The human vitamin D-binding protein gene contains locus control determinants sufficient for autonomous activation in hepatic chromatin

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ABSTRACT

The human vitamin D-binding protein (hDBP) gene is a member of a cluster that includes albumin, \( \alpha \)-fetoprotein and \( \alpha \)-albumin genes. The common origin, physical linkage and hepatic expression of these four genes predict shared regulatory element(s). However, separation of hDBP from the other three genes by 1.5 Mb argues that hDBP may be under autonomous control. To test for hDBP autonomy, mouse lines were generated with a transgene containing the hDBP gene along with extensive flanking sequences. Expression of this transgene was hepatic, robust and proportional to transgene copy number. DNase I hypersensitive site (HS) mapping revealed five liver-specific HS at the hDBP locus: HSI and HSIII at \(-2.1\) kb and \(-0.13\) kb upstream of the transcription initiation site, HSIV and HSV within intron 1 and HSVII located 3’ to the poly(A) site. A second transgene with minimal flanking sequences confirmed the sufficiency of these gene-proximal determinants for hepatic activation. The hepatic-specific HS aligned with segments of phylogenetically conserved non-coding sequences. These data demonstrate the autonomy of the hDBP locus and suggest that this control is mediated by chromatin-based locus control determinants in close proximity to, and within the transcription unit.

INTRODUCTION

The vitamin D-binding protein (DBP) gene is robustly expressed in the livers of all mammalian species. In humans the hDBP protein (also known as Gc-globulin) is secreted from hepatocytes as a polymorphic glycoprotein that constitutes one of the most abundant serum proteins (232–464 \( \mu \)g/ml) (1). The major function of serum DBP is the binding and transport of 25-hydroxyvitamin D, the major circulating form of vitamin D and 1,25-dihydroxyvitamin D, the most active vitamin D metabolite. DBP also binds tightly to monomeric G-actin, blocking the formation of F-actin networks that may occlude the vasculature following cellular damage. Several studies suggest that covalent derivatives of DBP have pivotal roles in the innate immune response by serving as a macrophage-activating factor and by enhancing C5a-mediated chemotaxis for macrophages and neutrophils [reviewed in ref. (1–3)]. DBP is predominantly produced in hepatocytes, although secondary minor sites of synthesis, such as the kidney, have been identified (4). Due to its high-level of activity and its wide spectrum of functions, the DBP gene represents an important model for the analysis of hepatic gene expression. DBP is a member of a family of four robustly expressed liver-specific genes. The conserved intron/exon organizations along with primary and secondary structural conservation of encoded proteins indicate a paralogous relationship of DBP to albumin (ALB), \( \alpha \)-fetoprotein (AFP) and \( \alpha \)-albumin/afamin (AFM). These four genes, formed by local duplications of a common progenitor gene, remain physically linked in the human and rodent genomes (2.5–10). Structural comparisons among these genes suggest that DBP was the oldest and most divergent member in this family (11–13). The developmental profiles of these genes have been described. During rodent embryonic development, expression of ALB, AFP and DBP is induced in yolk sac and is maintained in fetal liver (14), whereas the hepatic expression of AFM is initiated in the perinatal period. AFP is selectively silenced at the end of the fetal period, whereas ALB, AFM and DBP maintain high constitutive levels of expression in adult liver (9). The molecular mechanisms for the transcriptional activation of these genes in the native hepatic chromatin environment, and for their respective developmental controls, have yet to be determined.

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The close relationship among the four genes in the DBP-ALB cluster, and the fact that they are all expressed predominantly in liver, suggests that they may share one or more common cis-regulatory determinants. The possibility of shared regulatory control among these genes is supported by the identification of intergenic enhancers located between ALB and AFP that have the potential to regulate both gene promoters (15–18). Whether shared transcriptional control elements are more widely involved in this four-gene family has not been explored.

Models of regulatory elements controlling multi-gene families can be found in the locus control regions (LCRs) for the human and mouse β-globin gene clusters (19,20) and the human growth hormone gene cluster (21). In these cases, sets of regulatory elements, located quite distant from target promoters, appear to act in concert to generate ‘open’ chromatin structures or chromatin ‘hubs’ (22) conducive to local promoter access and transcription initiation. In some cases, the genomic regions harboring regulatory elements can be located as much as 1 Mb from the target promoter (23). Such remote determinants can be located 5′ or 3′ of the target gene and in some cases can reside within the introns of unrelated neighboring gene(s) [reviewed ref. (24)]. The mechanistic basis for such long-range control is presently under study. Whether the DBP-ALB gene family is controlled by a common set of chromatin regulatory elements remains unknown. With the recent clarification of the genomic organization of the DBP-ALB multi-gene cluster, these questions can now be directly addressed.

A recently refined physical and meiotic map of the 4q11–q13 region in the human genome reveals the organization of the cluster: centromere–3′-DBP–5′-AFP–3′-AFM–3′-telomere (Figure 1A). Of particular note is the inverse transcriptional orientation and wide (>1.5 Mb) separation of the DBP gene from the other three more tightly clustered genes in the family (25) (GenBank accession no. NT_006216). The rat DBP (rDBP) multi-gene cluster is located at 14p21–p22 in chromosome14, a region syntenic to human chromosome 4, and the order and transcriptional orientation of each gene correspond to the human cluster. These data allow us to propose a model in which the hDBP gene is independently regulated by a dedicated LCR. It further implies that the DBP-ALB gene cluster has evolved into at least two separable chromatin units that maintain common liver specificity.

### MATERIALS AND METHODS

#### Materials

Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA), Life Technologies (Rockville, MD), and Roche Molecular Biochemicals (Indianapolis, IN). [α-32P]dCTP and MicroSpin G-50 Columns were purchased from Amersham Biosciences (Piscataway, NJ). Random-primed DNA labeling kits and Taq DNA polymerase were from Roche Molecular Biochemicals. QIAEX II Kit was from QIAGEN and the GENE-CLEAN II Kit was from BIO 101, Inc (La Jolla, CA). Elutip columns were from Schleicher and Schuell (Keene, NH), and RNAzol B RNA isolation solvent was from TEL-TEST Inc. (Friendswood, TX). Zetabind membranes were from CUNO Inc. (Meriden, CT).

#### Oligonucleotides

The various oligonucleotides listed in Table 1, were synthesized by Life Technologies, Inc. or by the DNA Sequencing Facility of University of Pennsylvania.

#### Preparation of DNA probes

The DNA probes for genomic Southern blots and northern blots were released as EcoRI fragments from the rat (6) and human (26) DBP cDNA plasmids. The MX probe, which detects the unique sequence 3′-flanking region of the mβ-globin gene, was released as a 1.3 kb BamHI fragment from the pMX plasmid (27). The mouse ribosomal protein L32 (mrpL32) cDNA probe was released as 0.32 kb EcoRI and HindIII fragment from the mrpL32 plasmid (28). The probes used for DNase I mapping were generated by PCR using Taq DNA polymerase. The templates for the PCR were PAC clones, 231M2 or 45P24 (25). Each fragment was recovered using a QIAEX II kit from an agarose gel, and labeled by [α-32P]dCTP using a random-primed DNA labeling kit. Fragments were then purified on MicroSpin G-50 columns.

#### Generation and analysis of transgenic mice

The 105kb-hDBP fragment was released from vector sequences by NotI digestion of PAC clone, 231M2 plasmid (25) and the 51kb-hDBP fragment by double-digestion of PAC clone, 45P24 plasmid (25) with NotI and FspI. Fragments were separated by field-inversion gel electrophoresis (FIGE) using 1% SeaKem LE agarose gels (FMC BioProducts, Rockland, MA). Fragments were recovered with the GENECLEAN II Kit or by electrophoresis. Each fragment was purified by Elutip, diluted to 2 ng/μl in 10 mM Tris–HCl (pH 7.6), 0.1 mM EDTA and then microinjected into fertilized mouse oocytes (University of Pennsylvania Transgenic and Chimeric Mouse Core). Positive founders were detected by dot-blot analysis of tail DNAs using hDBP cDNA 771 bp EcoRI.
The transgene copy number for each line was determined by Southern blot analysis. All animal work was carried out under protocols approved by the University of Pennsylvania Institutional Animal Care and Usage Committee.

Southern blot analysis

An aliquot of 10–15 \( \mu \text{g} \) of restriction enzyme-digested mouse tail DNA was analyzed on 0.8% agarose gels, transferred to ZetaBlot nylon membrane with 10× SSC (1.5 M NaCl, 0.15 M sodium citrate), ultraviolet (UV) cross-linked to the membrane. The membrane was prehybridized [0.5 M NaPO$_4$ (pH 7.2), 7% SDS, 1% BSA and 200 \( \mu \text{g/ml} \) denatured salmon sperm DNA] at 65°C, hybridized at 65°C with 32P-labeled probe for 16 h, subsequently washed in 0.5× SSC, 0.1% SDS at room temperature, and finally 0.1× SSC, 0.1% SDS at 65°C. The washed membranes were exposed to XAR-5 films (Kodak), and signals were quantified by phosphorimaging (Molecular Dynamics, Inc., Sunnyvale, CA).
Table 1. Oligonucleotide primers and probes

| Probe | Primer sequence |
|-------|-----------------|
| P1    | 5'-TCGGCGCCAAATGGAATTGTTG-3' |
|       | 5'-CCGAGATGTGAAAACCACTTAAATG-3' |
| P2    | 5'-AGAAGACTGCTGCAACATTAAAGG-3' |
|       | 5'-CAGATGGGAAAAAATGGTG-3' |
| P3    | 5'-GCCCTGAGTTTTACCAACATCTG-3' |
|       | 5'-CCCGTGACTTAAATTTGGACGC-3' |
| P4    | 5'-CATCCTGGGAAAGAAGGACCTAC-3' |
|       | 5'-CTTATTGCTTCTCAGTTTCACA-3' |
| P5    | 5'-ACTCCCAACCTGGTGACAGAG-3' |
|       | 5'-GACTCCGAAACCCCGCCGAC-3' |
| P6    | 5'-GCTGGAGAGTTTACCAATTTCCA-3' |
|       | 5'-ACCTGATATATCAATGACTGATC-3' |
| pI    | 5'-GAATCACAGTTCAAGCTTACA-3' |
|       | 5'-CTTATCTCGTGGTTTGCACA-3' |
| pIII  | 5'-GCAAGGACTTCATGTCGAAACAG-3' |

**Northern blot analysis**

Total RNA was extracted from various tissues with RNAzol. 5–20 μg of total RNA were denatured at 55°C, separated in 1.5% agarose-formaldehyde gels, and transferred to Zetabind nylon membrane with 10× SSC. After UV cross-linking, the blots were prehybridized, and subsequently hybridized with 32P-labeled probes at 42°C for 16 h. The membranes were washed with 2× SSC, 0.1% SDS at room temperature, and finally 0.1× SSC, 0.1% SDS at 65°C. The washed membranes were exposed to XAR-5 films (Kodak), and signals were quantified by phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Radial immunodiffusion assay**

One percent agarose containing 3% rabbit polyclonal anti-hDBP (Cocalico Biologicals Inc., Reamstown, PA) was poured on to a glass backing, and circular wells were cut into the solidified matrix. Test mouse sera and the standard sera containing hDBP protein (Calbiochem Inc., San Diego, CA) were loaded into each well and allowed to diffuse for 40 h at room temperature. The gels were rinsed first with phosphate-buffered saline (PBS) for 16 h, then with distilled water for 20 h. Gels were stained with 0.1% Coomassie brilliant blue in 50% methanol and 10% acetic acid for 30 min, and subsequently de-stained with 50% methanol and 10% acetic acid for 1 h. The amount of hDBP in each serum sample was obtained by comparing the diameters of the stained immunodiffused circles of each test serum and the hDBP standard sera (50–500 μg/ml).

**Isolation of intact nuclei**

Livers of 105kb-hDBP transgenic mice were perfused with cold PBS and minced. Liver nuclei were isolated as described (29). The nuclei pellet was resuspended in buffer D [15 mM Tris–HCl (pH 7.4), 150 mM NaCl, 60 mM KCl, 0.5 mM EGTA, 0.5 mM β-mercaptoethanol, 0.5 mM spermidine and 0.5 mM spermine]. Brains of 105kb-hDBP transgenic mice were washed in PBS. Brain cells were dissociated in cell-free dissociation buffer (GIBCO-BRL, Grand Island, NY). Cells were lysed in NB3 buffer (320 mM sucrose, 1 mM MgCl2, 0.05% Triton X-100, 1 mM Pipes (pH 6.4) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Nuclei were washed in RB buffer [0.1 M NaCl, 50 mM Tris–HCl (pH 8.0), 3 mM MgCl2, 0.1 mM PMSF and 5 mM sodium butyrate], pelleted and resuspended in RB buffer.

**DNase I hypersensitivity mapping**

The concentrations of nuclei were estimated from measurements of A260/A280. 500 μg of liver nuclei were suspended in buffer D with 5 mM MgCl2 and were incubated on ice for 10 min with increasing amounts of DNase I (GIBCO-BRL). EDTA was added to 25–50 mM final concentration to terminate the reactions. The DNase I digested liver nuclei were incubated in lysis buffer (800 mM NaCl, 0.5% SDS and 100 μg/ml proteinase K) at 55°C overnight. 100 μg of brain nuclei were suspended in RB buffer with 1 mM CaCl2 and incubated on ice for 10 min with increasing amounts of DNase I (GIBCO-BRL). After terminating the reaction by adding EDTA to 25–50 mM and an equal volume of lysis buffer (1.6 M NaCl, 1% SDS and 200 μg/ml proteinase K), DNase I digested brain nuclei were incubated at 55°C overnight. The lysed liver or brain nuclei samples were extracted with phenol and chloroform, and the DNAs were precipitated with ethanol and suspended in TE buffer. The DNAs were subsequently digested with appropriate restriction enzymes, resolved by electrophoresis on 0.8–1.0% agarose gels, and transferred to Zetabind nylon membranes for Southern blot analysis.

**RESULTS**

**Generation of mouse lines carrying the hDBP locus with extensive flanking sequences (105kb-hDBP)**

In order to assess the ability of the isolated hDBP gene to establish an autonomous hepatic chromatin environment capable of supporting consistent and robust expression, a set of transgenic mouse lines were generated. To optimize the possibility of including all necessary regulatory elements, a large genomic fragment was used as transgene. The hDBP gene encompasses 13 exons and spans 42 kb (13). Four human genomic PAC clones containing the entire hDBP gene along with 37 kb of 5′-flanking region and 26 kb of 3′-flanking region (105kb-hDBP transgene; Figure 1A). This insert was released from the PAC vector, microinjected into fertilized mouse oocytes, and five founders were obtained. Each founder was crossed with a CD1 mate to generate F1 transgenic mice and five 105kb-hDBP lines were established. The integrity of the transgene in each line was determined by extensive Southern blot analysis of F1 or F2 tail DNA (Figure 1B and data not shown). Transgene copy numbers ranged from 2 to 12 as determined by Southern blot after the transgene signal was normalized to the hDBP signal in total human (HeLa) DNA (two copies) and to the endogenous mouse ζ-globin signal as a loading control.

**Expression of the 105kb-hDBP transgene**

The expression of the 105kb-hDBP transgene was assessed to determine whether the full complement of regulatory elements...
were included in this large transgene. A rigorous test of locus control was to determine whether these elements were able to establish a chromatin environment that was autonomous and independent of its site-of-insertion in the host genome. As background for these studies, it has been previously demonstrated that the endogenous rDBP locus is primarily expressed in the liver with 100- to 1000-fold lower levels observed in kidney, placenta/yolk sac, testis and abdominal fat and trace levels in intestine and spleen (4). Northern blot analyses of the 105kb-hDBP transgene detected high levels of human DBP mRNA in the liver of all lines, whereas signals of much lower intensity were consistently observed in the kidney (~2–10% of liver) (Figure 2A, upper panel and summary table). Even lower levels were detected in the intestines of 3 of 5 lines (~0.6–1.0% of liver), and sporadically in testes and spleen (Figure 2A and data not shown). The expression profile of the endogenous mouse DBP gene (mDBP) was established in parallel (Figure 2A, middle panel). Overall, the expression profile of the 105kb-hDBP transgene was consistent with that of the endogenous mDBP gene. These data indicated that the 105kb-hDBP transgene contains regulatory components sufficient to establish native patterns of expression and that the mouse has all the regulatory factors necessary to support the native expression pattern of the human DBP gene.

The ability of the 105kb-hDBP transgene to establish a fully productive and autonomous chromatin domain was further Figure 2. The 105kb-hDBP transgene programs full level, tissue-specific and copy number dependent expression. (A) Tissue specificity of 105kb-hDBP transgene expression. The autoradiograph shows a Northern blot surveying a set of tissues from a representative 105kb-hDBP transgenic line (mouse line 6). RNAs from the indicated tissues and control RNA from wild-type mouse liver (WT) were analyzed. The Northern blot was hybridized with an hDBP cDNA fragment and then with a mouse ribosomal protein L32 (mrpL32) cDNA fragment to normalize for RNA loading. The hDBP cDNA probe was specific for the human DBP mRNA and did not cross-hybridize to the mouse DBP mRNA. The blot was stripped then re-hybridized with rat DBP cDNA to detect the cross-hybridizing, endogenous mouse DBP mRNA. The 105kb-hDBP transgene was expressed predominantly in the livers and at lower level in the kidney of each line. The table summarizes the tissue distribution of transgene expression in an adult male mouse of each transgenic line (no signal detected). Expression in the liver is defined as 100 and all values in other tissues are calculated as using this as a reference. Very low-level expression was detected in intestines of three lines and occasionally in spleen or testis. (B) Copy number dependency of 105kb-hDBP transgene expression. Northern blots containing liver RNA from the five 105kb-hDBP transgenic lines were hybridized with hDBP cDNA and then mrpL32 cDNA. Three animals from each line were analyzed and a representative Northern blot is shown. hDBP mRNA was quantified for each line and normalized to the mrpL32 signal to correct for RNA loading. The corrected hDBP expression value was divided by transgene copy number and the mean ratio for line 1 was arbitrarily set to 1.0. The ratios from the remaining lines were normalized to line 1 and were plotted on the bar graph (±SD). All quantifications were done by phosphoimager analysis and all were in the linear range of detection (Materials and Methods). There were no statistically significant differences among the lines when hDBP expression was normalized to transgene copy number. A radial immunodiffusion assay was used to quantify levels of serum hDBP in each transgenic line. The serum hDBP levels per transgene copy number were plotted in the bottom panel using a logarithmic scale. The results demonstrated that the liver hDBP mRNA and serum hDBP levels were remarkably consistent from line to line in a copy number dependent fashion.
evaluated by determining the relationship between hDBP expression levels and 105kb-hDBP transgene copy number (Figure 2B). If the transgene contains locus control determinants sufficient to establish an autonomous chromatin domain, expression of the transgene at each independent insertion site should be directly related to transgene copy number. This was tested in the case of the hDBP transgene at both the levels of mRNA expression and protein production. hDBP mRNA levels in the livers of mice from each of the five 105kb-hDBP lines were quantified by northern analyses. Levels of hDBP mRNA were normalized to endogenous mouse ribosomal protein L32 (mrpL32) mRNA as a loading control and divided by transgene copy numbers to generate an expression per transgene copy value (Figure 2B, autoradiograph). A tight correlation was observed among values from the five lines, lying within a 2-fold range. The correlation between transgene copy number and levels of gene expression was confirmed by analyses of serum hDBP protein levels. The serum hDBP levels/transgene copy number in adult mice from the five lines fell within a 3-fold range, ranging from 85–233 μg/ml/copy. Remarkably, the levels of serum hDBP in these 105kb-hDBP mouse lines, when corrected for transgene copy number, approximated the range of normal human serum DBP levels (116–232 μg/ml/copy) (1) (Figure 2B, graph). There was no evidence for sexual dimorphism of transgene expression (data not shown). These results demonstrated site-of-integration independent, copy number dependent and tissue-specific expression of the 105kb-hDBP transgene. This was consistent with the establishment of an autonomous chromatin domain by the transgene and furthermore was consistent with the presence of determinants that constitute a LCR (19,30,31).

Liver-specific DNase I hypersensitive sites (HS) are established at the 105kb-hDBP chromatin locus

The transgene expression studies (Figures 1 and 2) indicated that determinants sufficient for establishment of a fully productive hDBP locus in native hepatic chromatin are encompassed within the 105kb-hDBP transgene. To identify candidate regions that contain the relevant LCR determinants, DNase I mapping was performed on the 105kb-hDBP transgenic liver chromatin. Regulatory sequences involved in such LCR function commonly localize to DNase I HS within the chromatin of expressing tissues [reviewed in ref. (32,33)]. With this tissue specificity in mind, chromatin from the brain (negative control) of 105kb-hDBP transgenic mice was also mapped with DNase I. A mapping approach was designed to cover the entire gene and its adjacent flanking regions. A representative analysis of the 26 kb BglI fragment that spans the 5' terminus of the gene (from -17.4 to +8.6 kb) is shown (Figure 3A). A probe hybridizing to the 3' terminus of this fragment (probe P1) revealed two strong liver-specific DNase I HS and one weak brain-specific HS in the promoter region (Figure 3A). Higher resolution analyses of these HS were obtained by probing a 6.7 kb HindIII fragment spanning sequences -3.0 to +3.7 kb (probe P2) (Figure 3B). This study confirmed the presence of the two liver-specific HS (HSI at -2.1 kb and HSIII at -0.13 kb) as well as the two additional HS specific to the brain (HSII-1 at -1.8 kb and HSII-2 at -1.5 kb).

DNase I mapping was extended throughout the hDBP gene and its 3'-flanking region (Figure 4 and data not shown). These analyses detected four additional HS in liver: HSIV, HSV, HSVI and HSVII. HSVI and HSV are located within intron 1 (Figure 4A and B). The broader and stronger HSVI was composed of at least three sub-signals, located at map coordinates +10.3 kb to +10.9 kb. A weaker HSV was located at +12.2 kb. Two additional HS were observed at the 3' terminus of the gene; HSVII (+42.7 kb) coincident with the polyA site was present in both liver and brain and hence was not liver-specific. In contrast HSVII (+43.9 kb), located immediately 3' to HSVI, was liver-specific (Figure 4C). The full HS mapping strategy and a summary of the results are shown (Figure 4D).

HSI is located -2.1 kb relative to the site of hDBP transcriptional initiation. It is of note that the sequences corresponding to HSI as well as HSIII are highly conserved. Comparison of the sequences at HSI demonstrated over 80% identity between human and rodent [GenBank accession no. L10641, L10642 (human), MW_047424 (rat) and NT_039308 (mouse)]. Such conservation of non-coding sequences in genomic alignments is a reliable indicator of regulatory determinants (34–36). The proximity of HSI with HSIII suggested that the two might work in concert to coordinate hDBP promoter function. Genomic sequence alignments also revealed a remarkable conservation of non-coding sequences within intron 1. This conserved segment is coincident with the 1.9 kb region bracketed by HSIV and HSV (Figure 5, upper). Sequence comparisons of the human and rodent DBP loci demonstrated that HSVI and HSVII in the 3' terminus region also correspond to segments with over 70% sequence similarity (data not shown). Thus, a set of liver-specific HS was identified within the 105kb-hDBP transgene locus in hepatic chromatin and these sites are coincident with segments of conserved non-coding sequences in the 5'-flanking region, in intron 1, and in the 3'-flanking region. This concordance of HS mapping and localized segments of phylogenetically conserved non-coding sequences supports functional roles for these regions in hDBP expression.

Generation of 51kb-hDBP transgenic mouse lines

Determinants involved in chromatin organization and LCR activity are characteristically located quite distant from a target gene. The HS identified in the preceding studies (Figures 3 and 4) are in close proximity to, or within, the hDBP transcription unit. To test whether these gene-proximal HS are sufficient for hDBP activation, a second set of mouse lines was generated using a transgene with minimal flanking sequences. This 51 kb genomic fragment, excised with NotI and FspI from the 123 kb human genomic PAC clone (PAC 45P24), encompassed the entire hDBP gene along with only 2.5 kb of 5'-flanking and 6.5 kb of 3'-flanking region (51kb-hDBP transgene; Figure 6A and B). Four transgenic founders were generated. Transgene integrity and copy numbers were determined for each line (Figure 6C).

Liver-specific copy number independent expression of the 51kb-hDBP transgene

High-level hDBP mRNA expression was detected in the livers of all four 51kb-hDBP transgenic lines (Figure 7A autoradiograph and data not shown). hDBP mRNA was also detected by
northern blot analysis at trace levels in the kidneys of two of the four 51kb-hDBP lines (0.05 and 0.04% of expression levels in livers, respectively); in the remaining two 51kb-hDBP lines expression in the kidney could not be detected (Figure 7A table and data not shown). Serum hDBP levels in the four 51kb-hDBP lines varied by less than 3-fold (80–189 mg/ml/transgene copy), indicating copy number dependent expression (Figure 7B). These levels were remarkably similar to those observed in the sera of 105kb-hDBP transgenic mice and similarly approximate the levels of expression of the native hDBP gene (1) (Figure 2B). These data demonstrated that the 51kb-hDBP transgene, containing only 2.5 kb of 5′-flanking region and 6.5 kb of 3′-flanking region, was able to overcome site-of-integration position effects and direct high levels of liver-specific hDBP gene expression from multiple random integration sites in transgenic mouse chromatin. In addition, we note a selective loss of hDBP transgene expression in the kidneys of the 51kb-hDBP mice when compared with the 105kb-hDBP transgene, suggesting that expression in the kidney may rely on a distinct set of determinants.

DISCUSSION

In the present report we have tested for autonomous regulation of the hDBP gene and have sought sites in the native hepatic chromatin that might encompass relevant regulatory elements. Many transgenes are not consistently or accurately expressed in the mouse due to the exclusion of determinants that are necessary to re-establish the native chromatin environment. Thus these transgenes are sensitive to the major influences, both positive and negative, at the foreign site-of-integration in the host (mouse) genome. An LCR is defined by its ability to re-establish a chromatin environment that is dominant over site-of-integration effects. Thus the LCR action allows the transgene to be expressed at a consistent level when compared among multiple lines, each representing a unique insertion site. The initial approach in our study was to introduce the hDBP gene with extensive flanking sequences into the mouse genome and determine whether it could establish an appropriate pattern and level of gene expression. The results clearly demonstrated that this was the case. The pattern of expression of the hDBP transgene encompassing 37 kb of 5′-flanking

![Figure 3](image-url)
Figure 4. Two sets of HS are formed within intron 1 and 3' of hDBP gene. (A) Mapping strategy. The BglII (B) and FspI (F) restriction map of the 105kb-hDBP transgene (positions -37 through +68 kb) indicates the locations of the probes (P3 and P6) used to map HS throughout the gene and into the 3'-flanking region. (B) Mapping the HS in intron 1. A 6 kb BglII fragment of intron 1 was mapped using probe P3 that is itself located in intron 1. Two HS were identified in liver chromatin, the more intense and broad HSIV at position +10.3 to +10.9 kb and HSV at position +12.2 kb (positions are relative to the transcription initiation site). These sites are placed on the map below the autoradiograph. Exon 2 (E2) is shown in this map as a filled rectangle. (C) Mapping the hDBP gene 3'-flanking region. Probe P6 was used to map a 9.0 kb BglII–FspI fragment spanning terminal exon 13 and the 3'-flanking region of hDBP gene. The map below the autoradiograph shows the location of probe P6 used to map HS within the 3' flanking region. HSVI, common to both liver and brain, and liver-specific HSVII were detected. Their positions are placed on the map below the autoradiograph. (D) Summary of DNase I mapping the 105kb-hDBP transgene. The results of the DNase I mapping of the entire 105kb-hDBP transgene (Figures 3 and 4 and data not shown) are summarized. The positions of probes used in the mapping and the specific restriction fragments mapped are indicated on the top diagram (P1–P6; probes, B; BglII, F; FspI, H; HindIII). The positions of each HS are shown in the lower map that also shows positions of the 13 exons (numbered black rectangles) and 12 introns of hDBP gene. Vertical arrows indicate the DNase I HS identified (liver, brain).
sequences and 26 kb of 3'-flanking sequences (105 kb-hDBP transgene) paralleled that of the endogenous DBP locus. This result represents the analysis of five independent lines. Not only was the robust expression primarily restricted to the liver, but the major secondary site of DBP expression in the kidney was also preserved (Figure 2A). Of particular note, the level of transgene expression, when monitored at the protein level and normalized to transgene copy number, was remarkably
From these data we concluded that the determinants sufficient to establish an autonomous and fully active chromatin locus capable of supporting full and accurate hDBP expression are within and/or closely linked to the hDBP gene. These determinants fulfill the operational definition of an LCR.

The second phase of the study was to map the determinants involved in the activation of the hDBP gene in hepatic chromatin. LCR determinants commonly map to sites of DNase I hypersensitivity (HS) in the chromatin of expressing cells. These HS reflect the localized displacement of histones or perturbation in chromatin structure by trans-acting factors binding at the site (37,38). Core elements that have been defined at individual HS are usually composed of arrays of multiple tissue-specific and ubiquitous transcription factor-binding sites [reviewed in ref. (32,33)]. With this in mind, the 105kb-hDBP transgene locus was mapped for HS in hepatic tissue as well as in brain, a non-expressing tissue. A set of five liver-specific HS was identified (Figure 4D). HSI and HSIII are located at −2.1 and −0.13 kb relative to the hDBP promoter, respectively, whereas HSIV and HSV are closely juxtaposed within a 1.9 kb region of intron 1 (10.3–10.9 kb and 12.2 kb, respectively). HSVII maps to a site 1.5 kb 3′ to the poly(A) addition site. HSVI, although detected in liver, was also present in brain chromatin and thus not tissue-specific. To support the sufficiency of these elements to activate of hDBP, we assessed the expression of a second transgene with minimal flanking sequences just long enough to encompass the full set of identified HS. High-level, copy number dependent and liver-specific expression of this shorter 51kb-hDBP transgene was observed in all four 51kb-hDBP transgenic lines. These results suggested that all determinants necessary for the establishment of a fully functional chromatin locus are present within the 51kb-hDBP transgene. These data support a model in which the set of determinants identified by the HS mapping constitute a liver-specific hDBP LCR and are sufficient for hDBP activation in the transgenic setting.

The exact structure and mode of action of the determinants at each of the HS remains to be established. Our prior assays in cell transfection systems had identified three functional HNF1-binding sites within the proximal 2 kb of the rat DBP gene (rDBP) 5′-flanking region (39). The region from −65 to −41 bp, the ‘A segment’, is the most proximal HNF1-binding site. Two more distal HNF1-binding sites, located in segments ‘B’ and ‘F-2’ (coordinates at −254 to −140 bp and −1844 to −1621 kb, respectively), function as classical enhancer elements. The B and F-2 segments both increase the activity of homologous and heterologous promoters when assayed in transfected HepG2 liver-derived cells. HNF1-α played a predominant role in the observed enhancer function. At the B and F-2 sites the HNF1β protein showed a trans-dominant negative effect on HNF1α-stimulated activity of the rDBP promoter suggesting that the relative levels of HNF1α and HNF1β in various tissues might contribute, in part, to tissue-specific expression of DBP in vivo (39). HSIII, detected in the present study, is located between the two HNF1-binding sites corresponding to the A and B binding sites described in the rDBP proximal promoter. This suggests that the formation of HSIII reflects local promoter complex assembly. HSIII is co-located to a highly conserved region among human, mouse and rat (Figure 5). The

consistent and similar to that of DBP levels in human serum (Figure 2B). Thus, the 105kb-hDBP transgene was expressed in a site-of-insertion independent and copy number dependent manner, at full levels, and with appropriate tissue specificity.
detection of HSIII in this region supports the previous conclusion that the two HNF1-binding sites in the proximal promoter are critical elements for hDBP activation. A comparison of the current studies with previous analyses of ALB and AFP genes highlights points of potential interest. Consistent with other numerous model systems, transgenic studies at the mouse ALB locus have revealed that the promoter region is insufficient for robust and consistent transgene expression. These studies identified a transcriptional enhancer located 10 kb upstream of the transcription start site that is essential for full liver-specific expression (40). This element co-maps with a strong liver-specific HS (40,41) and in vitro studies have demonstrated that this enhancer can trigger the activation of a compacted chromatin template (42). Analysis of the mouse AFP gene has identified three enhancer elements at −2.5, −5.0 and −6.5 kb relative to the promoter (43,44). All three sites were required to achieve high-level expression of ALB in fetal liver (45). In contrast to these findings for the ALB and AFP genes, the current report reveals that the hDBP transgene is robustly activated in vivo in the absence of remote flanking determinants. These data along with the HS mapping raise the possibility that HSIV, possibly in concert with the substantially weaker HSV, mediates enhancer functions comparable to the distal enhancers far upstream of ALB or AFP promoter. In light of other studies in the literature, the intronic location of HSIV and HSV is fully compatible with their functioning as dominant locus control element(s) (46–51). The broad and multipartite structure of HSIV is consistent with a fully autonomous chromatin domain are located in close proximity to, and within the hDBP gene. The combination of transgenic studies, HS mapping and phylogenetic sequence comparisons establish a model in which HSI, HSIII, HSIV, HSV and HS VII constitute components of a liver-specific LCR. How each element contributes to this activity can now be further explored. These data further establish a functional independence of hDBP from the other members of its cluster. This suggests that critical elements that trigger the hepatic expression of these genes have duplicated during evolution and are represented at multiple sites within the cluster. It remains possible, that at some higher level of nuclear organization, these elements may interact to facilitate and coordinate their respective developmental profiles.

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