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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, α-fetoprotein and α-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 noncoding 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 μg/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*), *α-fetoprotein* (*AFP*) and *α-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′-5′-*ALB*-3′-5′-*AFP*-3′-5′-*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. The distance between the *rDBP* gene and the remaining family members is also quite large (1.0 Mb) in rat (GenBank accession no. NW_047424). These data demonstrate that the organization, transcriptional orientation and expression patterns of the *DBP* multi-gene clusters in rat and human are highly conserved. Whereas the close juxtaposition of *ALB*, *AFP* and *AFM* is consistent with the presence of common transcriptional determinants, the substantial separation of *DBP* from the other members suggests an alternative model of autonomous control.

In the present report we test for autonomy of the *DBP* locus using two sets of transgenic mouse models. We further begin to explore its mechanistic basis by probing hepatic chromatin by DNase I mapping for sites of potential transfactor interactions. These studies indicate that determinants sufficient for establishment of an autonomous hepatic chromatin environment that supports robust *hDBP* expression *in vivo* are located in close proximity to, and within, the *hDBP* gene itself. In our transgenic setting, these locus control determinants are responsible for copy number dependent, site-of-integration independent, and robust tissue-specific expression of the *hDBP* locus. 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-^{32}P]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 GENECLEAN 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 *mζ-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-^{32}P] 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 electroelution. 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

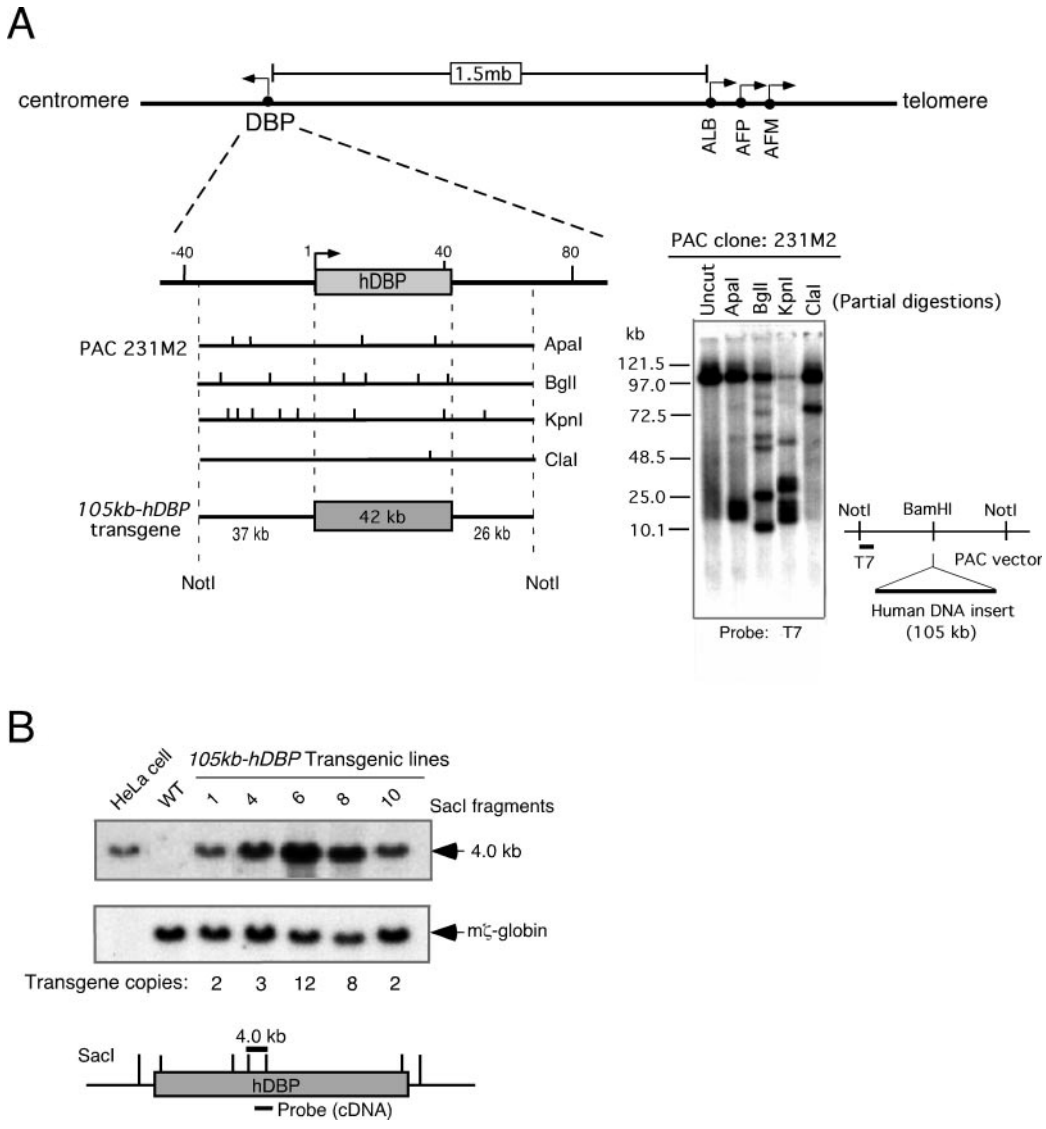


Figure 1. Generation of transgenic mouse lines carrying the *105kb-hDBP* transgene. (**A**) Isolation and characterization of the *105kb-hDBP* transgene. The arrangement of the human *DBP*, *ALB*, *AFP* and *AFM* genes and their transcriptional orientations (bent arrows) are shown at the top. Clones were isolated from a human PAC library by PCR with a series of *hDBP* probes and the insert ends were sequenced using T7 and SP6 primers (25). PAC clone 231M2 encompasses a 105 kb human genomic fragment containing the entire *42kb-hDBP* gene with extended 5′- and 3′-flanking regions. Representative partial digestion patterns of 231M2 using four restriction enzymes indirectly end-labeled with a T7 probe are shown in the autoradiograph to the right. The deduced restriction map of 231M2 and the position of *hDBP* within the 231M2 fragment derived from this analysis are summarized at the left. (**B**) Mapping and copy number determination in five independent *105kb-hDBP* transgenic lines. The *105kb-hDBP* transgene was released from PAC clone 231M2 by digestion with NotI (A), purified, and microinjected to generate transgenic founders. A series of five transgenic mouse lines were obtained. The transgene copy numbers were determined in the F1 generation of each line by Southern blot analysis. Tail genomic DNA was digested with SacI and hybridized with an *hDBP* cDNA probe (303 bp EcoRI fragment). Genomic DNA from HeLa cells and the tail of a wild-type mouse were used as positive and negative controls, respectively. The lower diagram shows the 4.0 kb SacI fragment expected to hybridize to the probe, as well as the location of this cDNA probe. The transgene-specific bands (4.0 kb, indicated by arrow) were quantified by phosphorimaging. Transgene copy numbers were estimated by normalizing these signals to those from human genomic DNA (HeLa cell; two endogenous copies). The mouse ζ-*globin* was used as the DNA loading control. The deduced transgene copy numbers in the five lines ranged from 2 to 12 and are indicated below the corresponding lanes.

fragment. 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 μg of restriction enzyme-digested mouse tail DNA was analyzed on 0.8% agarose gels, transferred to Zetabind 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 μg/ml denatured salmon sperm DNA] at 65°C, hybridized at 65°C with ^{32}P-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 phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Table 1. Oligonucleotide primers and probes

Probe	Primer sequence
P1	5′-TGGGGCACAATGGAATAGTTTG-3′, 5′-CCGAGATGCTGAAACAACCTAAGTG-3′
P2	5′-AGAAGACTGTCGCAGCATTAAAGG-3′, 5′-CAGATGGGGAAAAAATGGGTG-3′
P3	5′-CGCCTGAGTCTTTCAACCAATCTG-3′, 5′-CCCTGCATTTAATTTGGACGC-3′
P4	5′-CATCTGGGAAAGGAGGACTTCAC-3′, 5′-CTTAGGGCTTCCATCAAACCAATC-3′
P5	5′-ACTCCAACCTGGGTGACAGAGG-3′, 5′-GCTTTGCGAAACAATGAGGATG-3′
P6	5′-GCTTGAGAGTTTACCCAATTTCCA-3′, 5′-ACCTGATTATCAATGACTGAGATC-3′
pI	5′-GAATCACAGTAAGCAATCAA-3′, 5′-CTTTATCTCTGCTTTTGCAA-3′
pIII	5′-TCCCACTAAACAGATCTCAGTCAT-3′, 5′-GCAAGGACTTCATGTCTGAAACAG-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 membrane with 10× SSC. After UV cross-linking, the blots were prehybridized, and subsequently hybridized with ^{32}P-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) (2–4 μl), 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), 15 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 $MgCl_2$, 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 $MgCl_2$, 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 A_{260}/A_{280}. 500 μg of liver nuclei were suspended in buffer D with 5 mM $MgCl_2$ 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 $CaCl_2$ 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*)

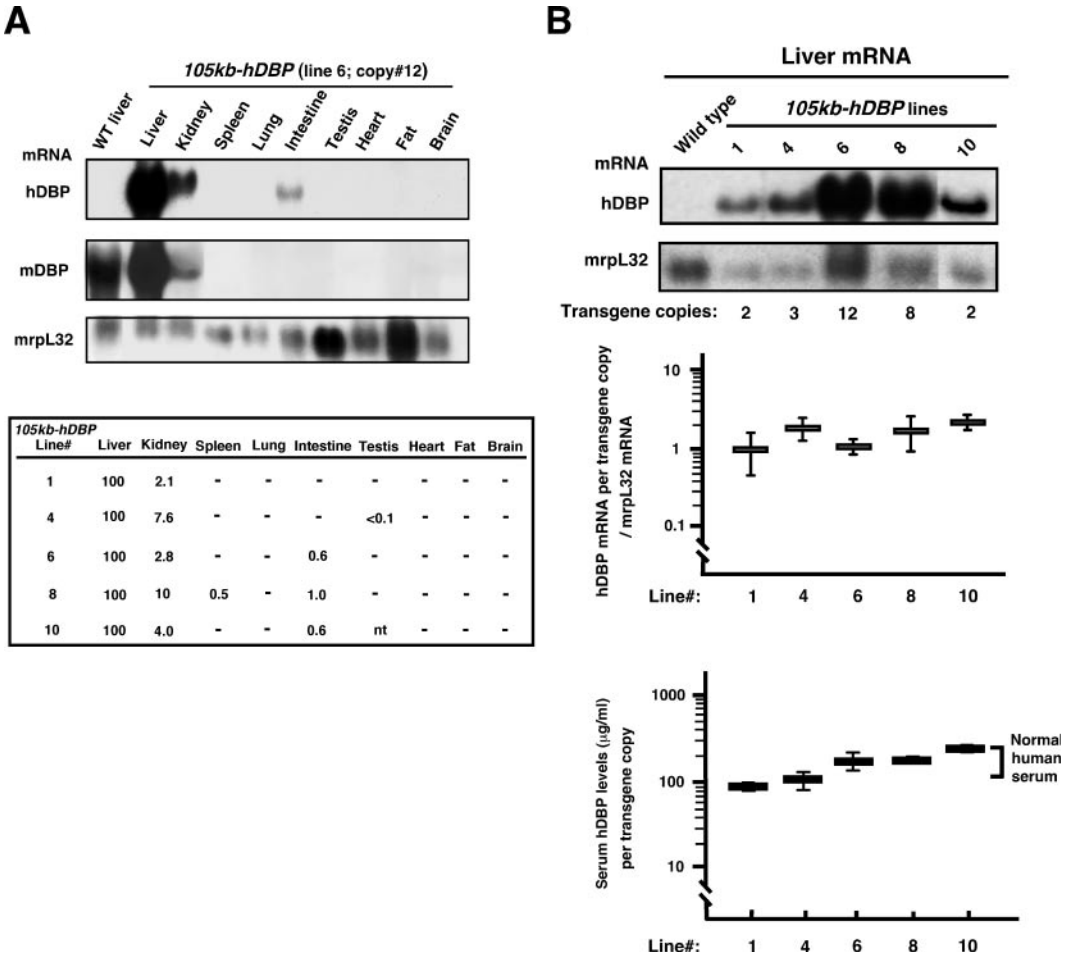
In order to assess the ability of the isolated *hDBP* gene to establish an autonomous hepatic chromatin environment capable of supporting consistent and robust expression, a set of transgenic mouse lines were generated. To optimize the possibility of including all necessary regulatory elements, a large 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 were isolated from a PAC-recombinant library (25). Among them, PAC clone 231M2 carried a 105 kb human genomic fragment encompassing 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



105kb-hDBP Line#	Liver	Kidney	Spleen	Lung	Intestine	Testis	Heart	Fat	Brain
1	100	2.1	-	-	-	-	-	-	-
4	100	7.6	-	-	-	<0.1	-	-	-
6	100	2.8	-	-	0.6	-	-	-	-
8	100	10	0.5	-	1.0	-	-	-	-
10	100	4.0	-	-	0.6	nt	-	-	-

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. HSIV and HSV are located within intron 1 (Figure 4A and B). The broader and stronger HSIV 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; HSVI (+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)], (Figure 5, upper). 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, lower). 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 at 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 and copy number dependent 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

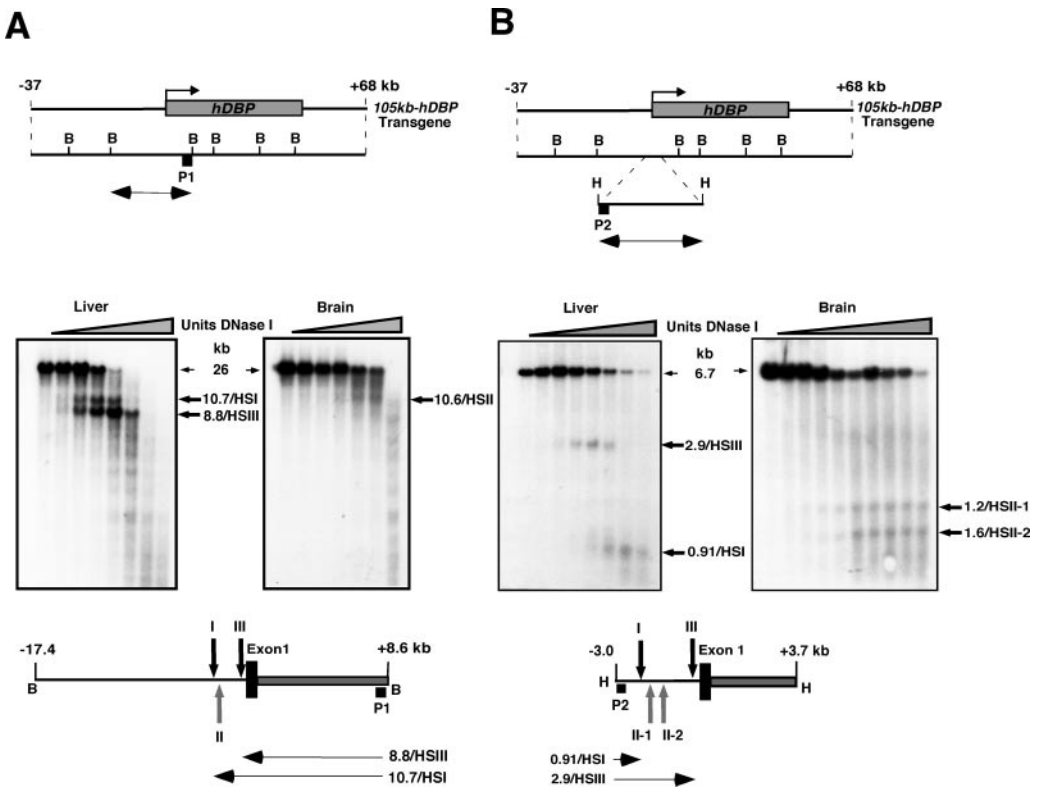


Figure 3. Two liver-specific DNase I HS are present 5′ to the *hDBP* gene. (**A**) Mapping the 5′-flanking DNase I HS using a downstream probe. A BglI (**B**) restriction map of the *hDBP* gene and flanking regions from position −37 to +68 kb is shown at the top. The position of hybridization probe P1 is labeled and the 26 kb BglI fragment subjected to mapping is indicated (horizontal double-headed arrow). DNase I HS mapping was carried out on chromatin isolated from *105kb-hDBP* liver and brain (negative control), left and right autoradiographs, respectively. Aliquots of chromatin were subjected to increasing amounts of DNase I (wedges). Genomic DNA from these aliquots were digested to completion with BglI and subjected to Southern analysis. The 26 kb BglI fragment spanning the 5′-flanking region, exon 1 and part of intron 1 was mapped by hybridization to probe P1 (see Table 1). Two prominent liver-specific DNase I HS (HSI and HSIII) were identified near the *hDBP*-proximal promoter region. A single weak HS (HSII) was identified only in the control brain sample. The results are summarized on the map under the autoradiographs. Vertical arrows indicate the positions of the DNase I HS (↓; liver, ↑; brain). The horizontal arrows below the map indicate fragment sizes (kb) and their designations. (**B**) Refined mapping of the 5′-flanking DNase I HS using an upstream probe. The DNase I mapping results in (A) were confirmed using a probe located upstream of the sites and at a higher level of resolution. The map at the top shows the 6.7 kb HindIII (H) fragment that encompasses the identified DNase I sites and the location of probe P2 (Table 1). The Southern analysis of liver chromatin samples as in (A) confirmed the presence of the two liver-specific DNase I HS (HSI and HSIII). In the analysis of this shorter fragment, the brain-specific site was resolved into two closely linked HS (designated HSII-1 and HSII-2). The results of the refined mapping are summarized on the map under the autoradiograph, as in (A).

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 μg/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

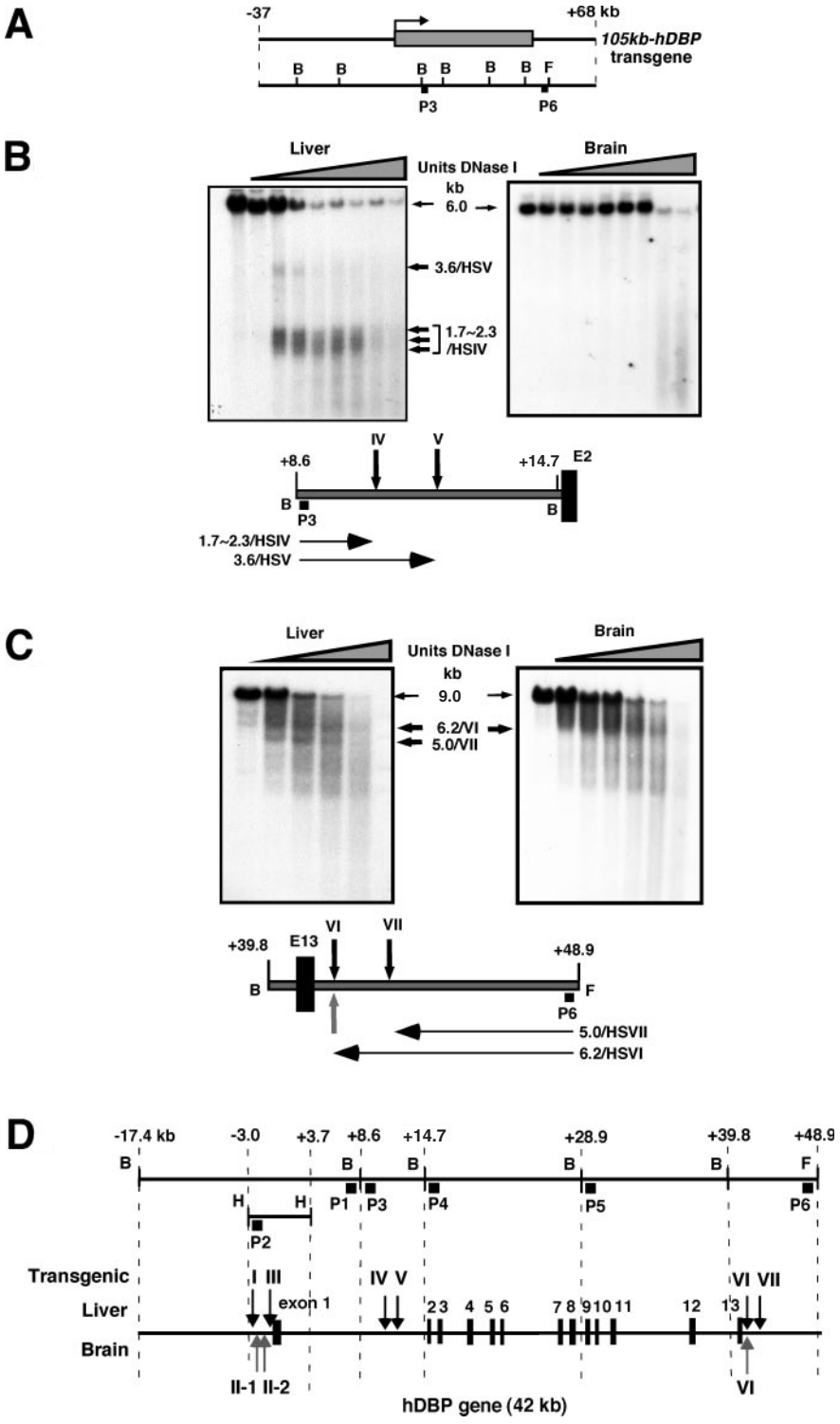


Figure 4. Two sets of HS are formed within intron 1 and 3′ of *hDBP* gene. (**A**) Mapping strategy. The BglI (B) and FspI (F) restriction map of the *105kb-hDBP* transgene (positions −37 through +68 kb) indicates the locations of the probes (P3 and P6) used to map HS throughout the gene and into the 3′-flanking region. (**B**) Mapping the HS in intron 1. A 6 kb BglI 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 BglI–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; BglI, 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).

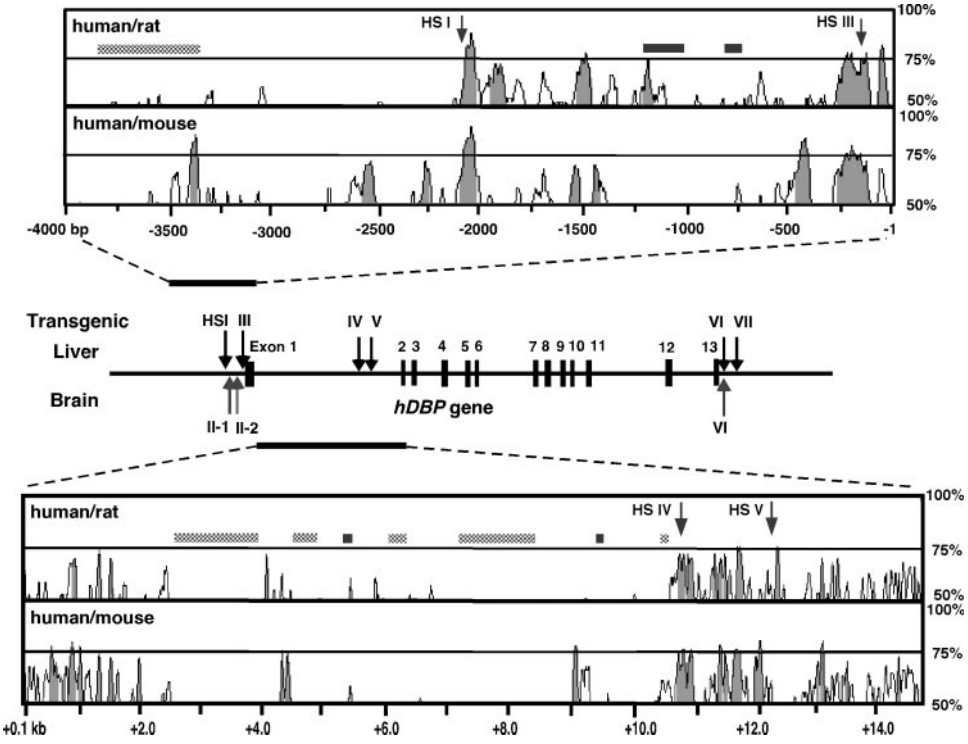


Figure 5. Comparisons between human and rodent genomic sequences in the vicinity of the DBP locus. A map of the *hDBP* gene is displayed in the center of the figure showing the exon-intron arrangement and location of the HS in liver (downward arrows) and brain (upward arrows). An expanded view of the sequence comparisons (human compared to rodent) in the 5′-flanking region (upper panel) and intron 1 (lower panel) are shown. The numbering on the *x*-axis refers to the position in the human sequence. The level of similarity in sequence is displayed on the *y*-axis as a percentage (mVISTA program; window size 50 bp, homology threshold 70%). The 75% level of sequence similarity is marked by a horizontal line and regions of the sequence representing repeated elements are indicated by the shaded rectangles, LINE elements (stippled) and SINE (solid). The positions of liver-specific HSI, HSIII, HSIV and HSV sequence comparisons are each indicated by the downward arrows. Note the peaks of sequence identity at each of the indicated DNase I HS.

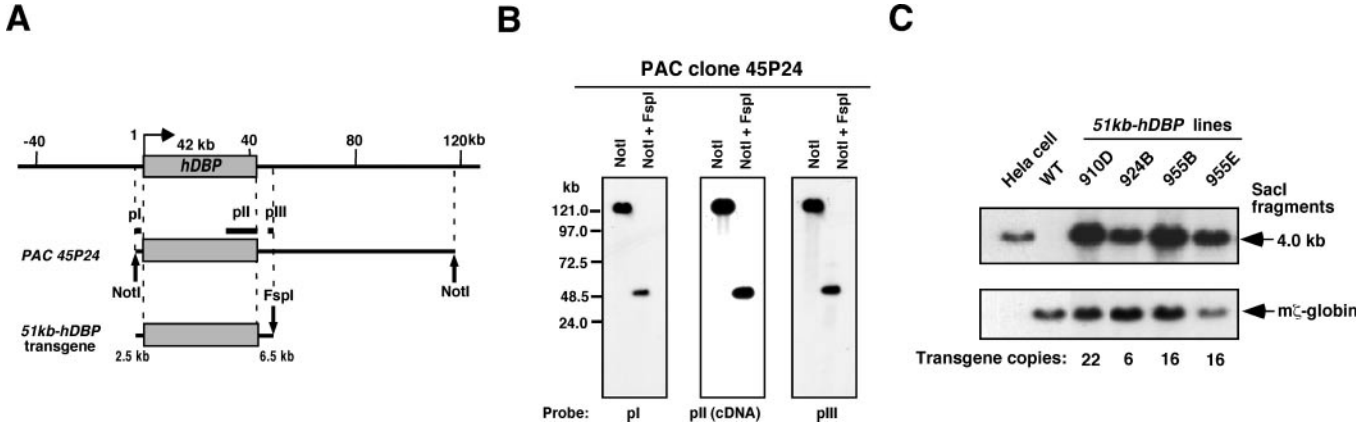
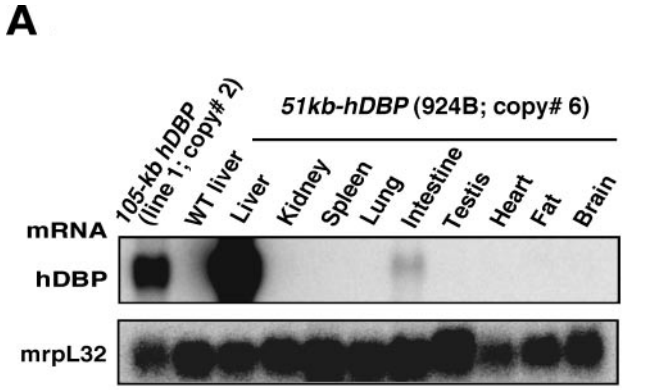


Figure 6. Generation of transgenic mouse lines using the *51kb-hDBP* transgene. (**A**) Map of the *51kb-hDBP* transgene. PAC clone 45P24 (25) was digested with NotI and FspI to generate the *51kb-hDBP* transgene. This 51 kb transgene encompasses the *hDBP* gene with 2.5 kb of 5′-flanking and 6.5 kb 3′-flanking regions, including all of the liver-specific DNase I HS detected. Positions of probes used to confirm the structure of the transgene are indicated (pI, pII, derived from the *hDBP* cDNA, and pIII; Table 1) (**B**) Southern blot analysis of PAC clone 45P24 confirmed the structure of the 51 kb fragment. Insert DNA was released with NotI digestion from the PAC vector and the 51 kb fragment was obtained with NotI and FspI double-digestions. Southern blots of these digestions were probed with radiolabeled pI, pII or pIII (A). A single 51 kb band was detected with each probe as expected. (**C**) Analysis of *51kb-hDBP* transgenic lines and copy number determinations. Four *51kb-hDBP* mouse lines were generated (910D, 924B, 955B and 955E). The transgene copy numbers were determined in F1 or F2 generations of each line by SacI digestion and Southern blot analysis as described (Figure 1B). The copy numbers determined (ranging from 6 to 22) are indicated below the corresponding lines.

sequences and 26 kb of 3′-flanking sequences (*105kb-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



51kb-hDBP Line#	Liver	Kidney	Spleen	Lung	Intestine	Testes	Heart	Fat	Brain
910D	100	0.05	-	-	0.2	-	-	-	-
924B	100	-	-	-	0.1	-	-	-	-
955B	100	-	-	-	0.06	-	-	-	-
955E	100	0.04	-	-	-	-	-	-	-

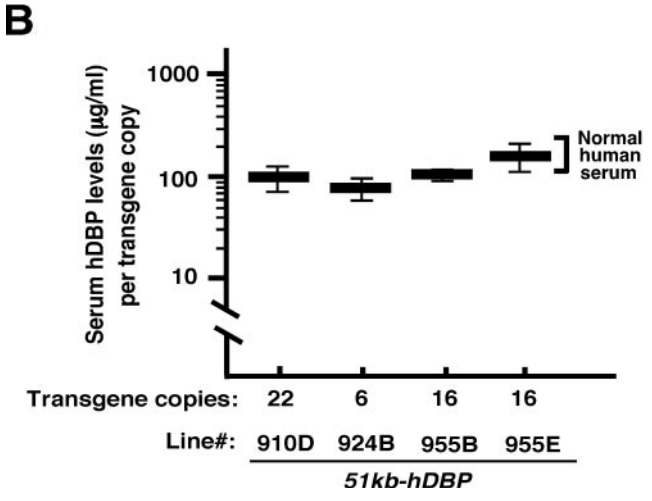


Figure 7. *51kb-hDBP* transgene expression is liver-specific and copy number dependent. (**A**) Tissue specificity of *51kb-hDBP* transgene expression. A Northern blot tissue survey from a representative *51kb-hDBP* transgenic mouse line (line 924B, transgene copy number 6) is shown. The probes used are indicated on the left of the autoradiograph. High-level liver-specific expression of the *51kb-hDBP* transgene was observed. The table summarizes the relative quantification of transgene expression in various tissues of an adult male mouse from each of the four *51kb-hDBP* lines; the expression of *hDBP* mRNA in the liver is defined as 100 in each line and expression in other tissues is related to this value. (**B**) Copy number dependency of *51kb-hDBP* expression. Serum hDBP level *per* transgene copy of each *51kb-hDBP* line was quantified. Means and SD were calculated from serum hDBP levels of at least eight mice in each line. Consistent and copy number dependent serum hDBP levels were observed. These levels were concordant with the levels in the *105kb-hDBP* lines carrying the more extensive *hDBP* gene flanking regions.

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. 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

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 multiple arrays of transcription factor-binding sites, a composition common among LCR elements (32,33).

In addition to its expression in the liver, human and rodent *DBPs* are also expressed at substantially lower levels in several secondary sites. The most prominent and well described of these is in the kidney. The local expression of *DBP* in the kidney may be important in vitamin D recycling via interactions with the renal scavenger protein megalin (52–54). While the transcriptional initiation site for *hDBP* in the two tissues appears to be identical (4), it is likely that the complex of determinants driving expression in this secondary site differ from those in the liver. The *105kb-hDBP* transgene paralleled the endogenous *mDBP* with respect to kidney expression; *hDBP* expression was observed at considerable levels (∼2–10% of liver) in the kidneys of all five *105kb-hDBP* lines. In contrast, kidney expression of the more truncated *51kb-hDBP* transgene was sporadic while liver expression remained robust and consistent. We have generated a single transgenic mouse line carrying a 123 kb insert of PAC clone *45P24* (Figure 6A). This transgene has the same 5′ terminus as the *51kb-hDBP* at −2.5 kb as well as a more extensive 78 kb 3′-flanking region. The tissue distribution of *hDBP* expression from this transgene by Northern blots showed robust liver expression and an absence of expression in the kidney (data not shown). These results suggest that one or more important elements for kidney *hDBP* expression are located in the region between +37 kb and +2.5 kb upstream from the transcription start site. Whether these elements will constitute a kidney-specific element of the *hDBP* LCR remains to be determined.

In conclusion, the present study demonstrates that elements critical to hepatic activation of the *hDBP* gene and establishment of 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 HSVII 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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REFERENCES

1. Cooke,N.E. and Haddad,J.G. (1997) Vitamin D-binding protein. In Feldman,D., Glorieux,F.H. and Pike,J.W. (eds), *Vitamin D*. Academic Press, San Diego, CA, pp. 87–101.
2. Cooke,N.E. and Haddad,J.G. (1995) Vitamin D-binding protein (Gc-globulin). *Endocr. Rev.*, **4**, 125–128.
3. White,P. and Cooke,N.E. (2000) The multifunctional properties and characteristics of vitamin D-binding protein. *Trends Endocrinol. Metab.*, **11**, 320–327.
4. McLeod,J.M. and Cooke,N.E. (1989) The vitamin D-binding protein, alpha-fetoprotein, albumin multigene family: detection of transcripts in multiple tissues. *J. Biol. Chem.*, **264**, 21760–21769.
5. Yang,F., Brune,J.L., Naylor,S.L., Cupples,R.L., Naberhaus,K.H. and Bowman,B.H. (1985) Human group-specific component (Gc) is a member of the albumin family. *Proc. Natl Acad. Sci. USA*, **82**, 7994–7998.
6. Cooke,N.E. (1986) Rat vitamin D binding protein. Determination of the full-length primary structure from cloned cDNA. *J. Biol. Chem.*, **261**, 3441–3450.
7. Cooke,N.E., Willard,H.F., David,E.V. and George,D.L. (1986) Direct regional assignment of the gene for vitamin D binding protein (Gc-globulin) to human chromosome 4q11–q13 and identification of an associated DNA polymorphism. *Hum. Genet.*, **73**, 225–229.
8. Yang,F., Bergeron,J.M., Linehan,L.A., Lalley,P.A., Sakaguchi,A.Y. and Bowman,B.H. (1990) Mapping and conservation of the group-specific component gene in mouse. *Genomics*, **7**, 509–516.
9. Belanger,L., Roy,S. and Allard,D. (1994) New albumin gene 3′ adjacent to the alpha 1-fetoprotein locus. *J. Biol. Chem.*, **269**, 5481–5484.
10. Lichenstein,H.S., Lyons,D.E., Wurfel,M.M., Johnson,D.A., McGinly,M.D., Leidli,J.C., Trollinger,D.B., Mayer,J.P., Wright,S.D. and Zukowski,M.M. (1994) Afamin is a new member of the albumin, alpha-fetoprotein, and vitamin D-binding protein gene family. *J. Biol. Chem.*, **269**, 18149–18154.
11. Haefliger,D.N., Moskaitis,J.E., Schoenberg,D.R. and Wahli,W. (1989) Amphibian albumins as members of the albumin, alpha-fetoprotein, vitamin D-binding protein multigene family. *J. Mol. Evol.*, **29**, 344–354.

12. Ray,K., Wang,X., Zhao,M. and Cooke,N.E. (1991) The rat vitamin D binding protein (Gc-globulin) gene. Structural analysis, functional and evolutionary correlations. *J. Biol. Chem.*, **266**, 6221–6229.
13. Witke,W.F., Gibbs,P.E., Zielinski,R., Yang,F., Bowman,B.H. and Dugaiczyk,A. (1993) Complete structure of the human Gc gene: differences and similarities between members of albumin gene family. *Genomics*, **16**, 751–754.
14. Muglia,L. and Locker,J. (1984) Developmental regulation of albumin and alpha-fetoprotein gene expression in the rat. *Nucleic Acids Res.*, **12**, 6751–6762.
15. Wen,P., Groupp,E.R., Buzard,G., Crawford,N. and Locker,J. (1991) Enhancer, repressor, and promoter specificities combine to regulate the rat α-fetoprotein gene. *DNA Cell. Biol.*, **10**, 525–536.
16. Vacher,J., Camper,S.A., Krumlauf,R., Compton,R.S. and Tilghman,S.M. (1992) *raf* regulates the postnatal repression of the mouse α-fetoprotein gene at the posttranscriptional level. *Mol. Cell. Biol.*, **12**, 856–864.
17. Jin,J.R., Wen,P. and Locker,J. (1995) Enhancer sharing in a plasmid model containing the α-fetoprotein and albumin promoters. *DNA Cell. Biol.*, **14**, 267–272.
18. Vorachek,W.R., Steppan,C.M., Lima,M., Black,H., Bhattacharya,R., Wen,P., Kajiyama,Y. and Locker,J. (2000) Distant enhancers stimulate the albumin promoter through complex proximal binding sites. *J. Biol. Chem.*, **275**, 29031–29041.
19. Grosveld,F., van Assendelft,G.B., Greaves,D.R. and Kollias,G. (1987) Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell*, **51**, 975–985.
20. Moon,A.M. and Ley,T.J. (1990) Conservation of the primary structure, organization, and function of the human and mouse β-globin locus-activating regions. *Proc. Natl Acad. Sci. USA*, **87**, 7693–7697.
21. Jones,B.K., Monks,B.R., Liebhaber,S.A. and Cooke,N.E. (1995) The human growth hormone gene is regulated by a multicomponent locus control region. *Mol. Cell. Biol.*, **15**, 7010–7021.
22. De Laat,W. and Grosveld,F. (2003) Spatial organization of gene expression: the active chromatin hub. *Chromosome Res.*, **11**, 447–459.
23. Lettice,L.A., Heaney,S.J., Purdie,L.A., Li,L., de Beer,P., Oostra,B.A., Goode,D., Elgar,G., Hill,R.E. and de Graaff,E. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum. Mol. Genet.*, **12**, 1725–1735.
24. Kleinjan,D.A. and van Heyningen,V. (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am. J. Hum. Genet.*, **76**, 8–32.
25. Song,Y.-H., Naumova,A.K., Liebhaber,S.A. and Cooke,N.E. (1999) Physical and meiotic mapping of the region of human chromosome 4q11-q13 encompassing the vitamin D binding protein DBP/Gc-globulin and albumin multigene cluster. *Genome Res.*, **9**, 581–587.
26. Cooke,N.E. and David,E.V. (1985) Serum vitamin D-binding protein is a third member of the albumin and α-fetoprotein gene family. *J. Clin. Invest.*, **76**, 2420–2424.
27. Liebhaber,S.A., Wang,Z., Cash,F.E., Monks,B. and Russell,J.E. (1996) Developmental silencing of the embryonic zeta-globin gene: concerted action of the promoter and the 3′-flanking region combined with stage-specific silencing by the transcribed segment. *Mol. Cell. Biol.*, **16**, 2637–2646.
28. Meyuhas,O. and Perry,R.P. (1980) Construction and identification of cDNA clones for mouse ribosomal proteins: application for the study of r-protein gene expression. *Gene*, **10**, 113–129.
29. Gorski,K., Carneiro,M. and Schibler,U. (1986) Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell*, **47**, 767–776.
30. Festenstein,R., Tolaini,M., Corbella,P., Mamalaki,C., Parrington,J., Fox,M., Miliou,A., Jones,M. and Kioussis,D. (1996) Locus control region function and heterochromatin-induced position effect variegation. *Science*, **271**, 1123–1125.
31. Fraser,P. and Grosveld,F. (1998) Locus control regions, chromatin activation and transcription. *Curr. Opin. Cell Biol.*, **10**, 361–365.
32. Li,Q., Harju,S. and Peterson,K.R. (1999) Locus control regions. *Trends Genet.*, **15**, 403–408.
33. Li,Q., Peterson,K.R., Fang,X. and Stamatoyannopoulos,G. (2002) Locus control regions. *Blood*, **100**, 3077–3086.
34. Pennacchio,L.A. and Rubin,E.M. (2001) Genomic strategies to identify mammalian regulatory sequences. *Nature Rev. Genet.*, **2**, 100–109.
35. Pennacchio,L.A. and Rubin,E.M. (2003) Comparative genomic tools and databases: providing insights into the human genome. *J. Clin. Invest.*, **111**, 1099–1106.
36. Vavouri,T., McEwen,G.K., Woolfe,A., Gilks,W.R. and Elgar,G. (2006) Defining a genomic radius for long-range enhancer action: duplicated conserved non-coding elements hold the key. *Trends Genet.*, **22**, 5–10.
37. Walter,P.P., Owen-Hughes,T.A., Cote,J. and Workman,J.L. (1995) Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol. Cell. Biol.*, **15**, 6178–6187.
38. Boyes,J., Omichinski,J., Clark,D., Pikaart,M. and Felsenfeld,G. (1998) Perturbation of nucleosome structure by the erythroid transcription factor GATA-1. *J. Mol. Biol.*, **279**, 529–544.
39. Song,Y.-H., Ray,K., Liebhaber,S.A. and Cooke,N.E. (1998) Vitamin D-binding protein gene transcription is regulated by the relative abundance of hepatocyte nuclear factors1α and 1β. *J. Biol. Chem.*, **273**, 28408–28418.
40. Pinkert,C.A., Ornitz,D.M., Brinster,R.L. and Palmiter,R.D. (1987) An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. *Genes Dev.*, **1**, 268–276.
41. Liu,J.K., Bergman,Y. and Zaret,K.S. (1988) The mouse albumin promoter and a distal upstream site are simultaneously DNase I hypersensitive in liver chromatin and bind similar liver-abundant factors *in vitro*. *Genes Dev.*, **25**, 528–541.
42. Cirillo,L.A., Lin,F.R., Cuesta,I., Friedman,D., Jarnik,M. and Zaret,K.S. (2002) Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol. Cell*, **92**, 279–289.
43. Godbout,R., Ingram,R. and Tilghman,S.M. (1986) Multiple regulatory elements in the intergenic region between the alpha-fetoprotein and albumin genes. *Mol. Cell. Biol.*, **6**, 477–487.
44. Godbout,R., Ingram,R.S. and Tilghman,S.M. (1988) Fine-structure mapping of the three mouse alpha-fetoprotein gene enhancers. *Mol. Cell. Biol.*, **8**, 1169–1178.
45. Hammer,R.E., Krumlauf,R., Camper,S.A., Brinster,R.L. and Tilghman,S.M. (1987) Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science*, **235**, 53–58.
46. Ernst,P. and Smale,S.T. (1995) Combinatorial regulation of transcription II: the immunoglobulin mu heavy chain gene. *Immunity*, **2**, 427–438.
47. Aronow,B.J., Silbiger,R.N., Dusing,M.R., Stock,J.L., Yager,K.L., Potter,S.S., Hutton,J.J. and Wiginton,D.A. (1992) Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-independent transgene expression. *Mol. Cell. Biol.*, **12**, 4170–4185.
48. Brooks,A.R., Nagy,B.P., Taylor,S., Simonet,W.S., Taylor,J.M. and Levy-Wilson,B. (1994) Sequences containing the second-intron enhancer are essential for transcription of the human apolipoprotein B gene in the livers of transgenic mice. *Mol. Cell. Biol.*, **14**, 2243–2256.
49. Elefant,F., Cooke,N.E. and Liebhaber,S.A. (2000) Targeted recruitment of histone acetyltransferase activity to a locus control region. *J. Biol. Chem.*, **275**, 13827–13834.
50. Higgs,D.R., Wood,W.G., Jarman,A.P., Sharpe,J., Lida,J., Pretorius,I.-M. and Ayyub,H. (1990) A major positive regulatory region located far upstream of the human alpha-globin gene locus. *Genes Dev.*, **4**, 1588–1601.
51. Vyas,P., Vickers,M.A., Picketts,D.J. and Higgs,D.R. (1995) Conservation of position and sequence of a novel, widely expressed gene containing the major human alpha-globin regulatory element. *Genomics*, **29**, 679–689.
52. Nykjaer,A., Dragun,D., Walther,D., Vorum,H., Jacobsen,C., Herz,J., Melsen,F., Christensen,E.I. and Willnow,T.E. (1999) An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell*, **96**, 507–515.
53. Willnow,T.E. and Nykjaer,A. (2002) Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D3. *Curr. Opin. Lipidol.*, **13**, 255–260.
54. Leheste,J.R., Melsen,F., Wellner,M., Jansen,P., Schlichting,U., Renner-Müller,I., Andreassen,T.T., Worf,E., Bachmann,S., Nykjaer,A. and Willnow,T.E. (2003) Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. *FASEB J.*, **17**, 247–249.