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Regulatory elements and DNA-binding proteins mediating transcription from the chicken very-low-density apolipoprotein II gene

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ABSTRACT

The chicken Very-Low-Density Apolipoprotein II (apoVLDL II) gene is specifically expressed in liver in response to estrogen. In this study, we performed a functional analysis of the 300-base pair region immediately 5′ to the gene by gene transfer of chloramphenicol acetyl transferase (CAT) constructs into chicken embryonic hepatocytes (CEH). Two estrogen response elements (EREs) could be distinguished which together form a potent estrogen response unit. Stimulation of transient expression by co-transfection with a plasmid expressing rat C/EBP confirmed that a similar protein in chicken liver may be involved in apoVLDL II transcription. *In vitro* DNaseI footprinting and band-shift analysis with liver, oviduct and spleen nuclear extract revealed the tissue distribution of the proteins binding to the promoter region. A liver-specific protein bound to multiple sites of which some resembled the recognition sequence of the CCAAT/Enhancer binding protein, C/EBP. Of the other proteins binding to the apoVLDL II promoter, one was identified as the liver-specific LF-A1 by mobility shift analysis, using purified bovine LF-A1, and another as the general COUP-transcription factor, using an antiserum against the human COUP-TF.

INTRODUCTION

Transcription of eukaryotic genes is governed by regulatory proteins that bind to specific DNA sequences in the vicinity of these genes. Typical examples are the steroid hormone receptors that mediate the steroid control of gene expression in steroid-responsive tissues. After binding their ligand, these intracellular receptors interact with specific DNA sequences and modulate the transcription of adjacent genes. Gene transfer experiments and *in vitro* binding studies have revealed the target sequences for these receptors (1–4). These cis-acting sequences are usually located upstream of the promoter and are called Hormone Response Elements (5,6).

Besides the temporary modulation through activatable transcription factors, such as steroid receptors, gene expression is subject to cell-type specific regulation which appears to be determined by the combination of transcription factors present in a particular cell (7). This has become clear from studies on genes specifically expressed in liver, for example serum albumin (8), α-1 antitrypsin, fibrinogen and transthyretin (9–11). Protein-DNA interaction and transcription studies *in vivo* as well *in vitro* (12,15) have revealed some of the elements involved in the liver-specific expression of these genes. Recognition sites for liver-specific or -enriched factors have been identified, as for C/EBP (16), LF-A1 (17) HNF-1 (10), DBP (18) and LAP (19).

We are investigating the regulation of the chicken apoVLDL II gene, encoding the egg-yolk precursor protein, very-low-density apolipoprotein II. The gene is only expressed in liver, and its expression is under strict estrogen control (20). The regulation is primarily exerted at the level of transcription. Premature activation of the gene can be elicited by administration of estradiol to chicks or embryos. The induction by estrogen coincides with the appearance of DNaseI-hypersensitive sites (21) in the apoVLDL II locus, notably within the 300-bp region 5′ flanking the gene. These changes in chromatin structure involve the binding to the DNA of several proteins, as was shown by high-resolution *in vivo* footprinting with either dimethylsulphate (22) or DNAseI (23). These protein-DNA interactions are only found in the expressing liver, whereas in other tissues containing an estradiol receptor, as for example oviduct where the gene is completely silent, no binding is observed. Most of the binding sites found in chromatin were confirmed and further delimited by *in vitro* DNaseI footprinting and electrophoretic mobility shift analysis using crude nuclear extract from liver (23). In the previous studies, eight different binding sites could be distinguished within the 300-bp sequence 5′ flanking to the apoVLDL II gene, which were designated A through F starting with the promoter proximal element. Similarity of the protected sequences to established recognition motifs allowed Wijnholds et al. (23) to predict the most likely transcription factors to bind to these sites. In this way, they could tentatively assign binding

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sites E_1 and E_2 to the estrogen receptor (5,6), sites B_1, B_2 and D to DBP (18) and/or C/EBP (19), site A to he COUP-transcription factor (24, 25) and sites A and C to LF-A1 (14,17). Although the previous studies (22,23) located two estrogen response elements (EREs), their functional significance was not established. Here we present data of DNA transfection experiments with chicken embryonic hepatocytes showing hat both elements indeed confer estrogen inducibility to the apoVLDL II promoter. Likewise, we show by transfection experiments that that C/EBP acts as a transcription factor for the apoVLDL II gene and identify element B_1 as the minimal sequence required for this factor. By band-shift analysis, using COUP-TF antiserum and purified LF-A1 respectively, we experimentally confirm the anticipated (23) binding of the ubiquitous COUP-TF to site A and of the liver-specific LF-A1 to sites A and C. Insight into the tissue distribution of the binding activities interacting with the apoVLDL II 5′ region was obtained by *in vitro* footprinting. The role of the factors in the liver-specificity of apoVLDL II gene expression is discussed.

MATERIALS AND METHODS

Cell culture and transfection

Chicken embryonic hepatocytes were cultured and transfected as described (27). Briefly: 4 h after isolation, the cells were transfected by the calcium phosphate precipitation technique with a mixture of 7 μg reporter plasmid, 2 μg estrogen receptor expression plasmid HEO (28), and 1 μg pCH110. The latter plasmid contains the bacterial β-galactosidase gene under control of the SV40 promoter/enhancer (Pharmacia). Co-transfections including the C/EBP expression plasmid pMSV-C/EBP (16) were performed with 5 μg reporter plasmid, 2 μg HEO, 2 μg pMSV-C/EBP and 1 μg pCH110. The precipitate was divided over two Petri dishes (diameter 5 cm). After an 18-h incubation in a CO_2-incubator at 37°C, the cells were placed in fresh medium. The estrogen-analogue moxestrol was present in the medium where indicated at a concentration of 1 μM. Cell-free extracts were made as described (29), 44 hours after the addition of the $CaPO_4$-DNA precipitate. Extracts were assayed for β-galactosidase activity (30). The amount of whole cell extract used for CAT assays was normalized to a fixed β-galactosidase activity and was in the range of 10−100 μg. A 30-min CAT assay was performed (29).

Plasmids used for transfection

The following plasmids were constructed using standard procedures (31). A 1289-bp XbaI fragment (coordinates −963/+326) covering the apoVLDL II promoter (32) was cloned in the XbaI site of pTKCAT18 (27). pTKCAT18 had been constructed by cloning the multiple cloning site of pUC18 in pTKCAT8 (33). This plasmid contains the Herpes simplex virus TK promoter (−105/+51) driving the transcription of the chloramphenicol acetyl transferase (CAT) gene.

To generate 3′ deletion mutants of the (−963/+326) apoVLDL II fragment, the VLDL(−963/+326)TKCAT18 plasmid was cleaved 3′ to the apoVLDL II insert with BamHI and KpnI. The linearized plasmid was treated with Exonuclease III, S1 nuclease and Klenow polymerase as described (34). After religation, transformation and isolation of plasmid DNA, putative deletion mutants were identified by double-stranded plasmid sequencing using a labelled primer. From a selection of these 3′ deletion constructs, the TK promoter was deleted by restriction with XhoI and SacI. The sites were blunted with T4 DNA polymerase and religated in the presence of a BamHI linker (CCGGATCCGG). To create 5′ deletion mutants, the construct VLDL(−963/+34) CAT18 was cleaved 5′ to the apoVLDL II insert with SalI and SphI and treated with Exonuclease III as described above.

A plasmid in which the minimal promoter of the apoVLDL II gene was linked to the CAT gene was created by cloning the sequence from −41 to +34 relative to the transcription initiation site into pBLCAT3 (35). For this purpose, the 5′ deletion mutant VLDL(−41/+34)CAT was cut with HindIII and BamHI, and the sticky ends were filled-in by Klenow polymerase. pBLCAT3 was linearized with XhoI and blunted by Klenow polymerase. The VLDL fragment was ligated to pBLCAT3 and the desired plasmid, P_{VLDL}-CAT, was identified by double-stranded sequencing. Synthetic oligonucleotides encompassing the EREs and binding site B_1 were synthesized. The oligonucleotides were phosphorylated by T4 polynucleotide kinase and ATP. For annealing, about 100 pmol of each of the two complementary oligonucleotides were mixed, heated to 100°C and allowed to cool slowly to room temperature over a period of several hours. They were ligated in BglII-digested P_{VLDL}-CAT. The resulting recombinants were checked by double-stranded DNA sequencing.

Mutations of pVLDL(−277/+34)CAT in element E_1 were generated by the Kunkel method (36). All plasmids used for transfection experiments were purified by CsCl density centrifugation.

DNaseI footprinting and gel retardation

Nuclear extracts from livers of laying hen, immature hen and estrogen-treated rooster (23), from oviduct and spleen were prepared essentially according to Lichtsteiner *et al.* (37). These extracts were used in DNaseI footprinting and gel retardation experiments using the conditions and probes as decribed earlier (23). The binding reactions with LF-A1 were performed in LF-A1 binding buffer (20 mM Tris pH 7.9, 8% glycerol, 50 mM KCl, 1 mM EDTA and 0.2 mM DTT) supplemented with 0.2 μg poly (dI-dC), reannealed DNA corresponding to 0.1 ng of labelled oligonucleotide and 0.4 ng unlabelled oligonucleotide, 2 μl purified bovine LF-A1 (17) in a final volume of 15 μl. The samples were analyzed on 4% (30:1) polyacrylamide gels using 0,5×TBE as running buffer. Antiserum (1 or 2 μl of five times diluted serum in binding buffer) against COUP-TF (25) was

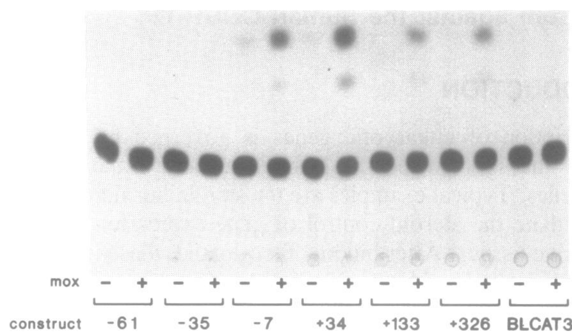

Figure 1. Determination of the 3′ border of the apoVLDL II promoter. Chicken embryonic hepatocytes were transfected with different VLDL-CAT plasmids, obtained by 3′ deletion of the VLDL(−963/+326)CAT construct, or with plasmid BLCAT3. Transient expression was in the absence (−) or presence (+) of moxestrol (mox; 1 μM). The autoradiogram of the CAT assay is shown; numbers refer to the 3′ end-point of the apoVLDL II sequence.

added after 10 min of pre-incubation of the labeled fragment and 3 μg of liver nuclear extract from a laying hen, and the incubation was allowed for another 10 min.

RESULTS

Minimal upstream sequences required for estrogen-dependent apoVLDL II expression

The minimal essential sequence for apoVLDL II promoter activity and estrogen-inducibility was delimited by transient expression of a linked chloramphenicol acetyl transferase (CAT) reporter gene in primary cultures of chicken embryonic hepatocytes (CEH). Starting from a gene construct which contained the apoVLDL II sequence from −963 to +326 and exhibited estrogen-dependent transcriptional activity in CEH cells (Fig. 1), 3′ deletions were generated. Activity was preserved when the 326-bp transcribed sequences were deleted, showing that they are not crucial for estrogen-dependent transcription. Activity was lost when the deletion proceeded from the −7 to the −35 position, which coincides with the removal of the TATA boxes for the major (+1) and minor (−11) transcription initiation sites (32). With a 5′ deletion series generated from the VLDL(−963/+34)CAT construct, activity was maintained up to position −256 (Fig. 2A). Upon further deleting apoVLDL II 5′ flanking sequences, the estrogen dependent activity was reduced in at least two steps, a large reduction when proceeding from −256 to −207 and loss of the remaining activity when deleting from −207 to −169. In each of the respective regions a sequence resembling the ERE consensus can be found; an imperfect palindromic sequence GGgCtCAGTGACC (mismatches in lower case), designated E2 and centered around −215, and a palindromic sequence GGTCAGACTGACC, designated E1 and centered around position −171. Mutating the sequence −177 to −165 resulted in a several fold lower activity of the −277 to +34 apoVLDL II fragment and allowed us to estimate the contribution of E1 to estrogen responsiveness (Fig. 2B). Together with the data from the 5′deletion series, this result suggest the presence further upstream of additional estrogen

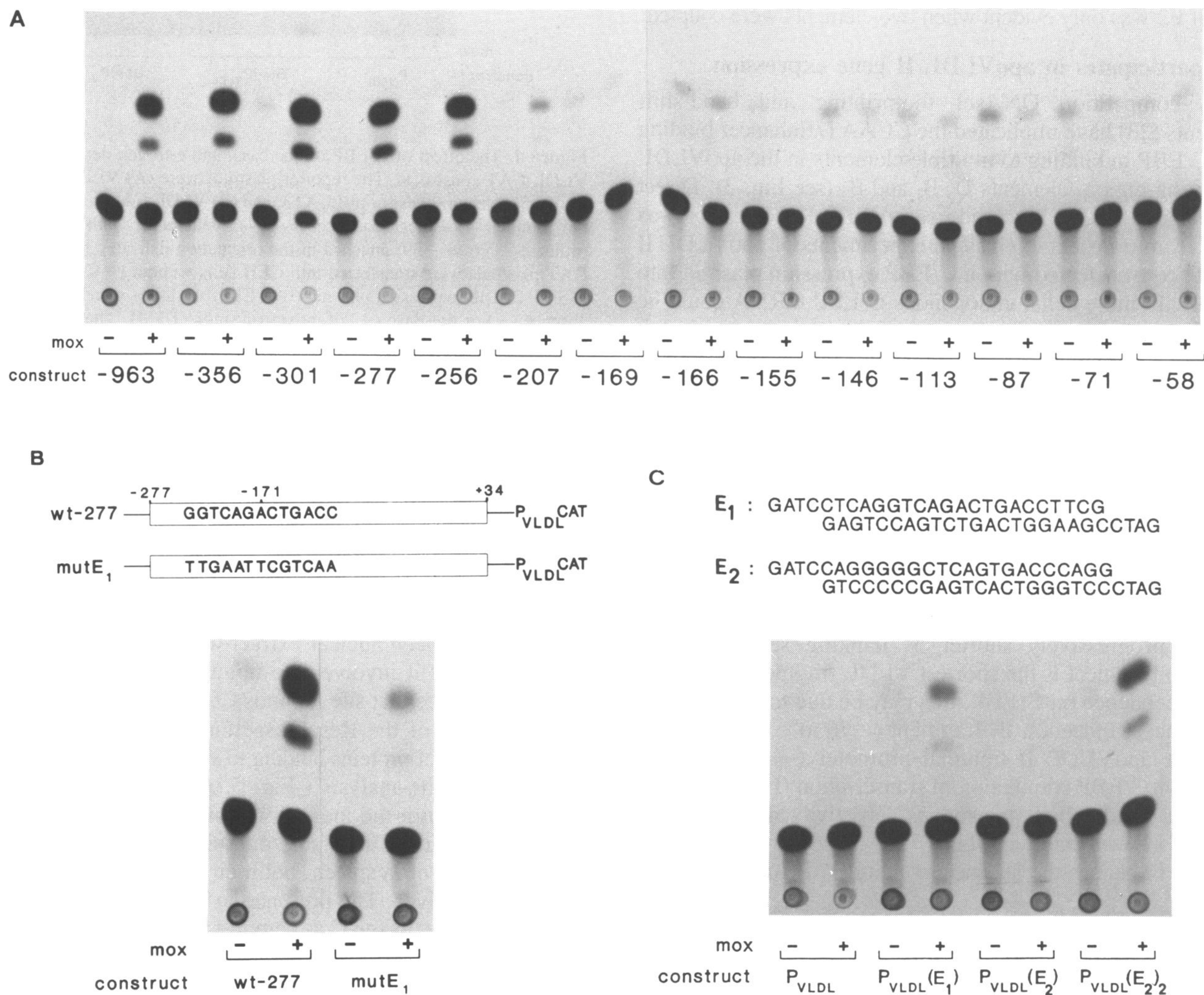

Figure 2. Analysis of the estrogen response elements upstream of the apoVLDL II promoter. Chicken embryonic hepatocytes were transfected with (**A**) a 5′ deletion series derived from the VLDL(−963/+34)CAT construct. Numbers refer to the 5′ end-points of the apoVLDL II sequence; (**B**) VLDL-CAT constructs containing the apoVLDL II sequence from −277 to +34 (wt-277) or a similar construct with the E1-sequence mutated as indicated (mut E1); (**C**) VLDL-CAT constructs containing the minimal promoter sequence −41 to +34 (P_{VLDL}), without or with oligonucleotides E_1 or E_2 ligated in front of it. All plasmids were co-transfected with the estrogen receptor expression plasmid, HEO (28). The cells were cultured for 44 hours without (−) or with (+) moxestrol (mox, 1 μM). An autoradiogram of the CAT assay is shown.

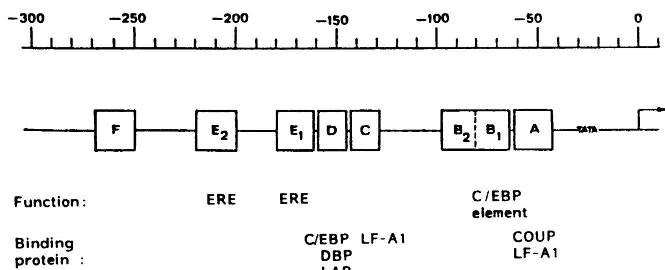

Figure 3. Schematic representation of the protein binding sites in the apoVLDL II promoter region. The binding sites (A through F) are boxed. The present study shows that E_1 and E_2 function as EREs, B_1 as a C/EBP element, that C binds LF-A1 and A binds COUP-TF and LF-A1. Based on its sequence, element D is indicated as a recognition site for C/EBP, DBP and LAP.

response sequences, possibly E2. Indeed, short oligonucleotides having these sequences incorporated conferred estrogen inducibility to a minimal apoVLDL II promoter-CAT construct (Fig. 2C). Whereas the activity of E1 was clearly visible, the activity of E2 was only evident when two elements were coupled.

C/EBP participates in apoVLDL II gene expression

Previous competition DNAseI footprinting and band-shift experiments (23) have implicated the CCAAT/Enhancer binding protein C/EBP in binding to multiple elements in the apoVLDL II 5′ flanking region, elements D, B_1 and B_2 (see Fig. 3). Direct evidence that C/EBP is involved in apoVLDL II expression was obtained from transient expression experiments of apoVLDL II constructs co-transfected with a C/EBP expression plasmid into CEH cells. In these cells, endogenous C/EBP mRNA could be detected but its concentration is considerably lower than in adult liver (data not shown) and is probably too low to maintain optimal C/EBP concentrations. Co-transfecting of the C/EBP effector gene (Figure 4A and Table 1) resulted in 5-fold stimulation of basal transcriptional activity from the VLDL(−277/+34)CAT construct. Estrogen-dependent transcriptional activity was stimulated to about the same proportion as basal activity. Since the effect of estrogen and C/EBP are more than additive, we conclude that C/EBP and the estrogen receptor have a co-operative action on apoVLDL II gene expression. C/EBP stimulation of basal transcription was maintained with constructs containing progressively shorter 5′ flanking sequence. The (−71/+34) fragment is the shortest VLDL fragment tested that showed dependence on C/EBP; this may be due to the presence of B sequences. Indeed, a B_1 fragment (−76 to −59) cloned in front of the apoVLDL II minimal promoter (−41/+34) was sufficient for C/EBP stimulation of transcription (Figure 4B and Table 1) and acted in both orientations. Its activity and stimulation by C/EBP is lower than with the −71/+34 construct (Table I), which may be due to the absence of the COUP/LF-A1 binding site A.

Taken together, the data provide evidence that the liver enriched transcription factor C/EBP participates in apoVLDL II expression and acts in co-operation with the estrogen receptor.

Tissue distribution of nuclear proteins binding to the apoVLDL II 5′ flanking region

The binding sites defined earlier (22,23) are schematically depicted in Figure 3. With liver nuclear nuclear extract, strong footprints were detected on sites A, B, D and F and weak

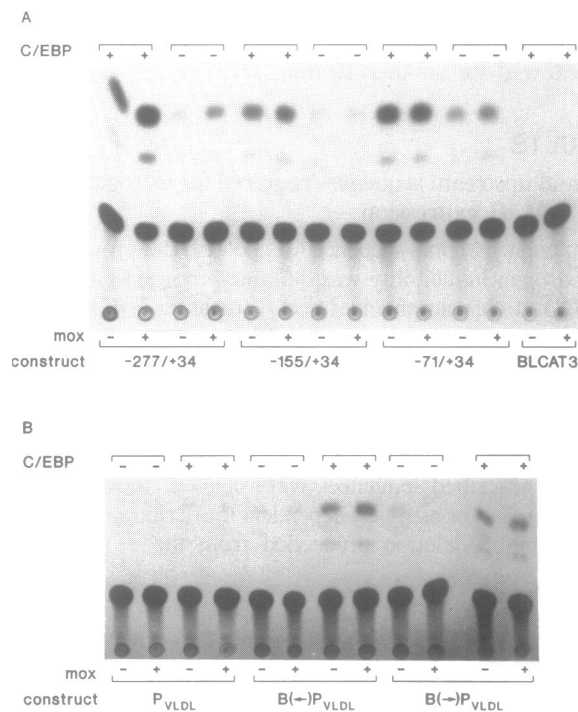

Figure 4. The effect of C/EBP on the basal and estrogen-dependent activity of VLDL-CAT constructs. The reporter plasmids were (**A**) VLDL-CAT plasmids from a 5′ deletion series, or pBLCAT3, and (**B**) VLDL-CAT constructs containing the minimal promoter sequence (P_{VLDL}), without or with element B_1 (co-ordinates −76 to −59) attached in the orientation indicated by the arrow. The CAT plasmids were transfected into CEH cells without (−C/EBP) or with the MSV−C/EBP expression plasmid (+C/EBP). Cultures were maintained in the absence (−) or presence (+) of moxestrol (mox; 1 μM). The autoradiogram of the CAT assay is shown.

footprints on sites C, E_1 and E_2 irrespective whether the extract was obtained from laying hen, hormone-treated or hormone-naive rooster (Fig. 5). With oviduct nuclear extract a strong footprint on element A and weak footprints on elements E_1, E_2 and the proximal moiety of element F were observed. The ability of liver as well as oviduct nuclear extract to interact with element A is confirmed by band-shift analysis showing two retarded bands (Fig. 6, panel A). A complex with the lower mobility was also formed with spleen nuclear extract which is consistent with the probability that it involves the ubiquitous protein, COUP-TF. Further evidence that site A binds COUP-TF is presented in the last paragraph of the Results section. The absence in oviduct nuclear extract of proteins binding to sites B and D was confirmed by the band-shift analysis (Fig. 5, panels B, C and E) and is in agreement with the indications that they represent the liver-enriched proteins C/EBP and/or DBP (23). Binding activities were also present in spleen, but their mobilities clearly differed from those in liver. The presence in liver and not in oviduct of a protein binding to site C as demonstrated by the *in vitro* DNaseI footprinting (Fig. 5) and band-shift analysis (Fig. 6, panel D) may indicate the involvement of still another liver-enriched protein. Evidence that is represents LF-A1 will be given in the following paragraph. Most of the *in vitro* footprints described here, cover the regions of protein-DNA interaction detected in liver chromatin by genomic footprinting (22,23). Whereas binding *in vivo* is restricted to the expressing liver, protection of the different sites *in vitro* was achieved with liver nuclear

Table 1. Effect of C/EBP on the basal and estrogen dependent activity of VLDL-CAT constructs

	CAT activity			
	−C/EBP		+ C/EBP	
VLDL	−mox	+mox	−mox	+mox
A. −277/+34	1.7	5.8	8.7	30.1
−155/+34	1.7	1.3	6.5	8.7
−71/+34	2.5	3.2	10.0	11.1
B. −41/+34	0.3	0.3	0.4	0.4
(B_1−)−41/+34	0.3	0.4	0.9	1.5
(B_1→)−41/+34	0.4	0.3	0.7	0.8

The CAT-activity from the experiments in Figure 3 is given as the percentage chloramphenicol acetylated. The data sets A and B were obtained in independent experiments. The co-ordinates of the VLDL fragment ligated to the CAT gene are given.

extracts from laying hen and estrogen stimulated rooster as well as from hormone-naive rooster. Thus, in non-expressing liver, the available proteins must in some way be prevented from binding to the DNA. Similarly, in oviduct the presumed COUP-TF must be excluded from binding to site A.

In summary, the apoVLDL II promoter region contains binding sites for general and liver-enriched transcription factors.

Element A is the target site for the ubiquitous COUP-transcription factor and liver-enriched LF-A1

The sequence of site A is reminiscent of the Chicken Ovalbumin Upstream Promoter element, binding the ubiquitous transcription factor COUP (22,23). Clear proof for binding of COUP-TF to element A was obtained in a super-bandshift assay with antibodies against the human COUP-TF (24,25). Incubation of liver nuclear extract of laying hen with labelled element A gave a distinct retarded complex that was upshifted when antibody against COUP-TF (COUP-ab) was included (Fig. 7A, lanes 3 and 4). The supershift complexes could be competed with a 100-fold excess of unlabeled element A (lane 5) or the authentic COUP-element (A′) (lane 6), but not with element C or D (lanes 7 and 8). Antibody alone gave an aspecific bandshift (lane 9). These results proof that element A is a recognition site for the COUP-TF. Super-shifted complexes could not be detected with element C (data not shown). Element A is recognized by still another factor, LF-A1 (17), as became clear when purified bovine LF-A1 was incubated with element A (lane 11). A similar, although weaker, signal is detected when LF-A1 is incubated with element C (lane 12). The position of the LF-A1/element A or C complexes in the gel is different from that of the complexes found with nuclear extracts (lanes 2 and 10, and data not shown). Possible explanations for this are: (1) the complexes detected with nuclear extracts may be homo- or hetero-multimeric protein-DNA complexes, whereas the complex with LF-A1 are monomer complexes, (2) the purified LF-A1 may be a degradation product of the authentic protein, (3) the chicken and bovine LF-A1 may differ in protein length or protein modification. The LF-A1/element A or element C complexes could be competed with a 100-fold excess of element A (Figure 7B, lanes 3 and 7) and element C (lanes 5 and 9), but not with element B_2 (lanes 4 and 8). The experiments in Figures 7A and 7B show that element A is a strong and element C is a weak recognition site for LF-A1. No LF-A1/COUP-ab/element A or C complexes were detected, when COUP-ab was added to the incubation mixture, what indicates that COUP-TF and LF-A1 are different proteins.

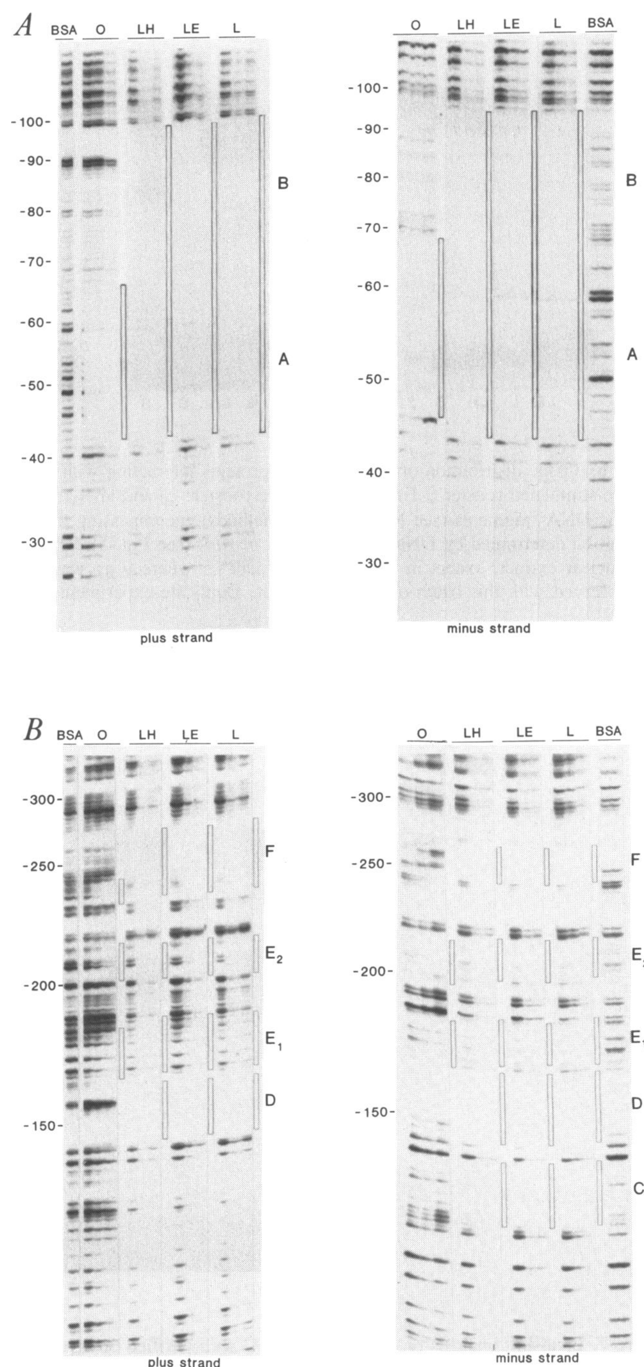

Figure 5. DNaseI footprinting of the apoVLDL II 5′flanking region. A DNA fragment extending from nt −555 to nt + 34 relative to the cap site was labelled at a BamHI site near position +34. DNaseI digestion was in the presence of three different concentrations (20 μg, 40 μg and 80 μg from left to right) nuclear extract from oviduct (O), laying hen liver (LH), estrogen-stimulated rooster liver (LE) and hormone-naive rooster liver (L) or 30 μg bovine serum albumin (BSA). The DNaseI pattern cleavage pattern of the +20 to −110 region (panel **A**) and the −100 to −300 region (panel **B**) are shown. Footprints observed are boxed.

DISCUSSION

The *in vitro* DNaseI footprinting described here and the *in vivo* footprinting reported earlier (22,23) show that the 300-bp sequence 5′to the apoVLDL II gene, contains several sites for DNA binding proteins. In the present study, we identified C/EBP

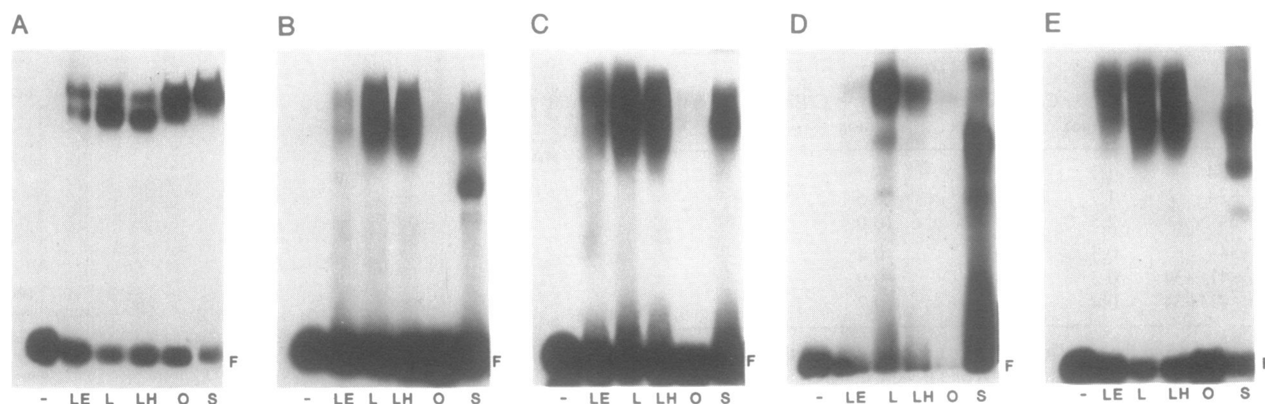

Figure 6. Tissue distribution of DNA binding proteins interacting with different DNA elements of the apoVLDL II promoter. Nuclear extracts from the livers of estrogen-stimulated rooster (LE), hormone-naive rooster (L) and laying hen (LH), from oviduct (O) and from spleen (S) were examined in band-shift analysis with synthetic DNA fragments (see Materials and Methods) encompassing the binding site A (panel **A**), site B_1 (panel **B**), site B_2 (panel **C**), site C (panel **D**) and site D (panel **E**) determined by DNaseI footprinting *in vitro* (see Fig. 5). The first lane in each panel is without nuclear extract. The experiments were performed with 3 μg nuclear extract, except in the case of element C, where 9 μg was used. Note: the weak binding as observed with LE nuclear extract and fragment C was only observed with this batch of nuclear extract. Duplicate experiments with other batches showed binding as strong as observed with the L and LH extracts.

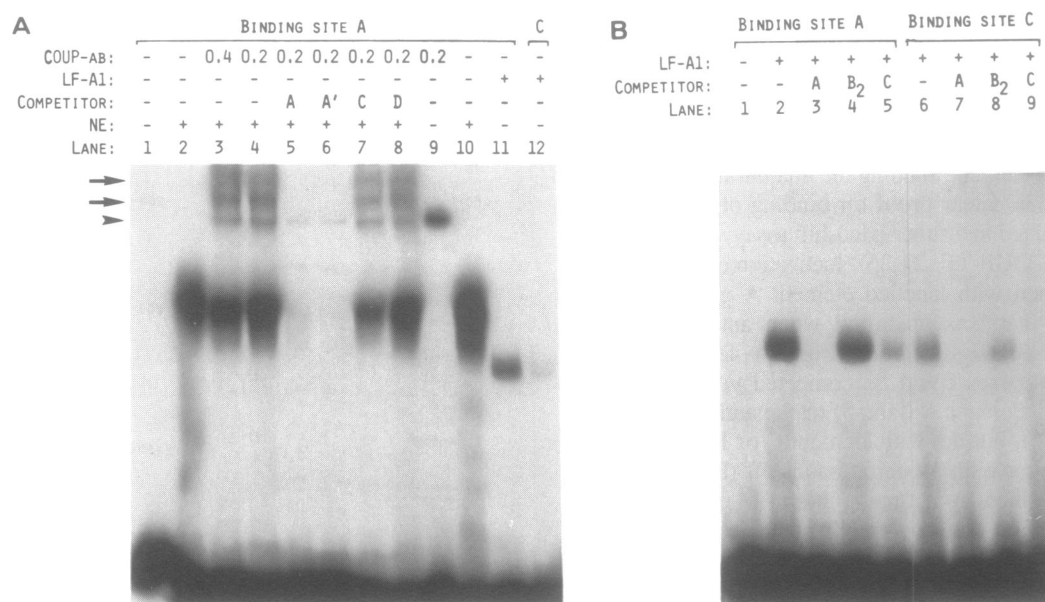

Figure 7. Bandshift analysis of elements A and C with purified bovine LF-A1 and antiserum against the COUP-TF. Panel (**A**), bandshift analysis using elements A (lanes 1–11) and C (lane 12), antiserum against the human COUP-TF (COUP-ab), purified bovine LF-A1, liver nuclear extracts (NE) from a laying hen, and unlabeled double-stranded oligonucleotides A, C, and D from the apoVLDL II promoter, and the COUP-element (A') from the ovalbumin promoter as competitors. The amount of COUP-ab added is indicated in μl serum; (–), absent; (+), present; arrows, supershifted COUP-ab/COUP-TF/element A complexes; arrowhead, aspecific complex detected in the COUP-ab serum. Panel (**B**), bandshift analysis using elements A (1–5) and C (6–9), and purified bovine LF-A1, and unlabeled elements A, B_2, and C as competitors. The binding-assay was performed as described in Materials and Methods, and incubated in standard binding buffer (Fig 7A, lanes 1–9), or in LF-A1 binding buffer (Fig. 7A, lanes 10–12 and Fig. 7B).

and the estrogen receptor as two of the proteins involved, show that they act as *trans*-acting factors and map their binding sites. In addition, we show that element A is recognized by the ubiquitous COUP-TF (24,25) and LF-A1 (14,17), and that element C is a (weak) recognition site for LF-A1 (Fig. 4).

Transfection experiments revealed an estrogen response unit (ERU) (5) located between −221 and −165. This unit consists of two estrogen response elements (EREs), a perfect palindromic sequence GGTCAGACTGACC (element E_1) at position −164 and an imperfect palindromic sequence GGGCTCAGTGACC (element E_2) at position −208. Both elements can confer estrogen inducibility to the apoVLDL II minimal promoter positioned *in cis*, showing that each of them is recognized by the estrogen receptor. Together they form a potent estrogen-inducible enhancer. Their combined action appears to be considerably larger than the sum of their individual activities, indicating that synergism between closely adjacent EREs (2) contributes to the total estrogen response of the apoVLDL II gene.

Co-transfection experiments with a rat C/EBP expression plasmid showed that C/EBP acts as a *trans*-acting transcription factor on the apoVLDL II promoter and that element B_1 (−76 to −59) is the minimal cis-acting sequence required to direct the factor to the gene. Birkenmeier *et al.* (39) showed that C/EBP is a liver-enriched transcription factor. Our *in vitro* DNaseI footprinting experiments in which we compared nuclear extracts from the estrogen target tissues liver and oviduct, showed that protection of element B_1 by nuclear extracts is restricted to liver. So, the apoVLDL II gene appears to be specifically expressed in liver under the control of C/EBP.

The combined action of the estrogen receptor and the liver-enriched transcription factor C/EBP would be sufficient to impose hormone-dependency and tissue-specificity on apoVLDL II expression. However, other DNA binding proteins are apparently involved including the COUP-TF recognizing site A, and LF-A1 recognizing sites A and C. We do not know whether the footprint on site A formed by liver nuclear extracts is due to COUP-TF or LF-A1. However, the different positions of the gene-proximal boundary of footprint A generated with liver and oviduct nuclear extracts (see Fig. 5) may indicate that the most effective binding activities in these tissues differ. Site C was identified as a second, less strong, binding site for LF-A1. Multiple binding sites for the same factor were also found for C/EBP. Based on cross-competition with oligonucleotides it is concluded that sites B_1, B_2 and D bind the same heat-stable protein, presumably C/EBP (23). Indeed, site D is very similar to the established C/EBP binding site of the rodent albumin genes (40). Recently, it has been shown that C/EBP shares its affinity for the albumin element D with two other liver-enriched DNA-binding proteins, DBP (18) and LAP (19).

Concerning the footprint located upstream of element E_2, no clear similarities between sequence F and known recognition sequences were noticed. The possible function of this site in apoVLDL II gene expression has not been investigated yet.

Under the *in vitro* conditions of protein-DNA interaction, all binding sites in front of the apoVLDL II gene become occupied by liver nuclear proteins, independent of the estrogen status of the organ. This contrasts with the situation *in vivo* where binding is only observed in the expressing livers of the laying hen and the estrogen stimulated rooster (23). In the hormone-naive liver, binding of nuclear proteins, that are apparently available must therefore in some way be prevented. A likely explanation is that *in vivo* the binding sites are not freely accessible to their proteins because the DNA is folded into a nucleosome structure (21). The activated estrogen receptor would then primarily act as a chromatin 'opener' to make the other sites accessible to their binding proteins. Recently, evidence for such a scheme was presented by Piña *et al.* (41), who showed that nucleosome positioning modulates the accessibility of regulatory proteins to the mouse mammary tumor virus promoter.

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