Transactivation of the Rat CYP2C13 Gene Promoter Involves HNF-1, HNF-3, and Members of the Orphan Receptor Subfamily[†,‡]

Catherine Legraverend,[§] Hidetaka Eguchi,[∥] Anders Ström, Olivier Lahuna, Agneta Mode, Petra Tollet, Stefan Westin, and Jan-Åke Gustafsson[*]

Department of Medical Nutrition, Karolinska Institute, F60 NOVUM, S-141 86, Huddinge, Sweden

Received April 14, 1994; Revised Manuscript Received June 22, 1994[⊕]

ABSTRACT: The rat CYP2C13 gene (2C13) encodes one of the constitutive male forms of cytochrome P-450 that are involved in steroid metabolism. In addition to being developmentally regulated, the expression of 2C13 is restricted to the liver and suppressed by the female pattern of growth hormone (GH) secretion at the transcriptional initiation level. In this study, we show that the liver-specific expression, but not the regulation by GH, can be reconstituted with 117 bp to 2 kb of 2C13 5′ flank. Transactivation of the 2C13 promoter requires both HNF-1 and HNF-3 and is influenced by members of the orphan receptor subfamily of transcription factors. Although HNF-4, ARP-1, EAR-2, and COUP-TF bind to the 2C13 promoter *in vitro*, overexpression of EAR-2 and COUP-TF, but not of HNF-4 or ARP-1, results in the potentiation of the HNF-3- and HNF-1-supported activity in non-liver cells.

The sexual dimorphism characterizing growth hormone (GH) secretion in normal as well as in GH-deficient dwarf rats is governed by an ultradian rhythm with a periodicity of approximately 3.3 h (7 surges/day) in males, whereas females display a higher frequency/lower amplitude pattern (Edén, 1979). This sexual dimorphism, manifest at puberty, is important for several sexually differentiated functions, including body growth (Edén et al., 1987), serum levels of cholesterol (Oscarsson et al., 1990), major urinary proteins (Edén et al., 1987), and steroid metabolism (Gustafsson et al., 1983). The molecular basis for hepatic steroid metabolism is a subset of microsomal cytochrome P-450 isozyme products of the CYP2C gene subfamily that catalyze the hydroxylation of steroids at specific positions (Morgan & Gustafsson, 1987). Using full-length cDNA clones for the 2C7, 2C11, 2C12, and 2C13 genes, we have previously shown that their positive or negative regulation by GH occurs at the transcriptional initiation level (Westin et al., 1990; Legraverend et al., 1992).

The present study, focused on the 2C13 gene, was designed to identify the *cis* elements and *trans*-acting factors controlling 2C13 gene expression, so as to gain insight into the mechanism of repression of this gene by GH. P450IIC13, also designated P450 g, is a constitutive male-specific enzyme for which no positive regulator has been identified so far and which, in a reconstituted system, catalyzes the hydroxylation of testosterone at the 6β and 15α positions (Ryan et al., 1984). The level of the male-specific P450IIC13 protein has been found to be low, intermediate, or high (Bandiera et al., 1986; Rampersaud & Walz, 1987; McClellan-Green et al., 1989b). Genomic Southern and Northern blot analyses have revealed the existence of two alleles encoding two different mRNAs (Yeowell et al., 1990; Eguchi et al., 1991). These mRNAs are translated into a stable protein (+g) and an unstable (–g) protein that differ at seven amino acids (Yeowell et al., 1990), but the instability of the –g protein can be accounted for by a single substitution of a Cys for the Ser[180] (Faletto et al., 1992). In contrast to the expression of the protein, which is polymorphic, the mRNA levels are similar in –g and +g male rats.

Hypophysectomy (Hx) of male rats does not affect the 2C13 mRNA level. In contrast, hypophysectomy of female rats raises the 2C13 mRNA level (and the protein for +g individuals) to 50% of the male levels, and this effect is reversed by treating Hx rats of either sex with GH so as to mimic the female physiological pattern of GH secretion (McClellan-Green et al., 1989a; Zaphiropoulos et al., 1990). Transcription of the 2C13 gene is thus repressed by GH in intact female rats. We have previously shown that this repressive role of the female pattern of GH secretion results from a direct action of GH at the level of the hepatocyte (Legraverend et al., 1992; Liddle et al., 1992). In the experiments reported here, we have delineated two adjacent regions of the 2C13 promoter located 5′ and 3′ from nucleotide –117 that act, respectively, as negative and positive regulatory elements in differentiated hepatoma cell lines, but are inactive in other cell lines. The positive element binds several liver-enriched factors, including HNF-1, HNF-3, and COUP-TF, *in vitro*. Using site-directed mutagenesis and a transient cotransfection scheme of expression, we demonstrate the functional significance of these interactions.

MATERIALS AND METHODS

Treatment of the Animals. Sprague–Dawley rats hypophysectomized (Hx) at 6 weeks of age and age-matched intact male and female rats were purchased from Møllegaards Avlslaboratorium (Skensved, Denmark). Recombinant bovine GH (bGH) was administered to Hx male rats starting 16 days after hypophysectomy, at the daily dose of 0.47 mg of bGH/kg, either by one subcutaneous (sc) injection for 7 days or by continuous infusion from osmotic minipumps (Model 2001, Alza Corp., Palo Alto, CA) for 7 days, so as to mimic the male and female patterns of GH secretion, respectively.

Synthetic Oligonucleotides. Complementary oligonucleotides were synthesized on an Applied Biosystems oligonucle-

[†]This work was supported by grants from the Swedish Medical Council (No. 03x-06807).

[‡] The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank, and DDBJ Nucleotide Sequence Databases under the accession number X79810 *R. norvegicus* CYP2C13 gene.

* Author to whom correspondence should be addressed. Fax: +46-8-711 6659. Telephone: +46-8-779 7100.

[§]Present address: Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, 34094 Montpellier Cedex 5, France.

[∥] Present address: Department of Biochemistry, Saitama Cancer Center Research Institute, INA-Machi, Kitaadachi-Gun, Saitama, 362, Japan.

[⊕] Abstract published in *Advance ACS Abstracts*, August 1, 1994.

0006-2960/94/0433-9889$04.50/0

Chart 1

2C13-MUT-C	-183 / -154	GTATCAGCAATTATGTCGCTATATTTATAG
2C13-D	-104 / -82	gatcGAAGTGAGTTAAACAGTGACTAGgatc
2C13-MUT-D	-110 / -81	CATTTTGAAGTGGGCAACACAGTGACTAGT
2C13-E	-126 / -104	gatcTCATCTGCACAAAGTTCATTTTGgatc
2C13-MUT-E	-130 / -101	CTTATCATCTGCTCTCAGGTCATTTTGAAG
rHNF-1-Alb	-64 / -42	tcgaGTGTGGTTAATGATCTACAGTTAtcga
mHNF-3-TTR	-111 / -85	tcgaGTTGACTAAGTCAATAATCAGAATCAGgatc
hHNF-4-ApoC3	-66 / -87	tcgaGCGCTGGGCAAAGGTCACCTGCtcga
mC/EBP-TTR	-1.8kb	ctgcaCCATCTTACTCAACATCCTCctgca
NF-1-Ad2	+21 / +45	ATTTTGGCTTGAAGCCAATATG
mNF-Y-Alb	-95/ -73	gtaggAACCAATGAAATGCGAGGgtagg
hAP-1-Col	-80/-62	ctagAAGCATGAGTCAGACACctag
pGL2-UP	+5524 / +5550	TTATGGTACTGTAACTGAGCTAACATA
pGL2-DOWN	+102 / +77	TTTCTTTATGTTTTTGGCGTCTTCCA
2C13-A	-56 / -37	gatcCAAAATATTGATTCTGATGAgatc
2C13-MUT-A	-60 / -31	ATCACAAAATAGCGATACTGATGATGCATT
2C13-B	-77 / -55	gatcCTGTGCAAACATTGCAAATCACAgatc
2C13-MUT-B	-85 / -56	CTAGTTGGCTGTGAAAACCTTGCAAATCAC
2C13-C	-176 / -157	gatcCAATTAAGTCAATATATTTAgatc

otide synthesizer (Foster City, CA). The 5′ to 3′ sequences of the sense strands of the annealed oligonucleotides are as shown in Chart 1 [lower case letters denote nucleotides present after a fill-in reaction, and underlined characters denote mutated bases or core binding sites for known transcription factors; rHNF-1-Alb (Cereghini et al., 1988), mHNF-3-TTR (Costa et al., 1989), hHNF-4-ApoC3 (Ogami et al., 1991), mC/EBP-TTR (Costa et al., 1988), NF-1.Ad2 (Chodosh et al., 1988), mNF-Y-Alb (Lichtsteiner et al., 1987), hAP-1-Col (Lee et al., 1987)].

Expression Vectors. The pBJ5 mHNF-1 vector contains the mouse HNF-1α cDNA driven by the SR-α promoter (Kuo et al., 1990). The CMV-HNF-3α, -3β, and -3γ plasmids contain cDNAs coding for the rat HNF-3α, -3β, and -3γ transcription factors, respectively (Pani et al., 1992). The pLEN 4S plasmid contains the rat HNF-4 cDNA driven by the human metallothionein promoter (Sladek et al., 1990). The pMT2-ARP-1 and pMT2-EAR-2 contain the cDNAs for human ARP-1 and EAR-2 driven by the adenovirus promoter (Mietus-Snyder et al., 1992). CMV-COUP contains the human COUP-TF cDNA (Wang et al., 1989). CMV-RXR contains the rat RXR cDNA (Gearing et al., 1993). The pCMV-GHR vector contains the rat GH receptor driven by the CMV promoter and enhancers (Francis et al., 1993). CMV-C/EBPα and pSV-C/EBPβ contain the mouse C/EBPα and C/EBPβ cDNAs put in front of the CMV and TK promoters, respectively (Legraverend et al., 1993). The pSCT-DBP vector contains the rat DBP cDNA driven by the CMV promoter (Mueller et al., 1990). The Spi2–175 vector contains the sequence –175/+8 fused in front of the CAT gene in the pEMBL vector (Paquereau et al., 1992).

Isolation of the 2C13 5′Flanking Sequence and Construction of 2C13 Promoter Deletions and Site-Directed Mutants. A 3.8-kb *Eco*RI–*Bam*HI 5′ flank fragment of CYP2C13 +g was isolated from a λ EMBL-3 clone (Eguchi et al., 1991) and subcloned into the pGEM3Z vector. In order to remove the native translation start site, this construct was cleaved with *Nco*I and *Sph*I before being subjected to unidirectional digestion with exonuclease III. A 1864-bp *Hin*dIII fragment isolated from one of the deletion constructs (+15 at the 3′ end) was subcloned into the promotorless luciferase (Luc) reporter pGL2 basic vector (Promega, Madison, WI). Progressive 5′ deletion was obtained by restriction enzyme digestion followed by self-ligation (–1430, –712, –538, –86, and –32) or unidirectional digestion of WT-538Luc with exonuclease III. Site-directed mutagenesis of the first 198 nucleotides of the 2C13 5′ flank was done by overlap extension using the polymerase chain reaction (Ho et al., 1989). The sequences containing single base substitutions are listed in Figure 4. The sequences of all constructs were determined by the dideoxy-mediated chain termination method with the Sequenase version 2.0 DNA-sequencing kit from USB (Cleveland, OH).

DNase I Footprinting and Gel Retardation Assays. Rat liver nuclei were isolated by centrifugation through buffered 2 M sucrose as described earlier (Legraverend et al., 1992). Nuclear extracts were prepared according to the method of Gorski et al. (1986) in the presence of protease and phosphatase inhibitors. Whole cell extracts were prepared by thawing snap-frozen cell pellets in 1 vol of extraction buffer (10 mM Hepes (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 5% glycerol, and 1 mM DTT), followed by centrifugation to remove debris

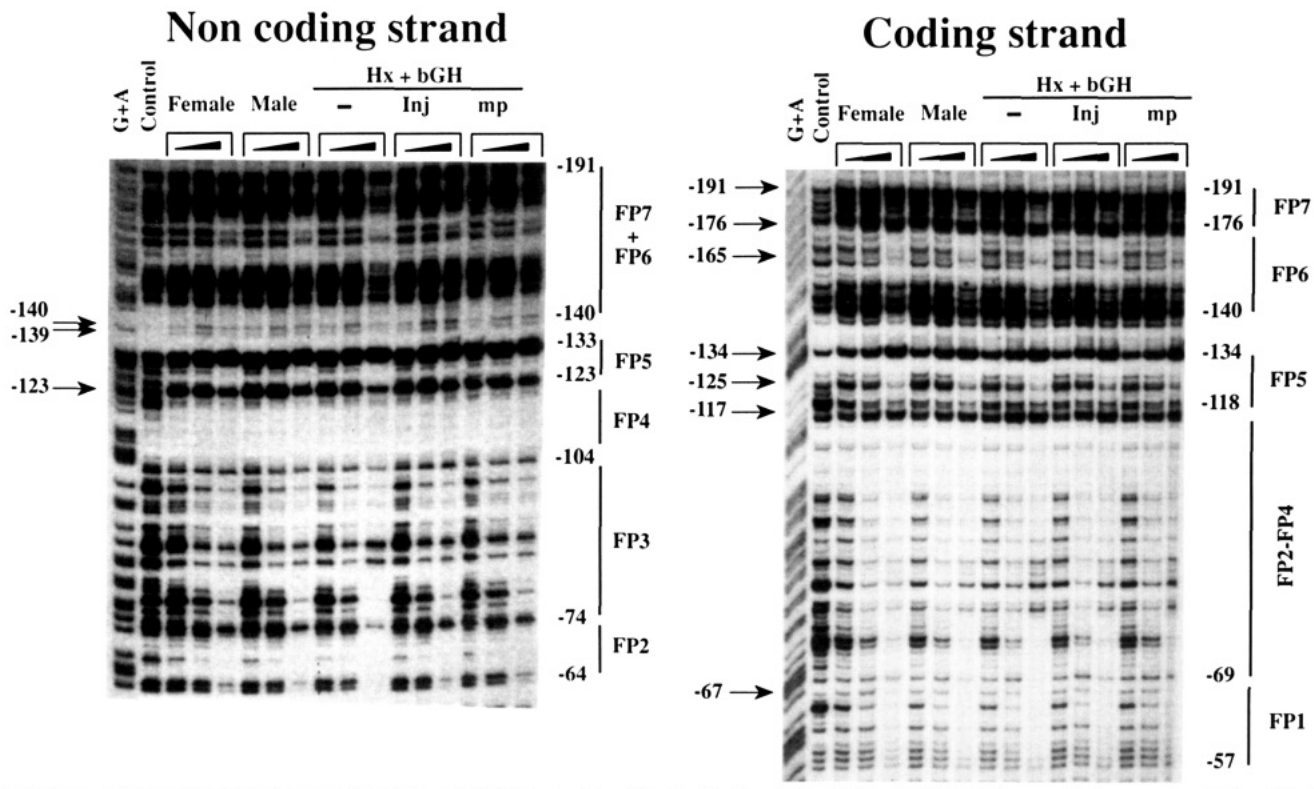

FIGURE 1: DNase I protection analysis of the rat 2C13 promoter. The *Dra*I–*Bam*HI (–363 to +24) 2C13 genomic fragment subcloned into pGEM3Z was labeled by 5′ end kination at the *Xba*I site of the polycloning site (noncoding strand, left) or by fill-in reaction at the same site with Klenow polymerase (coding strand, right). The ^{32}P-labeled probes were partially digested with DNase I in the absence (control) or the presence of increasing amounts (10–40 μg of protein) of rat LNE from intact females (F) and males (M) or from Hx males treated or not with bGH so as to mimic the male (inj) or the female (mp) pattern of GH secretion. The positions of the footprints (FP1–FP7) were determined by size fractionation of the DNase I digestion products on a denaturing polyacrylamide gel, along side the (G+A) Maxam–Gilbert degradation products of the same end-labeled fragment.

and storage of the supernatant at –135 °C until use. A DNA fragment of the 2C13 promoter was used to generate probes suitable for DNase I footprinting analysis as follows: the *Dra*I–*Bam*HI (–363/+24) fragment of the CYP2C13 gene was inserted between the *Sma*I and *Bam*HI sites of pGEM3Z. Labeling was done at the *Xba*I site of the polycloning site by 5′ end kination with T4 polynucleotide kinase (Figure 1A, non coding strand) or fill-in reaction of 3′ ends at the same site with Klenow polymerase (Figure 1B, coding strand). Following digestion with *Eco*RI, the labeled fragment was purified by electrophoresis through a 4% polyacrylamide gel. DNase I footprinting analysis was performed as previously described (Nelsen et al., 1990).

For gel retardation assay, double-stranded oligonucleotides were labeled to high specific activity by filling in with Klenow polymerase. Binding reactions were incubated at +4 °C for 30 min. The reaction mixture contained 0.5 ng of probe (25 000–50 000 cpm) with or without 50–100-fold molar excess of unlabeled oligonucleotide, crude rat liver nuclear extract, or whole cell extract corresponding to 1–6 μg of protein, 2–4 μg of poly(dI–dC) (Pharmacia, Sweden), 10 mM Hepes (pH 7.6), 4% Ficoll 400, 80 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT in a final volume of 20 μL. Protein–DNA complexes were resolved on preelectrophoresed 0.25 x TBE (1 x TBE: 0.09 M Tris–borate and 0.002 M EDTA) native 4% polyacrylamide gels (29:1) at +4 °C. Antisera produced in rabbits against HNF-3α, HNF-3β, and HNF-3γ (Lai et al., 1990) were used for "supershift" assays. In each case, 1 μL of undiluted to 10-fold-diluted antiserum was added to the reaction 15 min prior to the addition of the probe.

Cell Lines, Transfection, and Transient Expression Assays. The Fao (rat hepatoma) was a kind gift from M. C. Weiss of the Pasteur Institute. The HeLa (human cervix carcinoma), HepG2 (human hepatoma), CHO (Chinese hamster ovary), and Cos-7 (green monkey kidney) cell lines were obtained from the NIH ATCC repository (Bethesda, MD) and grown according to the ATCC recommendations. Cells were plated in duplicate 60-mm Petri dishes (Nunc, Norway) and transfected at 30% confluency. A DNA solution containing a total of 3 μg of DNA including 1 μg of luciferase reporter plasmid, various amounts of expression vectors ranging from 0.25 to 1 μg of DNA, and 0.4 μg of pSV2 CAT plasmid (Stratagene, La Jolla, CA) or 0.1 μg of pSV2-Luc in 20 μL of F12 medium was mixed with 20 μL of lipofectin reagent (BRL, Bethesda, MD) just before addition to the cultures overlaid with 2 mL of medium supplemented with 1% fetal calf serum. Luciferase and chloramphenicol acetyl transferase (CAT) activities were measured 24 h after transfection according to standard procedures (Maniatis et al., 1989). Normalized luciferase activity is expressed in light units (1 light unit is the recorded signal corresponding to 1 μV/cpm of ^{14}C acetylated chloramphenicol). Transfection has been repeated 2–6 times with different plasmid preparations, and data from a representative experiment are presented.

RESULTS

Footprinted Regions of the 2C13 Promoter Contain Putative Binding Sites for HNF-1, HNF-3, and HNF-4. Reviewing DNA sequences known to confer liver-specific or ubiquitous gene expression, we observed a significant level of identity between the –281 to –41 2C13 sequence and reported binding sequences for known transcription factors. These sequences and relevant references are listed in Table 1. Sites A and C are very similar to each other and to the HNF-3 binding sites found in the transthyretin (TTR), α1-antitrypsin,

Table 1: Sequence Comparison of the P4502C13 Proximal Promoter with Other DNA Regulatory Elements

2C13 gene		other gene/consensus		
site/footprint	sequence	sequence	site	factor
A (−51/−41)	TATTGATTCTG	TATTGAC/TTTA/TG	consensus	HNF-3[a]
C (−163/−173)/FP6	TATTGACTTAA	TATTGAC/TTTA/TG	consensus	HNF-3[a]
B (−67/−73)/FP2	TGTTTGC	TGTTTGC	consensus	HNF-3[b]
(−59/−67)/FP1	ATTTGCAAT	ATTTGCAAT (−2481/−2473)	TAT	C/EBP[b]
		TT/GNNGNAAT/G	consensus	C/EBP
D (−97/−81)/FP3	GTTAAACAGTGACTAGT	GTTTATCAGTGACTAGT (+846/+862)	ApoB	HNF-1[c]
E (−121/−109)/FP4	TGCACAAAGTTCA	TGGGCAAAGGTCA (−70/−80)	ApoC3	HNF-4[d]
F (−124/−138)/FP5	TGATAAGAATTTTTC	TGATAAGACTTATCT (−687/673)	GATA-1	GATA-1[e]

[a] Costa et al., 1988. [b] Grange et al., 1991. [c] Brooks & Levy-Wilson, 1992. [d] Ladias et al., 1992. [e] Tsai et al., 1991.

and α-fetoprotein genes. Site B contains the TGTTTGC HNF-3 binding sequence common to some liver-specific genes, including the tyrosine aminotransferase (TAT) and albumin genes. There is a 1-bp overlap between a putative C/EBP binding site ($^{-59}$ATTTGCAAT^{-67}) and site B ($^{-67}$TGTTTGC^{-73}). Site D is very similar to the HNF-1 binding site present in the second intron of the apolipoprotein B gene. Site E resembles a sequence present in the apolipoprotein C3 gene and is known to bind several orphan receptors that are members of the steroid hormone receptor superfamily, including HNF-4, ARP-1, EAR-2, and EAR-3/COUP-TF. Immediately 5′ from the putative orphan receptor binding site lies a sequence (site F) similar to that found in the promoter of the GATA-1 gene and known to bind the zinc-finger protein GATA-1.

As an initial approach to identify the transcription factors that interact with the 2C13 gene, we performed DNase I footprinting analysis of the promoter region spanning nucleotides −363 to +24 in the presence of increasing amounts (10–40 μg of protein) of liver nuclear extracts (LNE) from intact rats of both sexes or from Hx male rats treated or not with bGH so as to mimick the male (inj) or the female (mp) pattern of GH secretion. The regions protected from DNase I digestion were designated FP1–FP7, starting from the most proximal region with respect to the transcriptional start site of the gene. As shown in Figure 1, the extent of the footprints, as well as the appearance of the DNase I hypersensitive sites, changed upon the addition of increasing concentrations of proteins. On the noncoding strand, the −74/−64 (putative HNF-3 binding site B), −104/−74 (putative HNF-1 binding site D), −118/−104 (putative orphan receptor binding site E), and −133/−123 (GATA-1-like binding site F) regions were clearly protected at low protein input, with hypersensitive sites present at positions −140, −139, and −123. On the coding strand, the protected regions extended from −69 to −57 (putative C/EBP binding site), −118 to −69, −134 to −118 (putative GATA-1 binding sequence), −176 to −140 (putative HNF-3 binding site C), and −191 to −176, with hypersensitive sites at −191, −176, −165, −134, −125, −117, and −67. Heating the extracts at 80 °C for 10 min prevented the formation of all footprints (data not shown), suggesting that C/EBPα does not bind or is dependent on heat-labile factors for binding to the 2C13 promoter. None of the footprints or DNase I hypersensitive sites was sex- or GH-dependent. The binding of liver nuclear proteins at most of the sites predicted by computer analysis was thus confirmed by footprinting analysis. However, the dramatic sex difference in transcription observed with the endogenous 2C13 gene was not associated with any substantial difference in the protection of the analyzed region of the 2C13 promoter from the action of DNase I *in vitro*.

Liver-Specific Expression but Not Hormonal Regulation Can Be Reconstituted during Transient Transfection Experiments. To map the putative *cis*-acting elements mediating

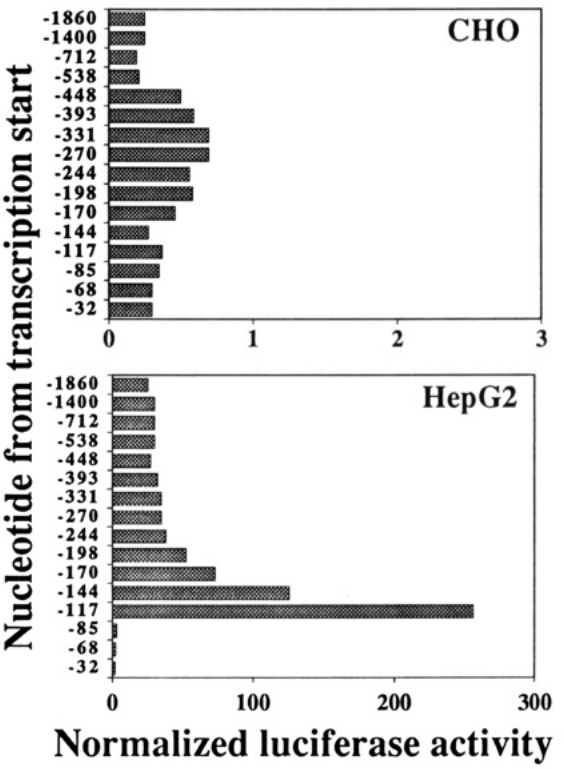

FIGURE 2: Functional analysis of the 2C13 5′ flank. Transient expression of truncated 2C13 promoter–luciferase constructs in CHO and HepG2 cells. 2C13–luciferase (3 μg) constructs and 0.4 μg of PsV2-CAT were transfected into the cells. PsV2-CAT was used as internal control for transfection with luciferase constructs. Means of duplicates of normalized luciferase activities are shown.

the liver-specific and hormonally regulated transcription of the 2C13 gene, chimeric constructs containing 32–1864 bp of 2C13 5′ flank fused to the luciferase gene were transfected into differentiated hepatoma (HepG2, FaO) and nonhepatic (HeLa, CHO) cell lines. The activity of the 2C13 promoter was found to be 13%, 1.3%, 0.04%, and 0.05% of that of the SV40 promoter in FaO, HepG2, CHO, and HeLa cells, respectively (data not shown). The 2C13 promoter is thus 30–300-fold more active in liver than in nonliver cell lines. In HepG2 (Figure 2) and in FaO (data not shown), maximal luciferase activity was achieved with only 117 bp of the 2C13 5′ flanking region. Deletion of part or all of the sequence spanning nucleotides −1920 to −171 did not influence the 2C13 promoter activity in hepatoma cells. In contrast, deletion of the segment spanning nucleotides -170 to −118 resulted in a 5-fold increase, whereas deletion of a 30-bp segment spanning nucleotides −117 to −86 led to a virtually complete suppression of promoter activity. This deletion analysis thus identified two adjacent regions located 5′ and 3′ from nucleotide −117 that act as negative and positive regulatory elements, respectively, in differentiated hepatoma cell lines.

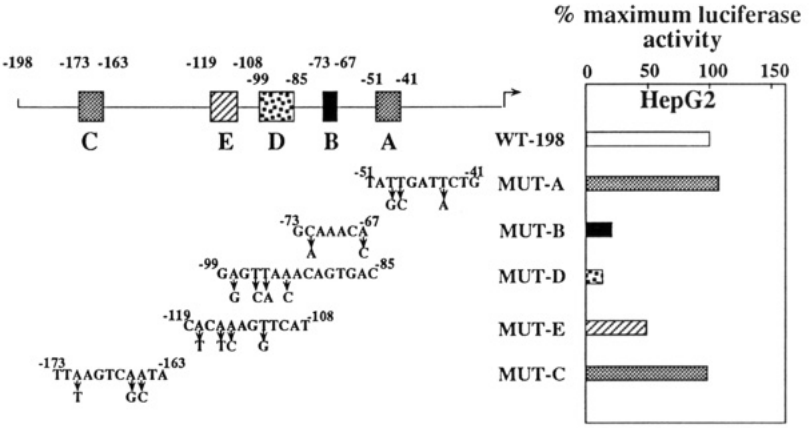

FIGURE 3: Analysis of the 2C13 promoter by site-directed mutagenesis. Luciferase vectors carrying the wild-type (WT-198) or mutated (MUT-A–MUT-E) versions of the 2C13 promotor were transiently transfected into HepG2 cells. The transcriptional activities of the mutants are expressed relative to that of the wild type.

The expression of the endogenous 2C13 gene previously has been shown to be suppressed, at the transcriptional initiation level, by the continuous presence of GH *in vivo* and in cultured hepatocytes (Legraverend et al., 1992). We thus decided to map the putative GH-responsive element(s) (GHRE) in the 5′ flanking region of the 2C13 gene. To this end, we cotransfected various 2C13 deletion constructs and a CMV promoter-driven expression vector for the rat GH receptor (GHR) into HepG2 cells. As a positive control for the GH effects we used a rat serine protease inhibitor–CAT fusion construct containing 175 bp of the Spi2–1 gene 5′ flank (Spi2-175). None of the 2C13 constructs was affected by treatment with GH, whereas the Spi2-175 construct was regulated by GH, Dex, and GH+Dex in a manner similar to the endogenous Spi2-1 gene (data not shown) (Le Cam et al., 1987). Similar results were obtained in primary cultures of hepatocytes, using rat endogenous GHR (data not shown).

A Composite Regulatory Element of the 2C13 Promoter Integrates the Signals from HNF-3, HNF-1, and Members of the Orphan Receptor Family of Transcription Factors. On the basis of the results of the deletion and DNase I footprinting experiments described above, the minimal region that confers liver-specific expression to the 2C13 promoter–luciferase constructs spans nucleotides –117 to –86 (see Figure 2) and contains a putative HNF-1 binding site corresponding to the FP2 footprint (see Figure 1). To clarify the role of the putative HNF-1 binding site and assess the contributions of the other putative binding sites to the liver-specific expression of the 2C13 gene, PCR-based mutations were introduced into the –198–Luc construct at sites A–E, and the effects of such mutations were tested in HepG2 cells. As shown in Figure 3, mutations of sites B (HNF-3 binding site), D (HNF-1 binding site), and E (orphan receptor binding site) brought the luciferase activity down to 20%, 15%, and 50%, respectively, of the wild-type value. In contrast, mutation of sites A or C had no effect. These results thus clearly identified the B+D+E cluster as a key regulatory region responsible for liver-specific expression of the 2C13 promoter. The highly deleterious effect of the B mutation was, however, at odds with the fact that the –85–Luc construct containing an intact B site was inactive in HepG2 cells (see Figure 2). This implied that site B may not serve its function as an HNF-3 binding site, but it may provide the right 5′ flanking sequence for the protein–DNA interactions occurring at the neighboring D site. The results of a cotransfection experiment in CHO cells show that this actually is not the case, since the 2C13 promoter is indeed activated by the α, β, and γ HNF-3 isoforms (Figure 4). Maximal activation by each isoform displays a somewhat different

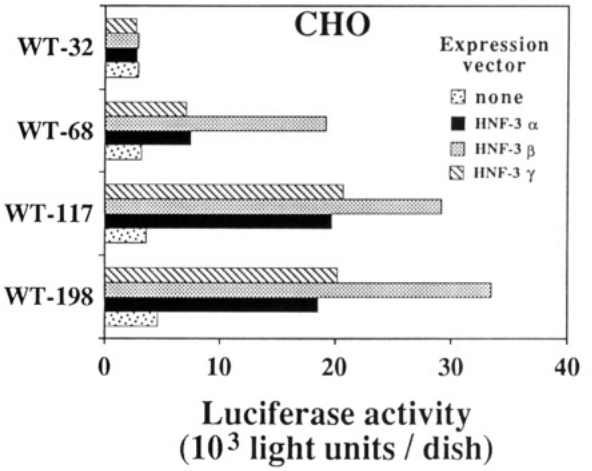

FIGURE 4: Transactivation of 2C13 promoter–luciferase constructs by HNF-3α, HNF-3β, and HNF-3γ in the non-liver CHO cell line. Amounts of DNA used for transfection: 1 μg of 2C13–luciferase construct and 0.5 μg of expression plasmid.

sequence requirement: –68 to –32 (containing site A) for HNF-3β and –117 to –68 (containing site B) for HNF-3α and -3γ. The results presented so far can thus be interpreted as sites A and B being *bona fide* HNF-3 binding sites with different specificities toward the three HNF-3 isoforms and depending on other promoter sequences (D and E) for function in liver cells.

If both sites B and D are functional and indispensable for liver-specific expression of the 2C13 promoter, how do they interact? What is the exact function of the E site? To answer these questions, a reconstitution experiment was designed during which optimum or suboptimum concentrations of plasmids expressing transcription factors likely to bind to sites A–E were transfected alone or in pair combinations together with the –198–Luc construct. The results show that HNF-1α, COUP-TF, HNF-3α, and HNF-3β are indeed potent transactivators of the –198–Luc construct when used alone (Figure 5A). The decreased transactivation seen with increasing amounts of several of the expression vectors may be attributable to squelching of the basal transcription factors. A weaker transactivation was obtained with DBP, HNF-4, and RXR, whereas C/EBPα, C/EBPβ, and ARP-1 were inactive. Furthermore, expression of COUP-TF at a level that does not activate the 2C13 promoter nor a CMV-driven CAT construct increased transactivation by HNF-1α (Figure 5B) and HNF-3β (Figure 5C) by 3- and 6-fold, respectively, whereas EAR-2 was less efficient. These observations are in consonance with the results presented earlier and support the

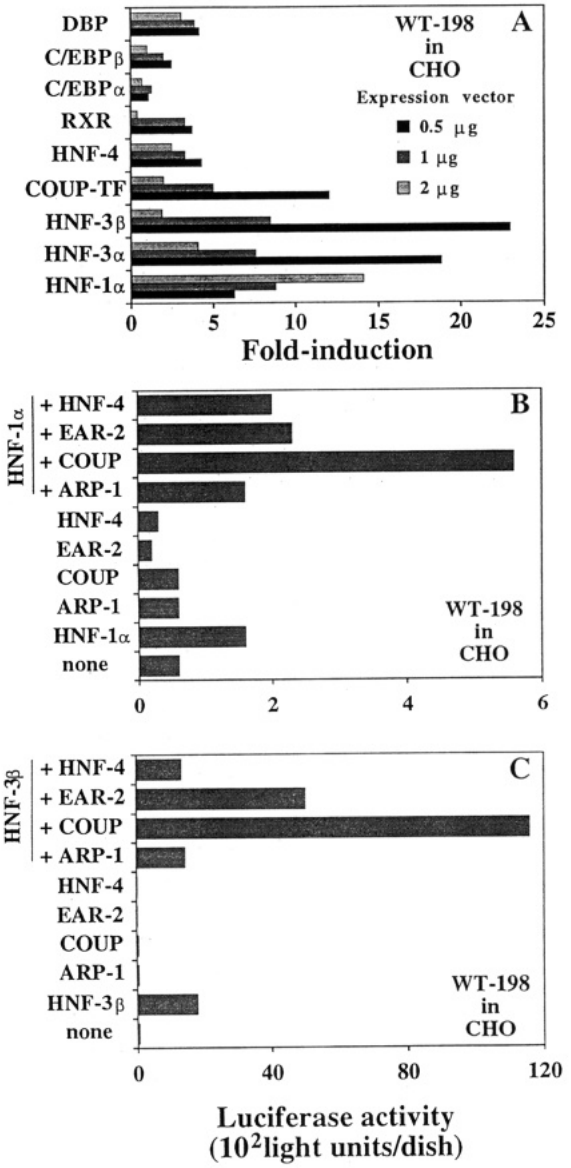

FIGURE 5: Reconstitution of the liver-specific activity of the 2C13 promotor. (A) Screening of known transcription factors for their potential to transactivate the wild-type 2C13 promoter. The WT-198 luciferase construct was transfected into the non-liver CHO cell line. Amounts of DNA used for transfection: 1 μg of reporter and 0.5–2 μg of expression vector. (B) Potentiation of HNF-1α-supported transactivation of the WT-198 construct by COUP-TF. (C) Potentiation of the HNF-3β-supported transactivation of the WT-198 construct by COUP-TF and EAR-2. Amounts of DNA used for transfection: 2 μg of WT-198 reporter and 0.25 μg of each expression plasmid.

notion that site B (HNF-3), which is necessary but not active *per se* in HepG2 (compare Figures 2 and 4), collaborates with sites D (HNF-1) and E (orphans) during the transactivation of the 2C13 promoter.

Characterization of the Proteins Interacting with the −119/−67 Region of the 2C13 Promoter. We have shown that protection of the 2C13 promoter from DNase I digestion was not influenced by the sex or the GH status of the donor rat. This observation implies that GH could modulate the activities of transcription factors without affecting their DNA binding properties (through posttranslational modification). Alternatively, GH could induce the replacement of some factors by others without any measurable change in sensitivity of the chromatin to nucleases. We thus decided to monitor DNA sequence-specific binding using the gel retardation assay with oligonucleotides (oligos) spanning the 2C13 sequences identified by DNase I footprinting, deletion analysis, and site-directed mutagenesis as being functionally important. For the sake of clarity, we have adopted a nomenclature whereby each protein–DNA complex is identified by the probe it binds to (2C13 probes B, D, and E), followed by an arabic number given in order of increasing electrophoretic mobility.

Table 2: Schematic Presentation of Competition in Gel Retardation Assays with Probes B, D, and E

protein–DNA complex										competitor oligo
B							D		E	
1	2	3	4	5	6	7	1	2		
+[a]				+			–		–	B
–				–			+		–	D
–				–			–		+	E
–				+			–		–	HNF-3-TTR
+				–			ND[b]		ND	AP-1-Col
–				–			+		–	HNF-1-Alb
–				–			–		+	HNF-4-ApoC3

[a] Indicates whether or not competition occurred. [b] Not determined.

Incubation of rat LNE with the B probe gave rise to a complex pattern consisting of at least seven bands (Figure 6A). An oligo containing the strong HNF-3 binding site of the mouse transthyretin gene (HNF-3-TTR) efficiently disrupted the formation of most complexes formed between rat LNE and probe B with the exception of B1, which was eliminated by competition with a 100-fold molar excess of B or AP-1-Col oligos (Table 2). Furthermore, extracts of Cos-7 cells overexpressing the HNF-3α, −3β, or −3γ isoforms were bound by probe B (Figure 6A) to the same extent as by the HNF-3-TTR probe (data not shown). This result confirms that site B is a *bona fide* HNF-3 binding site.

The only oligos capable of competing for protein binding to the D probe were D itself and the high-affinity HNF-1 binding site of the rat albumin gene promoter (Table 2). As shown in Figure 6B, one of the two complexes formed between probe D and rat LNE (D1) migrates in a manner similar to the complex formed with extracts of Cos-7 cells overexpressing HNF-1α. D1 thus is likely to contain HNF-1α homodimers, whereas the faster D2 complex displays the electrophoretic characteristics of an αβ–heterodimer-containing complex (Rey-Campos et al., 1991). More D1 than D2 was produced with the LNE of intact rats of both sexes. The D2 complex was increased substantially in proportion to D1, following the hypophysectomy of male rats. Following GH treatment of the Hx males, the D1/D2 ratio was shifted toward that of normal animals. Thus, it looks as if the GH status of the rat seems to be important for the maintenance of the αα/αβ ratio. Identical results were obtained with a probe containing the high-affinity HNF-1 binding site of the albumin promoter (data not shown).

In contrast to the banding pattern obtained with the D probe, that obtained with probe E was neither sex- nor GH-dependent (Figure 6C). Complexes formed with rat LNE and HepG2 extracts migrated similarly to those generated with extracts of Cos-7 cells overexpressing rat HNF-4, human ARP-1, and human COUP-TF, but not EAR-2 (data not shown). In addition, cross competition was achieved with a sequence present in the human ApoC3 gene promoter and known to bind all of these factors (Ladias et al., 1992) (data not shown and Table 2).

DISCUSSION

In this study, we have identified and characterized the protein binding sites that are responsible for liver-specific

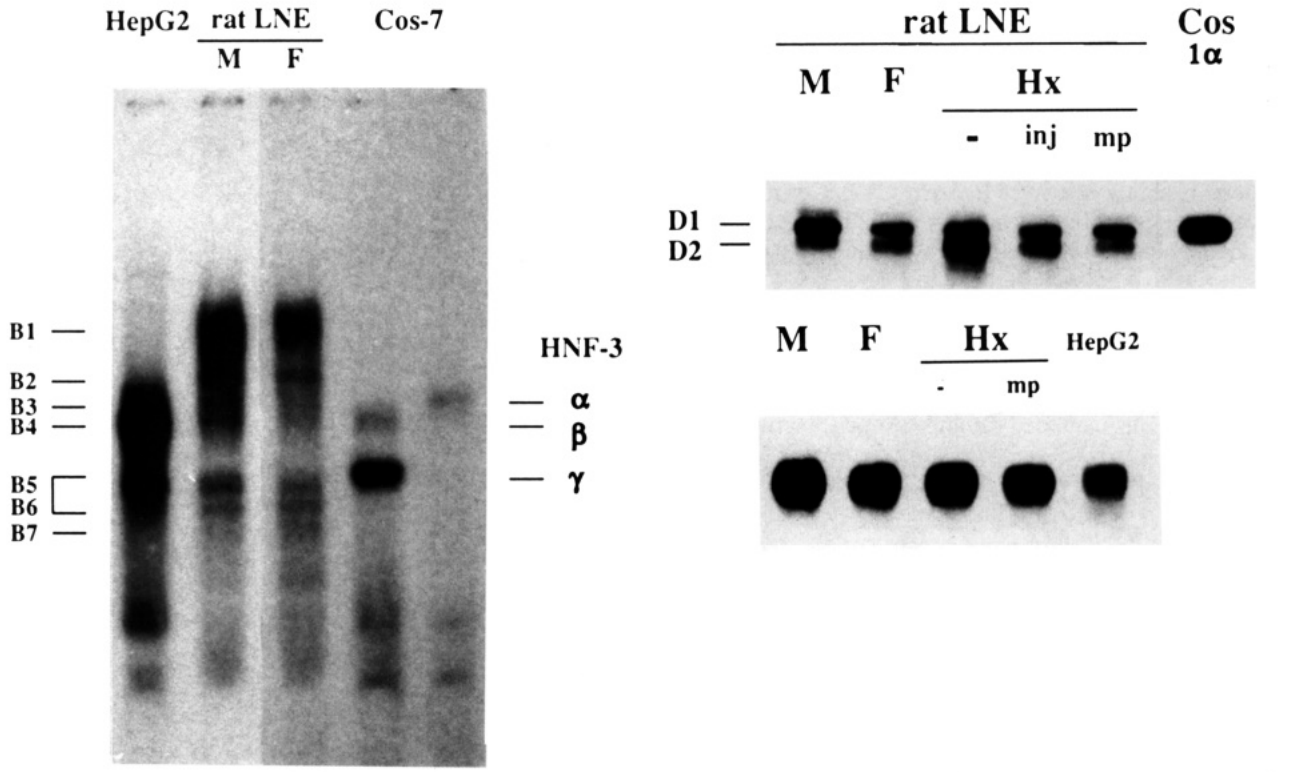

FIGURE 6: Analysis of the liver protein–DNA sequence-specific interactions by gel retardation assay. (A, left) Effects of sex on the binding properties of the 2C13 probe B with HepG2 whole cell extracts (lane HepG2), liver nuclear extracts (lane rat LNE) from male (lane M) and female (lane F) rats, and whole cell extracts from cos-7 cells overexpressing HNF-3 isoforms. (B, top right) Effects of sex (lane M, male; lane F, female), hypophysectomy (lane Hx, –), and GH substitution treatment of Hx rats (lane Hx, inj (injection of GH); lane Hx, mp (GH infusion via minipump)) on the binding of HNF-1 isoforms to 2C13 site D. (C, bottom right) Rat liver nuclear extracts from male (lane M) and female (lane F) rats, hypophysectomized rats (lane HX, –), GH-infused hypophysectomized rats (lane Hx, mp), and whole cell extracts from HepG2, interacting with 2C13 probe E.

expression of the rat 2C13 gene promoter. The evidence that HNF-1 binds to the 2C13 promoter is based on the results of the DNase I footprinting analysis, specific disruption of complex D formation by competition with the HNF-1 binding site of the rat albumin promoter, and transactivation of the 2C13–198–Luc construct by HNF-1α in nonliver cell lines. Using the same strategy, we have shown that site B, lying 10 base pairs downstream from site D, is a target for the transcription factors HNF-3α, -3β, and -3γ. Since mutation of either site D or B leads to a dramatic reduction in 2C13 promoter activity, it is possible that transactivation of the 2C13 promoter in HepG2 cells requires both HNF-1 and HNF-3. The HNF-1α and HNF-1β proteins are the most distant relatives of the homeoproteins identified so far. These proteins share dimerization and DNA binding but not activation domains (Mendel et al., 1991). Both proteins have been shown to heterodimerize *in vitro* (Mendel et al., 1991), and *in vivo* (Rey-Campos et al., 1991) and to transactivate several hepatic genes (Herbst et al., 1991; Ron & Habener, 1992). The HNF-3 family of transcription factors are evolutionarily ancient and related to the *Drosophila* homeotic protein forkhead (fkh), with which they share a recently characterized DNA binding motif (Clark et al., 1993). Because the forkhead is critical for the differentiation of the intestine in *Drosophila melanogaster* (Weigel et al., 1989), and because HNF-3 is expressed in organs of endodermal origin like the liver and intestine, HNF-3 is suspected to play an important role in mammalian liver development. HNF-3 also regulates the expression of HNF-1 and is therefore known to function at an early position within the hierarchy of factors involved in hepatocyte differentiation (Kuo et al., 1992). In view of the fact that GH modulates hepatic gene expression at a predominantly transcriptional level, it is conceivable that the expression, the DNA binding activity, or the activation potential of at least some of the liver-enriched transcription factors could be influenced by GH. Our finding that the GH status of a rat influences the HNF-1α/HNF-1β binding ratio renders plausible the hypothesis that modulation of the HNF-1α/HNF-1β ratio is implicated in the mechanism by which GH represses 2C13 gene expression.

Our transient transfection scheme of expression failed to reproduce the GH-mediated repression of 2C13 transcription observed in isolated cultured hepatocytes (Legraverend et al., 1992). The strong positive effect exerted by GH on a short Spi2-1 promoter construct (Spi2–175) contrasted with the lack of GH effect on 2C13 gene constructs containing up to 1.9 kb of 5′ flank and transiently transfected into HepG2 cells expressing the rat GHR or isolated hepatocytes expressing their endogenous GHR (data not shown). GH inducibility is conferred on the Spi2-1 promoter by the presence of a positive regulatory element to which as yet uncharacterized GH-dependent factors bind (Yoon et al., 1990; Rossi et al., 1992). Constructs containing the promoters of the insulin and somatostatin (Billestrup et al., personal communication), lipoprotein lipase (Francis et al., 1993), and ovine β-lactoglobulin (Goujon et al., personal communication) genes have also been shown to respond positively to GH in transient transfection experiments. Because similar experiments have failed to reproduce the transcriptional effects exerted by GH on the endogenous 2C11 and 2C12 genes (data not shown), we are inclined to believe that the putative GHREs of the 2C13, 2C11, and 2C12 genes may not be located in the first 2 kb of the 5′ flank of the genes or that collaboration between elements present in the first 2 kb of the 5′ flank and one or several elements located elsewhere may be required. The modulation of the CYP2C genes by GH could also be highly

dependent on specific changes in the chromatin structure that cannot be reconstituted in transient transfection experiments.

HNF-4, EAR-3/COUP-TF, and ARP-1 belong to a subgroup of the steroid receptor superfamily known as the orphan receptor group also comprising EAR-2 (Wang et al., 1989). The orphan receptor binding sequence of the 2C13 promoter is highly conserved in many CYP2C genes (Venepally et al., 1992). Interestingly, we have found the pattern of transactivation by HNF-4, ARP-1, EAR-2, and EAR-3 to be different for the 2C7, 2C11, 2C12, and 2C13 promoters (A. Ström and S. Westin, unpublished experiments). The positive transactivation of the 2C13 promoter by COUP-TF (Figure 5) contrasts with the negative effect of COUP-TF on the transcription of the apolipoprotein CIII gene, the latter presumably being mediated through competition for binding to the same site as HNF-4 (Mietus-Snyder et al., 1992). This is an interesting observation given the high level of sequence identity (10 out of 13 bases) that exists between the 2C13 site E and the ApoC3 HNF-4 binding site, since both HNF-4 and EAR-3 are expressed in liver. The finding that EAR-3/COUP-TF but not HNF-4 has the potential to collaborate with HNF-1 and HNF-3 to activate transcription of the 2C13 gene suggests that the intracellular balance between HNF-4 and EAR-3 may be important for the transcription of this gene. Since mutation of either the B or D site decreases the HepG2-supported activity of the 2C13 promoter by 75–80%, whereas that of the E site decreases it by only 50%, interaction of HNF-1 with HNF-3 at the D+B site probably acts as a master switch, whereas the E site plays the role of a strong modulator. The collaboration between sites B, D, and E in transactivating the 2C13 promoter thus represents a variation on a theme common to genes expressed in the liver, such as the human apolipoproteins (Ladias et al., 1992; Paulweber et al., 1993), α1-antitrypsin (Monaci et al., 1988; Tian & Schibler, 1991), and pyruvate kinase genes (Zhou & Benedict Yen, 1991).

ACKNOWLEDGMENT

We thank Akiko Eguchi for skillful technical assistance. We are indebted to Gerald Crabtree, Jim Darnell, Vincent Preziozo, Thierry Grange, John Ladias, Alphonse Le Cam, Gunnar Norstedt, Ueli Schibler, Lee-Ho Wang, and Kleanthis Xanthopoulos for the gifts of expression vectors.

REFERENCES

Bandiera, S., Ryan, D. E., Levin, W., & Thomas, P. E. (1986) *Arch. Biochem. Biophys. 248*, 658–676.

Brooks, A. R., & Levy-Wilson, B. (1992) *Mol. Cell. Biol. 12*, 1134–1148.

Cereghini, S., Blumenfeld, M., & Yaniv, M. (1988) *Genes Dev. 2*, 957–974.

Chodosh, L. A., Baldwin, A. S., Carthew, R. W., & Sharp, P. A. (1988) *Cell 53*, 11–24.

Clark, K. L., Halay, E. D., Lai, E., & Burley, S. K. (1993) *Nature 364*, 412–420.

Costa, R. H., Grayson, D. R., Xanthopoulos, K. G., & Darnell, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3840–3844.

Costa, R. H., Grayson, D. R., & Darnell, J. E. (1989) *Mol. Cell. Biol. 9*, 1415–1425.

Edén, S. (1979) *Endocrinology 105*, 555–560.

Edén, S., Jansson, J.-O., & Oscarsson, J. (1987) in *Growth hormone: basic and clinical aspects* (Isaksson, O., Binder, K., Hall, K., & Hökfelt, B., Eds.) pp 129–151, Excerpta Medica, Amsterdam.

Eguchi, H., Westin, S., Ström, A., Gustafsson, J.-Å., & Zaphiropoulos, P. G. (1991) *Biochemistry 30*, 10844–10849.

Faletto, M. B., Linko, P., & Goldstein, J. A. (1992) *J. Biol. Chem. 267*, 2032–2037.

Francis, S. M., Enerbäck, S., Möller, C., Enberg, B., & Norstedt, G. (1993) *Mol. Endocrinol. 7*, 972–8.

Gearing, K. L., Göttlicher, M., Teboul, M., Widmark, E., & Gustafsson, J.-Å. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1440–1444.

Gorski, K., Carneiro, M., & Schibler, U. (1986) *Cell 47*, 767–776.

Grange, T., Roux, J., Rigaud, G., & Pictet, R. (1991) *Nucleic Acids Res. 19*, 131–139.

Gustafsson, J.-Å., Mode, A., Norstedt, G., & Skett, P. (1983) *Annu. Rev. Physiol. 45*, 51–60.

Herbst, R. S., Nielsch, U., Sladek, F., Lai, E., Babiss, L. E., & Darnell, J. E. (1991) *New Biologist 3*, 289–296.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51–59.

Kuo, C. J., Conley, P. B., Hsieh, C.-L., Francke, U., & Crabtree, G. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9838–9842.

Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E., & Crabtree, G. R. (1992) *Nature (London) 355*, 457–461.

Ladias, J. A. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V., & Cladaras, C. (1992) *J. Biol. Chem. 267*, 15849–15860.

Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H., & Darnell, J. E. (1990) *Genes Dev. 4*, 1427–1436.

Le Cam, A., Pages, G., Auberger, P., Le Cam, G., Leopold, P., Benarous, R., & Glaichenhaus, N. (1987) *EMBO J. 6*, 1225–1232.

Lee, W., Mitchell, P., & Tjian, R. (1987) *Cell 49*, 741–752.

Legraverend, C., Mode, A., Westin, S., Ström, A., Eguchi, H., Zaphiropoulos, P. G. & Gustafsson, J.-Å. (1992) *Mol. Endocrinol. 6*, 259–266.

Legraverend, C., Antonson, P., Floby, P., & Xanthopoulos, K. G. (1993) *Nucleic Acids Res. 21*, 1735–1742.

Lichtsteiner, S., Wuarin, J., & Schibler, U. (1987) *Cell 51*, 963–973.

Liddle, C., Mode, A., Legraverend, C., & Gustafsson, J.-Å. (1992) *Arch. Biochem. Biophys. 298*, 159–166.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

McClellan-Green, P. D., Linko, P., Yeowell, H. N., & Goldstein, J. A. (1989a) *J. Biol. Chem. 264*, 18960–18965.

McClellan-Green, P. D., Negishi, M., & Goldstein, J. A. (1989b) *Biochemistry 28*, 5832–5839.

Mendel, D. B., Hansen, L. P., Graves, M. K., Conley, P. B., & Crabtree, G. R. (1991) *Genes Dev. 5*, 1042–1056.

Mietus-Snyder, M., Sladek, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A. A., Darnell, J. E., & Karathanasis, S. K. (1992) *Mol. Cell. Biol. 12*, 1708–1718.

Monaci, P., Nicosia, A., & R., C. (1988) *EMBO J. 7*, 2075–2087.

Morgan, E. T., & Gustafsson, J.-Å. (1987) *Steroids 49*, 213–245.

Mueller, C. R., Maire, P., & Schibler, U. (1990) *Cell 61*, 279–291.

Nelsen, B., Kadesch, T., & Sen, R. (1990) *Mol. Cell. Biol. 10*, 3145–3154.

Ogami, K., Kardassis, D., Cladaras, C., & Zannis, V. (1991) *J. Biol. Chem. 266*, 9640–9646.

Oscarsson, J., Carlsson, L. M. S., Bick, T., Lidell, A., Olofsson, S.-O., & Edén, S. (1990) *J. Endocrinol. 128*, 433–438.

Pani, L., Overdier, D. G., Porcella, A., & Qian, X. (1992) *Mol. Cell. Biol. 12*, 3723–3732.

Paquereau, L., Vilarem, M.-J., Rossi, V., Rouayrenc, J.-F., & Le Cam, A. (1992) *Eur. J. Biochem. 209*, 1053–1061.

Paulweber, B., Sandhofer, F., & Levy-Wilson, B. (1993) *Mol. Cell. Biol. 13*, 1534–1546.

Rampersaud, A., & Walz, F. G. (1987) *J. Biol. Chem. 262*, 5649–5653.

Rey-Campos, J., Chouard, T., Yaniv, M., & Cereghini, S. (1991) *EMBO J. 10*, 1445–1457.

Ron, D., & Habener, J. F. (1992) *Genes Dev. 6*, 439–453.

Rossi, V., Rouayrenc, J.-F., Paquereau, L., Vilarem, M.-J., & Le Cam, A. (1992) *Nucleic Acids Res. 20*, 1061–1068.

Ryan, D. E., Lida, S., Wood, A. W., Thomas, P. E., Lieber, C. S., & Levin, W. (1984) *J. Biol. Chem. 259*, 1239–1250.

Sladek, F. M., Zhong, W., Lai, E., & Darnell, J. E. (1990) *Genes Dev. 4*, 2353–2365.

Tian, J.-M., & Schibler, U. (1991) *Genes Dev. 5*, 225–2234.

Tsai, S.-F., Strauss, E., & Orkin, S. H. (1991) *Genes Dev. 5*, 919–931.

Venepally, P., Chen, D., & Kemper, B. (1992) *J. Biol. Chem. 267*, 17333–17338.

Wang, L.-H., Tsai, S. Y., Cook, R. G., Beattie, W. G., Tsai, M.-J., & O'Malley, B. W. (1989) *Nature (London) 340*, 163–166.

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E., & Jackle, H. (1989) *Cell 57*, 645–658.

Westin, S., Ström, A., Gustafsson, J.-Å., & Zaphiropoulos, P. G. (1990) *Mol. Pharmacol. 38*, 192–197.

Yeowell, H. N., McClellan-Green, P. D., Negishi, M., & Goldstein, J. A. (1990) *Biochemistry 29*, 713–718.

Yoon, Y.-B., Berry, S. A., Seelig, S., & Towle, H. C. (1990) *J. Biol. Chem. 265*, 19947–19954.

Zaphiropoulos, P. G., Ström, A., Robertson, J. A., & Gustafsson, J.-Å. (1990) *Mol. Endocrinol. 4*, 53–58.

Zhou, D.-X., & Benedict Yen, T. S. (1991) *Mol. Cell. Biol. 11*, 1353–1359.