Eur. J. Biochem. 249, 113–120 (1997)

Differential stimulation by CCAAT/enhancer-binding protein α isoforms of the estrogen-activated promoter of the very-low-density apolipoprotein II gene

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(Received 22 May/11 August 1997) – EJB 97 0729/2

The transcription factors CCAAT/enhancer-binding proteins α and β (C/EBPα and C/EBPβ) are highly expressed in liver and are believed to function in maintaining the differentiated state of the hepatocytes. C/EBPα appears to be a critical regulator of genes involved in metabolic processes. We are interested in the roles of C/EBP in the expression of the very-low-density apolipoprotein II (apoVLDL II) gene. This gene encodes an avian yolk protein, is induced by estrogens and is only expressed in liver. To examine the role of C/EBP in apoVLDL II expression, footprinting and electromobility-shift analysis were performed. For three of the protein-binding sites in the apoVLDL II promoter region, C/EBPα and C/EBPβ were identified as the major DNA-binding activities. For one of the C/EBP genes, C/EBPα, the effect of the gene products on apoVLDL II transcription was examined. From transfection experiments we conclude that maximal estrogen-dependent activity of the apoVLDL II promoter requires the dual action of the estrogen receptor and C/EBP. The level of activity is different depending on the nature of the C/EBPα translational isoform transfected, the full-length C/EBPα polypeptide being the most active isoform and the N-terminally truncated isoform being moderately active. The present results suggest a role of C/EBPα translational isoform ratio in the modulation of expression of C/EBP target genes, such as those involved in metabolic processes.

Keywords: CCAAT/enhancer-binding protein α; estrogen receptor; very-low-density apolipoprotein II; isoform.

Expression of the avian egg-yolk precursor protein very-low-density apolipoprotein II (apoVLDL II) is confined to liver and strictly dependent on estrogen [1]. Normally, the gene is only expressed in hen throughout vitellogenesis, but it can also be induced prematurely and in male by administration of estrogen. Expression of the chicken apoVLDL II gene is primarily regulated at the level of transcription and provides a convenient model to study control of liver-specific, steroid-inducible gene expression.

Transfection studies with deletion mutants revealed that the 300-bp 5′ region flanking the apoVLDL II gene has retained the capacity for estrogen-dependent transcription [2]. Two estrogen-response elements have been identified, which form a potent estrogen-response unit. Within the 300-bp gene-proximal region, multiple estrogen-inducible deoxyribonuclease-I-hypersensitive sites are present, indicating that this region is a major target for DNA-binding proteins and that binding occurs in an estrogen-dependent fashion [3]. By *in vivo* and *in vitro* footprinting and electromobility-shift analysis several binding activities in liver could be distinguished and their binding sites delineated [2, 4, 5]. In addition to binding sites for the estrogen receptor (ER), which correspond to the estrogen-response elements, sites for the ubiquitous chicken ovalbumin upstream promoter transcription factor and hepatocyte nuclear factor-4 (HNF-4) were identified. Three of the footprints in the upstream region of the apoVLDL II gene were tentatively assigned to the C/EBP family, because binding activity in liver resists heat treatment [5]. C/EBP are known to be heat-stable proteins. Transfection experiments by Beekman et al. [2] have shown that rat C/EBPα enhances the estrogen-dependent expression of an apoVLDL-II-promoter-driven reporter gene.

Of the C/EBP transcription factors, C/EBPα and C/EBPβ are the major C/EBP-site-binding proteins in liver [6]. C/EBPα is also found in several other terminally differentiated cells, among others in adipocytes [7], whereas C/EBPβ is most abundant in liver and lung [8, 9]. C/EBPα has been shown to promote the terminal differentiation of adipocytes, including their conversion from preadipocytes [10–12], and the transcription of fat-specific genes [13, 14]. Mature adipoctes appear to be kept in the quiescent state by the anti-mitotic activity of the C/EBPα protein [15]. In liver, C/EBPα is involved in the regulation of a subset of genes that are critical for glycogen synthesis and gluconeogenesis [16, 17]. The reduced C/EBPα levels during proliferation of hepatoma cells in culture and of hepatocytes in regenerating liver [18] suggest that C/EBPα may inhibit mitosis of hepatocytes as is does of adipocytes. In view of its effects in adipocytes and hepatocytes, C/EBPα has been described as the central regulator of energy metabolism [19]. Recently, this view has ob-

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Abbreviations. apoVLDL II, very-low-density apolipoprotein II; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; HNF, hepatic nuclear factor; K, T or G; NF-M, nuclear factor-myeloid.

tained support from the phenotype of C/EBPα knock-out mice, which are deficient in glycogen storage, due to reduced glycogen synthase expression, and do not accumulate lipid in adipose tissue [20]. During the acute-phase response, the steady-state levels of C/EBPα in liver and adipose tissue decrease whereas C/EBPβ is induced [21, 22]. The induction of tissue-specific genes by C/EBP appears to occur cooperatively with other transcription factors, for example, for C/EBPα with nuclear factor-Y and HNF-1 in the activation of the serum albumin promoter in liver [23, 24], and for C/EBPβ with the c-Myb proto-oncogene product in the induction of myeloid-specific genes [25].

A characteristic feature of C/EBPα and C/EBPβ is that their mRNAs are translated into distinct isoforms by translation from multiple in-frame start sites [6, 26, 27]. Two principal isoforms are generated: a full-length isoform containing the N-terminal domain responsible for *trans*activation and growth arrest; and an N-terminal truncated isoform lacking this domain. For C/EBPα it has been shown [28] that the generation of the smaller isoform is critically dependent on the presence of a small upstream ORF in its mRNA 5′ untranslated sequence. Depending on the promoter context, the smaller isoform behaves as a dominant-negative regulator or as transcriptional activator with reduced activity [27–29].

In this paper we show that chicken C/EBPα [30] and chicken C/EBPβ (nuclear factor-myeloid, NF-M) [31] are the principal proteins in adult chicken liver that interact with the C/EBP-binding sites in the apoVLDL II proximal region. For C/EBPα it was shown that it cooperates with ER in the synergistic activation of the apoVLDL II promoter. The level of estrogen induction is dependent on the C/EBPα isoform that participates in the activation.

MATERIALS AND METHODS

Nuclear-extract preparation. Nuclei were isolated from livers of Rhode Island Red hens as described by Lichtsteiner et al. [32]. Liver nuclear extracts were prepared following the ammonium sulphate procedure described earlier [5]. Heat-treated nuclear extract was prepared by 10-min incubation at 70°C, cooling on ice and removal of aggregated material by 3-min centrifugation in a microcentrifuge. Total nuclear lysate (in NaCl/urea/Nonidet P-40) from liver was prepared following a protocol of Lavery and Schibler [33]. The following proteinase inhibitors were included in the extraction buffers: 125 μM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin; 0.7 μg/ml pepstatin A; and 1% Trasylol.

Expression plasmids. Construction of expression plasmids in pSG5 with the C/EBPα gene driven by the simian virus 40 early promoter and site-directed mutagenesis were performed as described previously described in [28]. The truncated chicken C/EBPα expression construct (cC/EBP:cC/EBP:Δldr) has been described previously [28]. A full-length chicken C/EBPα pSG5 construct with the first chicken C/EBPα AUG start codon in an optimal Kozak sequence [34] and without the mRNA leader sequences was made by site-directed mutagenesis using primer 5′-GCA GGC TGT A*GG ACC* ACC ATG GAG CAA GCC-3′ (mutations affecting the start site underlined; *Ava*II site introduced for subcloning in italics). The pSG5-based expression plasmid encoding the chicken estrogen receptor was a gift of P. Chambon [35, 36]. The pVLDL (−301/+34)CAT reporter plasmid has been described previously [37].

Restriction sites for subcloning in pET prokaryotic expression vectors [38] were created by site-directed mutagenesis of the cC/EBPwt-pSG5 construct as described previously [28]. For *cC/EBPαFl-pET3c*, a *Bam*HI site was introduced just in front of the first ATG start codon using primer 5′-AGG CTG TAG GAT CCC CAT GGA GCA AGC-3′ (mutations underlined). The *Bam*HI fragment covering the chicken C/EBPα coding region was cloned into the *Bam*HI site of pET3c, and the clones with the proper orientation were selected by restriction analysis. For *cC/EBPβFl-pET3d*, an *Nco*I−*Bam*HI fragment from the NF-M-pBluescript (gift of A. Leutz, Max Delbrück Center, Berlin) containing the coding region of NF-M was cloned in the *Nco*I and *Bam*HI sites of pET3d.

Overexpression of recombinant proteins. For overexpression of recombinant C/EBP polypeptides in *Escherichia coli*, the cC/EBPα-pETc and NF-M.pETd constructs were introduced in strain BL21DE3.pLysE [38]. Cultures were grown at 28°C to A_{660} = 0.6 and induced with 1 mM isopropylthio β-D-galactopyranoside. Cells were harvested after 4 h cultivation at 28°C, washed once, and suspended in 0.01 vol. 100 mM KCl, 25 mM Hepes, pH 7.8, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol. Total protein extracts were made by sonication, and cleared from precipitated material by a 30-min centrifugation in an Eppendorf centrifuge. The soluble protein fraction contained 10–30% recombinant C/EBP. Because C/EBP proteins are heat stable, the recombinant C/EBP proteins could be further purified by incubating the total protein extract for 10 min at 70°C and removal of the precipitated material by a 3-min centrifugation in an Eppendorf centrifuge.

DNAse I footprinting. For DNase I footprinting, labelled fragments of the promoter region were obtained by PCR. One of the two primers used in the PCR reaction was 5′ labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The PCR contained 10 ng plasmid pVLDL-CAT, 125 ng upstream primer and 125 ng downstream primer. The PCR product was purified with a QIAquick-spin PCR purification column (Qiagen). 40000–60000 cpm (≈10–20 ng) DNA was used in each assay. The standard binding reaction was performed in 50 μl 20 mM Hepes, pH 7.9, 60 mM KCl, 0.06 mM EDTA, 0.6 mM dithiothreitol, 2 mM spermidine, 10% glycerol, containing 2.5 μg poly(dI-dC), 20–80 μg nuclear extract (native, or heat treated for 10 min at 70°C) or 15 μg chicken-C/EBPα-containing *E. coli* extract (heat treated). After incubation on ice for 10 min, the reaction mixture was supplemented with the DNA probe, incubated at 25°C for 20 min, placed on ice for 5 min, and incubated for 2 min at 0°C with 2.5 μl freshly diluted DNase I (50–150 μg/ml) in 10 mM Hepes pH 7.9, 25 mM $CaCl_2$, 117 mM $MgCl_2$, 100 μg/ml BSA. Reactions were stopped by adding 200 μl 20 mM Tris/HCl pH 7.5, 20 mM EDTA, 0.5% SDS, 2.5% yeast tRNA. The reaction mixture was extracted twice with phenol/chloroform, and twice with chloroform. The DNA was precipitated with ethanol, dissolved in 80% formamide, 10 mM EDTA and tracking dyes, denatured for 2 min at 90°C and loaded on a 6% urea/polyacrylamide gel. Sequence reactions were obtained with the primers used for PCR.

Electromobility shift assay (EMSA). The probes used in the EMSA were obtained by annealing synthetic complementary oligonucleotides (HPLC purified). For probe A, 5′-GAT CCC AGG ACC TTT GAC CCC TCA CG-3′ and 5′-GTA CCG TGA GGG GTC AAA GGT CCT GG-3′; for probe B1, 5′-GAT CCA TGG GGA AAC AAA GCA GGA CG-3′ and 5′-GAT CCG TCC TGC TTT GTT TCC CCA TG-3′; for probe B2, 5′-CTA GAA GAA TTT GCA TGC AAA AGA GT-3′ and 5′-CTA GAC TCT TTT GCA TGC AAA TTC TT-3′; for probe C, 5′-AGC TTA CAG GTC CAG AGT CCT ACA-3′ and 5′-AGC TTG TAG GAC TCT GGA CCT GTA-3′; for probe D, 5′-GGG ACC TTC CAT TAC CAA ATC CGA ACC C-3′ and 5′-GGG TTC GGA TTT GGT AAT GGA AGG TCC C-3′; for probe E1, 5′-GAT CCT CAG GTC AGA CTG ACC TTC G-3′ and 5′-GAG TCC AGT CTG ACT GGA AGC CTA G-3′; for probe F, 5′-

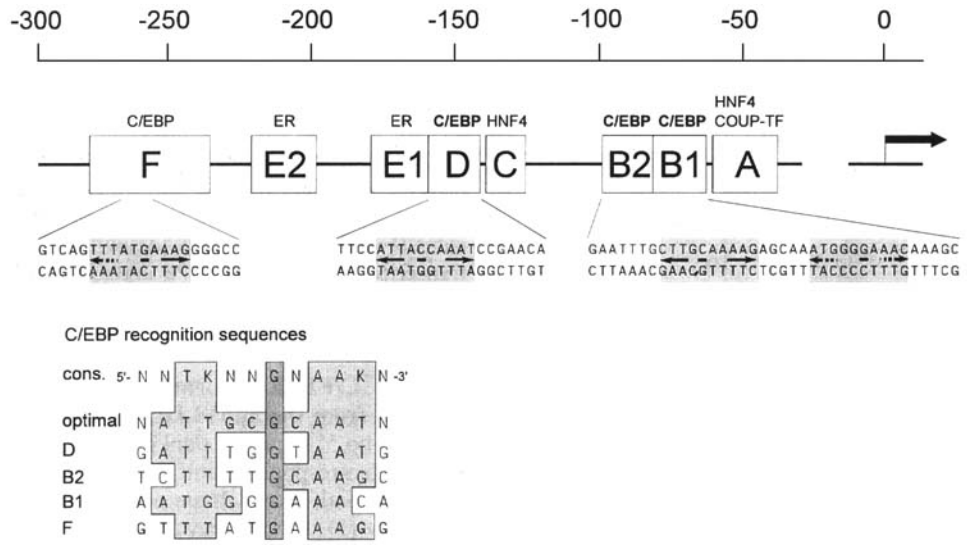

Fig. 1. Schematic representation of the protein-binding sites in the apoVLDL II promoter and the associated factors. The binding sites are indicated as lettered boxes and represent the footprints found in the present and previous studies [4, 5]. Sequences containing putative C/EBP-binding sites are specified. In the lower part of the figure the putative C/EBP-binding sites are aligned with the C/EBP consensus (cons.) and optimal target sequence [41, 42].

GAT CCG GAA TTT GGT CAG TTT ATG AAA GGG GCC TCT ATG ACA TGG TTG CCG-3′ and 5′-GAT CCG GCA ACC ATG TCA TAG AGG CCC CTT TCA TAA ACT GAC CAA ATT CCG-3′. 50 ng single-stranded oligonucleotide was ^{32}P-labelled with T4 polynucleotide kinase, separated from free ^{32}P by gel filtration on Sephadex G50, precipitated with ethanol in the presence of 2 μg poly(dI-dC), and washed with 96% ethanol. The labelled DNA was dissolved with a fourfold excess of the opposite DNA strand in 20 μl 10 mM Tris/HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol. Prior to annealing, the mixture was heated for 2 min at 90°C and cooled slowly to room temperature.

The binding reaction was in 15 μl 20 mM Hepes pH 7.9, 60 mM KCl, 0.06 mM EDTA, 0.6 mM or 2.5 mM dithiothreitol, 2 mM spermidine, 10% glycerol, containing 2 μg poly(dI-dC) and 0.1−0.5 ng labelled double-stranded oligonucleotides. After adding 1 μg bacterial extract or 0.5−2 μg liver nuclear extract, the mixture was kept for 10 min on ice, supplemented with the DNA probe, and incubated for 20 min at 25°C. The samples were analysed on a 4% (30:1) polyacrylamide gels with Tris/borate/EDTA electrophoresis buffer [40]. After air drying on Whatman paper, the gels were exposed to Kodak X-ray film or to a PhosphorImager plate (Molecular Dynamics) for visualisation.

The immune-EMSA procedure is essentially the same as the EMSA procedure except that chicken C/EBPα or chicken C/EBPβ/NF-M antiserum was added (1:150−1:15 dilutions) in the binding reaction. The C/EBPα polyclonal antiserum used has been described previously [28]. The C/EBPβ/NF-M polyclonal antiserum was a gift of A. Leutz (Max Delbrück Center, Berlin).

Cell culture, transfection, and promoter-activation assay. LMH cells [39] and HepG2 cells were cultured in six-well tissue-culture dishes in Dulbecco's modified Eagle's medium/F12/5% fetal calf serum (GibcoBRL) in a 10% CO_2/air mixture at 37°C. At about 60% confluency, cells were transfected with 1 μg pSG5-based expression plasmids and 2 μg VLDL(−301/+34)CAT-reporter plasmid [34] using 5−10 μl lipofectin (GibcoBRL) in serum-free medium. After 24 h, the transfection medium was replaced by serum-containing medium without or with addition of 0.1 μM moxestrol (a synthetic estrogen) for estrogen receptor activation. Cells were harvested after 1 day and chloramphenicol acetyltransferase (CAT) expression was measured by a CAT assay [40] using equal amounts of cell protein/assay. To account for transfection efficiency, all transfections were in duplicate and performed twice.

RESULTS

Binding sites for C/EBP transcription factors in the apoVLDL II promoter region. Of the eight binding sites for liver nuclear proteins that are present in the 5′ 300 bp flanking the apoVLDL II gene, three have been identified provisionally as C/EBP sites. These sites, termed B1, B2 and D, conform to the C/EBP consensus sequence TKNNGNAAK, where K ist T or G [41, 42] (Fig. 1) and are protected by heat-treated liver nuclear extract in a DNAse-I-footprint experiment [5]. To identify the effective binding activity, the binding characteristics of liver nuclear extract were compared with those of recombinant chicken C/EBPα and the complexes were analysed with antisera raised against chicken C/EBPα and C/EBPβ. Binding coordinates of recombinant chicken C/EBPα protein and heat-treated chicken liver nuclear extract were compared in an *in vitro* footprint analysis with DNA fragments covering the proximal promoter region (Fig. 2). Recombinant C/EBPα gave strong protection of two regions in the apoVLDL II promoter (Fig. 2), designated D (−144/−165) and B (−62/−98). Both sequences match exactly the regions protected by heat-treated nuclear extract (Fig. 2). Protected region B contains two distinct C/EBP-binding sites, called B1 and B2, as can be deduced from its sequence harbouring two consensus C/EBP-recognition sites (Fig. 1). EMSA with bacterially expressed recombinant C/EBPα and oligonucleotides encompassing the different protein-DNA−interaction sites (Fig. 1, elements A−F) of the apoVLDL II promoter region revealed that binding sites B1, B2 and D are high-affinity C/EBP-binding sites (Fig. 3A). In addition, weak binding was found with the large distal element F, which contains a possible C/EBP recognition sequence (Fig. 1). The absence of a defined footprint in the present DNase-I-protection experiment (Fig. 2) is probably due to extinction of the signal in the upper gel regions. EMSA with the chicken homologue of C/EBPβ (NF-M) [31] obtained from a prokaryotic expression system

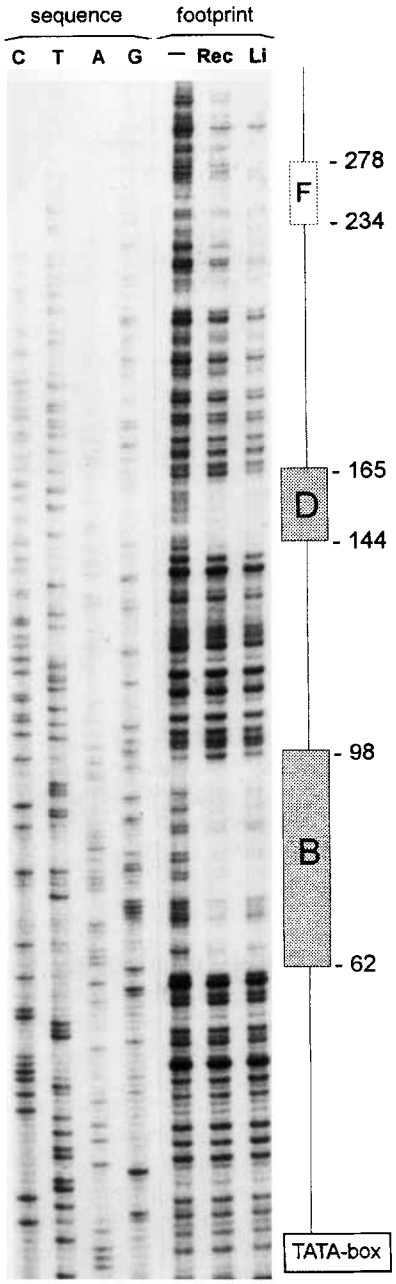

Fig. 2. *In vitro* DNase-I-footprinting of heat-stable liver nuclear proteins and recombinant chicken C/EBPα to the apoVLDL II promoter region. A fragment extending from nucleotide −553 to +34 was multiplied and end-labelled on the non-coding strand with a [γ-^{32}P]ATP-labelled primer using PCR. DNase I digestion was in the presence of heat-treated *E. coli* proteins as a control (−), recombinant C/EBPα (Rec) or heat-treated chicken liver nuclear proteins (Li). The position of the TATA box is indicated and protected regions B and D are indicated by lettered boxes with the positions relative to the start of transcription indicated. Box F indicates the position of an earlier detected footprint not visible in the present experiment. The chain-termination sequencing reaction was performed with the primer used for the labelling reaction.

showed identical binding characteristics (data not shown). The proteins from liver nuclear extract binding to site D also bind to sites B1 and B2, as shown by the competition of binding to labelled D by excess unlabelled B1 and B2 and not by unlabelled A (Fig. 3B). To unambiguously identify C/EBP proteins as the binding activity in chicken liver nuclear extract, immune-EMSA were carried out using antisera against recombinant C/EBPα [28] and C/EBPβ [31]. Both antisera have been shown to specifically interact with the respective recombinant C/EBP protein (data not shown). The site-D-binding complex formed with liver nuclear extract was partly shifted to complexes with lower electrophoretic mobility by the C/EBP antisera, most clearly with the C/EBPβ antiserum. The lesser capacity of the C/EBPα antiserum to generate a supershift may be caused by antibody interference with the DNA binding of C/EBPα, as the reduction in signal of the retarded protein-DNA complex indicates (Fig. 4). The data provide evidence that the liver-enriched transcription factors C/EBPα and C/EBPβ bind to sites B1, B2 and D, and possibly F, in the proximal promoter region of the apoVLDL II gene.

Chicken C/EBPα isoforms modulate estrogen-induced expression from the apoVLDL II promoter in hepatoma cells. The 300-bp 5′ sequence flanking the apoVLDL II gene contains an estrogen-responsive unit composed of two estrogen-response elements, both of which are necessary for maximal estrogen inducibility [2]. Of the transcription factors in liver that bind to specific positions within this region, C/EBPα and C/EBPβ occur as different isoforms [6, 26−28]. These translational isoforms of C/EBPα and C/EBPβ are functionally different in their *trans*-activation potential in liver cells. On a promoter containing solely C/EBP-binding sites the full-length C/EBP isoforms activate transcription and the smaller C/EBP isoforms act as repressors of *trans*activation [27, 28].

To examine the activities of the different C/EBPα isoforms in the context of the estrogen-inducible apoVLDL II promoter, transfection experiments in two hepatoma cell lines were performed. In the chicken hepatoma cell line LMH [39], which expresses endogenous estrogen receptor [43], addition of the synthetic estrogen moxestrol to the growth medium stimulates the transfected apoVLDL II promoter (bp −304/+34) ninefold (Fig. 5A). When an effector plasmid encoding full-length C/EBPα was cotransfected into estrogen-deprived cells, basal activity was stimulated sixfold. Addition of moxestrol enhanced the activity to 24-fold the basal level without C/EBPα (Fig. 5A). The magnitude of the latter response was considerably larger than the sum of the separate effects, suggesting that the cognate transcription factors, ER and C/EBPα, act synergistically. Truncated C/EBPα also enhanced apoVLDL II promoter activity (Fig. 5A). Although the stimulation by truncated C/EBPα was smaller than by full-length C/EBPα (3-fold and 16-fold of basal activity in the absence and presence of estrogen, respectively), synergism is clearly preserved. Further truncating C/EBPα, leaving only the basic leucine zipper DNA-binding domain intact, abolished all C/EBP-mediated activity (Fig. 5A). It is unlikely that the observed differences in the activities of the C/EBP isoforms are due to differences in expression levels or protein stability because in a previous study we showed that in COS-1 cells the C/EBPα-expression constructs gave similar expression levels with no detectable protein degradation [28].

Because of the presence of ER, the activity of this transcription factor in LMH cells could only be manipulated by ligand deprivation or addition. Therefore we switched to the human hepatoma cell line HepG2, which is known to contain HNF-4 [44], but has very little C/EBPα [29] and is devoid of ER. This system allowed us to study the effects of ER more directly. ApoVLDL II promoter activity was stimulated 50−70-fold by coexpression of full-length C/EBPα in an estrogen-independent manner (Fig. 5B). Cotransfection of ER only yielded 22-fold or 77-fold stimulation in the absence or presence of estrogen, respectively (Fig. 5B). The effect of C/EBPα and ER in the presence of estrogen was strongly synergistic, as can be concluded from the apoVLDL II promoter activity being 390-fold the basal activity without any effector plasmid co-transfected (Fig. 5B).

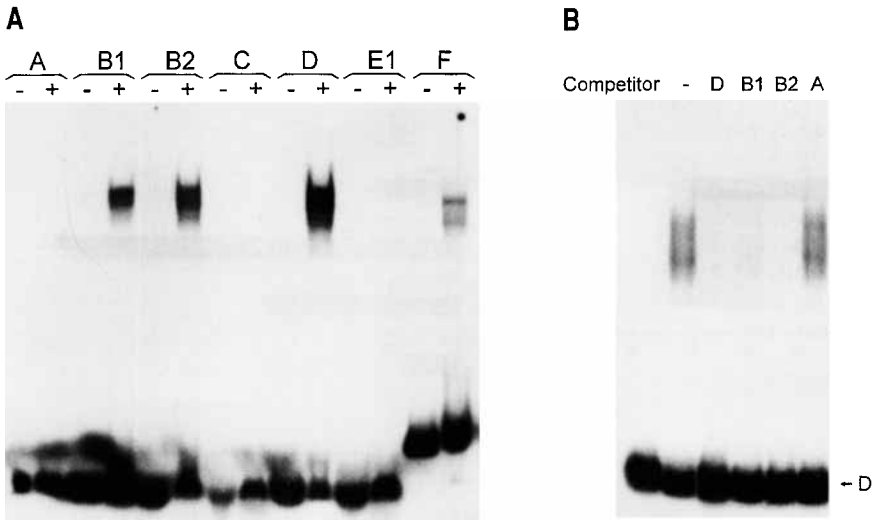

Fig. 3. *In vitro* binding of chicken C/EBPα to specific apoVLDL II binding sites. (A) EMSA with recombinant C/EBPα (1 μg) and labelled oligonucleotides covering the different binding sites identified in the apoVLDL II proximal regulatory DNA (Fig. 1). Binding reactions were without (−) or with (+) recombinant C/EBPα. The sites B1, B2 and D bind C/EBPα with high affinity, site F binds C/EBPα with low affinity. (B) Binding sites B1, B2 and D bind the same proteins in chicken liver nuclear extract. The binding reaction contained 0.5 μg nuclear extract, labelled element D and no (−) or 50-fold molar excess unlabelled competitor DNA (element B1, B2, D or A). Binding to element D can be competed by the C/EBP-elements D, B1 and B2 but not by the unrelated element A.

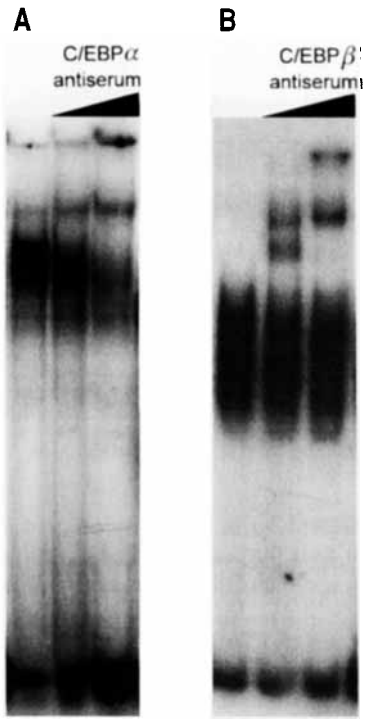

Fig. 4. Identification of chicken C/EBPα and C/EBPβ as the proteins in chicken liver that bind to element D. The EMSA binding reaction contained labelled site D, 3 μg liver nuclear extract and anti-C/EBPα serum (none, and 1:150 and 1:15 dilutions) (A) or labelled site D, 1.8 μg liver nuclear extract and anti-C/EBPβ serum (none, and 1:150 and 1:15 dilutions) (B). Total nuclear lysate [33] was used for C/EBPα detection because in the conventional nuclear-extract preparation full-length C/EBPα proteins are lost [28].

Although truncated C/EBPα was inactive when applied separately (Fig. 5B), it stimulated the apoVLDL II promoter when added in the presence of ligand-activated ER (Fig. 5B). As in LMH cells, in HepG2 cells the stimulation by truncated C/EBPα was smaller than by full-length C/EBPα, and synergism was preserved.

The data obtained in both hepatoma cell lines show that high-level, estrogen-dependent activation of the apoVLDL II promoter is dependent on the presence of ER and C/EBPα. The action of both factors is synergistic. The magnitude of the activation of the apoVLDL II promoter by ER and C/EBPα depends on the translational C/EBPα isoform participating. Activation is maximal in the presence of the full-length C/EBPα isoform and sub-optimal in the presence of the truncated C/EBPα isoform.

DISCUSSION

Tissue-specific expression of an estrogen-inducible gene is usually restricted to one or a limited subset of estrogen target cells. The specific expression of an estrogen target gene in a particular cell type is believed to depend on the action of ER in combination with a unique set of tissue-specific transcription factors. In chicken, the apoVLDL II gene is induced by estrogen. Induction only occurs in liver and not in other estrogen target tissues [3, 4]. The inactivity of the apoVLDL II gene, for example in oviduct, may be explained by the absence of transcription factors such as C/EBPα, C/EBPβ and HNF-4.

The chicken hepatoma cell line LMH was chosen for transfection studies of the apoVLDL II promoter because these cells most closely resemble the natural environment, including the presence of endogenous ER. In addition, the mammalian hepatoma HepG2 cell-line was used because these cells do not express ER and C/EBP proteins, allowing us to control their presence by transfection of the cognate expression plasmids. Earlier transfection studies have shown that estrogen induction was retained with constructs harbouring the 300-bp 5′ sequence of the apoVLDL II gene [2]. In the present study we showed that C/EBPα and C/EBPβ are responsible for occupation of three, possibly four, protein-binding sites within this region, that maximal transcriptional activation of the promoter is achieved by the synergistic action of ER and C/EBP proteins, and that the nature of the translational C/EBPα isoform participating in the estrogen induction determines the level of apoVLDL II expression.

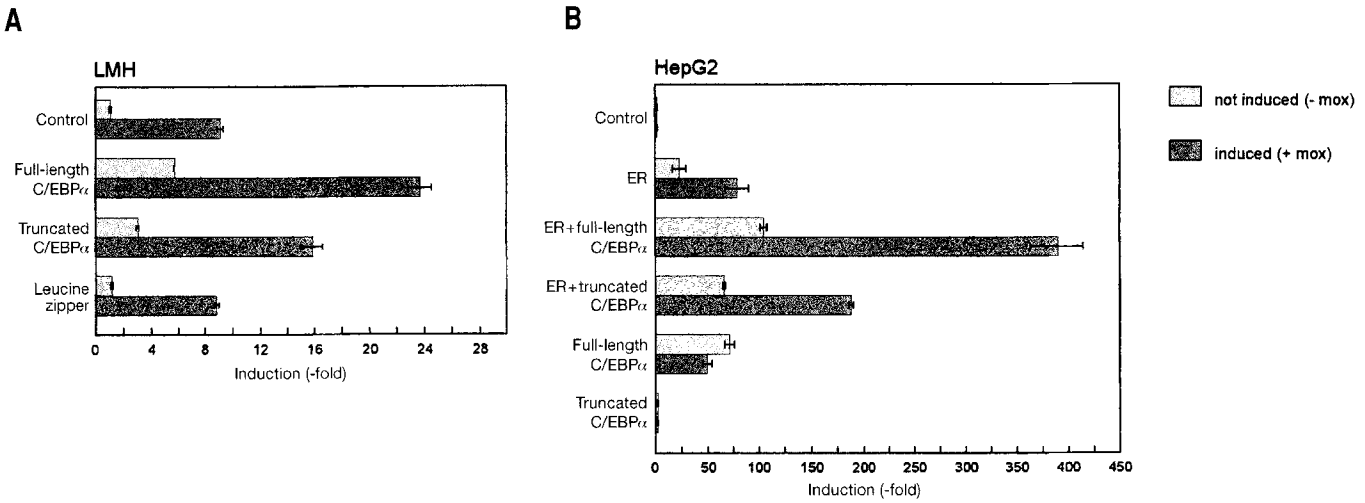

Fig. 5. Chicken C/EBPα isoforms modulate the estrogen induction of apoVLDL II expression. (A) LMH cells were transfected with 2 μg pVLDL(−301/+34)CAT reporter plasmid and 1 μg C/EBPα expression plasmid or empty pSG5 plasmid. (B) HepG2 cells were transfected with 2 μg pVLDL(−301/+34)CAT reporter plasmid and various combinations of expression plasmids or empty pSG5 plasmid. 1 μg expression vector was used, and 1 μg empty expression vector was added to obtain 2 μg DNA. The CAT activity was measured of unstimulated (not induced) cells and of cells treated with 10 μM moxestrol (estrogen induced). The induction levels were calculated relative to the CAT activity of cells transfected with 2 μg VLDL(−301/+34)CAT reporter plasmid alone.

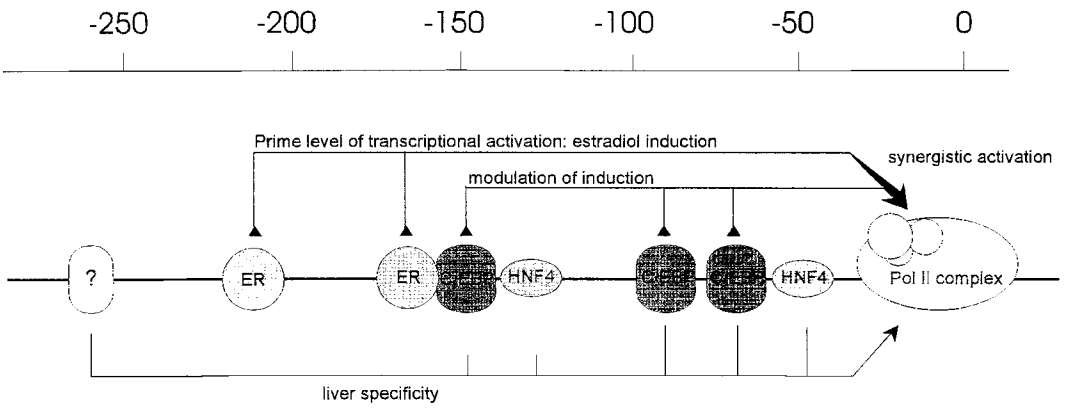

Fig. 6. Schematic representation of the dual regulation of the apoVLDL II promoter. The proximal regulatory region of the apoVLDL II from transcriptional start site to −300 bp is depicted. The binding of the transcription factors ER, C/EBP and HNF4 are indicated. PolII, RNA polymerase II.

The different roles of ER and C/EBPα in the regulation of the apoVLDL II gene are schematically depicted in Fig. 6. ER acts as an on/off switch through its activation by the estrogen ligand. Contrary to the endogenous gene, which is absolutely silent in the absence of the hormone, the transfected gene exhibits some promoter activity in the absence of activated ER. This basal activity, which is further enhanced by C/EBPα, is probably due to the transfected construct lacking the proper chromatin organisation of the endogenous apoVLDL II gene. The highest activity of the apoVLDL-II promoter was found in the presence of both transcription factors, indicating that ER and C/EBPα act in synergy. C/EBP proteins have also been shown to be necessary for glucocorticoid-receptor-mediated gene expression. During chick neural development, glucocorticoid hormone controls the expression of glutamine synthase, which is a marker gene for neural retina functional maturation. Binding of C/EBP to a *cis*-regulatory element just 5′ to the glucocorticoid-receptor-binding site is required for the glucocorticoid response [45, 46]. Furthermore the liver-enriched activating protein, which is the full-length rat C/EBPβ isoform, is required for maximal glucocorticoid induction of the rat α-1 acid glycoprotein gene [47]. C/EBP are also involved in the glucocorticoid induction of the angiotensinogen gene [48] and may be involved in the glucocorticoid-induced expression of the rat tyrosine aminotransferase gene [49].

The occurrence of C/EBPα as multiple isoforms with different *trans*-activating potential implies that transcription of the apoVLDL II gene will be modulated by changes in the isoform ratio (Fig. 6). This is concluded from the transfections in which the ER expression plasmid was transfected with plasmids encoding different C/EBPα polypeptides. Truncation of C/EBPα to the size of the truncated isoform most abundant *in vivo* reduces the estrogen-dependent apoVLDL II activity to roughly half the value with full-length C/EBPα. The activity of the different isoforms also depends on the context of the target promoter. Whereas the truncated C/EBPα isoform is inactive on a promoter driven by C/EBP sites only [28], it exhibits transcriptional activity when the sites are within the context of the apoVLDL II promoter. This is in agreement with the transcriptional activity that the truncated rat C/EBPα proteins have on the serum albumin promoter [29]. Recently, a number systems have been described in which C/EBP isoform ratios vary depending on the

circumstances and thus may play a role in modulation of C/EBP target gene expression. These systems include regenerating liver after partial hepatectomy [50, 51] and the acute-phase response [52]. Earlier we hypothesised [53] that the C/EBP isoforms may be part of a signalling route that leads from translation initiation factor 2, the central regulator of protein synthesis under conditions of, for example, growth-factor deprivation [54], downstream to C/EBP target genes. Within this route, reduction of initiation factor-2 activity would be sensed by the upstream ORF in the C/EBPα leader sequence, leading to a shift in the C/EBPα initiation codon usage, similarly as in yeast GCN4 mRNA translation [55], and subsequently to an alteration of the C/EBPα isoform ratio.

We thank Justin Heydenrijk, for EMSA analysis, Harm Nijveen, for making some of the mutant DNA constructs, Marten Smidt, for useful discussions, Jaap Bouwer, for assistance with the animal experiments, Dr Achim Leutz, for the C/EBPβ/NF-M clone and antiserum, Dr P. Chambon, for the estrogen receptor expression plasmid. This research is supported by the Netherlands Organisation for Scientific Research through the Council of Medical Sciences (grant no. 900-546-083).

REFERENCES

1. Wiskocil, R., Bensky, P., Dower, W., Goldberger, R. F., Gordon, J. I. & Deeley, R. G. (1980) Coordinate regulation of two estrogen-dependent genes in avian liver, *Proc. Natl Acad. Sci. USA 77*, 4474–4478.
2. Beekman, J. M., Wijnholds, J., Schippers, I. J., Pot, W., Gruber, M. & Ab, G. (1991) Regulatory elements and DNA-binding proteins mediating transcription from the chicken very-low-density apolipoprotein II gene, *Nucleic Acids Res. 19*, 5371–5377.
3. Kok, K., Snippe, L., Ab, G. & Gruber, M. (1985) Nuclease-hypersensitive sites in chromatin of the estrogen-inducible apoVLDL II gene of chicken, *Nucleic Acids Res. 14*, 5189–5202.
4. Wijnholds, J., Philipsen, J. N. J. & Ab, G. (1988) Tissue-specific and steroid-dependent interaction of transcription factors with the oestrogen-inducible apoVLDL II promoter *in vivo*, *EMBO J. 7*, 2757–2763.
5. Wijnholds, J., Muller, E. & Ab, G. (1991) Oestrogen facilitates the binding of ubiquitous and liver-enriched nuclear proteins to the apoVLDL II promoter *in vivo*, *Nucleic Acids Res. 19*, 33–41.
6. Ossipow, V., Descombes, P. & Schibler, U. (1993) CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials, *Proc. Natl Acad. Sci. USA 90*, 8219–8223.
7. Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H. & McKnight, S. L. (1989) Tissue-specific expression, developmental regualtion and genetic mapping of the gene encoding C/EBP, *Genes & Dev. 3*, 1146–1156.
8. Descombes, P., Choijkier, M., Lichtsteiner, S., Falvey, E. & Schibler, U. (1990) LAP, a novel member of the C/EBP gene family, a liver-enriched transcriptional activator protein, *Genes & Dev. 4*, 1541–1551.
9. Williams, S. C., Cantwell, C. A. & Johnson, P. F. (1991) A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers *in vitro*, *Genes & Dev. 5*, 1553–1567.
10. Samuelson, L., Strömberg, K., Vikma, K., Bjursell, G. & Enerbäck, S. (1991) The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte differentiation, *EMBO J. 10*, 3787–3793.
11. Lin, F.-T. & Lane, M. D. (1994) CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program, *Proc. Natl Acad. Sci. USA 91*, 8757–8761.
12. Freytag, S. O., Paeilli, D. L. & Geddes, T. M. (1994) Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells, *Genes & Dev. 8*, 1854–1863.
13. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J. & Lane, M. D. (1989) Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhanceer binding protein interacts with and activates the promoter of two adipose-specific genes, *Genes & Dev. 3*, 1323–1335.
14. Kaestner, K. H., Christy, R. J. & Lane, M. D. (1990) Mouse insuline-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein, *Proc. Natl Acad. Sci. USA 87*, 251–255.
15. Umek, R. M., Friedman, A. D. & McKnight, S. L. (1991) CCAAT-enhancer binding protein: a component of a differentiation switch, *Science 251*, 288–292.
16. Lemaigre, F. P. & Rousseau, G. G. (1994) Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver, *Biochem. J. 303*, 1–14.
17. Darlington, G. J., Wang, N. & Hanson, R. W. (1995) C/EBP alpha: a critical regulator of genes governing integrative metabolic processes, *Curr. Opin. Genet. Dev. 5*, 565–570.
18. Mischoulon, D., Rana, B., Bucher, N. & Farmer, S. (1992) Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBP alpha) gene expression during hepatocyte proliferation in the regenerating liver and in culture, *Mol. Cell. Biol. 12*, 2553–2560.
19. McKnight, S. L., Lane, M. D. & Gluecksohn-Waelsch, S. (1989) Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? *Genes & Dev. 3*, 2021–2024.
20. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R. & Darlington, G. J. (1995) Impaired energy homeostasis in C/EBP alpha knockout mice, *Science 269*, 1108–1112.
21. Alam, T., An, M. R. & Papaconstantinou, J. (1992) Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response, *J. Biol. Chem. 267*, 5021–5024.
22. Ray, A. & Ray, B. K. (1994) Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-beta and C/EBP-delta and their activation by phosphorylation, *Mol. Cell. Biol. 14*, 4324–4332.
23. Milos, P. M. & Zaret, K. S. (1992) A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment *in vitro*, *Genes & Dev. 6*, 991–1004.
24. Wu, K., Wilson, D. R., Shih, C. & Darlington, G. J. (1994) The transcription factor HNF1 acts with C/EBP alpha to synergistically activate the human albumin promoter through a novel domain, *J. Biol. Chem. 269*, 1177–1182.
25. Ness, S. A., Kowenz, L. E., Casini, T., Graf, T. & Leutz, A. (1993) Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types, *Genes & Dev. 7*, 749–759.
26. Lin, F.-T., MacDougald, O. A., Diehl, A. M. & Lane, M. D. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity, *Proc. Natl Acad. Sci. USA 90*, 9606–9610.
27. Descombes, P. & Schibler, U. (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA, *Cell 67*, 569–579.
28. Calkhoven, C. F., Bouwman, P. R. J., Snippe, L. & Ab, G. (1994) Translation start site multiplicity of the CCAAT/enhancer binding protein alpha mRNA is dictated by a small 5′ open reading frame, *Nucleic Acids Res. 22*, 5540–5547.
29. Nerlov, C. & Ziff, E. B. (1994) Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter, *Genes & Dev. 8*, 350–362.
30. Calkhoven, C. F., Ab, G. & Wijnholds, J. (1992) cC/EBP, a chicken transcription factor of the leucine-zipper C/EBP family, *Nucleic Acids Res. 20*, 4093.
31. Katz, S., Kowentz-Leutz, E., Müller, C., Meese, K., Ness, S. A. & Leutz, A (1993) The NF-M transcription factor is related to C/EBP beta and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells, *EMBO J. 12*, 1321–1332.
32. Lichtsteiner, S., Wuarin, J. & Schibler, U. (1987) The interplay of DNA-binding proteins on the promoter of the mouse albumin gene, *Cell 51*, 963–973.
33. Lavery, D. J. & Schibler, U. (1993) Circadian transcription of the cholesterol 7 alpha hydroxylase gene may involve the liver-enriched bZIP protein DBP, *Genes & Dev. 7*, 1871–1884.

34. Kozak, M. (1989) The scanning model for translation: an update, *J. Cell. Biol. 108*, 229–241.
35. Krust, A., Green, S., Argos, P., Vijay, K., Walter, P., Bornert, J.-M. & Chambon, P. (1986) The chicken oestrogen receptor sequence: homology with *v-erbA* and the human oestrogen and glucocorticoid receptors, *EMBO J. 5*, 891–897.
36. Green, S., Issemann, I. & Sheer, E. (1988) A versatile *in vivo* and *in vitro* eukaryotic expression vector for protein engineering, *Nucleic Acids Res. 16*, 369.
37. Schippers, I. J., Kloppenburg, M., van Waardenburg, R. & Ab, G. (1994) Cis-acting elements reinforcing the activity of the estrogen-response element in the very-low-density apolipoprotein II gene promoter, *Eur. J. Biochem. 221*, 43–51.
38. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol. 185*, 60–89.
39. Kawaguchi, T., Nomura, K., Hirayama, Y. & Kitagawa, T. (1987) Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH, *Cancer Res. 47*, 4460–4464.
40. Sambrook, L., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Press, Cold Spring Harbor NY.
41. Ryden, T. A. & Beemon, K. (1989) Avian retroviral long terminal repeats bind CCAAT/enhancer-binding protein, *Mol. Cell. Biol. 9*, 1155–1164.
42. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. & Kishimoto, T. (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family, *EMBO J. 9*, 1897–1906.
43. Berkowitz, E. A. & Evans, M. I. (1992) Estrogen-dependent expression of the chicken very low density apolipoprotein II gene in serum-free cultures of LMH cells, *In Vitro Cell. Dev. Biol. 28A*, 391–396.
44. Mietus-Snyder, M., Sladek, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A., Darnell, J. E. & Karathanasis, S. K. (1992) Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells, *Mol. Cell. Biol. 12*, 1708–1718.
45. Ben-Or, S. & Okret, S. (1993) Involvement of a C/EBP-like protein in the acquisition of responsiveness to glucocorticoid hormones during chick neural retina development, *Mol. Cell. Biol. 13*, 331–340.
46. Zhang, H. & Young, A. P. (1991) A single upstream glucocorticoid response element juxtaposed to an AP1/ATF/CRE-like site renders the chicken glutamine synthetase gene hormonally inducible in transfected retina, *J. Biol. Chem. 266*, 24332–24338.
47. Williams, P. M., Ratajczak, T., Lee, S. C. & Ringold, G. M. (1991) AGP/EBP(LAP) expressed in rat hepatoma cells interacts with multiple promoter sites and is necessary for maximal glucocorticoid induction of the rat alpha-1 acid glycoprotein gene, *Mol. Cell. Biol. 11*, 4959–4965.
48. Brasier, A. R., Ron, D., Tate, J. E. & Habener, J. F. (1990) A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1 alpha induced, NF kappa B mediated trans-activation of the angiotensinogen gene acute-phase response element, *Mol. Endocrinol. 4*, 1921–1933.
49. Grange, T., Roux, J., Rigaud, G. & Pictet, R. (1991) Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor, *Nucleic Acids Res. 19*, 131–139.
50. Diehl, A. M. & Yang, S. Q. (1994) Regenerative changes in C/EBPα and C/EBPβ expression modulate binding to the C/EBP site in the *c-fos* promoter, *Hepatology 19*, 447–456.
51. Rana, B., Xie, Y., Mischoulon, D., Bucher, N. L. R. & Farmer, S. R. (1995) The DNA binding activity of C/EBP transcription factors in regulating the G1 phase of the hepatocyte cell cycle, *J. Biol. Chem. 270*, 18123–18132.
52. An, M. R., Hsieh, C.-C., Reisner, P. D., Rabek, J. P., Scott, S. G., Kuninger, D. T. & Papaconstantinou, J. (1996) Evidence for posttranscriptional regulation of C/EBPα and C/EBPβ isoform expression during the lipopolysaccharide-mediated acute phase response, *Mol. Cell. Biol. 16*, 2295–2306.
53. Calkhoven, C. F. & Ab, G. (1996) Multiple steps in the regulation of transcription-factor level and activity, *Biochem. J. 317*, 329–342.
54. Rhoads, R. E. (1993) Regulation of eukaryotic protein synthesis by initiation factors, *J. Biol. Chem. 268*, 3017–3020.
55. Hinnebusch, A. G. (1990) Involvement of an initiation factor and proein phosphorylation in translation control of GCN4 mRNA, *Trends Biochem. Sci. 15*, 148–152.