Circadian transcription of the cholesterol 7 α hydroxylase gene may involve the liver-enriched bZIP protein DBP

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The liver-enriched transcription factor DBP is expressed with a stringent circadian rhythm. We present evidence that DBP is a regulator of the circadian expression of the rat gene encoding cholesterol 7 α hydroxylase (C7 α H), the rate-limiting enzyme in the conversion of cholesterol to bile acids. As with DBP, C7 α H mRNA reaches peak levels in the evening, and its cycling is independent of daily food and light cues. As predicted for a DBP target gene, the primary level of C7 α H circadian expression is at the transcriptional level. DBP can activate the C7 α H promoter in cotransfection assays through a cognate DNA site centered around -225. In nuclear extracts prepared by a novel method that, in contrast to conventional techniques, yields near-quantitative recovery of DBP and other non-histone proteins, the DNA site required for DBP activation is the predominant site of occupancy by nuclear factors on the C7 α H promoter. At this site, the predominant binding activity is an evening-specific complex of which DBP is a component. These data suggest that DBP may play an important role in cholesterol homeostasis through circadian transcriptional regulation of cholesterol 7 α hydroxylase.

[Key Words: DBP; circadian rhythm; cholesterol; transcription factor; nuclear extract]

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In vertebrates, the liver plays an essential role in many diverse physiological processes, including production of serum proteins, xenobiotic detoxification, and the regulation of cholesterol homeostasis. Although some of these processes are expressed constitutively, the expression of others may be regulated by a wide array of extrinsic and intrinsic cues, including diet, hormones, and circadian rhythms. Determination of how these different cues might converge on a common target gene to mediate its regulation may give us insight into how "cross-talk" between pathways can lead to a carefully controlled physiological response.

The rat albumin D-element-binding protein (DBP) provides a valuable tool to study target genes under diverse physiological regulation. DBP is a liver-enriched transcription activator protein that was cloned by its ability to interact with the albumin promoter D-element (Mueller et al. 1990). DBP belongs to the basic leucine zipper (bZIP) family of transcription factors, whose members dimerize by carboxy-terminal leucine zipper domains and bind DNA through a domain rich in basic residues (for review, see Baxeavanis and Vinson 1993). Although DBP mRNA is detected in most tissues, the protein ac-

cumulates to high levels only in the nuclei of adult rat liver (Mueller et al. 1990).

The most remarkable aspect of DBP regulation, however, is that its expression follows a stringent circadian rhythm (Wuarin and Schibler 1990). The daily cycle of DBP protein levels in rats is ~100-fold in magnitude, with maximal levels detected ~8 p.m. The DBP cycle is "free-running," that is, independent of food and light cues, and primarily regulated at the level of transcription initiation. DBP mRNA levels cycle in nonliver tissues, suggesting that a systemic regulator contributes to DBP oscillation. One such regulator may be corticosterone; serum levels of this glucocorticoid hormone display a circadian cycle (Guillemin et al. 1959). Furthermore, dexamethasone, an analog of corticosterone, can repress accumulation of DBP RNA and protein in rats (Wuarin and Schibler 1990).

What is the physiological significance of the robust circadian rhythm in DBP expression? Investigation of this question requires the identification of DBP target genes whose expression also displays a circadian rhythm. One candidate for DBP regulation is the albumin gene, as DBP can activate the albumin promoter in cotransfection assays (Mueller et al. 1990). Although albumin gene transcription was found to cycle throughout the day, levels of albumin mRNA did not vary significantly (Wuarin

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and Schibler 1990), presumably because of high mRNA stability (Wuarin et al. 1992). Thus, whereas DBP may contribute to the pattern of albumin gene transcription, its circadian rhythm does not appear to be of physiological significance to albumin expression. Better candidates for study as DBP target genes might be found among those liver-enriched enzymes for whom diurnal variations have already been demonstrated. Such variations have been reported for several liver-expressed enzymes (e.g., McVerry and Kim 1972; Clarke et al. 1984), including rat cholesterol 7 α hydroxylase (C7 α H) (Mitropoulos et al. 1972). This member of the cytochrome P450 gene superfamily encodes the rate-limiting enzyme in the conversion of cholesterol to bile acids, which is the major route of cholesterol metabolism in rats and humans (Turley and Dietschy 1988). C7 α H mRNA exhibits liver-restricted accumulation (Jelinek et al. 1990) and a diurnal cycle at the levels of mRNA, protein, and enzyme activity, with an amplitude of approximately five-fold (Mitropoulos et al. 1972; Chiang et al. 1990; Noshiro et al. 1990). Peak levels of C7 α H expression were detected around midnight (Chiang et al. 1990; Noshiro et al. 1990), shortly after the peak in DBP accumulation. These observations led us to test C7 α H as a potential DBP target gene. We demonstrate that similar to DBP cycling, C7 α H RNA cycling is truly circadian in that it is independent of external time cues. Moreover, it is primarily regulated at the transcriptional level. We find that DBP can induce C7 α H promoter activity through a high-affinity DBP-binding site, and that DBP is a component of the major DNA-binding activity in the C7 α H promoter. Thus, DBP appears to be an important regulator of the circadian expression of C7 α H and, therefore, of cholesterol homeostasis.

Results

Circadian accumulation of C7 α H RNA

Several groups have reported diurnal cycling of C7 α H mRNA accumulation in rat liver (Noshiro et al. 1990; Sundseth and Waxman 1990). To confirm the results of these studies in our Lewis rat strain, we analyzed C7 α H RNA accumulation in livers harvested at 4-hr intervals over a 24-hr period from rats kept on a 12-hr light-dark cycle, with the light-dark switch at 6 p.m. RNA levels were determined by RNase protection assays, using a riboprobe spanning the major RNA start sites mapped by Jelinek and Russell (1990), derived from the cloned rat C7 α H gene (see Materials and methods). Accumulation of C7 α H poly(A)⁺ mRNA detected with this probe was found to cycle with an amplitude of 10-fold (Fig. 1A), with a peak ~10 p.m. and a trough ~10 a.m. As a control, no significant change was found in levels of mRNA for the cytochrome P450 gene CYP2C6 (Fig. 1A; Gonzalez et al. 1986). Cycling of C7 α H RNA accumulation was also found when analyzing total RNA. Although the amplitude of C7 α H RNA cycling in total RNA was found to vary somewhat between experiments (three- to eight-fold), peak accumulation was always found at ~10 p.m.

These results indicate that the diurnal pattern of C7 α H RNA accumulation is consistent with its regulation by DBP, whose maximal accumulation occurs ~8 p.m. (Wuarin and Schibler 1990).

To determine whether C7 α H RNA cycling is independent of daily food intake cues, rats were starved for 3 days before being sacrificed, and livers from starved and control rats were harvested at 9 a.m. or 9 p.m. Livers of starved animals were found to be reduced approximately one-third in mass. Levels of C7 α H mRNA and DBP protein were determined by RNase protection and Western blotting, respectively. As shown in Figure 1B, C7 α H RNA accumulation in total RNA was found to cycle with similar amplitude in livers of starved and control rats, as is also the case for DBP protein levels (Wuarin and Schibler 1990). Similar results on food-independent C7 α H RNA cycles were reported by Noshiro et al. (1990). Thus, cycling of C7 α H RNA and DBP protein does not require daily food cues. C7 α H RNA and DBP protein cycling persist as well after 7 days of treatment with cholestyramine (data not shown), a bile acid sequesterer that increases C7 α H RNA levels (Jelinek et al. 1990; Noshiro et al. 1990; Sundseth and Waxman 1990). Taken together, these data suggest that similar to the case for DBP cycling (Wuarin and Schibler 1990; Fig. 1B), food intake cues do not override the underlying circadian rhythm in C7 α H RNA cycling.

To examine whether C7 α H RNA cycling is independent of light cues, C7 α H RNA levels at 9 a.m. and 9 p.m. were assessed in samples of livers taken from rats that had been kept in constant darkness for 5 days. During this time, rats remained cyclic in their locomotor activity (data not shown). In these rats, C7 α H RNA and DBP protein levels continued to cycle (Fig. 1B), indicating that both of these cycles are free-running. Therefore, the persistence of the daily rhythm in C7 α H RNA accumulation in the absence of daily dietary or light cues suggests that its cycling, like that of DBP (Wuarin and Schibler 1990), is truly circadian.

Circadian transcription and tissue-specific expression of the rat C7 α H gene are correlated with DBP accumulation

If the circadian rhythm in C7 α H RNA accumulation is influenced by DBP, one would expect that the rate of C7 α H gene transcription would fluctuate with DBP accumulation. To investigate this, rat liver nuclei isolated at 4-hr intervals throughout the day were used in nuclear run-on assays to assess polymerase density on the C7 α H gene. Although transcription of the gene for a randomly selected rat liver cDNA clone of unknown coding specificity, R8, remained relatively constant throughout the day, C7 α H transcription demonstrated a cyclical pattern (Fig. 2). The relative C7 α H transcription rates closely follow the DBP protein accumulation peak, which is delayed by ~4 hr with regard to DBP gene transcription (Fig. 2; Wuarin and Schibler 1990). Quantitation of the signals by densitometry indicated a cycling in C7 α H transcription of approximately sixfold when normalized

Figure 1. Circadian accumulation of rat C7 α H mRNA and DBP protein. (A) RNase protection analysis of C7 α H RNA throughout the day. Poly(A)⁺ liver RNA (2 μ g) isolated from rats sacrificed at the hours indicated was analyzed by ribonuclease protection using probes for C7 α H (top) and, as a control, the cytochrome P450 gene CYP2C6 (bottom). (P) 1/400 dilution of undigested probe; (Y) yeast RNA control. Numbers at left represent molecular size in nucleotides, determined by comparison with migration of labeled DNA markers. (B) Circadian variation in C7 α H RNA and DBP protein accumulation during starvation and constant darkness. (Top) C7 α H RNA accumulation in rats fed ad libitum (Control), starved (Starved), or kept in constant darkness (C. Dark). Rats were sacrificed at the hours indicated, and total liver RNA was extracted and assayed by ribonuclease protection as in A, using 100 μ g of total RNA. Molecular sizes in nucleotides indicated at left were determined as in A. (Bottom) Accumulation of DBP protein detected by immunoblotting. Equivalent samples of sonicated liver nuclear lysates (Control, Starved) or nuclear extracts (C. Dark) from the same animals as in top panel were analyzed for DBP accumulation by Western blotting. Migration of protein molecular mass standards of the indicated molecular size are shown at left in kilodaltons.

to R8 gene transcription. Therefore, although we do not rule out that post-transcriptional regulation may also contribute to circadian C7 α H expression, the primary level of circadian C7 α H gene regulation is transcriptional. This is the first demonstration of circadian regulation of C7 α H gene expression at the transcriptional level.

As the relative C7 α H transcription rates closely parallel DBP protein accumulation (Wuarin and Schibler 1990), these data further support the hypothesis that the transcription factor DBP may participate in C7 α H gene activation. We note, however, that C7 α H transcription does not fall to background levels, even at times of the day when DBP is nearly undetectable, such as 8 a.m. (Wuarin and Schibler 1990). This suggests that addi-

tional, perhaps constitutively expressed, transcription factors contribute to the establishment of a basal level of C7 α H transcription.

Previously, Northern blot experiments indicated that evening levels of C7 α H mRNA accumulate specifically in the liver, whereas no detectable C7 α H accumulation was found in any of the other tissues examined (Jelinek et al. 1990). Furthermore, nuclear run-on experiments indicated that liver-restricted C7 α H expression is primarily effected at the transcriptional level (Russell and Setchell 1992). We find similar results for C7 α H mRNA accumulation in both morning and evening RNA samples isolated from five different tissues and analyzed by RNase protection (Fig. 3). In nonhepatic tissues examined (brain, kidney, lung, and spleen), C7 α H RNA was

Figure 2. Circadian transcription of the C7 α H gene. Liver nuclei purified from rats sacrificed at the hours indicated were used in nuclear run-on assays. Probes were used for the following genes: DBP, C7 α H, and R8, which encodes a randomly selected rat liver cDNA clone of unknown coding specificity. Plasmid pKS+ was included as a control for nonspecific background signal. Autoradiographic signals from probe hybridizations were quantitated by densitometric analysis of multiple exposures and are presented in the *bottom* panel as fold increase over minimal daily levels.

not detected above background levels, in samples from either morning or evening. The integrity of these RNAs was ascertained by an RNase protection experiment with a cDNA probe for RNA encoding the b subunit of the ubiquitous nuclear factor NF-Y (NF-Yb; Hooft van Huijsduijnen et al. 1990). As expected, NF-Yb mRNA was detected at similar levels in all samples (Fig. 3). Thus, C7 α H RNA, like DBP protein (Mueller et al. 1990; Wuarin and Schibler 1990), is highly liver enriched.

Figure 3. Tissue-specific accumulation of C7 α H RNA. Ribonuclease protection assays for C7 α H (*top*) and the ubiquitously expressed NF-Yb (*bottom*) mRNAs were performed with 100 μ g (C7 α H) or 50 μ g (NF-Yb) of RNA isolated from indicated tissues of 14-week-old rats sacrificed at morning or evening hours, as indicated. The molecular sizes in nucleotides of undigested probe RNAs and of the major protected fragments are indicated.

DBP activates C7 α H promoter activity in HepG2 cells

Given the close correlation between circadian DBP protein accumulation and C7 α H transcription, we sought to determine whether DBP could activate expression of C7 α H promoter-reporter gene fusion constructs in cotransfection experiments. Transfection experiments were performed using the human hepatoma cell line HepG2, which expresses endogenous C7 α H enzyme activity at a level similar to that observed in morning rat liver (Leighton et al. 1991). Plasmids were constructed in which segments of the C7 α H promoter beginning at nucleotide +47 and including various amounts of sequences 5' to the major RNA start sites were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene. These plasmids were transfected into HepG2 cells with or without a DBP expression vector, pCMV-DBP (Mueller et al. 1990), to determine promoter regions required for basal and DBP-induced expression of the C7 α H promoter in HepG2 cells. As a positive control, HepG2 cells were transfected with the plasmid pSV2CAT (Gorman et al. 1982), which contains the CAT gene under the control of the SV40 virus promoter-enhancer. In all transfections, a plasmid containing the firefly luciferase gene under control of the Rous sarcoma virus long terminal repeat (RSV LTR) (deWet et al. 1987) was included to control for transfection efficiency.

Cotransfection of the DBP expression vector with the different promoter constructs demonstrated that DBP can strongly activate the C7 α H promoter. Induction is at least 7-fold in magnitude (Fig. 4), and often exceeds 10-fold in magnitude, depending on DBP concentration (e.g., see Fig. 6, below). Cotransfections with progressive 5'-deletion promoter mutations indicated that activation of the C7 α H promoter by DBP required DNA sequences between -340 and -131 (Fig. 4) and was not increased by inclusion of sequences to -1790 (data not shown). Thus, the sequences from -340 to -131 appear to be sufficient for DBP activation of the C7 α H promoter.

Figure 4. DBP activation of C7 α H promoter constructs by cotransfection in HepG2 cells. C7 α H promoter-CAT fusion plasmids (lower left) were cotransfected with (+) or without (-) 5 μ g of the DBP expression vector, pCMV-DBP. Plasmid pSV2CAT, which expresses the CAT gene under the control of the SV40 enhancer, and pKS+CAT are included as positive and negative controls, respectively. CAT activity was quantitated and corrected for transfection efficiency as described in Materials and methods and represented in the lower right panel as percentage of values for positive control plasmid pSV2CAT.

The C7 α H promoter contains a DBP-binding site

Given that the C7 α H promoter region from nucleotide -340 to -131 was sufficient for DBP activation, the nucleotide sequence within this region was examined to identify any sequence elements similar to the previously determined, partially palindromic DBP consensus sequence, 5'-A/G TTATGTAA C/T-3'. This sequence has been deduced by comparing >60 high-affinity binding sites that were selected with recombinant DBP from a mixture of double-stranded oligonucleotides containing the random sequence N₁₆ [E. Falvey, L. Marcacci and U. Schibler, in prep.].

We found a sequence, 5'-GTTATGTcAg-3' (mismatched nucleotides in lowercase letters), with 8 of 10 nucleotide identity to the DBP consensus sequence, centered ~225 nucleotides upstream of the C7 α H transcription start site. To determine whether this sequence element in the C7 α H promoter region to -340 might function as a DBP-binding site, DNase I footprinting studies were performed using purified full-length recombinant DBP and a radioactively end-labeled DNA fragment extending from -340 to -30. As presented in Figure 5, a single DBP-binding site was found, even at high concentrations of recombinant protein. Comparison with chemical sequencing products of the DNA probe indicated that the region protected by recombinant DBP was centered at nucleotide -225 and included the sequence similar to the DBP consensus sequence.

We have observed recently that each DBP-binding site also has affinity for CCAAT/enhancer-binding protein (C/EBP) family proteins C/EBP α and liver-enriched transcriptional activator protein (LAP), which are highly expressed in the liver [Ossipow et al. 1993; L. Marcacci and U. Schibler, unpubl.]. Although accumulation of C/EBP α and LAP does not vary throughout the day (P.

Descombes, unpubl.), the C/EBP family has been implicated in the control of many liver-expressed genes (for review, see Crabtree et al. 1992) and may contribute to basal C7 α H expression. To determine whether members of this family might interact with the C7 α H promoter, DNase I footprinting experiments were performed with purified recombinant LAP, which displays sequence specificity in DNA binding representative of the C/EBP family [Descombes et al. 1990]. Three regions were protected by LAP in the promoter region between -340 and -30, centered around nucleotides -295 (referred to as FP-1), -225 (FP-2), and -185 (FP-3). Thus, the DBP-binding site in this promoter region, FP-2, is also a C/EBP protein family-binding site, although the C/EBP family-binding sites FP-1 and FP-3 are not bound by DBP. These observations are in keeping with previous results demonstrating that DBP has a higher sequence specificity than C/EBP family members [see also Discussion; Mueller et al. 1990].

The intact FP-2-binding site is required for efficient DBP-mediated activation of transcription from the C7 α H promoter

To determine more directly whether DBP can activate transcription of the C7 α H promoter through the FP-2 site, this site was mutated by site-directed mutagenesis (Fig. 6A). These mutations were chosen to disrupt the putative DBP consensus sequence efficiently. Electrophoretic mobility-shift assay (EMSA) experiments indicated that binding of recombinant DBP to this mutant sequence was undetectable (data not shown). The resulting FP-2 mutant-containing plasmid, pCH-340M₁CAT, or the wild-type plasmid pCH-340CAT, was transfected into HepG2 cells, along with increasing amounts of the

Figure 5. DNase I footprint analysis of C7 α H promoter binding by recombinant DBP and LAP. Reactions were performed with purified recombinant DBP or recombinant LAP using increasing concentrations in lanes from *right to left*, as indicated. Reactions of probe alone with dilution buffer (–) or in chemical sequencing reactions (A+G, C+T) for orientation of the promoter are indicated. Promoter regions strongly protected from DNase I digestion by DBP or LAP (FP-1, FP-2, and FP-3) are indicated at *right*, as well as a region weakly protected by LAP (bracket), and their orientation in the promoter sequence is indicated at *left*.

DBP expression vector, pCMV–DBP. As presented in Figure 6B, basal promoter activity in the absence of cotransfected pCMV–DBP was not affected significantly by the FP-2 mutation. However, DBP-mediated activation of the FP-2 mutant promoter-driven reporter gene was reduced to a very low level relative to the wild-type promoter construct. Impairment of C7 α H promoter induction by DBP after mutation within the DBP consensus sequence in FP-2 implies that FP-2 is an important element for DBP activation of the C7 α H promoter.

DBP is the most prominent nuclear factor binding to the C7 α H promoter

Preparation of conventional ammonium sulfate nuclear extracts (Gorski et al. 1986) results in the loss of the bulk (67%) of DBP protein from its sedimentation with the

chromatin pellet during centrifugation (Fig. 7). Therefore, we developed a new extraction procedure to increase recovery of nonhistone proteins using a solution (NUN) containing 0.3 M NaCl, 1 M urea, and 1% nonionic detergent Nonidet P-40, which destabilize salt bridges, hydrogen bonds, and hydrophobic interactions, respectively. Extraction of total nonhistone proteins is at least twofold greater with this method, and recovery of DBP is increased to >80% (Fig. 7). These conditions also allow for efficient extraction of other nuclear factors, such as C/EBP α , LAP, HNF-1, HNF-4, and NF-Y, from the chromatin (data not shown). Because core histones, as well as histone H1, remain associated with the DNA (Fig. 7), the chromatin remains compact and can be sedimented by low-speed centrifugation in a conventional microcentrifuge. Also, as precipitation or dialysis is not required for use of these extracts in EMSA or DNase I footprint assays, the entire reaction can be completed within 30 min and can be performed in a volume of a few microliters. This method has already been proven very useful in experiments where tissue sources are limiting, such as with cell culture samples or analysis of small tissues, or with transgenic animals (E. Schmidt, P. Descombes, and U. Schibler, unpubl.).

To examine the interactions of rat liver nuclear factors with the C7 α H promoter, NUN nuclear extracts were prepared from rat livers harvested at 8 a.m. or 8 p.m., and used in DNase I footprinting experiments with the radiolabeled C7 α H promoter fragment from –340 to –30. With both morning and evening liver extracts, three sites are protected in this promoter region, with the most prominent protection centered at approximately –225 and with lesser protection centered around –295 and –185 (Fig. 8). These protected sites correspond to the regions protected by recombinant DBP and LAP (FP-1, FP-2, and FP-3; cf. with Fig. 5). Interestingly, the most prominent nuclear factor-binding site corresponds to the site bound by recombinant DBP (FP-2), which is necessary for DBP activation of the C7 α H promoter in cotransfection experiments (see Fig. 6). Only a slight difference was detected in the degree of protection of these sites by evening versus morning nuclear extracts in the absence of nonspecific competitor DNA (Fig. 8, lane 0). However, upon addition of increasing amounts of salmon sperm DNA, which served to partially mimic the high concentration of nonspecific competitor DNA in the cell nucleus, protection of site FP-2 by evening nuclear extracts was found to be more resistant to competition than FP-2 protection by morning nuclear extracts, even with as little as 10 ng of salmon sperm DNA (Fig. 8, cf. lanes 10 in panels 8h and 20h). These results suggest that the factor (or factors) in evening extracts interacting with site FP-2 binds with greater affinity and/or are more abundant than the factor (or factors) interacting with FP-2 in the morning.

Cotransfection and DNase I footprinting experiments indicated that the FP-2 site might be a site of action for DBP on the C7 α H promoter. We reasoned, therefore, that if DBP represents a significant fraction of FP-2 DNA-binding activity in evening rat liver nuclear extracts, an

Figure 6. Mutation of site FP-2 reduces C7 α H promoter activation by DBP. (A) Sequences of the wild-type and mutant FP-2 elements of the C7 α H promoter in plasmids pCH-340CAT and pCH-340MCAT, respectively. The sequences that were mutated are boxed. The promoter elements FP-1, FP-2, and FP-3, the putative TATA element, and the transcription initiation sites are indicated (not to scale). (B) Wild-type C7 α H promoter-reporter plasmid pCH-340CAT or FP-2 site mutant pCH-340MCAT was cotransfected into HepG2 cells with increasing amounts of DBP expression vector pCMV-DBP (0–4 μ g; Mueller et al. 1990), indicated on the x axis. Percent conversion of chloramphenicol substrate relative to the positive control, pSV2CAT (100%), is presented for both the wild-type (○) and mutant (▲) promoter, as a function of the amount of cotransfected DBP expression vector, corrected for transfection efficiency as in Fig. 4.

evening-specific complex of nuclear factors with the FP-2 site should be detectable by EMSA experiments. Furthermore, the presence of DBP in such a complex could be detected by incubation with anti-DBP antiserum; antibody binding to this complex should impede its migration and produce a "supershift."

EMSA experiments were performed in which a radiolabeled FP-2 oligonucleotide probe was incubated with NUN nuclear extracts prepared from rat liver nuclei isolated at 4-hr intervals around the clock. As demonstrated in Figure 9A, a prominent complex of factor (or factors) with FP-2 (marked with an asterisk) was apparent by 4 p.m., which increased to represent the major DNA-binding complex by 8 p.m. After 8 p.m., the complex was reduced in intensity, and by 8 a.m. it was no longer discernible. At least two other complexes were detected with the FP-2 oligonucleotide probe (arrowheads): one that migrates faster and one slower than the evening-specific complex. These complexes were of reduced intensity relative to the evening complex and do not appear to change in intensity throughout the day. Thus, the appearance of the major FP-2-binding activity in rat liver nuclear extracts closely parallels the patterns of both C7 α H gene transcription (see Fig. 2) and DBP accumulation (Wuarin and Schibler 1990). In control experiments, no difference in DNA-binding activity was detected with these 24-hr extracts using either an FP-1 oligonucleotide or an FP-3 oligonucleotide as a probe (data not shown). Likewise, no difference was seen in the binding pattern of spleen nuclear factors to the FP-2 probe when using extracts prepared at 8 a.m. or 8 p.m. (data not shown). Furthermore, the appearance of the evening-specific complex is free-running: EMSA experiments using nuclear extracts prepared from rats kept in constant dark

for 5 days (Fig. 9C) again demonstrated the appearance of the evening-specific complex in extracts prepared at 10 p.m., which was absent in liver nuclear extracts prepared at 10 a.m. from rats kept in constant dark. The lower band in lane 22h, which is not detected in lane 10h, is not seen consistently and appears to be the result of non-specific DNA-binding activity. No difference was detected with these constant dark extracts in binding to an FP-3-containing oligonucleotide probe (data not shown).

To determine whether the evening-specific DNA-binding activity contained DBP (or DBP-like epitopes), supershift experiments were performed in which morning or evening NUN nuclear extracts were first incubated with radiolabeled FP-2 oligonucleotides, followed by incubation with either rabbit preimmune serum or dilutions of rabbit antiserum directed against recombinant DBP (Wuarin and Schibler 1990). As demonstrated in Figure 9B, incubation of anti-DBP antiserum with the FP-2 probe and evening nuclear extracts resulted in the appearance of a slower migrating species, and the diminution of the evening-specific major complex. The appearance of this supershift was dependent on the concentration of antiserum used; use of the highest antiserum concentration resulted in the aggregation of FP-2-protein complexes in the well, presumably attributable to network formation by the antibodies. The pattern of FP-2 complexes with evening extracts was not modified by incubation with rabbit preimmune serum, nor was the pattern with morning extracts altered by the addition of either rabbit preimmune serum or anti-DBP antiserum. Supershift experiments were also performed with the liver nuclear extracts prepared from rats maintained in constant dark (Fig. 9C). Addition of anti-DBP antiserum affected the migration of only the evening-specific

Figure 7. DBP recovery in NUN and standard nuclear extracts. (A) Total proteins in different extract fractions from 8 p.m. rat liver nuclei detected by staining with Coomassie blue. Fractions analyzed were from ammonium sulfate extraction $(\text{NH}_4)_2\text{SO}_4$, (T) total nuclear lysate; (P) chromatin pellet after ammonium sulfate lysis; (D) precipitate formed during dialysis; and (E) dialyzed extract; and from NUN extraction, (T) total nuclear lysate; (P) NUN chromatin pellet; and (E) NUN extract. Protein molecular mass markers (M), with the corresponding molecular mass in kilodaltons are noted at left. Brackets at left indicate the position of core histone proteins (Core) and histone H1 proteins (H1). (B) Western blot analysis of DBP protein in fractions of conventional and NUN nuclear extracts. Proteins in A were electrotransferred to nitrocellulose and probed for DBP accumulation. Lanes are marked as in A. The 48-kD DBP protein and smaller reactive species are indicated by the bracket at right, and the migration of molecular mass markers and their corresponding molecular masses in kilodaltons are indicated at left.

complex (data not shown). Thus, the appearance of the evening-specific complex that reacts with the anti-DBP antiserum is also free-running, as is the case for C7 α H mRNA and DBP detected in Western blot analyses (Wuarin and Schibler 1990; see Fig. 1B). Taken together, these experiments indicate that DBP is a component of the major DNA-binding activity at FP-2, the most prominent site of nuclear factor interactions on the C7 α H promoter.

Discussion

DBP as a potential regulator of cholesterol homeostasis

We present evidence that DBP is a circadian regulator of the liver-specific enzyme, C7 α H. The diurnal and tissue-specific expression patterns of both C7 α H RNA and DBP are compatible. Both rhythms are free-running, being independent of daily food and light cues. For the first time we demonstrated that the primary level of circadian C7 α H expression is transcriptional, with the peak in C7 α H gene transcription following the peak of DBP accumulation. Furthermore, DBP can activate C7 α H promoter expression in transient cotransfection assays in HepG2 cells. This activation is mediated by a high-affin-

ity DBP-binding site, which is also the site of the most prominent interaction of rat liver nuclear factors with the C7 α H promoter, as detected by DNase I footprint analysis. As DBP is detected in the major FP-2-binding activity in evening liver nuclear extracts, by extension DBP can be considered to be the most prominent factor interacting with the C7 α H promoter. Taken together, these observations give us confidence that DBP is indeed an important regulator of C7 α H expression.

C7 α H is the rate-limiting enzyme for the conversion of cholesterol into bile acids. This reaction is responsible for the elimination of the majority of cholesterol from the body, and as a result, the level of C7 α H expression can significantly influence cholesterol levels not only in the liver but in the entire organism (Turley and Dietsch 1988). Although cholesterol is required continuously for membrane synthesis, elevated levels can be toxic to cells and can influence pathological states such as atherosclerosis (Vlahcevic et al. 1986). On the other hand, large amounts of bile acids are required for efficient digestion and for absorption of dietary cholesterol. Clearly, C7 α H expression must be balanced adequately to satisfy both dietary requirements for bile acids and maintenance of cholesterol homeostasis. Perhaps this is the benefit of a

Figure 8. DNase I footprinting analysis on the C7 α H promoter with morning and evening NUN liver nuclear extracts. The C7 α H promoter probe used in Fig. 5 was incubated with constant amounts of extracts prepared from rats sacrificed at 8 a.m. (8h) or 8 p.m. (20h), and increasing amounts of nonspecific competitor DNA (0–50 ng, as indicated). Extracts used gave equivalent DNA-binding activity on the albumin D site. Products of a chemical sequencing reaction (G + A) of the probe permit orientation to the promoter sequence, indicated at *left*. Promoter regions corresponding to FP-1, FP-2, and FP-3 in Fig. 5 are indicated at *right*.

circadian regulation system that has evolved to program peak synthesis of C7 α H in the evening, when demand for bile acids would be greatest in an evening feeder such as the rat, with basal C7 α H expression during the inactive period. The rate-limiting enzyme for cholesterol synthesis, HMG CoA reductase, also displays a circadian rhythm in accumulation of its RNA (Clarke et al. 1984). It is intriguing to speculate that perhaps the expression of this enzyme is also under the control of DBP. This could be accomplished either through direct activation of the HMG CoA reductase gene, or indirectly, as a consequence of C7 α H-stimulated turnover of cholesterol. Cholesterol has been established as a feedback inhibitor of HMG CoA reductase expression (Brown and Goldstein 1980). Thus, DBP could have a central role in the coordinate regulation of both cholesterol synthesis and breakdown.

We have found that in cotransfection experiments with the human hepatoma cell line HepG2, DBP

strongly activates expression from the C7 α H promoter. HepG2 cells express the endogenous C7 α H enzyme at a level near that expressed in rat liver during the morning (Leighton et al. 1991). Interestingly, transfection experiments in HeLa cells indicated that the activity of the C7 α H promoter is reduced greatly compared with that in HepG2 cells, with or without cotransfection of a DBP expression vector (data not shown), suggesting that other liver-enriched factors are necessary for efficient C7 α H expression. Expression of the human C7 α H promoter in HepG2 cells was increased greatly by cotransfection with an expression vector for hepatocyte nuclear factor 3 (HNF-3; Molowa et al. 1992).

Because the DBP-binding site within the C7 α H promoter also has affinity for C/EBP family proteins, and as factors in morning nuclear extracts can interact with this site, one has to consider that members of the C/EBP family may also participate in the control of C7 α H expression. However, for several reasons, we judge DBP to be a more attractive candidate as a key regulator. First, C/EBP α and LAP accumulate constitutively throughout the day and thus cannot readily account for circadian C7 α H transcription. Second, the transcription activation potential of both of these proteins is dampened by the concomitant expression of two *trans*-dominant inhibitory proteins, LIP (Descombes and Schibler 1991) and C/EBP α 30 (Ossipow et al. 1993). Third, owing to their relaxed DNA-binding specificity, C/EBP family homo- and heterodimers are expected to encounter much greater difficulties in finding their cognate sequences within the C7 α H promoter than DBP. Although DBP, C/EBP α , and LAP are approximately equally abundant in liver nuclei at 8 p.m. [$\sim 1 \times 10^5$ monomers each (F. Fleury-Olela and U. Schibler, unpubl.)], the number of binding sites in rat genomic DNA is ~ 20 -fold higher for the C/EBP proteins than for DBP. The presence of C/EBP family binding sites within 340 nucleotides of examined promoter sequences is not surprising and may be purely fortuitous: DNase I protection studies with randomly selected DNA fragments encompassing a total of 10 kb of rat genomic DNA have revealed two DBP-binding sites but as many as 40 C/EBP family member cognate sequences with an affinity comparable with that observed for the FP-2 element (L. Marcacci and U. Schibler, unpubl.).

Glucocorticoids have also been proposed as possible regulators of C7 α H expression (Gielen et al. 1975; Princen et al. 1989). Serum concentrations of glucocorticoid cycle with a circadian rhythm, reaching a peak at the light–dark switch (Guillemin et al. 1959), shortly before maximal C7 α H gene transcription. Furthermore, endogenous glucocorticoid cycling begins after weaning in the rat (Ader 1969), as does the cycling of both C7 α H RNA and DBP (data not shown). However, it is not clear whether transcriptional, post-transcriptional, translational, or post-translational controls account for this increase. With regard to transcriptional C7 α H regulation, our preliminary experiments suggest a negative rather than a positive role for glucocorticoids. At least in cotransfection experiments, C7 α H promoter activity is

Figure 9. EMSA analysis of circadian FP-2 nuclear factor complexes. (A) Rat liver nuclear extracts prepared every 4 hr as indicated were incubated with a radiolabeled FP-2 oligonucleotide probe and analyzed by EMSA. Asterisk (*) indicates the evening-specific complex, and arrowheads indicate other major noncycling complexes. (B) Supershift analysis of FP-2 complexes using anti-DBP antiserum. NUN nuclear extracts from 8 a.m. (8h) and 8 p.m. (20h) were incubated with radiolabeled FP-2 probe as in A, followed by incubation with preimmune serum diluted 1 : 20 (PI), or anti-DBP antiserum diluted 1 : 100, 1 : 50, or 1 : 20 (α -DBP, with antiserum concentration increasing in lanes from left to right), and analyzed by EMSA as in A. (C) NUN liver nuclear extracts from rats kept in constant dark for 5 days and sacrificed at the hours indicated (10 or 22) were used in EMSA with radiolabeled FP-2 oligonucleotides. The evening-specific complex is marked by an asterisk (*).

reduced by the addition of dexamethasone. In the animal, high levels of adrenal hormones may also impair C7 α H transcription indirectly, by down-regulating DBP expression (Wuarin and Schibler 1990).

At present, we do not know whether DBP activates C7 α H transcription as a homodimer or as a heterodimer with other dimerization-compatible proteins. Possible dimerization partners include hepatocyte leukemia factor (HLF; Hunger et al. 1992; Inaba et al. 1992) and thyrotroph embryonic factor (TEF; Drolet et al. 1991). The mRNAs from both of these genes are detected in liver (Hunger et al. 1992; Inaba et al. 1992; P. Fonjallaz and U. Schibler, unpubl.); moreover, their protein products can heterodimerize with DBP and can bind to the albumin promoter D element as homo- and heterodimers in vitro.

Pacemakers and circadian outputs

Circadian rhythms have been described in nearly every eukaryotic system, from fungi to humans, as well as several prokaryotic systems (Dunlap 1990). In each of these organisms, pacemakers help to anticipate the needs of the organism through regulation of specific target genes. Thus, in *Neurospora*, one morning-specific target gene, *cgc-2*, encodes a conidial protein required for more effective dissemination of spores (Bell-Pedersen et al. 1992). Because *cgc-2* transcription appears to be under the control of the *frequency* (*frq*) gene (Loros and Dunlap 1991), a clock gene of *Neurospora* (Dunlap 1990), the characterization of *cgc-2* regulatory factors may establish the regulatory connection between *frq* and *cgc-2*. Circadian transcriptional regulation has also been established for the wheat chlorophyll a/b binding peptide gene *cab-1* (Nagy et al. 1988). Circadian expression of the gene encoding this component of the light harvesting complex

may help prepare the plant for efficient photosynthesis. Interestingly, the *Drosophila* clock gene *per* is itself a target gene for circadian transcription regulation (Hardin et al. 1992); in addition, *per* expression has been shown to be autoregulated (Hardin et al. 1990).

In mammals, the pacemaker resides in the suprachiasmatic nucleus (SCN) within the hypothalamus (for review, see Moore 1992). Many circadian outputs are regulated by the pacemaker through the hypothalamus–pituitary axis of the endocrine system (Frohman 1988). How this regulatory axis might influence circadian gene expression in the liver remains unclear. One possible mechanism is the circadian activation of glucocorticoid production by ACTH, which may exert negative effects on DBP expression.

The characterization of C7 α H as a DBP target gene suggests that the robust circadian rhythm in DBP expression is of physiological significance. Whether DBP is a global regulator of all circadian-regulated liver-specific genes or is restricted to a subset of genes such as the cytochrome P450 superfamily remains to be determined. However, the circadian expression of DBP provides a novel opportunity to study liver gene regulation in a system where the concentration of a transcriptional regulator changes by two orders of magnitude within 12 hr. Further characterization of the interactions of DBP with other nuclear factors on the C7 α H promoter may shed light on how a promoter responds to diverse physiological cues, including those involved in circadian gene regulation.

Materials and methods

Cloning of the rat genomic fragment containing the C7 α H promoter

Two overlapping oligonucleotides containing the sequence of

the C7 α H cDNA corresponding to positions +31 to +78 in the rat C7 α H cDNA (Jelinek and Russell 1990; Noshiro et al. 1990) were synthesized:

5'-TCCCCTTTGGAAATTTCTGCTTTTGC-3'
3'-CGAAACGTTTACTACTGATAAAGAAA-5'

After annealing and filling in with the *Escherichia coli* DNA polymerase I Klenow fragment and [α -³²P]dATP, the resulting 48-bp probe was used to screen an EMBL3 rat genomic DNA library, as described (Tian and Schibler 1991). Screening of 5×10^5 recombinant phages resulted in the isolation and purification of two overlapping phages that hybridized with the 48-bp C7 α H probe. Inserts of these phages were subcloned into plasmid vector pBluescript KS+ (pKS+; Stratagene), and dideoxy sequencing of a 4.8-kb *EcoRI* fragment in plasmid pE4.8 revealed identity with the published genomic sequence from -590 upstream of the major RNA start site (Jelinek and Russell 1990) to the first exon-intron junction (+154; Jelinek et al. 1990; Noshiro et al. 1990).

Ribonuclease protection assays

C7 α H RNA was detected using a radiolabeled RNA probe spanning the 5' cap sites to the *NdeI* site at +133. This probe was generated by cutting plasmid pE4.8 with *NdeI*, blunting the ends with T4 DNA polymerase, and cutting again with *HindIII*. This fragment was cloned into pKS+, which had been first cut with *KpnI*, blunted with T4 DNA polymerase, and recut with *HindIII*. Digestion of the resulting plasmid, pCH5', with *Alw44I* and transcription with T3 RNA polymerase generates an RNA probe of 167 nucleotides, which gives rise to major protected RNAs of 133 and 131 nucleotides, corresponding to the two major transcription start sites identified by Jelinek and Russell (1990). Smaller fragments (115–125 nucleotides) were also detected, presumably arising from the "breathing" of hybrids within an A/T-rich region 3' to the start sites.

The RNA of NF-Yb, a subunit of the ubiquitous nuclear factor NF-Y, whose RNA is expressed at equivalent levels per microgram of RNA in all mouse and rat tissues examined (E. Schmidt and U. Schibler, in prep.; E. Schmidt, pers. comm.), was detected using a 250-nucleotide radiolabeled RNA probe from the mouse NF-Yb cDNA (kindly provided by Drs. R. Mantovani, U. Pes-sara, X.-Y. Li, and C. Benoist, INSERM, Strasbourg, France). Hybridization of this probe to rat RNA yields a protected fragment of 225 nucleotides when digested at 24°C (E. Schmidt, unpubl.).

The mRNA from the cytochrome P450 superfamily gene CYP2C6 (formerly P450 PB1) was detected using an internal *SphI*-*EcoRI* fragment from the rat cDNA (kindly provided by Dr. F. Gonzalez (Gonzalez et al. 1986). The *SphI* site was blunted with T4 DNA polymerase, and the resulting fragment was subcloned into *PstI*-cut, T4 DNA polymerase-blunted, *EcoRI*-cut plasmid pKS+. After digestion with *BamHI*, T7 RNA polymerase was used to generate RNA transcripts of 200 nucleotides, which gives a major protected band of 163 nucleotides after hybridization to rat liver RNA and RNase digestion.

RNase protection assays were performed essentially as described (Schmidt and Merrill 1989), using 50–100 μ g of total RNA or 2 μ g of poly(A)⁺ RNA per reaction, in hybridization mix containing 70% formamide (C7 α H and CYP2C6) or 80% formamide (NF-Yb). As controls, reactions were also assembled with yeast RNA plus probe RNA (negative control) and with yeast RNA plus diluted probe RNA (probe control). Hybridization was allowed to proceed for 12–24 hr at 52°C (NF-Yb) or 37°C (C7 α H, CYP2C6). Ribonuclease T1/A digestions were per-

formed at 24°C (NF-Yb), 37°C (C7 α H), or 45°C (CYP2C6), except for the probe control, which was incubated without ribonucleases. Samples were then treated with proteinase K and extracted with phenol-chloroform. After ethanol precipitation, the samples were separated on an 8% polyacrylamide-8 M urea sequencing gel, fixed, dried, and exposed to X-ray film.

Isolation of nuclei and NUN extract preparation

Nuclei were purified from rat liver as described previously (Tian and Schibler 1991), by homogenization in a 1.9 M final concentration sucrose solution, followed by pelleting once through a 2 M sucrose cushion. DNA concentrations were determined by spectrophotometric absorbance at 260 nm, and nuclei were used either directly or flash-frozen in nuclear storage buffer [NSB: 20 mM Tris-Cl (pH 7.9), 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol] and stored in liquid nitrogen.

Thawed or fresh nuclei in NSB were concentrated to 25 mg/ml of DNA content, which is essentially a nuclear slurry, in a 1.5-ml plastic microcentrifuge tube or in a glass Corex tube. After resuspension by gentle agitation, nine volumes of a 1.1 M NUN solution was added to give final concentrations of 1 M urea, 0.3 M NaCl, 1% Nonidet-P40, 25 mM HEPES (pH 7.6), and 1 mM DTT. After vigorous vortex agitation for 5 sec, the tubes were left on ice for 15 min, during which time a string-like precipitate of chromatin and associated structures appeared. The chromatin precipitate was sedimented by centrifugation (10 min at 4°C in a microcentrifuge, or 15 min at 10,000g and 4°C in Corex tubes), allowing nearly quantitative removal of the supernatant. Glycerol was added to the supernatant to a final concentration of 10%, and small aliquots were flash-frozen and stored in liquid nitrogen. Protein concentrations in dialyzed NUN nuclear extracts were at least twofold greater than those in extracts prepared by ammonium sulfate extraction.

Western blot analysis

Detection of DBP in liver nuclear lysates by immunoblotting was performed using a polyclonal rabbit anti-DBP antiserum, as described (Wuarin and Schibler 1990). For nuclear extracts, volumes equivalent to 0.5–1 OD₂₆₀ unit of starting material were diluted with an equal volume of 2 $\times$ loading buffer (Laemmli 1970) and analyzed as described above. Immune complexes were detected using ¹²⁵I-labeled protein A (Amersham).

Nuclear run-on assays

Nuclei were purified from livers of adult male rats by sucrose homogenization (Tian and Schibler 1991) at 4-hr intervals over 24 hr, flash-frozen in liquid nitrogen, and stored at -70°C until use, as described previously (Wuarin and Schibler 1990). In vitro polymerase elongation and extraction of labeled RNAs were performed as described (Wuarin and Schibler 1990), using 1×10^7 nuclei per time point, and labeling with 250 μ Ci of [α -³²P]UTP for 10 min.

Linearized, denatured plasmids containing probes for genes of interest were applied to nitrocellulose filters with a slot blot apparatus (Schleicher & Schuell), using 10 μ g of DNA per slot, followed by baking for 2 hr at 80°C in vacuo. DNA probes used were as follows: pE4.8, which contains the rat C7 α H RNA start sites and 4.2 kb of transcribed sequences; pBRP, an *EcoRI*-*PstI* rat genomic fragment containing the DBP major RNA start sites (E. Falvey and J. Wuarin, unpubl.); R8, a cloned rat liver cDNA of unknown coding specificity; and pKS+, the cloning vector, included as a background control plasmid. Hybridizations were

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performed with equivalent input radioactivity for each time point, as described previously (Schibler et al. 1983; Chen-Kiang and Lavery 1989), except that hybridization was allowed to proceed for 48 hr. Filters were washed stringently, treated with RNase A as described (Wuarin and Schibler 1990), and exposed to X-ray film. Autoradiographic signals were quantitated from different exposures using a Shimadzu CS-9000 densitometric scanner.

C7αH promoter–CAT reporter constructions and transient transfection experiments

Fusion of C7αH promoter sequences to a bacterial CAT reporter gene was accomplished by insertion of a *Hind*III site into the C7αH 5′-untranslated region at +47, using PCR amplification (Saiki 1989) of plasmid pE4.8 (see above). Primers used were 5′-CAAAAGCAGGAAGCTTTCCAAAGGGG-3′ (mutated bases are underlined) and the M13 reverse sequencing primer. The resulting 762-bp PCR product was cut with *Hind*III and cloned into the *Hind*III site of pKS+CAT, which contains the *Hind*III–*Bam*HI fragment of the bacterial CAT gene (Gorman et al. 1982) cloned into pKS+. The resulting plasmid, pCH-340CAT, was used to create other promoter–CAT fusions. Partial cleavage of pCH-340CAT with *Alw*44I or *Hae*III was followed by religation to generate plasmids pCH-30CAT and pCH-131CAT, respectively. The 1.7-kb *Alw*44I–*Bam*HI fragment of pCH-340CAT was cloned into *Bam*HI-digested, *Alw*44I partially digested pE4.8 to generate pCH-590CAT. Insertion of a 1.2-kb *Hind*III–*Eco*RI upstream fragment into *Eco*RI partially digested pCH-590CAT resulted in pCH-1790CAT.

Mutation of FP-2, the putative DBP-binding site centered at approximately –225 in the C7αH promoter, was accomplished using an in vitro oligonucleotide-directed mutagenesis kit (Amersham), in which the sequence 5′-ATGTTATG-3′ from –232 to –225 from the first major RNA start site was replaced with 5′-TACCGCAC-3′ in the plasmid pCH-340CAT to generate pCH-340MCAT. The sequence of the mutated promoter region was confirmed by sequencing.

Plasmids purified twice by CsCl gradient centrifugation were used in transient transfection assays in the human hepatoma cell line HepG2 as described previously (Maire et al. 1989). Subconfluent cells were transfected by the calcium phosphate method using 8 μg of CAT reporter constructs, with or without the DBP eukaryotic expression vector pCMV–DBP (Mueller et al. 1990), along with a plasmid containing a retrovirus LTR-driven luciferase gene (deWet et al. 1987), and carrier plasmid to equalize total DNA added. CAT assays were performed as described, using [¹⁴C]chloramphenicol and analyzing reaction products by silica gel thin layer chromatography (TLC) (Gorman et al. 1982). CAT values were quantitated using a Berthold Tracemaster 20 TLC linear analyzer and Chroma software package. Luciferase assays were performed as described (deWet et al. 1987) and quantitated using a Bio-Orbit 1250 luminometer to provide values for standardization of transfection efficiency. Transfection results are presented as the percentage of signal relative to the positive control signal, pSV2CAT (Gorman et al. 1982), after normalizing for transfection efficiency by dividing CAT values by relative luciferase values.

DNase I footprinting assays

DNase I footprinting was performed as described (Lichtsteiner et al. 1987), using a *Hind*III–*Alw*44I C7αH promoter fragment (–340 to –30 bp from the first major RNA start site) radiolabeled at the *Hind*III site using the *E. coli* DNA polymerase I Klenow fragment and [α -³²P]dATP. Approximately 1×10^4 to 5×10^4

cpm of probe DNA was incubated with serial dilutions of recombinant DBP or recombinant LAP, which were overproduced in *E. coli* and purified by heparin–agarose column chromatography (Descombes et al. 1990; E. Falvey, unpubl.), or incubated with 20 μg of morning or evening rat liver NUN extracts. Samples were then digested on ice with 1 μg/ml of DNase I for 2 min, followed by proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation. The resulting DNA fragments were displayed on an 8% polyacrylamide–8 M urea sequencing gel, along with (A + G) and (C + T) chemical sequencing reactions of the probe (Maxam and Gilbert 1980) to orient the protected regions. For DNase I footprinting reactions with nuclear extracts, protected regions persisting in the presence of increasing amounts of nonspecific competitor DNA were identified by the addition of 0–50 ng of sonicated salmon sperm DNA to the binding reaction.

EMSA and supershift assays

Two overlapping oligonucleotides corresponding to the C7αH promoter element FP-2, from –240 to –216, were synthesized, purified, and annealed. This gives the following FP-2 probe when filled in with the *E. coli* DNA polymerase I Klenow fragment and radioactive nucleotides:

5′-CTTTGGATGTTATGTCAGCACATGACTTTC-3′
3′-GAAACCTACAATACAGTCGTGTACTGAAAG-5′

Radiolabeled double-stranded oligonucleotides (5–10 ng per reaction) were incubated on ice for 15 min with 4 μl of NUN nuclear extracts, and complexes were separated on 6% polyacrylamide–0.25× TBE gels, as described previously (Wuarin and Schibler 1990), with the addition of 50 μg/ml of sonicated salmon sperm DNA as nonspecific competitor DNA. Because of the presence of urea in the NUN nuclear extracts, protein concentrations in nondialyzed extracts cannot be conveniently measured. Therefore, the amount of extract used was normalized to equivalent binding activity with the albumin promoter D site (Wuarin and Schibler 1990). In practice, the high reproducibility of protein extraction by this method requires essentially no variation in extract volumes to give nearly identical D site-binding activities (data not shown).

For supershift experiments, binding reactions were performed as described above. After 15 min on ice, dilutions of a rabbit polyclonal anti-rat DBP antiserum (Wuarin and Schibler 1990) or rabbit preimmune serum were added (2 μl per 20-μl reaction) and incubated on ice for an additional 30 min. Samples were then electrophoresed on a nondenaturing polyacrylamide gel, as above.

Other methods

Cytoplasmic RNA and nuclear extracts were prepared from the same tissues by homogenizing liver tissues in homogenization buffer containing 0.5 M sucrose, sedimenting nuclei by low-speed centrifugation, and extracting RNA from the supernatant by guanidine thiocyanate homogenization, followed by pelleting through a 5.7 M CsCl cushion. The nuclear pellet was resuspended in 2.0 M sucrose and centrifuged through a 2.0 M cushion. NUN nuclear extracts were then prepared as described above. Total RNA was prepared from different tissues by direct homogenization in a solution of 4 M guanidine thiocyanate (5 ml/gram of tissue), followed by centrifugation through a 5.7 M CsCl cushion (E. Schmidt and U. Schibler, in prep.).

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