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An enhancer element 6 kb upstream of the mouse HNF4α1 promoter is activated by glucocorticoids and liver-enriched transcription factors

Alain Bailly, Maria Elena Torres-Padilla, Antoine P. Tinel and Mary C. Weiss*

Unité de Génétique de la Différenciation, FRE 2364 du CNRS, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France

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ABSTRACT

We have characterized a 700 bp enhancer element around –6 kb relative to the HNF4α1 transcription start. This element increases activity and confers glucocorticoid induction to a heterologous as well as the homologous promoters in differentiated hepatoma cells and is transactivated by HNF4α1, HNF4α7, HNF1α and HNF1β in dedifferentiated hepatoma cells. A 240 bp sub-region conserves basal and hormone-induced enhancer activity. It contains HNF1, HNF4, HNF3 and C/EBP binding sites as shown by DNase I footprinting and electrophoretic mobility shift assays using nuclear extracts and/or recombinant HNF1α and HNF4α1. Mutation analyses showed that the HNF1 site is essential for HNF1α transactivation and is required for full basal enhancer activity, as is the C/EBP site. Glucocorticoid response element consensus sites which overlap the C/EBP, HNF4 and HNF3 sites are crucial for optimal hormonal induction. We present a model that accounts for weak expression of HNF4α1 in the embryonic liver and strong expression in the newborn/adult liver via the binding sites identified in the enhancer.

INTRODUCTION

HNF4α, a member of the steroid hormone receptor superfamily (1), is one of the first liver-enriched transcription factors (LETF) to be expressed during the course of development. Indeed, transcripts are observed in the mouse at embryonic day (E)4.5 in primitive endoderm, at E5.5 in the extra-embryonic visceral endoderm and in embryonic tissues in the liver and gut primordia at around E8.5 (2). A somewhat earlier and overlapping expression pattern characterizes HNF3β and HNF1β, respectively, the only other LETF to have such precocious expression (3–6). Each of the three factors is essential for normal embryogenesis: targeted mutation of the corresponding genes causes an early embryonic lethal phenotype (3–7), due in the cases of HNF4α and HNF1β to disruption of visceral endoderm function, essential for the progression of gastrulation (4,5,8). In addition, HNF4α is required for the expression of a number of essential liver functions of differentiated hepatocytes (9). Alteration of some of these functions may be involved in maturity-onset diabetes of the young 1 (MODY1), linked to HNF4α insufficiency (10,11). HNF4α mutations linked to MODY1 affect the coding region of the protein. However, mutations in regulatory regions of the gene may yet be identified (reviewed in 12), providing a strong impetus to locate and characterize these sequences. In view of the importance of HNF4α for execution of the hepatocyte differentiation program, as well as its early expression pattern, the search for regulators of the HNF4α gene should inform us of the factors that are critical not only for hepatocyte differentation and function, but for endoderm formation as well.

Relatively little information is available concerning the regulation of expression of the HNF4α gene. A series of up to 10 DNase I hypersensitive sites (HS) in adult liver chromatin, spread out over 15 kb of DNA upstream of the start of transcription of HNF4α1, the initially isolated isoform (1), implies that its regulation is complex (13,14). Zhong *et al*. (14) used a 7.5 kb fragment of DNA upstream of the transcription start site and its 5′ deletions to analyze expression in transient transfection assays in hepatoma cells as well as in transgenic mice. While the long fragment and deletions down to –228 bp showed similar activity in transient expression assays, only the long fragment conferred appropriate activity to a reporter gene in transgenic mice. These results imply that the distal HS harbor important regulatory sites for HNF4α1 expression. Several splice variants of HNF4α have been described (reviewed in 12), so it is assumed that all of the HNF4α variants concern alternative splicing events within a single gene region. However, the most recently described of them, HNF4α7, contains a unique sequence encoding the N-terminal portion of the protein and the 5′-untranslated region (5′-UTR), implying that it is transcribed from a separate promoter (15).

We have analyzed the regulatory role of distal sequences upstream of the HNF4α1 promoter and characterized a 700 bp enhancer conferring high activity and glucocorticoid induction to both a heterologous and the HNF4α1 promoter. We have found LETF binding sites and glucocorticoid-responsive sequences essential for enhancer activity in a 240 bp sub-region. Our results are in line with the requirement for upstream sequences for tissue-specific activity of the HNF4α1 promoter

*To whom correspondence should be addressed. Tel: +33 1 45 68 85 00; Fax: +33 1 40 61 32 31; Email: mweiss@pasteur.fr

(14) and with glucocorticoid induction of HNF4α1 expression (11,15) and lead us to propose a model of biphasic regulation of this enhancer involved in HNF4α1 expression during liver development.

MATERIALS AND METHODS

Nucleotide sequence accession number

The GenBank accession number for the mouse 700 bp HNF4α1 enhancer is AF320052.

Cell lines and culture conditions

FGC4 rat hepatoma cells (16) are well differentiated whereas H5 cells (17) are dedifferentiated variants. Cells were grown in modified Ham's F12 medium (18) containing 5% fetal calf serum. Human epithelial carcinoma C33 cells (19) were maintained in Dulbecco's modified Eagle's medium containing 7% fetal calf serum. All cells were cultured under humidified 7% CO_2.

Transient transfections

Fragments of the mouse HNF4α1 5′-region (see Figs 1A and 2A) derived from the λSBII2.1 clone (a kind gift of W.Zhong, Yale University, New Haven, CT) were cloned in front of the herpes simplex virus thymidine kinase (tk) promoter driving transcription of the chloramphenicol acetyltransferase gene (CAT) in pBLCAT5 (20). The 700 bp enhancer was cloned in –34pZLUC (14). –6.8pZLUC is described in Zhong *et al*. (14). Constructs with the 700 bp enhancer and its sub-regions were checked by DNA sequencing. Standard transfections were performed by the calcium phosphate procedure as previously described (21) using 5 µg each of reporter plasmid and internal control plasmid RSVlacZ. Cells were harvested 64 h later. Where indicated, 1 µM dexamethasone (Dex) was added 48 h before harvest. In co-transfection experiments in C33 cells (15 or 6×10^5), test and control plasmids were used at 0.165 µg each and pBluescript plasmid was added to 3.3 µg total DNA per dish. In some experiments, FGC4 cells (5×10^6 cells in 150 µl of medium) were electroporated with a Bio-Rad gene pulser and capacitance extender (960 µF, 0.2 kV), using 5 µg each of test and control plasmid. In co-transfections, expression vectors for HNF1α (RSV-HNF1; 22), HNF1β (RSV-vHNF1; 23), HNF4α1 (HNF4tag; 24), HNF4α7 (M.E.Torres-Padilla, C.Fougère-Deschatrette and M.C.Weiss, submitted for publication) and HNF3α (a kind gift of R.H.Costa, University of Illinois, Chicago, IL) were used. The expression vector promoter was kept constant by addition of appropriate vector DNA. CAT and luciferase activities were determined as described (25,26) and β-galactosidase activity by the standard colorimetric method. CAT and luciferase activities were normalized to β-galactosidase activity and calculated relative to that of pBLCAT5 or –34pZLUC, respectively.

DNase I footprinting and electrophoretic mobility shift assays (EMSA)

Nuclear protein extracts from FGC4 and H5 cells, treated or not with Dex (1 µM) for 2 days, and from rat liver were prepared as described previously (27) except that $(NH_4)_2SO_4$ precipitation and dialysis were omitted. Protein concentrations were measured with the Bio-Rad protein assay. Purified recombinant truncated rat HNF1α (HNt) and rat HNF4α1 were kind gifts of C.Cheret (Institut Pasteur, Paris, France) and F.Ringeisen (Institut Pasteur), respectively. The DNase I footprinting assay was performed essentially as described previously (28), except that the binding reaction contained 85 mM NaCl and 10 mM KCl. Proteins were incubated with labeled DNA probe (1 ng) in 12 µl for 1 h on ice before adding DNase I. Products were analyzed on 6% polyacrylamide–7 M urea gels.

EMSA was carried out as described previously (27), with 50 mM NaCl added to the reaction mix. Binding reactions were performed with 10 µg of nuclear protein extracted from FGC4 or H5 and 0.4 ng of labeled oligonucleotide. Double-stranded oligonucleotides used contained HNF4α1 upstream sequences binding to HNF1α (oligo BS1, 5′-GCAAACTCATGCCCAGTTAATTTTTTAAAGCAAGA CCAACAGGCTC) or protected in DNase I footprinting experiments as well as binding sites of HNF1α in the rat albumin promoter (PE56; 27), HNF3/HNF6 in the mouse transthyretin promoter, from –85 to –110 (TTR; 29–31), HNF3/Oct-1 and HNF6 in the 6-phosphofructo-2-kinase promoter (PFK-III and PFK-IV, respectively; 32), Oct-1 in the mouse κ light chain gene promoter (Oct-Igκ; 32), HNF4α in the human apolipoprotein CIII promoter, from –67 to –92 (ApoCIII; 33), and in the rat (34) and mouse (35) HNF1α promoter (H1H4, 5′-GATCGGCTGAAGTCCAAAGTTCA-GTCC), C/EBP in the rat albumin promoter, from –86 to –109 (DEI; 28), and of the glucocorticoid receptor (GR) in the rat tyrosine aminotransferase gene, from –2491 to –2510 (GRE; 36). A double-stranded oligonucleotide (oligo BS1m) containing four point mutations in the HNF1 consensus site of oligoBS1 as described in the mutant PE DS 34 of PE 56 (27) was also used. In competition experiments, 20 ng of non-labeled double-stranded oligonucleotide was added to the reaction mix except as otherwise stated. A C/EBPα-specific antibody (sc-61 X; Santa Cruz Biotechnology) and an antibody raised against the N-terminus of rat HNF4α1 (a kind gift of F.M.Sladek, University of California, Riverside, CA; 37) were used.

Site-directed mutagenesis

Site-directed mutagenesis of the B1E5B2 reporter was performed using the QuickChange kit (Stratagene). All isolated clones were analyzed by DNA sequencing. We introduced a clustered point mutation (38) (mutant F1m1, 5′-GGAGAGGC-CAATGGCCTCGAAGCTTCCCCAGGAGGCCTCCTGCCC) or two point mutations (mutant F1m2, 5′-GGCCAAT-GGCCTCTAGAATAACCCAGGAGGCC) at the C/EBP consensus site in F1. The HNF1 binding site BS1 was mutated using oligo BS1m. Clustered point mutations according to the mutation of the HNF3 binding site in the TTR promoter (29) were introduced at a HNF3 consensus site in F3 (mutant F3mH3, 5′-GCTGCCTTTATCTCCCTGTGGTAAAGATCT-GAATATACTCAGGCCCTGGAGCAGGGC). Three point mutations were introduced at the center of a HNF4 consensus site (12) in F2 (mutant F2mH4, 5′-GCTGTTGACTCTT-GAGCTCCGTCTTCCCTCCGGAGG).

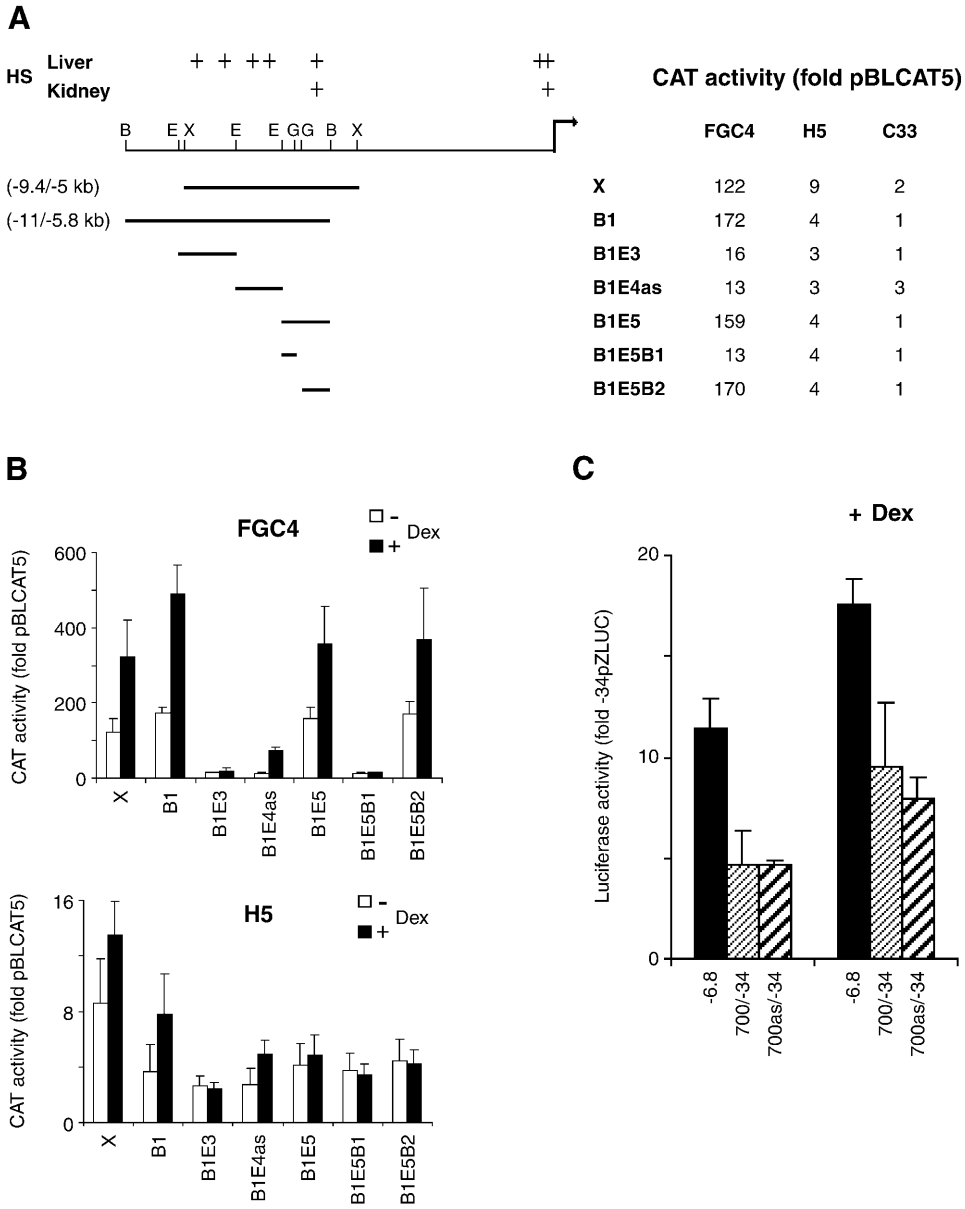

Figure 1. Enhancer activity and Dex inducibility of distal sequences upstream of the HNF4α1 transcription start. (**A**) (Left) Scheme of the HNF4α1 5′-region showing positions of liver- and kidney-specific HS (13,14), a partial restriction map (B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; X, *Xba*I) and the transcription start (arrow). (Below) Cloned restriction fragments (B1E4 was cloned in the antisense direction). (Right) CAT activity of the corresponding reporter plasmids transfected in differentiated (FGC4) or dedifferentiated (H5) hepatoma cells or in non-hepatic cells (C33). Values are means of three (FGC4), two to three (H5) or one to four (C33) independent experiments. (**B**) Activity of the same constructs transfected in FGC4 and H5 cells treated or not with Dex. Means ± SD of three (FGC4) and two to three (H5) independent experiments. (**C**) Activity in transfected FGC4 cells, treated or not with Dex, of a minimal HNF4α1 promoter (–34) controlled by the 700 bp enhancer, cloned in both orientations, or by HNF4α1 5′-sequences up to −6.8 kb. Means ± SD of two independent experiments.

RESULTS

A 700 bp enhancer located 6 kb upstream of the HNF4α1 transcription start increases activity and confers Dex inducibility to heterologous and homologous promoters

For transfection studies we used differentiated FGC4 rat hepatoma cells containing HNF4α transcripts synthesized only from the HNF4α1 promoter (M.E.Torres-Padilla, C.Fougère-Deschatrette and M.C.Weiss, submitted for publication) and therefore appropriate to analyze HNF4α1 regulatory sequences. Activity of two fragments covering sequences from –5 to –11 kb upstream of the HNF4α1 transcription start and cloned upstream of the tk promoter was examined in these cells and in dedifferentiated H5 and non-hepatic C33 cells (Fig. 1A). Both fragments exhibited strong enhancer activity, but only in FGC4 cells. The more active fragment was subdivided into three fragments and tested. Essentially full activity, again limited to FGC4 cells, was conserved by the proximal fragment, B1E5. Finally, B1E5 was divided into two fragments and full activity in FGC4 cells was retained by the more proximal

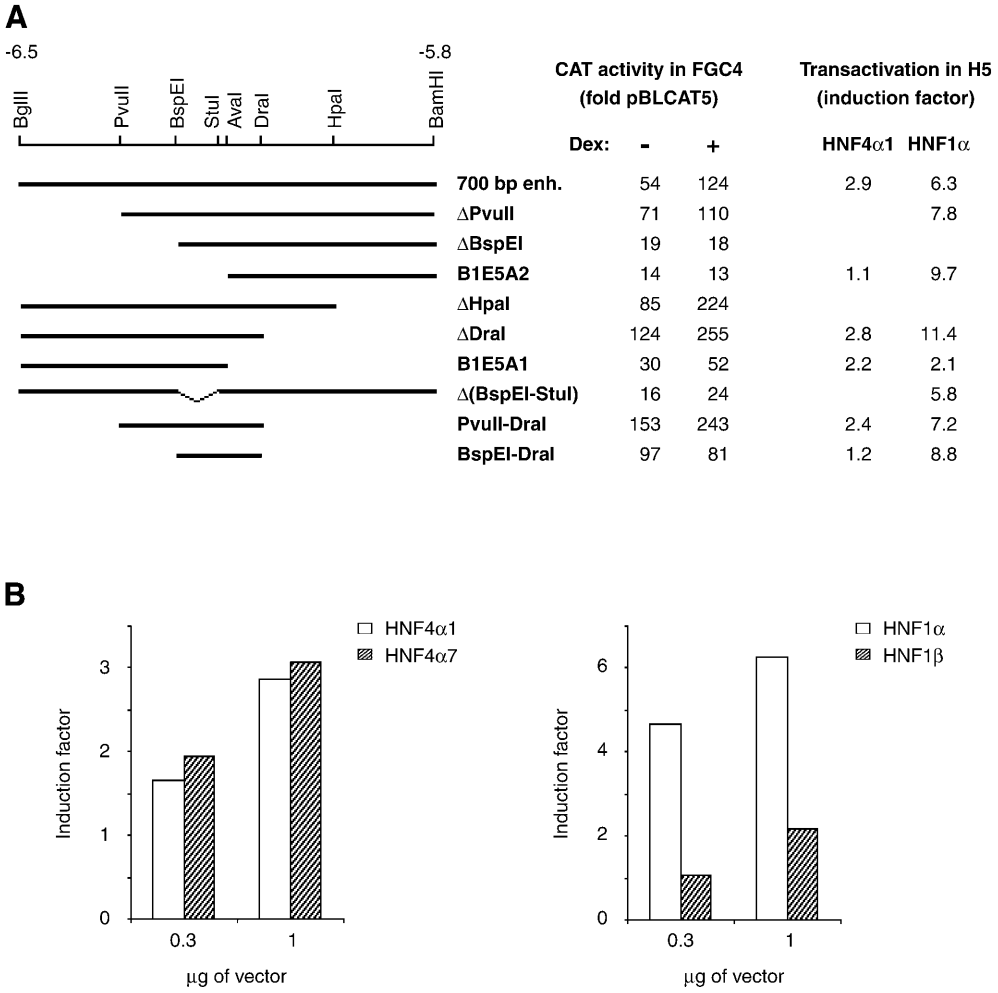

Figure 2. (**A**) A 240 bp region of the 700 bp enhancer shows basal and Dex-induced enhancer activities and is transactivated by HNF4α1 and HNF1α. (Left) Restriction map of the enhancer with positions (in kb) relative to the transcription start and schemes of cloned sub-regions. (Middle and right) Activities of the corresponding reporter plasmids either after transfection in FGC4, treated or not with Dex, or co-transfection of H5 with 1 μg of HNF4α1 or HNF1α expression vector. Induction factor represents the ratio of CAT activity in the presence of the factor to that in the presence of the control expression vector. For the 700 bp enhancer, the induction factor is the mean of four to five independent experiments. Induction factors for the other fragments have been normalized to this mean value. Blanks indicate that fragments were not tested for activity. (**B**) The 700 bp enhancer is transactivated by HNF4α7 (left) and HNF1β (right). H5 cells were co-transfected by the B1E5B2 reporter plasmid and 0.3 or 1 μg of expression vector for HNF4α1, HNF4α7, HNF1β or HNF1α as indicated. CAT activity is expressed as induction factor [see (A)].

fragment, B1E5B2. This fragment, covering one HS (13,14), therefore acts as a cell-specific enhancer and is referred to as the 700 bp enhancer.

Since we had observed that transcription of the endogenous HNF4α gene of rat hepatoma cells is induced by Dex treatment (data not shown), each of the fragments was tested for Dex inducibility (Fig. 1B). Dex induction was observed only in FGC4 (2–3-fold) and not in H5 cells and, with one exception, it was found only for the most active fragments. The only fragment with low basal activity that showed inducibility was B1E4, which covers HS V (13). This fragment has not yet been studied further.

To test whether the 700 bp enhancer can act on the homologous promoter, we cloned it in front of the –34/+182 HNF4α1 sequence in plasmid –34pZLUC (14). The 5′ deletion to –34 retained non-tissue-specific promoter activity (14). In FGC4 (Fig. 1C) the 700 bp enhancer, whatever its orientation, increased the activity of –34pZLUC 5-fold, while the relative activity of –6.8pZLUC was increased 11-fold. This last plasmid contains the 6.8 kb upstream of HNF4α1 (14). Moreover, Dex treatment increased the activity of the 700/–34pZLUC constructs ~2-fold, which accounts totally for the Dex induction of –6.8pZLUC (Fig. 1C).

A 240 bp region is sufficient for enhancer activity and Dex inducibility and is transactivated by HNF1α and HNF4α1

Deletions were performed in the 700 bp enhancer to delineate the sequences that are critical for basal and inducible expression (Fig. 2A). Concerning the 5′ and 3′ deletions, in Δ*Bsp*EI and B1E5A2 basal activity was strongly reduced compared to that of the 700 bp enhancer and hormone inducibility was abolished. The sequences deleted between Δ*Hpa*I and Δ*Dra*I had a negative effect on both basal and induced activity because their loss led to increased activity. Further deletion from *Dra*I to *Ava*I caused a strong decline in both basal and hormone-induced activity. An internal deletion, Δ(*Bsp*EI–*Stu*I), also strongly reduced both basal and induced activities compared to the 700 bp enhancer. Therefore, three adjacent blocks of

sequence lying between the *Pvu*II and *Dra*I sites are important for full basal and hormone-induced activity of the 700 bp enhancer, with the most 5′ one (*Pvu*II–*Bsp*EI) being the most critical for the Dex response. Analysis of two central fragments, *Pvu*II–*Dra*I and *Bsp*EI–*Dra*I, confirmed that an element required for hormone response is located between the *Pvu*II and *Bsp*EI sites. None of the fragments shown in Figure 2A exhibited strong enhancer activity in dedifferentiated H5 cells in the absence or presence of Dex (data not shown).

Because it is known that the HNF4α gene is subject to cross-regulation by HNF1α and to auto-regulation (24,39–42), several of the fragments were tested for transactivation by HNF1α and HNF4α1. The 700 bp enhancer is transactivated by both factors in H5 (Fig. 2A) and C33 (data not shown) cells. Analysis of deleted constructs in H5 cells (Fig. 2A) shows that the HNF4α response element is located between the *Pvu*II and *Bsp*EI sites and the HNF1α element between the *Ava*I and *Dra*I sites. In addition, in H5 cells the 700 bp enhancer is transactivated similarly by HNF4α1 and HNF4α7 and less efficiently by HNF1β than HNF1α (Fig. 2B).

In conclusion, these results show that the 700 bp enhancer is transactivated by HNF4α1, HNF4α7, HNF1α and HNF1β. Furthermore, the 240 bp *Pvu*II–*Dra*I sequence contains HNF4α and HNF1α response elements and acts as a cell-specific enhancer whose activity is increased by glucocorticoids.

Hepatoma cell nuclear proteins bind to the 240 bp enhancer

DNase I footprint experiments were performed to search for binding sites of nuclear proteins in the 700 bp enhancer. Figure 3A shows four footprints (F1–F4), revealed with nuclear extract from FGC4 and H5 cells. Due to the location of F1 very close to the labeled end of the probe, its 3′-limit is not clearly visible. However, using a probe labeled further downstream on the same strand we precisely located the 3′-end of F1 (data not shown). There is a surprising similarity between the footprints obtained with the two extracts. For F1 (Fig. 3A and data not shown) the H5 extract is clearly less active than that of FGC4 and for F2 a greater protection by FGC4 extract is evident only at the 3′-extremity of the footprint. As only F1–F3 lie within the 240 bp enhancer region characterized above (see Fig. 3C), we mapped these three footprints on the opposite strand using FGC4 extract (Fig. 3B). The autoradiogram shows F2 and F3 at higher resolution. In addition, Figure 3A and B shows F1 as a particularly strong footprint on both strands. Positions and sequences of F1–F3 are given in Figure 3C and D, along with sequences of the oligonucleotides used in the EMSA.

In F1 a C/EBP binding site is essential for basal enhancer activity and overlaps with Dex-responsive sequences

EMSA were performed with nuclear extracts from FGC4 and H5 cells treated or not with Dex and an oligonucleotide containing F1 (oligo F1) (Fig. 4A). In FGC4 extracts a complex pattern of six bands was observed, two of which (designated 1 and 3) were restricted to these extracts. The H5 extract showed strong bands corresponding to complexes 2 and 4 only after Dex treatment (even though Dex inducibility was restricted to FGC4 cells), while hormone treatment did not change the pattern in FGC4. Interestingly, three of the six bands observed in FGC4 were strongly represented in liver extract (Fig. 4B). Moreover, complexes 1 and 3 appeared common between liver and FGC4. In competition experiments

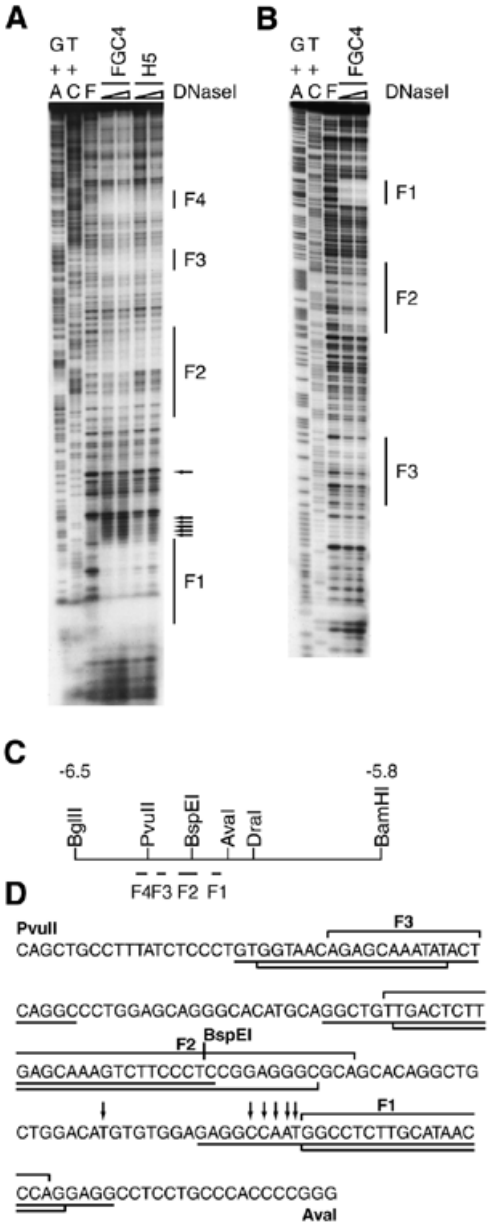

Figure 3. DNase I footprints generated by FGC4 and H5 nuclear extracts on the 700 bp enhancer. (**A**) The *Bgl*II–*Ava*I fragment [see (C)], ^{32}P-labeled on the upper strand at the 3′-end of the *Ava*I site was incubated in the absence (F) or presence of nuclear extract (20 µg protein) before adding DNase I at final concentrations of 0.7 (F) or 13 and 20 U/ml (FGC4 and H5). G+A and T+C show chemical sequencing reaction products. Footprints (bars) and hyper-digestion sites (arrows) generated by the FGC4 extract are indicated. There are some differences in the DNase I digestion pattern using H5 extract (see text). (**B**) The same experiment using a *Hin*dIII–*Bam*HI fragment ^{32}P-labeled at the 3′-end of the *Hin*dIII site (sites in the vector) and containing the *Pvu*II–*Dra*I fragment [see (C); lower strand labeled], and FGC4 nuclear extract. (**C**) Positions of the DNase I footprints generated by FGC4 nuclear extracts in the enhancer. Results are from (A) and (B) and data not shown. (**D**) Sequences of the *Pvu*II–*Ava*I fragment with DNase I footprints (bracketed lines) and hyper-digestion sites (arrows) on the upper and lower strand (above and below the sequence, respectively) indicated. Oligonucleotides used for further analysis are underlined.

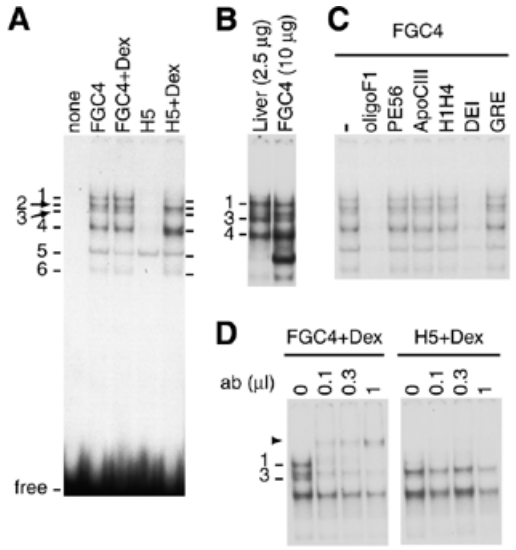

Figure 4. C/EBPα binds to F1 in FGC4 cells and rat liver nuclear extracts. (**A**) Nuclear extracts from cells treated or not with Dex were incubated with labeled oligo F1 in EMSA as indicated. Numbers designate retarded protein–DNA complexes. Free DNA is indicated. (**B**) The same experiment using nuclear extracts from rat liver and FGC4 cells. Protein concentration is indicated. (**C**) EMSA of labeled oligo F1 and FGC4 nuclear proteins without (minus) or with competitor oligo F1 or oligonucleotides containing binding sites for HNF1 (PE56), HNF4 (ApoCIII and H1H4), C/EBP (DEI) and GR (GRE). (**D**) EMSA with labeled oligo F1, FGC4 and H5 nuclear extracts and increasing amounts of anti-C/EBPα antibody. Antibody (1–3 µl, undiluted or 10-fold diluted) was preincubated with nuclear extracts as indicated for 10 min at room temperature, then reactions were completed and incubated for 30 min on ice. Cells were treated with Dex to observe complexes 2 and 4 in H5 cells. Complexes 1 and 3 were supershifted by the antibody (arrowhead).

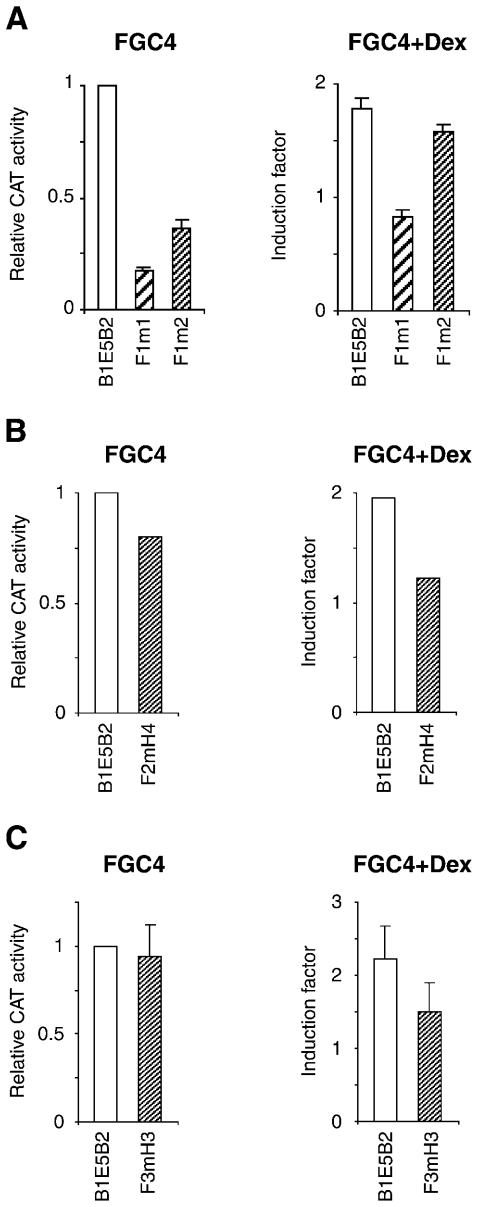

Figure 5. The C/EBP site is required for full enhancer activity and functional GRE consensus sites overlap with the C/EBP, HNF4 and HNF3 sites. (**A**) (Left) FGC4 cells were transfected with B1E5B2 reporter plasmids, either wild-type or bearing two different mutations in the C/EBP binding site of F1. An overlapping half-GRE consensus site is affected by mutation in the F1m1 but not in the F1m2 mutant. Activity is expressed relative to B1E5B2. The means ± SD of three (F1m1) or two (F1m2) independent experiments are shown. (Right) Same experiment except that the transfected cells were treated with Dex. Induction factor represents the ratio of CAT activity in the presence and absence of Dex. The means ± SD of three (B1E5B2, F1m1) or two (F1m2) independent experiments are shown. (**B**) The same experiment except that the B1E5B2 reporter plasmid was mutated in the HNF4 consensus site of F2 (F2mH4). (**C**) The same experiment except that the B1E5B2 reporter plasmid was mutated in the HNF3 consensus site of F3 (F3mH3). In both the F2mH4 and F3mH3 mutants GRE consensus sites are affected. For the F3mH3 mutant, the mean ± SD of two independent experiments is shown.

oligos F1 and DEI, containing the C/EBP site of the albumin promoter, displaced the bands in the three types of extracts (Fig. 4C and data not shown). In contrast, binding sites for the other LETF or GR did not compete (Fig. 4C and data not shown). Therefore, C/EBP family proteins bind to F1. In agreement with this, a sequence homologous to the C/EBP binding site consensus (43) was found in the center of F1. The two bands specific to FGC4 and liver extracts were shown to contain C/EBPα by supershift assays: a specific antibody displaced only bands 1 and 3 in FGC4 and liver extracts and had no effect in the H5 extract (Fig. 4D and data not shown). Consequently, we conclude that C/EBPα binds to F1 in FGC4 cells and the liver but not in H5 cells.

To determine whether C/EBPα plays a role in enhancer activity, critical bases were mutated in the C/EBP consensus site (38) in the 700 bp enhancer. The activity of the mutant reporter plasmid (F1m1) was then tested in FGC4 cells. The mutant showed a low basal activity, reduced ~6-fold compared to the wild-type (Fig. 5A, left). In addition, Dex inducibility was entirely destroyed by the mutation (Fig. 5A, right). The mutant sequence failed to bind proteins of FGC4 or H5 extracts, as shown by EMSA and DNase I footprint experiments (data not shown). However, three of the seven mutated nucleotides in the F1m1 mutant belong to a glucocorticoid response element half-site (half-GRE) consensus overlapping with the C/EBP consensus (data not shown). Consequently, the mutation could impair binding of GR linked to the hormone. We then performed two point mutations in the C/EBP

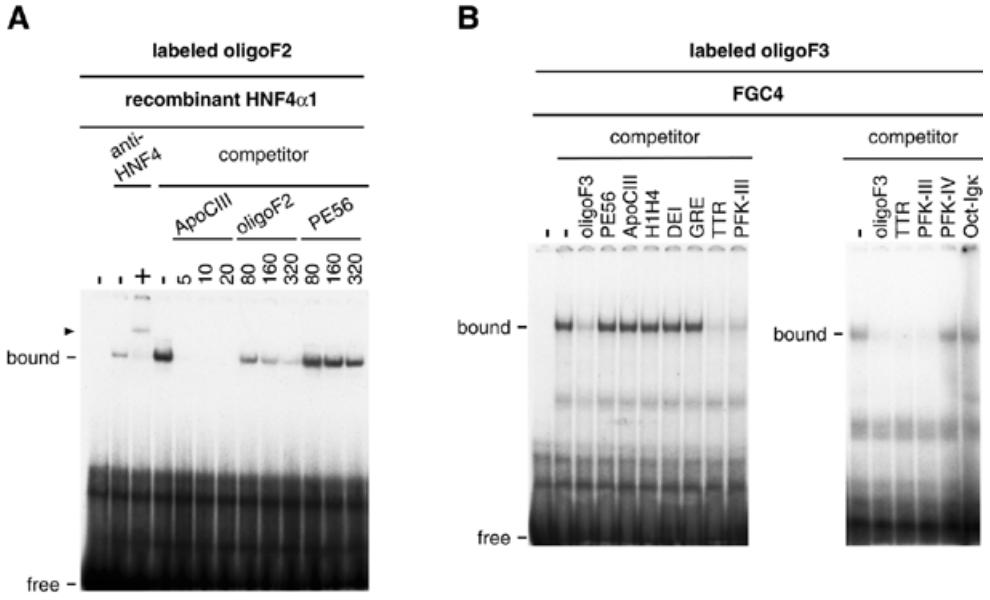

Figure 6. Recombinant HNF4α1 binds to F2 (A) and HNF3 to F3 (B). (**A**) Labeled oligo F2 and recombinant rat HNF4α1 (0.7 µg) were incubated without (minus) or with (plus) an anti-HNF4α1 antibody and without (minus) or with competitor oligonucleotides. The first lane shows migration without protein. For the reaction with the antibody and the control, 1 µl of 10-fold diluted antibody in PBS (plus) or PBS alone (minus) was added to reaction mix that had been preincubated for 15 min on ice. Incubations were carried out for 25 min at room temperature. Other reactions were incubated on ice for 30 min. The arrowhead designates the ternary complex antibody–HNF4–DNA. Oligonucleotide amounts are given in nanograms. Protein–DNA complexes are indicated. (**B**) Labeled oligo F3 was incubated without (minus) or with FGC4 nuclear extract and competitor oligonucleotides. TTR and PFK-III contain binding sites for HNF3 and HNF6 and for HNF3 and Oct-1, respectively, whereas PFK-IV and Oct-Igκ bind HNF6 and Oct-1, respectively.

consensus site that do not affect the half-GRE consensus but impair C/EBP binding as analyzed by competition experiments in EMSA (data not shown). This mutation diminished basal activity (mutant F1m2, Fig. 5A, left) but caused only a weak inhibition of the Dex effect (Fig. 5A, right). We conclude that the C/EBP binding site is required for full basal enhancer activity and overlaps with a half-GRE consensus site involved in hormone inducibility. These data indicate that C/EBPα is involved in enhancer activity in FGC4 cells.

F2 and F3 contain HNF4α and HNF3 binding sites, respectively, both overlapping with Dex-responsive sequences

An oligonucleotide spanning the more protected part of F2 (oligo F2) was used in EMSA with FGC4 extract. Competition experiments suggested that F2 binds HNF4α weakly (data not shown). To confirm that HNF4α binds to F2, we used purified recombinant rat HNF4α1 and labeled oligo F2 in EMSA. Figure 6A shows a complex which was displaced by unlabeled ApoCIII and oligo F2 but not by unlabeled HNF1 site PE56. Moreover, antibody against rat HNF4α1 supershifted nearly all the complex (compare lanes anti-HNF4 + and –). This figure also shows that oligo F2 binds HNF4α1 with an efficiency >60-fold lower than ApoCIII.

EMSA was carried out with FGC4 extract and an oligonucleotide containing F3 (oligo F3). One complex formed, which was competed by oligo F3 and by oligos TTR and PFK-III, containing HNF3/HNF6 (30,31) and HNF3/Oct-1 (32) binding sites, respectively (Fig. 6B, left), but not by oligos PFK-IV and Oct-Igκ (Fig. 6B, right), binding to HNF6 and Oct-1, respectively (32). This shows that HNF3 binds to F3. In support of a functional role of HNF3 binding, HNF3α transactivates the 700 bp enhancer in co-transfection experiments in C33 cells (data not shown).

HNF4 and HNF3 consensus sites were found in oligos F2 and F3, respectively. To examine the functional roles of these sites, we introduced point mutations in each of them in the 700 bp enhancer and tested the resulting plasmids for basal and Dex-induced activity in FGC4 cells. In F2 the HNF4 mutation (Fig. 5B) caused a slight reduction in basal activity (left) and a significant reduction in inducibility (right). A slight reduction in inducibility was obtained for the HNF3 mutation in F3 while the basal activity was not affected (Fig. 5C). However, these mutations reduced but did not abolish HNF4α1 and HNF3 binding as analyzed by competition experiments in EMSA (data not shown). Furthermore, the HNF4 consensus site is included in a GRE consensus site which is affected by the HNF4 mutation while the HNF3 mutation touches a half-GRE consensus site. These data show that GRE consensus sites intermingled with the LETF sites are required for maximal Dex inducibility of the 700 bp enhancer, as is the case for the C/EBP site in F1.

A functional HNF1 binding site is essential for basal enhancer activity

The 700 and 240 bp enhancers are similarly transactivated by HNF1α. A computer search for putative HNF1 binding sites in the 700 bp sequence using a weighted matrix (44) revealed three sites with high scores. The most upstream contains the *Dra*I site and the three last bases of the consensus (15 bp) were replaced by vector sequence in cloned fragments cut by *Dra*I. The second HNF1 site is located a few base pairs downstream of the *Dra*I site and the third lies 70 bp further downstream (data not shown). In DNase I footprinting experiments using purified recombinant truncated HNF1α protein (HNt) we

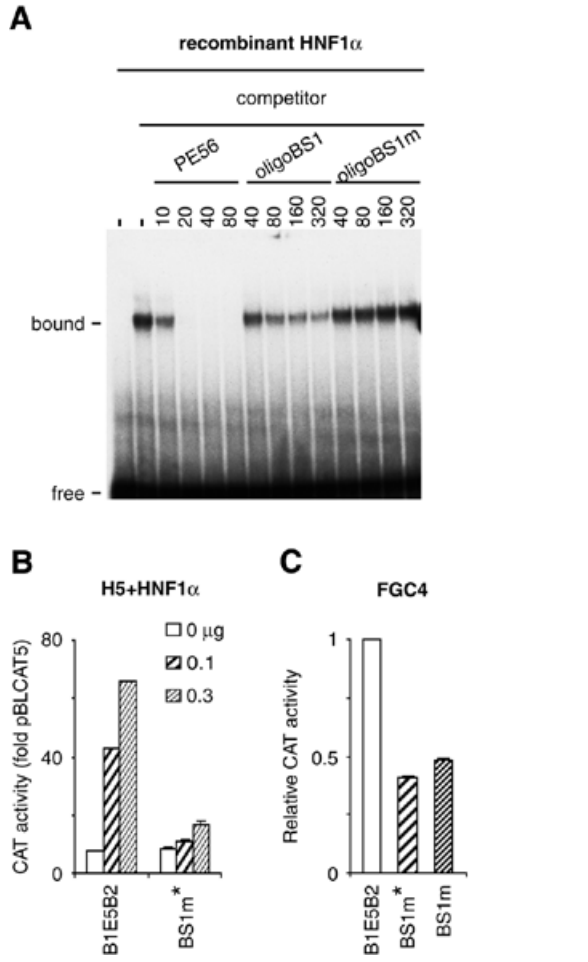

Figure 7. Recombinant HNF1α binds to a site at the 3′-end of the 240 bp enhancer (A) and this site is required for HNF1α transactivation (B) and full enhancer activity (C). (**A**) Purified recombinant truncated rat HNF1α was used at 0.5 µg in EMSA with the labeled oligo BS1 in the absence or presence of increasing amounts (indicated in ng) of competitor oligo PE56, BS1 or BS1m (mutated in BS1). (**B**) CAT activity of reporter plasmid B1E5B2, wild-type or mutated in BS1 (BS1m*, see text), in H5 co-transfected with HNF1α. For the mutant reporter, the mean ± SD of three independent experiments is shown. Expression vector amounts are indicated. (**C**) CAT activity in FGC4 of reporter plasmid B1E5B2, wild-type or mutated in BS1 (BS1m* or BS1m). The mean ± SD of two independent experiments is shown.

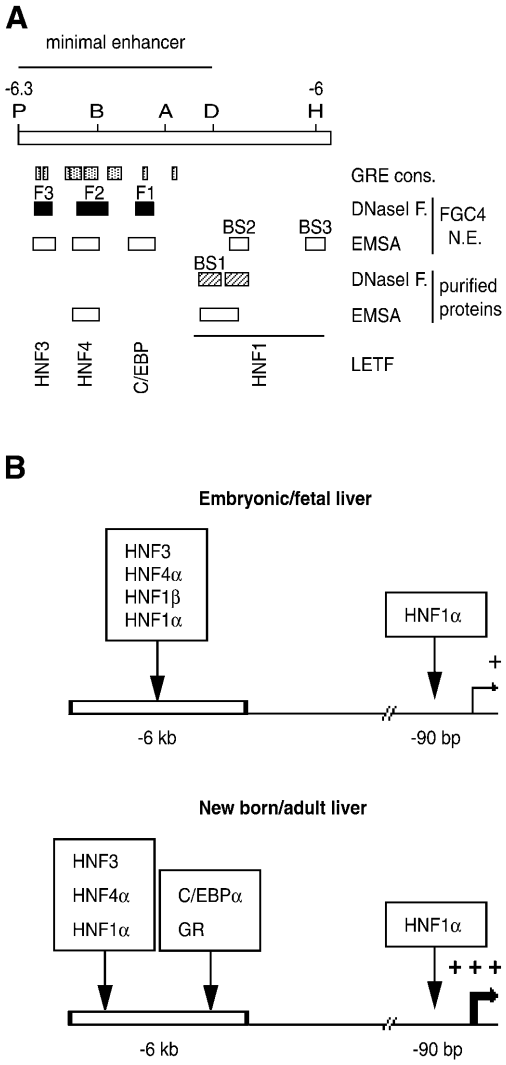

Figure 8. Overview of the –6 kb enhancer (A) and a model for HNF4α1 regulation (B). (**A**) A restriction map is shown (P, *Pvu*II; B, *Bsp*EI; A, *Ava*I; D, *Dra*I; H, *Hpa*I). Positions of three imperfect whole and five imperfect half-GRE consensus sites in the minimal enhancer are indicated. Protein binding sites characterized using DNase I footprinting assay and EMSA with FGC4 nuclear extract or purified recombinant proteins are represented. Footprints F1–F3, HNF1 binding sites and LETF binding to the sites are indicated. (**B**) Schematic representation showing a model of the factors that are expressed and could bind to the HNF4α1 –6 kb enhancer and promoter (14) regions, resulting in low expression during early liver development and a strong up-regulation at birth.

demonstrated that the two most upstream consensus sites correspond to authentic HNF1 binding sites (BS1 and BS2, respectively; data not shown). Then we tested the recombinant HNF1α protein by EMSA using double-stranded oligonucleotides containing BS1, wild-type (oligo BS1) or mutated (oligo BS1m) in order to abolish HNF1 binding (27). A complex between HNt and labeled oligo BS1 formed which was competed by PE56 and oligo BS1, but not by oligo BS1m (Fig. 7A). From these experiments we conclude that BS1 binds HNF1α, but with an efficiency >16-fold lower than the high affinity PE56 oligonucleotide. In the constructs 3′ deleted at the *Dra*I site, modified BS1 still binds to HNt but with a lower affinity (data not shown). In EMSA using FGC4 extracts the third consensus (BS3) proved also to bind HNF1α.

To investigate whether BS1 could mediate transactivation by HNF1α we used oligo BS1m to mutate BS1 in the 700 bp enhancer. We obtained two mutant reporter plasmids of which one (BS1m*) contains an insertion of two G residues within BS1 in addition to the expected mutation. As shown in Figure 7B, in H5 cells this mutant plasmid exhibited the same low basal activity as the B1E5B2 reporter plasmid but transactivation by co-transfected HNF1α was severely compromised, in spite of the continued presence of intact BS2 and BS3 sites. Moreover, activity of both mutant plasmids in FGC4 was strongly and similarly reduced compared to the wild-type (Fig. 7C). These results thus show that BS1 is required not only for transactivation by HNF1α, but also for enhancer activity of the 700 bp region in differentiated hepatoma cells.

In summary, we have characterized several LETF binding sites (Fig. 8A) within the 700 bp enhancer. C/EBP, HNF4 and

HNF3 binding sites are located in the 240 bp minimal enhancer, *Pvu*II–*Dra*I, whereas an HNF1 binding site overlaps its 3′-end and two other HNF1 binding sites lie further downstream. The C/EBP site and the first (BS1) HNF1 site are required for full basal activity. Several imperfect whole and half-GRE consensus sites were found in the minimal enhancer (Fig. 8A). Three of them, overlapping with LETF sites, are crucial for maximal hormone inducibility of the 700 bp enhancer.

DISCUSSION

In this work we have defined an enhancer element upstream of the HNF4α1 promoter that is active in well-differentiated FGC4 rat hepatoma cells and not in dedifferentiated variant hepatoma and non-hepatic cells. Fragments covering –5 to –11 kb upstream of the transcription start site have been tested upstream of the tk promoter and full activity can be localized to a fragment of 700 bp (700 bp enhancer) at around –6 kb, which corresponds to a HS of both liver and kidney chromatin (13,14). This fragment increases the activity of a minimal HNF4α1 promoter from which the HNF1 site (14) has been deleted. This result agrees with the requirement of upstream sequences up to –7 kb to obtain liver- and kidney-restricted activity of the HNF4α1 promoter in transgenic mice (14). Moreover, the 700 bp enhancer mediates glucocorticoid induction, but only in differentiated hepatoma cells. This agrees with the reported glucocorticoid induction of HNF4α expression (11,15). The 700 bp enhancer is thus implicated in physiological regulation of HNF4α1 activity.

The 700 bp enhancer contains a 240 bp core sequence sufficient for both enhancer activity and Dex induction and within which three footprints (F1–F3) have been identified. C/EBP binds to F1, HNF4 to F2 and HNF3 to F3. Finally, a functional HNF1 binding site is at the 3′-end of the core sequence. Figure 8A summarizes these data. The sequence comprising the minimal enhancer is conserved. Blast analysis of the mouse and human (NCBI database accession no. AL117382) sequences showed an overall 86% identity. The F1–F3 and HNF1 sites exhibit the highest scores (90–100%). This fact implies that the HNF4α1 enhancer is an important element that plays a similar role in humans.

The C/EBP site is the strongest footprint in the enhancer. Its mutation significantly reduces enhancer activity but only in cells that produce C/EBPα, implying that this site responds specifically to binding of C/EBPα. Previously we proposed that HNF4α regulates its own expression based on studies of stable transfectants (24,42). Our data here support such a direct auto-regulation mechanism because the enhancer core sequence contains a functional HNF4 response element. In addition, HNF3α transactivates the enhancer. However, while mutation of the HNF4 and HNF3 sites had only small effects, the introduced mutations impaired binding of the factors only partially. The HNF4α1 enhancer is strongly transactivated by HNF1α and more weakly by HNF1β. Transactivation by HNF1α is mediated by a weak HNF1 binding site, which is also required for high basal activity of the enhancer. A similar functional role of weak HNF1 binding sites has been reported for the mouse phenylalanine hydroxylase gene enhancer (21). A functional HNF1 binding site in the HNF4α1 enhancer, in addition to the site described earlier in the promoter region (14), accords with earlier reports of cross-regulation between HNF4α and HNF1α (39–42). Based on the strong response to HNF1α, we propose that this factor plays a critical role in enhancer activity.

In agreement with our observations, glucocorticoids have been shown to activate HNF4α expression in primary cultured rat hepatocytes (11). Moreover, HNF4α1, but not HNF4α7, is induced by glucocorticoids in murine hepatic cells (15). Maximal Dex induction of the enhancer requires the F1–F3 region where three imperfect GRE and five imperfect half-GRE consensus sites were found, some overlapping with the F1–F3 footprints (see Fig. 8A). Although competition EMSAs using GRE failed to provide evidence for binding of GR, the presence of GRE consensus sites and responsiveness to Dex imply that GR binds to the enhancer. Indeed, mutations introduced into LETF binding sites that affect GRE whole sites or half-sites compromised Dex induction, implying that these sites are functional. Moreover, induction of the 700 bp enhancer was restricted to differentiated hepatoma cells, implying that a liver-specific protein(s) is required for the glucocorticoid effect. Indeed, the LETF can synergize with GR to achieve glucocorticoid induction of the tyrosine aminotransferase (TAT) (45–47) and the phosphoenolpyruvate carboxykinase (PEPCK) genes (48). A similar situation appears to exist for the HNF4α1 enhancer.

At first sight, it is surprising to observe that the major transactivators for the HNF4α1 enhancer are hormone-activated GR and LETF that are expressed late in development, such as C/EBPα and HNF1α, even though it is known that HNF4α transcripts are first detected very early (2). However, probes that do not distinguish between the HNF4α1 and HNF4α7 isoforms were used for analysis of early embryos. Recent work from our laboratory has revealed that expression of the HNF4α1 isoform is strong in the liver only at the end of fetal life and at birth, whereas the HNF4α7 isoform is the predominant form of HNF4α in embryonic liver (M.E.Torres-Padilla, C.Fougère-Deschatrette and M.C.Weiss, submitted for publication). The dramatic activation of HNF4α1 that occurs just before birth could be assured, on the one hand, by C/EBPα, whose expression is also activated at birth (49), and, on the other, by glucocorticoids. Indeed, the TAT and PEPCK genes, which like HNF4α1 have enhancers in which glucocorticoid hormone response modules overlap LETF sites (46–48), are strongly induced by glucocorticoids at birth.

HNF4α1 is thus expressed at low levels in embryonic liver. Binding sites in the HNF4α1 enhancer do include transcription factors that are present in the developing liver. HNF3α and β are among the first LETF to be expressed in this tissue (50). HNF1β, known to be expressed before HNF1α in the embryonic liver (4,5), is a transactivator of the enhancer, as is HNF4α7.

Although many studies have contributed to the notion that the group of LETF is subject to cross-regulation and to autoregulation (4,5,8,9,14,24,30,31,34,35,39–42,51–63), this is the first detailed study of an enhancer regulating expression of one of these factors. Furthermore, this analysis concerns one of the first of these factors to be expressed, HNF4α (2). The results of this study are entirely consistent with the observed behavior of the HNF4α gene *in vivo* and provide a basis to explain its expression pattern. Indeed, binding sites for essentially all of the LETF so far identified are present, including factors expressed early during development, such as HNF3α and β,

HNF1β and HNF4α7, and others that are expressed a little later, such as HNF1α, and in particular those that are up-regulated at birth, including C/EBPα, HNF4α1 and, perhaps most importantly, activated GR. Consequently, the properties of the –6 kb enhancer are consistent with it being involved both in the initiation of expression of HNF4α1 and in its maintenance. Figure 8B presents our model of biphasic regulation of the HNF4α1 enhancer during liver development.

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