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Cloning and characterisation of a nuclear, site specific ssDNA binding protein

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ABSTRACT

Estradiol inducible, liver-specific expression of the apoVLDL II gene is mediated through the estrogen receptor and a variety of other DNA-binding proteins. In the present study we report the cloning and characterisation of a single-strand DNA binding protein that interacts with the lower strand of a complex regulatory site, which includes the major estrogen responsive element and a site that resembles the rat albumin site D (apoVLDL II site D). Based on its binding specificity determined with electro-mobility shift assays, the protein is named single-strand D-box binding factor (ssDBF). Analysis of the deduced 302 amino acid sequence revealed that the protein belongs to the heteronuclear ribonucleoprotein A/B family (hnRNP A/B) and resembles other known eukaryotic single-strand DNA binding proteins. Transient transfection experiments in a chicken liver cell-line showed that the protein represses estrogen-induced transcription. A protein with similar binding characteristics is present in liver nuclear extract. The relevance of the occurrence of this protein to the expression of the apoVLDL II gene is discussed.

INTRODUCTION

Transcription of the chicken apoVLDL II (apoII) gene is liver-specific and estrogen-dependent. The gene is rapidly induced by estradiol and stays active as long as the hormone is present (1). The stimulation is mediated via the estrogen receptor (ER), which binds to estrogen response elements (EREs) in the 300 bp 5′ flanking to the gene. Of these elements, the ERE at 164 nucleotides in front of the gene appears to be of major importance for the estrogen response (2).

Since the apoVLDL II gene is not expressed in estrogen target tissues other than liver, such as oviduct, its transcription must be under the control of additional components besides the estrogen receptor. Probably tissue-specific factors are involved, either factors that are restricted to liver and activate apoVLDL II expression, or conversely, inhibitory factors that are present in non-liver tissues. Such factors are believed to constitute part of the DNA-binding proteins that interact with the 300 bp upstream gene region (3). The main question is how these factors modulate the activity of the estrogen receptor binding to the same promoter region.

In the present study, we have focused on a particular sequence within the apoVLDL II promoter region that may be the site of interplay between the estrogen receptor and (an)other transcription factor(s). It concerns the E1D site which is composed of two overlapping sites, the major estrogen response element (ERE) E1 and site D which is recognised by the bZIP proteins, VBP and C/EBP (4,5). *In vitro* footprinting has revealed that both sites can be occupied simultaneously, but that there is at least one protein binding to site D which interferes with the occupation of site E1 (3). With these complex binding characteristics in mind we have started a search for proteins binding to site D that may interfere with ER binding.

Here, we report the cloning of a novel factor from a cDNA expression library using a recognition site D oligonucleotide probe. The protein is named single-strand D-box binding factor (ssDBF) because it binds specifically to the lower strand of site D. Sequence analysis revealed homology to the hnRNP-A/B class of heterogeneous nuclear ribonucleoproteins (6) and to single-strand DNA binding proteins, like the CArG-box binding factor A, CBF-A (7), the human hepatitis B virus enhancer II binding protein, E2BP (8) and human hnRNP A/B type protein (9). Whereas these proteins share a conserved central domain, their N-and C-terminal sequences differ and may be involved in specific functions. A preliminary characterisation of ssDBF is described.

MATERIALS AND METHODS

Cloning of ssDBF

Screening of an adult rooster liver cDNA library in phage λgt11 (Clontech, Palo Alto, CA) with a recognition site DNA probe was performed as described (10) with some modifications (11). The probe was a trimer of the apoVLDL II site D. The insert of the positive clone was excised with *Eco*RI, subcloned in pEMBL18 and sequenced (12). A *Bam*HI–*Hin*dIII (bp position 649–1093) fragment was used to re-screen an embryonic cDNA library (Venström) (13). The inserts of positive clones were excised with *Eco*RI, subcloned in pGem7zf+ and sequenced (12).

Gel retardation assay

Labelling of probes and conditions of the assay were essentially as described (3) with some modifications. When single-strand oligos were used as probes, single-strand aspecific competitor (poly dI/dC, Bhoeringer) was added. To stabilise diluted protein

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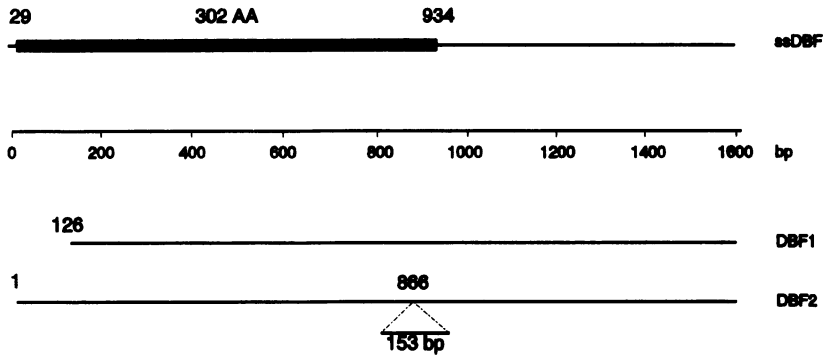


Figure 1. Schematic overview of cloned cDNAs of the ssDBF gene. The two clones are identical except for the in-frame insertion of 153 bp and the extended 5′ sequence in the DBF2 clone. The ssDBF gene was reconstructed by fusing the first 126 bp from the DBF2 clone to the DBF1 clone. This resulted in a complete reading frame of 302 AA with a short leader sequence. See Material and Methods for further cloning details.

extracts, 10 µg of bovine serum albumin (BSA, Boehringer) was added to the binding reaction.

Nucleotide sequences of oligonucleotides

Single-strand DNA fragments corresponding to the different binding sites upstream of the apoVLDL II gene were the following synthetic oligonucleotides:

For Site E_1D (–144/–184)

5′-GATCTCAGGTCAGACTGACCTTCCATTACCAAATCCGAACA-3′ (upper)

5′-GATCTGTTCGGATTTGGTAATGGAAGGTCAGTCTGACCTGA-3′ (lower)

For site D (–144/–165)

5′-GGGACCTTCCATTACCAAATCCGAACCCC-3′ (upper)

5′-GGGTTCGGATTTGGTAATGGAAGGTCCCC-3′ (lower)

For site EIIb of the hepatitis enhancer (8)

5′-GATCCTGGGAGGAGTTGGGGGAGGAGATTAGG-3′ [1]

5′-GACCCTCCTCAACCCCCTCCTCTAATCCCTAG-3′ [2]

For the CArG-box (7)

5′-GATCCTTTTACCTAATTAGGAAATGG-3′ (upper)

5′-GATCCCATTTCCTAATTAGGTAAAAG-3′ (lower)

To obtain double-strand probes the complementary oligos of a recognition sequence were annealed. The recognition site D probe used for expression library screening was obtained by ligating double-strand oligonucleotide D into the *Sma*I site of pEMBL18. A clone containing three copies of the apoVLDL II D-box sequence GACCTTCCATTACCAAATCCGAAC in a head-to-tail-to-head orientation connected by CCGG-linker sequences was digested with *Kpn*I and *Bam*HI to excise the insert, yielding the trimeric oligo D probe. The oligonucleotide used for primer extension was as follows:

5′-CTGCTCCGCTTCGGACATGCT-3′.

Overexpression of ssDBF

The expression plasmid was made by cloning a blunted (Klenow) *Eco*RI fragment, containing the ssDBF partial sequence (DBF1), into the blunted (Klenow) *Bam*HI site of pET3B (14). The fusion gene encoding amino acid sequence 34–302 of ssDBF (Fig. 1) was checked by sequencing. The ssDBF sequence was preceded by the phage sequence MASMTGGQQMGRDQFP. The plasmid was transformed into an *Escherichia coli* BL31DE3pLysE strain. Growth and induction was performed as described earlier (4). Total cell extract was made by sonication of the *E.coli* cells in 20 mM HEPES pH 7.9, 200 mM NaCl, 2 mM EDTA, 2 mM DTT and 2 mM PMSF at 0°C. Cell debris was removed by centrifugation (4°C) and the supernatant was frozen with 10% glycerol in liquid nitrogen and stored at –80°C.

Primer extension analysis

The oligonucleotide primer was labelled with [γ-^{32}P]dATP and polynucleotide kinase, purified by gel filtration (Sephadex G50) and ethanol precipitated. Five ng of primer was annealed to 20 µg total chicken embryonic RNA in hybridisation buffer (0.01 M Tris-Cl pH 8.3; 0.05 M $MgCl_2$) for 90 min at 65°C. Next 33.3 µl primer extension mix (30 mM Tris-Cl pH 8.3; 15 mM $MgCl_2$; 8 mM DTT; 0.2 mM dNTPs; 5 U AMV reverse transcriptase) was added and incubation was continued for 1 h at 42°C. The reaction was terminated by addition of RNase A (15 min at 37°C), the mixture extracted with phenol/chloroform and the cDNA precipitated with ethanol. The pellet was resuspended in 8 µl of formamide dye and incubated for 5 min at 65°C before loading on a 6% PAA/urea sequence gel.

RT–PCR

Approximately 200 ng of total RNA, isolated by the guanidinium method (15), was used in a reverse transcriptase PCR reaction (Tth DNA polymerase, Boehringer). Primers from position 786–807 and 1040–1019 of the ssDBF sequence were used to generate a fragment of 252 bp on the ssDBF gene and a fragment of 405 bp on the DBF2 gene. The reverse transcriptase reaction was carried out at 65°C for 25 min with the downstream primer. The PCR reaction was performed with the following conditions: 30 s at 94°C, 30 s at 45°C and 1 min at 72°C for 35 cycles. For the control cDNAs the same conditions were used except for the number of cycles, which was reduced to 25.

Cell culture and transfection

Leghorn strain M hepatoma (LMH) cells (16) were grown in flasks containing Waymouth's MB 752/1 medium supplemented with 10% foetal calf serum, 50 µg/ml streptomycin and 50 U/ml penicillin under conditions of 10% CO_2/air mixture at 37°C. Cell culture media were from Gibco. DNA transfection was carried out following the Ca-phosphate co-precipitation method (17). The LMH cells were plated at 40% confluence on a 6 cm dish one day prior to transfection and cultured in medium with serum. The DNA precipitate, containing 4 µg of reporter- and 20 µg of

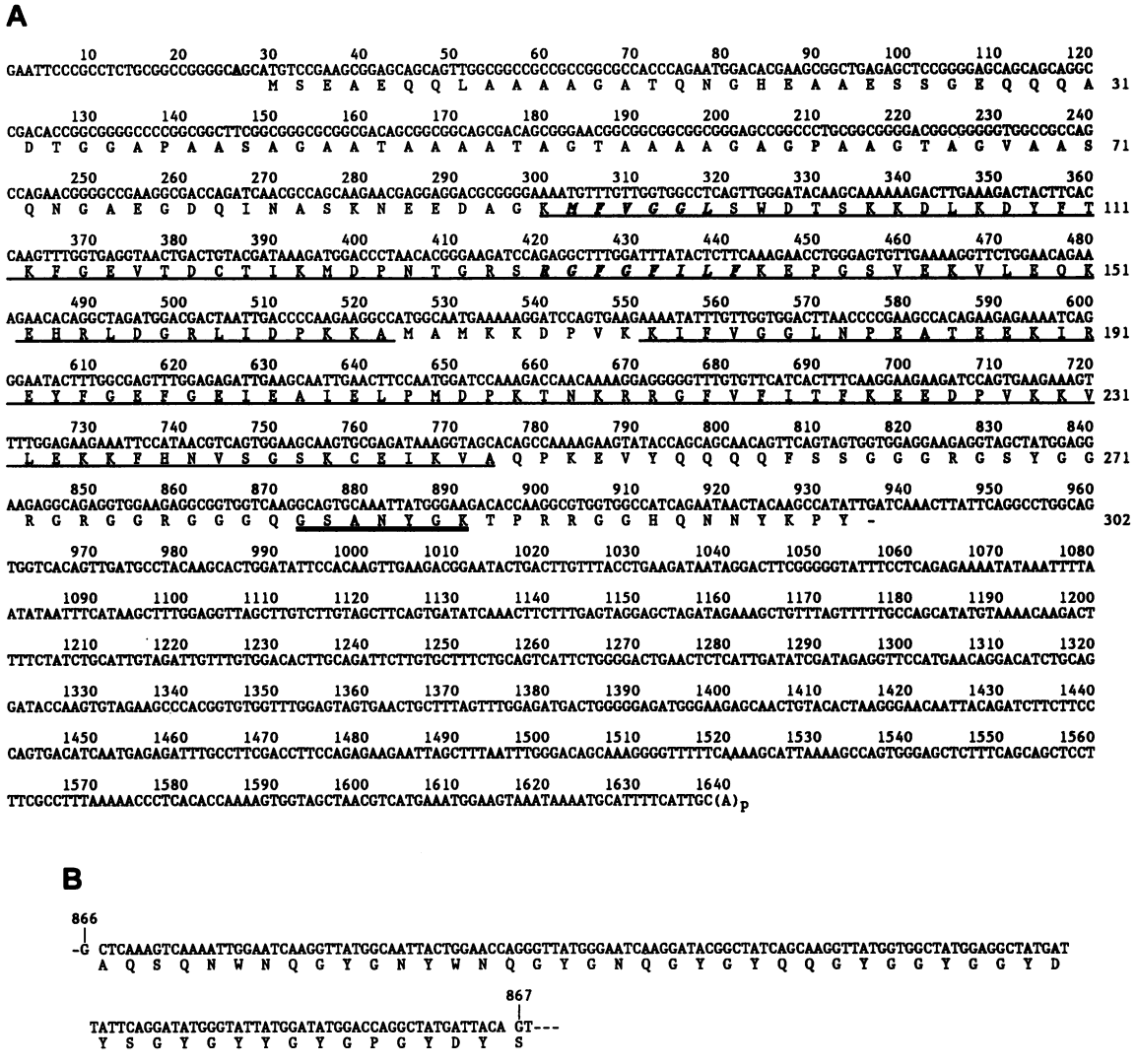


Figure 2. (**A**) Nucleotide sequence of the ssDBF gene and deduced amino acid sequence. The consensus RNA-binding motifs (RNP-1 and RNP-2) are shown in bold italics. The consensus ATP/GTP binding site P-loop (21) in the C-terminal region is double underlined. The internal duplication in the central region of the gene is underlined. (**B**) Nucleotide sequence and deduced amino acid sequence of the insertion present in the DBF2 clone, including the altered border sequences.

effector plasmid unless stated otherwise, was spread across the cells. To obtain equal DNA concentrations in all precipitates, the mixtures were adjusted, to end concentrations of 40 μg/ml DNA, by addition of the bacterial plasmid pGem7zf+ (Promega). After 4 h the cells received a glycerol (15%) shock for 1 min. The cells were washed with 10 ml medium and supplemented with 5 ml medium without serum. Cells were hormonally induced with 10^{-7} M moxestrol, immediately and 24 h after transfection. Cells were harvested at ~48 h after transfection and cell extracts were obtained by three freeze–thaw cycles. The CAT activity was assayed by a standard non-TLC procedure (18). All transfections were carried out at least three times to ensure that the results can be reproduced. For the CAT assay, the extracts were normalised with respect to the protein concentration.

Nuclear extract preparation

A liver from a laying hen was harvested at 08:00 hours. Nuclear extracts were prepared according to the NUN-extract procedure (19) and were dialysed twice during 30 min in 1000 vol extraction buffer without urea.

RESULTS

Cloning and structural features of DBF1

Screening of 6×10^5 plaques of an adult liver rooster cDNA library with a trimer of the apoVLDL II site D yielded three clones (4). Subcloning of their *Eco*RI inserts and sequencing revealed that one of the clones resembled the mouse CBF-A gene, which encodes a transcription factor that binds to a single-strand sequence, called CArG for CC(A/T-rich)$_6$GG-sequence and represses transcription (7). From the sequence alignment with the CBF-A cDNA (7), it is concluded that the cDNA clone is a partial, not complete at the 5′ end (Fig. 1, DBF1). Screening of a chicken embryonic cDNA library with a probe containing sequences from position 649 (*Bam*HI) to 1093 (*Hin*dIII) yielded a clone which contains 125 additional nucleotides 5′ to the initial DBF1 cDNA

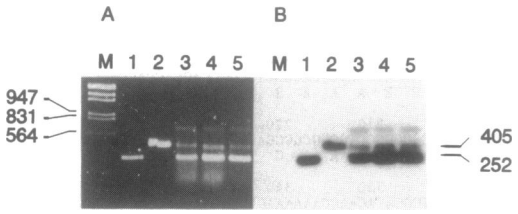


Figure 3. Agarose gel electrophoresis of RT–PCR products from DBF RNA. RT–PCR products from LMH cells (lane 3); total embryo (lane 4) and laying hen liver (lane 5). PCR products generated from the ssDBF cDNA (lane 1) and the DBF2 cDNA (lane 2) serve as size markers. (**A**) Ethidium bromide stained gel [M: λ(*Hin*dIII/*Eco*RI)-marker]; (**B**) Autoradiogram of ^{32}P-labelled bands after Southern blotting. A ^{32}P-random-primed-labelled *Bam*HI–*Hin*dIII (bp position 649–1093) fragment of the ssDBF cDNA was used as a probe.

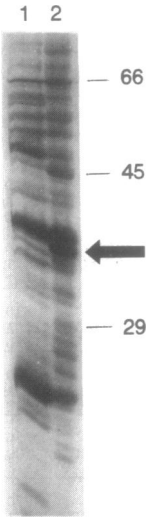


Figure 4. Overexpression of a ssDBF fusion protein in *E.coli*. The expression vector contained the full-length DBF1 cDNA sequence. Equal amounts of bacterial extract from transformed *E.coli* before (lane 1) and after induction (lane 2) for 3 h with 0.4 mM IPTG were loaded on a 12.5 % PAA gel. The induced protein, with a size of ~36 kDa, is indicated by the arrow.

sequence, including a possible initiation codon (Fig. 1, DBF2). As inferred from primer extension analysis, about 130 bp of the 5′ untranslated region could still be lacking from the cloned sequences as was confirmed by the sequence from the cloned promoter region of the ssDBF gene (data not shown).

Interestingly, the DBF2 clone contains an insertion of 153 bp between position bp 866 and 867 in the C-terminal moiety of the ssDBF sequence. This leads to a 51 amino acid insertion in the polypeptide chain and the replacement of a glycine (Gly-280) by an alanine (Fig. 2A/B). The DBF2 clone possibly represents a splicing variant (see Discussion).

The reconstructed gene (Fig. 2A) has an open reading frame which begins at nucleotide 29 and encodes a peptide of 302 amino acids with a predicted molecular weight of 31 861. We called the gene single-strand D-box binding factor, ssDBF (see below). The sequence around the first AUG has an adenine at position –3 making it a favourable context for translation initiation (20). Analysis of the primary structure of ssDBF revealed that the central section contains two 75 amino acid repeats of 44% mutual similarity. Each repeat resembles the consensus RNA-binding domain in hnRNP proteins of which two motifs, RNP-1 and RNP-2 (6), lying about 30 amino acids apart, are the hallmarks. The sequences RGFGFILF (131/138) and RGFVFITF (214/222) resemble the RNP-1 consensus sequence K/R-G-F/Y-G/A-F-V-X-F/Y and the sequences MFVGGL (92/97) and IFVGGL (173/180) resemble the aromatic and aliphatic amino acid-rich hexapeptide sequence RNP-2. Further, ssDBF contains a sequence GSA-NYGKT (282/289) which resembles the consensus sequence A/G-X_4-G-K-S/T for the P-loop of an ATP/GTP binding site (21).

mRNA analysis

The isolation of the different clones, DBF1 and DBF2, implies that at least two variant mRNAs occur. To determine whether they accumulate to different ratios depending on the tissue, total RNA from embryo, LMH cells and liver was subjected to RT–PCR (Fig. 3). Two of the bands have the expected size (252 and 405 bp) for PCR products generated on DBF1 and DBF2 mRNA, respectively, showing that both variants are present in all three samples examined and that they occur in a similar ratio. The experiment also yielded a third, larger PCR product of about 700 bp. Repeated PCR of the excised band and sequencing of the cloned product revealed no sequences related to the reported ssDBF gene, suggesting that the product is a PCR artefact.

ssDBF binds to the lower strand of the apoVLDL II site D

In order to study the DNA-binding characteristics of the ssDBF, the protein was overexpressed in *E.coli* transformed with a T7-promoter-driven fusion gene (14). The recombinant ssDBF which is a fusion of the entire DBF1 sequence linked to a short N-terminal phage peptide is visible as an induced protein of ~36 kDa upon SDS–PAGE analysis of the bacterial cell extract (Fig. 4, arrow). Electrophoretic mobility shift assay (EMSA) using the crude recombinant ssDBF preparation shows that the protein binds solely to the E1D lower-strand probe (E1Dl), not to the upper-strand probe(E1Du) nor to reannealed double-strand probe(E1Dds). Furthermore, no specific binding of ssDBF to any of the EIIB probes could be found (Fig. 5). The E1D oligonucleotide consists of two different, partially overlapping recognition sequences. To determine the recognition sequence of the ssDBF, EMSA was performed with the site E1 and site D probes, separately. Whereas no shift was found with single- or double-strand E1 probes (not shown), a specific complex was formed with the lower strand of oligo D (Fig. 6A and B, first lane). The protein also binds to the lower strand of the CBF-A recognition site CArG box [TCCTAATTAGGTAA;(7)] which shares 10 out of 14 nucleotides with the D box sequence (TTCGGATTTGGTAA). From oligonucleotide competition experiments it can be concluded that the ssDBF has about equal affinities for the D- and the CArG sequence (Fig. 6). In liver there appears to be two proteins binding to the D-box sequence, a major complex with about the same mobility as the recombinant protein and a minor complex with higher mobility. Based on its position in the gel, the minor band probably does not represent the DBF2 but rather a shorter peptide of ssDBF. However, we cannot exclude that the protein is encoded by another related gene. Whatever the explanation of the heterogeneity is, both retarded complexes have the same binding characteristics and represent highly related proteins.

Whereas ssDBF recognised the CArG site of the related CBF-A protein, it did not bind to oligos containing the recognition

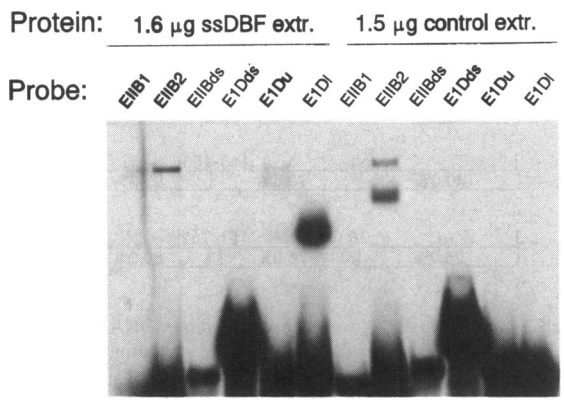


Figure 5. Analysis of the binding specificity of ssDBF by electrophoretic mobility shift assay (EMSA). Cell-free extracts of *E.coli*, overexpressing the recombinant ssDBF protein, were used in binding reactions. Both strands of the hepatitis B virus (HBV) enhancer II region (EII1, EII2), of the apoVLDL II E1D region (E1Du, E1Dl) and the respective double-strand probes (EIIds, E1Dds) were used. The binding reactions were in the presence of 1 µg single strand poly dI/dC competitor (Boerhringer). Approximately 0.1 ng of probe was added to the reaction. Only with the lower strand of the E1D probe a specific retardation is observed. The control extract consist of an *E.coli* extract without ssDBF.

sequence of E2BP, the homologous human hepatitis B virus enhancer II binding protein. This is in accordance with the absence of extensive sequence similarity between site D and the E2BP recognition sequence.

ssDBF functions as a transcriptional silencer

The activity of ssDBF on transcription was examined in transient transfection assays with an apoVLDL II-CAT construct as a reporter plasmid. This plasmid contains the –301/+34 apoVLDL II promoter region which is activated by estrogen in cells containing the estrogen receptor such as the chicken hepatoma cell line, LMH (Fig. 7A). Co-transfection of the cells with an effector plasmid containing the ssDBF coding sequence behind the RSV promoter (ssDBF-N) showed dramatic reduction of the estrogen-dependent CAT activity compared to the transfections with the vector without the ssDBF gene (pRC/RSV) or a plasmid with the ssDBF gene in the reverse orientation (ssDBF-R). This repression was shown to be dose dependent with respect to the amount of effector plasmid (Fig. 7B).

The inhibition mediated by the ssDBF was not specific for the apoVLDL II promoter as was shown in a transient transfection experiment using a reporter which contained a heterologous viral promoter with several CArG-sites (RSV-CAT)(22) (Fig. 7C).

DISCUSSION

The ssDBF protein binds to single-strand DNA molecules in a sequence specific manner. The cloning of the DBF1 cDNA from an expression library with the trimeric site D oligonucleotide indicates that the probe must have contained non-annealed strands. The truncated DBF1 cDNA starting at position 126 of the reconstructed gene is most likely the result of a library preparation with premature termination of the first cDNA strand synthesis. This may have been promoted by the high GC content of the 5′ mRNA sequence.

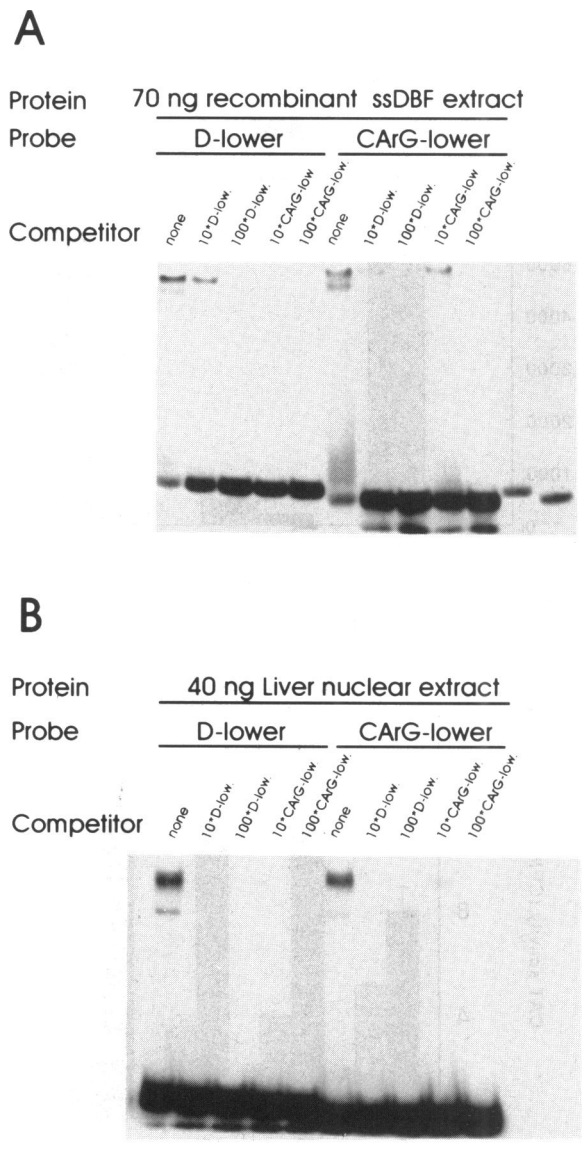
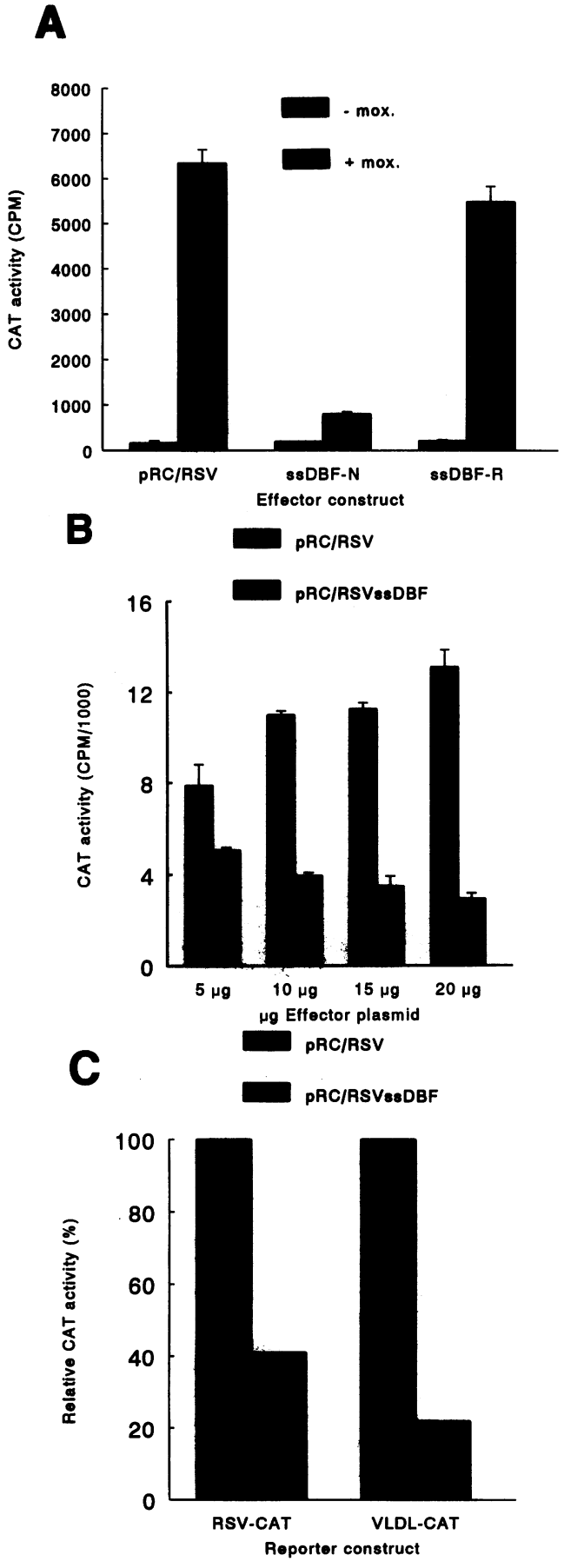


Figure 6. Competition EMSA experiments with recombinant ssDBF protein (**A**) and laying hen liver nuclear extracts (**B**), using sites D and CArG as probes and specific competitor. The reactions were performed with the addition of 1 µg single strand poly dI/dC (Boerhringer) as aspecific competitor and 10 µg of bovine serum albumin (Boehringer) to stabilise the protein extracts. The amount of protein is indicated. The excess of specific competitor is indicated in molar ratio over the labelled probe.

The deduced amino acid sequence identifies ssDBF as a member of the heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B) family (6). This classification is based on the following criteria: (i) the presence of two ~80 amino acid RNA-binding domains, each containing copies of the highly conserved RNP-1 and RNP-2 consensus sequences; (ii) the presence of a Gly-rich C-terminal domain (16 Gly/39 amino acids). Because of these criteria this family is also referred to as the 2*RNA binding domain Gly-rich auxiliary domain (2*RBD-Gly) (6). A gene bank search identified three proteins with considerable similarity to ssDBF: human hnRNP A/B type protein (HSRNPC, according to former classification as C-type protein) (9) (79.6 %), mouse CArG box binding protein CBF-A



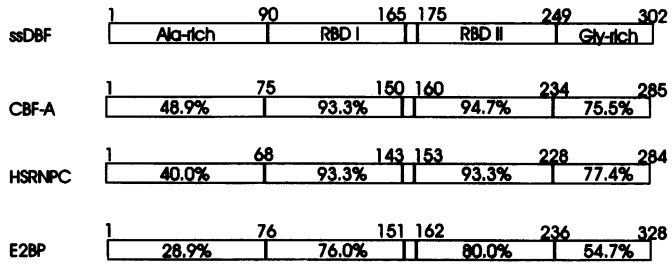


Figure 8. Similarity of ssDBF to other genes encoding ssDNA binding proteins. The domain structure is schematically represented and positional identities are indicated (%).

(6) (81.8 %) and the human hepatitis B virus enhancer II binding protein E2BP (8) (58.9 %) (Fig. 8). The latter two proteins have erroneously been referred to as hnRNP C-type proteins on the basis of the initial size classification of HSRNPC. These proteins clearly lack the characteristics of C-type proteins which have only one RBD domain and contain a negatively charged C-terminal segment (6).

Unlike the N-terminal and C-terminal domains, the central domain in the hnRNP A/B family is conserved between family members, including ssDBF, CBF-A and E2BP and is probably necessary for the interaction with single-strand nucleic acids. The Ala-rich N-terminal domain (amino acids 36–70, 60% alanine) of ssDBF may be involved in the repression function because such domains are found in other factors with known repressing function like Tup1 (40% alanine, amino acids 120–144) Krüppel, Engrailed and Even skipped (23). Another N-terminal Ala-rich domain containing protein, UP2, has been found to bind single-strand DNA. This E2BP homologue lacks the corresponding first RNA binding domain and the Gly-rich C-terminus (24). At present the function of UP2 has not been elucidated. The C-terminal NTP-binding site (P-loop) found in ssDBF is present in the hnRNP A/B proteins and in hnRNP C type proteins (6). The C-terminal domain of hnRNP A/B proteins is not very conserved between family members. Interestingly, the insertion found in DBF2 resembles the insertion in the human hnRNP $A1^B$ gene [hnRNP $A1^B$, (25)] with respect to the number of amino acids (51 and 52 residues, respectively) and the C-terminal position.

The DNA binding activity of the ssDBF protein was examined using the recombinant protein. The presented data show that it can bind site-specific to single-strand DNA. A target site for the protein is the lower-strand of the apoVLDL II D-box, which is not surprising because the same region was used to clone the cDNA initially. Comparison of our target site (the D-box) with the lower strand of the CArG-box motif, the target site of the homologous

Figure 7. Inhibition by the ssDBF of transiently expressed promoter-CAT constructs in a chicken hepatoma cell line. All the experiments were performed at least three times. Shown are the means with standard deviation of duplicate experiments. (**A**) Expression of the –301/+34 apoVLDL II-CAT reporter, induced by moxestrol and using the endogenous ER, in the presence of the expression vector without the ssDBF cDNA (pRc/RSV), the expression vector with the ssDBF gene in the sense orientation (ssDBF-N) and the expression vector with the ssDBF gene in the antisense orientation (ssDBF-R). (**B**) Estrogen-dependent activity of the –301/+34 apoVLDL II promoter with increasing amounts of vector (pRC/RSV) and ssDBF cDNA containing effector plasmid. (**C**) Comparison of the inhibitory effect of ssDBF on the –301/+34 apoVLDL II (–301/+34-CAT) and the RSV promoter (RSV-CAT).

CBF-A protein, shows that there is considerable similarity. The E2BP target site [HBV enhancer II region; (8)] differs significantly from the apoVLDL II D- or the Sm-α actin gene first intron CArG sequence (7). As expected, no specific binding of ssDBF to either strand of the EIIb region could be found (Fig. 5). Whether the 51 amino acid insertion found in the DBF2 cDNA influences the binding characteristic is at present not known.

We showed that ssDBF can have a strong repressing effect on different promoters in transient transfection assays, most pronounced on the –301/+34 bp apoVLDL II promoter. CBF-A has similar inhibiting characteristics with respect to the α-actin promoter (7). The E2BP gene product on the contrary enhances the transcription from the enhancer II promoter (8). This altered function may be due to the fact that E2BP lacks the N-terminal putative repressing domain characterised as the alanine rich sequence in ssDBF and CBF-A.

Several single-strand DNA binding proteins have recently been implicated in regulation of gene expression. (i) The lytic control element (LCE) binding protein (LCP-1) activates the neurotrophic JC virus enhancer. The protein is believed to be involved in early gene transcription during the early phase of the lytic cycle through a presumed single-strand region within the LCE allowing LCP-1 to bind and interact with the general transcription machinery (26). (ii) The pyrimidine tract binding protein (PTB) is involved in liver-specific activation of the tyrosine aminotransferase promoter. A dual function both in RNA-processing and transcription regulation has been proposed (27). (iii) Two single-strand DNA binding proteins have been implicated in repression of the β-casein promoter, through a repressing region at position –221/–183 relative to the transcription start site (28). (iv) A far upstream element (FUSE) binding protein (FBP) with site specific single-strand DNA binding activity has been identified stimulating the c-Myc proto oncogene. The minimal DNA binding domain was mapped and the DNA target sequence was associated *in vivo* with single-strand DNA structures (29). (v) A ubiquitous 45 kDa protein from yeast has been purified that binds specifically to the lower strand of an estrogen responsive element (ERE) and facilitates the binding of a purified Baculovirus-produced human estrogen receptor (30). This was particularly interesting because it was shown that the ER can bind to a single-strand ERE (31,32).

In summary, the ssDBF binds to the lower strand of the apoVLDL II D-region and represses transcription when using reporters containing the ssDBF recognition sequence. Although these results and the above cited literature concerning ssDNA binding proteins indicate that ssDBF may exert its action via DNA binding, such a mechanism would imply a single-strand or otherwise distorted DNA target sequence. However in previous *in vitro* experiments it was shown that the ER binds simultaneously with a bZip protein (vittelogenin binding protein, VBP) to the E1D element (3), indicating that the DNA recognition site is in a normal double-strand conformation. This indicates that the ssDBF may repress transcription via a different mechanism, for example via direct protein–protein interaction with specific or general transcription factors. Transfection experiments using mutated reporter constructs or ssDNA-binding deficient mutants of the ssDBF should clarify this. The high level of apoVLDL II expression in adult laying hen liver (1), despite the expression of the ssDBF, indicates that its concentration is insufficient to repress transcription. To study the possible function of the ssDBF in gene regulation, we are presently investigating the relevance of the DNA-binding function and are trying to localise the ssDBF repressing domain.

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