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Chicken Vitellogenin Gene-Binding Protein, a Leucine Zipper Transcription Factor That Binds to an Important Control Element in the Chicken Vitellogenin II Promoter, Is Related to Rat DBP

SUBRAMANIAN V. IYER, DORENE L. DAVIS, SAMAR N. SEAL, AND JOHN B. E. BURCH*

Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

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We screened a chicken liver cDNA expression library with a probe spanning the distal region of the chicken vitellogenin II (VTGII) gene promoter and isolated clones for a transcription factor that we have named VBP (for vitellogenin gene-binding protein). VBP binds to one of the most important positive elements in the VTGII promoter and appears to play a pivotal role in the estrogen-dependent regulation of this gene. The protein sequence of VBP was deduced from a nearly full length cDNA copy and was found to contain a basic/zipper (bZIP) motif. As expected for a bZIP factor, VBP binds to its target DNA site as a dimer. Moreover, VBP is a stable dimer free in solution. A data base search revealed that VBP is related to rat DBP. However, despite the fact that the basic/hinge regions of VBP and DBP differ at only three amino acid positions, the DBP binding site in the rat albumin promoter is a relatively poor binding site for VBP. Thus, the optimal binding sites for VBP and DBP may be distinct. Similarities between the VBP and DBP leucine zippers are largely confined to only four of the seven helical spokes. Nevertheless, these leucine zippers are functionally compatible and appear to define a novel subfamily. In contrast to the bZIP regions, other portions of VBP and DBP are markedly different, as are the expression profiles for these two genes. In particular, expression of the VBP gene commences early in liver ontogeny and is not subject to circadian control.

Genetic and biochemical studies have established that transcription by RNA polymerase II requires the assembly of a stable preinitiation complex over the proximal promoter region of each target gene (6, 32). This event is subject to regulation by various combinations of more distal control regions that may be generally classified as either upstream promoter regions, enhancers, or locus activation regions. Further studies have revealed that these control regions have modular suborganizations, with the individual elements corresponding to transcription factor binding sites (reviewed in references 15 and 26). While the information content of a control region is thus restricted by the diversity of the resident elements, a simple description of these elements may not suffice to account for the observed activity. For example, an element may be subject to positive or negative influences by neighboring elements, and in addition, qualitatively similar elements may differ markedly in their ability to compete for a factor because of differences in binding affinities. Detailed studies are thus required to evaluate the activity of individual control regions. An even more imposing challenge for complex genes is to determine how the activities of a number of contributory control regions are integrated to yield precise patterns of gene expression.

Further insights regarding the complexity of transcriptional control have come with the cloning of transcription factors (reviewed in references 16 and 26). For example, a single element may represent a target for several unrelated factors or distinct combinations of heteromeric complexes. Several dimerization motifs (such as the leucine zipper and the helix-loop-helix) have been shown to be involved in both homotypic and heterotypic interactions. Some heterodimers are competent to mediate transcriptional effects, but examples of dominant negative effects have also been described in which a nonfunctional partner suppresses the activity of a functional partner. In addition to DNA binding domains (and, in some cases, dimerization domains), transcription factors also contain one or more transactivation domains that are required to mediate positive effects on the general transcriptional machinery. Subclasses of transactivation domains have been identified, and it is likely that other novel domains will be found as more transcription factors are cloned and analyzed. Much current work is directed at understanding how these activation domains function, and the notion that additional bridging factors may be involved has been advanced from several recent studies (reviewed in reference 25).

Studies in our laboratory are focused on a molecular understanding of the estrogen-dependent and liver-specific transcriptional regulation of the chicken vitellogenin II (VTGII) gene. The estrogen-dependent aspect of this regulation was shown to be due to the presence of two upstream estrogen response elements, and the ability of the VTGII promoter to be activated by these elements was found to be cell type specific (5, 7). A linker scanner mutational analysis of the VTGII promoter using transient expression assays in chicken hepatoma (LMH) cells (18) and chicken embryo fibroblast cells revealed that this promoter has multiple positive elements as well as a negative element (35). Whereas factors have been identified that bind to four of these elements, we previously did not find evidence for a factor that binds to the palindromic element spanning the BC region (−100 GTTTACATAAAC −89) of the VTGII promoter (35). We now show that LMH cells do indeed express a factor that binds to this palindromic target site, and we infer that the binding of SP1 to an overlapping site precluded our earlier attempts to detect this factor. Moreover, we have isolated nearly full length cDNA clones for a factor that has the same binding specificity for this VTGII promoter element. This factor, which we have named VBP (for vitello-

* Corresponding author.

genin gene-binding protein), is a member of the basic/zipper (bZIP) family of transcription factors. We show that VBP is most closely related to a liver-specific factor known as DBP, which binds to an important control element in the rat albumin promoter (27), and we present evidence that VBP and DBP define a novel subfamily of bZIP transcription factors.

MATERIALS AND METHODS

Isolation and characterization of VBP cDNA clones. A λgt11 chicken liver cDNA library (kindly provided by Todd Kirschgessner) was screened by using a standard protocol (37) to identify clones that express β-galactosidase fusion proteins capable of binding an oligomerized probe of the −113 to −79 region of the VTGII promoter (see Fig. 2). To prepare the probe, 500 ng each of sense and antisense oligonucleotides spanning this region of the VTGII promoter (5′-AGCT TCAAAAAGAGGAGTGTTTACATAAACTGATAAAAAA G-3′ and 5′-GATCCTTTTTTATCAGTTTATGTAAACACT CCTCTTTTTGA-3′, respectively) were first 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol). To make sure that essentially all of the ends were phosphorylated (which was critical for the subsequent oligomerization step), unlabeled ATP was added to a final concentration of 50 mM during the last 10 min of the 40-min kinase reaction. Equal amounts (250 ng) of the two labeled oligonucleotides were then annealed, and the double-stranded probe was self-ligated by using T4 DNA ligase to yield oligomerized probes with ~10 binding sites per fragment (33). The specific activities of these probes were typically in the range of 2×10^8 to 4×10^8 cpm/μg.

Six large (150-mm) plates each containing 10^5 phage were incubated for 3 h at 42°C, then overlaid with nitrocellulose filters (Schleicher & Schull) that had been soaked for 10 min in 10 mM isopropyl-β-D-thiogalactoside (IPTG), and incubated for an additional 6 h at 37°C. The nitrocellulose filters were then air dried, denatured in 6 M guanidinium hydrochloride, renatured with decreasing amounts of guanidinium hydrochloride, and then incubated at 22°C for 60 min in binding buffer (200 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 30 mM $MgCl_2$, 400 mM KCl, 1 mM dithiothreitol, 5% nonfat dry milk). The filters were washed in the same buffer containing 0.25% nonfat dry milk and incubated for 2 h at 22°C with 2×10^6 cpm of the oligomerized probe (described above) per ml in binding buffer containing 0.25% nonfat dry milk. After three 10-min washes at 4°C in binding buffer, the filters were blotted dry and exposed to film (Kodak X-Omat AR) at −70°C for 16 h between two intensifying screens (Dupont Lightning-Plus). Twelve identical positives that contained a 0.72-kb VBP cDNA insert were obtained from this primary screen.

Seven additional overlapping VBP cDNA clones were isolated after screening of 10^6 phage from each of five different λgt11 libraries, using the original 0.72-kb VBP cDNA insert as a hybridization probe (33). The *Eco*RI inserts from these seven clones, as well as the original 0.72-kb insert, were subcloned into pGEM-3 (Promega) and characterized by restriction mapping and sequencing. The VBP cDNA sequence presented in Fig. 4 was determined on both strands, using SP6 and T7 primers and supercoiled (9) Bal31 nuclease-deleted templates (7). The standard Sequenase nucleotide mix and a mix containing deazaguanosine (U.S. Biochemical) were used to sequence each template.

Cloning and sequencing of a segment of rat DBP cDNA. A 2-μg quantity of rat liver poly(A)$^+$ RNA (prepared as described below) was used to synthesize single-stranded cDNA, using a dT_{17} adaptor (13) as the primer and avian myeloblastosis virus reverse transcriptase. The reaction was carried out at 42°C for 2 h, after which it was terminated by heating at 95°C for 5 min. An aliquot (1/10) of the total cDNA was then used as the template to program a polymerase chain reaction (PCR) with two synthetic oligonucleotides which contained *Eco*RI sites near their 5′ ends and were directed against a portion of the rat DBP cDNA sequence (5′-CGCGAATTCGTGCCCGAGGAACAGAAGGAT-3′ and 5′-CGCGAATTCGTGGCCTCACAGTGTCCCGTG-3′, respectively). Standard PCR conditions were used (1 min at 95°C; 1 min at 50°C; 2 min at 72°C; 30 cycles; followed by 4 min at 72°C) with 50 pmol of each primer. The resultant PCR fragment (which contains DBP cDNA sequences spanning 1111 to 1350 [27]) was digested with *Eco*RI and cloned into pGEM-3. Four independent clones were sequenced, using SP6 and T7 primers and Sequenase reagents (U.S. Biochemical).

Construction of vectors for use in coupled in vitro transcription/translation reactions. The long-form pT7βglobin-VBP (sense and antisense) plasmids (designated 4-7 and 3-13 in Fig. 1) were constructed by transferring the original 0.72-kb VBP cDNA insert into the *Eco*RI site of pT7βglobin (kindly provided by Tom Kadesch). This cloning site is preceded by an *Nco*I site, which encodes an ATG initiation codon, and an upstream recognition site for T7 RNA polymerase (4).

The PCR method was used to make the short-form pT7βglobin-VBP(sense and antisense) plasmids. Two synthetic oligonucleotides were used in a PCR reaction to copy the 722-to-1206 region of the original VBP cDNA clone (see Fig. 4). One oligonucleotide contained the 722-to-739 region of VBP with an *Eco*RI site near its 5′ end (5′-GCGGAAT TCCCTGACCCTGCTGATTTA-3′); the other oligonucleotide contained sequences from λgt11 immediately downstream of the *Eco*RI cloning site (5′-TTGACACCAGACCA ACTGGTAATG-3′). The template (10^7 PFU of phage stock) was boiled in 50 μl of water for 5 min, cooled to 4°C, and then used in a standard PCR procedure (see above). The product of this reaction was digested with *Eco*RI and ligated into the *Eco*RI site of pT7βglobin.

A two-step PCR method (42) was used to change the leucines in the second and third heptad repeats of the VBP leucine zipper (encoded by nucleotides 971 to 973 and 992 to 994 in the sequence shown in Fig. 4) to valines within the context of the long-form pT7βglobin-VBP(sense) plasmid. In the first step, the two point mutations were introduced into overlapping left and right portions of the 0.72-kb VBP cDNA sequence. The left portion was copied from recombinant phage stock as described above, using a λgt11 primer directed immediately upstream of the *Eco*RI cloning site (5′-GGTGGCGACTCCTGGAGCCCG-3′) and an antisense mutated VBP primer (5′-GCAGCCTTCGTTGAGAAAGAG AATACGGCCGTGAGGACG-3′; the mutated positions are underlined). The right portion was copied by using a λgt11 primer directed immediately downstream of the *Eco*RI cloning site (5′-TTGACACCAGACCAACTGGTAATG-3′) and a sense mutated VBP primer (5′-CGTCCTCACGGCCGTATT CTCTTTCTCAACGAAGGCTGC 3′; the mutated positions are again underlined). These two products were purified through PrimeErase columns (Stratagene), and the concentration of each was determined in a fluorometric assay (21). Equivalent amounts (1 ng) of the two products were carried though 8 PCR cycles without addition of oligonucleotides to generate a full-length template, which was then amplified by

20 additional PCR cycles after addition of 50 pmol each of the two λgt11 primers. This final PCR product was then digested with *Eco*RI and subcloned into pT7βglobin.

The VBP-DBP(ZIP) chimeric vector was prepared by a similar two-step PCR protocol. In the first standard PCR reaction (see above), two oligonucleotides (5′-AAGAACAA TGTAGCAGCCAA-3′ and 5′-CGCGAATTCGTGGCCTCA CAGTGTCCCGTG-3′) were used as primers along with the DBP cDNA template (described above) to amplify the leucine zipper domain of DBP. In a parallel PCR reaction, two oligonucleotides (5′-TTGGCTGCTACATTGTTCTT-3′ and 5′-GGTGGCGACGACTCCTGGAGCCCG-3′) were used as primers along with the recombinant VBP phage stock as template to amplify the left end of the original VBP cDNA clone. These two first-round PCR products were isolated through PrimeErase columns (Stratagene), and the concentration of each was determined (21). Equal amounts (1 ng) of each were mixed together and carried through 8 PCR cycles without addition of oligonucleotides to generate a full-length VBP-DBP(ZIP) chimeric sequence, which was then amplified by 20 additional PCR cycles after addition of 50 pmol each of the terminal primers (directed against the flanking λgt11 sequences) used in the first-round PCR reactions. This final PCR product was digested with *Eco*RI and subcloned into pT7βglobin.

In vitro transcription and translation. Each of the various pT7βglobin and pT7βglobin-VBP vectors described above was digested with *Sph*I (which cuts downstream of the cloning site), and 5-μg aliquots were used to program 100-μl T7 RNA polymerase reactions (Promega) according to the manufacturer's specifications. Following sequential extractions with phenol-chloroform (1:1) and chloroform, RNA samples were ethanol precipitated and resuspended in 25 μl of diethypyrocarbonate-treated water. Aliquots of RNA (5 μl) were used to program 50 μl (final volume) in vitro translational reaction mixtures (Promega) containing 35 μl of rabbit reticulocyte lysate, 1 μl (20 U) of RNasin, 1 mM amino acid mix minus methionine, 10 mM KCl, and either 1 mM methionine (to prepare unlabeled protein for gel shift assays) or 5 μl of [^{35}S]methionine (to prepare labeled protein for polyacrylamide gel analysis [22]). The reactions were carried out at 30°C for 1 h, and the translated proteins were stored in aliquots at −70°C until further use.

Gel mobility shift assays. Gel shift assays were performed by using either the BCD probe (which spans the −113 to −79 region of the VTGII promoter; see Fig. 3) or the D-box probe (which spans the −95 to −112 region of the rat albumin promoter; see Fig. 3). In each case, equal amounts (250 ng) of the sense and antisense oligonucleotides were annealed after labeling of the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (33). After isolation from a 12% nondenaturing polyacrylamide gel (33), probe aliquots (0.1 ng) were mixed with the indicated amounts of in vitro-synthesized protein or LMH cell nuclear extract (35) in a final volume of 30 μl of either modified binding buffer [250 mM KCl, 12 mM Tris (pH 7.8), 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 mM $CaCl_2$, 5 mM NaCl, 0.1 mg of bovine serum albumin (BSA) per ml, 5% glycerol, 8 μg of poly(dI-dC); Fig. 2, 3, 5, 8, 9, and 11] or HotFoot buffer (Stratagene; Fig. 1). After incubation for 1 h at 4°C, free DNA and protein-DNA complexes were resolved on a 6% nondenaturing polyacrylamide gel that had been prerun for 1 h at 4°C. Gels were run at a constant voltage of 100 V, and the electrophoresis buffer (0.25× TBE [33]) was recirculated manually every 30 min. Competition experiments were carried out as described above except that 100-fold molar excesses of unlabeled fragments were added prior to addition of the translated protein.

Methylation interference assays. Methylation interference assays were performed by using the BCD probe (which spans the −113 to −79 region of the VTGII promoter) and the D-box probe (which spans the −95 to −112 region of the rat albumin promoter); these sequences are shown in Fig. 3. The probes were prepared as described above for the gel shift assays except that only one oligonucleotide was labeled for each double-stranded probe. An aliquot (12 ng) of each probe was methylated for 2 min at 37°C in a 250-μl reaction volume containing 50 mM sodium cacodylate (pH 8.0), 1 μg of tRNA, and 1 μl of dimethylsulfate. The reactions were terminated by addition of 50 μl of stop buffer (1.5 M sodium acetate [pH 7.0], 1 M β-mercaptoethanol), and the DNA was ethanol precipitated twice prior to use in a gel shift assay. The gel shift assay was performed as described above except that the reactions were scaled up fivefold. Following electrophoresis, the gel was covered with Saran Wrap and exposed to film (Kodak X-Omat AR) overnight at 4°C, and then the free DNA and DNA-protein complexes were excised and eluted overnight into a buffer containing 0.5 M ammonium acetate–1 mM EDTA buffer. The samples were extracted with phenol-chloroform (1:1), and the DNA was ethanol precipitated and then cleaved in 50 μl of 0.5 M piperidine at 90°C for 30 min. The DNA samples were then dried under vacuum, and equivalent amounts of radioactive material were analyzed on a 15% acrylamide sequencing gel (33).

RNA preparation and analysis. Total cellular RNA was prepared according to a standard protocol (33). Briefly, frozen or fresh tissues were homogenized in 10 volumes of 3.7 M guanidine isothiocyanate containing 5 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.5% Sarkosyl. Aliquots (28 ml) of the homogenate were layered over 10-ml pads containing 5.7 M cesium chloride and 0.1 mM EDTA (pH 7.5) and centrifuged at 112,000 × *g* for 16 h at 25°C. The RNA pellets were dissolved in 4 ml of buffer (10 mM Tris-HCl [pH 7.6], 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], extracted once with an equal volume of phenol-chloroform and once with chloroform alone, and precipitated with ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The RNA pellets were dissolved in diethylpyrocarbonate-treated water and stored at −20°C until further use.

Poly(A)$^+$ RNA samples were isolated from aliquots (3 to 4 mg) of total RNA on oligo(dT) columns (Stratagene) according to a standard protocol (33). Following elution from the columns and ethanol precipitation, the poly(A)$^+$ RNA samples were resuspended in diethylpyrocarbonate-treated water and stored at −70°C until further use. RNA concentrations were determined by UV absorbance.

Aliquots (1 μg) of poly(A)$^+$ RNA were resolved on 1% agarose gels in MOPS buffer (33) and transferred to Nytran membranes (Schleicher & Schuell). The filters were baked for 30 min at 80°C in a vacuum oven, prehybridized at 55°C for 2 h in Stark's buffer containing 50% formamide, and hybridized at 55°C for 18 h in the same buffer containing 10^6 cpm of riboprobe per ml (33). Riboprobes (specific activities of 0.5×10^8 to 1.0×10^8 cpm/μg) were prepared by using SP6 or T7 polymerase as specified by the manufacturer (Promega). Following hybridization, the blots were washed twice for 30 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% SDS and then twice for 30 min at 65°C in 0.1× SSC containing 1% SDS (33) before exposure to film (Kodak

X-Omat) for approximately 18 h at −70°C between two intensifying screens (Dupont Lightning-Plus).

RESULTS

Isolation of a clone for a transcription factor (VBP) that binds to a conserved motif in the chicken VTGII and *Xenopus* vitellogenin A2 promoters. The chicken VTGII and *Xenopus laevis* A2 vitellogenin gene promoters contain a number of similar sequence motifs that have been implicated in the estrogen-dependent liver-specific transcription of these evolutionarily related genes (39). For example, each promoter contains a consensus binding site for the ubiquitous transcription factor USF (34), and we showed that a linker scanner mutation over the USF binding site in the VTGII promoter reduced transient estrogen-dependent transcription from the VTGII promoter 10-fold when assayed in chicken LMH hepatoma cells (35). The upstream promoter regions are also very similar and have been shown in each case to contain multiple positive control elements. For example, a palindromic sequence in the distal region of the VTGII promoter (−100 GTTTAcaTAAAC −89) and a similar sequence in the distal activator region of the frog A2 promoter (−108 GTTTAcaCAACC −97) have both been shown to be functionally important (17, 35). Although C/EBP can bind to both of these sequences (17, 35), we suspect that this transcription factor may not be relevant to the activity of the chicken element since binding could be detected only with use of relatively large amounts of recombinant C/EBP.

In light of the critical importance of this conserved element, we used a binding assay (37) to search for factors other than C/EBP that might be capable of binding to this palindromic sequence. Using an oligomerized probe (denoted BCD) spanning the −113 to −79 region of the VTGII promoter (the sequence of the VTGII promoter is shown in Fig. 2), we identified 12 positive clones from a screen of ~2 × 10^6 λgt11 clones bearing chicken liver cDNA inserts. All 12 clones proved to be identical after plaque purification and sequence analysis. The sequence of the common 0.72-kb cDNA insert is shown in Fig. 4. For the sake of convenience, we will henceforth refer to this cDNA insert as coding for VBP in light of its ability to bind to the distal promoter regions of the chicken VTGII and frog A2 vitellogenin genes (see below).

To characterize the binding properties of VBP in greater detail, the 0.72-kb cDNA fragment was subcloned in both orientations into an expression vector (pT7βglobin) that utilizes the same reading frame (relative to the *Eco*RI ends) as used by λgt11 (4). As shown in Fig. 1A, the pT7βglobin-VBP(sense; 4-7) clone yielded a 25-kDa protein in a coupled in vitro transcription/translation reaction, whereas the pT7βglobin-VBP(antisense; 3-13) clone gave rise to only the same pattern of weak translation products seen with the mock translation control.

The pT7βglobin-VBP(sense and antisense) clones were next used as templates in coupled in vitro transcription/translation reactions to prepare unlabeled proteins for gel shift assays. The probe used in this assay (BCD) contains the −113 to −79 sequence from the VTGII promoter (Fig. 2); the oligomerized version of this probe was used in the initial λgt11 screen described above. As shown in Fig. 1B, the VBP(sense; 4-7) translation product bound to the BCD probe, as evidenced by the novel band of reduced mobility that was not seen with either the VBP(antisense; 3-13) or mock translation products. In comparing the results of this gel shift assay with those presented below, it is important to point out that the latter was performed with use of a modified binding buffer (see Materials and Methods) which was found to increase the ratio of specific to nonspecific bands seen in gel shift assays (e.g., Fig. 2).

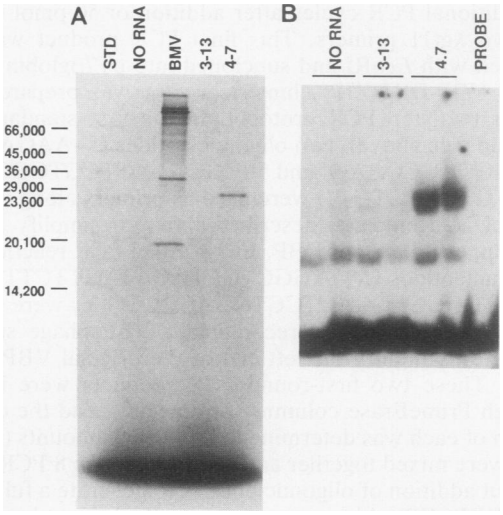

FIG. 1. In vitro expression and binding of VBP. The 0.72-kb VBP cDNA insert from the initial positive λgt11 clone (identified on the basis of its ability to bind to the concatemerized BCD probe; see Fig. 2) was subcloned into the pT7βglobin expression vector (4). (A) ^{35}S-labeled proteins obtained when linearized versions of the VBP sense (4-7) and antisense (3-13) pT7βglobin-VBP plasmids were used as templates to program coupled in vitro transcription/translation reactions. The VBP(sense; 4-7) vector is shown to encode a 25-kDa protein, whereas the VBP(antisense; 3-13) vector yielded only the pattern of weak bands seen with the mock-translated control. In vitro translation products from a control Brome mosaic virus (BMV) RNA template are also shown, as are molecular weight standards (STD). (B) Results of a gel shift assay in which the BCD probe was incubated with duplicate preparations of unlabeled translation products from the VBP(sense; 4-7) and VBP(antisense; 3-13) orientation vectors as well as mock translation product. The last lane shows the mobility of the free probe.

To verify that the retarded band observed in gel shift assays programmed with in vitro-translated VBP was indicative of a specific interaction with the BCD probe, we performed a competition gel shift assay using a set of unlabeled fragments spanning the −117 to −24 region of the VTGII promoter (Fig. 2). Of the large promoter fragments tested (ABCD, CDEF, EFGH, and GHIJ) it is clear the ABCD fragment (which spans the −117 to −80 region and thus includes the probe sequence) was the only one that competed for VBP binding. Thus, the binding is specific. Competition with smaller fragments from the VTGII promoter revealed that the VBP binding site is confined within the BC portion (−107 to −89) of the VTGII promoter; this region contains the palindromic sequence (−100 GTTTAc/aTAAAC −89) that is related to a segment of the frog A2 activator module (−108 GTTTAc/aCAACC −97). As shown in the penultimate lane of Fig. 2, this frog sequence competed for VBP binding, albeit less efficiently than the chicken BC box fragment. For reasons that will become apparent (see Fig. 7), we also tested whether the D-box region of the rat albumin promoter (27), which is similar (−92 CATTAc/aAAATC −115) to the VTGII BC box, could compete for VBP binding. As shown in the rightmost lane of Fig. 2, this

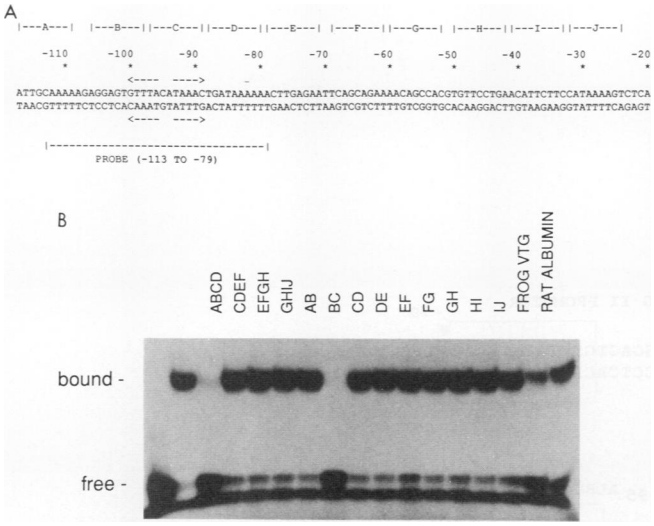

FIG. 2. Evidence that VBP binds specifically to a single site in the VTGII promoter. Gel shift assays (B) were preformed using the BCD probe (A) and VBP that was translated in vitro from the pT7βglobin-VBP(sense) clone described in the legend to Fig. 1. The first two lanes show, respectively, the free probe and the VBP gel-shifted probe. Note that the incubation buffer used in this and all subsequent gel shift assays differs from the nonoptimal conditions used for Fig. 1 (see text for details). The additional lanes show the results of adding 100-fold excesses of various unlabeled DNA fragments as competitors. The letter designations refer to different segments of the VTGII promoter sequence (A). The DNA competitor fragments used in the last two lanes correspond to the activator module from the frog vitellogenin A2 gene promoter (frog VTG) and the rat albumin D box (rat albumin) (see text for details).

element competed only weakly with the BCD probe and thus appears to represent a poor binding site for VBP.

Methylation interference experiments using both the BCD probe from the VTGII promoter and the rat albumin D-box probe were carried out to gain insights regarding the binding of VBP to these high- and low-affinity sites, respectively. As shown in Fig. 3, VBP binding to the BCD probe was inhibited by methylation of the G residue at position −95 on the lower stand, which corresponds to position −1 of the palindrome (−100 GTTTAc/aTAAAC −89). We were surprised to find that methylation of the G residues at the ends of the palindrome (positions −100 and −89) did not affect VBP binding, especially in light of the fact that methylation of the G that is in a comparable position of the more degenerate rat albumin D-box palindrome (position −109 on the lower strand) did interfere with VBP binding. The other methylation-sensitive G residue in the rat albumin D box was on the lower strand at position −113, which corresponds to position −1 of the degenerate palindrome and thus would appear to be comparable to the strong contact site detected in the VTGII promoter.

VBP is a member of the bZIP family of transcription factors. The original 0.72-kb VBP cDNA insert was sequenced and found to contain a poly(A) tract preceded by a partial open reading frame. This open reading frame was extended in the 5′ direction by isolating and sequencing seven additional overlapping VBP cDNA clones (see Materials and Methods). A total of 1,179 bp of VBP cDNA sequence [exclusive of the poly(A) tract] was thus obtained. This size agrees well with that of the 1.2-kb VBP mRNA visualized on Northern (RNA) blots (see Fig. 10) and thus must comprise a nearly full length copy of this message. This sequence is presented in Fig. 4. On the basis of the fact that translational start sites are almost always preceded by a purine in the −3 position relative to the ATG (20), we infer that the VBP open reading frame likely begins at the methionine codon at position 140. As shown in Fig. 4, this predicts an open reading frame of 313 codons for VBP.

One feature that was immediately obvious upon inspection of the predicted VBP protein sequence is that it contains a bZIP motif (38) near the carboxy-terminal end (codons 246 through 298). A comparison of this region of VBP with the consensus bZIP sequence is shown in Fig. 4B. It is clear that the putative basic region of VBP deviates from the consensus in only a single position. Similarly, the putative leucine zipper region of VBP is superficially atypical only in having an isoleucine in place of a leucine in the first heptad repeat (however, see Discussion).

The palindromic nature of the VBP binding site in the VTGII promoter (Fig. 2) is noteworthy in light of the fact that bZIP factors bind to their target sites as dimers. As a test of whether VBP is indeed a bZIP factor, we performed a gel shift assay using long and short forms of VBP (see Materials and Methods). As a control, we also used long and short forms of C/EBP, as this is a well-characterized bZIP factor and we previously showed that it also can bind to the VBP target site in the VTGII promoter (35). As expected (24), when long and short forms of C/EBP were incubated with the probe at 4°C and assayed on a nondenaturing gel, a novel band was observed that has a mobility intermediate to those seen when the short and long forms of C/EBP were assayed separately (Fig. 5C). This band is diagnostic of binding by a heterodimer containing one copy each of the long and short forms of C/EBP. In contrast, when the short and long forms of VBP were incubated together with the probe at 4°C, no evidence for heterodimer formation was seen in a gel shift assay (Fig. 5A). However, as shown in Fig. 5B, heterodimers between the long and short forms of VBP were formed when these two proteins were first preincubated at 37°C before addition of probe at 4°C. These results confirm that VBP does indeed bind to its target palindromic sequence as a dimer and furthermore reveal that VBP exists as a stable dimer in the absence of DNA. The observation that VBP dimers are more stable than C/EBP dimers in solution was unexpected, given that C/EBP has a more typical and extended leucine zipper than does VBP.

One further prediction, based on mutational studies of other bZIP factors (15, 24), is that leucine-to-valine substitutions in the second and third heptad repeats of the putative VBP leucine zipper should abolish binding activity if VBP is indeed a member of the bZIP class of transcription factors. To address this matter, we used a PCR method to introduce these two codon changes into the VBP sequence of the pT7βglobin-VBP(sense; 4-7) clone described above. This mutated vector, as well as the corresponding wild-type pT7βglobin-VBP vector, were used in coupled in vitro transcription/translation reactions. As shown in Fig. 6A, when [^{35}S]methionine was used in the translation reactions, products of the expected size were observed on a protein gel. However, as shown in Fig. 6B, when the corresponding unlabeled products were used to program a gel shift assay, only the unmutated VBP product was found to bind to the BCD probe from the VTGII promoter (−113 to −79; Fig. 2). Thus, VBP binding activity was abolished by two modest mutations in the putative leucine zipper region as expected of a bZIP factor.

The leucine zipper of rat DBP can functionally substitute for

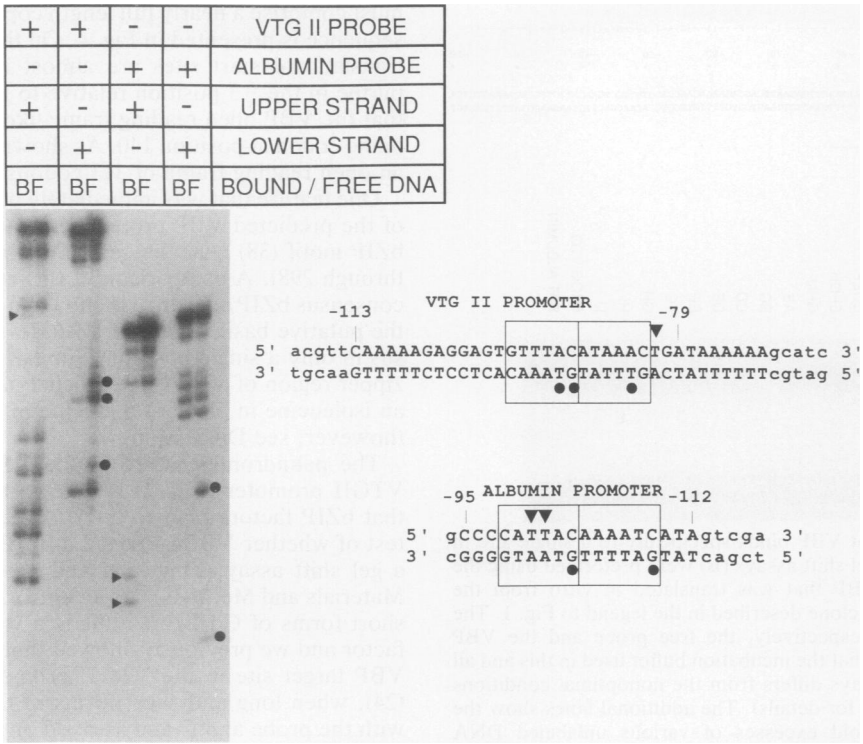

FIG. 3. Methylation-sensitive contacts in high- and low-affinity VBP binding sites. The sequences of the chicken VTGII BCD probe and the rat albumin D-box probe are shown at the right. Methylation-sensitive residues are indicated by circles; residues that result in enhanced binding when modified are indicated by triangles. The data that support these assignments are shown on the left. Each probe was labeled at the 5′ end of either the upper or lower strand, methylated to a limited degree with dimethylsulfate, and then incubated with in vitro-translated VBP. The free and VBP-bound probes were then isolated from a nondenaturing gel and cleaved with piperidine, and the fragments were resolved on a sequencing gel before being exposed to film (see Materials and Methods for details).

the leucine zipper of VBP. A search of the data base revealed that VBP is strikingly similar to a rat transcription factor that was designated DBP on the basis of its ability to bind to the D box of the rat albumin promoter (27). However, in light of the data for VBP presented above, we were initially puzzled by the reported absence of a leucine zipper for DBP. A comparison of the carboxy-terminal regions of the VBP and DBP open reading frames revealed that the similarity between these sequences deviated abruptly within the middle of the leucine zipper region of VBP. In examining the DBP sequence more closely, we noticed that the similarity between VBP and DBP could be extended through to a common termination site with the introduction of a frameshift in the DBP sequence between positions 1236 and 1237 of the published sequence (27). To resolve this issue, we isolated four independent DBP cDNA clones spanning this region (see Materials and Methods) and sequenced each. The results of this analysis (data not shown) indicated that a nucleotide (G) had indeed been overlooked in the published sequence at the expected position.

A comparison of the predicted amino acid sequence of VBP with the corrected amino acid sequence of DBP is shown in Fig. 7. It is clear that some regions of VBP and DBP are very similar (most notably over the respective basic/hinge regions), whereas others (such as the amino-terminal regions) are quite dissimilar. It is striking that of the 21 amino acids that define the basic regions of VBP (codons 246 to 267) and DBP (codons 258 to 279), there are only three differences between VBP and DBP. However, the two nonconservative amino acid differences are likely to be significant since VBP binds with a much higher affinity to the VBP binding site in the VTGII promoter than to the DBP binding site in the rat albumin promoter (see above).

The primary amino acid sequence comparison of VBP and DBP shown in Fig. 7 indicates that the leucine zippers of VBP and DBP are markedly different in the second, third, and four heptad repeats. An interesting insight regarding these differences was revealed, however, by comparing these two leucine zippers on a helical wheel projection. As shown in Fig. 8, it is remarkable that all of the nonconservative amino acid differences between the leucine zippers of VBP and DBP are confined to only three of the helical spokes: the four spokes that are predicted to project toward the dimerization surface are nearly identical.

To test whether the limited sequence similarities between the VBP and DBP leucine zippers might be sufficient to allow the DBP zipper to function in place of the VBP zipper, we took advantage of the fact that the nucleotide sequences of these two factors are nearly identical within a portion of their basic regions. This enabled us to use a PCR protocol (see Materials and Methods) to replace the VBP zipper with the DBP zipper in the pT7βglobin-VBP(long form) vector that we used in previous mixing experiments (Fig. 5). The chimeric expression vector that we obtained, pT7βglobin-VBP-DBP(ZIP), as well as the vector coding for the short form of the wild-type VBP, pT7βglobin-VBP, were used to program in vitro transcription/translation reactions for use in gel shift assays with the BCD probe (Fig. 9). Firstly, it is

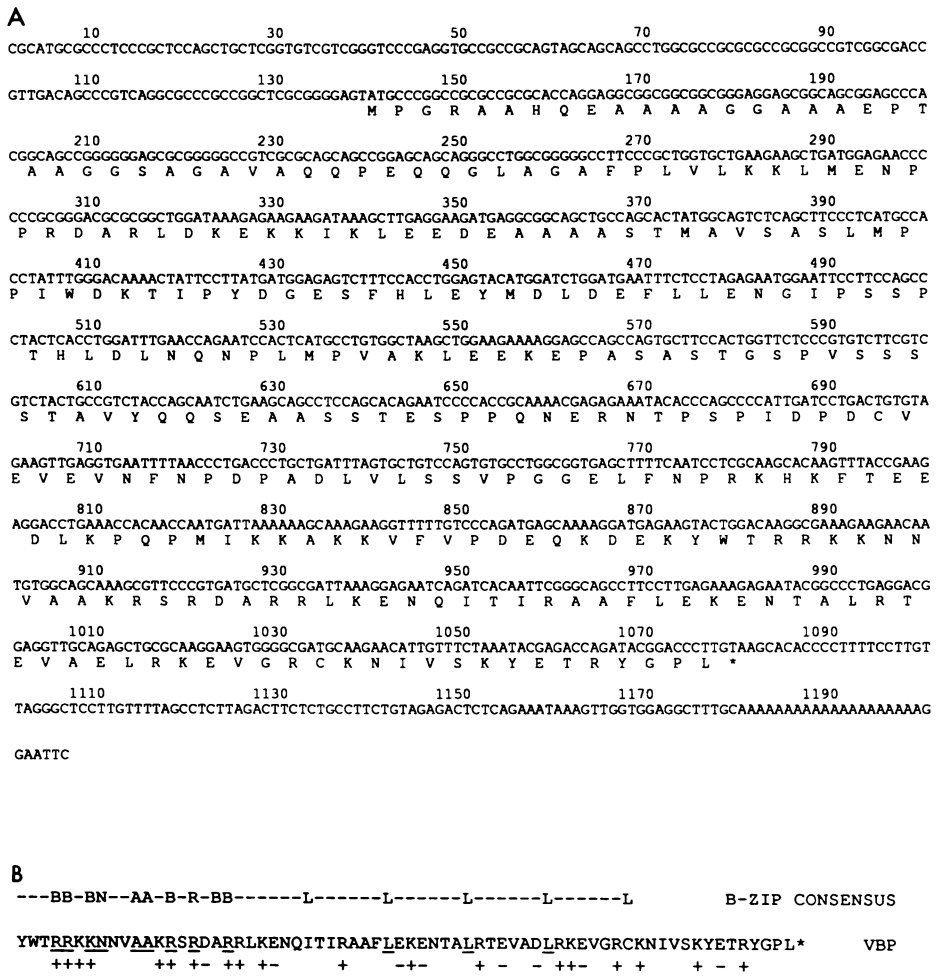

FIG. 4. Sequence of VBP cDNA. The 1,179-bp VBP cDNA sequence [exclusive of the poly(A) tract] (A) was determined from overlapping cDNA clones. The cDNA insert from the initial λgt11 clone that was identified on the basis of its ability to bind the concatemerized BCD probe (spanning the −113 to −79 region of the VTGII promoter) included sequences from position 486 through the poly(A) tract. The VBP open reading frame is predicted to extend from the Kozak box (20) at position 140 to the termination codon at position 1,079, as indicated. The carboxy-terminal portion of this open reading frame (encoded by nucleotides 875 through 1033) contains a bZIP consensus motif (38), as highlighted by the comparison shown in panel B. The amino acid positions that match the bZIP consensus sequence are underlined (N, A, and L denote codons for asparagine, alanine, and leucine, respectively; B denotes a basic amino acid). The charged amino acids within this portion of the VBP open reading frame are also indicated.

clear that homodimers of VBP-DBP(ZIP) bind to the BCD probe (lane 6). Second, and more important, heterodimers between the short form of VBP and the long form of VBP-DBP(ZIP) also bind to this probe (lanes 3 to 5), indicating that these two leucine zippers are functionally compatible.

VBP RNA expression occurs early in liver ontogeny and does not display circadian fluctuations. Given the relationship between chicken VBP and rat DBP, it was of interest to address whether VBP gene expression in the chicken is similar to the novel pattern of DBP gene expression in the rat (27, 41). Three parameters were tested: whether VBP expression in the chicken liver is restricted to adults, whether the VBP gene is expressed at the RNA level in a number of somatic tissues but not in testes, and whether expression of the VBP gene is subject to circadian control.

As shown by the Northern blot presented in Fig. 10A, the onset of VBP gene expression in the liver occurs early in development. VBP mRNAs of 1.2 and 3.5 kb are evident as early as day 15 of embryonic development. In fact, the VBP gene must be expressed even earlier than this stage, since we have isolated VBP cDNA clones from a cDNA library prepared from day 10 embryos (data not shown). A nearly full length cDNA copy of the 1.2-kb VBP mRNA sequence is shown in Fig. 4. On the basis of an analysis of two additional VBP cDNA clones, we infer that the 3.5-kb VBP mRNA results from a failure to use the polyadenylation site that gives rise to the 1.2-kb VBP mRNA (data not shown). Since the 1.2- and 3.5-kb VBP mRNAs are colinear over the first 1.2 kb, both encode the open reading frame shown in Fig. 4. A clone containing a nearly full length copy of the long 3′ untranslated region of the 3.5-kb VBP mRNA has been isolated, but only a portion of this clone has been sequenced (data not shown).

The Northern blot shown in Fig. 10B reveals that VBP mRNAs are present in a variety of somatic tissues, with the highest levels being seen in the oviduct (lane 2). We presume that the higher-molecular-weight VBP RNAs that are seen in

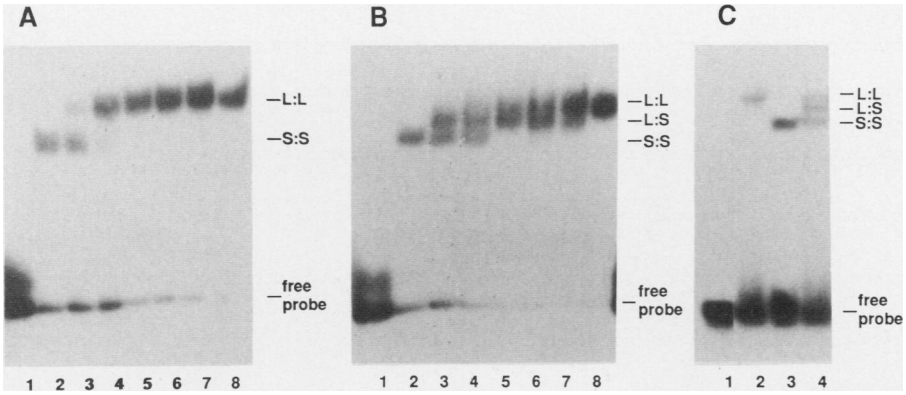

FIG. 5. Evidence that VBP exists as a stable dimer in solution and binds to its target site as a dimer. Coupled in vitro transcription/translation reactions were used to prepare long and short forms of VBP for gel shift assays. These proteins were preincubated in binding buffer at either 4°C (A) or 37°C (B) before incubation with the BCD probe (see Fig. 2) at 4°C. The long (lanes 8) and short (lanes 2) forms of VBP were each competent to bind to the BCD probe. In lanes 3 to 7 of both panels, increasing amounts of the long form of VBP were added to a constant amount of the short form of VBP during the preincubation step. It is clear that a novel band of intermediate mobility (denoted L:S) resulted from preincubating the mixture of long and short proteins at 37°C (B) but that no such heterodimeric complex was generated when the preincubation mix was kept at 4°C (A). In contrast, long and short forms of C/EBP freely exchanged at 4°C and bound to the BCD probe (C). The mobility of the BCD probe is shown in lane 1 of each panel. The various types of dimers are indicated; S and L refer to short and long forms, respectively, of either VBP (A and B) or C/EBP (C).

several of the samples are due to processing intermediates, but we have not addressed this matter critically. We note, however, that this probe did not cross-react with other related genes when genomic Southern blots were analyzed (data not shown). The 1.2- and 3.5-kb VBP mRNAs are present in each somatic tissue examined although not in the same relative proportions. Whereas the testes sample (lane 3) is somewhat degraded, it is clear that the VBP gene is also expressed in this tissue. It is noteworthy that VBP mRNAs are evident in both chicken hepatoma (LMH) cells and chicken embryo fibroblast cells (lanes 4 and 5, respectively), since these were the two recipient cell types used in previous transient expression assays with linker scanner mutated VTGII promoter constructs (35). Indeed, below we present evidence that the VBP protein is also expressed in LMH cells.

The Northern blot shown in Fig. 10C demonstrates that VBP mRNA levels do not fluctuate appreciably as a function of time of day (the chickens used in this experiment were caged under conditions of 12 h of light [6 a.m. to 6 p.m.] and 12 h of dark [6 p.m. to 6 a.m.]). This result is significant because the rat DBP gene, which is clearly related to the chicken VBP gene, was found to be expressed only during a narrow period of time each day (41). Since the VTGII gene, which is one of the presumptive targets for VBP, is estrogen inducible, we also tested whether the VBP gene might itself be regulated by estrogen. As shown in Fig. 10C, VBP mRNA levels did not differ appreciably in hormone-naive birds (odd-numbered lanes) and birds that had been injected with estrogen 24 h before sacrificing (even-numbered lanes). Thus, the VBP gene is not obviously regulated by estrogen, although our data do not rule out the possibility of either a transient or delayed response to estrogen.

A factor that has the binding characteristics of recombinant VBP is present in chicken LMH hepatoma cells. As noted above, VBP mRNAs are expressed in chicken LMH hepatoma cells at levels comparable to those seen in the liver (Fig. 10B). This observation supports the notion that VBP is critical to VTGII promoter function, since a linker scanner mutation over (what we now know to be) the VBP binding

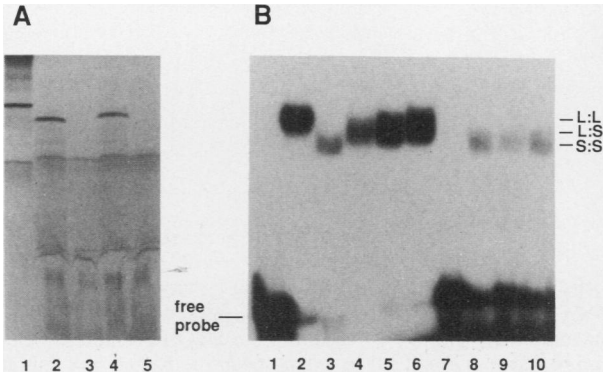

FIG. 6. Evidence that changing the leucines in the second and third heptad repeats within the putative leucine zipper region of VBP to valines abolishes binding to the BCD probe. A PCR protocol was used to introduce the two desired codon changes in the pT7βglobin-VBP vector to yield pT7βglobin-VBP(mut). This vector and the unmutated pT7βglobin-VBP vector were then used to program coupled in vitro transcription/translation reactions. (A) Assay in which the products obtained when [^{35}S]methionine was used in the translation reactions were resolved on a denaturing protein gel and imaged on film. Lanes 1, products of a control Brome mosaic virus (BMV) template; 2 and 4, VBP and VBP(mut), respectively; 3 and 5, translation products encoded by antisense constructs of VBP and VBP(mut), respectively. (B) Gel shift assay in which unlabeled long forms of VBP and VBP(mut) as well as a short form of VBP (see text) were made in parallel in vitro translation reactions. The retarded complexes seen with the long (L) and short (S) forms of VBP are shown in lanes 2 and 3, respectively. Heterodimeric binding complexes of long and short forms of VBP were also evident when increasing amounts of the long form of VBP were preincubated at 37°C with a constant amount of the short form of VBP (lanes 4 to 6; see also Fig. 5). In contrast, when the analogous mixing experiment was done using the short form of VBP and the long form of VBP(mut), no heterodimers were evident (lanes 8 to 10). This mixing experiment also shows that the addition of VBP(mut) did not in any way interfere with the short form of VBP binding to the BCD probe. As shown in lane 7, the VBP(mut) protein also failed to bind to the BCD probe as a homodimer. The mobility of free probe is shown in lane 1.

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  1 MPGRAAHQEAAAAGGAAAEPTAAGGSAGAVAQQPEQQGLAGAFPLVLKK. 49
          | : :: .::|   ||.|||.:....  || : :.   |.
  1 ........MARPLSDRTPGPLLLGGPAGAPPGGGALLGLRSLLQGNSKPK 42

 50 .....LMENPPRDARLDKE..KKIKLEEDEAAAASTMAVSAS........ 84
       |:.:..|.| |...  .   ||..::|.|.| |||::
 43 EPASCLLKEKERKATLPSAPVPGPVLETAGPADAPTGAVSGGGSPRGRSG 92

 85 .......LMPPIWDKTIPYDGESFHLEYMDLDEFLLENGIPSSPTHL.DL 126
         : | :|::|:|::: :||:|||.||||:|:|.||..  :|
 93 PVAGPSLFAPLLWERTLPFGD....VEYVDLDAFLLEHGLPPSPPPPGGL 138

127 NQNPLMPVAKLEEKEPASASTGSPVSSSSTAVYQQSEAASSTESPPQNER 176
   ...|  : .. ...:|:|.|.:|| ||.: |. ... :|.:...:. ..|
139 SPAPSPARTPAPSPGPGSCSSSSPRSSPGHAPARATLGAAGGHRAGLTSR 188

177 NTPSPIDPDCVEVEVNFNPDPADLVLSSVPGGELFNPRKHKFTEEDLKPQ 226
    :||||:|||.||| :.|:|||||.|||:|| | |:||:|:|.||:||||
189 DTPSPVDPDTVEVLMTFEPDPADLALSSIPGHETFDPRRHRFSEEELKPQ 238

227 PMIKKAKKVFVPDEQKDEKYWTRRKKNNVAAKRSRDARRLKENQITIRAA 276
    |::|||:|| ||:|||||||.|| ||| ||||||||||||||||.:|||
239 PIMKKARKVQVPEEQKDEKYWSRRYKNNEAAKRSRDARRLKENQISVRAA 288

277 FLEKENTALRTEVAELRKEVGRCKNIVSKYETRYGPL* 314
    ||||||. || ||..:|.|:::::.::|:|:...|.||
289 FLEKENALLRQEVVAVRQELSHYRAVLSRYQAQHGTL* 326
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FIG. 7. Relatedness of chicken VBP to rat DBP. The predicted open reading frames of chicken VBP and the corrected version of rat DBP (see text) were compared by using a BESTFIT alignment program (11). As shown, the most notable similarity spans the basic/hinge regions of VBP (amino acids 238 to 270) and DBP (amino acids 250 to 282). The leucine zippers appear from this comparison to be much less similar, especially in the third and fourth heptad repeats (amino acids 285 to 298 for VBP; amino acids 297 to 210 for DBP). As shown in Fig. 9, however, these differences do not preclude functional interactions between these leucine zippers.

site drastically reduced estrogen-dependent transcription from the VTGII promoter in transient expression assays with LMH cells (35).

In light of these two results, we were initially puzzled by our previous failure to detect a VBP-like binding activity in LMH cell extracts (35). The fact that the VBP binding site identified in this study (Fig. 2) overlaps with the high-affinity SP1 binding site that we previously identified led us to

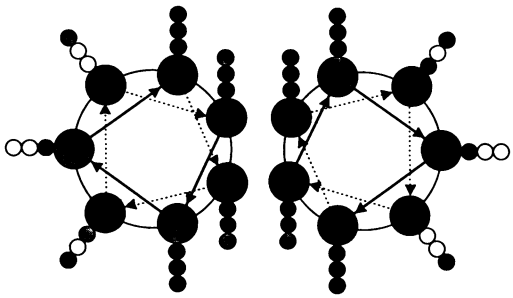

FIG. 8. Diagram showing that similarities between the leucine zippers of chicken VBP and rat DBP are largely confined to four of the seven helical spokes. Helical projections of two leucine zippers are shown apposed at the putative dimerization surface; the leucine spoke in the left helix is located at 2 o'clock, whereas the leucine spoke in the right helix is located at 8 o'clock. Analogous positions of the VBP and DBP leucine zippers were compared and characterized as to whether they have identical amino acids (solid circles), conservative changes (A/T, G/S, H/R, I/V, L/V, S/T; stippled circles), or nonconservative changes (A/E, A/L, A/V, K/Q, T/Q; open circles). It is clear from this projection that the four helical spokes that project toward the dimerization surface are very similar for VBP and DBP but that the other three spokes are markedly dissimilar. The functional significance of this limited homology is supported by the experiment presented in Fig. 9.

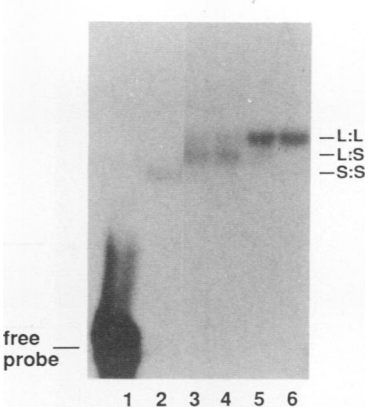

FIG. 9. Evidence that the leucine zipper from DBP can functionally substitute for the leucine zipper of VBP. A PCR protocol (see Materials and Methods) was used to introduce the leucine zipper coding region of DBP in place of the VBP zipper in the long form (L) of the pT7βglobin-VBP vector. The chimeric VBP-DBP(ZIP) protein encoded by this vector as well as the short form (S) of wild-type VBP (see Fig. 6) were independently translated in vitro and analyzed in a gel shift assay with the BCD probe. As shown in lanes 2 (VBP) and 6 [VBP-DBP(ZIP)], both proteins were competent to bind to the BCD probe as homodimers. To test whether VBP and VBP-DBP(ZIP) can also heterodimerize and bind the BCD probe, a constant amount of VBP was mixed with increasing amounts of VBP-DBP(ZIP) and preincubated at 37°C before addition of the BCD probe at 4°C. As shown in lanes 3 to 5, a band of intermediate mobility was evident in this gel shift assay that was not seen when the two proteins were assayed separately. This result demonstrates that the leucine zippers of VBP and DBP can heterodimerize to yield binding-competent complexes. The mobility of the free DNA probe is shown in lane 1.

suspect that SP1 binding may have precluded VBP binding to the ABCD probe that was used in our previous study. To circumvent this complication, we used a smaller gel shift probe that spans only the BC region of the VTGII promoter; this region is sufficient for VBP binding (Fig. 2) but lacks the SP1 binding site. Finally, to compare directly the results of the present analysis with our earlier published gel shift assays, we used the same lot of LMH cell nuclear extract that was previously characterized (35).

Interestingly, as shown in Fig. 11, the LMH extract gave rise to three retarded bands when the BC probe was used in a gel shift assay. Each of these retarded bands (designated F1, F2, and F3) is indicative of a specific protein-DNA complex, since they were each competed for by a 100-fold excess of the homologous unlabeled BC fragment (lane 4) but not by a comparable excesses of any of the unlabeled CD, DE, and EF fragments (lanes 5 to 7, respectively), which span other regions of the promoter (Fig. 2).

We infer that the F1 complex seen in Fig. 11 cannot be attributable to VBP since the F1 band is efficiently competed for by the AB fragment from the VTGII promoter (which does not bind recombinant VBP; Fig. 2), the rat albumin D box (which binds recombinant VBP only weakly; Fig. 2), and the chicken apoVLDLII D box (which also binds recombinant VBP only weakly; data not shown) (lanes 3 and 8 to 10, respectively). Instead, it is likely that the F1 complex involves a member of the C/EBP family of transcription factors, since the fragments that compete for the F1 band are each known to bind C/EBP (17, 27, 35, 40). It is interesting that this C/EBP-like factor was not detected with the larger ABCD probe in our previous gel shift assays (35), presum-

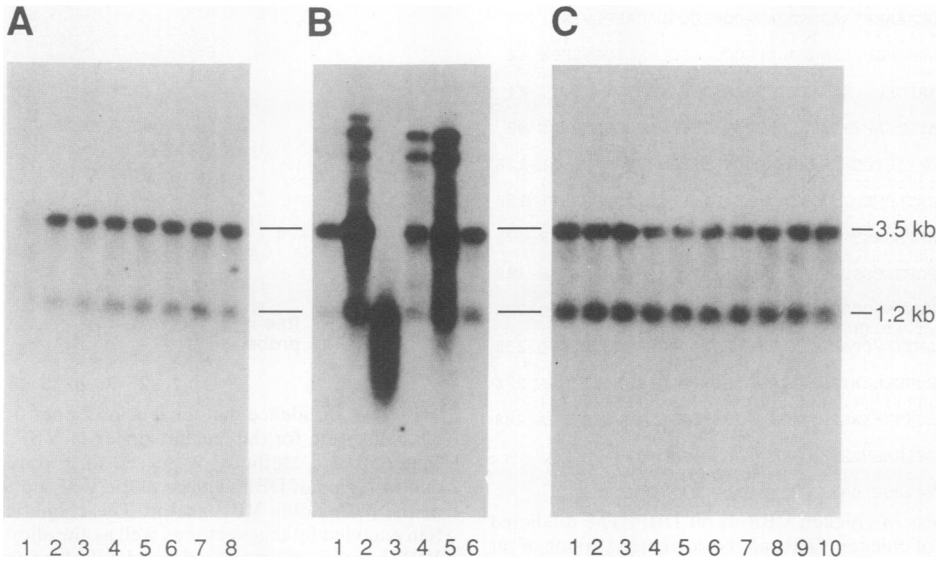

FIG. 10. Demonstration that VBP gene expression is evident in a variety of somatic tissues and occurs early in liver ontogeny. A 0.72-kb antisense RNA probe corresponding to the 3′ end of the VBP cDNA sequence shown in Fig. 4 was used to identify VBP mRNAs on Northern blots. (A) Ontogeny of VBP gene expression in the liver. A sample of total RNA from a laying hen is shown in lane 1; poly(A)$^+$ RNA was used in the other lanes and was obtained at embryonic day 15 (lane 2) or 1 day (lane 3), 1 week (lane 4), 2 weeks (lane 5), 3 weeks (lane 6), or 6 weeks (lane 7) after hatching, or from a laying hen (lane 8). (B) VBP mRNAs in poly(A)$^+$ RNA from various tissues and cell types: spleen (lane 1), oviduct (lane 2), testes (lane 3), LMH hepatoma cells (lane 4), chicken embryo fibroblast cells (lane 5), and liver (lane 6). (C) VBP mRNAs in liver poly(A)$^+$ RNA from birds sacrificed at different times of the day: 8 a.m. (lanes 1 and 2), noon (lanes 3 and 4), 4 p.m. (lanes 5 and 6), 8 p.m. (lanes 7 and 8), and midnight (lanes 9 and 10). The poly(A)$^+$ RNA samples were obtained either from hormone-naïve birds (odd-numbered lanes) or from birds that had been injected with estadiol 24 h prior to sacrifice (even-numbered lanes). Equivalent amounts (1 μg) of poly(A)$^+$ RNA were used for all lanes except that 10 μg of total RNA was loaded in lane 1 of panel A. The two VBP mRNAs observed are estimated to be 3.5 and 1.2 kb, respectively, as indicated. The cDNA sequence for the 1.2-kb VBP mRNA is shown in Fig. 4. The 3.5-kb VBP mRNA contains additional 3′ untranslated sequence due to a failure to use the polyadenylation site that gives rise to the 1.2-kb VBP mRNA (see text for details).

ably because of competition between this factor and SP1 for overlapping binding sites.

The factors responsible for the F2 and F3 complexes display binding characteristics akin to those of recombinant VBP (Fig. 2). Since proteolytic fragments of other bZIP factors have been found in crude extracts and shown to bind to their target DNA sequences, we suspect that F3 may be attributable to a proteolytic fragment of the factor (presumably VBP) that is responsible for the F2 complex. Obviously, a critical test of this prediction will require the production of anti-VBP antibodies. As can be seen in Fig. 11, the F2 and F3 bands were competed for efficiently by the homologous BC fragment (lane 4) and to a lesser extent by the frog A2 activator fragment (lane 8). In contrast, the rat albumin D box (lane 9) and the apoVLDLII D box (lane 10), which differs from the albumin D box at only one position (39), were relatively poor competitors.

DISCUSSION

In this study we have described the cloning and characterization of a new member of the bZIP family of transcription factors. We have named this factor VBP since it was identified by screening a cDNA expression library with a probe spanning the distal promoter region of the chicken VTGII gene. Chicken LMH hepatoma cells express a factor that has the same binding properties as recombinant VBP, and we previously showed that a linker scanner mutation over the VBP site within the VTGII promoter resulted in a 10-fold reduction in transient estrogen-dependent expression from this promoter in LMH cells (35). This same linker

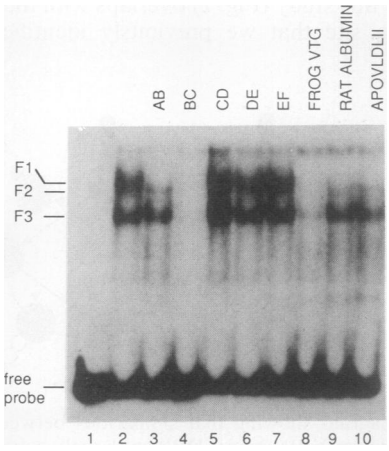

FIG. 11. Expression in chicken LMH hepatoma cells of a factor with the same binding properties as recombinant VBP. A probe spanning the BC region of the VTGII promoter (−106 to −89; see Fig. 2) was used to program gel shift assays with either BSA (lane 1) or crude nuclear extract prepared from chicken LMH hepatoma cells (lanes 2 to 10). The three gel-retarded complexes observed with use of the BC probe and LMH extract are indicated as F1, F2, and F3. In lanes 3 to 10, a 100-fold molar excess of the indicated fragment was included as a competitor. These fragments correspond to segment AB (lane 3), BC (lane 4), CD (lane 5), DE (lane 6), or EF (lane 7) of the VTGII promoter (see Fig. 2), the frog A2 activator (lane 8) (17), the rat albumin D box (lane 9) (27), or the chicken apoVLDLII D box (lane 10) (40). See text for details.

scanner mutation essentially abolishes binding to recombinant VBP in vitro (data not shown). These results provide compelling evidence that VBP plays a pivotal role in the estrogen-dependent transcription of the VTGII gene. In addition, our finding that recombinant VBP binds to the activator element from the frog A2 vitellogenin gene promoter further suggests that the frog homolog of VBP may similarly be involved in inducing expression from this promoter in response to estrogen.

Of the large number of bZIP transcription factors that have been described, VBP is clearly most closely related to rat DBP. The most striking similarity between VBP and DBP spans the respective basic/hinge regions: within a stretch of 33 consecutive amino acids (from the conserved proline immediately upstream of the basic region [38] up to the first heptad repeat), there are only four differences between VBP and DBP. Our finding that the hinge regions are identical was unexpected and indicates that there may be strict constraints in the proper coupling of information between this class of leucine zipper (see below) and basic region. Considering the near identity between the VBP and DBP basic regions, it is not surprising that VBP is able to bind to the DBP binding site (the D box) within the rat albumin promoter. However, the fact that the rat albumin D box is a relatively poor binding site for VBP indicates that the optimal binding sites for VBP and DBP are probably distinct.

Site-directed mutational studies have delineated important residues in the basic regions of several mammalian bZIP proteins. In most cases, blocks of two or more residues were changed or deleted, which resulted in the reduction or elimination of DNA binding activity (12, 14, 19, 24, 28, 36). Replacements of single basic amino acids with uncharged or acidic amino acids have also been shown to effect various alterations in DNA binding activity (12, 14, 28, 30). On the basis of these results, it has been suggested that the overall distribution of positive charge within the basic region of a bZIP factor may be a critical in determining binding specificities (3). In this context we note that although the basic regions of VBP and DBP differ in only three amino acid positions, the basic region of VBP has a positive charge of +2 relative to the basic region of DBP. Given that these differences are highlighted between two wild-type factors which have a related target site specificity, site-directed mutational studies should yield novel insights regarding the coevolution of bZIP factors and their target sites.

The basic region of each bZIP factor is obviously of critical importance in determining target site specificity and depends on a proper alignment dictated by the adjacent leucine zipper region (see below). In the case of VBP, several observations indicate that binding affinity is also profoundly influenced by a more amino terminal region of the protein. In particular, the so-called short form of VBP (which includes amino acids 195 through the carboxy-terminal end) was consistently found to bind to the VTGII promoter site much less avidly than the so-called long form of VBP (which contains an additional 79 residues in the amino-terminal direction). An even more dramatic distinction was obtained when the rat albumin D box was used as a probe in gel shift assays: whereas the long form of VBP did bind to this probe (e.g., Fig. 3), the short form of VBP was unable to do so under any conditions tested (data not shown). We also expressed the analogous short form of DBP (which includes amino acids 207 through the carboxy-terminal end; Fig. 7) and found that this protein was unable to bind either the VTGII promoter probe or the rat albumin D-box probe (data not shown). This critical dependence for binding on sequences quite far removed from the bZIP motif was unexpected in light of the fact that most bZIP factors can be truncated to very near the bZIP motif and still retain binding activity.

The leucine zipper regions of VBP and DBP are unusual in a number of ways. The most obvious feature is that neither has a canonical leucine heptad repeat. VBP has leucines in three of four positions (I-L-L-L), whereas DBP has only two leucines (I-L-L-V). Another unusual feature is that VBP contains only three possible intrahelical salt bridges, whereas DBP has none. This is in marked contrast to other bZIP factors that have been studied (23). It is thus surprising that homodimers of VBP were found to be even more stable in solution (in the absence of DNA) than C/EBP dimers, considering that the leucine zipper of C/EBP is longer and has numerous intrahelical salt bridges. An insight into this paradox may be provided by the helical wheel comparison of the VBP and DBP leucine zippers (Fig. 8), which highlights the fact that all four of the helical spokes that project toward the dimerization surface are very similar for these two bZIP factors. Whereas the conservation of the leucine spoke and the adjacent aliphatic spoke is not surprising, the striking conservation of the two flanking charged spokes suggests that the exact information content of these spokes may also be important in defining this tight dimerization interface. A cursory inspection of the amino acid sequence of these two spokes reveals that oppositely charged amino acids are opposed across the dimerization interface, which raises the possibility that interhelical salt bridges contribute to the stability of these dimers.

It is also intriguing that the leucine zipper of VBP contains a cysteine immediately following the fourth heptad repeat since, in the context of the leucine zipper interface, the two copies of this residue (one from each helix) would be predicted to be juxtaposed. In support of this view, we note that when recombinant VBP was isolated from bacteria, it was found to be almost exclusively in the form of a covalent dimer (40a). Heterodimeric interactions between VBP and other compatible bZIP factors might thus be restricted in vivo, depending on whether VBP exists as a covalent dimer.

Previous studies with the rat albumin promoter indicated that DBP binding sites represent a subset of the C/EBP binding sites (27). An analogous result for VBP was obtained in the present analysis of the chicken VTGII promoter, in which only one of the two previously identified C/EBP binding sites (35) was found to be a binding site for VBP. The realization that VBP, DBP and C/EBP all contain leucine zippers prompted us to consider whether the situation might be further complicated by heterodimer formation between VBP (or DBP) and C/EBP (or other members of the C/EBP subfamily [1, 8, 10, 15a, 29, 31]). Whereas all of the possible combinations of heterodimers between various members of the C/EBP subfamily of bZIP factors (C/EBP; NF-IL6, AGP/EBP, LAP, IL-6DBP, Ig/EBP-1, CRP2; CRP1) were able to bind to the VBP site from the VTGII promoter in gel shift assays, no evidence was obtained for heterodimeric complexes between VBP and any of the four C/EBP-related factors tested (40b). Thus, the leucine zippers of the VBP/DBP subfamily appear to be incompatible with leucine zippers of the C/EBP subfamily. We therefore infer that these two subfamilies of bZIP factors probably coexist in parallel in tissues such as the liver in which members of both subfamilies are represented, rather than directly interacting with each other as heterodimers.

The observation that VBP mRNAs are present in all tissues examined (Fig. 10) suggests that VBP may be a

ubiquitous factor. However, since VBP is closely related to DBP, we are cautious to draw such an inference at present. In particular, DBP protein can be detected only in the liver despite the fact that DBP mRNA is expressed in many somatic tissues (27). Although the basis for this restricted expression if DBP is not known, it has been suggested that the two weak Kozak boxes that precede the initiation codon may contribute to regulating DBP expression at the level of translational initiation. It may therefore be significant that the predicted open reading frame of VBP is also preceded by a poor Kozak box (at position 4; Fig. 4) in a reading frame that is distinct from the one defined by the initiation codon at position 140. In considering other levels at which DBP protein expression might be regulated, we noticed that the open reading frame of DBP has a disproportionate number of rare codons (2) for alanine (GCG) and proline (CCG) in the amino-terminal portion. This would be expected to promote translational pausing and could conceivably have been exploited to restrict DBP translation in nonliver cells. Interestingly, even though the amino acid sequences of VBP and DBP are quite different in their amino-terminal halves (Fig. 7), the same bias is seen for the VBP open reading frame. For both VBP and DBP, 1/3 of the alanine and proline codons in the amino-terminal halves are rare codons, as compared with only 1/20 for the carboxy-terminal halves. This asymmetry is peculiar. It should be noted that this observation is not simply due to the GC content of these regions, since cytosines in the third base positions would result in preferred codons in each case.

Further studies to map the transactivation domains of VBP and DBP should also be quite interesting. Although DBP contains a candidate transactivation motif (13 of the 26 amino acids over positions 129 to 154 are proline; Fig. 7), VBP is devoid of a comparable proline-rich region and lacks any obvious glutamine-rich and acidic regions as well (26). Experiments to address the possibility that VBP encodes a novel transactivation domain will be the focus of future studies.

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ADDENDUM IN PROOF

The existence of a transactivation domain(s) located upstream of the bZIP region of VBP has been demonstrated with two different expression/reporter systems in transient expression assays.

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