first at a restriction site near the region of interest and subsequently at the methylated guanosines by piperidine treatment (Maxam and Gilbert, 1980). The fragments were separated on a sequencing gel, electroblotted and UV cross-linked onto nylon membranes, and the G ladder was displayed by indirect end labelling, using a probe abutting the above restriction site. The footprinting strategy is depicted in Figure 1. The sequence extending from 1300 nucleotides in front of the gene up to 200 nucleotides within the transcribed region was analysed in both strands.

Figure 2 shows an example of a genomic sequence, covering the region extending from nt −30 to −177. Methylated DNAs from different chicken tissues were analysed and compared to reveal sites of protein contact. Naked genomic DNA cannot serve as a suitable standard for comparisons of DMS reactivity as was pointed out by Becker *et al.* (1986). We compared liver of the laying hen and oestrogen-treated cockerel expressing the apoVLDL II gene (Wiscocil *et al.*, 1980) with different non-expressing tissues. The latter tissues, which included the committed liver of hormone-naive cockerel and the non-inducible erythrocytes and oviduct tubular gland, all have the same methylation pattern reflecting the inactive state of the apoVLDL II gene. This is in accordance with the absence of nuclease hypersensitive sites near the apoVLDL II gene within these tissues (Kok *et al.*, 1985). On the other hand, livers actually

expressing the apoVLDL II gene differ from the other tissues in the reactivity of several guanosines. The guanosines with reduced or increased intensity shown in Figure 2, as well as those observed elsewhere in the 1.5-kb region analysed, are summarized in Figure 3. The sites of interaction are interspersed and flanked by extended stretches of DNA that do not show a sign of protein binding (data not shown). Each protected or enhanced guanosine depicted was observed in five independent samples of apoVLDL-II-expressing livers, three from cockerels and two from laying hens, and mapped from at least two different restriction sites. Most G contacts map within the 230 bp upstream of the cap site in accordance with the positions of the hypersensitive sites. Most contacts appear to be grouped into distinct patches, indicating the presence of multiple protein-binding sites. Hormonal induction of expression is a precondition for the altered reactivity which is strictly limited to liver and does not occur in other hormone-responsive tissues not expressing apoVLDL II. The different sites will be shown and discussed in more detail in the following sections.

Protein–DNA contacts occur in palindromic sequences resembling the ERE

Two clusters of guanosines with altered DMS reactivity coincide with palindromic sequences, centred around positions −171 and −215. Both sequences have previously been recognized as copies of a common motif that is found in the 5′ end region of oestrogen-regulated genes and conforms to the consensus sequence GGTCANNNTGACC (Walker *et al.*, 1984). The protected Gs −177, −176 and −168 in the upper strand and Gs −174, −166 and −165 in the lower strand (Figures 2 and 4) lie in the perfect palindromic sequence, 5′-GGTCAGACTGACC-3′. The other cluster comprises four contacts, as appears from a strong enhancement of G −219 in the upper (Figure 5) and protection of Gs −210, −209 and −208 in the lower strand (Figure 4), is within the imperfect palindromic sequence 5′-GGGCTCAGTGACC-3′ (deviations from the consensus are underlined). The protein contacts in both elements differ quantitatively and qualitatively. This may indicate a different positioning of the protein molecule bound, caused by variations in the DNA sequence.

The contact points within the first palindromic sequence are positioned in a perfectly symmetrical configuration around the 2-fold axis, whereas in the other element this is not the case. The factor that interacts with the perfect palindromic sequence makes contacts at one side of the DNA helix. The consensus motif has recently been shown to be contained in the EREs of two *Xenopus laevis* (Seiler-Tuyns *et al.*, 1986; Klein-Hitpass *et al.*, 1986) and one chicken vitellogenin gene (Klein-Hitpass *et al.*, 1988). Although the particular elements within the apoVLDL II upstream region have not been tested in transfection experiments yet, our observation that they constitute protein-binding sites that are occupied *in vivo* in a tissue-specific and oestrogen-dependent manner argues in favour of a role in transcriptional regulation.

In conclusion, protein–DNA contacts are observed in two, 44-bp spaced sequence elements resembling the ERE, GGTCANNNTGACC.

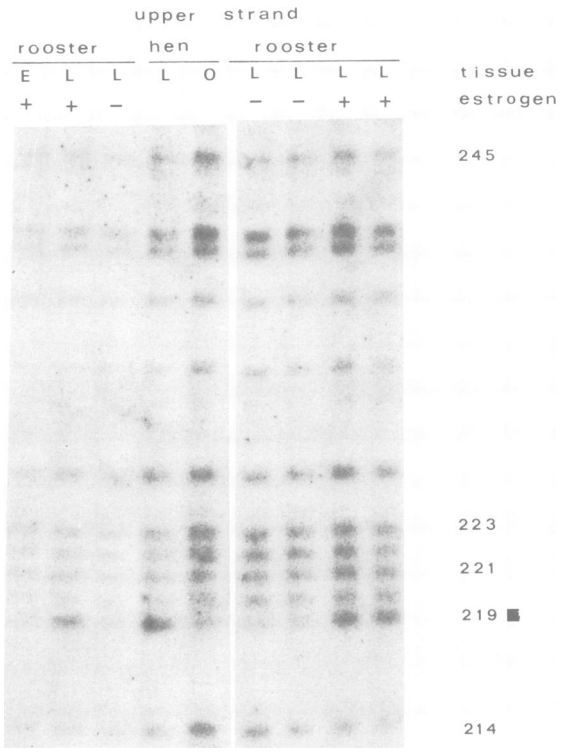

Fig. 5. DMS footprinting of protein–DNA interactions in the ERE 2 sequence *in vivo*. Restriction of methylated DNAs from DMS-treated tissues was performed with *Hin*fI and hybridization was carried out with the *Pvu*II(−300)–*Hin*fI(−134) probe complementary to the upper strand as in Figure 2. Symbols and numbering are as described in Figure 2.

Protein–DNA contact points occur in a sequence resembling the COUP box

A third protein-binding site is found at position −48 to −60 of the apoVLDL II upstream region (Figure 6). All guanosines within this region appear to be bound to a protein in the expressing liver (protected Gs −52 and −59 in the upper and Gs −48, −49, −50, −56 and −57 in the lower strand) or in close proximity to a hydrophobic protein patch (enhanced G −60 in the upper strand), as we infer from their altered reactivities against DMS in the oestrogen-stimulated liver versus non-expressing tissues. The protein binding sequence 5′-GGACCTTTGACCC-3′ is similar, apart from two mismatches (underlined) to the sequence 5′-TGACCTTTGACAC-3′, which is present in the COUP element (Sagami *et al.*, 1986; Pastorcic *et al.*, 1986; Tsai *et al.*, 1987). The COUP element has recently been shown to be the target site of a transcription factor that is present in oviduct as well as in HeLa cells (Bagchi *et al.*, 1987). Its purine base and backbone contact sites have been determined by *in vitro* methylation and ethylation interference experiments with purified COUP-transcription factor (Tsai *et al.*, 1987). All guanosines appear to be important for binding. The similarity in sequence and guanosine contacts of the ovalbumin and apoVLDL II elements (Figure 7) suggests that the same protein might be binding. As long as the relationship of the liver protein to the COUP-transcription factor has not been firmly established, we will

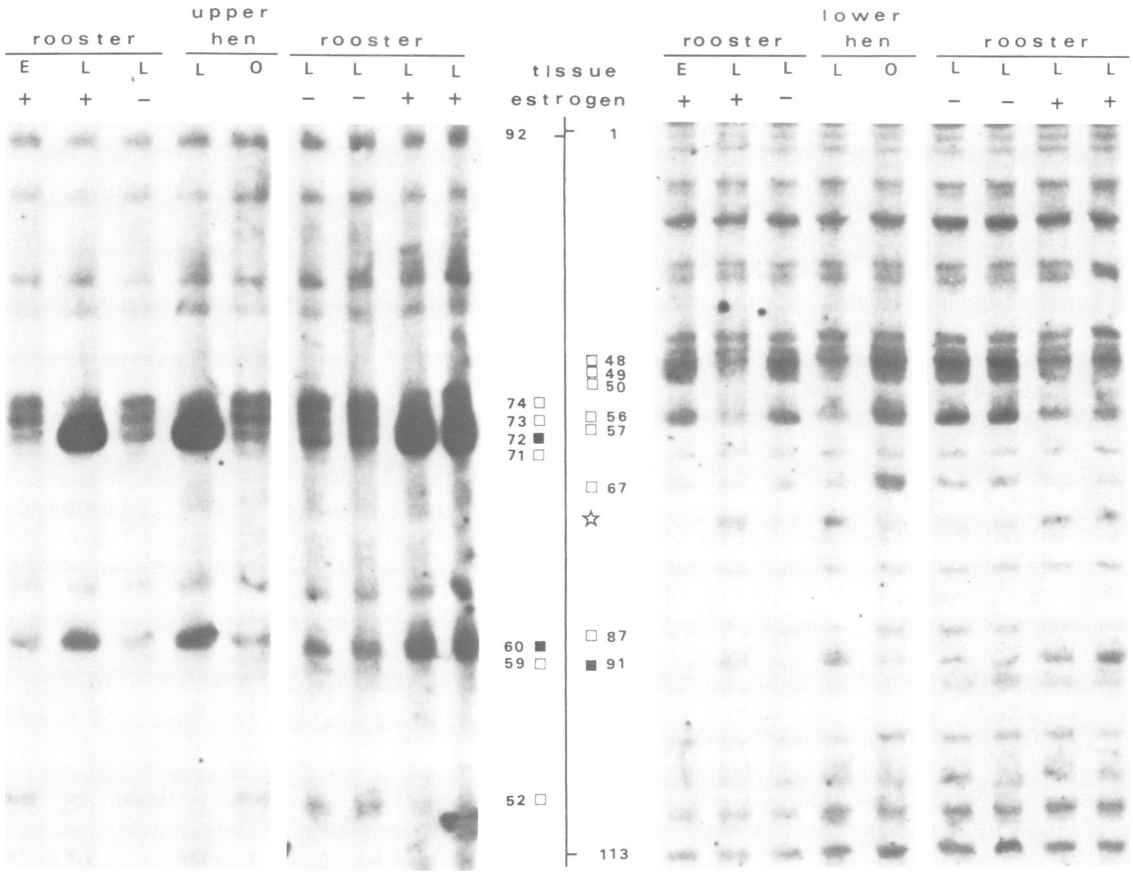

Fig. 6. DMS footprinting of protein−DNA interaction in the COUP sequences *in vivo*. Restriction of methylated DNAs from DMS-treated tissues was performed with *Bst*EII and hybridization was carried out with a *Hin*fI(−134)−*Bst*EII(+400) probe complementary to the upper strand (**left panel**). For the corresponding region of the lower strand, a section of Figure 2 is shown in close-up (**right panel**). Symbols and numbering are as described in Figure 2.

refer to its target site in front of the apoVLDL II gene as the COUP-like element. If the element serves a similar function in the expression of the apoVLDL II gene in liver as of the ovalbumin gene in oviduct, it should do so irrespective of its orientation towards the gene since its direction is opposite in both cases.

Just in front of the element, we find additional contact sites. Most pronounced is the enhancement of guanosine −72, lying in between three protected Gs (upper strand) and the protection of G at position −67 (lower strand). At position −74 in the lower strand an enhanced band appears which is not a guanosine. Whether it originates from the DMS reaction with a different base (cytosine) or represents a strand break by endogenous nuclease activity is now under investigation. Taken as a whole, the −74 to −48 region displays a weak palindromic structure. Whether the sequence next to the COUP-like element binds the same or a different factor, must await further analysis by *in vitro* binding experiments with the purified proteins.

In conclusion, protein−DNA contacts are observed in an apoVLDL II promoter element resembling the chicken ovalbumin promoter element.

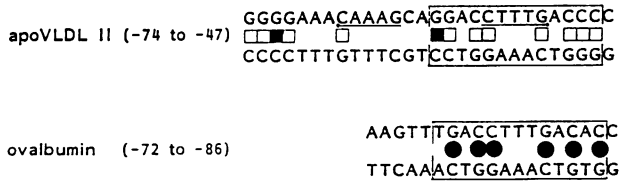

Fig. 7. Comparison of the methylation data for apoVLDL II and ovalbumin COUP elements. The numbers give the position to the cap site. **Squares**: *in vivo* methylation protection data from this study (open symbols, protected Gs; closed symbols, enhanced Gs). **Circles**: *in vitro* methylation interference data from Tsai *et al.* (1987): G residues which, after methylation, interfered with protein−DNA complex formation. The COUP element is boxed. The sequences are depicted in opposite orientation to show their similarity.

Additional protein-binding sites disclosed by DMS footprinting

Besides the elements discussed above, clearly resembling the ERE and the COUP element, other protein contact sites were revealed by DMS footprinting. A clear protection of G −87 and an enhancement of G −91, is found in the imperfect palindromic sequence, 5′-ATTTGCTTGCAAAA-3′

(see Figures 2 and 6). It overlaps a pair of sequence elements, AGAATTT and TTGCAAA, originally recognized (Burch, 1984) as elements that are shared between the 5′-flanking sequences of the apoVLDL II gene and another oestrogen-controlled gene of liver encoding the major vitellogenin. Moreover, some resemblance of its complementary sequence with the octamer motif ATGCAAAT (Falkner *et al.*, 1986) can be noticed.

A second sequence, consisting of a group of six protected Gs between positions −140 and −129 does not resemble any known protein recognition sequence (see Figure 2). Further upstream and beyond the region of DNase I-hypersensitive sites we find enhanced Gs (positions −590 and −591), located within the sequence 5′-GGGCACAGTTCCC-3′ which shows some similarity to the ERE (data not shown).

Discussion

We have carried out high-resolution mapping of sites of DNA−protein interaction in avian cell chromatin *in vivo* by genomic footprinting. A region extending up to 600 bp upstream of the oestrogen-inducible apoVLDL II gene from chicken contains several sites of protein binding in expressing liver. Of these sites, two resemble a palindromic element with the consensus sequence 5′-GGTCANNNTGACC-3′ found in the 5′ end region of oestrogen-regulated genes (Walker *et al.*, 1984). Recently, evidence has accumulated that this sequence represents or forms part of the ERE. Martinez *et al.* (1987) have shown that a single, 13-bp perfect palindromic copy, 5′-GGTCAC-TGTGACC-3′, of the consensus is capable of conferring oestrogen inducibility to the heterologous thymidine kinase promoter and behaves as an inducible enhancer since it acts in a relative position- and orientation-independent manner. Moreover, two imperfect palindromic copies <69 bp apart were shown to form a functional ERE. We infer from our footprinting experiments that both palindromic sequences resembling the ERE consensus sequence, lying upstream of the apoVLDL II gene, are involved in the expression of the gene since their Gs make protein contacts. Given their 44-bp spacing, the elements could well cooperate. Direct evidence for the interaction between the oestrogen receptor and an ERE has recently been obtained for the rat prolactin promoter by *in vitro* binding experiments (Maurer and Notides, 1987). However, the requirement of the hormone for binding of the receptor to DNA has not been established in these experiments. Our data demonstrate, for the first time, the hormone-dependent occupation of an ERE *in vivo* by a protein which is presumably the oestrogen receptor. Similarly, Becker *et al.* (1986) have recently reported that *in vivo* protein−DNA interactions in a glucocorticoid-responsive element lying in front of the rat tyrosine amino transferase gene require the presence of the hormone.

A third protein-binding site within the apoVLDL II 5′-flanking region resembles the chicken ovalbumin upstream promoter element which is recognized by the COUP transcription factor (Tsai *et al.*, 1987). By *in vitro* reconstitution and DNA-binding experiments, it has been shown that the COUP-transcription factor is essential for efficient transcription of the ovalbumin gene *in vitro* and binds specifically to the COUP sequence. Transcription of the ovalbumin gene requires a second transcription factor, termed S300II, which probably acts by stabilizing COUP−promoter complexes via interaction with the COUP factor (Tsai *et al.*, 1987). The COUP factor has been purified from chicken oviduct cells and human HeLa cells, and the purines important for binding have been determined by methylation interference experiments *in vitro*. The obtained arrangement of important guanosines resembles the guanosine contacts which we find in the apoVLDL II promoter *in vivo*. This suggests that the COUP-transcription factor is present in liver, and involved in the expression of the apoVLDL II gene there.

Occupation of the ERE- and the COUP-like elements as well as the other sites, for which the identity of the binding protein is less obvious, appears to be restricted to the liver and dependent upon oestrogen. However, the inability to detect contacts in the inactive promoter does not necessarily imply the absence of proteins on the DNA. The absence of hypersensitive sites in non-expressing tissues (Kok *et al.*, 1985) in fact suggests that the DNA of the inactive promoter is organized in a common nucleosomal structure. Activation of the apoVLDL II promoter is not only accompanied by binding of the oestrogen receptor to the ERE, but also of other factors to different binding sites. Loading of the transcription factors to the promoter might occur in a cascade fashion initiated by receptor binding as has been proposed by Cordingley *et al.* (1987) for the glucocorticoid-regulated MMTV promoter.

The tissue specificity of the observed protein interactions in the apoVLDL II promoter region raises a number of questions, in particular, when liver and oviduct are compared. The oestrogen receptor and the COUP-transcription factor, which are present in oviduct, appear to be excluded from the apoVLDL II promoter in this tissue. The tissue specificity may be controlled by tissue-specific factors or by the accessibility of protein-binding sites. Firstly, tissue-specific factors involved in the expression of liver-specific genes have recently been described (Grayton *et al.*, 1988). It is not excluded that one or more of the non-identified proteins interacting with the apoVLDL II promoter fulfil such a function. Secondly, Becker *et al.* (1987) have shown that methylation of cytosine residues within a protein-binding site of the tyrosine amino transferase promoter prevents the factor from binding. Expression of the apoVLDL II gene in the oviduct might be prevented in a similar way. Finally, differences in the chromatin structure between different tissues, notably between liver and oviduct, may play a role in the tissue specificity of expression of the apoVLDL II gene. A difference between liver and oviduct in the hypersensitive sites located ~1.7 kb upstream of the cap site has been described (Kok *et al.*, 1985). The possible involvement of this region in tissue-specific gene expression will be analysed in more detail by *in vivo* and *in vitro* footprinting techniques.

Materials and methods

Animals and hormonal induction

Rhode Island Red roosters were 6−10 weeks and laying hens 8 months old. Oestrogen stimulation of roosters was achieved by a s.c. injection of 50 mg/kg body weight of diethylstilbestrol (DES) 3 days before being killed.

In vivo methylation of cells and preparation of DNA

Livers were perfused with ice-cold PBS (140 mM NaCl, 2.5 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5) containing 5 μM DES when oestrogen-induced animals were used. Liver and oviduct tissues were

mildly dispersed in DMEM (2 ml/g) containing 10 mM Hepes, pH 7.4, using an Ultra-turrax. Methylation with DMS was according to the protocol of Ephrussi *et al.* (1985) with the following modifications. Aliquots of 3 ml of tissue homogenate or erythrocyte suspension were incubated with DMS (15 μl) for 2–5 min on ice, subsequently diluted 10-fold in PBS containing 140 mM 2-mercaptoethanol, and centrifuged for 5 min at 3000 r.p.m. The cells were washed with 30 ml PBS, resuspended in 4.5 ml 0.3 M sucrose containing 60 mM KCl, 15 mM NaCl, 60 mM Tris–HCl, pH 8.2, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, and treated with detergent by adding 4.5 ml of 1% NP-40 in the same buffer. After 5 min on ice, nuclei were centrifuged for 5 min at 3000 r.p.m., washed with 15 ml and resuspended in 2.5 ml of 0.3 M sucrose-buffer. The suspension was supplemented with 2.5 ml 0.5 M EDTA, 50 μl 1% RNase A, 125 μl 20% Sarcosyl or 250 μl 10% SDS, and incubated for 3 h at 37°C. Proteinase K was then added to 100 μg/ml and incubation continued overnight. DNA was deproteinized with phenol and phenol/chloroform (1:1), dialysed overnight against 10 mm Tris–HCl pH 7, 1 mM EDTA and precipitated with ethanol.

DNA cleavage, electrophoretic separation and electroblotting

Thirty micrograms of genomic DNA was digested with 60 U of restriction enzyme overnight. Digestion was stopped by adding EDTA to 10 mM; DNA was recovered by precipitation and subjected to piperidine treatment (Maxam and Gilbert, 1980). After 30 min, the solution was transferred to a fresh tube, DNA was precipitated, washed three times with 80% ethanol, and dried for at least 30 min in a SpeedVac concentrator. DNA was resuspended in 5 μl water and 2.5 μl formamide, boiled for 3 min and separated on a 6% denaturing polyacrylamide gel (7 M urea) in 1 × TBE (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA, pH 8.3) and electroblotted in 0.5 × TBE onto Schleicher & Schuell Nytran membrane (Nytran N13, N13N, 0.45 μm; N13N, 0.1 μm) as described by Church and Gilbert (1984). After the transfer was completed, the membrane was air-dried, baked for 20 min at 80°C *in vacuo* followed by UV crosslinking of the DNA to the membrane. The irradiation time depended on the batch and the type of membrane used.

Probes

The *Bgl*II(−553)−*Pvu*II(−367), *Pvu*II(−300)−*Hin*fI(−134), *Hin*fI(−134)−*Bst*EII(−100) fragments of the chicken apoVLDL II gene were filled in with Klenow polymerase and cloned in both orientations into the *Sma*I site of M13mp18 (Messing and Vieira, 1985). The *Hae*III (−1099)−*Hin*fI(−911) fragment was Klenow-filled and cloned in both orientations into the *Hin*cII site of M13mp18. All constructs were analysed by DNA sequencing (Sanger *et al.*, 1977). Other M13 probes used were described by Van het Schip *et al.* (1986). Probes used for genomic blots are indicated in Figure 1. Radio-labelled probes were synthesized from single-stranded M13 templates as described by Church and Gilbert (1984) with modifications (Becker *et al.*, 1986).

Hybridizations

Hybridizations were performed as detailed by Church and Gilbert (1984) with the following modifications. After the ssDNA probe was recovered by isotachophoresis (Ofversted *et al.*, 1984), it was added directly to the hybridization mix. Hybridizations were carried out at 65°C in glass cylinders with polypropylene caps in a home-made rotary incubator. Washes were as described (Becker *et al.*, 1987). For rehybridizations, membranes were washed for 20 min in 100 ml 10 mM Tris–HCl, 1 mM EDTA (pH 7.5) containing 0.1% SDS at 65°C before they were subjected to a new probe. Autoradiography was on Kodak XAR film with an intensifying screen (Kodak) at −80°C for 4–10 days.

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